Session 4:

Bioconversion and Byproduct Use

Chapter 22

Fermentation in Cassava Bioconversion[1]

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Introduction

Cassava fermentation is traditionally practiced in the tropics. But both technology and product characteristics differ according to region and sociocultural conditions: *gari* in East and West Africa, *chikwangue* or *fufu* in Central Africa, and sour starch in Latin America. But they have in common the aim to eliminate the poisonous cyanide components and conserve cassava by lactic acidification.

The essential role of lactic acid bacteria in the three products was demonstrated by studies carried out by the Institut français de recherche scientifique pour le développement en coopération (ORSTOM) through the STD2 Program of the European Union (EU), otherwise known as "Improving the Quality of Traditional Foods Processed from Fermented Cassava" (Raimbault, 1992; Saucedo et al., 1990).

When producing *gari*, lactic acidification of cassava is rapid and detoxification is sometimes incomplete. Controlling through inoculation would improve quality. For *fufu* or *chikwangue*, retting is essential for texturing and detoxifying the cassava. Lactic acid fermentation is heterolactic, operating in association with secondary alcoholic and anaerobic fermentation to produce alcohol and organic acids such as butyrate, acetate, and propionate that develop special aromatic and organoleptic characteristics. As for *gari*, fermentation for sour starch (especially in Colombia and Brazil) is homolactic, but takes 3 or 4 weeks. Amylolytic lactic acid bacteria have been isolated from *chikwangue* by ORSTOM scientists and from sour starch by CIRAD scientists.

A. Brauman isolated a new strain, *Lactobacillus plantarum* A6, which was described by Giraud et al. (1991). Its physiological and enzymological characteristics for cultivation on cassava starch media, amylase production, and biochemical

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1. No abstract was provided by the authors.

properties have now been described (Giraud et al., 1992; 1993a; 1993b).

ORSTOM scientists have been researching solid fermentation cultivation of fungi on cassava and amylaceous components for more than 10 years. Soccol et al. (1994) showed that protein enrichment is possible by cultivating various strains of *Rhizopus*, even on crude, nongelatinized cassava flours. Saucedo et al. (1992a; 1992b; 1992c) studied, at the ORSTOM Laboratory, Montpellier, the growth and alcohol fermentation of cassava starch in solid-state fermentation, using a highly promising amylolytic yeast.

Swedish and African researchers have described the beneficial effects of lactic acid fermentation on the prophylactic and keeping characteristics of those traditional foodstuffs made from fermented cassava, maize, and mixed cereals, and of baby foods. These foods tend to increase children's resistance to diarrhoea.

All these studies are being continued in new projects comprising the EU-STD3 Program. Other EU studies are being conducted on cassava quality, environment, physical processing, and transformation at a low industrial scale to take advantage of the economic and commercial opportunities in Latin America.

Solid-State Fermentation of Cassava and Starchy Products

For more than 15 years, an ORSTOM group has worked on a solid-state fermentation process for improving the protein content of cassava, potatoes, bananas, and other starchy commodities used for animal feed. Fungi, especially from the *Aspergillus* group, are used to transform starch and mineral salts into fungal proteins (Oriol et al., 1988a; 1988b; Raimbault and Alazard, 1980; Raimbault and Viniegra, 1991; Raimbault et al., 1985). Table 1 shows the overall changes in composition between the initial substrate and final products. Through such techniques a cassava-fermented product with an 18%-20% protein content (dry matter basis) was obtained.

More recently, Soccol et al. (1993a; 1993b), also at the ORSTOM Laboratory, obtained good results with the *Rhizopus* fungi, of special interest in traditionally fermented foods. In particular, they studied the effect of cooking before fermentation on the availability of starch, protein content, and the rate of starch's bioconversion into protein (Table 2). They found that a selected strain of *Rhizopus oryzae* could transform uncooked cassava, which contains only 1.68% protein, into a fermented cassava containing 10.89% protein.

Table 1. Effects of *Aspergillus niger* on protein and sugar contents of different starches (percentage of dry matter) after 30 h of fermentation in solid-state culture.

Substrate	Initial composition		Final composition	
	Proteins	Sugar	Proteins	Sugar
Cassava	2.5	90	18	30
Banana	6.4	80	20	25
Banana waste	6.5	72	17	33
Potato	5.1	90	20	35
Potato waste	5.1	65	18	28

Table 2. Growth of *Rhizopus oryzae* in solid-state cultivation on cassava granules after various cooking treatments.

Treatment[a]	Dry matter[b]		Total sugar[c]		Proteins[c]	
	Initial	Final	Initial	Final	Initial	Final
I	60.90	46.48	80.01	46.78	1.20	11.69
II	59.18	45.35	84.11	60.72	1.61	12.40
III	57.95	42.12	82.44	52.57	1.56	13.93
IV	55.63	43.88	82.49	56.62	1.47	11.89
V	45.57	37.88	82.04	56.62	1.68	10.89

a. Treatment:

I = Cassava autoclaved for 30 min at 120 °C, frozen, dried, and ground

II = Cassava flour (40% water) autoclaved for 30 min at 120 °C

III = Cassava flour (30% water) autoclaved for 30 min at 120 °C

IV = Cassava flour (30% water) vapor cooked for 30 min at 100 °C

V = Untreated crude cassava flour

b. g/100 g total weight.

c. g/100 g dry matter.

SOURCE: Soccol et al., 1994.

Table 3 shows results of amylase biosynthesis in solid or liquid culture, using raw or cooked cassava. The amount of glucoamylase was 10 to 15 times higher in solid than in liquid culture, and higher in raw starch medium than in cooked cassava.

This work is being continued in the EU-STD3 Program at the Bioconversion Laboratory of the Universidad del Valle, Cali, Colombia. It focuses on simplifying cassava processing by learning more about the specificity of *Rhizopus* strains in degrading the raw starch granule. But clean flours of raw cassava are needed. The common flours of cassava contain too much natural microflora to allow microbial studies with fungi; they must first be sterilized and (unfortunately) gelatinized. Ramírez et al. (1994) developed raw cassava flour with a very low content of bacteria and fungi, and little gelatinization.

To measure gelatinization, the simple method of Wotton et al. (1971) was adopted and a good correlation coefficient for the calibration curve was obtained. Table 4 shows the effect of thermic treatment and microwaves on starch gelatinization in cassava flour (water content typically lower than 10%). Where water content was very low, gelatinization was also low.

The same thermic treatment of dry cassava flour eliminated the natural microflora contained in raw flour, from 10^9 bacteria/g of dry flour to fewer than 10^3 bacteria/g after heating the flour for 30 min at 90 °C. With gelatinization limited to less than 5% under such conditions, obtaining clean, raw cassava flour is possible in the laboratory.

Figures 1 and 2 show the effects of various physical and thermic treatments on the bacteria content of cassava flour. Cassava flour will be used as a solid substrate for cultivating *Rhizopus* strains, and to compare the capacity of selected strains to grow on raw or gelatinized cassava starch.

Table 3. Effect of cooking and type of culture on the growth and amylases of various strains of *Rhizopus oryzae* cultivated on cassava granules.

Strain of *Rhizopus*	Liquid-state culture[a]						Solid-state culture[a]					
	Raw cassava			Cooked cassava			Raw cassava			Cooked cassava		
	α–amylase (U/g DM)	Gluco-amylase (U/g DM)	Protein (g/100 g DM)	α–amylase (U/g DM)	Gluco-amylase (U/g DM)	Protein (g/100 g DM)	α–amylase (U/g DM)	Gluco-amylase (U/g DM)	Protein (g/100 g DM)	α–amylase (U/g DM)	Gluco-amylase (U/g DM)	Protein (g/100 g DM)
28168	42.20	9.60	3.90	157.20	3.10	10.00	39.30	55.30	10.60	178.40	46.22	12.30
34612	40.40	7.30	4.60	168.50	5.70	9.30	55.00	70.00	12.60	170.00	47.00	14.10
28627	76.00	7.80	4.00	145.40	3.30	9.60	98.00	108.00	11.40	167.00	37.00	13.80

a. DM = dry matter; U = enzyme units.

SOURCE: Soccol et al., 1994.

Table 4. Effect of temperature and microwaves on starch gelatinization of cassava flour.

Temperature	Time (min)	Gelatinization rate (%)[a]			
		Exp. 1	Exp. 2	Exp. 3	Mean
Test 1 (80% gel.)		75.439	84.063	88.911	82.80
Test 2 (20% gel.)		25.411	26.184	29.702	27.10
80 °C	60	3.529	3.444	2.714	3.23
85 °C	30	3.529	3.357	3.487	3.46
85 °C		3.444	3.486	3.444	3.46
90 °C	30	3.572	3.444	3.572	3.53
90 °C	60	9.454	9.064	9.107	9.21
95 °C	30	6.961	5.546	5.803	6.10
100 °C	30	4.965	4.602	4.001	4.52
105 °C	30	6.961	5.503	5.301	5.92
120 °C	30	4.816	4.730	4.473	4.67
140 °C	30	4.773	3.100	3.100	3.66
160 °C	30	3.529	3.487	4.301	3.77
Autoclaving (121 °C)	15	3.572	3.100	4.301	3.66
Microwaves (Pot. 70)	5	2.886	2.410	2.842	2.71
Microwaves (Pot. 100)	5	2.971	2.242	2.242	2.49
Microwaves (Pot. 30)	15	3.879	3.057	3.915	3.62

a. Exp. = Experiment. Mean is across the experiments.

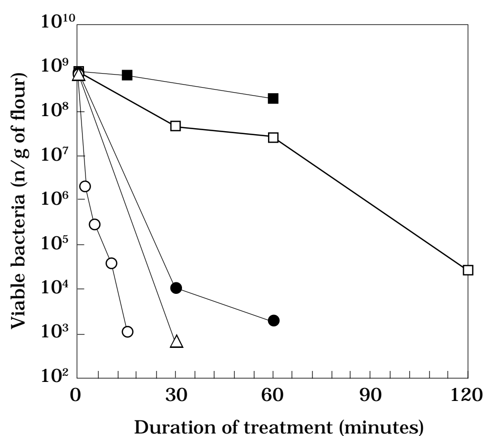

Figure 1. Total microflora (plate count analysis) in cassava flour, according to treatment. (■ = ultra-violet radiation; ○ = microwaves; □ = 80 °C; ● = 85 °C; △ = 90 °C.)

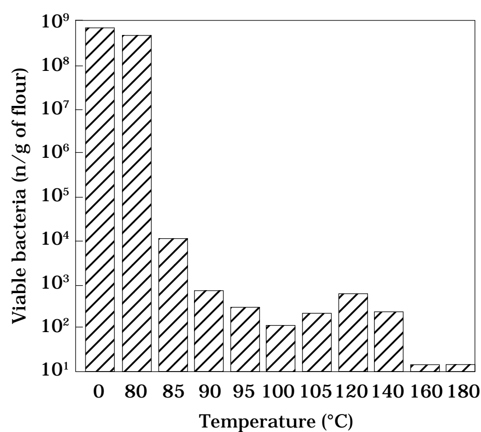

Figure 2. Effect of temperature on bacterial population in cassava flour.

Lactic Acid Fermentation of Cassava

Lactic acid fermentation is important for many traditional fermented foods, silage, and animal feed, and for recycling agroindustrial byproducts. Because of its acid, bacteriostatic, and bactericidal properties, fermentation prevents microorganisms, whether parasitic, saprophytic, or pathogenic, from breaking down vegetable material.

In tropical countries, lactic fermentation not only plays an important role in the traditional transformation of starchy foods, such as cassava, but also in the transformation and conservation of other foods, and fish and its byproducts. Two types of lactic fermentation exist:

(1) Homolactic, when more than 80% of total acidity and metabolites formed consists of lactic acid, and
(2) Heterolactic, when the percentage of acetic acid, propionic acid, and ethanol is more significant, and lactic acid represents 50%-80% of total acidity.

Lactic bacteria produce two types of lactic acid: L(+) and D(-). Only the L(+) form is assimilated by humans.

Previous studies, realized during the EU-STD2 Program in 1988-1991 (Raimbault, 1992), consisted of improving traditional fermented food made from cassava in Africa and Latin America. Three kinds of traditional foods were considered: *gari*, *chikwangue*, and sour starch. We demonstrated the essential role of lactic acid bacteria in all traditional processes.

Amylolytic lactic bacteria were isolated from fermented cassava. The first strain of *Lactobacillus plantarum* to be described as having very high amylolytic capacity was obtained from fermented cassava by A. Brauman in the Congo. Detailed physiological and biochemical characterization of this new strain is expected to be published soon by E. Giraud.

