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Production of a *Lactobacillus plantarum* Starter with Linamarase and Amylase Activities for Cassava Fermentation

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Abstract: *Lactobacillus plantarum* strain A6 isolated from cassava, cultured on cellobiose MRS medium showed a growth rate of 0·41 h^{-1}, a biomass yield of 0·22 g g^{-1}, and produced simultaneously an intracellular linamarase (76·4 U g^{-1} of biomass) and an extracellular amylase (36 U ml^{-1}). The synthesis of both enzymes was repressed by glucose. The use of such a strain as a cassava fermentation starter for gari production had the following influences: a change from a heterofermentative pattern observed in natural fermentation to a homofermentation, a lower final pH, a faster pH decline rate and a greater production of lactic acid (50 g kg^{-1} DM). However, this starter did not appear to play a significant role in cassava detoxification, since it was observed that the level of endogenous linamarase released during the grating of the roots was sufficient to permit the complete and rapid breakdown of linamarin.

Key words: fermented cassava, lactic acid bacteria, starter, *Lactobacillus plantarum*, amylase activity, linamarase activity, cyanide, *Manihot esculenta* Crantz, gari.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple food for 500 million people (Cock 1982). However, its use as a food is influenced by its toxicity. Cassava roots contain two cyanoglucosides, linamarin (2-(β-D-glucopyranosyloxyl) isobutyronitrile) and lotaustralin (2-(β-D-glucopyranosyloxyl) methylbutyronitrile)). Although much of these toxic components is removed during processing, in various foods a quantity still remains, depending on the process used (Nambisan and Sundaresan 1985). Daily consumption of these foodstuffs which still contain residual levels of cyanogenic compounds can result in chronic diseases such as goitre, cretinism, tropical atoxic neuropathy and tropical diabetes (Cock 1982).

During gari preparation, two stages appear to have a significant role in cassava detoxification (Nambisan and Sundaresan 1985; Vasconcelos *et al* 1990): (i) the grating or mincing of the roots permits, through the cell structure damage, the releasing of endogenous linamarase (EC 3.2.1.21, linamarin β-D glucoside glucohydrolase) able to hydrolyse linamarin into glucose and cyanohydrin (Conn 1969); and (ii) the roasting allows the removal of residual free cyanide (acetone cyanohydrin and HCN). On the other hand, the natural fermentation stage, through the development of lactic acid microflora, contributes to the sensory qualities of the final product (Ngaba and Lee 1979; Dougan *et al* 1983).

As reported by Mkpong *et al* (1989) and Ikediobi and Onyike (1982), the endogenous linamarase content could not permit the complete breakdown of linamarin. However, it was demonstrated (Ikediobi and Onyike 1982) that it is possible to reduce the gari toxicity by the addition of an exogenous linamarase during the fermentation. Many authors (Ikediobi and Onyike, 1982; Padmaja and Balagopal 1985; Okafor and Ejiofor 1990) have suggested the inoculation of fermenting cassava with a linamarase-producing microorganism. Bearing

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this in mind, the authors have recently reported that various lactic acid bacteria have the ability to hydrolyse linamarin (Giraud *et al* 1992). *Lactobacillus plantarum* strain A6, isolated from retted cassava for its amylolytic activity (Giraud *et al* 1991a), appeared to be the more suitable. Indeed, after culture on cellobiose MRS medium this strain showed a strong linamarase activity.

The purpose of this work was to study the physiology of this microorganism in order to produce a starter with high linamarase and amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) activities and evaluate the potential for use of such a strain in cassava fermentation.

EXPERIMENTAL

Strains

The microorganisms used were *Lactobacillus plantarum* Lacto-Labo (Dange Saint Romain, France) and *L plantarum* strain A6 isolated from retted cassava (Giraud *et al* 1991a). The strains were conserved in glycerol at −80°C in 1 ml Nunc tubes.

