

CASSAVA LACTIC FERMENTATION IN CENTRAL AFRICA: MICROBIOLOGICAL AND BIOCHEMICAL ASPECTS

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Summary

Retting is a lactic fermentation during which cassava roots are soaked for long periods in water. Despite the importance of this fermentation, no kinetic study of it has been undertaken. Our study therefore examined the biological and physical changes of cassava roots during retting to provide a basis for its possible mechanization.

The study was carried out to (1) enumerate and characterize the main microorganisms of the process; (2) determine the evolution of physicochemical parameters during retting; and (3) measure the production of organic products and some principal enzyme activities.

Retting can be characterized by three essential transformations of the roots: (1) a degradation of endogenous

cyanogenic compounds (e.g., concentration decreased from 400 ppm in fresh cassava to 20 ppm in fermented mash); (2) a significant lysis of cassava cell walls due to the simultaneous action of endogenous pectin methylesterase and bacterial pectin lyase; and (3) the production of organic acids (C₂ to C₄), mainly lactate and butyrate, that contribute to the typical flavors of *chikwangue* and *fufu*.

In the study, most microflora involved in retting were facultative, anaerobic, fermentative bacteria, among which lactic bacteria were predominant. From the second day of fermentation, endogenous *Lactobacillus* species were totally supplanted by *Leuconostoc mesenteroides* and *Lactococcus lactis*. Anaerobic bacteria such as *Clostridium butyricum* were also found and seemed responsible for initiating butyrate production. Yeasts played no significant role, but their increasing number at the end of the process (*Candida* species) probably influenced the conservation of end products.

Despite the significant number of amylolytic bacteria (10⁵-10⁶ b/ml), the amylase activity found in the retting juice came from the roots and disappeared after 48 h of fermentation. The main enzymes of

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this process were cassava pectin methylesterase, bacterial pectinase, and endogenous linamarase.

The pH became stable at about 4.5 after 48 h and the partial oxygen pressure dropped to 0.2 mg/L after 10 h.

These results suggested that retting is a typical heterolactic fermentation with a significant production of butyrate.

Introduction

Processed cassava (*Manihot esculenta* Crantz) is eaten in West and Central Africa in such forms as *gari*, *lafun*, *fufu*, *chikwangue*, and tapioca. In the Congo, the world's second largest cassava consumer after Zaïre (Trèche, n.d.), cassava roots account for 47% of the calorie intake (Trèche and Massamba, n.d.b).

The two main products associated with fermented cassava are *fufu* and *chikwangue*. The former is a flour obtained from sun-dried cassava mash that is pulverized. This flour may be mixed with boiling water and served in bowls with sauce and fish or meat. *Chikwangue*, a cassava bread, is obtained after multiple postfermentation steps, including defibering and pugging (Trèche and Massamba, n.d.a).

Both products require a fermentation in which the roots soak for 3 to 6 days in tap water. During this process, cyanogenic compounds are eliminated, flavor compounds are elaborated, and the roots soften (Okafor et al., 1984; Oladele Ogunsa, 1980). Softening is indispensable for further root processing but the mechanisms involved are not yet fully understood.

Significant differences exist in retting processes throughout Central Africa and even in the Congo. Peeled or unpeeled roots are retted in rivers, standing water, large barrels of water, or even buried in soil. The fermentation temperature varies with season and location. Such differences, combined with the low reproducibility of the local processors, lead to a variability in quality and taste of cassava foods (Trèche and Massamba, n.d.a).

To increase the quality of these traditional products and provide a basis for the possible mechanization of the process, the European Union (EU) Program-STD2, known as "Improving the Quality of Traditional Foods Processed from Fermented Cassava" was set up in 1990 in Central Africa and South America. Our laboratory was to describe the mechanisms of root transformation during retting with a view to optimizing product quality and fermentation speed.

In this paper, we present the main results obtained during this EU program, describe the microbiological and biochemical evolution throughout the process, and define the origin (vegetal or microbial) of the main enzymes.

Material and Methods

Origin of plant material

Cassava roots (*Manihot esculenta* var. MM 86, or 'Ngansa') were harvested near Brazzaville, Congo, 18 months after planting.

Retting procedures

About 100 kg of washed and peeled roots were placed in a barrel and the volume made up to 50 L with rain water. A second barrel, filled only

with rain water, was used as control for physicochemical measurements ($T^{\circ}\text{C}$, pH, $p\text{O}_2$). Samples were taken every 12 h for the first 2 days and then every 24 h until retting was completed.

