Appl Microbiol Biotechnol (1990) 33:529–533

Applied and Microbiology Biotechnology

Detoxification of cassava pulp using *Brevibacterium* sp. R312

J. L. Legras, M. Jory, A. Arnaud, and P. Galzy

Chaire de Microbiologie Industrielle et de Génétique des Microorganismes, Ecole Nationale Supérieure Agronomique de Montpellier, Place Viala, 34060 Montpellier Cedex, France

Received 2 January 1990/Accepted 6 April 1990

Summary. *Brevibacterium* sp. strain R312 has an endocellular β-glucosidase, a nitrile hydratase and an amidase that can break down some cyanoglucosides. Nonsterile cassava pulp suspensions were fermented using this strain and 70%–80% reduction of nitrile compounds, in particular cyanoglucosides and α-hydroxynitriles, was observed. This type of nitrile-hydratase-active microorganism could be a solution for the detoxification of cassava. Experiments conducted with the yeasts *Candida molischiana* and *C. wickerhamii* showed no improvement in detoxification.

Introduction

Processed cassava roots represent the staple food for 200–300 million people in developing countries. This crop can be successfully grown with high yield on poor soil and under difficult climatic conditions. However, the consumption of these roots results in a number of nutritional problems due to their low protein content and the presence of cyanoglucosides (linamarin and lotaustralin). Following the crushing of the roots, these cyanoglucosides decompose in a two-stage process: first, an endogenous β-glucosidase (linamarase) liberates glucose and an α-hydroxynitrile that is unstable above pH 5.5, then the nitriles break down yielding a ketone and cyanide.

The local populations are aware of the toxic nature of cassava roots and have developed a number of processes (maceration, soaking, fermentation, boiling, etc.) to eliminate most of the nitrile compounds. However, residual cyanide in the commercially available product varies widely and is far from being harmless. Nartey (1981) and Rosling (1988) described many chronic health troubles observed in populations using cassava as the staple component of their diet, especially under food shortage conditions.

Offprint requests to: A. Arnaud

Several studies have been undertaken to solve this problem either by plant variety selection, or by modifying the traditional processing technology. Ikediobi and Onyike (1982a, b) showed that the addition of exogenous β-glucosidase was effective in reducing the amounts of residual nitrile compounds. However, no studies have been undertaken to verify the possible application of nitrile-hydratase-active microbial strains such as *Brevibacterium* sp. R312 (Arnaud et al. 1976a, b; Bui et al. 1984) for the detoxification of cassava. This strain also possesses β-glucosidase activity (Legras et al. 1989a) and could break down cyanoglucosides (Legras et al. 1989b). The present paper reports on the results obtained when cassava pulp was fermented with *Brevibacterium* sp. strain R312.

Materials and methods

Organisms, media and culture conditions. Brevibacterium sp. R312 was isolated from soil by Arnaud et al. (1976a, b) among other strains and was found to have the greatest nitrile hydratase activity by L'Homme et al. (1981). The two yeast strains were obtained from the Centraalbureau Voor Schimmelculture (Baarn, the Netherlands): *Candida molischiana* (Zikes) Meyer and Yarrow CBS 136 and *C. wickerhamii* (Capriotti) Meyer and Yarrow CBS 2928.

Brevibacterium sp. R312 was grown on the liquid medium described by Legras et al. (1989a) whereas the yeast strains were grown on the glucose medium described by Galzy (1964). The β-glucosidase enzyme extract from *C. molischiana* was produced from medium with added cellobiose (5 $g \cdot l^{-1}$) as the sole carbon source according to the method described by Gondé et al. (1985). All cultures were performed in erlenmeyer flasks filled to 10% of their capacity and shaken (80 oscillations/min, 8 cm amplitude) at 28° C.

