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Essers, A.J.A.; Bennik, M.H.J.; Nout, M.J.R.

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Mechanisms of increased linamarin degradation during solid-substrate fermentation of cassava

A.J.A. Essers,* M.H.J. Bennik and M.J.R. Nout

Several fungi and bacteria, isolated from Ugandan domestic fermented cassava, released HCN from linamarin in defined growth media. In 72 h, a *Bacillus* sp. decreased the linamarin to 1% of initial concentrations, *Mucor racemosus* to 7%, *Rhizopus oryzae* and *R. stolonifer* to 30%, but *Neurospora sitophila* and *Geotrichum candidum* hardly degraded the linamarin. Adding pectolytic and cellulolytic enzymes, but not linamarase, to root pieces under aseptic conditions, led to root softening and significantly lower linamarin contents. *Neurospora sitophila* showed no linamarase activity, in contrast to *M. racemosus* and *Bacillus* sp., both of which were less effective in root softening and decreasing the root linamarin content. The most important contribution of microorganisms to linamarin decrease in the solid-substrate fermentation of cassava is their cell-wall-degrading activity, which enhances the contact between endogenous linamarase and linamarin.

Key words: Cassava, cell-wall-degrading enzymes, fungi, linamarin, *Neurospora sitophila*, solid-substrate fermentation.

The tropical starchy root crop cassava (*Manihot esculenta* Crantz) contains small amounts of cyanogenic glucosides, especially linamarin (Butler 1965). Disintegration of the tissue structure results in contact of linamarin with linamarase, which is located in the cell walls (Mkpong *et al.* 1990), and subsequent hydrolysis to glucose and cyanohydrins, which easily break down to ketones and HCN (Cooke 1978). To prevent toxicity, cassava roots with high cyanogen concentration are usually processed before consumption. Many processing methods have been developed by the cassava-farming population (Lancaster *et al.* 1982). One of these, which is used in several African countries and has been studied recently (Essers *et al.* 1995a) involves a 3- to 4-day solid-substrate fermentation in covered heaps after a short initial sun-drying. During incubation, a varied and abundant microflora develops; the roots become covered with a blanket of fungal mycelia and gradually soften from

the outside towards the centre. Subsequently, the mycelia are removed and the roots are crushed and submitted to final sun-drying before being pounded into flour. Incubation alone reduces linamarin contents but the microflora causes an additional effect, associated with root softening (Essers *et al.* 1995b). Two possible mechanisms contributing to this effect are introduction of microbial linamarase to the cassava, and cell-wall-degrading enzymes that permit contact between the compartmentally separated linamarin and endogenous linamarase.

The aim of the present study was to clarify the mechanism(s) of the solid-substrate fermentation process that enhance breakdown of cyanogenic glucosides. The linamarase activity of several microbial strains was evaluated and the effects of enzymatically induced root softening and exogenous linamarase on the cyanogenic glucosides were compared.

A.J.A. Essers and M.H.J. Bennik were and M.J.R. Nout is with the Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703HD Wageningen, The Netherlands. A.J.A. Essers is now with the Department of Toxicology, Wageningen Agricultural University, PO Box 8000, 6700EA Wageningen, The Netherlands; fax: 31 837084931. M.H.J. Bennik is now with the Agrotechnological Research Institute, PO Box 17, 6700AA Wageningen, The Netherlands.

*Corresponding author.

Materials and Methods

Roots, Enzymes and Picrate Paper

Cassava roots from Costa Rica, protected by a wax layer, were obtained commercially in the Netherlands. Linamarase (7.6 U/ml)

was extracted from fresh Ugandan roots according to Cooke (1979). One unit (U) was defined as the amount hydrolysing 1 μmol linamarin/min at 30°C in 0.1 M phosphate, pH 6.0. Picrate-impregnated paper was made by immersing strips of filter paper in a solution of equal volumes of saturated picric acid and 5% (w/v) Na_2CO_3 . Pectinex Ultra-sp (Novo Nordisk, Denmark) (0.5%, w/v) was used for cell-wall degradation.

Growth Media

The yeasts and moulds, bacilli, and lactic acid bacteria were pre-cultured on yeast nitrogen base (YNB; Difco) plus 1% (w/v) agar and 5% (w/v) glucose, at 25°C for 48 h, BHI broth (Gibco) at 30°C for 24 h, and MRS agar (Merck) plus 1% (w/v) agar at 30°C for 24 h, respectively.