Mbugua and Njenga (1991) and Svanberg (1991a; 1991b), working in Tanzania and at the Uppsala University, respectively, have reported on the effect of lactic acid fermentation on the pathogen microflora content of traditional African foods.

Some of their results are reported in Table 5 and Figure 3, which show how lactic acid bacteria reduce the number of food-poisoning pathogens such as species of *Staphylococcus*, *Salmonella*, and *Shigella*, and *Escherichia coli*. High levels of such pathogens are sometimes found in traditional foods after processing under unhygienic conditions, especially those for malting maize during the rainy season in parts of tropical Africa.

Lactic fermentation of traditional foods reduces pathogenic bacteria from 10^8 to 10^3. The same authors also found a significant correlation between the resistance of young children to diarrhoea and eating acidified gruels.

We are bioconverting, through probiotics and bactericides, cassava flour and starch containing amylolytic lactic acid bacteria to isolate new strains from traditional foods. At the same time, we are broadening knowledge on the cultivation of lactic acid bacteria in starchy substrates. We hope such information will help elaborate new food and feed products.

Table 5. Effect of lactic acid fermentation on the content of pathogenic bacteria in traditional fermented foods in Africa.

Time (h)	Log number of bacteria/g food			
	Control	Nonfermented, acidified food	Fermented food	
			Flour (nonviable)	Gruel (viable)
Shigella flexneri				
0	6.8	6.7	6.4	6.0
3	6.6	5.8	5.1	4.0
7	7.0	4.2	5.5	3.3
24	7.0	4.1	3.7	2.7
Salmonella typhimurium				
0	8.5	8.1	8.3	7.7
3	8.0	6.7	6.0	7.1
7	7.9	5.3	4.4	6.3
24	8.9	4.0	2.0	2.0

SOURCE: Lorri and Svanberg, 1988.

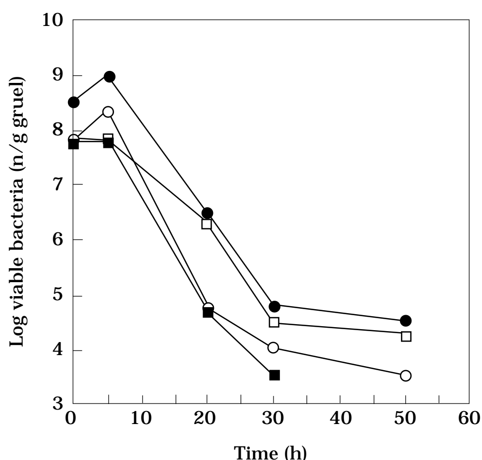

Figure 3. Evolution of pathogenic bacteria during the lactic fermentation of *uji*, a fermented cassava gruel (after Mbugua and Njenga, 1991). (□ = *Staphylococcus aureus*; ● = *Salmonella typhimurium*; ○ = *Escherichia coli*; ■ = *Shigella dysenteriae*.)

Alcoholic Fermentation of Cassava and Starch Products

Cassava is a potential producer of ethanol, considering its potentially high yields and low costs. Yet few reports concern the industrial application of cassava for ethanol production. This may be because, first, cassava cultivation yields relatively few, commercially significant byproducts, compared with, for example, sugarcane which yields enormous quantities of bagasse, a valuable source of energy for distillation. Second, cassava starch needs to be hydrolyzed into sugar for bioconversion into ethanol by the common *Saccharomyces cerevisiae*. This implies an additional, costly step.

For cassava to be an economically viable energy source, its processing costs must be reduced. Solid-state fermentation is one, simple, and new method of reducing costs: the use of an amylolytic yeast that eliminates hydrolysis.

At the ORSTOM Laboratory, Saucedo et al. (1992a; 1992c) developed a new process for the solid culture of an amylolytic yeast, *Schwanniomyces castelii* (Figure 4). The main advantage of this technique is its continuous recuperation of ethanol in a cold trap condenser. The gas produced in the reactor is pumped throughout the system, thus ensuring its continual removal from the medium

and limiting its toxic effects on the yeast's metabolism. The results obtained by Saucedo et al. (1992a; 1992b; 1992c) were promising, but the technology and feasibility of the process for commercial operation need further research.

Table 6 shows the results obtained by various authors on the potential of cassava as a substrate for ethanol production. The solid-state technique has to be carefully considered. Results obtained with the fungus *Rhizopus koji* are particularly significant. The potential of *Schwanniomyces* is also interesting because amylolytic yeast would be easier to control at the small-scale industrial level.

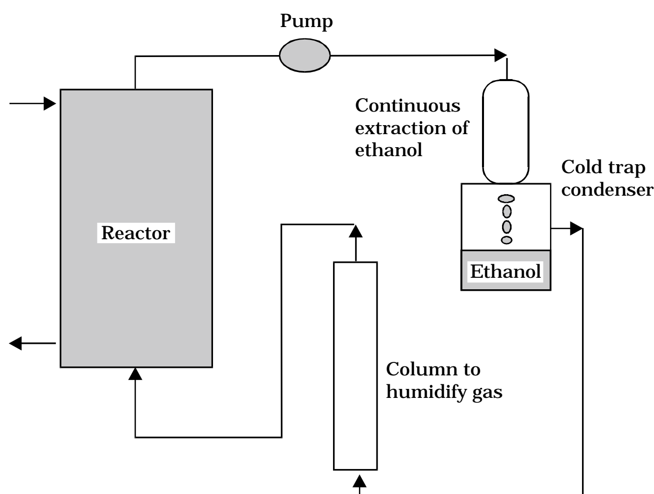

Figure 4. Producing ethanol through solid-substrate fermentation of cassava starch. The reactor contains a solid support impregnated with a starchy suspension and inoculated with the fermentation agent, an amylolytic yeast known as *Schwanniomyces castelii*. The resulting gas is pumped to a condenser where ethanol is extracted. The residual gas is sent to a humidifier.

Table 6. Comparison of various processes for ethanol production from cassava in liquid or solid substrate.

Process	Hydrolysis	Sugar (g/L)	Ethanol (g/L)	Recovered (g/L)	Theoretical (%)
Liquid substrate, using *S. cerevisiae* [a, b]	+	145	72.50	72.50	83.2
Solid substrate, using *S. cerevisiae* [b, c]	+	165	41.73	41.73	65.0
Solid substrate, using *Rhizopus koji* [d]	-	200	110.00	110.00	83.0
Solid substrate, using *Schw. castelii* [e, f]	-	300	68.40	212.60	64.0

a. Saraswati, 1988.
b. *S.* = *Saccharomyces*.
c. Jaleel et al., 1988.
d. Jujio et al., 1984.
e. Schw. = *Schwanniomyces*.
f. Saucedo et al., 1992a.

Conclusions on Bioconverting Cassava and Potential Products

To bioconvert cassava starch and flour to elaborate new products, ORSTOM, CIRAD, and collaborating institutes are emphasizing two approaches: solid-state fermentation, and lactic acid fermentation.

The first is of great interest because of its potential to simplify processes and reduce costs, and its large reactor volume. Both *Rhizopus* and *Schwanniomyces* (or other amylolytic) yeasts can be used in a solid-state cultivation process. This implies a three-phase reactor with a solid fiber support, a liquid phase containing the substrate in suspension and salts, and a gaseous phase for exchanging volatile components, that is, oxygen, water, and ethanol.

In lactic acid fermentation, we are investigating the culture control of amylolytic lactic acid bacteria in mixed and composite starters able to remain competitive in a natural, nonaxenic environment. The prophylactic role of lactic acid bacteria is also of great interest.

Finally, we are studying microorganisms able to degrade native cassava starches without need of gelatinization, as in in natural biotransformation and biodegradation. We will also study the amylolytic capacity of *Rhizopus* spp., yeasts, and lactic acid bacteria.

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Chapter 23

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 (C_2 to C_4), 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 (10^5-10^6 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 °C, pH, pO_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 Na_2S-$9H_2O$ (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 m*M* 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 µl of extract, 100 µl of 50 m*M* linamarin in 0.1 *M* citrate buffer (pH = 6.0) were added. At regular intervals, 50 µl aliquots were added to 50 µl of 0.1 *M* NaOH to stop the reaction, and stored at 4 °C. Cyanide was liberated by adding 50 µl of 0.1 *M* H_2SO_4 and 850 µl 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 m*M* 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 m*M* 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 m*M* 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

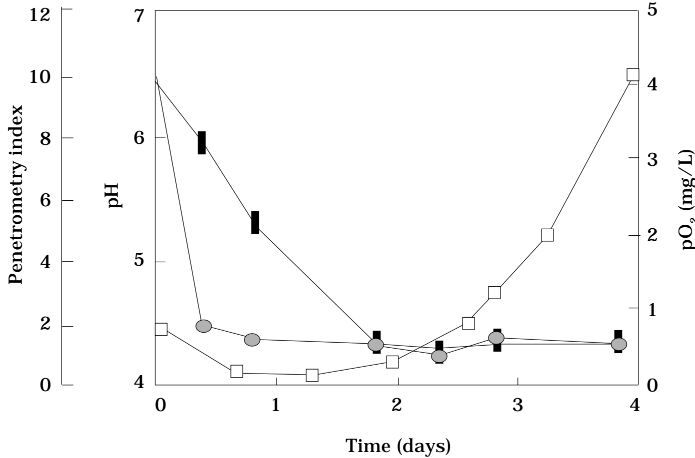

Figure 1. The evolution of physicochemical parameters during retting. (▌ = pH; ⬭ = pO_2; □ = penetrometry index.)

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 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.

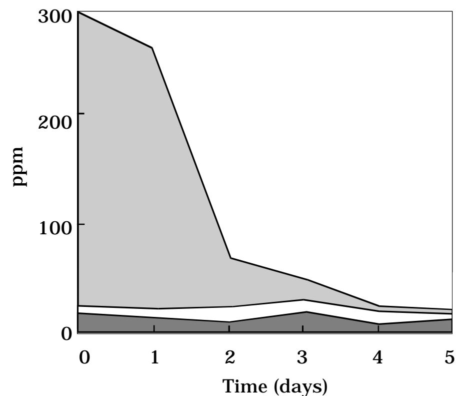

Figure 2. Total cyanide evolution. (□ = linamarin; □ = cyanhydrines + free cyanides; ■ = free cyanides.)

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

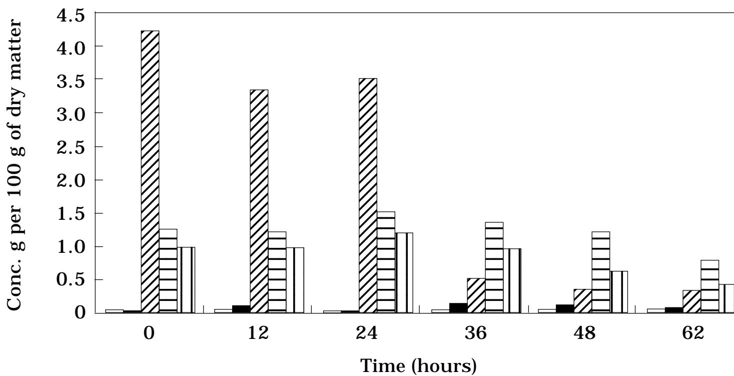

Figure 3. Oligo- and monosaccharide evolution during retting. (□ = maltotriose; ■ = maltose; ▨ = saccharose; ▤ = glucose; ▥ = fructose.)

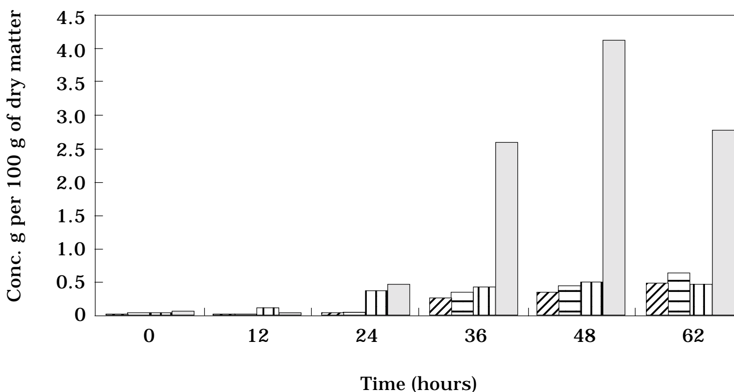

Figure 4. Organic acids and alcohol evolution during retting. (▨ = butyrate; ▤ = ethanol; ▥ = acetate; □ = lactate.)

