MAKING SAFE FLOUR FROM BITTER CASSAVA BY INDIGENOUS SOLID SUBSTRATE FERMENTATION

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Abstract

In some African communities, a stage of solid substrate fermentation is included in the sun-drying process of transforming cassava roots into flour. The dynamics of cyanogen removal, the role of the microflora involved, and cyanogen and mycotoxin contamination of the final products were studied in Uganda. Dominant mycelial growth was from the fungi *Neurospora sitophila*, *Geotrichum candidum* and *Rhizopus oryzae*. No mutagenicity, cytotoxicity or aflatoxins could be detected in the flours. The fermentation process was significantly more effective in reducing cyanogen levels than sun-drying alone, but did not always result in innocuous levels of cyanogens.

Laboratory experiments showed that non-inoculated incubation of disinfected root pieces reduced cyanogenic glucoside levels, and separate inoculation with fungi and a *Bacillus* sp. isolated from on-farm fermented cassava caused significant additional reduction. Several of the micro-organisms involved were capable of displaying linamarase activity. However, enhanced glucoside breakdown in root pieces was associated with root softening but not with the micro-organisms' linamarase capacity. Addition of a cell wall-degrading enzyme preparation under aseptic conditions caused softening of root pieces and a significant reduction of cyanogenic glucosides, but adding linamarase did not. It is proposed that the main contribution of micro-organisms to linamarin level reduction in solid substrate fermenting cassava is their cell wall-degrading capacity which facilitates the contact between endogenous linamarase and the cyanogenic glucosides.

Inclusion of a stage of solid substrate fermentation during processing into flour appeared instrumental in reducing the potential toxicity of cassava roots and was more effective than sun-drying alone; there is room, however, for optimization to ensure safer products.

Key words: Cyanogenic glucosides, cyanogen removal, heap fermentation

Introduction

In many African cultures south of the Sahara, the staple food eaten is often in the form of a paste, prepared from a flour (Lancaster *et al.*, 1982). Processing the bulky root crop cassava into flour involves a stage of sun-drying of whole or fractionated peeled roots. The practice to include a sheltered incubation stage with high humidity, provoking an impressive mold growth, was reported from Kenya by Anderson (1944), from Tanzania by Childs (1961), from Uganda by Goode (1974), from Mozambique by Essers *et al.* (1992), and from Uganda by Essers *et al.* (1994). In Mozambique, the processors said that they had recently adopted the method for processing bitter, toxic roots as it was more effective in reducing bitterness and toxicity than sun-drying alone, and the limited availability of water prevented them from applying the known alternative of soaking. People of the Alur tribe in north-west Uganda traditionally apply a process

involving solid substrate fermentation in covered heaps on their predominantly bitter cassava roots. The aim is to reduce toxicity and improve palatability of the paste that they prepare from the resulting flour (Essers *et al.*, 1994).

The potential toxicity of cassava is due to the presence of cyanogenic glucosides, mainly linamarin, in the plant cells. Linamarin can be split by the enzyme linamarase, located in the cell walls (Mkpong *et al.*, 1990). The cyanohydrins formed are increasingly unstable at a pH greater than 5, yielding the volatile and toxic hydrogen cyanide (HCN) (Cooke, 1978). Dietary cyanide exposure from insufficiently processed cassava is a factor in some public health problems (Rosling *et al.*, 1993), but the dynamics of cyanogen removal during processing are not yet fully understood.

Interest in the fungal fermentation came from authorities of the health sector (Essers and Nout, 1989) and the agricultural sector (Essers *et al.*, 1994), as the process seems an uncontrolled rotting of a formerly attractive white root crop, that may bring about food losses and contamination with mycotoxins, and results in 'a most unappetizing looking article' (sic, Anderson, 1944). Interest may also come from food scientists who see possibilities for microbial protein enrichment, improved utilization of root wastes for animal feed and development of new cassava-based products.

