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Reduction of cyanide levels during anaerobic digestion of cassava

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Summary

During the methanogenic fermentation of cassava peel in a plug flow digester, cyanide bound as linamarin in cassava was released as HCN in the fermentation liquor, and then eliminated by the activity of free enzymes and by non-enzymatic reactions. The raw cassava peel contained (1) the enzyme permitting the hydrolysis of linamarin and the liberation of HCN (linamarase), and (2) a cyanide detoxification enzyme (β-cyanoalanine synthase). Cyanide removal was sufficiently fast to maintain a cyanide concentration in the fermentation liquor which was non-inhibitory for the methanogenic microflora.

Keywords

Cassava peel, methanogenic fermentation, *Manihot esculenta* Crantz, linamarase, β-cyanoalanine synthase.

Introduction

Cassava is a major source of calories in developing tropical countries. In 1986, FAO estimated that 35% of the 137.4-million ton world production was produced in African countries. The solid wastes from cassava processing can be used to produce biogas via anaerobic digestion, and thus generate part of the energy needed for drying cassava meal (Cuzin *et al.*, 1992). Cassava contains cyanogenic glucosides, mainly linamarin (92–98%), which release hydrogen cyanide after hydrolysis by an endogenous linamarase (Butler *et al.*, 1965; Conn, 1969). Anaerobic digestion can be inhibited by cyanide, because of the high sensitivity of methanogenic bacteria to this compound ($<1\,mg\,l^{-1}$ in pure culture) (Eikmanns & Thauer, 1984; Smith *et al.*, 1985). During the methanogenic fermentation of cassava peel in a plug flow digester, successive loads of cassava peel progressively increased cyanide concentration in the liquid phase, which is not renewed, although this never exceeded $6\,mg\,l^{-1}$, and no disturbance of methane production was observed (Cuzin *et al.*, 1989). The solid wastes discharged after digestion were free of cyanide (HCN, cyanohydrin or linamarin). This suggested a complete release of cyanide from the solid phase into the liquid phase.

We investigated the ability of liquor from a cassava peel anaerobic digester to

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release cyanide from linamarin by the action of linamarase present in cassava peel (Cooke *et al.*, 1978), and by elimination of cyanide added as KCN by the action of the detoxification enzymes β-cyanoalanine synthase and rhodanese which have been detected in cassava seedling or cassava leaf (Nartey, 1973; Boey *et al.*, 1976).

Materials and methods

Materials

Substrate for anaerobic digestion and reactor operation. Fresh cassava (*Manihot esculenta* Crantz, variety Mpembe) was collected in Central Africa from a small cassava transformation plant (Brazzaville, Congo). Cassava roots were peeled by hand after harvesting, and the peels were stored at 4°C until use. Raw cassava peel (3.3 kg) was loaded once every 2 days into a 'Transpaille' (128-l) digester (Farinet *et al.*, 1987; Cuzin *et al.*, 1992), operating in the mesophilic range (35–39°C). The digester used a continuous process patented by IRAT/CIRAD (France). It is based on the transfer of an heterogeneous substrate immersed in water in an horizontal cylindrical tank (Farinet *et al.*, 1987). In the fermenter used for the experiment the solid phase is moved as a plug-flow by means of a hydraulic jack, and is fermented at about 460 g volatile solids per day in a non-renewed liquid phase established once at the beginning of the process (Fadlalla, 1989). Three gas sampling points (G1, G2, G3) and three liquid sampling points (C1, C2, C3) were set up along the tank (Fig. 1).

Methods

Batch culture toxicity tests. Fermentation liquor (pH 7) was collected at C2 point of the digester in anaerobic sterilized bottle, immediatly distributed in 16 ml 'Hungate type' tubes (13 ml tube^{-1}) in an anaerobic glove box. Tubes were screw-capped and sealed with flanged rubber stoppers. Solutions (0.1 ml) of KCN or linamarin (BDH

