Contribution of Selected Fungi to the Reduction of Cyanogen Levels During Solid-Substrate Fermentation of Cassava.

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Abstract

The effect of six individual strains of the dominant microflora in solid substrate fermenting cassava on cyanogen levels was examined. Six out of eight batches of disinfected cassava root pieces were incubated for 72 hrs after inoculation with either of the fungi *Geotrichum candidum, Mucor racemous, Neurospora sitophila, Rhizopus oryzae* and *Rhizopus stolonifer,* or a *Bacillus sp.*, isolated from on-farm fermented cassava flours from Uganda. One non-inoculated batch was incubated as a reference. Levels of initial and final moisture and cynogens were assayed. The experiment was done in quadruplicate.

Incubation of disinfected root pieces reduced cyanogenic glucoside levels significantly to a mean (±SD) of 62.7% (±2.8) of the initial value. Microbial growth resulted in significant additional reduction of the cyanogenic glucoside levels to 29.8% (±18.9) of the ones which were obtained after non-inoculated incubation. Among the tested strains, *N. sitophila* reduced cyanogenic glocuside levels most effectively, followed by *R. Stolonifer* and *R. oryzae.* Of all fermented samples, both *Rhizopus spp.* showed highest proportion of residual cynagoens in the cyanohydrin form, followed by the *Bacillus sp.* Flours showed similar patterns of cyanogens as the batches they were prepared from. Cyanogenic glucoside level reduction was significantly correlated (r=0.86) with the extent of root softening.

It is concluded that both incubation and microbial activity are instrumental in reducing the potential toxicity of cassava during the solid substrate fermentation and that effectiveness varies considerably between the species of microorganisms applied. [2]

Introduction

The tropical root crop cassava contains cyanogenic glucosides, which are potentially toxic. Cleavage of the glucosidic bond by the compartmentally separated enzyme linamarase, located in the cell-wall, renders glucose and cynohydrin (Mkpong *et al.* 1990). Cyanohydrins can be degraded enzymatically, but also decompose spontaneously at pH > 5 into acetone and the volatile HCN (Cooke, 1978). Toxicity of the latter is indicated by the estimated minimal lethal oral dose of 0.5 - 3.5 mg per kg human body weight (Montgomery, 1969). Cyanohydrins may decompose at the pH prevailing in the gut, yielding equal molar amounts of HCN. The toxicity of the glucosides is not yet well understood. Several health problems related to cyanogen uptake from insufficiently processed cassava have been reported from Africa (Rosling, 1993).

The required reduction of cyanogen levels before consumption is achieved by processing and preparation. A processing method applied in several parts of Africa includes a step of solid substrate fermentation (Essers *et al.*, 1992, Essers et al., 1995, Gidamis, 1993). In Uganda (Essers *et al.*, 1995) a common process is as follows: After superficial drying, the peeled roots are heaped and covered to incubate for 3 days to enable produce mould growth. After removal of the fungal mycelium, crushing and sun-drying, the resulting crumbs are pounded into flour. Although the fermentation stage appeared functional in cyanogen removal, the contribution of the microflora remained unclear. Analyses of the microflora associated with this process in Uganda showed the frequent occurrence and abundance of several fungal species. The pH of the resulting flours was generally higher than 7, in contrast with other fermented products from cassava (Vasconcelos *et al.*, 1990, O'brien *et al.*, 1992).

The scope of this work is to study the contribution of several representatives fungal and bacterial strains, isolated from Ugandan on-farm fermented cassava, to the changes in cyanogen levels by solid substrate fermentation. We examined whether incubation alone of disinfected cassava leads to reduction of the cyanogen levels; whether microbial growth leads to additional reduction of these levels; and whether effectiveness differs among micro-organisms.

Materials and Methods

Sample/microbiological preparation

Disinfection of cassava pieces was required to suppress the outgrowth of microbial contamination, without hindering the intended growth of the inoculated strains, and without affecting the tissue structure. After preliminary comparison of options, the following procedure was selected. Cassava roots from the Costa Rica were peeled and cut into 15 g pieces, using sterilised equipment. The pieces destined for fungal fermentation were submerged in oxytetracycline (0.5 g/l water) for 60 sec., the ones for growth of bacteria in ethanol (70% v/v) for 60 sec., while the control batches were submitted to both treatments for 30 sec. Subsequently, the pieces were forced-air dried at 55° C for $1^1/_2$ - 2 h, to reach a moisture content of 55 - 60 %.