The method and its suggested function to reduce toxicity were recently studied in Uganda. Papers with detailed information on the study methods and results have just been submitted for publication. Here, the findings on the cyanogen removal and the role of the microflora in the heap-fermentation process will be summarized, and the safety of the products obtained discussed.

Research outline

In Uganda, the heap-fermentation process was studied, initially by key informants and household interviews, and the information obtained verified by observations and focus group interviews in a village. The entire process was closely observed at rural households, and samples were taken at each stage for analysis of cyanogens, pH and moisture. The process was repeated at a laboratory site in Kampala and compared with simultaneous sun-drying of root segments. Cyanogen levels were determined (Essers *et al.*, 1993) also in samples of fermented cassava collected from rural households. The microflora was determined in samples of root surface scrapings taken during the fermentation stage and in flour from cassava roots processed by heap fermentation in rural settings. Mycotoxin contamination was assayed by measuring aflatoxins, mutagenicity and cytotoxicity in fermented cassava samples.

Isolates of the micro-organisms were screened for their capacity to release HCN from a linamarin-containing defined medium, and linamarase capacity of 5 dominant micro-organisms was quantified. Their capacity to reduce linamarin levels in cassava was evaluated on disinfected cassava root pieces. The importance of root softening for linamarin breakdown was examined by incubation of submerged cassava root pieces with an added cell wall-degrading enzyme complex or with added linamarase under aseptic conditions.

The heap-fermentation process in Uganda

Description of the process

Key informant and household interviews, verified by observations and focus group interviews with the Alur in Kiryandongo village, revealed the following: bitter cassava roots always undergo heap fermentation to reduce toxicity, bitterness and to improve texture characteristics of the paste made from the resulting flour, and as a preparative step in the processing into a strong distilled alcoholic beverage. The Alur consider the process as part of their tradition and it is scheduled as follows: after superficial drying, the peeled

roots are heaped and covered to incubate for 3 days to induce profuse mold growth. After removal of the fungal mycelium, crushing and sun-drying, the resulting dry crumbs are eventually pounded into flour. The schedule needs adaptation to climatic conditions. Initially, white molds appear, but near the end of the heap phase, the roots should be covered with molds with a black or (less-preferred) orange appearance, or both. Greenish molds were considered as a sign of spoilage by the processors, caused by an insufficient first drying step. The crucial criterion for ending the heap phase was the softness of the roots. Shortening the period of fermentation or final drying, considered to increase the risk of intoxications, was said to be used only exceptionally when people were extremely short of time.

Microflora, mutagenicity and aflatoxins

The microflora in the 6 flours obtained from the monitored cassava batches at rural households was abundant and diverse. The following fungi were isolated from at least 3 flours: *Neurospora sitophila*, *Geotrichum candidum*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Aspergillus oryzae*, *Aspergillus fumigatus* and *Mucor racemosus*. The first 3 were the most prevalent ones. On the fermenting cassava, green-blue spots, probably indicating conidia of *Aspergillus* or *Penicillium* spp., were observed only sporadically. From samples taken during the process, microscopic inspection showed that initially several fungi were present which were not recovered from the resulting flour, indicating that they lost competition. During the incubation in heaps, the roots started softening from the outside towards the inside, coinciding with the profuse mold growth, suggesting cellulolytic and pectolytic enzyme activity by the microflora (Okolie and Ugochukwu, 1988).

Except for *N. sitophila* and *G. candidum*, the 12 other fungi we isolated are reported to be able to produce mycotoxins under pure culture laboratory conditions (Northolt, 1988; Frisvad, 1988). This does not imply that they would produce mycotoxins on cassava in the presence of competing fast-growing microflora on cassava. Contamination with mycotoxins was estimated by assaying mutagenicity and cytotoxicity by the Ames test in 25 samples of fermented crumbs, collected at rural households and in one sample of dried scraped-off mycelia. Aflatoxins were assayed by HPTLC in 10 of the samples. The Ames test was negative for all 25 tested crumb samples. A slight mutagenicity was observed only in the sample of dried scraped-off molds. Aflatoxins were absent in all 10 screened samples. The absence of aflatoxins, mutagenicity and cytotoxicity in the screened flours suggests that mycotoxins were not formed in quantities that are detrimental to public health. The occurrence of some suspect fungi as well as a slight mutagenicity in the scraped-off molds indicate that some mutagenicity might develop during this process.