Sample preparation for bacterial enumeration

Sampling was carried out by randomly selecting six root sections, which were then cut into 0.5-cm cubes and mixed under sterile conditions. Of this mixture, 60 g were extracted and diluted in 540 ml of sterile, peptonized water (dilution 10^{-1}). The solution was then mixed in a Blendor (Turnmix ME 88, SOFRACA, France) and serially diluted in sterile, peptonized water for aerobic counts and in anaerobic Hungate tubes containing sterile, reduced water, flushed with 20% CO_2 and 80% N_2 for anaerobic counts.

Methods of bacterial quantification

Two types of enumeration were performed: "most probable number" (MPN) enumeration and plate counts on solid medium. The MPN method was used to either ascertain the growth of fermentative and pectinolytic bacteria or count the metabolites produced during growth on appropriate media for anaerobic, lactate-using bacteria. For each MPN determination, four successive dilutions of root samples were inoculated in three or four tubes per dilution. Results were calculated according to the McCready tables (McCready, 1918).

For plate counts, 0.1 ml samples of appropriate dilutions were inoculated in triplicate on agar medium in plates. All the plates were incubated at 30°C and the number of colony-forming units determined after 48 or 72 h of incubation.

Bacterial enumeration

Lactic acid bacteria (l.a.b.).

The l.a.b. were enumerated on MRS agar medium (de Man et al., 1960), supplemented with 0.1% of aniline blue. In each petri dish, 0.1 ml of appropriate root sample dilution was covered with medium and kept at 45°C . Enumeration was carried out after a 48-h incubation at 30°C . Subcultures were further purified by repeated plating.

Strains were differentiated into various bacterial groups by the following tests: microscopy examination, gram reaction, catalase test, and oxygen metabolism (fermentative or oxidative) test in soft MRS agar. Strains which were gram positive, catalase and oxidase negative, nonmotile rods or cocci, and colored by aniline blue were considered as lactic bacteria.

Glucose- and lactate-fermenting bacteria.

These bacteria (g.f.b. and l.f.b., respectively) were enumerated on a basal medium that contained the equivalent of 2 g/L glucose or 5 g/L of lactate (used as a carbohydrate source); 0.5 g/L of trypticase and yeast extract; 0.5 g/L of cysteine HCl (used as a reductive agent); 0.1 g/L of sodium acetate; 0.005 g/L of resazurine; 20 ml of Widdel mineral solution (Widdel and Pfennig, 1984); and 1 ml of Widdel trace element solution (Widdel and Pfennig, 1984).

The Hungate technique (Hungate, 1969), modified for using syringes (Macy et al., 1972), was used throughout the study. After boiling, the medium was cooled under a continuous flow of oxygen-free N_2 , adjusted to a pH of 7.2 with NaOH solution, and distributed anaerobically into Hungate tubes. The medium was sterilized for 35 min at 110°C . Before inoculation, 1% of $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (5%) was added as a

reductive agent to each tube. Inoculations were performed with syringes filled with oxygen-free N_2 , using a gas manifold.

Yeast. A potato-dextrose agar medium (PDA, DIFCO Laboratory) was prepared, containing 0.05 g/L of chloramphenicol and with a final pH of 3.5, adjusted with tartaric acid (10%). The agar's surface was then dried. From an appropriate microbial dilution, 0.1 ml was spread, in triplicate, on plates containing the medium. The plates were then incubated for 72 h at 30 °C. Subcultures were further purified by repeated plating on PDA. Isolates were characterized to the genus level, and Api tests (API 5030 strips Biomerieux, France) were used to determine fermentation carbohydrate sources.

Physicochemical parameters

Penetrometry index.

Penetrometry was used to indicate root softening during retting. A previous study showed that a penetrometry index of 15 mm/5 s corresponded to the end of retting as it is traditionally evaluated (Brauman et al., n.d.). A penetrometer (PNR 10-SUR, Berlin) was used to measure the consistency of the roots. Every 10 h, and for each experiment, six root sections were randomly chosen. Penetrometry depth was estimated with six repetitions for each root section.

The pH and partial oxygen pressure of the retting juice. Every 10 h, 50 ml of retting juice was extracted to test the pH (measured with CG 838 pH-meter from SCHOTT Geräte, Germany) and estimate partial oxygen pressure (measured with OXI 91 from WTW, Germany).