Defined Growth Media for Testing Linamarase Activity

Linamarin-containing media with a low glucose content were used to screen linamarase activity. For yeasts and moulds, YNB plus 0.5% (w/v) glucose, plus 1% (w/v) agar and 0.01% (w/v) linamarin (Sigma) in 0.1 M sodium phosphate buffer, pH 6.15, was used. For bacilli, 0.5% (w/v) neutralized bacterial peptone (Oxoid) plus 0.2% (w/v) Lablemco meat extract (Oxoid), 0.1% (w/v) Tween 80 (Merck), 0.02% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0056% (w/v) $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5% (w/v) glucose, 1.0% (w/v) agar and 0.01% (w/v) linamarin (Sigma) in 0.1 M sodium phosphate buffer, pH 6.0, was used. For lactic acid bacteria, the medium for bacilli was used, without agar. For quantification of linamarase activity, the same media were used without agar.

Screening Microorganisms for Linamarase Capacity

The breakdown of linamarin and subsequent volatilization of HCN were examined with picrate-impregnated paper, fixed in the headspace of test tubes. Microorganisms, isolated from Ugandan, domestic, solid-substrate fermented cassava, described in Essers *et al.* (1995a), were inoculated on the relevant medium. After 4 days at 25°C (yeasts and moulds) or 30°C (bacilli and lactobacilli), the colour of the picrate-impregnated papers was compared with a standard range.

Linamarase Activity of the Dominant Microorganisms

Five 250-ml Erlenmeyer flasks, each containing 20 ml of the corresponding linamarin-containing medium, were inoculated with *Rhizopus oryzae*, *Mucor racemosus*, *Neurospora sitophila*, *Geotrichum candidum* or a *Bacillus* sp. (one species/flask). A control was left uninoculated. A strip of picrate-impregnated paper was fixed in the headspace as a qualitative indicator of HCN. After 72 h at 26°C, residual cyanogens and mycelial weight were assayed. The incubation time and temperature are similar to the conditions observed in domestic processing in rural Uganda. The experiment was performed in quadruplicate.

Enzyme-induced Cassava Structure Loss and Linamarin Degradation

The effect of tissue-structure loss on linamarin breakdown was examined by incubation of submerged cassava root pieces with a cell-wall-degrading enzyme complex, with and without additional linamarase, under aseptic conditions.

Preliminary testing of several commercial enzyme preparations indicated that the cellulolytic and pectolytic activity of Pectinex Ultra-sp was able to soften cassava root tissue. Linamarase activity, measured according to the procedure of Cooke (1979), was absent from this preparation. To determine if the proteolytic capacity of Pectinex Ultra-sp (Tableros *et al.* 1987) would affect linamarase activity, the activity of succinate buffer (0.05 M, pH 5.5) initially containing 0.26 U linamarase/ml, with and without addition of

0.5% (w/v) Ultra-sp, was measured according to Cooke (1979) after 0, 4 and 23 h. No interference of Pectinex Ultra-sp with linamarase was found. The 0.04% (w/v) sodium azide used as preservative had no interference on the cyanogen assay.

A cassava root was peeled aseptically and cut into two pieces. These pieces were immersed for 30 min in 70% (v/v) ethanol, then 1 min in sterile water, and, after discarding the 0.5 cm diam. central column, diced aseptically into cubes of about 1 cm³. Five batches of disinfected cubes (approx. 11 g) were transferred to 30-ml sterile cups and submerged under 15 ml succinate buffer (0.05 M, pH 5.5) containing 0.02% (w/v) sodium azide to prevent microbial activity. One cup was assayed immediately (control); the remaining four received no addition, 0.5% (w/v) Pectinex Ultra-sp, crude linamarase (0.26 U/ml), or 0.5% (w/v) Pectinex Ultra-sp and crude linamarase (0.26 U/ml). The amount of linamarase used approximates to the average linamarase activity in 11 g fresh root, according to De Bruijn (1973). After incubation for 16 h at 40°C, cyanogens and microbial counts were determined. The experiment was performed in quadruplicate.

Analyses

Semi-quantitative determination of HCN release was by picrate-impregnated paper. This method was derived from the leaf-picric acid test (Sadik *et al.* 1974) and is based on the change of picric acid from yellow to brown-red after contact with HCN (Guignard 1906). Tubes, containing standards of 0 to 100 mg linamarin/l phosphate buffer (0.1 M, pH 6.0), to which 1 U linamarase was added, were fitted with picrate-impregnated filter paper in the headspace and incubated for 24 h at 30°C. This resulted in six pieces of coloured paper with increasing redness and these were photographed to give a semi-quantitative reference scale.