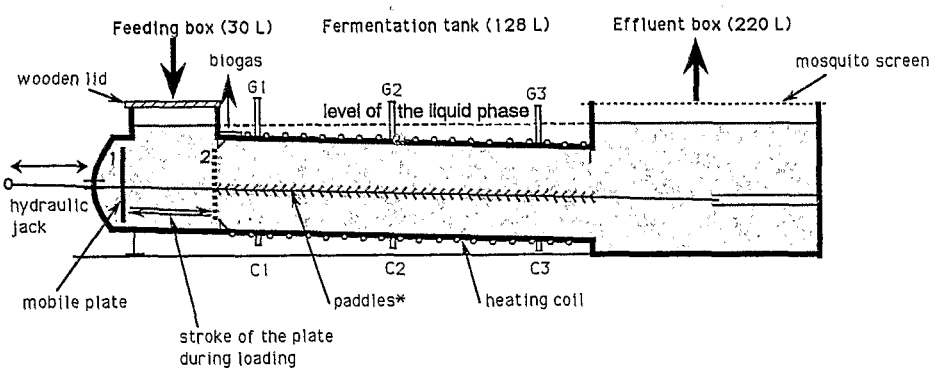

Figure 1. Transpaille fermenter — longitudinal cross-section. G1, G2, G3: gas sampling point. C1, C2 C3: liquid sampling points. 1: position for loading. 2: position during fermentation.
*paddles attached to the jack axis help to move forward the solid phase in the fermentation tank during loading.

Chemicals, London) or distilled water were added to a final concentration of 0, 5, 10 and 25 mg. l^{-1} CN. The tubes were incubated in duplicate at 37°C in a water bath under agitation (50 rpm min^{-1}). Triplicate samples, taken anaerobically with a hypodermic syringe, were analysed daily for free and total cyanide over 5 days.

Three controls, tested under the same conditions, were (1) distilled water, (2) supernatant after double centrifugation (8500 g for 20 min) of the fermentation liquor, and (3) supernatant inactivated by heating at 90°C for 30 min.

Two other series of tubes with fermentation liquor were prepared in the same conditions to test the effects of cyanide on methane production.

Analyses. To measure the cyanide content, cassava extracts were prepared in orthophosphoric acid (0.1 M) using the method of Cooke (1978). In order to uniformize experimental conditions, batch culture toxicity tests were performed on aliquots (fermentation liquor or controls) containing 0.1 M orthophosphoric acid, that were centrifuged (12500 g for 5 min), before measuring the cyanide content of the supernatant. Total cyanide content was measured after linamarase (BDH Chemicals, London) action (from a stock solution of 2 units ml^{-1} in 0.1 M phosphate buffer pH 6) and NaOH addition (Cooke, 1979). Free cyanide and HCN were measured by omitting linamarase in the assay, and both linamarase and NaOH respectively. Linamarin was calculated as the difference between free cyanide (cyanohydrin and HCN) and total cyanide. Cyanohydrin was calculated as the difference between the free cyanide and HCN.

Gas samples (0.1 ml) were taken with a syringe from the headspace of the tube and analysed for methane by gas chromatography with a Delsi Instruments Chromatograph, equipped with a flame ionization detector (Column HAYE SEP-D-1 m × 1/8″ stainless steel-80°C-carrier gas N_2 25 ml min^{-1}).

Enzyme extracts. Cassava peel (30 g) was diced (0.5 cm^3) and homogenized in a blender for 5 × 1 min at high speed with 0.1 M acetate buffer pH 5.5 (100 ml) for linamarase extraction, and with 0.05 M Tris HCl pH 8.5 (100 ml) for β-cyanoalanine synthase and rhodanese extraction. After rough filtration (mesh 0.5 mm), the filtrate was centrifuged at 27 000 g for 20 min. The supernatant was used for enzyme assays. All steps were carried out below 4°C.

Enzyme assays. Linamarase activity was assayed using 50 µl linamarin 10 mM (BDH Chemicals, London, UK) added to 50 µl aliquot of enzyme extract in 0.1 M acetate buffer pH 5.5 (final volume 0.5 ml) at 30°C. After 15 min the liberated glucose was determined using the DNS method (Miller, 1959). The standard curve was prepared for concentrations of glucose ranging from 0.5 to 4 micromoles. Linamarase activity was expressed in micromoles of glucose produced per min. β-cyanoalanine synthase was assayed according to Svenson & Andersson (1977). Its activity was calculated from the time curve for hydrogen sulphide production. The standard curve of sodium sulphide was linear up to 25 nmoles. Rhodanese activity was assayed according to Singleton & Smith (1988). Standard curve was prepared for concentrations of potassium thiocyanate ranging from 0.1 to 0.7 micromoles.