The pH and partial oxygen pressure of the roots. A 20-g

sample was added to a Waring blender and mixed with 120 ml distilled water at low speed for 15 s and at high speed for 1 min. The mixture was then filtered through a GF/A filter and the volume made up to 200 ml with distilled water. Extracts were taken in duplicate at 0 h, 48 h, and at the end of retting. Acidity was titrated with 0.01 M NaOH.

Biochemical analysis

Enzyme assays. A sample of 40 g of cassava mash was added to a Waring blender, together with 80 ml of 0.1 M citrate buffer (pH = 6.5) and the mixture homogenized. The mixture was held overnight at 4 °C and centrifuged at 12,000 g for 30 min. The supernatant was lyophilized and resuspended in 1/10 volume of citrate buffer.

β -glucosidase activity. This was measured with a chromogen, *p*-nitrophenol- β -D-glucopyranoside, at 20 mM in 0.1 M of Na-phosphate buffer (pH = 6.8) for 1 h at 25 °C. The reaction was stopped by adding an equal volume of 0.2 M sodium borate (pH = 9.8), and *p*-nitrophenol was determined with a spectrophotometer at 400 nm (Hosel and Bartz, 1975).

Linamarase. This was assayed with linamarin as substrate and by measuring the appearance of CN^- (Giraud et al., 1992). To 400 μ l of extract, 100 μ l of 50 mM linamarin in 0.1 M citrate buffer (pH = 6.0) were added. At regular intervals, 50 μ l aliquots were added to 50 μ l of 0.1 M NaOH to stop the reaction, and stored at 4 °C. Cyanide was liberated by adding 50 μ l of 0.1 M H_2SO_4 and 850 μ l distilled water to each aliquot, and was measured with a spectroquant kit (Merck, Darmstadt, Germany). One unit of linamarase was defined as the

amount of enzyme that released 1 μmol of CN^- per minute.

Activity of pectinesterase (PE; pectin pectylhydrolase, EC 3.1.1.11). This was assayed by titrating 1 ml of extract in 1% pectin at 30 °C (Grindsted RS400-DM 74%), and in 0.1 M NaCl and 1 mM NaN_3 . pH was increased to 7.0 with 0.01 M NaOH. One unit corresponds to the neutralization of 1 μmol of COO^-/min .

Polygalacturonate lyase (PGL) activity. PGL activity was assayed by the Starr et al. (1977) procedure. This assay does not differentiate between endo-PGL (poly (1,4- α -D-galacturonide) lyase, EC 4.2.2.2) and exo-PGL (poly (1,4- α -D-galacturonide) exolyase, EC 4.2.2.9). One unit of PGL corresponds to the formation of 1 μmol of one unsaturated bond in galacturonide between C4 and C5.

Polygalacturonase (PG; poly (1,4- α -D-galacturonide) glycanohydrolase, EC 3.2.1.15). This was assayed by viscometry. To 40 ml of 1% pectin in 100 mM of acetate buffer (pH = 4.7), 0.5 ml of extract was added. The rate of reduction in viscosity was measured at 25 °C in a viscometer (Haake model; VT 500, rotation: 150.93 s^{-1} and system MV-MV1). One unit corresponds to the release of 1 μmol of hexose/min. Total activities are expressed as units per 100 g of cassava.

Action of pectic enzymes in vivo. Sterilized slices of cassava were inoculated with 50 μl of enzyme extract or 5 μl of purified pectolytic enzymes (endopolygalacturonase P-5146 from *Aspergillus niger*, pectolyase P-3026 from *A. japonicum*; and pectinesterase P-0764 from orange peel) (Sigma, Saint-Quentin Fallavier, France). The inoculated slices were placed in sterile beakers containing 10 ml of 0.01 M of citrate

buffer (pH = 5.0). Penetrometer readings were estimated after 24 h and 48 h at 30 °C.

Cellulase, amylase, and xylanase activities. These activities were also assayed at 37 °C and pH of 5.8, using the Somogyi procedure (Somogyi, 1945). The substrates were microcrystalline cellulose (100 mg) and xylan (18 mg/ml).

Other analytical methods

Total and free cyanides were assayed by the Cooke et al. method (1978). Protein was determined with a modified Lowry procedure (Bensadoun and Weinstein, 1976).

Organic compounds

Sugars, volatile fatty acids (VFA), and lactate and ethanol concentrations in the roots were determined by high-performance liquid chromatography (HPLC) of the supernatant, as described by Giraud et al. (1991). The resulting columns (BioRad Laboratories, Richmond, California) were:

- (1) Fast carbohydrate column for monosugars analysis (100 x 7, 8 min) with 0.6 ml flow of milliQ water (pH = 6.0) at 70 °C;
- (2) Aminex HP 42 A (300 x 7.8 min Biorad) for polyosides analysis with 0.3 ml flow of milliQ water (pH = 6.0) at 70 °C;
- (3) Aminex HP x 87H column with 0.8 ml/min flow of H_2SO_4 6 mM at 60 °C.