Cyanogens were extracted and assayed according to Essers *et al.* (1993). In the experiment on enzyme-induced linamarin degradation, each sample of 11 g cassava pieces with 15 ml soak solution was homogenized in 44 ml 0.1 M H_3PO_4 . Moisture in fresh cassava was determined in quadruplicate by oven-drying at 105°C to constant weight. Total counts of mesophilic aerobic microorganisms were carried out by surface plating on plate count agar (Oxoid) and incubating at 30°C for 3 days. The dry weight of mycelial biomass was assayed by filtering the culture broth through a pre-weighed filter paper (Schleicher & Schuell 595 $\frac{1}{2}$) and subsequent drying at 100°C overnight. The bacterial biomass was determined in the same way after centrifugation for 10 min at 2000 $\times$ g. Root softening was considered complete when a 3 mm diam. glass rod could be pushed through a 1-cm thick root piece without noticeable hindrance.

Statistical Analyses

Treatment effects were compared by the Protected Least Significant Difference method (PSD) (Snedecor & Cochran 1980). Significance of differences is presented as two-tailed probability (P) values.

Results and Discussion

Mucor and *Rhizopus* sp. were able to release HCN from linamarin but *Geotrichum candidum*, *Aspergillus*, *Neurospora*, *Penicillium* and *Scopulariopsis* sp. were unable to do so. Most of the yeasts and lactobacilli released HCN, and two of the four bacilli released a small amount of HCN (Table 1).

Quantification of linamarin breakdown by microorgan-

Table 1. HCN release from a linamarin-containing, defined growth medium after 4 days' incubation with isolates of microorganisms from Ugandan, on-farm fermented cassava.

Organism	No. of isolates showing:		
	No release	Moderate release	Strong release
<i>Aspergillus fumigatus</i>	1		
<i>Aspergillus niger</i>	1		
<i>Aspergillus oryzae</i>	1		
<i>Aspergillus parasiticus</i>	5		
<i>Fusarium sporotrichoides</i>	1		
<i>Geotrichum candidum</i>	11		
<i>Mucor circinelloides</i>			2
<i>Mucor racemosus</i>			10
<i>Neurospora crassa</i>	1		
<i>Neurospora sitophila</i>	9		
<i>Penicillium frequentans</i>	1		
<i>Penicillium funiculosum</i>	1		
<i>Penicillium grisofulvum</i>	1		
<i>Penicillium rugulosum</i>	1		
<i>Penicillium waksmanii</i>	1		
<i>Rhizopus oryzae</i>		1	
<i>Rhizopus stolonifer</i>		7	
<i>Scopulariopsis brevicaulis</i>	1		
<i>Scopulariopsis fuxa</i>	1		
<i>Lactobacillus</i> spp.	5	12	3
<i>Bacillus</i> spp.	2	2	
Yeasts	2	10	2

isms (Figure 1) showed that *Neurospora sitophila* and *Geotrichum candidum* did not hydrolyse appreciable amounts of linamarin but *Bacillus* sp. hydrolysed virtually all. *Mucor racemosus* hydrolysed at least 90% of the linamarin and *Rhizopus oryzae* 45% to 84%. Although *R. stolonifer* was not included in this experiment, earlier experiments showed similar linamarin breakdown by *R. oryzae* and *R. stolonifer* under the same conditions. The picrate-impregnated paper indicated HCN escape from the media cultured with *Mucor*, *Rhizopus* and *Bacillus* but not from the ones with *Geotrichum* or *Neurospora*.

The findings of the quantitative experiment were consistent with the findings from the screening, except for *Bacillus* sp. The HCN release by the *Bacillus* sp. was lower than one would expect from the residual linamarin measured. A possible explanation for this is that the *Bacillus* sp. assimilated part of the HCN. It is known that several microorganisms are capable of metabolizing HCN (Knowles & Bunch 1986). Padmaja & Balagopal (1985) described linamarase and rhodanese activity, as well as cyanide assimilation, by *Rhizopus oryzae* isolated from rotting cassava. Rhodanese can form thiocyanate (SCN^-) from CN^- if suitable sulphur sources are available. The thiocyanate formed may then be used for mycelium formation but, if accumulated, would be assayed as non-glucosidic cyanogen.

Although *Neurospora sitophila* is known to be a producer of β -glucosidase (Oguntimein & Moo-Young 1991), the strain tested did not break down significant amounts of the linamarin under the prevailing conditions. β -Glucosidases are substrate specific and therefore not all can degrade linamarin. The pH of the medium after incubation remained between 5.0 and 6.3, which is adequate for linamarase activity from various sources (Cooke *et al.* 1978; Okafor & Ejiofor 1985).