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

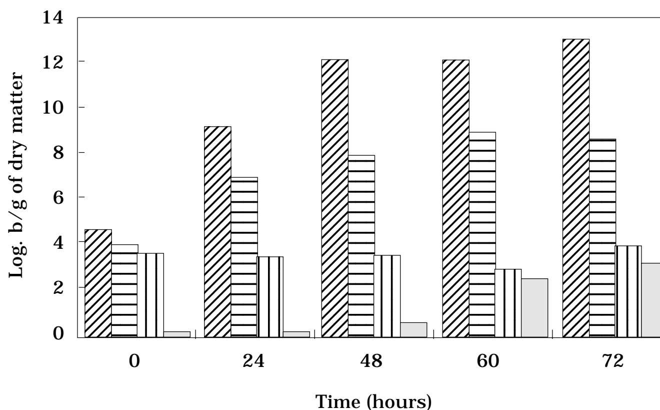

Figure 5. Evolution of fermentative microflora during retting. (= glucose-fermenting bacteria; = lactic acid bacteria; = lactate-fermenting bacteria; = yeast.)

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.

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.

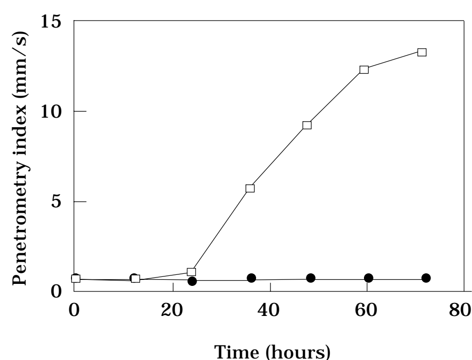

Figure 6. Comparative evolution of softening between a sterile (●) and a natural retting (□).

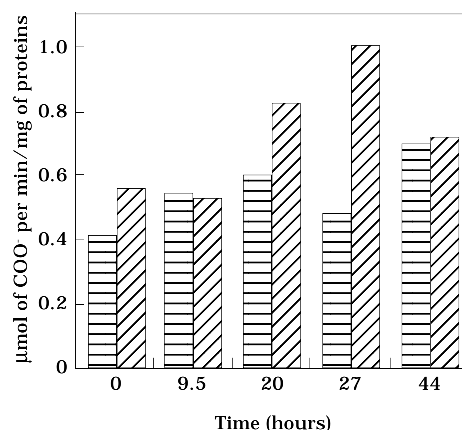

Figure 7. Pectinesterase activity during retting. (▤ = sterile fermentation; ▨ = control fermentation.)

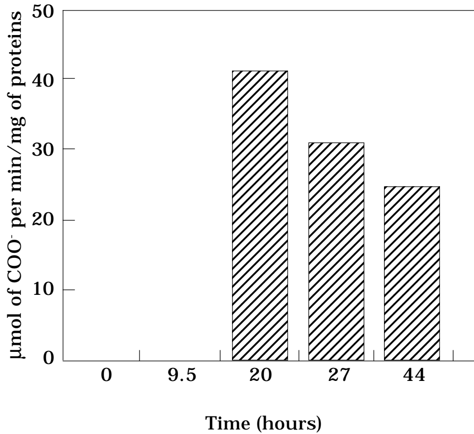

Figure 8. Pectate lyase activity during "natural" fermentation.

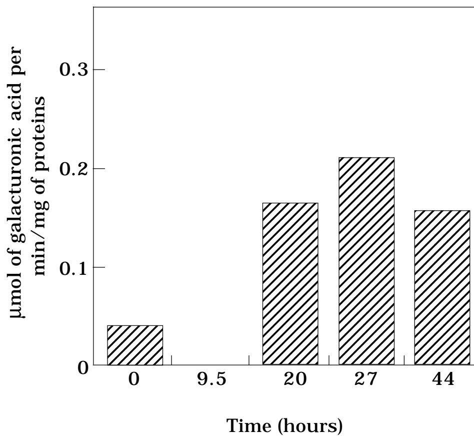

Figure 9. Endopolygalacturonase activity during "natural" fermentation.

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).

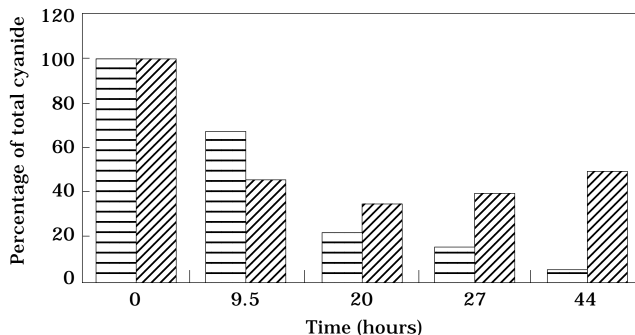

Figure 10. Total cyanide evolution in control (▤) and sterile (▨) fermentations.

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

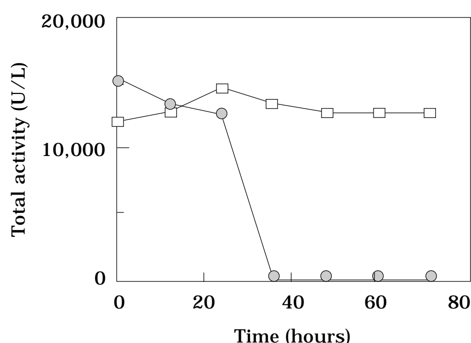

Figure 11. Amylase activity in control (○) and sterile (□) fermentations.

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 amylolitic *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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Chapter 24

A Lactic Acid Bacterium with Potential Application in Cassava Fermentation

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Abstract

An amylolytic lactic acid bacterium, identified as *Lactobacillus plantarum*, was isolated from cassava roots (*Manihot esculenta* var. Ngansa) during retting. Cultured on starch, the strain displayed a growth rate of 0.43 per hour, a biomass yield of 0.19 g/g, and a lactate yield of 0.81 g/g. The growth kinetics were similar on starch and glucose. Enough enzyme was synthesized, and starch hydrolysis was not a limiting factor for growth. The synthesized amylolytic enzyme was purified by fractionated precipitation with ammonium sulfate and by anion exchange chromatography. It was identified as an α-amylase with an optimal pH of 5.5 and an optimal temperature of 65 °C. The use of such a strain as a cassava fermentation starter for *gari* production had the following effects: a change from a heterofermentative pattern observed in natural fermentation to a homofermentation one, a lower final pH, a faster pH decline rate, and a greater production of lactic acid (50 g/kg of dry matter).

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Introduction

Lactic microflora play an important role in the preparation of traditional foods based on fermented cassava, such as *gari*, *chikwangue*, *fufu*, and sour starch. But this microflora's function in preserving foods, eliminating cyanogenic compounds, and improving organoleptic qualities is not yet clear. Traditional technologies are still used to manufacture these foods. As fermentation occurs naturally with lactic microflora, the quality of the food products is not uniform.

The mass inoculation of cassava roots with one or several selected strains would permit a better control over natural fermentation, thus resulting in a product of improved quality. Because cassava contains mainly starch (more than 80% of dry matter), the selection of a lactic acid bacterium capable of metabolizing starch (i.e., amylolytic) is essential.

But few lactic acid bacteria can convert starch into lactic acid. Examples of amylolytic lactic acid bacteria are *Streptococcus bovis*, *S. equinus*, *Lactobacillus amylophilus*, *L. amylovorus*, *L. acidophilus*, *L. cellobiosus*, and others isolated from animal digestive tracts and plant wastes (Champ et al., 1983; Cotta, 1988; Nakaruma, 1981; Nakaruma and Crowell, 1979; Sen and

Chakrabarty, 1986; Sneath, 1986). Almost no information exists on the physiology of these microorganisms.

Below we describe how we isolated and identified a new amylolytic lactic acid bacterium from fermenting cassava roots. We also investigated the physiology of this bacterium and the properties of the amylase produced.

Methods

Isolating and identifying strains

Peeled roots were immersed in rain water. Sampling was carried out 4 days after fermentation by randomly selecting six roots cut into 0.5-cm cubes and mixed under sterile conditions. A sample of 60 g was diluted in 540 ml of sterile peptone solution. Then 0.1 ml of decimal dilutions were spread on JP2 medium (see below) in petri dishes. After incubation for 48 h at 30 °C, the dishes were exposed to iodine vapor to detect the starch hydrolysis areas. Isolated strains were then purified by three successive transfers on JP2 medium, and cultures routinely checked for purity by microscopic observation.

Microorganisms were identified by:

(1) the configuration of the lactic acid produced after treatment (Ivorec-Szylit and Szylit, 1965) with the enzymes dehydrogenase l and d (Boerhinger Mannheim);
(2) the microorganisms' homolactic or heterolactic character, as determined by acetic acid or
(3) presence or absence of catalase;
(4) microscopic and macroscopic examination of morphology, mobility, and spores;
(5) Gram stain;
(6) arginine dissemination;
(7) growth at 15 and 45 °C; and
(8) fermentation of different carbon sources (API 50CH #5030 strips, Biomérieux, France).

"Bergey's Manual" (Sneath, 1986) was used to evaluate results and identify the different strains.

Strains and culture media

Three strains were used as reference: *Lactobacillus plantarum* (Lacto Labo, France), *Streptococcus equinus* CNCM 103233, and *Lactobacillus amylophilus* CNCM 102988T.

JP2 medium (g/L). This consisted of:

Component	g/L
M66 universal peptone	2.5
Soya peptone obtained by papain digestion	5
Casein peptone obtained by pancreatic digestion	2.5
Yeast extract	5
Meat extract	2.5
$MgSO_4,7H_2O$	0.1
NaCl	3
$(NH_4)_2SO_4$	2
K_2HPO_4	0.2
Prolabo soluble starch	3
Tween 80 (in ml)	0.4

The pH was adjusted to 6.75 before sterilization.

Physiological studies were performed, using a de Man-Rogosa-Sharpe (MRS) basal medium (de Man et al., 1960) and changing the carbon sources to 5% glucose and 5% starch.

Culture conditions. Strains were cultured in a 2-L bioreactor (Biolafitte, France) at 30 °C and agitated at 200 rpm. The pH was adjusted to 6.0 by adding NaOH (5 *N*). Inoculation at 10% v/v was performed with a 20-h pre-culture in the same medium used for fermentation.

Analytical methods

The biomass concentration was determined by measuring the optical density (OD) at 540 nm related to the dry weight measured after two washing and centrifugation cycles and drying at 105 °C for 24 h. For starch cultures, hydrolysis of residual starch was performed with a mixture of amylases (thermamyl + dextrosyme, supplied by Novo). The dry weight and OD were then determined as above. Lactic acid, glucose, acetic acid, and ethanol concentrations in the supernatant were assayed by high-performance liquid chromatography (HPLC). Compounds were separated by using an Aminex HPX 87H column (Bio Rad Laboratory) with a 0.8 ml/min flow (pump LDC 3200) of H_2SO_4 (0.012 *N*) solution at 65 °C. Analyses were carried out with a refractive index detector (Philips PU 4026). Total sugars in media containing starch were also determined with anthrone, using the Dubois et al. (1956) method.

Amylase assay. The α-amylase activity was measured by incubating 0.1 ml of appropriately diluted enzyme solution with 0.8 ml of a solution containing 1.2% of Prolabo soluble starch in 0.1 mol/L citrate-phosphate buffer (pH = 5.5) at 55 °C. The reaction was stopped by adding 0.1 ml of 1 mol/L H_2SO_4. After incubation, residual starch contents were determined colorimetrically after different periods at 620 nm by adding 0.1 ml of the reaction mixture to 2.4 ml of an iodine solution containing 30 g/L of KI and 3 g/L of I_2 and diluted to 4% with distilled water.

An enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions described above. Protein concentration was estimated with the Bradford (1976) method, using a Biorad Kit (Cat No. 500-0001, Ivry-sur-Seine, France) and bovine serum albumin as standard.

Purification of amylase. Fermentation was stopped after culture for 9 h. Cells were removed by centrifugation (at 15,000 g for 15 min at 4 °C), and the supernatant fluid (750 ml) filtered through a cellulose filter (0.45 µm pore size, HAWP type, Millipore, Saint Quentin les Yvelines, France) to remove cell debris.

Powdered ammonium sulfate was then slowly added to the supernatant fluid under constant stirring at 4 °C. Most of the amylase activity was precipitated at between 50% and 70% saturation.