A high number of *Escherichia coli* found in one of the flour samples is indicative of an unhygienic process.

Cyanogen removal

Monitoring of the process at 6 households showed similar changes in cyanogen levels, moisture and pH over time. Duration of the process varied from 5 to 9 days. The initial total cyanogen level varied from 237 to 559 mg CN eq./kg dry weight and the residual level in the flours varied from 8 to 41 mg CN eq./kg dry weight. Residual levels of cyanohydrins plus HCN in the flours were less than 5 mg CN eq./kg dry weight.

The process was then repeated in quadruplicate at a laboratory site in Kampala by an experienced Alur farmer and compared with simultaneous sun-drying of roots from the same lot. Cyanogen extraction and pH determination were done within 4 h after collection.

Levels of cyanogenic glucosides showed an exponential decline during the processes. Cyanohydrin levels rose, due to the breakdown of the glucosides. The conversion rate of glucosides to cyanohydrins was overtaken by that of cyanohydrins to HCN after 1 or 2 days of incubation. HCN disappeared nearly simultaneously and was partly lost during extraction and extract storage. The reduction of cyanogen levels during the short cooking step when preparing *ugali* was negligible. Laboratory-site experimental processing showed significantly lower levels ($p < 0.001$, two-tailed *t*-test) of residual glucosides in the heap-fermented batches (3.4 ± 1.3 mg HCN equivalent kg^{-1}, dry weight basis) than in the sun-dried cassava (40.3 ± 2.7). Also, cyanohydrin levels were significantly lower ($p < 0.025$, two-tailed *t*-test) in fermented batches (12.3 ± 2.8 mg HCN equivalent kg^{-1}) than in the sun-dried cassava (37.8 ± 1.2 mg HCN equivalent kg^{-1}).

It seems that linamarin is degraded in the drying cassava until the water activity has dropped below a certain level that is critical for linamarin-linamarase interaction. In that case, the incubation stage with high humidity may extend the period of linamarase activity.

Residual cyanogen levels in collected flours

Dried fermented cassava crumbs of the bitter variety *karangwa*, considered ready for conversion to flour and preparation, were collected at consecutive Alur households in Kiryandongo village in April and September 1990 (23 samples) and in October 1992 (21 samples).

Residual total cyanogen levels in the 1990 samples ranged from 1.4 to 65.8 mg CN eq./kg dry weight, with a mean of 20.3 (SD 16.8). This was similar to the mean level of the 6 flours resulting from the domestic processing monitored in the same month and village, and very low compared to levels (133 ±71 mg CN eq. kg-1 dry wt) found in sun-dried flours collected in a normal year at households in Masasi, Tanzania (Mlingi *et al.*, 1992).

Residual cyanogenic glucoside levels in the 1992 samples varied between 7.1 and 202 mg CN eq./kg dry weight, with a mean of 56.5 (SD 53.3). Residual levels of cyanohydrins plus HCN varied between 1.2 and 33.4 mg CN eq./kg dry weight, with a mean of 9.1 (SD 8.7). Moisture levels varied between 13.0% and 19.1%. In 6 batches that were destined for sale, cyanogenic glucoside levels were significantly higher than in the 15 batches for home consumption.

