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DEGRADATION OF CASSAVA LINAMARIN BY LACTIC ACID BACTERIA

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Summary

Six out of ten lactic acid bacteria strains tested displayed linamarase activity. *Lactobacillus plantarum* strain A6 displayed the greatest activity affecting 36 U/g cells on MRS cellobiose. The strain also broke down in less than 2 hours the linamarin extracted from cassava juice. HPLC analysis of the products of the reaction showed that the bacteria converted the linamarin into lactic acid and acetone cyanohydrin.

Introduction

The roots of cassava (*Manihot esculenta* Crantz) form the staple diet of over 500 million people in developing countries (Cock, 1985). However, they have a high level of toxicity as they contain large amounts of two cyanoglucosides, linamarin (96%) and lotaustrolin (4%) (Butler, 1965). Most processes for preparing traditional cassava-based foods therefore include natural fermentation stages of varying lengths to eliminate the two substances (El Tinay, 1984; Ayenor, 1985) and also to preserve the cassava through the production of large amounts of lactic acid (Okafor *et al.*, 1984 ; Regez *et al.*, 1988). This detoxication is mainly related to the presence of linamarase, an endogenous ß-glucosidase, which is released when the cell structure is damaged and enables the hydrolysis of linamarin into glucose and acetone cyanohydrin (Conn, 1969). It would appear that the amount of enzyme released or the highly acid conditions during fermentation do not permit the complete breakdown of linamarin (Ikediobi and Onyike, 1982a and 1982b). Daily consumption of these foodstuffs which still contain residual levels of cyanogenic compounds can result in chronic toxicity causing neurological and metabolic disturbances.

Our aim in the work reported here was to show that lactic acid bacteria can be used to break down the linamarin in cassava and contribute to better understanding of the mechanisms involved.

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Materials and Methods

Strains: The microorganisms used were *Lactobacillus plantarum* (Lactolabo, Dange Saint Romain, France), *L. plantarum* A6 and A43 isolated from retted cassava (Giraud *et al.*, 1991a), *Lactobacillus brevis* CNCM 102806, *Lactobacillus amylophilus* CNCM 102988T, *Streptococcus lactis* CNRZ 145, *Streptococcus equinus* CNCM 103233, *Leuconostoc mesenteroides* INRA 20G, *Leuconostoc cremoris* CNCM 103009 and *Pediococcus pentosaceus* CNCM 102260. The strains were conserved in glycerol at -80°C in 1 ml Numc tubes.

Media and culture conditions: The composition of the basic medium was identical to that of MRS medium (Man *et al.*, 1960). According to the experiment, glucose was replaced by 2% cellobiose or another carbon substrate. The pH values of the various media were adjusted to 6.7-6.8 before autoclaving. Culture media seeded from Numc tubes were incubated at 28°C for 20 hours (50 ml of medium in a 125 ml Erlenmeyer flask).

Analytical methods: Biomass was determined by measuring OD at 540 nm. Lactic acid, glucose, acetic acid, ethanol, linamarin and acetone cyanohydrin contents were determined in the supernatant by HPLC under the conditions described by Giraud et *al.* (1991b).

Determination of enzymatic activities: Aryl-ß-glucosidase was measured in whole cells resuspended in acetate 0.1 M buffer, pH 5.5, at 30°C with 4-nitrophenyl ß-D-glucopyranoside (pNPG) substrate at a final concentration of 0.01M. Determination of the p-nitrophenol released was performed according to Blondin *et al.* (1983). Linamarase activity was measured under the same conditions with pNPG replaced by linamarin (Sigma ref. L9131). The cyanides produced in the basic medium by spontaneous breakdown of the acetone cyanohydrin released by the enzyme were assayed using a Merck Spectroquant kit (ref. 14800). One enzymatic unit is defined as the quantity of enzymes which enables the hydrolysis of one µmole of substrate (pNPG or linamarin) per min.

Breakdown by *L. plantarum* A6 of linamarin extracted from cassava juice: 200 g of fresh cassava was grated in 64 ml of orthophosphoric acid 0.5M and then ground twice for 1 min in a Warren blender to extract linamarin. The juice was recovered by pressing, adjusted to pH 9 with KOH 10N and boiled for 5 min to inactivate endogenous linamarase. The pH was adjusted to 6 after cooling. The following were added to different 3 ml aliquots of this extract: 1 mg of linamarin (Assay 1), 0.5 IU of linamarase (Assay 2), a pellet from 5 ml of an *L. plantarum* A6 culture on MRS cellobiose (Assay3) and 1 mg of linamarin + a pellet from 5 ml of an *L. plantarum* A6 culture (Assay 4). The appearance of cyanide was monitored in the various fractions thus prepared.