Inoculation was similar for all microbial cultures and was carried out by pressing thus pre-treated root pieces directly on plates overgrown with pure cultures microorganisms, isolated by the authors from traditionally heap-fermented cassava in Kiryandongo village, Masindi district, in rural Uganda (Essers *et al.*, 1995). Filamentous fungi, identified on the basis of their macro and microscopic morphology according to Samson & Van Reenen Hoekstra (1988), and a Gram-positive endospore forming rod-shaped bacterium classified as *Bacillus* sp., not further identified, were deposited in the culture collection of the Department of Food Science of Wageningen Agricultural University (LU). These included the fungi *Geotrichum candidum* (LU243), *Mucor racemosus* (LU360), *Neurospora sitophila* (LU420), *Rhizopus oryzae*

(LU582) and *Rhizopus stolonifer* (LU590), which were cultured on Malt Extract Agar, Oxoid CM59, and a *Bacillus* sp. (LU809) which was cultured on Nutrient Agar, Oxoid CM3. Incubation was in sterilised jars of 720 ml during 72 h at 25° C. Screwcaps were left open to avoid anaerobiosis.

Cyanogen analysis

Extraction and analysis of the cyanogenic compounds was by homogenisation of about 70 g in 250 ml 0.1 M orthophosphoric acid, followed by conversion to HCN, which was measured spectrophotometrically after coloration (Essers *et al.*, 1993).

Experimental

Peeled cassava roots were cut into $1^1/_2$ cm thick discs, which were split into several segments, having near to equal cyanogen levels (De Bruijn, 1971). The adjacent segments were matched to obtain 8 batches of about 350 g with similar cyanogen levels. After disinfection pre-treatment, from one batch 4 sub-samples were taken and extracted immediately (t=0) to serve as a reference for the cyanogen content; one non-inoculated batch and 6 batches inoculated with either of the microorganisms mentioned above were incubated for 72 h at 25° C. Treatment codes are shown in **Table 1**. After these treatments, a part of each batch was processed into flour by oven drying at 55° C for one day and subsequent milling (Fritsch Pulverisette type 14.702, Laborgeschaft, Idar-Oberstein, Germany) through sieves of, subsequently, 4 and 1mm. Extraction and subsequent analysis for cyanogens took place immediately after incubation and after flour preparation.

Specificity of microbial growth was evaluated macroscopically and by characteristic odour of the fermenting products. The extent of growth was monitored semi quantitatively, using a subjective classification scale from 0 (no visible growth) to 5 (completely covered). Quantitative measurement of fungal propagules was omitted, as this would mainly refer to sporulation of the fungi and not to the more relevant mycelial growth and physiological activity (Nout *et al.*, 1987). Extent of softening of after manually probing with a round tipped 3 mm of glass bar.

The experiment was carried out 4 times with intervals of one week.

Statistical analysis

Treatment effects were judged with the Protected Least Significant Difference method (PSD) (Snedecor & Cochran, 1980, p 234). First, the overall treatment effect was examined by an F-test in a 2 way analysis of variance with 7 df for treatments and 21 for residuals. In case of a significant F, the differences between treatment effects were tested by t- test (Snedecor & Cochran, 1980). In order to stabilize the variance, a differentiate between low residual levels.

Significance of differences are related to two-tailed probability (*P*) values.

Results and Discussion

Microbial growth was good in the inoculated batches and nearly absent in the non-inoculated ones. Although the disinfection treatment does not guarantee exclusive growth of the inoculated micro-organism, macroscopic inspection an characteristic odour release did not indicate significant outgrowth of contaminating microorganisms. **Table 1** presents the absolute data on residual cyanogen levels, as well as the softening, in the moist cassava batches and their flours. As the initial total cyanogen levels varied considerably between 4 experiments, compilation of the results for presentation in Figures 1 and 2 was after calculating the residual cyanogen levels as % of the initial total cyanogen levels. The pH was significantly reduced ($P<0.0001$) to 4.8 (SD 0.5) after fermentation by *Rhizopus oryzae*, but remained at 6.7 (SD 0.2) after the other treatments (results not shown).

Cyanogenic glucoside levels after incubation only (treatment 2) were significantly lower than those of fresh roots (treatment 1), with $P<0.0001$ for the moist products and $P = 0.001$ for the flours, indicating that incubation alone led to reduction of cyanogenic glucoside levels. The cause of this decrease is probably associated with the physiological deterioration of the cell-wall and membrane structure (Padmaja & Balagopal, 1985) which, by leaking, allow contact between endogenous linamarase and the cyanogenic glucosides, resulting in breakdown of the latter.