Media and culture conditions

The composition of the basic medium was identical to that of MRS medium (de Man *et al* 1960). For our purposes glucose was replaced by (a) cellobiose 20 g $litre^{-1}$; (b) cellobiose 10 g $litre^{-1}$ and glucose 10 g $litre^{-1}$; and (c) cellobiose 10 g $litre^{-1}$ and soluble starch 10 g $litre^{-1}$ (Prolabo). Strains were cultured in 2-litre bioreactors (Biolaffite, Poissy, France) at 30°C and agitated to 200 rpm; pH was adjusted to 6·0 by addition of 5 M NaOH. Inoculation at 100 ml $litre^{-1}$ was performed with a 20 h pre-culture in the same medium as used for fermentation.

Analytical methods

The biomass, the lactic acid, glucose and cellobiose contents, as well as total sugars were determined by the methods described by Giraud *et al* (1991b).

Enzyme assays

Amylase activity was assayed by adding 0·1 ml of culture supernatant to 0·8 ml of a solution containing 12 g $litre^{-1}$ of Prolabo soluble starch (Paris, France) in 0·1 M citrate/phosphate buffer pH 5·5. The reaction was stopped by addition of 0·1 ml of 1 M H_2SO_4. After incubation at 55°C, residual starch at different time periods was determined colorimetrically at 620 nm by adding 0·1 ml of the reaction mixture to 2·4 ml of an iodine solution (KI, 1·2 g; I_2, 0·12 g; distilled water 1 litre). One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions given above.

Linamarase activity was assayed on whole cells by the method described by Giraud *et al* (1992).

Assays on gari

Fresh imported cassava tubers from Cameroon were obtained from Anarex (Paris, France). Gari was prepared from peeled washed cassava roots, chopped and minced in a food mixer. The pulp obtained was packed tightly into plastic sterile screw-capped containers (60 ml; OSI, A12.160.56) and held at 30°C. Three batches were prepared: (a) natural fermentation, using the endogenous microflora present; (b) fermentation after inoculation with *L plantarum* A6 (10^8 cfu g^{-1} of dry cassava) previously cultured in bioreactors on cellobiose MRS medium; (c) fermentation after inoculation with *L plantarum* Lacto-Labo (10^8 cfu g^{-1} of dry cassava) previously 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 parameters as follows.

- The pH was measured on a sample (10 g) homogenised in distilled water (20 ml). Moisture was measured by drying a sample (10 g) at 105°C for 24 h.
- The linamarase activity indigenous in the cassava was determined by the method of Giraud *et al* (1992) on the supernatant (0·1 ml) from a sample (10 g) homogenised in 10 ml 0·1 M phosphate buffer pH 6·0, chopped and mixed for 1 min at 4°C using an Ultra-Turrax (Janke and Kunkel, Ika-Werk, Staufen, Germany) and centrifuged (10000 × *g*, 10 min).
- Lactic acid bacteria (LAB) were estimated on a sample (10 g) homogenised in 90 ml of sterile physiological solution; colonies were counted on MRS agar using a spread plate technique on Petri dishes, after incubation (30°C, 48 h).
- To measure cyanide compounds and organic acids, 10 g of sample were homogenised in 10 ml 0·05 M H_2SO_4 and chopped and mixed for 1 min at 4°C using an Ultra-Turrax; the assays were carried out on the supernatant obtained from centrifugation.

Organic acids were determined by HPLC under the conditions described by Giraud *et al* (1991b). Cyanide compounds were measured by a procedure based on the method of Cooke (1978); HCN was assayed using a Merck Spectroquant Kit (ref. 14800) by omitting the linamarase (a pellet from 1 ml of an *L plantarum* A6 cultured on MRS cellobiose medium) and NaOH from

the assay. Linamarin was determined as the difference between free and total cyanides, and cyanohydrin as the difference between free cyanides and HCN.