The cassava roots (2 kg) were peeled, washed and aseptically blended with sterile water (2.5 l) in a Moulinex blender. The resulting suspension contained 10% dry matter and was divided into 500-ml fractions in seven 5-l erlenmeyer flasks. The erlenmeyers were then inoculated according to the following scheme: mode 1, pulp, control (Fig. 1a); mode 2, pulp, *Brevibacterium* sp. R312 (Fig. 1b); mode 3, pulp, *C. wickerhamii* (Fig. 2a); mode 4, pulp, *C. wickerhamii*, *Brevibacterium* sp. R312 (Fig. 2b); mode 5, pulp, *C. molischiana* (Fig. 3a); mode 6, *C. molischiana*, *Brevibacterium* sp. R312 (Fig. 3b); mode 7, pulp, 250 units of β-glucosidase from *C.*

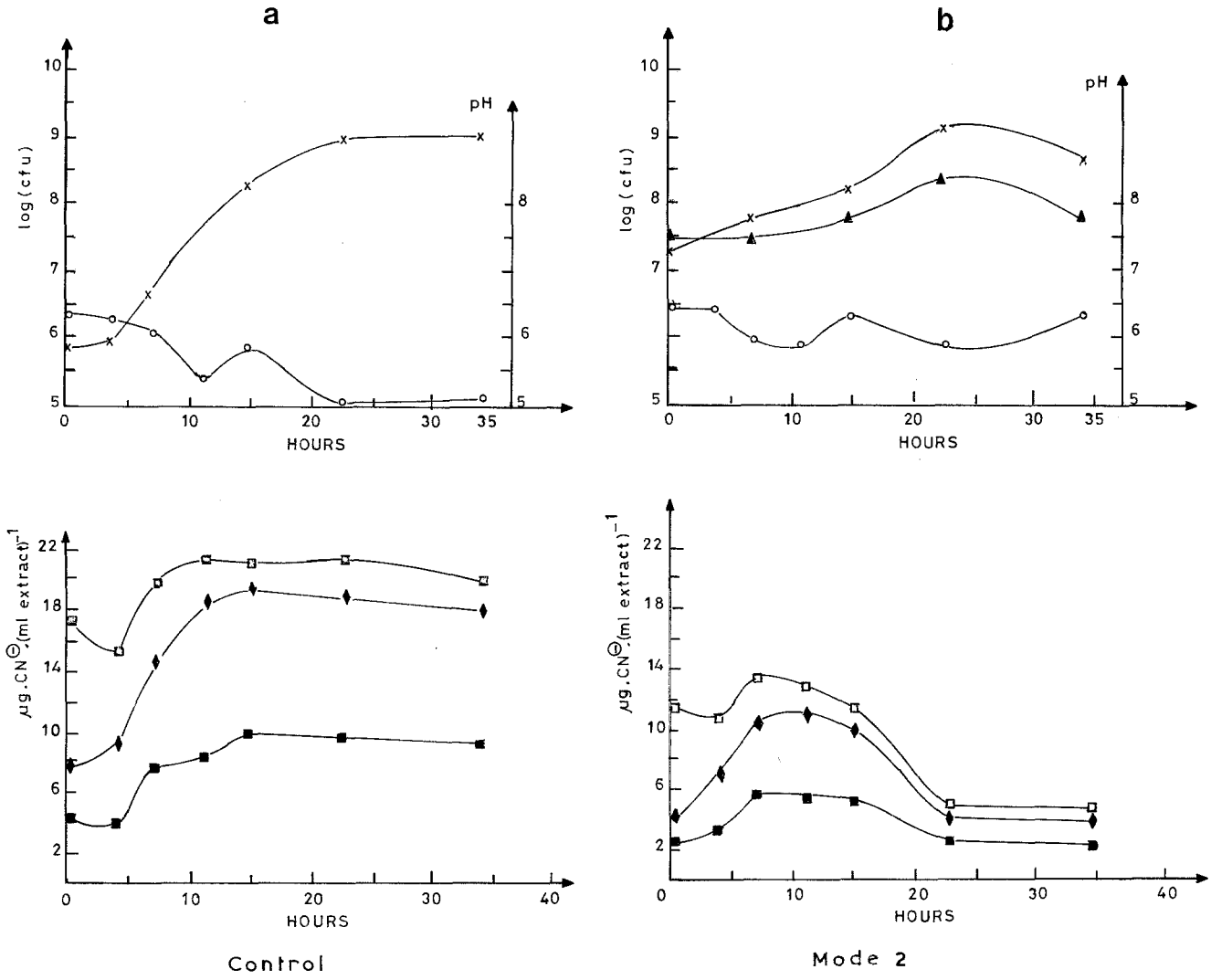

Fig. 1a, b. Fermentation of cassava pulp. **a** Mode 1 (control). **b** Mode 2 (*Brevibacterium* sp. R312): O—O, pH; □—□, total cyanide compounds (CT); ◆—◆, cyanoglucosides + cyanohydrins (CG + CH); ■—■, cyanoglucosides; ×—×, log of colony-forming units (cfu) counted on MRS (see Materials and methods) medium: lactic flora; ▲—▲, log of *Brevibacterium* sp. R312 cfu counted on minimal medium with added 1% acetonitrile