Results and Discussion

Kinetic studies of retting

We now present the results of our global study of lactic fermentation. Kinetic parameters such as total and fermentative microflora, physicochemical parameters, and

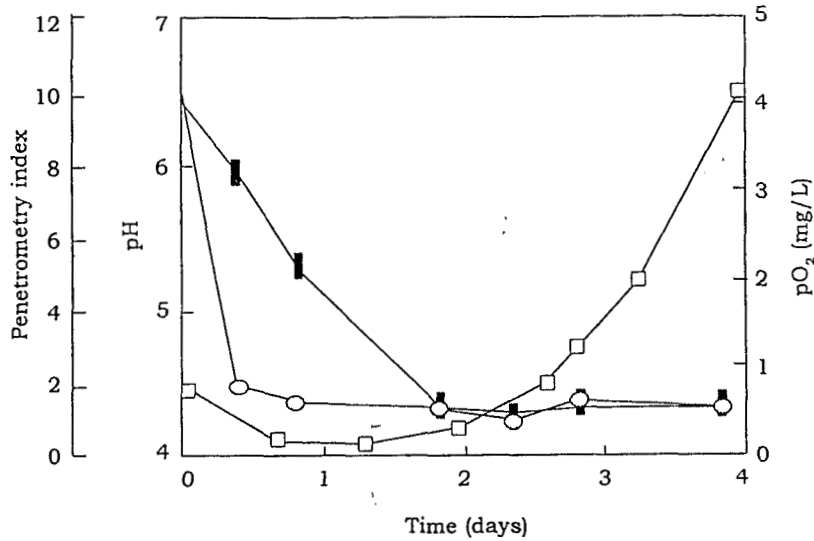


Figure 1. The evolution of physicochemical parameters during retting. (■ = pH; ○ = pO₂; □ = penetrometry index.)

substrates and metabolites produced have been measured throughout the process. These results are the mean of seven rettings performed in barrels under the same conditions.

Evolution of physicochemical parameters

The main physicochemical parameters were assayed throughout the process (Figure 1). The partial oxygen pressure dropped to well below 1 mg/L after 10 h and the pH became stable (at 4.5) within 48 h. Conversely, root softening, indicated by the penetrometry index, appeared after 2 days of fermentation and evolved exponentially. This process seems to require anaerobic and acidic conditions to proceed. Microscopic examination shows that the cassava cell walls were extensively disrupted at the end of the process, demonstrating the attack of depolymerizing enzymes.

The concentration of endogenous cyanogenic compounds decreased from 300 mg/kg as HCN (dry matter

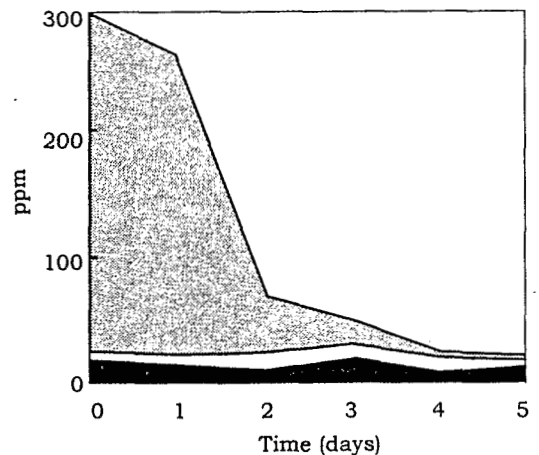


Figure 2. Total cyanide evolution. (□ = linamarin; □ = cyanhydrines + free cyanides; ■ = free cyanides.)

basis) in fresh cassava to 20 in the fermented mash (Figure 2). In all assays, total cyanogens were almost eliminated (90%). These results demonstrated that, under the standard conditions of local transformations in Central Africa, detoxification occurred normally without need of an additional process.

Evolution of substrates and metabolites

The main substrates degraded (Figure 3) were oligosaccharides (fructose, glucose, and saccharose). The low level of polyosides generated by starch degradation (e.g., maltotriose and maltose) underline the weak degradation of the starchy mass during retting. Saccharose seems to be the main substrate degraded by the fermentative microflora.