The cyanogen levels in the products collected in 1992 were much higher than in the ones collected in 1990. High residual levels may have been caused by a high initial level of cyanogens or an inadequate execution of the process. As cyanohydrins are considered to decompose at the pH prevailing in the gut, we must consider the levels of cyanohydrins plus HCN in 5 out of 21 samples exceeding 10 mg HCN equivalent kg^{-1} dry weight as being higher than the FAO/WHO (1991) recommended upper limit (10 mg HCN equivalent kg^{-1}) for hydrocyanic acid content in cassava flour. The total cyanogen levels in all 21 samples were higher than this limit. However, the relation of the linamarin levels to this recommended limit for hydrocyanic acid is unclear. If this limit is meant to include linamarin levels, it seems to contain ample safety margins, considering that no acute toxic effects on a large scale were noticed, and in accordance with calculations by Rosling *et al.* (1993). Probably, other cassava varieties or other processing techniques would be necessary to achieve total cyanogen levels (mean + 2SD) below 10 mg HCN equivalent kg^{-1} flour.

Apparently, although in principle useful, heap-fermentation as applied in the studied village did not guarantee sufficient reduction of cyanogens. The reasons for differences in effectiveness between households requires further study.

Role of the microflora

Screening of micro-organisms for linamarase activity

Microbial strains, isolated from Ugandan domestic fermented cassava, were screened for their capacity to release HCN from a linamarin-containing defined medium. Isolates of the micro-organisms were inoculated on a pH 6 buffered linamarin containing minimum growth medium in agar slants. After 4 days of incubation at 25°C (yeasts and molds) or 30°C (bacillus spp. and lactobacilli), the color of picrate-impregnated paper strips, fixed in the headspace of the test tubes, was compared with a standard range.

The fungi *Mucor* spp. and *Rhizopus* spp. were able to release HCN from the linamarin- containing medium, while *Geotrichum candidum, Aspergillus, Neurospora, Penicillium,* and *Scopulariopsis* spp. were unable to do so. Most of the yeasts and lactobacilli were able to release HCN, and two of the four bacillus spp. released a small amount of HCN.

Quantitative comparison of microbial linamarase activity

Quantification of the linamarase activity of 5 dominant micro-organisms was done in quadruplicate by culturing the micro-organisms for 72 h at 26°C on linamarin-containing growth media, and determining the residual linamarin levels. This experiment showed that *Neurospora sitophila* and *Geotrichum candidum* did not break down appreciable amounts of linamarin, in contrast to the *Bacillus* sp., which virtually broke it all down. *Mucor racemosus* had broken down at least 90% of the linamarin and *Rhizopus oryzae* 45-84%.

The pH after the incubation remained between 5.0 and 6.3, which is adequate for linamarase activity (Cooke *et al.*, 1978). The mean fungal biomass developed in 72 h was of the same order for all fungi. Although biomass development and enzyme excretion do not necessarily coincide, this at least suggests that the superior linamarase activity exhibited by *Mucor* cannot be contributed to the growth conditions being specifically favorable for this fungus.

Enzyme-induced cassava structure loss and linamarin degradation

The pectolytic/cellulolytic enzyme complex Pectinex Ultra-sp (Novo Nordisk, Denmark) appeared well able to soften cassava root, but did not show significant linamarase activity when tested. Disinfected cassava root pieces were diced aseptically in cubes of about 1 cm^3. Five batches (11 g) of disinfected cubes were transferred to sterile cups and submerged with succinate buffer containing sodium azide to prevent any microbial activity. One cup was assayed immediately (control), and 3 cups were incubated for 16 h at 40°C, of which one cup was without addition, one was supplemented with 0.5% (w/v) Pectinex Ultra-sp, and one was supplemented with crude linamarase (0.26 E.U./mL). After incubation, cyanogens were assayed in the homogenate of the cubes plus soak solution.

Softening of diced root, induced by the added pectinase/cellulase mixture, led to significantly lower linamarin levels, while the addition of linamarase did not. Facilitating the contact between the substrate and the endogenous linamarase, through cell wall degradation by the Ultra-sp, thus proved more effective in linamarin level reduction than supplementing linamarase. This can be explained as the exogenous linamarase was unable to enter the cassava cells and come into contact with the linamarin. Once the cells are disrupted, additional linamarase may accelerate the linamarin breakdown, as was shown by Ikediobi and Onyike (1982).