RESULTS AND DISCUSSION

Production of an *L plantarum* starter

Figures 1–3 represent growth kinetics, sugar consumption, and linamarase and amylase production of *L plantarum* A6 cultured on different media as indicated in the experimental section. The main fermentation parameters are shown in Table 1. In all three tested media, biomass productivity and growth rates were high and practically identical. However, linamarase and amylase amounts differed with the medium used.

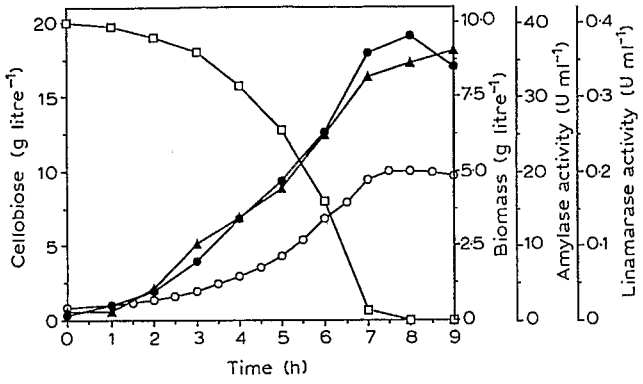

Fig 1. Fermentation of *L plantarum* A6 on cellobiose MRS medium at 30°C and pH 6·0: □, cellobiose; ○, biomass; ▲, amylase activity; ●, linamarase activity.

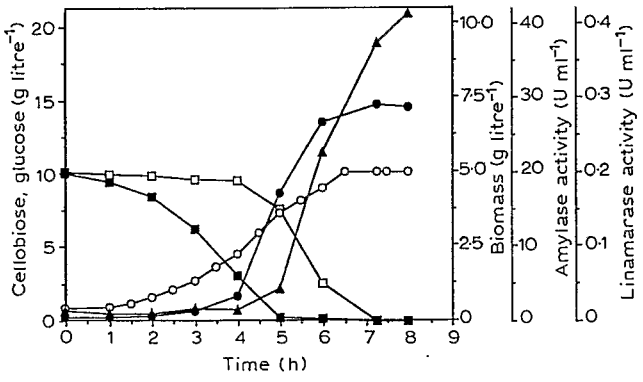

Fig 2. Fermentation of *L plantarum* A6 on cellobiose–glucose MRS medium at 30°C and pH 6·0: ■, glucose; □, cellobiose; ○, biomass; ▲, amylase activity; ●, linamarase activity.

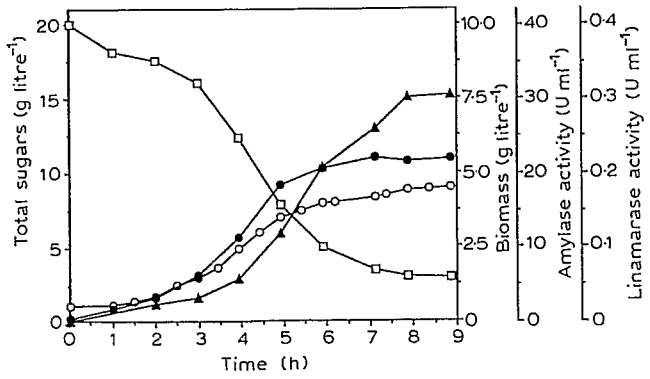

Fig 3. Fermentation of *L plantarum* A6 on cellobiose–starch MRS medium at 30°C and pH 6·0: □, total sugars; ○, biomass; ▲, amylase activity; ●, linamarase activity.

On cellobiose MRS medium

Linamarase and amylase synthesis occurred at the start of fermentation and seemed to be related to biomass formation. Linamarase concentration at the end of the fermentation was 76·4 U g^{-1} of biomass. A recent study (Giraud *et al* 1992), carried out in flasks on the same medium, demonstrated that the amount of linamarase produced was 29 U g^{-1} of biomass. In the bioreactor, under controlled conditions, it increased 2·6 times. It was noticed that the strain produced an amylase while there was no starch in the medium, moreover, the amount was higher than that obtained on cellobiose–starch MRS medium.