The mean ($\pm$ standard deviation) fungal biomass developed in 72 h was 171 ± 21 mg and was the same order for all fungi, while the bacillus biomass was 475 ± 205 mg. Although biomass development and enzyme excretion do not necessarily coincide, the results indicate that the superior linamarase activity exhibited by *Mucor* cannot be attributed to the growth conditions being specifically favourable for this fungus, nor to its higher biomass.

From earlier experiments (Essers *et al.* 1995b), we know that *Neurospora sitophila* was best at reducing linamarin in fermenting cassava, followed by *Rhizopus* sp. *Bacillus* sp., *Geotrichum candidum* and *Mucor racemosus* were less effective and also caused less root softening than the *Neurospora* and *Rhizopus* sp. In the present experiments, *N. sitophila* showed no linamarase activity, and there is no indication that this would be different in cassava. This indicates that the structure loss, caused by the enzyme system of *N. sitophila*, is of greater importance for linamarin degradation than the combination of low cell-wall-degrading and high linamarase activity of *M. racemosus* and the *Bacillus* sp. Comparing *N. sitophila* and *R. oryzae*, the latter proved at least as effective in softening cassava root tissue (Essers *et al.* 1995b) and displayed linamarase activity (present study) but was less effective in diminishing linamarin levels in cassava (Essers *et al.* 1995b). Therefore, it seems that the type of cell-wall degradation produced by *N. sitophila* is better suited to enable endogenous linamarase–linamarin contact.

In the experiment on enzyme-induced linamarin degradation, microbial counts in root pieces were $< 10^3/\text{g}$ in all batches. This excludes interference from microbial contamination. The cassava pieces treated with Pectinex Ultra-sp or Ultra-sp plus linamarase were completely soft after 16 h. Cassava root softening is caused by cell-wall degradation through the combined activity of pectinases and cellulases (Okolie & Ugochukwu 1988). The residual cyanogen concentrations after the treatments are given in Table 2. Treatment with Pectinex Ultra-sp alone resulted in the lowest linamarin concentrations, both in the absolute sense, as well as relative to total cyanogen concentrations. These concentrations were significantly lower ($P < 0.05$) than those after other treatments. Second in terms of effectiveness was the combination of Pectinex Ultra-sp and linamarase. This also resulted in absolute and relative linamarin concentrations that were significantly lower ($P < 0.05$)

Table 2. Cyanogens in cassava pieces before and after incubation with enzyme supplements.

Enzyme added	Incubation (h)	Total cyanogens* (mg CN equivalent/kg dry wt)	Cyanogenic glucosides*† (mg CN equivalent/kg dry wt)	Ratio† (Cyanogenic-glucosides : cyanogens)
None	0	118 ± 12	112 ± 14 ^a	0.95 ^a
None	16	131 ± 8	64 ± 3 ^b	0.49 ^b
Pectinex Ultra-sp (0.68 mg/g)	16	93 ± 4	12 ± 5 ^c	0.13 ^c
Linamarase (0.36 U/g)	16	123 ± 10	59 ± 4 ^b	0.48 ^b
Pectinex Ultra-sp (0.68 mg/g) + linamarase (0.36 U/g)	16	107 ± 6	28 ± 3 ^d	0.26 ^d

* Values are means ± standard deviations for four sets of replicates.

† Within-columns, values with different superscript letters differ significantly ($P < 0.05$).

Figure 1. Mean ($N = 4$) residual cyanogen levels, as % of initial linamarin concentration, in defined growth media after 72 h at 26°C. Error bars (standard deviations) are given for linamarin concentrations. □— Non-glucosidic cyanogens; ▨— linamarin.

than those in the other treatments. The concentrations after treatment with linamarase alone were not significantly different from those in the incubated controls.

Facilitating the contact between the substrate and the endogenous linamarase, through cell-wall degradation by Pectinex Ultra-sp, thus proved more effective in reducing the linamarin concentration than supplementing with linamarase. This is due to the inability of the exogenous

linamarase to enter intact cassava cells and come in contact with its substrate. Once the cells are disrupted, additional linamarase may accelerate the linamarin breakdown, as was shown by Ikediobi & Onyike (1982). The treatment with both linamarase and Pectinex Ultra-sp was therefore expected to be more effective than Pectinex Ultra-sp alone, but it was not. The reason for this is not clear.

The higher efficacy achieved by adding exogenous cell-wall-degrading enzymes than by adding exogenous linamarase to intact cassava tissue was in line with the finding that a root-softening, but linamarase-deficient fungus (*N. sitophila*) could be more effective in diminishing linamarin content than microorganisms displaying linamarase activity.