molischiana, *Brevibacterium* sp. R312 (Fig. 4). Since the pulp used was the same for all manipulations, only one control was done.

The activity of the enzyme extract was assayed using 5 m*M* *p*-nitrophenyl-β-D-glucopyranoside (pNPG) at pH 4.0 and 30° C according to the method of Gondé et al. (1985).

Enumeration methods. The microbial populations were estimated using selective media: *Brevibacterium* sp. R312 was counted on the minimal medium with 1% v/v acetonitrile as the sole nitrogen source (Arnaud et al. 1976a, b). *Candida molischiana* and *C. wickerhamii* were enumerated using potato dextrose agar (PDA) containing 0.5 $g \cdot l^{-1}$ chloramphenicol. The lactic flora was counted on MRS medium containing: glucose, 20 $g \cdot l^{-1}$; peptone, 10 $g \cdot l^{-1}$; beef extract, 10 $g \cdot l^{-1}$: yeast extract, 5 $g \cdot l^{-1}$; sodium acetate, 5 $g \cdot l^{-1}$; K_2HPO_4, 2 $g \cdot l^{-1}$; triammonium citrate, 2 $g \cdot l^{-1}$; $MgSO_4$, 0.1 $g \cdot l^{-1}$; $MnSO_4$, 0.05 $g \cdot l^{-1}$ and Tween 80, 80 $ml \cdot l^{-1}$. The pH was adjusted to 6.0 with 1 *N* HCl solution.

Assay methods for cyanide and nitrile compounds. Cyanide, α-hydroxynitrile and cyanoglucosides (linamarin and lotaustralin) were assayed according to the method of Nambisan and Sundaresan (1984): CT represents the total nitrile compounds (cyanide + cyanohydrins + cyanoglucosides), CG the cyanoglucosides, and CH the cyanohydrins. The results are expressed as µmoles of CN^- per millilitre of suspension.

Results and discussion

In order to assess the action of *Brevibacterium* sp. R312, seven fermentations were conducted in parallel as described above. *Candida molischiana* and *C. wickerhamii* are known to produce exocellular β-glucosidases (Gondé et al. 1985; Leclerc et al. 1984) which could complete the action of endogenous linamarase. The results are shown in Figs. 1, 2, 3, 4.

Enumeration results showed that generally there was an increase in the lactic flora. However, no media acidification seemed to occur except in the control; there was no satisfactory lactic fermentation in the other erlenmeyer flasks. The fact that the pH did not drop might be the result of assimilation, by *Brevibacterium* sp. R312 cells, of the lactic acid produced.