On cellobiose–glucose MRS medium

The kinetics indicated that glucose was rapidly consumed during the first 4 h of fermentation, while cellobiose content remained constant. It appeared that linamarase was not formed as long as the glucose concentration remained at about 3 g $litre^{-1}$. Extremely rapid uptake of

TABLE 1
Fermentation parameters of *L plantarum* A6 cultured on various media at pH 6·0 and 30°C

Medium	*Growth rate* (h^{-1})	*Biomass*		*Enzyme activity*	
		Concn. ($g\ litre^{-1}$)	*Yield* ($g\ g^{-1}$)	*Linamarase* ($U\ g^{-1}$)	*Amylase* ($U\ ml^{-1}$)
MRS cellobiose	0·41	5	0·22	76	36
MRS cellobiose–glucose	0·46	5	0·23	59	42
MRS cellobiose–starch	0·44	4·5	0·23	49	30

cellobiose was then observed and it correlated well with considerable synthesis of linamarase (β-glucosidase). Amylase synthesis was slightly retarded and occurred when the glucose concentration was approximately zero; about 80–90% of the enzymes was synthesised during this stage. It is interesting to note that the production of amylase reached a level higher than that observed on the two other tested media.

Glucose had a repressive effect on the synthesis of the two enzymes and the effect was more significant on amylase synthesis. Previous work by Abalaka and Garba (1989) also provided evidence of the strong influence of glucose content on the linamarase production for various fungi. This repressive effect of glucose on the amylase synthesis has already been reported (Giraud *et al.* 1991a).

On cellobiose–starch MRS medium

The growth profile differed from those in the other two media tested, and the biomass production was slightly lower (10%). A linear growth phase was observed after 5 h of fermentation and it seemed to be related to a difficulty on the part of the microorganism to utilise starch degradation by-products with consequent retardation of total sugar consumption.

Concerning enzyme synthesis, linamarase and amylase production was noted from the initial stages of the fermentation. Nevertheless, while linamarase production appeared to be related to the biomass formation as observed on cellobiose MRS medium, amylase was produced mainly during linear growth. Thus, the amount of amylase produced rose by 250% while biomass only increased by 30%.

This study showed that the substrate used influenced strongly the amounts produced, and the production kinetics, of both linamarase and amylase. The high biomass yield, and the strong linamarase and amylase activities observed after culture on cellobiose MRS medium, suggests that *L plantarum* strain A6 can be used as a very suitable starter for cassava fermentation.

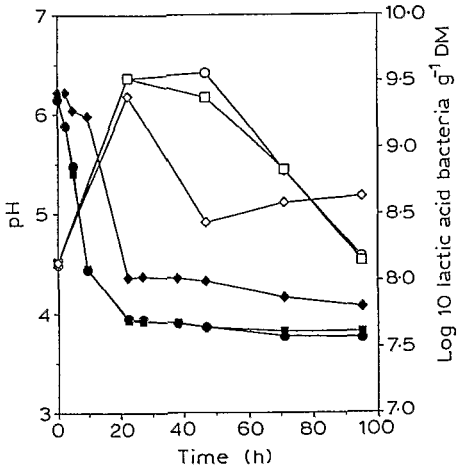

Fig 4. Changes in pH and numbers of lactic acid bacteria (LAB) during cassava fermentation. Natural fermentation (◆, pH; ◇, LAB); inoculated with *L plantarum* A6 (●, pH; ○, LAB); inoculated with *L plantarum* Lacto-Labo (■, pH; □, LAB).