Results and Discussion

Linamarase and aryl-ß-glucosidase activities in different lactic acid bacteria

Linamarase and aryl-ß-glucosidase activities were measured after culture of 10 lactic acid bacteria on MRS cellobiose; 7 of the bacteria were chosen at random from a collection (Table 1). Of the 10 strains tested, 6 displayed linamarase activity (*L. plantarum* (Lactolabo), *L. plantarum* A6, *L. plantarum* A43, *Streptococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*). The ability of lactic acid bacteria to break down linamarin does not therefore appear to be an exceptional characteristic.

Table 1. Comparison of linamarase and aryl-ß-glucosidase activities of different lactic acid bacteria cultured on MRS cellobiose medium.

Organism	Linamarase U/g dry biomass	Aryl-ß-glucosidase U/g dry biomass
L. plantarum A6	35.5	12.1
L. plantarum A43	31.4	12.0
L plantarum (Lactolabo)	30.5	9.9
L. brevis	0.0	0.9
L. amylophilus	0.0	0.0
S. lactis	19.0	19.1
S. equinus	0.0	0.0
L. mesenteroides	4.4	5.7
L. cremoris	0.0	0.0
P. pentosaceus	26.6	41.2

No activity was detected in the culture supernatant of any of the strains tested. It is noted that in general (except for *L. brevis*) the strains with aryl-ß-glucosidase activity also possessed linamarase activity. This is in agreement with the results of Okafor and Ejiofor (1986). However, we cannot claim on the basis of these results that the hydrolysis of pNPG and linamarin are caused by the same enzyme.

The *L. plantarum* strains seem to be the most interesting of the 6 strains. They displayed the strongest linamarase activities measured (over 30 U/g) and the most growth (3 g/l). *L. plantarum* A6 was chosen for the subsequent part of the study because of its strongly amylolytic nature (Giraud *et al.*, 1991a).

Induction of linamarase and aryl-ß-glucosidase activities in *L. plantarum* A6

Enzymatic activity was measured after growth on different carbon sources to determine the most suitable carbon substrate for maximum linamarase production. The results are shown in Table 2. Linamarase activity was observed with all the carbon sources but different quantities of enzyme were observed. Linamarase production was low on maltose, glucose, lactose, sucrose and starch. The amount was larger on melibiose but still a third of that produced with cellobiose as substrate. It would seem that linamarase is a constitutive enzyme in *L. plantarum* A6 and the amount of enzyme can be increased by induction. These results are different to those of Okafor (1985) for *L. mesenteroides* in which linamarase activity is present only if linamarin is used as carbon source.

Table 2. Pattern of linamarase and ß-glucosidase activities of *L.plantarum* A6 cultured on different carbon sources

Carbon source	Linamarase U/g dry biomass	aryl-ß-glucosidase U/g dry biomass	Ratio of Linamarase to aryl-ß-Glucosidase
Cellobiose	35.5	12.1	2.9
Melibiose	12.1	15.1	0.8
Lactose	6.2	8.6	0.7
Starch	5.6	7.4	0.8
Sucrose	3.4	7.0	0.5
Maltose	1.5	2.7	0.6
Glucose	1.5	3.4	0.4

The ratio of linamarase and aryl-ß-glucosidase activities was less than 1 for all the substrates studied except cellobiose, for which the ratio was about 3. Cellobiose may therefore induce 2 ß-glucosidases, as was observed in *Saccharomyces cerevisiae* (Kaplan and Tacreiter, 1966). In the present case, the ß-glucosidases may have different affinities for pNPG and linamarin. Purification tests of *L. plantarum* ß-glucosidases are therefore in progress to determine their biochemical characteristics more accurately.

HPLC analysis of the products of degradation of linamarin by *L. plantarum* A6

L. plantarum cells cultured on MRS cellobiose were washed and resuspended in an acetate buffer containing linamarin. The reaction mixture was analysed by HPLC after different incubation times. The chromatograms are shown in Figure 1.