Protein determination. Protein was determined by the colorimetric method of Bradford (1976) with bovine serum albumin (Sigma Chemical Co, Saint Louis, MO, USA) as standard.

Results and discusion

Cyanide in cassava peel and in fermentation liquor from the 128-l reactor

The total cyanide content of the cassava peel was ranged from 150 to 360 mg CN kg^{-1} fresh weight, mainly in the form of cyanohydrin (34–64% w/w) and HCN (12–66% w/w). Cyanide content depends greatly on the cassava variety (de Bruijn, 1971). We detected only a small amount of bound form probably because cassava was peeled manually, permitting release and contact between the linamarase and the linamarin (de Bruijn, 1971), leading to early hydrolysis of the cyanogenic glucoside to cyanohydrin and HCN. The cassava peel remained for a few hours in air before being stored in a refrigerator for several weeks. The pH was about 4 in this substrate, giving the cyanohydrin great stability (Cooke, 1978; Fomunyan *et al.*, 1985).

The fermentation liquor contained low amounts of cyanide (up to 6 mg l^{-1}) mainly in the HCN form (more than 90%). Cyanide content varied depending on the regularity of loading and decreased from the peel load feeding box to the solid waste effluent box (Figs 1 and 2).

Release of HCN from linamarin by cassava fermentation liquor

In this assay, the initial CN concentration in the liquor varied from 0 to 6 mg l^{-1} and remained constant during the experiments. Cyanide contents were corrected by substracting the endogenous cyanide level measured in a control.

The centrifugation of the liquor removed most micro-organisms from the supernatant as shown by the absence of methane production. It was not possible to filter the liquor. Heating the supernatant at 90°C inactivated the enzymes.

In distilled water, the total cyanide remained constant, whatever the amount of cyanide added, HCN was only released after 24 h, and all the cyanide was in the HCN form within 250 h (Fig. 3, only the 25 mg l^{-1} curve is shown).

In the unheated liquors (fermenter liquor and supernatant), the release of HCN was immediate and increased quickly. No accumulation of cyanohydrin was observed. All the cyanide was in the HCN form within 24 h, after which the levels decreased slowly (Fig. 3). In the heated juice (inactivated supernatant), HCN appeared only after 24 h. The release time of free cyanide from linamarin was the same regardless of the amount of added cyanide.

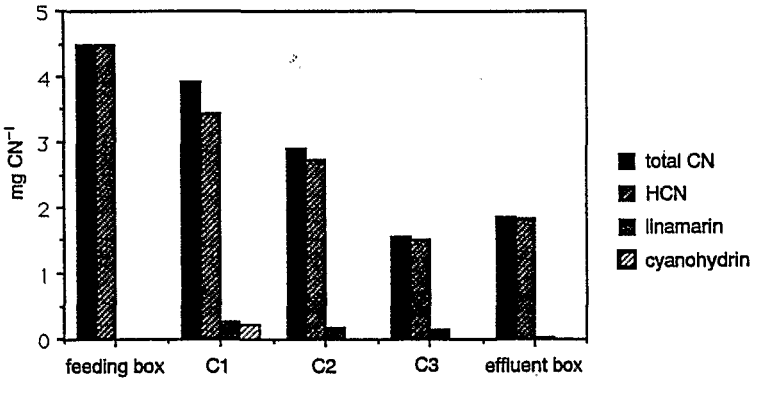

Figure 2. Cyanide content in the liquid phase of the fermenter from the feeding box to the effluent box, during the methanogenic fermentation of cassava peel.