The main organic acid produced was lactate. However, significant levels of ethanol, acetate, and butyrate were also found (Figure 4). They seem to be generated mostly by the heterolactic fermentation of the oligosaccharides present in the cassava roots, except for butyrate, which could have come from an anaerobic fermentation mediated by *Clostridium* species. Butyrate concentration could vary from 0.4 to 2.5 g/100 of dry matter in different fermentations carried out under the

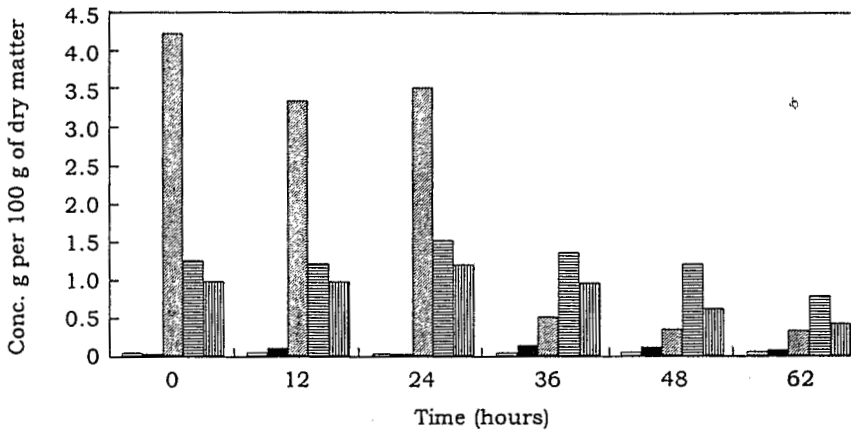


Figure 3. Oligo- and monosaccharide evolution during retting. (□ = maltotriose; ■ = maltose; ▣ = saccharose; ▤ = glucose; ▥ = fructose.)

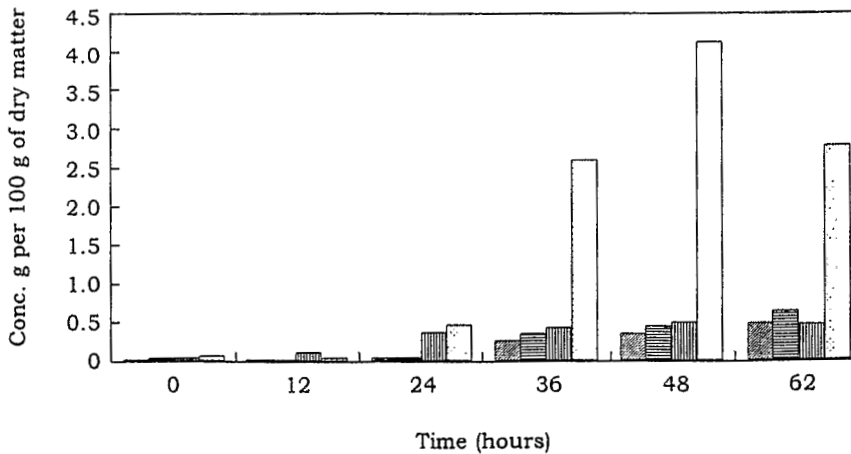


Figure 4. Organic acids and alcohol evolution during retting. (▧ = butyrate; ▨ = ethanol; ▩ = acetate; □ = lactate.)

same conditions. Because of their organoleptic qualities, butyrate and lactate seem to be the most typical products of this process.

Microflora evolution

Fermentative and lactic microflora. In the enumerations, only fermentative bacteria were counted because retting was seen as largely anaerobic (Figure 1). The fermentative microflora evolved during the first 2 days of fermentation and remained stable to the end. The total fermentative microflora represented by the glucose-fermenting bacteria was dense, reaching 10^{12} b/g after 48 h of fermentation. The next most predominant flora were the l.a.b. (Figure 5), reaching 10^4 to 10^8 b/g of DM on fresh roots. The variation of endogenous l.a.b., composed mainly of *Lactococcus* and heterolactic *Lactobacillus* species, did not influence the evolution of l.a.b. during fermentation.

Lactate-fermenting bacteria.

One metabolite formed during fermentation is butyrate (Figure 4). This compound is a typical product of carbohydrate fermentation by

anaerobic spore formers (*Clostridium* species). To evaluate this population, enumeration was done anaerobically on lactate because (1) lactate is the major substrate found in retting; and (2) it is not used as a substrate by the l.a.b. Surprisingly, the results of this enumeration showed that the population of lactate-fermenting bacteria remained constant and at low levels (10^3 b/g of DM) throughout the retting (Figure 5). The presence of butyrate and acetate in the positive tubes, and the isolation of strictly anaerobic, sporulating, gram-positive rods with the same fermentation pattern as *Clostridium butyricum*, confirmed that *Clostridium* species are present in retting. However, their role in the process remains to be studied because of their reduced numbers in the enumeration and lactate does not seem to be their natural substrate in retting.