Contribution of selected fungi to the reduction of cyanogen levels

The effect on cassava cyanogen levels of 5 individual strains of the dominant microflora was examined. Six out of 8 batches (approximately 350 g) of disinfected cassava root pieces with similar cyanogen levels were incubated for 72 h at 26°C after inoculation with either of 5 fungi or a *Bacillus* sp., isolated from domestic fermented cassava flours from Uganda. One non-inoculated batch was incubated as a reference. Levels of initial and final moisture and cyanogens were assayed.

Incubation of disinfected root pieces reduced cyanogenic glucoside levels significantly to 63 % (SD=3%) of the initial value. Inoculation of cassava and subsequent fungal growth resulted in significant additional reduction of the cyanogenic glucoside levels to 30 % (SD=19%) of the levels which were obtained after non-inoculated incubation. The most effective reduction of linamarin level in fermenting cassava was by *Neurospora sitophila*, followed by *Rhizopus stolonifer* and *R. oryzae*; the least effective was by the *Bacillus* sp., *Geotrichum candidum* and *Mucor racemosus*. Root softening was significantly ($p<0.05$) more effective by the *Rhizopus* spp., followed by *N. sitophila*, than by the other micro-organisms. Overall, the root softening was significantly correlated with the linamarin degradation but did not explain all treatment effects.

In a similar experiment, cyanogen level reductions by incubation were compared after inoculation with *N. sitophila*, a fungal mix of *G. candidum*, *N. sitophila*, *R. oryzae* and *R. stolonifer*, and 4 complex inoculum mixes of dried microflora which had been scraped off from 4 different batches of locally on-farm fermented roots. Cyanogenic glucoside levels were significantly more reduced in the *N. sitophila* and fungal mix fermented batches than in 3 out of the 4 batches inoculated with the more complex microflora.

This experiment showed that both incubation and microbial activity were instrumental in reducing cassava linamarin levels during the solid substrate fermentation. It also showed that the effectiveness varied considerably between the species of micro-organisms applied, and that it was correlated with the degree of root softening. Inoculation with mixtures of different micro-organisms did not result in more effective linamarin-level reduction than with the most effective single strain.

Role of the microflora concluded

Neurospora sitophila caused significant root softening and effectuated the highest linamarin- level reduction in cassava, but showed no linamarase activity. Conversely, *M. racemosus* and *Bacillus* spp. hardly softened the roots and were less effective in linamarin level reduction, in spite of showing ample linamarase activity. This indicates that the structure loss, caused by the pectolytic and cellulolytic enzyme system (Okolie and Ugochukwu, 1988) of *N. sitophila* was of greater importance than the combination of low cell wall degrading capacity and high linamarase activity of *M. racemosus* and the *Bacillus* sp.

The separate experiment, indicating higher efficacy from added exogenous cell wall-degrading enzymes than from exogenous linamarase in intact cassava tissue, was in line with the findings that a cell wall-degrading but linamarase-deficient fungus could be more effective than linamarase activity displaying micro-organisms. We therefore propose that the most important contribution of micro-organisms to linamarin-level reduction in solid substrate-fermenting cassava is their cell wall-degrading activity which enhances the contact between endogenous linamarase and the linamarin.

Conclusions and recommendations

The indigenous solid substrate fermentation method, as practiced by the Alur in Uganda, reduces the cyanogen levels of cassava roots more efficiently than sun-drying alone, but it does not guarantee reduction to the FAO/WHO suggested limit. The fermentation stage plays an obvious role in the reduction of cyanogen levels. The microflora contributes to the removal of the cyanogenic glucosides mainly through enabling contact between the compartmentally separated endogenous linamarase and the linamarin. This is attained by enzymic cell wall degradation. We conclude that the process of heap fermentation is in principle useful in the reduction of cassava's potential toxicity, but the method and its application need optimization to ensure still safer products. Improvements should be sought in better ensuring an effective cyanogen removal and a more hygienic product with fewer suspected fungi, and in shortening the time necessary for the process. These objectives might be met by optimizing processing conditions, including the use of appropriate starter cultures.

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