

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*, *chikwangué*, *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*, *S. 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 (Boehringer 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:

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 ₂ O	0.1
NaCl	3
(NH ₄) ₂ SO ₄	2
K ₂ HPO 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 M). 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 H₂SO₄ (0.012 M) 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 H₂SO₄. 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 I₂ 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 μ m 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 KH₂PO₄/Na₂HPO₄ 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 nondenaturing 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

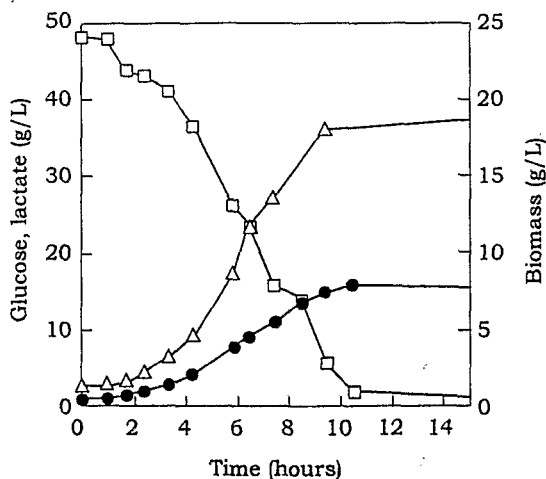


Figure 1. Fermentation of *Lactobacillus plantarum* A6 on MRS glucose (\square = glucose; Δ = lactic acid; \bullet = biomass). Temperature = 30 °C; pH = 6.0.

almost identical. The strain therefore does not seem to require nutrients other than those of the common strain, suggesting that mass production is possible.

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

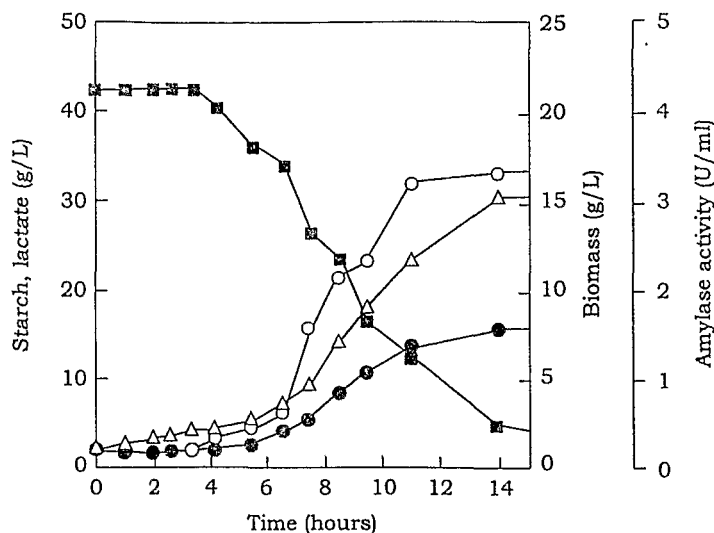


Figure 2. Fermentation of *Lactobacillus plantarum* A6 on starch MRS medium (■ = starch ; Δ = lactic acid; ● = biomass; ○ = amylase activity). Temperature = 30 °C; pH = 6.0.

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 $(\text{NH}_4)_2\text{SO}_4$ 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
$(\text{NH}_4)_2\text{SO}_4$ (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

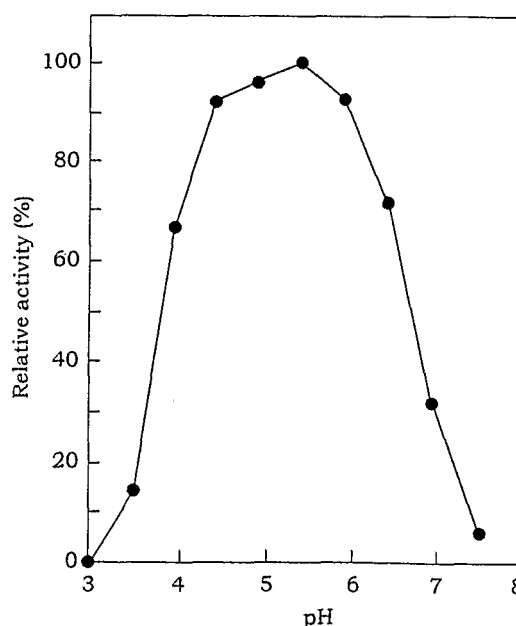


Figure 3. Effects of pH on amylase activity at 55 °C.