Yeasts. The only flora that appeared after 48 h of fermentation and still developed until the end of retting were yeasts. Their metabolisms allow them to grow at the low pH imposed by the l.a.b. Their numbers remained low during the fermentation (about 10^3 b/g of

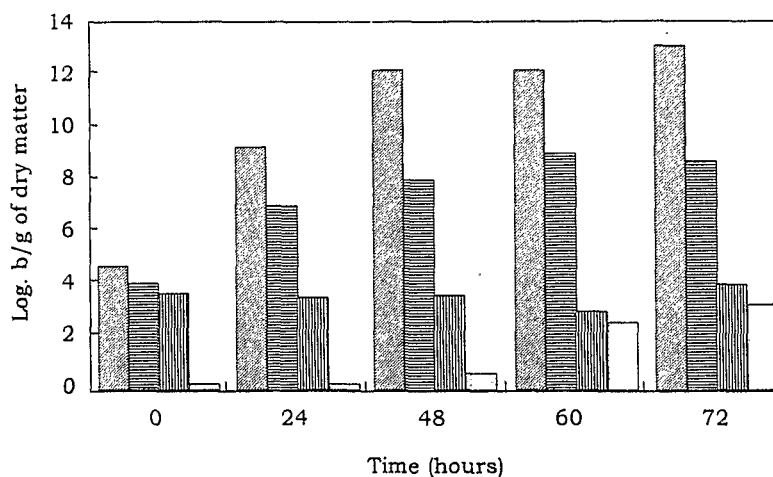


Figure 5. Evolution of fermentative microflora during retting. (■ = glucose-fermenting bacteria; ▨ = lactic acid bacteria; ▩ = lactate-fermenting bacteria; □ = yeast.)

DM), suggesting that they do not play a significant role in retting. When the retting finished, the yeasts covered the entire water surface and became the main flora of the postretting stage. Their increasing numbers at the end of the process (mostly *Candida* species) may therefore influence the conservation of end products.

Origin of enzymes involved in retting. The main enzymes found in this process were pectinase and linamarase, and to a lesser extent, amylase (data not shown). No cellulase or xylanase activities were found in retting. To elucidate the origin of cyanogen elimination and the mechanism of root softening, two fermentations were carried out simultaneously: one "natural," used as a control (CF), and one sterile (SF). pH and oxygen pressure of SF were set on those of CF. Pectinase and linamarase activities were assayed throughout the experiment. For SF, cassava roots were sterilized with HgCl_2 and soaked in sterile water.

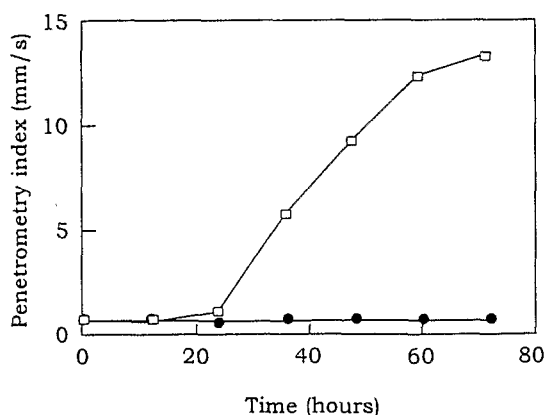


Figure 6. Comparative evolution of softening between a sterile (●) and a natural retting (□).

Origin of softening. No softening was obtained in sterile fermentation (Figure 6). High endogenous pectin methyl esterase activities were found in cassava extracts from both fermentations (Figure 7). Depolymerizing enzymes, endopolygalacturonase (active at low pH), and pectate lyase were found only in the "natural" fermentation (Figures 8 and 9). No other depolymerizing enzymes, such as cellulase or xylanase, nor other hydrolases were found. Moreover, softening could be performed by inoculating commercial pectinesterase and depolymerizing pectolytic enzymes on fresh and sterile cassava roots.

We suggest, therefore, that root softening is a result of the combined action of both endogenous pectin methyl esterase and exogenous bacterial depolymerizing enzymes. But further studies are needed to show the precise contribution of each pectic enzyme to root softening.