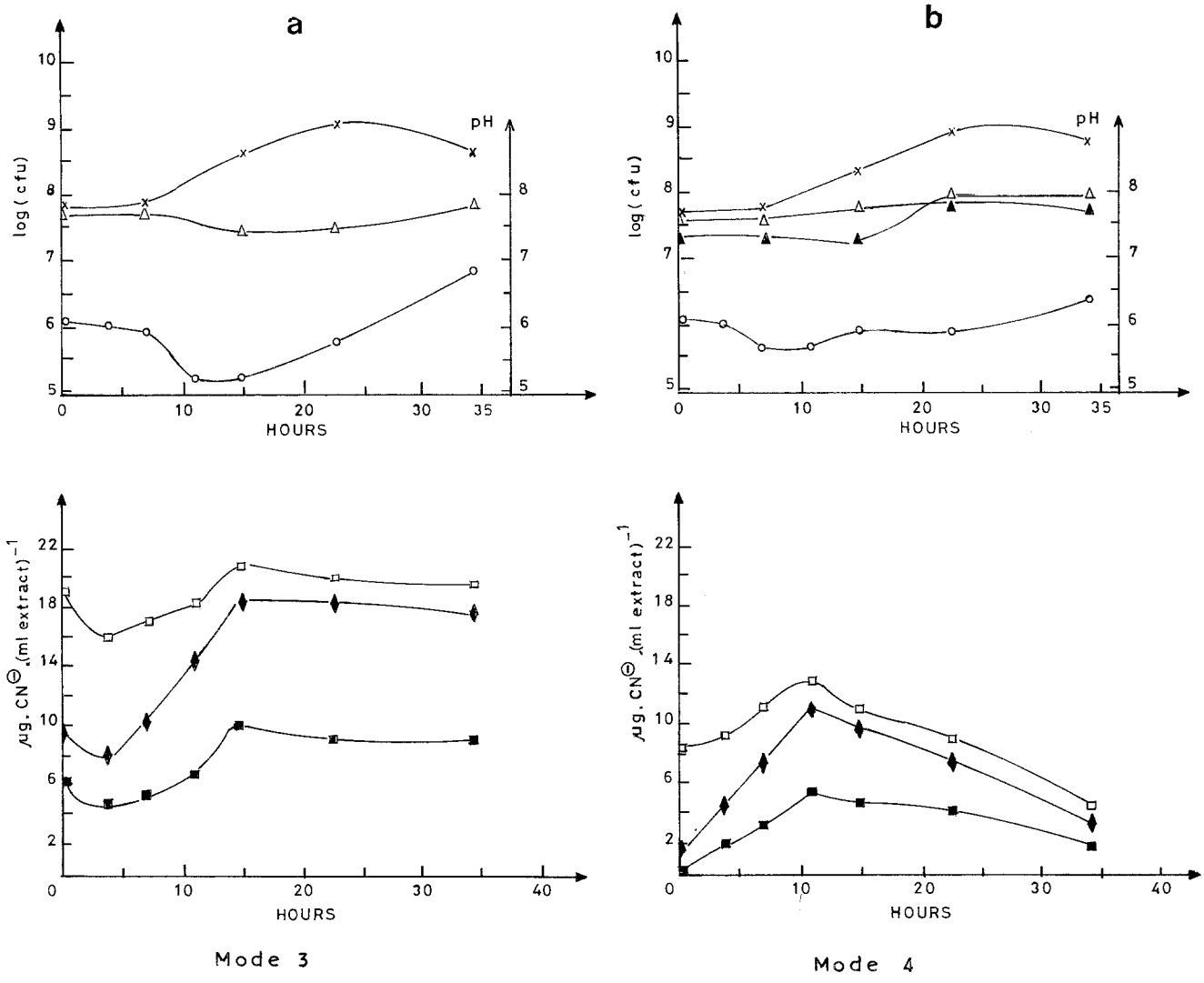

Fig. 2a, b. Fermentation of cassava pulp. **a** Mode 3 (*Candida wickerhamii*). **b** Mode 4 (*C. wickerhamii* + *Brevibacterium* sp. R312): ○—○, pH; □—□, CT; ◆—◆, CG + CH; ■—■, cyanoglucosides; ×—×, log cfu counted on MRS medium: lactic flora; △—△, log cfu counted on potato dextrose agar (PDA) medium + chloramphenicol: yeasts; ▲—▲, log of *Brevibacterium* sp. R312 cfu counted on minimal medium with added 1% acetonitrile

The exogenous strains varied in their development. *Brevibacterium* sp. R312 showed moderate growth following a lag phase that lasted about 15 h. *Candida molischiana* and *C. wickerhamii* did not grow on the medium and thus did not play their expected roles.

The pattern of CT production showed that the fermentation tests could be divided into two groups: those with or without *Brevibacterium* sp. R312. This division was confirmed by Student's *t* test (Scherrer 1984) for the mean CT obtained from either of the two groups. The means of the two groups were significantly different ($\alpha = 0.01$) whereas within the same group the means were not significantly different (Table 1). For each experiment, we have calculated the mean of the seven values obtained for CT (Table 1, column 2). The variance (Table 1, column 3) is an indicator of the variation between those seven values and the calculated mean. In the fourth column are reported the values obtained for the Student's *t* test, which allow us to know if the calculated means are significantly different with a level of significance, α, chosen to equal 1%.

