MIRCEN Journal, 1989, **5**, 169–179

Cassava-fermenting organisms

Donald Nwankwo, Edith Anadu* and Ralph Usoro

Department of Biological Sciences, University of Calabar, Calabar, Nigeria

Received as revised 13 December 1988; accepted 24 December 1988

Introduction

Cassava (*Manihot esculenta* Crantz) is a tropical root tuber which contains starch and belongs to the family *Euphorbiaceae* (Akinrele 1964). Processed cassava is widely eaten in Nigeria and other West African countries as 'fufu', 'garri', 'lafun', tapioca and in many other forms (Akinrele 1964; Okafor 1977; Ngaba & Lee 1979). Despite its importance as a major staple food, not much is known about the fermenting organisms or the by-products of fermentation.

The bitter varieties of cassava tubers contain the cyanogenic glucosides, linamarin and lotaustralin (Knowles 1976; Onwueme 1978). These water-soluble glucosides produce highly poisonous HCN on hydrolysis. The rate of hydrolysis of these glucosides is accelerated when cassava tubers are soaked in water (Okafor 1983). Continuous consumption of improperly detoxified cassava could give rise to ailments such as tropical ataxic neuropathy and goitre (Delange *et al.* 1976).

A two-stage fermentation of cassava was suggested to occur during 'garri' production by Collard & Levi (1959). In the first stage, *Corynebacterium manihot* hydrolyzes the starch present in tubers producing organic acids which are in turn converted into esters and aldehydes by the fungus *Geotricum candida* to impart the characteristic aroma and taste to 'garri'.

Recent studies however, attribute degradation of cassava tubers to bacterial activity. The predominant organisms isolated from cassava fermentation were bacteria, mainly *Leuconostoc* and to a lesser extent yeasts (Okafor 1977). Abe & Lindsay (1978) reported the isolation of lactic acid producing streptococci. Ngaba & Lee (1979) isolated *Lactobacillus plantarum* and *Streptococcus* species from cassava fermented for 'garri' production.

More emphasis has been laid on fermentation studies for 'garri' production to the neglect of fermentation studies for 'fufu' production. It is still not clear whether degradation of the cyanogenic glucoside proceeds by action of endogenous enzymes or by bacterial activity (Okafor 1977). It is also not known whether the genes involved in cassava fermentation are found on plasmids or are chromosomally located. The

*Corresponding author and present address: Department of Food Science and Technology, Anambra State University of Technology, Abakiliki Campus, Nigeria.

actual role played by individual organisms in the fermentation process is also not known.

The need to document organisms involved in cassava fermentation and to begin the search for the genes whose protein products are involved in the break-down of starch and the cyanogenic glucosides present in cassava tubers motivated this study. For two consecutive years, therefore, different strains of cassava tubers have been used in the study of cassava fermenting organisms. All organisms isolated produce α-amylase.

Materials and methods

Cassava tubers

Tubers of cassava varieties Tms 30211, Tms 30555, Tms 30572, Tms 40764 and a local variety, 'Akumbe' (Anon 1980) were collected from Agricultural Services Division, Ministry of Agriculture, Ikot Efanga, Calabar, Nigeria. *Bacterial strains. Escherichia coli* K12 strain RR1 (Bolivar & Backman 1979) obtained from G. Wilson (New England Biolabs Inc. Beverly, MA, USA) was used as control for α-amylase activity.

Enzymes

Restriction endonucleases were obtained from New England Biolabs Inc., Beverly (MA, USA) and used according to manufacturer's recommendations.

Cassava fermentation

Methods used locally to ferment cassava tubers for 'fufu' production were employed. The two outer layers were removed from 800 $\times$ *g* of cassava tubers and washed with clean but not necessarily sterile tap water and steeped in small fermentation vats for 5 days. The vats were covered except during sample collection. Fermentation was carried out at room temperature (about 28°C).