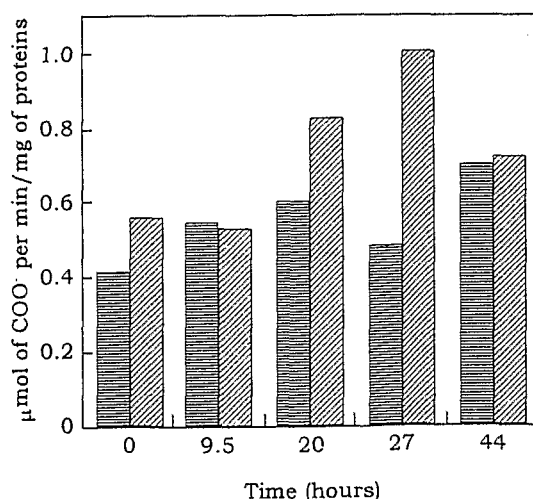


Figure 7. Pectinesterase activity during retting. (■ = sterile fermentation; ▨ = control fermentation.)

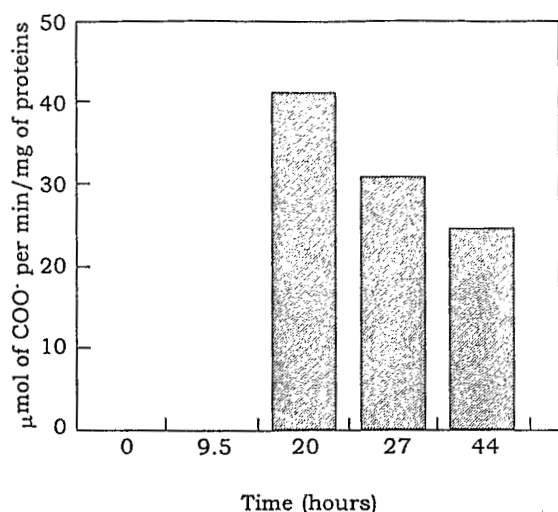


Figure 8. Pectate lyase activity during "natural" fermentation.

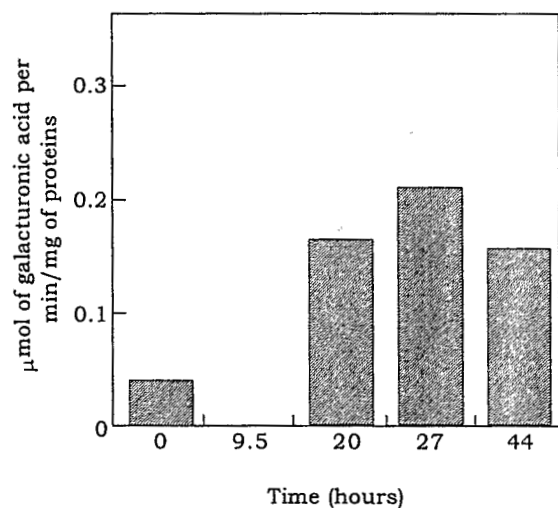


Figure 9. Endopolygalacturonase activity during "natural" fermentation.

Origin of cyanogen elimination.

Of total cyanogenic compounds, 50% were eliminated in SF and 97% in CF (Figure 10). Enzyme assays further confirmed endogenous linamarase activity (Table 1). Linamarase activity (measured as β -glucosidase activity) in CF was significant in fresh roots (specific activity 9.4 units/mg protein). This total activity then decreased after a few hours. In SF, total activity remained constant, but at a low level. The difference in β -glucosidase activity in the fresh roots between SF and CF may be attributed to the inhibitory effect of the HgCl_2 used to sterilize the roots. However, as nearly 25% (Table 1) of the total β -glucosidase activity present in the sterile roots can degrade more than 50% of the total cyanide content of the fresh roots, we can assume that the level of linamarase activity present in the intact roots was sufficient to detoxify the roots.

Origin of the amylolytic activity.

The amylase activity remained constant in SF, but disappeared after 36 h of fermentation in CF (Figure 11). Our data suggest that the amylase activity detected in retting does not have a bacterial origin as suggested by different authors (Collard and Levi, 1959; Oyewole and Odunfa, 1992; Regez et al., 1987).