After the ammonium sulfate fractionation, the precipitated protein collected by centrifugation (at 15,000 *g* for 30 min at 4 °C) was resuspended in 50 mmol/L KH_2PO_4/ Na_2HPO_4 standard buffer (pH = 6.8). The enzyme solution was washed and concentrated with a PM-10 Amicon ultrafiltration membrane. It was then loaded onto a diethylaminoethyl (DEAE) cellulose column (DE-52; Whatman Laboratory Sales, Hillsboro, Oregon, USA). The column (25 x 250 mm, flow rate 2.5 ml/min, 25 °C) was previously equilibrated with the standard buffer. The enzyme was eluted, using a concave, sodium chloride gradient (0-1.0 mol/L). Fractions (5 ml) were collected. The fractions that were enzymatically the most active were pooled, dialyzed overnight at 4 °C against the standard buffer, and used for further studies. They were kept at -30 °C. No activity was lost for at least 3 months under such conditions.

Polyacrylamide gel electrophoresis. This was carried out according to Laemmli's method (1970), with a 10% running gel and 4%

stacking gel. Electrophoresis under nondenaturating conditions was performed in the absence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol in any buffer. Gels were run at a constant 150 V for 1 h at 25 °C. Proteins were stained by the silver method (Oakley et al., 1980).

Amylase stain. After electrophoresis, gel was incubated for 1 h at 30 °C in 0.1 mol/L citrate-phosphate buffer (pH = 5.5), containing 1% of soluble starch. After two washes with distilled water, light lanes (representing starch hydrolysis areas of amylase activity) were detected by immersing the gel in Lugol's solution.

Molecular mass determination. SDS-PAGE electrophoresis was used to determine the approximate molecular mass of amylase. Marker proteins (Biorad, Cat. No. 161-0315) used were myosin (200,000), β–galactosidase (116,250), phosphorylase-b (97,400), bovine serum albumin (67,000), and ovalbumin (45,000).

Assays on gari. Fresh imported cassava roots from Cameroon were obtained from Anarex (Paris, France). *Gari* was prepared from peeled, washed cassava roots, which were chopped and minced in a food mixer (SEB). The pulp obtained was packed tightly into plastic, sterile, screw-capped containers (60 ml; OSI, A12.160.56) and placed at 30 °C.

Three batches were prepared:
(1) natural fermentation, using the endogenous microflora present;
(2) fermentation after inoculation with *L. plantarum* A6 (10^8 cfu/g of dried cassava), which had been cultured in bioreactors on cellobiose MRS medium;
(3) fermentation after inoculation with *L. plantarum* Lactolabo (10^8 cfu/g of dried cassava), which had been cultured in bioreactors on MRS cellobiose. Cells were washed in physiological solution before cassava inoculation.

A container from each batch was monitored every day to test the following parameters:

(1) The pH was measured on a 10-g sample and homogenized in distilled water (20 ml). Moisture was measured by drying a 10-g sample at 105 °C for 24 h.
(2) The number of lactic acid bacteria (l.a.b.) was estimated on a 10-g sample homogenized in 90 ml of physiological sterile solution. Colonies were counted on MRS agar, using a spread-plate technique on petri dishes and after incubation at 30 °C and 48 h.

Results and Discussion

Isolation and identification of Lactobacillus plantarum *A6*

Seven amylolytic microorganisms were isolated on JP2 medium from retted cassava roots. Two were revealed by HPLC to have a capacity to produce lactic acid from starch. Table 1 lists their morphological, physiological, and biochemical characteristics. The ability of these cultures to use 49 different carbohydrates was studied with API 50CH #5030 strips. The results were compared, by computer, with the percentage of positive reactions of different *Lactobacillus* species as per API. A 99.9% rate of similarity with *L. plantarum* was observed and hence identifying these cultures as strains of *L. plantarum*. The two strains, A6 and A43, displayed precisely the same sugar degradation profiles, which suggests that they are probably the same.

The amylolytic activities on JP2 medium of *L. plantarum* A6, *S. equinus*, and *L. amylophilus* indicated that the

Table 1. Characteristics of *Lactobacillus plantarum* strains A6, A43, and Lacto Labo (check).

Strain	A6	A43	Check
Ratio of d:l lactic acid	69:31	66:34	73:27
Homolactic	+	+	+
Catalase	-	-	-
Bacterium shape	Short rod	Short rod	Short rod
Gram stain	+	+	+
Spore	-	-	-
Mobility	-	-	-
Dissemination of arginine	-	-	-
Growth at 15 °C	+	+	+
Growth at 45 °C	-	-	-

starch hydrolysis zone was largest for *L. plantarum* A6. It was therefore selected for further studies.

Lactobacillus plantarum *A6 growth kinetics*

The growth of *L. plantarum* A6 on glucose MRS medium (Figure 1) is fully comparable with that of *L. plantarum* (Lacto Labo). The growth rate (0.43/h) and biomass productivity (0.75 g/L per hour) were slightly lower than those of the standard (Lacto Labo) strain, but the biomass and lactate yields were almost identical. The strain therefore does not seem to require nutrients other than those of the common strain, suggesting that mass production is possible.

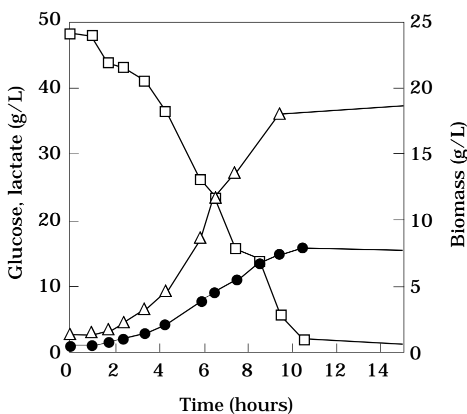

Figure 1. Fermentation of *Lactobacillus plantarum* A6 on MRS glucose (□ = glucose; △ = lactic acid; ● = biomass). Temperature = 30 °C; pH = 6.0.

On starch MRS medium, the strain exhibits the same kinetic profiles (Figure 2) and the same yields as the standard strain. The rate of starch hydrolysis was greater than the uptake rate, leading to a 3 g/L maltose peak during the seventh hour of fermentation (results not shown). Thus, hydrolysis of starch is not a limiting factor.

Characterizing the amylolytic enzyme

To characterize the amylolytic activity exhibited by *L. plantarum* A6, a comparison was made of the HPLC profiles after starch hydrolysis by the cell-free extract and commercial amylolytic enzymes (*Aspergillus oryzae* α-amylase, Sigma A0273; potato β-amylase, Sigma A7005, and *Aspergillus niger* amyloglucosidase, Sigma A3514). Under these conditions, the main products of starch hydrolysis analyzed by HPLC were glucose from amyloglucosidase, maltose from β-amylase, and a mixture of glucose, maltose, and oligosaccharide (retention time of 5.2 min) from α-amylase. The

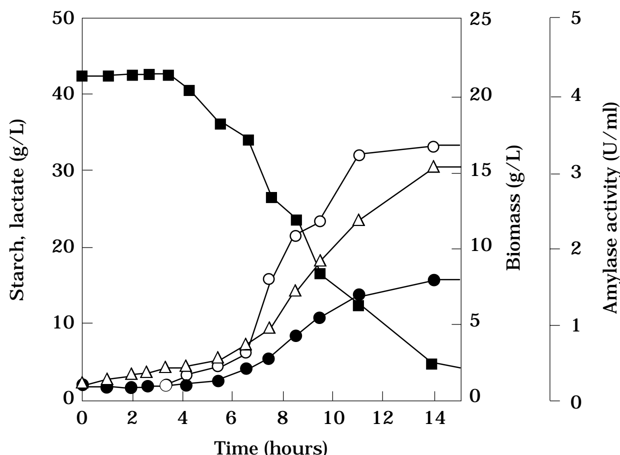

Figure 2. Fermentation of *Lactobacillus plantarum* A6 on starch MRS medium (■ = starch ; △ = lactic acid; ● = biomass; ○ = amylase activity). Temperature = 30 °C; pH = 6.0.

breakdown profile of starch by the enzyme from *L. plantarum* A6 is similar to that of α-amylase, thereby indicating that the enzyme synthesized by *L. plantarum* A6 is extracellular α-amylase.

Purification of amylase

The results of purifying the amylase produced by the strain *L. plantarum* A6 are summarized in Table 2. The first step in purification was conventional $(NH_4)_2SO_4$ fractionation. The 50%-70% fraction revealed maximum enzyme activity and was selected for further purification by DEAE-cellulose. The elution profile displayed only one amylase activity peak. The purification procedure described above makes it possible, in only two stages, to obtain a protein fraction containing most of the amylase activity of *L. plantarum* A6 enriched by a factor of nearly 20.

Testing the homogeneity of the fraction by electrophoresis under native conditions revealed a major protein and three others that were quantitatively unimportant. However, all the proteins detected in the purified fraction possessed an amylase activity. These procedures were therefore considered sufficient for purifying the extracellular amylase activity of *L. plantarum* A6. The SDS-PAGE analysis of the purified fraction resulted in a distribution between a clearly defined band (50 kDa) and a diffuse band with a molecular weight of close to 150 kDa.

Hypotheses. Several hypotheses can explain these many amylase forms. We find the most satisfactory is that which suggests that the purified extract consists of a population of aggregates of a 50-kDa amylase. This interpretation is based on the fact that most of the bacterial amylases described have a molecular weight of this order (Fogarty, 1983). This type of aggregation of purified enzyme was observed in *Bacillus subtilis* amylase (Robyt and Ackerman, 1973), with zinc being the factor inducing clumping. The clumping factor remains to be defined in our case.

Further study is needed to support this hypothesis. The

Table 2. Purification of α-amylase of *Lactobacillus plantarum* strain A6 cultivated in a modified MRS medium containing 2% (w/v) soluble starch and 0.5 g/L $CaCl_2$ at 30 °C.

Materials	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	750.0	82.5	35100	425	100.0	1.0
$(NH_4)_2SO_4$ (50%-70% fraction)	39.0	18.1	25935	1433	73.9	3.4
Ultrafiltrate	8.8	10.4	16016	1540	45.6	3.6
DEAE-cellulose (117-130 fractions)	61.8	1.5	12484	8270	35.6	19.5

amount of enzyme isolated was not large enough for further investigation. Immunological characterization would probably determine the type of relation between the different amylase forms observed and thus confirm the hypothesis.

Effects of pH and temperature on amylase activity. The effect of pH on enzyme activity was studied in a 3.0 to 7.5 pH range with 0.1 mol/L citrate-phosphate buffer at 55 °C. The enzymatic activity profile according to temperature was determined within a 10 to 80 °C temperature range under standard conditions (see above). The optimal pH was 5.5 and the optimal temperature was 65 °C (Figures 3 and 4).

Compared with the characteristics of the lactic acid bacterial amylases described in the literature, the properties of the enzyme synthesized by *L. plantarum* A6 are different. The enzyme from a *Leuconostoc* spp. studied by Lindgren and Refai (1984) had a pH optimum of 6.0 and a temperature optimum of 40 °C. Two active enzyme fractions were clearly separable by isoelectric focusing. The enzyme isolated from *L. cellobiosus* (Sen and Chakrabarty, 1986) had a molecular weight of 22.5 to 24 kDa, a pH optimum from 6.3 to 7.9, and a temperature optimum of 40 to 50 °C. But the characteristics of the amylase from *L. plantarum* A6 are very similar to those of *Bacillus subtilis* (Fischer and Stein, 1960; Fogarty, 1983; Robyt and Ackerman, 1973; and Welker and Campbell, 1967): extracellular enzyme, identical optimal pH (5.5), identical optimal temperature (65 °C), presence of tyrosyl phenolic groups at the active site, and presence of multiple forms (aggregates).

We speculated that the exceptional capacity of *L. plantarum* A6 to break down starch might have

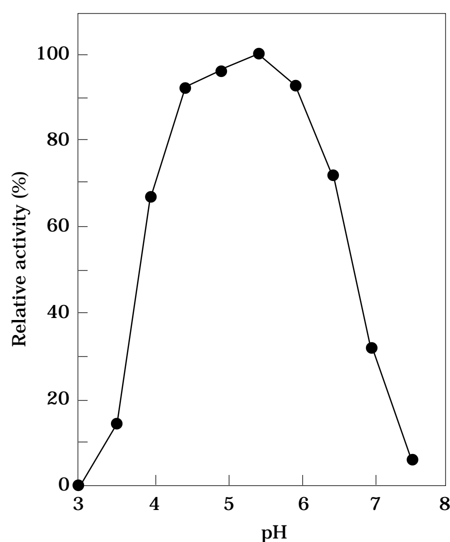

Figure 3. Effects of pH on amylase activity at 55 °C.