Isolation and characterization of cassava fermenting organisms

Dilutions of water samples from steeped cassava were plated out at 24 h intervals on Nutrient agar plates (0.1% Lablemco powder, 0.2% yeast extract, 0.5% peptone, 0.5% NaCl, 1.5% agar) or Tryptone/yeast extract broth (Tryptone 1.0%, yeast extract 0.5%, NaCl 0.5%, glucose 0.1%) supplemented with 1.5% (w/v) agar. Representative colonies were purified by repeated plating on same media used for isolation. Pure cultures of the isolates were transferred into soft agar in screw capped vials and maintained at room temperature. Broth cultures of the isolates were also frozen at −22°C in 12% glycerol.

Biochemical tests

Sugars, in basal peptone water were inoculated from an overnight broth culture and incubated at 37°C for 24 to 48 h. Indole production, acetoin production, gelatin liquefaction, urea hydrolysis were carried out as described by Cowan & Steel (1974). Citrate utilization was tested on Simmon's citrate slopes. Tests for oxidase (Kovac's method) and catalase were carried out as described by Cowan & Steel (1974).

Antibiotic sensitivity
Isolated organisms were each inoculated into 3 ml soft agar (0.75%) at about 45°C and overlaid on nutrient agar plates. Upon setting, oxoid antibiotic multodisks (2023E) were placed on them and incubated overnight at 37°C. Sensitivity was determined by presence or absence of a zone from the edge of the disk to the edge of the bacterial growth.

Test for starch hydrolysis
α-Amylase production was determined by inoculation of test organisms on 1% starch agar plates using a thin needle. Inoculated plates were incubated for 24 h at 37°C. Amylase activity was detected by flooding of incubated plates with Lugol's iodine. Size of halos surrounding the colonies reflect α-amylase activity.

Purification of plasmids
Plasmids were purified either by a modified version of the alkaline procedure of Birnboim & Doly (1979) or by hydroxyapatite chromatography procedure of Colman *et al.* (1978).

Restriction analysis of purified plasmid DNA
Purified plasmid DNA were digested with the restriction endonuclease *Bam* Hl using buffers and reaction conditions recommended by the manufacturers. Digested DNA molecules were then analysed by electrophoresis on 1% agarose gel. Molecular weight of the plasmid DNA molecules were estimated by determining the total weight of fragments generated by digestion with *Bam* Hl. Phage λ DNA digested with *Hind* III and $\phi X174$ DNA digested with *Hae* III were used as size markers. Bands were visualized in the dark using an ultra-violet lamp at 302 nm.

Transformation of E. coli *cells with plasmids purified from cassava fermenting organisms*
Plasmids purified from cassava fermenting organisms were used in transforming *E. coli* RR1 cells. The $CaCl_2$ heat shock method of Cohen *et al.* (1972) were employed.

Results

Organisms isolated
The most frequently isolated organisms from the four different varieties of cassava tubers were (in order of predominance): *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Corynebacterium manihot*, *Bacillus subtilis* and *Pseudomonas alcaligens*. *Pseudomonas aeruginosa* was isolated from cassava varieties Tms 30211 and Tms 30555. *Klebsiella pneumoniae* was isolated from cassava variety Tms 30572.

A pattern emerged during the fermentation: during the first 24 h, *Leuconostoc* and *Lactobacillus* appeared in very low numbers but the other organisms *Bacillus*, *Pseudomonas* and *Corynebacterium* were readily isolated. However their numbers started declining and at about 48 to 72 h the most frequently isolated organisms were *Leuconostoc* and *Lactobacillus*.

Pseudomonas aeruginosa and *Staphyloococcus aureus* were isolated as contaminants, probably from water, soil or from peeling of the cassava tubers.

pH of fermentation medium

Acidity of fermenting medium was monitored every 12 h for 5 days. The pH dropped from about 6.80 to 3.90 within 24 h of fermentation. The final pH of fermenting mediums after 5 days was between 3.60 and 3.70. This compares with results of Ngaba & Lee (1979) where the final pH at the end of cassava fermentation for 'garri' production was between 3.65 and 3.95. pH profile from fermentation of other varieties of cassava were similar (data not shown).