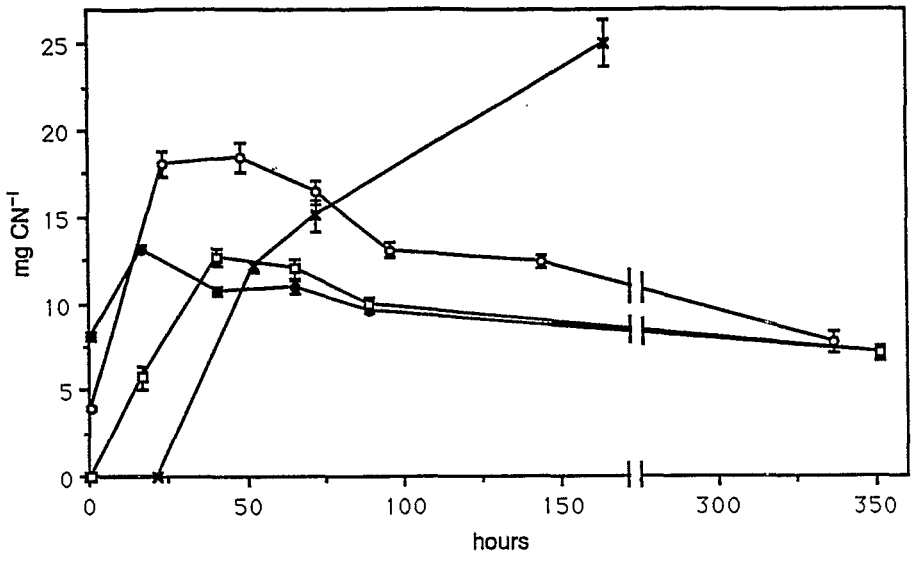

Figure 3. Release pattern of cyanide after linamarin addition (25 mg l^{-1} CN) in distilled water (x), fermentation liquor (○), supernatant (●), and inactivated supernatant (□).

The difference of release pattern between the fermentation liquor and supernatant indicated the contribution of the micro-organisms to hydrolysis of linamarin (Fig. 3).

Enzymatic activity in the fermentation liquor was responsible for the early production of free cyanide. Cassava cyanogens are hydrolysed by the plant enzyme linamarase (β-glucosidase), which occurs in large amounts in cassava (Cooke *et al.*, 1978), but the microbial breakdown of linamarin in fermenting cassava pulp was reported by Ejiofor & Okafor (1984).

Removal of HCN by cassava fermentation liquor

The same experiments were repeated using various concentration of KCN. Controls with distilled water showed a very small decrease in cyanide concentration, whatever the amount added (Fig. 4, only the 25 mg l^{-1} curve is shown).

In tests with heated and unheated mixtures a rapid loss of cyanide occurred within the first hour (Fig. 4). The cyanide may have reacted with compounds present in the liquor. Thereafter, in heated supernatant the rate of cyanide removal remained approximately constant. In fermentation liquor and supernatant, the rate of decrease was double that of heat treated liquor during the first 24 h and was followed by a levelling out (Fig. 4). The same pattern of removal was observed with 5 or 10 mg l^{-1} (data not shown) but in an attenuated form. The similarity between the dynamics of cyanide concentration obtained with the fermentation liquor and supernatant indicated that the microflora does not play a major role in the reduction of free cyanide, unless through extracellular enzymes.

The occurrence of cyanide detoxification enzymes in cassava might explain the first 24 h of high degradation in non-heated liquors. β-cyanoalanine synthase, catalysing the formation of β-cyanoalanine from HCN and cysteine has been detected in higher plants (Miller & Conn, 1980). Rhodanese activity has been identified in a reaction between cyanide and a sulphur donor (thiosulphate), where cyanide has been con-