Cyanogenic glucoside levels in moist products and flours from batches that were incubated after inoculation (treatments 3 to 8) were significantly lower ($P<0.0001$) than samples after

Table 1
Levels of cyanogenic glucosides and cyanohydrins in moist cassava and their flours, and softness, after different treatments in the experimental blocks

	Moist cassava		Flours		
Treat-ment	Cyanogenic glucosides[b]	Cyanohydrins[b]	Cyanogenic glucosides[b]	Cyanohydrins[b]	Softness
1	92.7	10.3	46.6	5.4	0
2	60.5	4.0	39.8	2.1	1
3	26.3	15.3	14.7	2.5	2
4	25.1	11.7	15.2	1.6	2
5	3.4	1.5	1.4	0.2	5
6	12.1	43.1	14.7	10.8	5
7	11.7	33.8	2.0	1.7	5
8	14.8	40.8	8.4	0.9	3
1	209.9	16.4	79.5	6.3	0
2	123.3	3.4	68.5	1.9	0
3	62.9	15.7	32.6	3.3	2
4	81.9	2.0	41.0	2.0	1
5	14.9	1.4	22.0	0.9	4
6	24.1	42.2	22.8	17.2	5
7	19.9	44.2	4.2	1.1	5
8	44.0	48.2	29.9	2.5	3
1	219.3	6.1	108.0	15.6	0
2	138.1	49.4	86.7	5.1	1
3	48.2	9.4	37.7	2.6	2
4	53.6	22.8	30.0	3.1	2
5	3.6	0.7	7.2	0.7	4
6	15.0	59.0	31.4	8.5	5
7	11.5	71.6	11.7	4.7	5
8	65.3	77.6	17.9	3.3	2
1	321.5	6.5	120.9	10.9	0
2	204.6	43.3	107.1	4.3	1
3	101.6	8.9	46.6	4.0	2
4	112.1	14.1	63.5	2.6	2
5	27.2	4.6	[d]	1.6	3
6	45.5	64.5	31.2	14.9	5
7	31.6	86.1	12.1	4.3	5
8	127.8	86.5	44.6	9.3	2

[a] Treatment: 1 Control t= 0 h, 2 Control t = 72 h, 3-8 are incubated for 72 h with: 3 Geotrichum candidum, 4 Mucor racemosus, 5 Neurospora sitophila, 6 Rhizopus oryzae, 7 Rhizopus stolonifer, 8 Bacillus sp. [b] Values in mg CN- equivalent per kg sample on dry weight base. [c] 0 = no softening, 5 = completely soft. [d] not examined

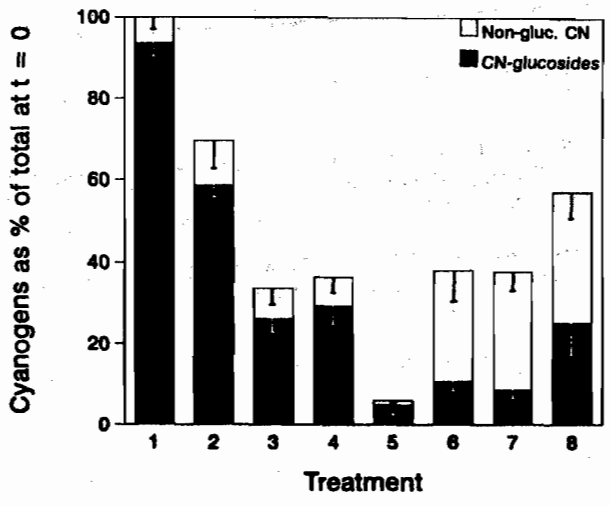

FIGURE 1 Mean (-SD) residual cyanogen levels in moist cassava after treatments, as % of total cynogens at t=0, from 4 experiments (treatment coding as Table 1)

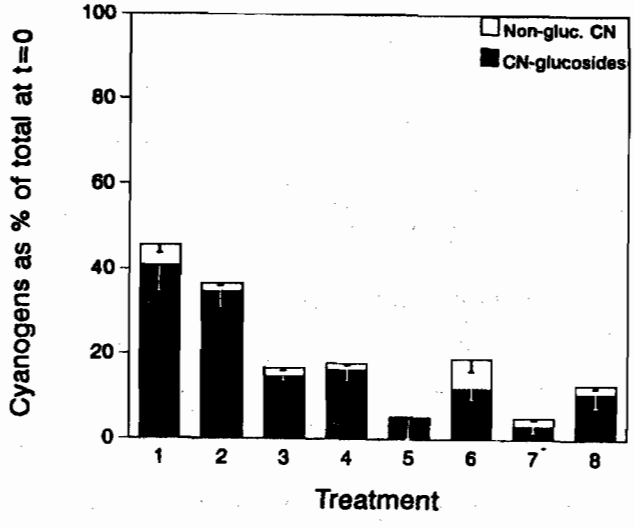

FIGURE 2 Mean (-SD) residual cyanogen levels in cassava flours made after treatments, as % of total cynogens at t=0, from 4 experiments (treatment coding as Table 1)

incubation only (treatment 2), indicating an additional effect of the microflora. The effect may have been caused by increased endogenous linamarase - linamarin contact through disintegration of cell-wall and membrane structures, or by introducing additional microbial linamarase, or by both.