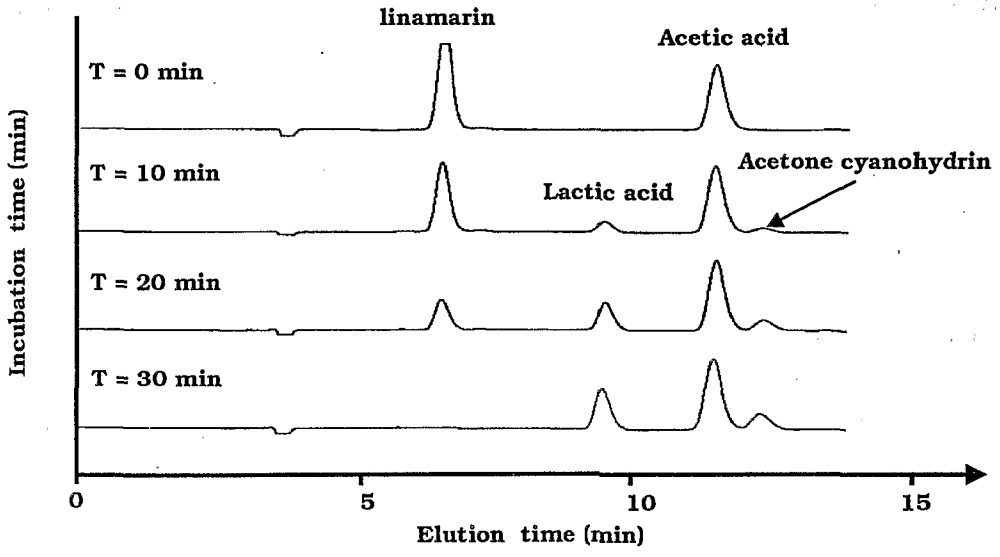

Fig. 1. HPLC analysis of linamarin degradation products by *L.plantarum* A6 cells after diffent incubation times. Temp: 30°C, pH: 5.5 .

linamarin was fully broken down into lactic acid and acetone cyanohydrin after incubation for 30 min. The glucose produced by linamarin hydrolysis was thus converted immediately into lactic acid by the bacteria which appeared to function as resting cells. This shows unambiguously that this strain can break down linamarin.

Degradation by L. *plantarum* A6 of linamarin extracted from cassava juice

This study was performed to confirm the ability of *L. plantarum* A6 to break down the linamarin in cassava juice. The endogenous linamarase in cassava was first inactivated (cf. Materials and Methods) to observe the linamarase activity of the bacterium studied. The results are shown in Figure 2.

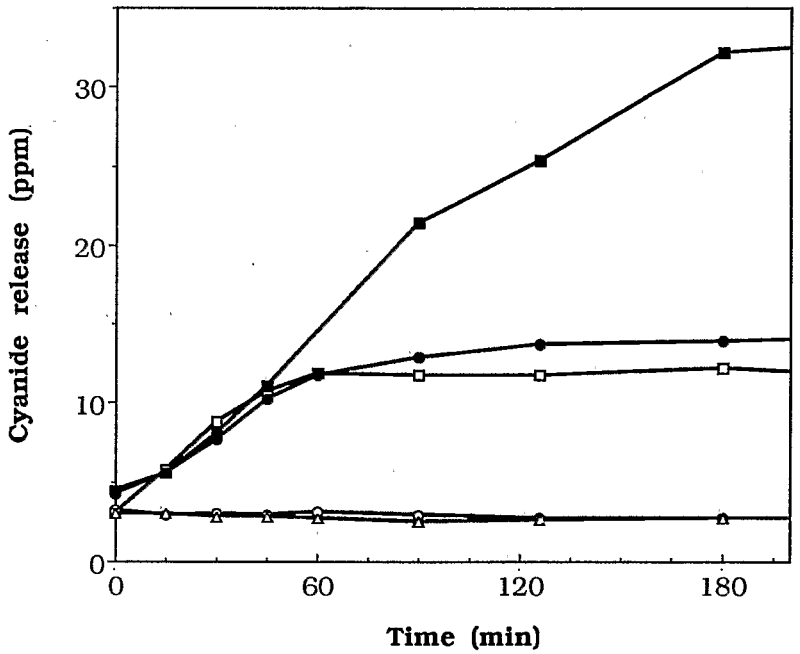

Fig. 2. Release of cyanide from a cassava extract after inhibition of endogenous linamarase. △ , Extract (control) ; ○ , Extract + linamarin (assay 1) ; □ , Extract + linamarase (assay 2) ; ● , Extract + *L. plantarum* A6 (assay 3) ; ■ , Extract + *L. plantarum* A6 + linamarin (assay 4).

It can be seen in the cassava extract (control) that the endogenous linamarase was inactivated as no free cyanide was detected even after addition of linamarin (Assay 1). However, the extract contained linamarin since cyanide release after addition of linamarase increased rapidly and then levelled after 60 min (Assay 2). The addition of *L. plantarum* A6 cells to cassava extract (Assay 3) resulted in release of cyanide comparable to that previously observed. Likewise, the bacterium was able to break down a larger amount of linamarin (Assay 4).

The results of this work thus demonstrate the ability of certain strains of lactic acid bacteria to break down cassava linamarin. The massive inoculation of cassava roots with a lactic acid bacterium strain chosen for its high linamarin breakdown capacity can be envisaged, as has already been suggested (Okafor and Ejiofor 1990; Ikediobi and Onyike 1982b; Padmaja and Balagopal 1985). This would enable better control of natural fermentation of cassava and lead to the production of a standardised, non-toxic and non-perishable foodstuff. Research is currently being performed on this aspect.

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