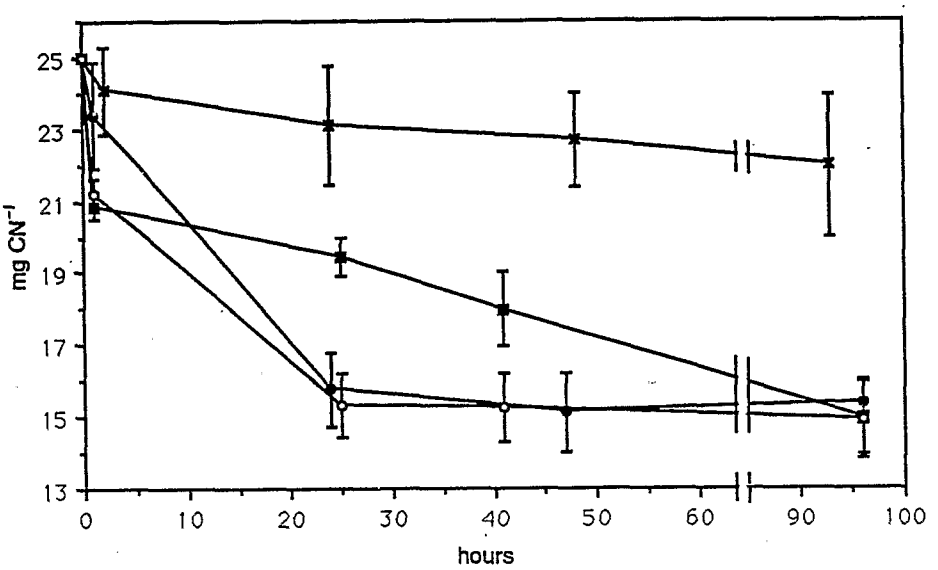

Figure 4. Dynamics of cyanide concentration after KCN addition (25 mg l^{-1} CN) in distilled water (x), fermentation liquor (o), supernatant (•), and inactivated supernatant (■).

verted into less toxic thiocyanate. Rhodanese occurs far less commonly in plants (Miller & Conn, 1980). Boey *et al.* (1976) have described the purification of rhodanese from cassava leaf. Micro-organisms posses enzymes for detoxifying cyanide (Knowles, 1976; Burton & Akagi, 1971), but little is known about cyanide assimilation by anaerobic micro-organisms (Knowles, 1976).

Enzymatic activities in cassava peel

The occurence of linamarase, β-cyanoalanine synthase, and rhodanese was investigated. Linamarase, the β-glucosidase which catalyses the hydrolysis of linamarin, was identified in cassava peel extracts by measuring the liberated glucose (29.8 μmoles glucose $min^{-1} mg^{-1}$ protein). This extract also showed the presence of a slight β-cyanoalanine synthase activity (9.33 nmoles thiocyanate $min^{-1} mg^{-1}$ protein), the enzyme which catalyses cyanide detoxification. The assay for β-cyanoalanine synthase in cassava peel extracts was complicated by an apparent inhibitor, whose specificity and function are unknown. Activity per unit volume decreased as the amount of extract increased. Attempts to overcome this apparent inhibition by the addition of bovine serum albumin, or gel filtration through Sephadex G-25, were unsucessful.

Whereas cassava linamarase was extensively investigated (Cooke *et al.*, 1978; Eksittikul & Chulavatnol, 1988; Mkpong *et al.*, 1990), only Nartey (1973) reported the detection of the β-cyanoalanine synthase in cassava seedling extracts. The slight β-cyanoalanine synthase activity determined in this study was of the same order as in other vegetal crude extracts (Cooney *et al.*, 1980; Miller & Conn, 1980; Goudey *et al.*, 1989). The occurence of a β-cyanoalanine synthase assay inhibitor was reported by Manning (1986), Mizutani *et al.* (1988) and Macadam & Knowles (1984). Using the calculation method of Lieberei & Selmar (1990), rhodanese activity was not detected in cassava peel.

Effects of cyanide on methane production

Additions of 5, 10, and 25 mg l^{-1} cyanide (KCN or linamarin) temporarily inhibited methanogenesis but when the concentration of cyanide reached its initial value (before KCN or linamarin addition), methane production recovered. The digester methanogenic microflora was sensitive to cyanide addition, but tolerated the low concentrations (6 mg l^{-1}) normally present in the fermenter.

Each new loading of cassava peel in the fermenter progressively increased cyanide concentration in the fermentation liquor. Substantial amounts of cyanide were removed by non-enzymatic processes (possible mechanisms are: complexation with metals, volatilization etc), but activity of free enzymes appeared to be important. The enzymatic systems are derived from cassava and/or the microflora. Bacteria appeared not to play an important role in cyanide removal. However, Fallon *et al.* (1991) suggested that methanogenic bacteria could favour the cyanide hydrolysis ($HCN + 2\,H_2O \rightarrow HCOO^- + NH_4^+$) by consuming formate thus making the reaction thermodynamically more favourable.

Thus the occurence of enzymes which remove cyanide from cassava peel ensures that the HCN released from linamarin by linamarase does not exceed 6 mg l^{-1} in the fermentation liquor, which does not inhibit methane production, in the anaerobic digester.

Acknowledgments

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