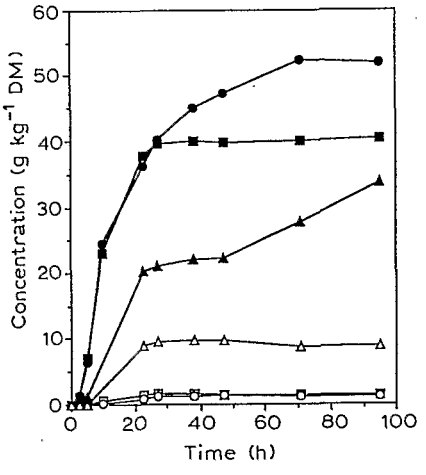

Fig 5. Evolution of lactate and acetate concentration during cassava fermentation. Natural fermentation (▲, lactate; △, acetate); inoculated with *L plantarum* A6 (●, lactate; ○, acetate); inoculated with *L plantarum* Lacto-Labo (■, lactate; □, acetate).

Inoculation effect of *L plantarum* on cassava fermentation

Three different types of fermentation were carried out: (a) natural cassava fermentation, (b) cassava inoculated with *L plantarum* A6, (c) cassava inoculated with a control strain *L plantarum* Lacto-Labo.

Variation of pH, organic acids and LAB

In all three types, a rapid pH decrease was observed from the start (Fig 4). The naturally fermented cassava (type 1) showed a steep fall from 6·2 to 4·3 and types 2 and 3 both fell from 6·2 to 3·9. This pH shift was correlated with lactic acid, the principal metabolite produced (Fig 5). These data confirm that the LAB are the predominant fermentative microflora, reaching 5×10^9 cfu g^{-1} after 24 h of fermentation in all three types of fermentation (Fig 4).

In natural cassava fermentation simultaneous production of lactic and acetic acids, and traces of propionic and butyric acids and ethanol, were observed within the first 24 h. However, while the acetate content reached its maximum level (10 g kg^{-1} DM) and remained constant after the first day of fermentation, an increase in lactate concentration was noticed from the second day. This may suggest that natural fermentation was initiated by heterolactic bacteria and was later supplanted by homolactic bacteria which are more acid-tolerant. This hypothesis is in agreement with the results of Oyewole

and Odunfa (1990) who reported a predominant development of *Leuconostoc mesenteroides*—in the study of characterisation and distribution of the lactic acid microflora during the preparation of fufu—replaced subsequently by *L plantarum*. Therefore, they suggested that this sequence is due to the inability of *L mesenteroides* to tolerate an increase in acidity.

In the inoculated fermentations the lactic acid content was higher. The production kinetics of this acid were identical for both *L plantarum* tested strains during the first 24 h. However, while for the control strain this concentration reached its maximum (40 g kg^{-1} DM) and then remained at that level, for the amylolytic strain (*L plantarum* A6) we found that the lactate production continued to rise, increasing by 25%.

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

Development of cyanide compounds and endogenous linamarase

In all three types of fermentation cyanides, present initially as linamarin, were transformed in less than 5 h to acetone cyanohydrin and HCN (Fig 6). The amount of free cyanide then remained constant until the end of the fermentation. Nevertheless, in the inoculated fermentations, it was observed that the proportion of acetone cyanohydrin was higher. This phenomenon appears to be related to a faster pH decrease that results, as reported by Cooke (1978), in a lower rate of acetone cyanohydrin breakdown to acetone and HCN.

Here, it appeared that the amount of cassava-indigenous-linamarase released during the grating stage was sufficient to permit the complete and rapid hydrolysis of linamarin. It was observed that this enzymatic activity decreased during the fermentation period (Fig 7). In inoculated fermentations this was more significant and may be associated with protein hydrolysis by *L plantarum* or by a higher and faster pH decrease promoting denaturation of cassava-indigenous-linamarase.