Starch hydrolysis

Carbohydrates found in cassava tubers consist mainly of starch (84%), the rest being low molecular weight sugars (4%) (Ketiku & Oyenuga 1970). Of the sugars found in cassava, sucrose constitutes 71%, glucose 13%, fructose 9% and maltose 3%. Isolated organisms were tested for α-amylase activity.

All the isolates displayed α-amylase activity (data not shown). The extent of starch hydrolysis depicted by size of halos surrounding the colonies were greatest for *Leuconostoc mesenteroides* (CFOC42), followed closely by *Pseudomonas alcaligens*, (CFOB21). Size of halos surrounding *Corynebacterium manihot* (CFOB23) and *Lactobacilus plantarum* (CFOA33) were about equal in size. This was followed by *Bacillus subtilis* (CFOC12) and then *Klebsiella* (CFOD11). Pseudomonad isolate (CFOC13) had the least α-amylase activity.

Antibiotic sensitivity of cassava fermenting organisms

The end-product of cassava fermentation is still loaded with the fermenting organisms and ingestion of such food products, if not properly treated, could produce a public health problem particularly if the organisms carry antibiotic resistance genes or are toxigenic. We therefore tested isolated organisms for resistance to several antibiotics (Table 1). Almost all the organisms isolated were resistant to ampicillin, sulfamethoxazole/trimethoprim, penicillin G, methicillin and sulfadiazine. Erythromycin, tetracycline and chloramphenicol inhibited the growth of almost all the organisms.

Plasmids of cassava fermenting organisms

As all organisms associated with cassava fermentation displayed α-amylase activity, and as the α-amylase gene could occur on plasmids, as is found in *Bacillus stearothermophilus* (Mielenz 1983) or on host chromosomes (Yamaguchi *et al.* 1974; Henner and Hoch 1980), we purified the plasmids from the isolates (Fig. 1). Except for *Klebsiella* which contained more than one plasmid (lane F), all other isolates contained single plasmids with size greater than 15 kb.

Test of purified plasmids for α-amylase activity

Purified plasmids were transformed into *E. coli* RR1 cells and plated on Luria-agar plates containing 50 μg/ampicillin/ml. Transformation efficiency of about $10^2/\mu$g of

Table 1 Antibiotic sensitivity of cassava fermenting organisms*

Isolate	Sulfamethoxazole/ trimethoprim (25 μg)	Antibiotics						
		Ampicillin (2 μg)	Penicillin Chloramphenicol (10 μg)	Penicillin G (1.5 Units)	Methicillin (10 μg)	Erythromycin (10 μg)	Tetracycline (10 μg)	Sulfadiazine (50 μg)
CFOA 12 *Bacillus subtilis*	+++	+++	−	+++	+++	+	−	+++
CFOA 33 *Lactobacillus plantarum*	+++	+++	−	+++	+++	+	−	+++
CFOA 42 *Leuconostoc mesenteroides*	+++	+++	−	+++	+++	−	−	+++
CFOB 23 *Pseudomonas alcaligens*	+++	+++	−	+++	+++	−	−	+++
CFOB 31 *Leuconostoc mesenteroides*	+++	++	−	++	++	−−	++	+++
CFOC 13 *Pseudomonas alcaligens*	−	++	−	++	++	++	−	++
CFOC 21 *Corynebacterium manihot*	++	++	−	++	++	−−	−−	++
CFOC 42 *Leuconostoc mesenteroides*	++	++	−	++	++	−−	−−	++
CFOD 11 *Klebsiella pneumoniae*	++	++	−	++	++	−	−	++
E. coli 2053	−	++	++	++	−−	++	−−	−−
E. coli RR1	−	−	−	−	−	−	−	−

* Key: − sensitive; −− very sensitive; + mildly resistant; ++ resistant; +++ very resistant.