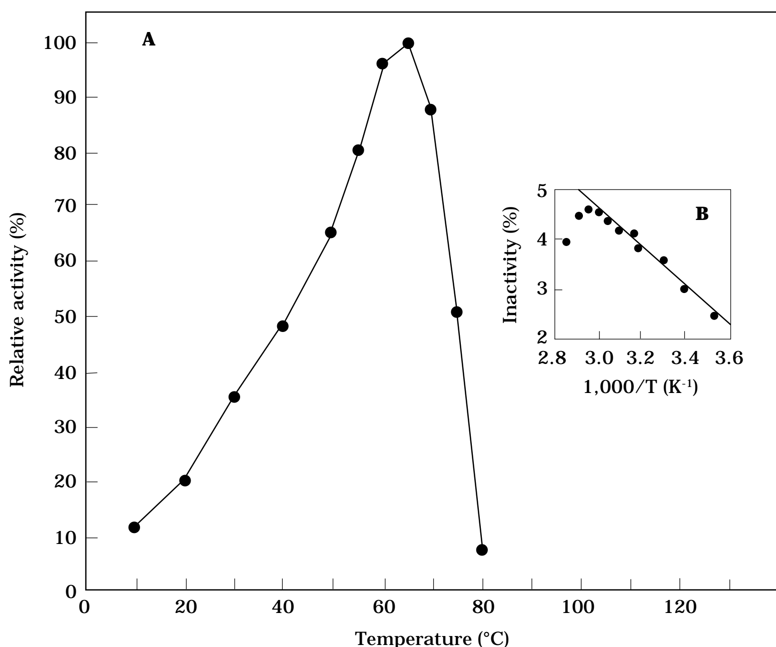

Figure 4. Effects of temperature on amylase activity at pH = 5.5. (A) Relative activity versus temperature; (B) Arrhenius plot.

been a result of transfer of genetic material between *Bacillus subtilis* and *L. plantarum*, which could be possible, because both are microorganisms found in the natural microflora of fermented cassava (Nwanko et al., 1989), and whose amylase activities are very similar. Further investigation would answer this question.

Inoculation effect of Lactobacillus plantarum *A6 on cassava fermentation*

Three different assays were carried out: (1) natural cassava fermentation, (2) cassava inoculated with *L. plantarum* A6, and (3) cassava inoculated with a control strain, *L. plantarum* Lacto Labo.

Evolution of pH, organic acids, and lactic acid bacteria. In all three assays, a rapid pH decrease was observed from the start of fermentation (Figure 5). The naturally fermented cassava showed a steep fall from 6.2 to 4.3 (assay 1), and both inoculation assays (2 and 3) from 6.2 to 3.9. This pH shift was correlated with lactic acid production, which was the principal metabolite produced (Figure 6). These data confirm that the lactic acid bacteria are the predominant fermentative microflora. In all three assays, this flora reached 5.10^9 cfu/g after 24 h of fermentation (Figure 5).

In the natural cassava fermentation, within the first 24 h, a simultaneous production of lactic and acetic acids and traces of propionic and butyric acids and ethanol were observed. But, although the acetate content reached its maximum level (10 g/kg DM) and remained constant after the first day of fermentation, the lactate concentration began increasing from the second day of the process. This suggests that fermentation is primarily related to

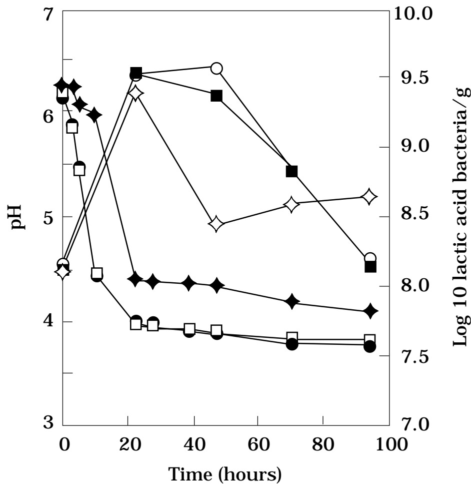

Figure 5. Changes in pH and numbers of lactic acid bacteria (l.a.b.) during cassava fermentation. (✦ = pH and ✧ = l.a.b. in natural fermentation; ● = pH and ○ = l.a.b. in fermentation inoculated with *Lactobacillus plantarum* A6; □ = pH and ■ = l.a.b. in fermentation inoculated with *L. plantarum* Lacto Labo.)

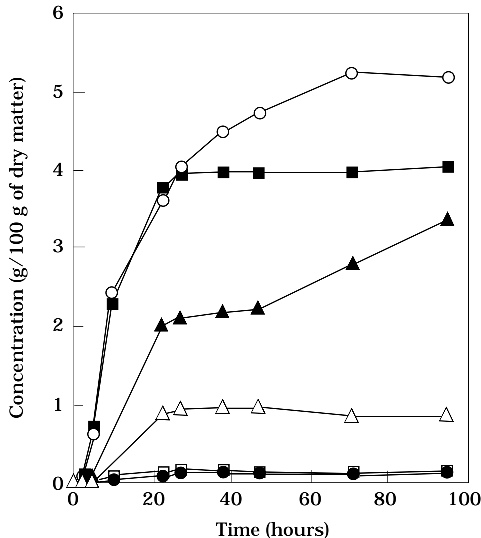

Figure 6. Evolution of lactate and acetate concentration during cassava fermentation. (▲ = lactate and △ = acetate in natural fermentation; ○ = lactate and ● = acetate in fermentation inoculated with *Lactobacillus plantarum* A6; ■ = lactate and □ = acetate in fermentation inoculated with *L. plantarum* Lacto Labo.)

an heterolactic flora growth, which is supplanted by a more acid-tolerant homolactic flora.

This hypothesis is supported by Oyewole and Odunfa (1990), who studied the characteristics and distribution of lactic acid microflora during the preparation of *fufu*. They reported a predominant development of *Leuconostoc mesenteroides*, which was subsequently replaced by *L. plantarum*. They suggested that this sequence resulted because *L. mesenteroides* was unable to tolerate increasing acidity.

In the inoculated fermentations, the lactic acid content was higher. The production kinetics of this acid were identical in both *L. plantarum* strains during the first 24 h. But, on the second day, this concentration reached its maximum (40 g/kg DM) and remained constant in the control strain. In contrast, in the amylolytic strain (*L. plantarum* A6), lactate production continued to rise, increasing by 25%.

Traces of ethanol, propionate, and butyrate were also found in the inoculated fermentation assays. Furthermore, the lower acetate production showed that a massive inoculation with an *L. plantarum* strain inhibited the development of the natural heterolactic microflora.

Conclusions

The presence of amylase in lactic acid bacteria has already been reported. But, as far as we know, no author has described any amylolytic strain of *L. plantarum*. When investigating the bacterial microflora of fermented cassava roots, Regez et al. (1988) isolated numerous *L. plantarum* strains, but did not report any amylolytic strains. Scheirlinck et al. (1989) studied the integration of the

α-amylase gene of *Bacillus stearothermophilus* in the genome of an *L. plantarum* strain, but did not verify the expression, stability, and competitiveness of the transformed strain in a natural medium.

In our research, we had isolated a natural amylolytic strain of *L. plantarum* from cassava roots. Our data, as reported here, suggest that this new lactic acid bacterium is of particular interest, not only for its taxonomy, but also for its capacity to develop rapidly and massively in starch-based media.

Finally, preliminary trials of inoculating cassava with *L. plantarum* A6 for *gari* production demonstrate that this strain may play a significant role in developing organoleptic qualities, and in standardizing and preserving the final product because of the large amounts of lactic acid produced and the resulting faster and significant drop in pH values.

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CHAPTER 25

CASSAVA WASTES: THEIR CHARACTERIZATION, AND USES AND TREATMENT IN BRAZIL[1]

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Introduction

Cassava is widely grown in Brazil. It is used fresh, that is, directly, in cooking; processed into a typical flour, known as *farinha*; and for starch extraction. All the resulting food products have no or nontoxic levels of cyanide (Table 1). Most cyanide is carried away by the wastes, whether liquid or solid.

The crop is grown in diverse production systems, ranging from small farms to plantations. Depending on their quantity and composition, cassava residues can damage the environment and even constitute profit losses. Culinary use, for example, does not produce significant amounts of residues. In contrast, industrial use may cause environmental problems. Even tiny factories such as the *casas de farinha* can produce significant quantities of residues, because of their tendency to cluster in certain areas or cities. For example, sour or fermented-starch factories are concentrated by the hundreds in two districts of Minas Gerais State: Divinópolis and Pouso Alegre. Paranaví, a district of Paraná State, has a concentration of about 150 flour factories of different sizes.

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1. No abstract was provided by the authors.

Cassava Structure and Composition

The literature on cassava's structure and chemical composition is variable. Nevertheless, the data overall suggest that the cassava root is caloric, and generates about 1,500 cal/kg from about 350 g/kg of carbohydrates. The average values of other components are about 50 g/kg. Phosphorus and calcium contents are higher. Iron may occur, but in low quantities. Hegarty and Wadsworth (1968) state that raw cassava usually has an iron content of 1 to 2 mg/100 g of dry matter, but warn that if the analytical equipment used is made of iron, then such content may reach as high as 3.2 mg/100 g.

Table 2 shows the differences in composition when cassava leaves are considered. Oke (1968) details cassava root composition, mainly as mineral contents, as follows:

Humidity: 71.50%

Dry matter (g/100 g):
- crude protein = 2.60
- crude fiber = 0.43
- ash = 2.40
- lipids = 0.46
- carbohydrates = 94.10

Ash minerals (g/kg):
- nitrogen = 0.84
- potassium = 1.38
- phosphorus = 0.15
- calcium = 0.13

Other minerals (mg/kg):
- sodium = 56.00
- iron = 18.00
- boron = 3.30
- molybdenum = 0.90
- magnesium = 12.00
- copper = 8.40
- zinc = 24.00
- aluminum = 19.00

Other components:
- oxalate = 0.32%
- phytic acid = 76.00%
- HCN = 38.00 mg/100 g

The potassium content is greater than that of calcium, phosphorus, and iron. The idea that cultivating cassava weakens the soil is probably based on this fact.

Group B vitamins occur in cassava varieties with yellow pulp. These varieties are normally used by factories only in the northern states of Brazil. Cassava roots have high vitamin C content, but it can be destroyed in factory processing or cooking.

The literature differs on the nitrogen fraction of cassava roots. The traditional methodology evaluates proteins by multiplying crude nitrogen by a factor. Cereal or other, vegetable, factors are calculated in this way. Oke (1968) does not consider this accurate enough because a factor for cassava amino acids has yet to be established. Despite being low, cassava proteins are overestimated because root nitrogen fractions include both a proteinic fraction and nonproteinic compounds. The nitrogen of the linamarin radical (CN), for example, could be wrongly considered as part of the protein evaluation of raw cassava or cassava fractions.

Sreeramamurthy (1945) reports that the traditional solvents of protein methodology fail to extract some nitrogen, part of which is of a proteinic nature. For example, copper hydroxide separates and precipitates only 10% of total protein. The cassava proteinic fraction contains arginine, tryptophan, and cystine, and important amino acids. Cassava root protein is small in quantity rather than low in quality when compared with casein, egg albumin, and the protein fractions of cabbage and sweetpotatoes. In contrast, Rogers (1965) suggests that cassava protein is low in histidine, proline, glycine, and amino acids containing sulfur (e.g.,

Table 1. Composition (in percentage) of some typical cassava products, Brazil. Numbers are rounded.

Component	Product			
	Farinha flour	Starch	Sour starch	Chips
Humidity	1.2	1.1	1.6	0.9
Dry matter:				
Carbohydrates	93.0	97.3	95.6	94.0
Proteins	1.3	0.6	1.5	1.7
Lipids	0.1	0.3	0.3	0.3
Crude fiber	3.3	0.6	0.7	1.1
Ash	1.1	0.1	0.3	0.4
Cyanide	0	0	0	1.6

SOURCE: Faculdade de Ciências Agronômicas (FCA), Universidade Estadual Paulista (UNESP), unpublished data.

Table 2. Central American cassava cultivars: root and leaf composition. Numbers are rounded.

Component	Root size			Leaves
	Long, thin	Medium	Short, thick	
Humidity (%)	62.10	61.10	62.10	77.20
Dry matter (g/100 g):				
Fiber	1.60	1.25	1.14	2.54
Lipids	0.65	0.20	0.24	1.31
Nitrogen	0.32	0.17	0.11	1.10
Carbohydrates	32.95	34.18	34.70	10.33
Ash	1.20	1.20	0.86	1.77
Other components (mg/kg):				
Calcium	46.00	27.00	27.00	206.00
Phosphorus	78.00	66.00	43.00	95.00
Iron	1.60	0.50	0.50	3.50
Carotene	0.01	0.01	0.01	4.53
Thiamine	0.09	0.06	0.05	0.15
Riboflavin	0.04	0.04	0.30	0.30
Niacin	0.82	0.72	0.60	2.02
Ascorbic acid	32.00	40.75	41.40	211.00

SOURCE: Calculated from Martelli, 1951.

methionine, cystine, threonine, isoleucine, and tryptophan).