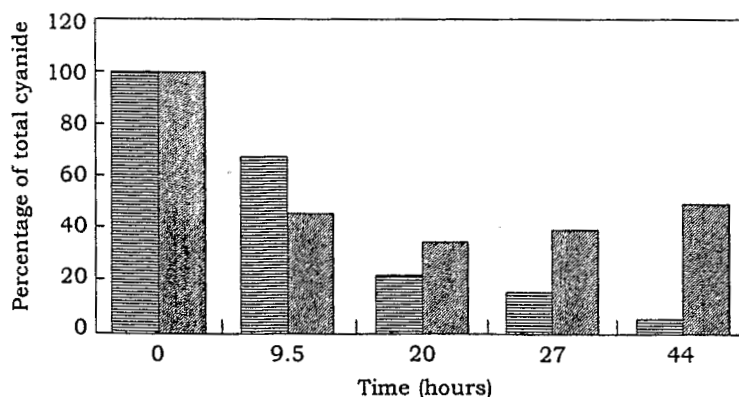


Figure 10. Total cyanide evolution in control (▨) and sterile (■) fermentations.

Table 1. β -glucosidase activities in control and sterile fermentations. (Activities are expressed in mmol per min/100 g of dry matter).

Time (h)	Fermentation	
	Control	Sterile
0	9.12	2.15
9.5	5.58	2.55
20.0	6.10	1.75
27.0	7.68	2.30
44.0	7.24	1.38

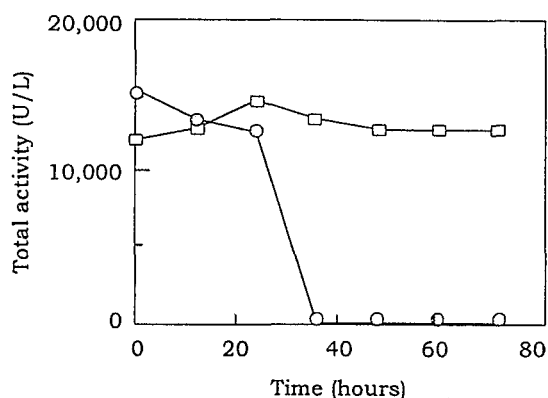


Figure 11. Amylase activity in control (○) and sterile (□) fermentations.

Conclusions

These results suggest that retting is a complex heterolactic fermentation, with an interaction between lactic bacteria, *Clostridium* species, and possibly *Bacillus* species. Heterolactic bacteria (such as *Leuconostoc mesenteroides*) are the most important and numerous microflora in the process; they are responsible for the physicochemical properties of retting (e.g., pO_2 and pH) and the production of the main organic acids (acetate and lactate). *Clostridium* species seem to be involved in butyrate formation, which is essential for the organoleptic properties of the final products. Moreover, recent

results (S. Kéléke, 1994, personal communication) suggest that *Clostridium* species (such as *Clostridium butyricum*) could be involved with *Bacillus* species (such as *Bacillus polymyxa*) in root softening as pectinase producers. We did not see any involvement of *Geotrichum* spp. or *Corynebacterium* spp., as have other authors (Collard and Levi, 1959; Okafor et al., 1984; Regez et al., 1987). Yeasts (mostly *Candida* species) were more involved in postretting.

Our biochemical analyses showed that retting is a fermentation in which both endogenous and microbial enzymes coact to soften the roots and degrade cyanogenic, endogenous compounds. Our results suggested that cell-wall degradation is initiated by endogenous pectinesterase, located in intercellular spaces and released by pH decrease. This is followed by microbial polygalacturonase and lyase depolymerizing pectic chains. The presence of pectic enzymes in cassava retting has previously been reported (Okafor et al., 1984; Oyewole and Odunfa, 1992). But this work gives the first evidence of the vegetal origin of pectinesterase and of the in vivo activity of depolymerizing enzymes.

The amylase activity measured in retting seems to be of vegetal origin. But its low level of activity and disappearance within the first 30 h of retting suggest that it is not important to the retting process.

Results of cyanide measurements indicate that endogenous linamarase (measured as β -glucosidase activity) is the main enzyme responsible for detoxification. We can assume, as Maduagwu (1983) suggested, that the level of linamarase activity present in intact roots is sufficient to detoxify them of their cyanogen content without help from any microbial linamarase. Nevertheless, if bacteria

do not directly detoxify cassava roots, they could help degrade linamarin by destroying cell walls.

Findings from our study have helped other researchers:

- (1) Isolate and characterize the first amylolytic *Lactobacillus plantarum* (strain A6) (Giraud et al., 1991);
- (2) Improve *fufu* processing by significantly reducing retting time, and increase the organoleptic qualities of the final product (Ampe et al., 1994);
- (3) Adapt the process for areas with low water availability (Miambi et al., n.d.).

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