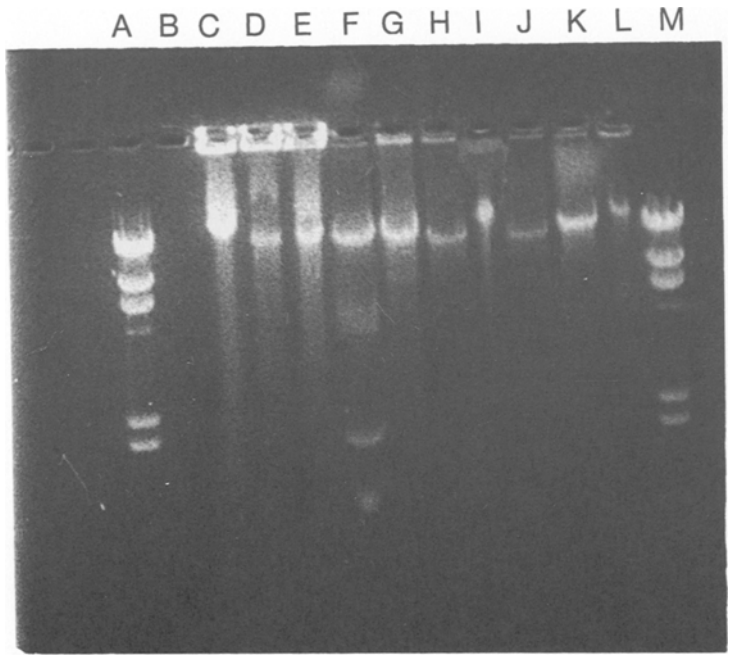

Fig. 1 Agarose gel electrophoresis of plasmids purified from cassava fermenting organisms. Lanes A and M are phage λ DNA digested with *Hin*d III. Lanes C–E, plasmids purified from transformants DT 100, DT 118 and DT 121; F, *Klebsiella pneumoniae*; G, *Leuconostoc mesenteroides*; H, *Corynebacterium manihot*; I, *Pseudomonas alcaligens*; J, *Bacillus subtilis*; K, *Lactobacillus plantarum*; L, *Bacillus subtilis*.

plasmid DNA were recovered only when plasmids purified from *Klebsiella* were used. Transformation efficiency of about $10^6/\mu g$ was obtained when pBR322 (Bolivar *et al.* 1977) was used in similar transformation experiments. Repeated transformation experiments produced similar results with occasional survivors which harboured no plasmids.

Transformants were tested for α-amylase activity by plating on Luria-agar plates containing 1% starch. None of the transformants displayed α-amylase activity (not shown).

Restriction analysis of purified plasmids

We sought to establish if plasmids purified from the various isolates produced identical restriction fragments, more so when almost all the plasmids could not be transferred and or maintained in *E. coli* RR1 cells. Plasmids purified from the original isolates, and the transformants were digested with various restriction enzymes. There were no restriction sites for the enzymes *Hin*d III, *Pst* I, and *Eco*RI on any of the plasmids (not shown). Most of the plasmids were digested with *Bam* H1 (Fig. 2). There were no similarities in the digestion pattern except amongst the transformants DT100, DT118 and DT121. Two common fragments, a 23 kb and a 4.80 kb were generated with *Bam* H1. CFOD11 from which the transformants were obtained produced larger sized fragments, a 6.5 kb and another fragment at about 25 kb.