Cassava juice is milky, smells of cyanide, and consists of 91.00% water, 0.13% essential oils containing sulfur, 2.30% gum, 1.14% saponins, 1.66% glycosides, and 3.80% nonspecified components.

Oke (1968) reported cassava lipids from 0.1% to 1.0%, made up of 35% palmitic, 3% stearic, 39% oleic, 18% linoleic, and 5% linolenic acids.

The literature rarely mentions cassava fiber. Despite cassava roots being fibrous, the processing method that uses acid and alkaline hydrolysis yields only about 2.0% fiber, whereas other methods (such as neutral detergent analysis or enzymatic analysis) yield almost 20%.

Carbohydrate is the highest fraction of cassava root composition, with starch constituting the largest part. Oke (1968) puts the nonstarchy fraction of the carbohydrates at 3.5%, of which 1.79% is composed of reducing sugars (0.93% glucose, 0.43% fructose, and 0.43% maltose) and 1.71% nonreducing sugars (1.70% saccharose and 0.01% raffinose). Oke considers starch content as being 35% of the fresh matter, and possibly higher if total carbohydrates are calculated by difference. Amylase hydrolyzes cassava starch to 48% in its granular or raw form and to 78% when previously boiled.

Sugar originating from starch may increase if fermentation takes place. According to Amido (Um novo caminho..., 1973), fermentation during starch extraction and purification causes loss of starch because it turns into soluble sugars.

Sreeramamurthy (1945) concluded that cassava roots are mainly starchy. They contain less than 1% protein, have a very low lipid content, and are poor in minerals and group B vitamins, although fresh roots have considerable vitamin C content.

Toxic Cassava Glycosides

Cooke (1979) describes both lotaustralin and linamarin, the toxic glycosides found in the cassava plant (Table 3), as being able to generate hydrocyanic acid. Although free cyanide is well known to be toxic, the toxicity of glycocyanide is still unknown.

Oke (1969) reported that linamarin is a β-glucoside of acetone cyanohydrin, and lotaustralin of ethyl-methyl-ketone-cyanohydrin. The more representative glucoside is linamarin, which constitutes 80% of total glucosides. He also suggested that glucosides in linked form are not toxic to the plants themselves.

Oke hypothesized that the glucosides are intermediate compounds in protein synthesis, such as from amino acids that are constituted from the nitrate absorbed by roots. Thus, the cyanogenic glucosides are stable intermediates that do not accumulate if conditions for protein synthesis are favorable. Glucoside synthesis probably starts with glycine.

The toxic action of cyanide (released when cell walls are damaged) on animals is explained by the cyanide's affinity to iron, combining with hemoglobin to form cyanohemoglobin. In higher plants and microorganisms (Cereda et al., 1981), cyanide interferes with the oxidative phosphorylation pathway, combining with cytochrome-oxidase to inhibit electronic transportation and thus the formation of adenosine triphosphate (ATP).

Table 3. Cyanogen glycoside concentration (mg/kg of tissue) in cassava tissues of sweet and bitter cultivars (*Manihot esculenta* Crantz).

Cultivars	Seeds	10-day-old plantlets	Mature leaves	Roots
Sweet	0	285.0	468.0	125.0
Bitter	7.5	245.0	310.0	185.0

SOURCE: Nartey, 1981.

For animals, calculating the quantity sufficient to cause death (lethal dose) is done by experiment and expressed in mg per kilo of live weight. Oke (1969) mentions that 1 mg/kg of live weight is considered the limit for humans, and is used to classify cassava roots into poisonous or nonpoisonous, according to the amount of cyanogenic potential in the root. The literature mentions values ranging from 15 to 400 mg of hydrocyanic acid per kg of fresh cassava roots, although average values are 30 to 150 ppm (Carvalho and Carvalho, 1979).

Oke (1969) suggests that, in processed foods made from cassava, the hydrolytic enzyme of the plant linamarase remains active and catalyzes a reaction that releases molecules of glucose, acetone, and hydrocyanic acid in proportions of 1:1:1. Linamarase has an optimal pH of 5.5 to 6.0. Glucose can act as an antidote because it changes the reaction's direction and cooperates with glucoside synthesis.

Microorganisms consume free glucose in preference to glucoside. Coop and Blakey (1948), cited by Oke (1969), confirmed this hypothesis. When an extract of cassava in a solution containing 1 to 3 ppm of HCl with a pH of 6.5, was placed in the presence of 2% glucose, the released cyanide content did not change. Nor was the extract toxic when incubated with rumen liquid. Determining the pH is important, because reaction rate depends on it. Animals, in general, have a detoxification mechanism that can prevent death when reaction is slow. It operates in swine, whose stomachs are

monogastric, with a pH of 3.0, but does not effectively prevent death in bovines, which have polygastric stomachs, with a pH of 7.0.

Microorganisms can develop on substrates that contain cyanide if they have an anaerobic metabolism—an alternative mechanism to the respiratory chain (Cereda et al., 1981)—or if they can detoxify cyanide by splitting the radical into carbon and nitrogen (Jensen and Abdel-Ghaffar, 1969). This fact may explain the related fertilizing effect of waste-water spillage from cassava processing.

Cassava Wastes

Cassava wastes are plant residues generated by processing. Waste quality and quantity vary greatly because of such factors as plant age, time after harvesting, kind of industrial equipment, and its adjustment.

In Brazil, cassava roots are mostly processed into flour (which generates more solid residues) and starch (more liquid residues). Some solid wastes are brown peel, inner peel, unusable roots, crude bran, bran, bagasse, and flour refuse. Among the liquid wastes is *manipueira*, which is formed during flour making by pressing bulk quantities of cassava roots. It is also formed during starch extraction, but the water used in the process dilutes the *manipueira*, reducing its organic load and cyanide content, but vastly increasing its output. Water from washing roots is also considered as liquid waste. Figures 1 to 5 show the relationships between cassava

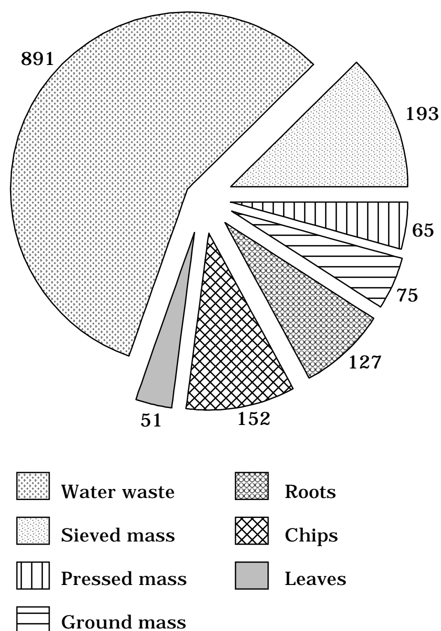
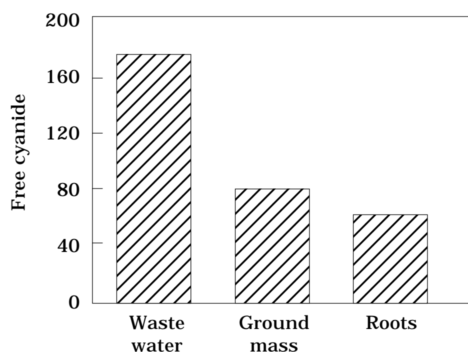


Figure 1. Cyanide content (ppm of HCN) of plant parts, products, and wastes of processing cassava cultivar IAC 12 829.



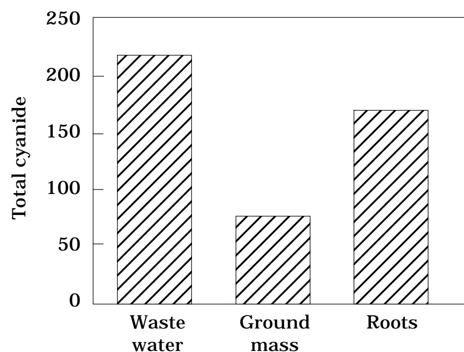


Figure 2. Free and total cyanogens (ppm of HCN) in products of a cassava flour factory (Equipamento Zaccharias), using cassava cultivar IAC 12 829 at 24 months old, São Paulo, Brazil.

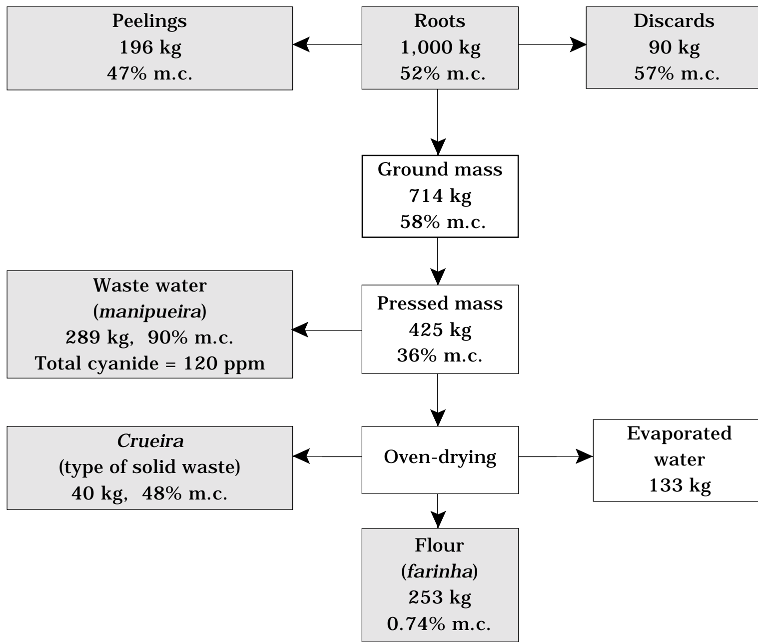


Figure 3. Mass balance of a fermented-starch factory, Colombia. (m.c. = moisture content.) (After Arguedas and Cooke, 1982.)

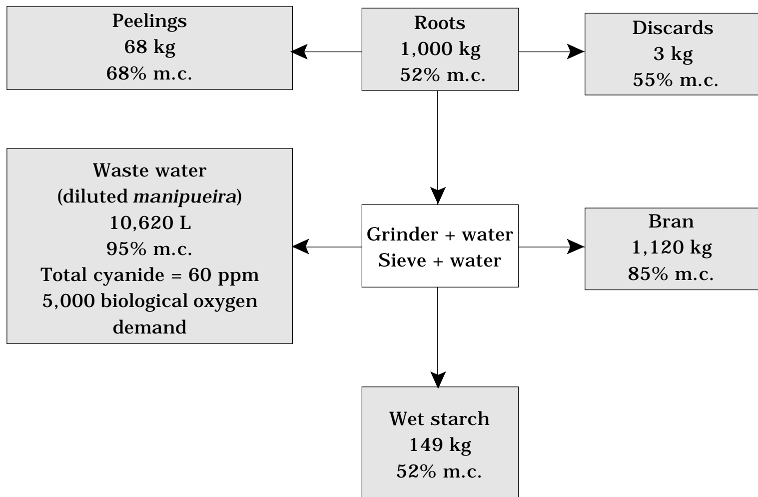


Figure 4. Mass balance of a fermented-starch factory, which used cassava variety Branca de Santa Catarina at 24 months old, Minas Gerais State, Brazil. (m.c. = moisture content.)

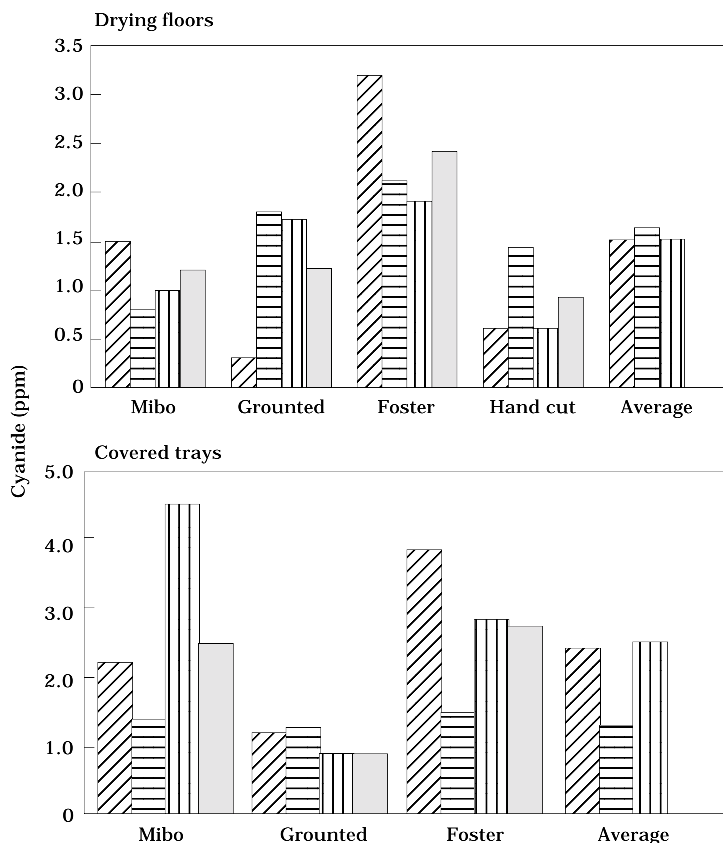


Figure 5. Cyanide reduction in cassava chips processed at three factories and by hand cutting, Brazil. (= 5 kg/m² = 10 kg/m² = 15 kg/m² = Average.)

processing, cultivars, and wastes in the material balance of cassava flour and sour starch production.