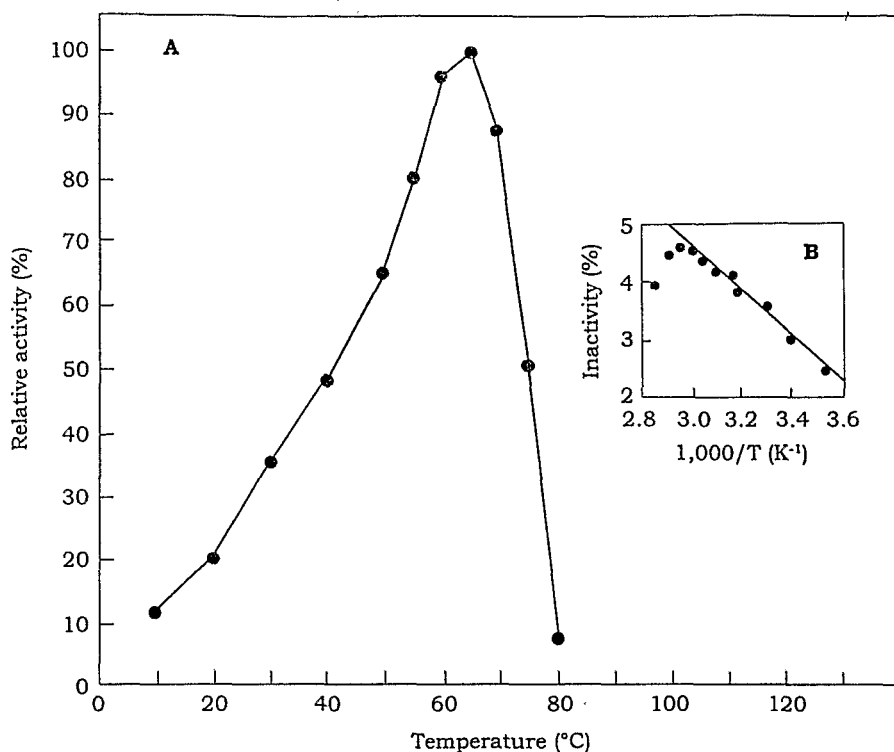


Figure 4. Effects of temperature on amylase activity at pH = 5.5. (A) Relative activity versus temperature; (B) Arrhenius plot.

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

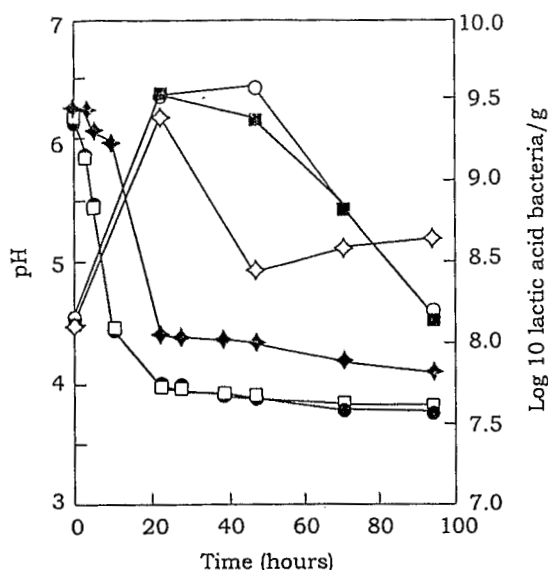


Figure 5. Changes in pH and numbers of lactic acid bacteria (l.a.b.) during cassava fermentation. (◆ = pH and ◇ = l.a.b. in natural fermentation; ● = pH and ○ = l.a.b. in fermentation inoculated with *Lactobacillus plantarum* A6; □ = pH and ■ = l.a.b. in fermentation inoculated with *L. plantarum* Lacto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

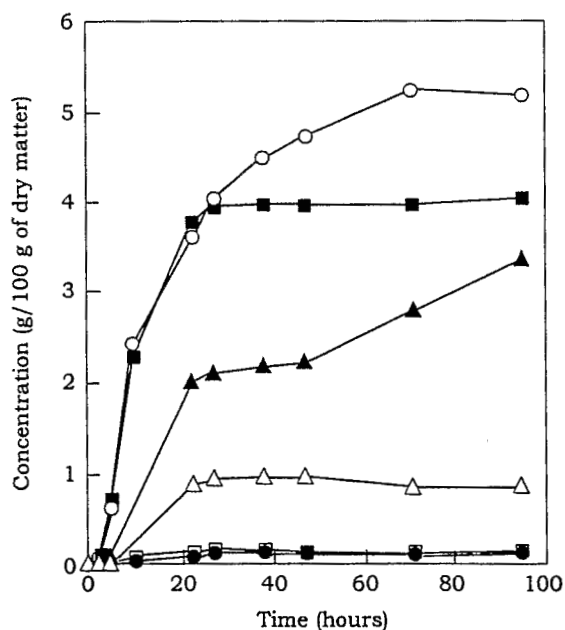


Figure 6. Evolution of lactate and acetate concentration during cassava fermentation. (▲ = lactate and △ = acetate in natural fermentation; ○ = lactate and ● = acetate in fermentation inoculated with *Lactobacillus plantarum* A6; ■ = lactate and □ = acetate in fermentation inoculated with *L. plantarum*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