The work reported here showed that the inoculation of cassava pulp with a strain of LAB possessing a strong linamarase activity does not appear to contribute to cassava detoxification, and may have a mostly antagonistic effect. This result is in agreement with Vasconcelos *et al* (1990) who reported that 95% of initial linamarin is hydrolysed 3 h after grating the roots. In contrast, it differs from those showed by Ikediobi and Onyike (1982) and Okafor and Ejiofor (1990) who observed that linamarase addition, or the inoculation with a strain having linamarase activity, may improve detoxification. The observed differences may be explained by the use of non-traditional means for the preparation of gari, particularly during the grating of the

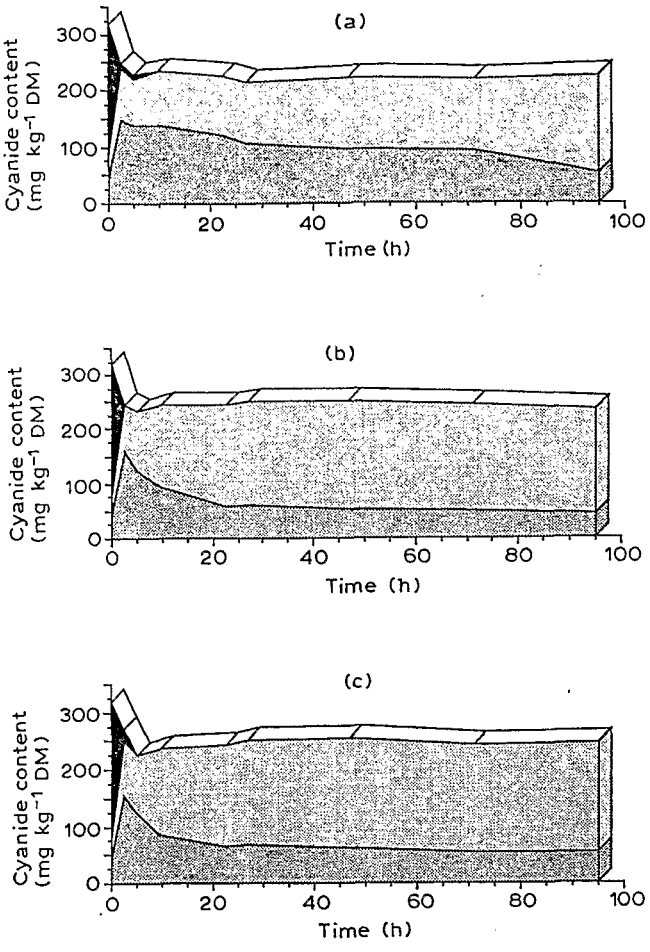

Fig 6. Changes in linamarin (dark shading), cyanohydrin (light shading) and HCN (medium shading) during cassava fermentation. (a) Natural fermentation; (b) inoculated with *L plantarum* A6; (c) inoculated with *L plantarum* Lacto-Labo.

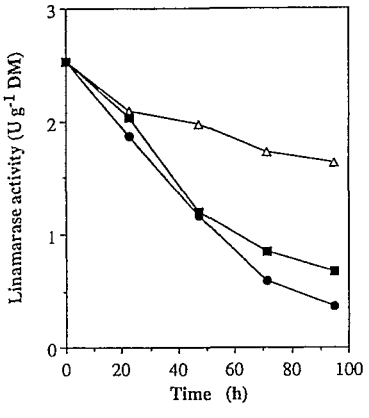

Fig 7. Change in linamarase activity during cassava fermentation. △, Natural fermentation; ●, inoculated with *L plantarum* A6; ■, inoculated with *L plantarum* Lacto-Labo.

cassava roots or as Mkpong *et al* (1990) noticed, by the utilisation of cassava varieties showing different levels of indigenous linamarase.

However, despite this, our work demonstrates that the utilisation of *L plantarum* A6 as a starter may play a

significant role in the development of sensory qualities, and in the standardisation and preservation of the final product by the large amounts of lactic acid produced and the resultant faster and more significant pH decrease.

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