Solid wastes

Peelings. The brown peel, sometimes called bark, of cassava roots corresponds, in technical terms, to the periderm and varies between 2% and 5% of the root total. It is thin and cellulosic, and although usually dark brown, can be white or cream-colored. A small quantity of inner peel, or cortical parenchyma, may come off with the bark, causing losses in starch factories. In *farinha* factories, if the inner peel is highly fibrous, it is best taken off. In industrial terms, peelings are residues and refer to the mixture of both inner peel and bark. Table 4 shows the average composition of several samples of peelings. Peelings can be used as fertilizer or animal feed.

Discards. These are produced during selection, so as not to overwork the rasper. Their composition is similar to that of cassava roots but is more fibrous because they contain the peduncle. Moisture content is 55%-60%. The

Table 4. Chemical composition of cassava peelings. Average values of several samples are given. Dashes indicate that no data were available.

Component	Peelings		
	Outer (bark)	Inner	Mixture
Humidity (%)	48.3	65.6	72.3
Dry matter:			
Volatile solids (%)	-	-	26.2
Ash (%)	4.0	3.0	1.4
Soluble carbohydrates (%)	-	-	7.9
Starch (%)	0	58.0	32.0
Lipids (%)	3.0	2.0	0.6
Nitrogen (%)	0.6	1.3	2.1
Fiber (%)	41.0	6.0	-
Lignin (% SV)	-	-	6.5
Free cyanide (ppm)	-	-	23.9
Total cyanide (ppm)	0	320.0	120.0
Phosphorus (ppm)	60.0	-	60.0
Potassium (ppm)	430.0	-	430.0
Calcium (ppm)	280.0	-	280.0
Magnesium (ppm)	80.0	-	80.0
Iron (ppm)	5,538.0	-	26.0
Copper (ppm)	9.0	-	9.0
Zinc (ppm)	21.0	-	21.0
Manganese (ppm)	104.0	-	103.0
Sulfur (ppm)	110.0	-	320.0
Boron (ppm)	18.0	-	18.0
Volatile acidity (mg acetic acid/L)	-	-	5,548.0
Alkalinity (mg bicarbonate/L)	-	-	2,191.0
C/N ratio	-	-	6.4
C/P ratio	-	-	0.3

SOURCE: Motta, 1985.

quality of discards depends on the cultivar and on root age. Together with bran, discards may be used raw as animal feed, thus bringing extra income for the industry. The values shown in Figures 3 and 4 may be overestimated, because the process is still being investigated.

Bran or bagasse. This solid waste is made up of fibrous root material, and contains starch that physically could not be extracted. It is produced as starch is separated. It has a large absorption capacity and may contain about 75% moisture. Table 5 shows the chemical composition of bran after partial drying, with differences according to the technology used. Table 6 shows the composition of sun-dried bran from fermented-starch factories in Minas Gerais, Brazil.

Crude bran. Another type of solid waste is crude bran (*farinhão* or *crueira*), which is made up of pieces of root and inner peel. In cassava-flour processing (at Equipamento Zaccharias, São Paulo State), these are separated out by sieving before being oven-dried. Table 7 (p. 231) shows the composition of such waste. At other factories (e.g., Mádia, Paraná State), these residues are replaced by fine threads (*fiapos*) made up of cassava fibers. Another solid residue is cassava-flour refuse, the grated mass that daily falls and collects on the floor.

Table 5. Differences in chemical composition of bran according to technology adopted. Dashes indicate that no data were available.

Component[a]	Type of technology		
	Royal[b]	Minas[c]	*Fiapos*[b]
Humidity (%)	9.42	14.82	9.52
Dry matter:			
Ash (%)	0.83	3.77	0.66
Soluble carbohydrates (%)	0.01	-	-
Starch (%)	69.76	74.99	63.85
Lipids (%)	0.65	0.28	0.83
Nitrogen (%)	0.24	1.86	0.32
Fiber (%)	11.08	7.81	14.88
Total cyanide (ppm)	0	0	-
Phosphorus (ppm)	-	30.00	-
Potassium (ppm)	-	280.00	-
Calcium (ppm)	-	90.00	-
pH	4.00	-	-

a. No data were available for the following components: volatile solids, lignin, free cyanide, magnesium, iron, copper, zinc, manganese, sulfur, boron, volatile acidity, alkalinity, C/N and C/P ratios, chemical oxygen demand, or titratable acid.

b. Large factory.

c. Small, traditional factory.

Table 6. Average composition of sun-dried bran from 20 fermented-starch factories (traditional) from Pouso Alegre and Divinópolis, Minais Gerais, Brazil.

Component	Average values (%)[a]	
	Pouso Alegre	Divinópolis
Starch	63.6	2.78
Soluble carbohydrates[b]	0.2	0.10
Protein	2.3	0.34
Phosphorus	0	0.01
Calcium	0.1	0.03
Potassium	0.3	0.06
Lipids	0.6	0.35
Fiber	8.3	2.06

a. Numbers are rounded.

b. Expressed in percentage of glucose.

SOURCE: Escola Superior de Agricultura de Lavras (ESAL), unpublished data.

Liquid wastes

Lagoon mud. Table 7 also shows the composition of sedimented lagoon mud and liquid wastes. Sometimes these are sun-dried and used as fertilizer. The use of thin starch is also uncommon.

Manipueira. Diluted *manipueira* is a liquid waste from cassava starch extraction and sour-starch manufacture. It may be waste water from root washing, after the washer/husker has removed soil and peelings and the water is decanted or filtered. The average factory volume is 2.62 m^3. Waste water may also be extracted from pressed and grated cake in flour manufacturing and from the roots themselves. It is also a byproduct of starch extraction (average factory volume is 3.68 m^3).

The average composition of *manipueira* sampled from different starch factories in São Paulo State is variable, as shown in the following list (numbers are rounded):

Component	Value
Humidity (%)	93.7
Dry matter (g %):	
Total solids	6.3
Volatile solids	5.2
Starch	0
Soluble carbohydrates	0.5
Lipids	0.5
Ash	1.1
Crude nitrogen	0.5
Fiber	0.3
Lignin	6.0
Free cyanide	43.7
Total cyanide	444.0
Dry matter (ppm):	
Phosphorus	160.8
Potassium	1,863.5
Calcium	227.5
Magnesium	405.0
Iron	15.3
Copper	1.1
Zinc	4.2
Manganese	3.7
Sulfur	19.5
Boron	5.0
Chemical oxygen demand	6,365.5
Volatile acidity (mg acetic acid/L)	2,703.7
Alkalinity (mg bicarbonate/L)	1,628.0
C/N ratio	7.6
C/P ratio	34.4
Titratable acidity (ml NaOH N%)	3.3
pH	4.1

Cyanogen content tends to be high, but varies according to cultivar. The organic load is also high, and varies with the type of processing used (Table 8). All residual starch is removed from *manipueira* before treatment. It has most soluble and some insoluble substances in suspension and this residue carries almost all the cyanogenic glycosides existing in the disintegrated root mass.

The water used in starch factories carries high concentrations of these glycosides (linamarin and lotaustralin) (Sobrinho, 1975). They are hydrolyzed by linamarase enzyme and acid, making the cyanide a free radicle (CN) (Williams, 1979).

According to Sobrinho (1975), liquid waste thrown onto soil or into waterways causes pollution. If the pollution rate of starch factories is expressed as biological oxygen demand (BOD) over 5 days, at 20 °C, and calculated as 24 g per habitant per day, it would be equivalent to that caused by 150-250 habitants per day—very high. In Santa Catarina State, the pollution caused by these wastes corresponds to 460 habitants per day (Anrain, 1983).

Conclusions

Cassava wastes can be used in different ways. The solid residues can be used as animal feed; the literature shows that cassava waste can replace a part or all of the feed components. *Manipueira* can be used in agriculture as a herbicide, nematicide, insecticide, or fertilizer. Anaerobic digestion is well studied in Brazil and is more advantageous than aerobic digestion. *Manipueira* comes from flour industries, and the best processing method uses the separated phases reactor. We now need to study how to optimize the acidic phase.

Cassava waste can also be used for biomass production. The yeast *Trichosporon* sp. can be isolated by natural fermentation with a cyanide-resistant respiration pathway, and potentially can produce both a proteinic and a fat biomass.

Table 7. Chemical composition of different types of cassava wastes, averaged over several analyses. Numbers are rounded. Dashes indicate no data were available.

Component[a]	Type of waste		
	Farinhão[b]	*Varredura*[c]	Lagoon mud
Humidity (%)	11.7	-	4.9
Dry matter (g %):			
Soluble carbohydrates	1.1	-	61.4
Lipids	68.5	-	1.8
Nitrogen	1.7	-	0.1
Fiber	0.5	0.5	1.8
Lignin (% SV)	-	-	9.7
Dry matter (ppm):			
Free cyanide	-	-	0
Total cyanide	-	-	0
Phosphorus	70.0	70.0	540.0
Potassium	700.0	640.0	240.0
Calcium	130.0	90.0	140.0
Magnesium	60.0	50.0	60.0
Iron	41.0	32.0	23,800.0
Copper	2.0	3.0	63.0
Zinc	8.0	8.0	75.0
Manganese	20.0	18.0	105.0
Sulfur	30.0	30.0	46.0
Boron	20.0	7.0	14.0
pH	5.4	-	5.4
Titratable acidity (ml NaOH N %)	3.7	-	3.9

a. No data were available for volatile solids, ash, and starch.
b. *Farinhão* = solid waste made of pieces of cassava roots and inner peels.
c. *Editor's note:* No explanation of this term was provided by the authors.

Table 8. Composition of extraction water (mg/L) from the Fleischmann-Royal factory, Conchal, São Paulo State, Brazil.

Component	Range measured
Chemical oxygen demand	6,280 - 51,200
Biological oxygen demand	1,400 - 34,300
Total solids	5,800 - 56,460
Soluble solids	4,900 - 20,460
Suspended solids	950 - 16,000
Fixed solids	1,800 - 20,460
Organic matter	1,500 - 30,000
Reducing sugars	2,800 - 8,200
Total phosphate	155 - 598
Total nitrogen	140 - 1,150
Ash	350 - 800
Sedimentable solids (1 h)	11 - 33
Cyanide content	22.0 - 27.1
pH	3.8 - 5.2

SOURCE: Lamo and Menezes, 1979.

The microorganisms can also be used to produce such biomasses as organic acids (citric or lactic), biological insecticides, and enzymes.

Solid wastes also have a potential use for foodstuffs. The production of high-fiber biscuits from bagasse is being studied at the Faculdade de Ciências Agronômicas (FCA) of the Universidade Estadual Paulista (UNESP).

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CHAPTER 26

CASSAVA STARCH EXTRACTION: A TYPICAL RURAL AGROINDUSTRY WITH A HIGH CONTAMINATION POTENTIAL

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Abstract

Every year, about 5,500 t of starch are produced in Colombia from about 27,000 t of cassava roots. Starch production usually involves simple technology, consuming an average of 23 m^3 of water per ton of cassava. This generates a contaminating load of about 180 kg of chemical oxygen demand (COD) per ton of roots. An average of 13.5 t of COD is discharged into Colombian rivers each day.

Processing generates two liquid residues: the first results from the washing and peeling of cassava roots, and generally contains a large amount of inert material with low COD; the second results from draining the starch sedimentation tank, and has a high contaminating load of COD and biochemical oxygen demand (BOD).