It's noticeable that in mode 5 (Fig. 3a) without *Brevibacterium* sp. R312, there was a drop in the total cyanide concentration. If such a phenomenon was confirmed, it might be the result of a hydrolysis of these compounds by the β-glucosidase of *C. molischiana* followed by assimilation of the products obtained. Nevertheless, being confined only to the last points of the fermentation (corresponding to 35 h), we cannot rule out the hypothesis that it resulted from a manipulation error.

From 70% to 80% of CT was eliminated in the erlenmeyer flasks by *Brevibacterium* sp. R312, mainly the cyanoglucosides and cyanohydrins difficult to eliminate by traditional processing technologies. The degradation of these compounds was particularly apparent following inoculation and towards the end of the fermentation process. The initial degradation was likely

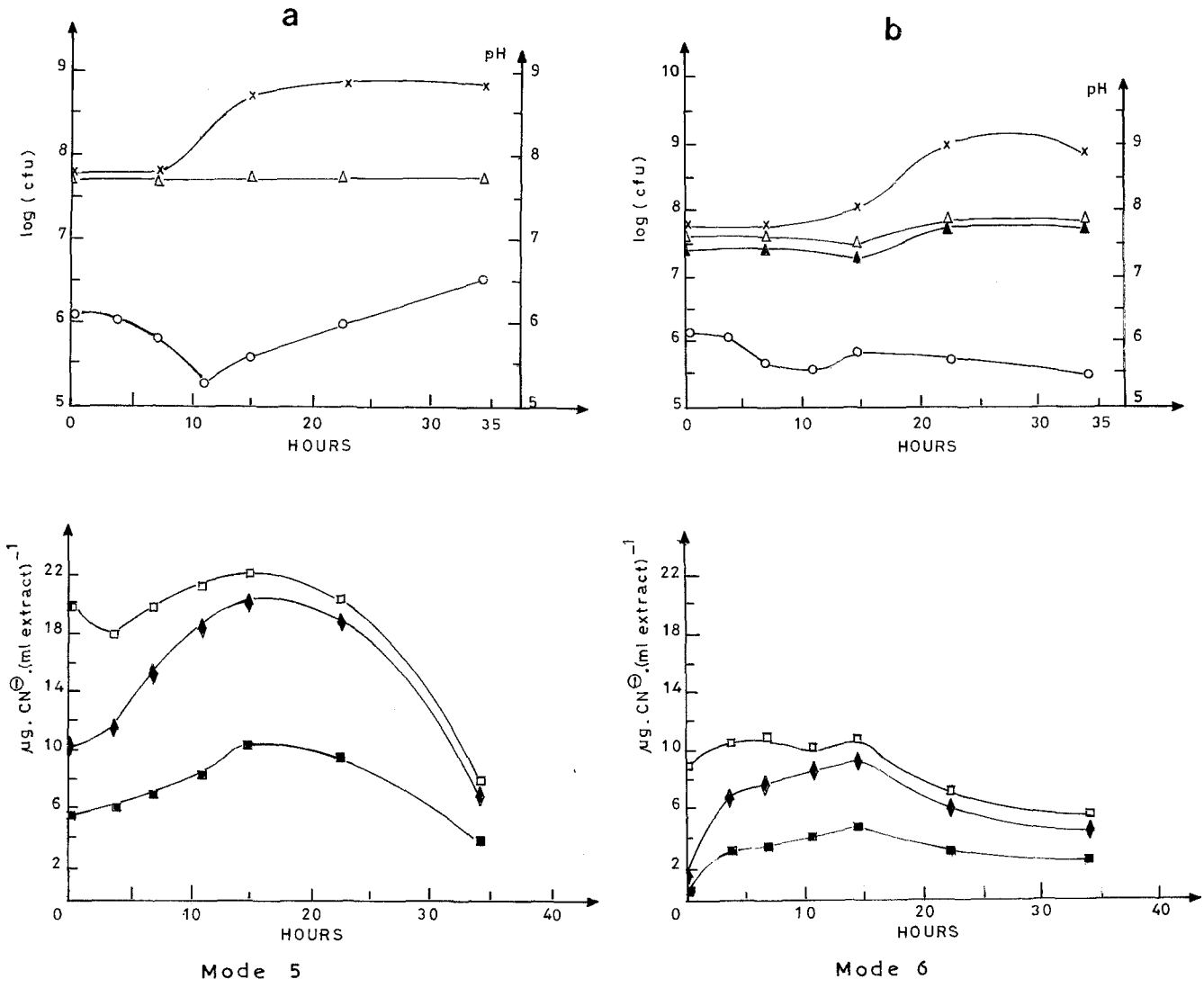

Fig. 3 a, b. Fermentation of cassava pulp. **a** Mode 5 (*C. molischiana*). **b** Mode 6 (*C. molischiana* + *Brevibacterium* sp. R312): O—O, pH; □—□, CT; ◆—◆, CG + CH; ■—■, cyanoglucosides; ×—×, log cfu counted on MRS medium: lactic flora; △—△, log cfu counted on PDA medium + chloramphenicol: yeasts; ▲—▲, log of *Brevibacterium* sp. R312 cfu counted on minimal medium with added 1% acetonitrile

to be due to the high activity of the inoculum whereas the second phase of degradation coincided with the growth of *Brevibacterium* sp. R312. The increase in the nitrile compounds between 5 and 15 h following the start of fermentation was thought to be due to the time required for these compounds to diffuse out of the pulp tissues into the liquid phase of the medium.

The positive action of *Brevibacterium* sp. R312 on the evolution of nitrile compounds from cassava pulp was similarly demonstrated in an aerobic trial in two identical 2-l fermentors. In this test, the difference in CT in the fermentor with the nitrile-hydratase-active strain compared to the control was most apparent at the beginning (30 min following inoculation). Later, volatile nitrile compounds were removed by forced aeration of the fermentors. This was confirmed by evaluating the HCN trapped in NaOH at the exit of the fermentor.

Our study thus shows that the nitrile hydratase activity of *Brevibacterium* sp. R312 could be useful for the detoxification of cassava. However, this process must occur under conditions that are compatible with traditional processes, for example the lactic fermentation of cassava during the preparation of gari and under sanitary conditions. Many microorganisms with this particular activity have already been isolated (Legras et al. 1990). The systematic study of these strains for the purpose of treating cassava would contribute greatly to finding a solution to the toxicity problem of this staple food.

References

Arnaud A, Galzy P, Jallageas JC (1976a) Remarques sur l'activité nitrilasique de quelques bactéries. CR Acad Sci 283:571-573

Arnaud A, Galzy P, Jallageas JC (1976b) Etude de l'activité nitrilasique de quelques bactéries. Rev Ferment Ind Aliment 31:39-44