Overall, the results of the present study indicate that the main contribution of the microflora to the reduction of linamarin concentrations in solid-substrate fermentations of cassava is their cell-wall-degrading activity, which allows contact between endogenous linamarase and the linamarin. Their linamarase capacity is of lesser importance.

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References

- Butler, G.W. 1965 The distribution of the cyanoglucosides linamarin and lotaustralin in higher plants. *Phytochemistry* **4**, 127–131.
- Cooke, R.D. 1978 An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). *Journal of the Science of Food and Agriculture* **29**, 345–352.
- Cooke, R.D. 1979 Enzymatic assay for determining the cyanide content of cassava and cassava products. In *CIAT Series 05 EC*, eds Ekelbaum, T. & Gómez, G. pp. 3–14. Bogota: CIAT.
- Cooke, R.D., Blake, G.G. & Battershill, J.M. 1978 Purification of cassava linamarase. *Phytochemistry* **17**, 381–383.
- De Bruijn, G.H. 1973 The cyanogenic character of cassava (*Mani-*

- hot esculenta). In *Chronic Cassava Toxicity: Proceedings of an Interdisciplinary Workshop, London, England*, eds Nestel, B. & MacIntyre, R. pp. 43–48. Ottawa: International Development Research Centre.
- Essers, A.J.A., Bosveld, M., Van Der Grift, R.M. & Voragen, A.G.J. 1993 Studies on the quantification of specific cyanogens in cassava products and introduction of a new chromogen. *Journal of the Science of Food and Agriculture* **63**, 287–296.
- Essers, A.J.A., Ebong, C., Van Der Grift, R.M. Otim-Nape, W., Nout, M.J.R. & Rosling, H. 1995a Reducing cassava toxicity by heap-fermentation in Uganda. *International Journal of Food Sciences and Nutrition* **45**, in press.
- Essers, A.J.A., Jurgens, C.M.G.A. & Nout, M.J.R. 1995b Contribution of selected fungi to the reduction of cyanogen levels during solid substrate fermentation of cassava. *International Journal of Food Microbiology* **24**, in press.
- Guignard, M.L. 1906 Chimie végétale — le haricot à acide cyanohydrique, *Phaseolus lunatus* L. *Comptes Rendus Hebdomadaire des Seances de l'Academie des Sciences* **142**, 545–553.
- Ikediobi, C.O. & Onyike, E. 1982 The use of linamarase in gari production. *Process Biochemistry* **17**, 2–5.
- Knowles, C.J. & Bunch, W. 1986 Microbial cyanide metabolism. *Advances in Microbiology and Physiology* **27**, 73–111.
- Lancaster, P.A., Ingram, J.S., Lim, M.Y. & Coursey, D.G. 1982 Traditional cassava based foods: survey of processing techniques. *Economic Botany* **36**, 12–45.
- Mkpong, O., Hua-Yan, E., Chism, G. & Sayre, R.T. 1990 Purification, characterization and location of linamarase in cassava. *Plant Physiology* **93**, 176–181.
- Oguntimein, G. & Moo-Young, M. 1991 Production and properties of β -glucosidases by *Neurospora sitophila*. *World Journal of Microbiology and Biotechnology* **7**, 4–11.
- Okafor, N. & Ejiofor, M.A.N. 1985 The linamarase of *Leuconostoc mesenteroides*. *Journal of the Science of Food and Agriculture* **36**, 669–276.
- Okolie, N.P. & Ugochukwu, E.N. 1988 Changes in activities of cell-wall-degrading enzymes during fermentation of cassava (*Manihot esculenta* Crantz) with *Citrobacter freundii*. *Journal of the Science of Food and Agriculture* **44**, 51–61.
- Padmaja, G. & Balagopal, C. 1985 Cyanide degradation by *Rhizopus oryzae*. *Canadian Journal of Microbiology* **31**, 663–669.
- Sadik, S., Okerere, O.U. & Hahn, S.K. 1974 Screening for Acyanogenesis in Cassava. Ibadan, Nigeria: IITA.
- Snedecor, G.W. & Cochran, W.G. 1980 *Statistical Methods*, 7th edn. Iowa State University.
- Tableros, M.A., Endress, H.U., Bannert, E., Finger, C., Shen, Z. & Gierschner, K. 1987 Bestimmung verschiedener glycosidischer aktivitäten in enzympräparaten des handels. *Industrielle Obst- und Gemüseverwertung* **72**, 351–355.

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