The effects of the microbial species (treatments 3 to 8) on cyanogenic glucoside levels could be differentiated statistically (P<0.05, PSD) as follows: *N. sitophila* fermented batches had lowest levels, followed by the *Rhizopus* spp. fermented ones. Of the flours, *R. stolonifer* and *N. sitophila* fermented batches had significantly lower (P<0.05) cyanogenic glucoside levels than the others.

The level of cyanohydrins, as well as their proportion of total cyanogens, was significantly different (P<0.0001) after the different treatments, both for moist products and flours. These levels and proportions were significantly higher (P<0.05, PSD) in the *R. oryzae*, R. *stolonifer* and *Bacillus* sp. fermented batches than in the others, and after drying remained significantly higher in the flour from the *R. oryzae* fermented cassava. As the loss of the cyanogenic glucosides in this process is through the stage of cyanohydrins, these compounds were apparently better preserved in these last three fermented products. As cyanohydrins are more stable at a lower pH (Cooke, 1978), the higher level of cyanohydrins and their proportion of total cyanogens in the *R. oryzae* fermented products may be explained by the lower pH attained by that fermentation. We cannot explain the high cyanohydrin to total cyanogen ratios in the *R. stolonifer* and *Bacillus* sp. fermented batches.

HCN levels in the moist products ranges from 0.1 - 5.7 mg CN equivalent per kg dry weight with a mean of 2.1 (SD 1.7, n=32). In the flours, these levels did not in each flour sample was below 10 mg CN equivalent per kg dry weight. These low levels are explained by the rigorous final drying step leading to moisture levels of about 5%. It has been observed earlier by Banea *et al.*, (1992) that cyanohydrins are lost at low moisture levels.

The treatments had a significant effect (P<0.0001) on the softness of the products. Softening of the pieces was most advanced (P<0.05, PSD) after fermentation by the *Rhizopus* spp., followed by *Neurospora. Geotrichum, Mucor* and *Bacillus* only caused superficial softening (Table 1). In moist products and flours, residual cyanogenic glucoside levels differed significantly (P<0.0001) among the softness classes of the fermented root pieces. The reduction in glucoside levels was significantly correlated (r=0.86) with the extent of root softening. Comparing the F values for treatments and softness in moist products after correcting each for the effect of the other, the effect of treatment (P=0.003) and softness (P=0.035) on the glucoside levels remained significant. For flours, the treatment effect remained significant (P=0.0003) after correction for softness of the moist products, but the effects of softness on the residual glucoside levels just lost significance (P=0.102) after correcting for treatment effect. These findings indicate that treatment is more powerful than softness alone for explaining the glucoside level reduction.

The cyanohydrin proportion of total cyanogens in the moist products was significantly related (P<0.0001) and correlated (r=0.730 to softness after fermentation, but not in the flours. The sequence suggested that the softer the product, the higher this proportion, except for *N. sitophila* fermented cassava. Also here, it appears that although softening has a substantial effect on the cyanohydrin proportion, it does not fully explain the treatment effect.

Softness or structure loss of cassava tissue was induced by the treatments. The fact that correction for the treatment effect reduced the statistical significance of the softness effect on the glucoside levels and cyanohydrins to total cyanogens rations far more than vice versa, indicates that treatment as such is a stronger determining factor than softness only. It is likely that, in addition to the extent of structure loss, also the type of cell-wall and membrane structure degradation is of importance. The possibility of an additional effect from microbial linamarase activity can not be excluded. Okafor & Ejiofor (1986) found a cyanogen lowering effect from microorganisms that were selected for their linamarase capacity, when seeded on fresh pulp. Maduagwu (1983) and Vasconselos *et al.* (1990) found no such effect in spontaneous fermenting cassava pulp. In the latter cases, the major part may already have been hydrolysed by endogenous enzyme liberated by the cell disruption, before the relevant microorganisms were in sufficient

number to cause significant effect. Neither of those studies compare to the present experiment, however, as here no mechanical disruption occurred to liberate the endogenous linamarase. The mechanisms of the microflora bringing about the linamarin level reduction in these experiments is subject of further study.

Although the culture conditions during these experiments were made to resemble average in-situ conditions, the growth and enzyme production of these microorganisms may be favoured differently according to the environmental conditions at household level.

Concluding, we found that both incubation and microbial growth were instrumental in reducing cyanogenic glucoside levels and changing the cyanogen composition in cassava root pieces. The effectiveness differed between the species of microorganisms and was also associated with the root softening obtained. From the perspective of reducing cassava's potential toxicity, *N. sitophila* was most successful of the here tested strains, followed by *R. stolonifer* and *R. oryzae*.

Acknowledgement

This study was financed by the Netherlands Ministry for Development Co-operation.

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