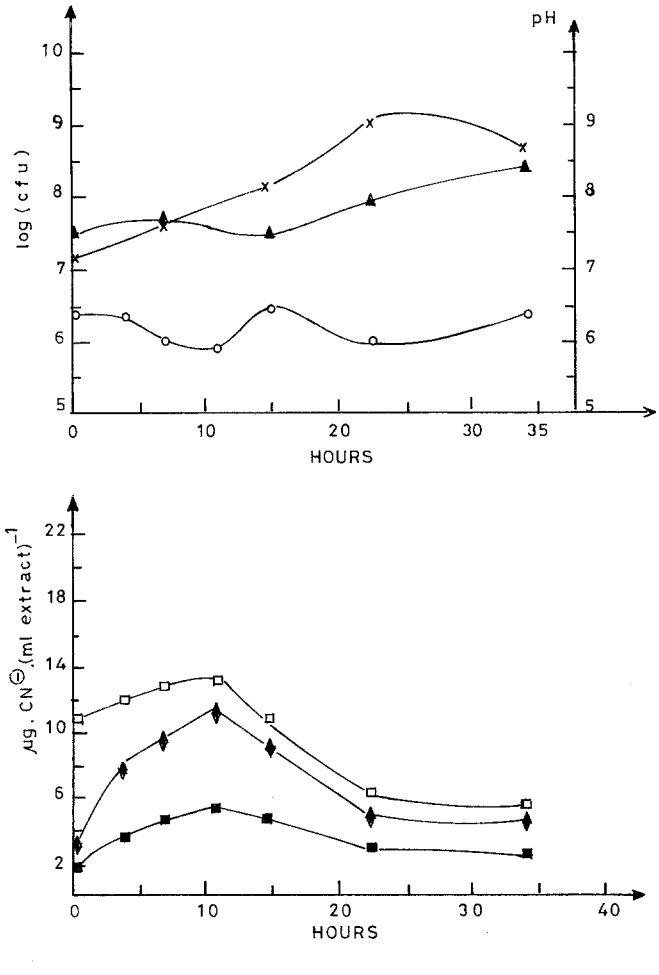

Fig. 4. Fermentation of cassava pulp: mode 7 (β-glucosidase of *C. molischiana* + *Brevibacterium* sp. R312): ○—○, pH; □—□, CT; ◆—◆, CG + CH; ■—■, cyanoglucosides; ×—×, log cfu counted on MRS medium: lactic flora; ▲—▲, log of *Brevibacterium* sp. R312 cfu counted on minimal medium with added 1% acetonitrile

Table 1. Comparison of total cyanide (CT) content in the different fermentation tests

Treatment	Mean (CT) $CN^- \cdot ml^{-1}$	Variance	t^a
Control	19.21	4.24	6.54
Mode 2	9.64	10.73	
Mode 3	18.57	2.62	9.81
Mode 4	9.29	3.65	
Mode 5	18.57	19.28	5.40
Mode 6	8.93	3.04	
Control	19.21	4.24	7.50
Mode 7	9.71	6.9	

For modes 1-7, see Materials and methods

[a] Modes 2 and 7 were compared to the control; modes 3 and 4 and modes 5 and 6 were compared to each other

Bui K, Fradet H, Arnaud A, Galzy P (1984) A ntirile hydratase with a wide substrate spectrum produced by a *Brevibacterium* sp. J Gen Microbiol 130:89-93

Galzy P (1964) Etude génetique et physiologique du métabolisme de l'acide lactique chez *Saccharomyces cerevisiae* Hansen. Thesis, Faculty of Sciences, Paris

Gondé P, Ratomahenina R, Arnaud A, Galzy P (1985) Purification and properties of the exocellular β-glucosidase of *Candida molischiana* (Zikes) Meyer and Yarrow capable of hydrolyzing soluble cellodextrins. Can J Biochem Cell Biol 63:1160-1166

Ikediobi CO, Onyike E (1982a) Linamarase activity and detoxification of cassava (*Maninhot esculenta* Grantz) during fermentation of gari production. Agric Biol Chem 46:1667-1669

Ikediobi CO, Onyike E (1982b) The use of linamarase in gari production. Process Biochem 17:2-5

Leclerc M, Gondé P, Arnaud A, Ratomahenina R, Galzy P, Nicolas M (1984) The enzyme systems in a strain of *Candida molischiana* Meyer and Yarrow participating in the hydrolysis of cellodextrins. J Gen Appl Microbiol 30:509-521

Legras JL, Kaaked MR, Arnaud A, Galzy P (1989a) Purification and properties of the β-glucosidase from a *Brevibacterium* sp. R312 strain with nitrile hydratase activity. J Basic Microbiol 29:655-669

Legras JL, Kaaked MR, Arnaud A, Galzy P (1989b) Degradation of cyanoglucosides by a *Brevibacterium* sp. R312 strain. J Gen Appl Microbiol 35:451-461

Legras JL, Chuzel G, Arnaud A, Galzy P (1990) Natural nitriles and their metabolism. MIRCEN Journal (in press)

L'Homme Ch, Arnaud A, Galzy P, Jallageas JC (1981) Contribution à l'étude de l'activité acétonitrilasique de diverses souches bactériennes. Rev Ferm Ind Alim 36:10-16

Nambisan B, Sundaresan S (1984) Plant toxins. Spectrophotometric determination of cyanoglucosides in cassava. J Assoc Off Anal Chem 67:641-643

Nartey F (1981) Cyanogenesis in tropical feeds and foodstuffs. In: Vennesland B, Conn E, Knowles CJ, Westley J, Wissing F (eds) Cyanide in Biology. Academic Press, London, pp 115-132

Rosling H (1988) Cassava toxicity and food security. A report for UNICEF's African household food security programme. Tryck Kontakt, Uppsala

Scherrer B (1984) Biostatistique. Morin G (ed) Québec, Canada, pp 401-422