A pilot project was proposed to treat waste waters, using an anaerobic filter and a transfilter. The transfilter technology has been tested in France, yielding good results with household effluents. Its operating principle is based on immobilizing microorganisms on a lignocellulose support. The hydrodynamic characteristics of three types of supports—sugarcane bagasse, bamboo, and *paja de monte* (underbrush straw)—were determined in the laboratory. *Paja de monte* showed the best characteristics.

These studies will be complemented with the monitoring of existing microflora as changes occur in operational parameters.

Introduction

Agroindustrial processes generate large volumes of waste waters and solid residues whose quality varies according to the process used. Generally, farm activities use abundant water to wash and treat products, at which point the water is loaded with harmful elements and compounds. These are directly discharged into rivers and streams, representing a risk for the environment, and the reduced quality makes the water less suitable for other uses.

Colombia is a mainly agricultural country, with small and medium-sized agroindustries widely scattered. This makes conventional water treatment

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systems onerous to use, given the small volumes of products processed. But versatile water treatment systems are now available at low cost and are attractive alternatives within the reach of small industries.

The feasibility of applying transfilter systems (anaerobic processing) to purify these discharges is being studied by the Universidad del Valle (UV), Cali, Colombia, in collaboration with the Institut français de recherche scientifique pour le développement en coopération (ORSTOM), France, and is financially supported by the European Union (EU). A pilot reactor will be located in a starch factory in the Cauca Department. The Corporación Autónoma Regional del Valle del Cauca (CVC) will build a pilot anaerobic filter at the same site, so both can be evaluated from technical and economic viewpoints.

Waste Waters from Cassava Starch Extraction

Production and identification of waste waters

About 200 cassava-starch production factories are located in the Cauca Department, most in the north. Their annual consumption of cassava roots is about 27,000 t, from which they extract about 5,500 t of starch. Plant processing capacity ranges from 500 to 2,500 kg fresh roots per day (Janssen and De Jong, 1981).

Cassava-starch extraction involves several stages: root washing and peeling, rasping, screening, starch sedimentation, and, for sour starch, fermentation (Figure 1). Roots are washed in a tank or drum. They can also be peeled in a drum, but this operation removes only 60% to 70% of the peel, the remainder being peeled

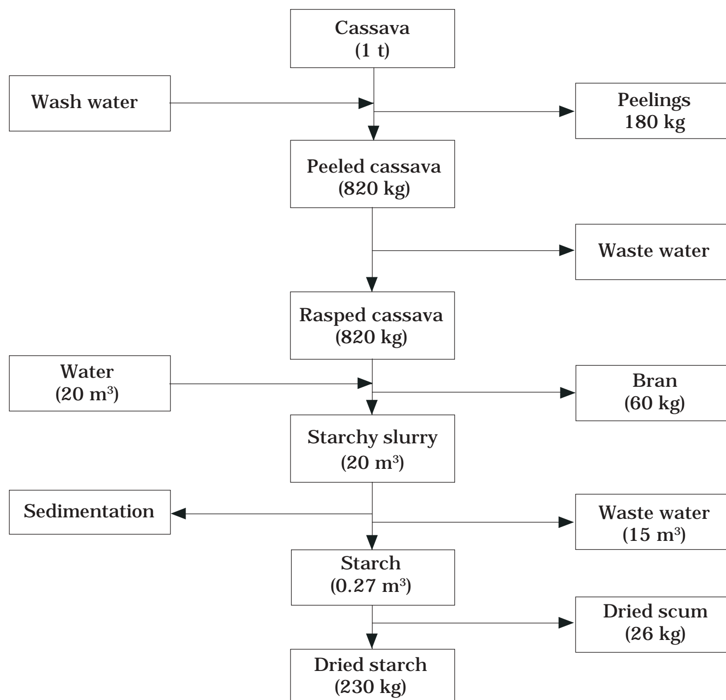

Figure 1. Characterization of waste water from cassava starch extraction.

by hand. This first stage of washing and peeling generates the first waste waters.

The roots are rasped, and the pulp sieved through a nylon mesh that covers the inside of the screening drum. The starchy slurry is left to settle for 20 to 24 h in sedimentation tanks, until the starch layer is 25 to 30 cm thick. The liquid is drained and discarded, and the extracted starch passes to fermentation tanks, which are completely filled and then covered with a thin layer of water. Fermentation takes about 4 weeks (Pinto, 1978).

Use of solid residues

When water is separated from starch in the sedimentation tank, a layer of greyish material is formed over the starch, called *mancha* by starch manufacturers. This film, or proteinaceous fraction, can be easily removed and dried. It is frequently used as animal feed and is widely accepted in the market.

Characteristics of waste waters

Waste water results from two processing stages: the washing of peeled cassava and the draining of the sedimentation tanks (Figure 1). The former contains a large amount of inert material and has a low COD, and the latter, high organic loads of BOD and COD.

Analyses of waste water samples taken from sedimentation tanks at different starch factories were carried out by the UV and the CVC. Average values were obtained to indicate the approximate composition of such water. According to this information, the volume of waste water discharged per processing plant per day ranges from 18 to 48 m^3. The average overall contaminant load is about 13.5 t of COD per day, or 3.45 t of BOD per day.

The use of anaerobic digestion to purify waste waters from cassava starch extraction

The results of characterizing the drainage water from sedimentation tanks permitted an analysis of the possibility of applying anaerobic treatment to this type of residue. The average COD value (900 mg/L) is high compared with that of BOD (300 mg/L), suggesting a high COD-to-BOD ratio and the presence of a high COD content, resistant to biological degradation. But the UV and CVC's preliminary research indicates that this factor is less important in terms of anaerobic biodegradation. When specifically tested to determine the percentage of organic matter biologically degradable under anaerobic conditions, the percentage of biodegradability was found to be 83%.

The results of waste water analyses show that a sufficient amount of nitrogen, a major element in biomass growth, is present in the residues. But the amount of phosphorus, another essential macronutrient, is deficient (Table 1).

The pH of drainage water from the sedimentation tank ranges from 3.9 to 4.7, which means the residue must be neutralized before being fed to the reactor.

The low cyanide concentration in the waste water (average 2.12 mg/L) suggests that the microbial biomass can adapt to this inhibitor. Methanogenic bacteria first react by reducing methane (CH_4) production, but, within a few days, they adapt to the cyanide and finally decompose it.

Based on this finding, the UV and the CVC conducted studies to see if anaerobic processes are applicable for treating this type of discharge (Table 2). Additional studies are now being conducted on a pilot scale.

Table 1. Characteristics of waste waters resulting from cassava starch extraction (average values).

Parameter	Average value	Range[a]
COD[b] (mg O_2/L)	9,100	4,000 - 12,800
BOD[c] (mg O_2/L)	3,100	1,500 - 8,600
COD/BOD (ratio)	2.9	-
Cyanides (mg CN^-/L)	2.12	1.2 - 4.04
Total solids (mg/L)	5,740	2,680 - 10,020
Volatile solids (mg/L)	4,870	2,020 - 9,320
Total organic carbon	2,420	870 - 5,300
pH (units)	-	3.9 - 4.7
Temperature (°C)	-	19 - 22
Sedimentation (ml/L)	29	15 - 47
Nitrogen (mg.N/L)	105	29 - 233
Phosphorus (mg.P/L)	2.34	0.3 - 6.0

a. This range is broad due to the amount of material processed.
b. COD = Chemical oxygen demand.
c. BOD = Biochemical oxygen demand.

SOURCE: Raddatz, 1986.

Table 2. Results of laboratory and pilot studies on the feasibility of anaerobic treatment of waste water from cassava processing, carried out by the Universidad del Valle and the Corporación Autónoma Regional del Valle del Cauca, Colombia.

Description[a]	12-liter reactor			23-liter reactor[b]		
	n	Average	∂n-1	n	Average	∂n-1
Effluent flow (ml/min)[c]	52	16.8	6.40	14	10.8	4.6
COD_{Af} (mg/L)[c]	52	3,294	2,732	14	1,640	1,263
COD_{Ef} (mg/L)[c]	52	659	56	12	105	26
OVL (kg COD/m^3.day)[c]	52	5.02	2.46	12	4.31	3.2
Removal COD (%)[c]	36	95	2	12	85.4	12.1
Biogas production (L/day)	53	16.4	8.4	-	-	-

a. COD_{Af} = chemical oxygen demand in affluent flow; COD_{Ef} = chemical oxygen demand in effluent flow; OVL = organic volume load.
b. The effluent was recycled by about 30%.
c. These units refer to average values of the COD.

SOURCES: Escandón, 1988; Hernández, 1987.

Methanogenic activity in mud was measured in the 12-liter reactor, increasing significantly from an initial rate of 0.063 kg COD-CH_4/kg VSS (volatile suspended solids) to a rate of 0.188 kg COD-CH_4/kg VSS. Although cyanide concentration was measured only a few times, it was calculated as decreasing by about 69%.

Transfiltering

This is a type of anaerobic treatment, using a lignocellulose base to filter particulate material present in the waste water, and to fix those microorganisms responsible for biodecomposing waste organic matter. The support bed decomposes with time so it has to be changed regularly; this also prevents silting.

Three operations occur simultaneously within the transfilter: (1) waste waters are purified by filtration and the organic material present in the waste is digested; (2) biogas (an energy resource) is produced; and (3) the lignocellulose

material (a solid waste appropriate for compost) is digested. Figure 2 is a diagram of a transfilter reactor (Farinet, 1993).

The UV is conducting laboratory research on the transfilter process based on waste waters from cassava starch extraction. A pilot reactor will later be built at a starch factory in the Cauca Department.

So far, with sugarcane bagasse, *paja de monte*, and bamboo as support beds, the following hydrodynamic characteristics were determined: volume of waste water eliminated from the supports, and load loss from clean water flowing through the filtering medium, as affected by water velocity and density of medium (compression).

Results showed very low load losses for higher velocities (35 m/h), and for stronger compressions (120 kg/m^3 for bagasse and 100 kg/m^3 for *paja de monte*). Maximum load loss was 7 cm for bagasse 1 m long and 6.3 cm for *paja de monte*. No load loss was observed in bamboo (Gotin, 1993). Technically, any of these materials can therefore be used, if due attention is paid to the operating criteria.

To complement the research on types of support, further studies on the filtration capacity of *paja de monte* will be made at a pilot starch factory. The aim is to determine the maximum compression of the support at which optimal filtration efficiency is obtained for a given volume and period in relation to time of silting the filter. The supernatant effluent from the starch sedimentation tanks will be the waste water treated.

To define constraints to designing the pilot reactor, feasibility studies on methanizing the filter effluent will be conducted in the laboratory, based on previous results.

Bacterial microflora will also be studied for their composition, distribution, and nature of the different groups of bacteria involved

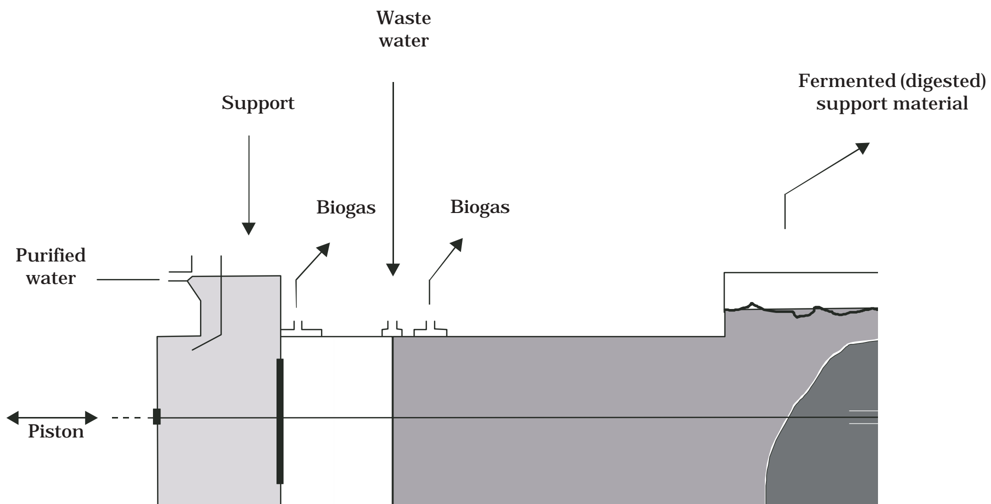

Figure 2. A transfilter reactor, used for the anaerobic treatment of waste water from cassava starch extraction. (From Farinet, 1993.)

and their interactions. Considerable knowledge is already available on the metabolic pathways of the microbiological process of anaerobic fermentation (hydrolysis, acidogenesis, acetogenesis, and methanogenesis).

We will quantify and characterize the active bacterial microflora responsible for biodegrading the effluent at each stage of the process. The evolution, and the density, of each group of bacteria will also be assessed throughout the operation of the digestor.

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