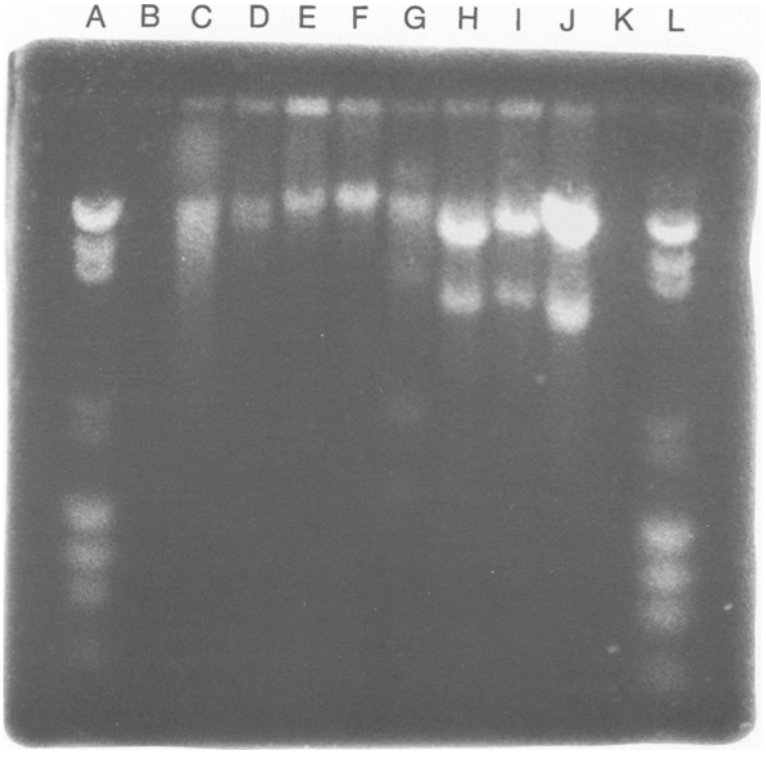

Fig. 2 Restriction analysis of plasmids purified from cassava fermenting organisms. About 1 μg each of the purified plasmids were digested with *Bam* H1. Digested plasmids were analysed by electrophoresis on 1% agarose gel.Lanes A and L, *Hin*d III digest of phage λ DNA and *Hae* III digest of φX 174 DNA used as size markers. Plasmid from *Lactobacillus plantarum*, lane C; *Leuconostoc/mesenteroides*, lane D; *Pseudomonas alcaligens* lane E; *Corynebacterium manihot*, lane F; *Klebsiella pneumoniae* lane G; transformants DT 100, DT 118 and DT 121 Lanes H, I and J, respectively.

Altered antibiotic sensitivity of transformants

The antibiotic sensitivity of the transformants were compared with the parental strain CFOD11. Transformants were selected on the basis of resistance to 50 μg/ml ampicillin. Table 2 shows there were slight differences in the antibiotic sensitivity of the parental strain CFOD11 when compared with transformants DT100 and DT118 derived using plasmids purified from CFOD11. Where as all were resistant to ampicillin, penicillin G and methicillin, the parental strain was resistant to sulfamethoxazole/trimethoprim while the transformants were sensitive.

Although the two transformants DT100 and DT118 displayed identical fragments on digestion with *Bam* H1, DT100 was resistant to sulfadiazine but was sensitive to tetracycline and chloramphenicol, where as DT118 was resistant to both tetracycline and chloramphenicol.

Discussion

The flora of organisms involved in fermentation of different strains of cassava were similar in each case. There are, however, differences in the isolates reported here and

Table 2 Antibiotic sensitivity of *E. coli* RR1 cells transformed with plasmids purified from the *Klebsiella* isolate CFOD11*

	Antibiotics							
Isolate	Sulfamethoxazole/ trimethoprim (25 μg)	Ampicillin (2 μg)	Chloramphenicol (10 μg)	Penicillin G (1.5 Units)	Methicillin (10 μg)	Erythromycin (10 μg)	Tetracycline (10 μg)	Sulfadiazine (50 μg)
CFOD 11	+++	+++	−−	+++	+++	−−	−−	+++
CFODT 100	−−	+++	−−	+++	+++	+++	−−	+++
CFODT 118	−−	+++	+++	+++	+++	++	++	−−−
E. coli RR1	−	−	−	−	−	−	− +	−

* Key: − sensitive; −− very sensitive; + mildly resistant; ++ resistant; +++ very resistant.

those reported by other groups during fermentation of cassava for 'garri' production. Collard & Levi (1959) reported isolation of *Corynebacterium manihot* and a fungus *Geotricum candida*. Okafor (1977) reported isolation of *Leuconostoc*, *Alcaligenes*, *Lactobacillus* and *Corynebacterium*. *Bacillus subtilis* and *Pseudomonas alcaligenes* have been isolated along with *Corynebacterium manihot*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. Since *Bacillus* and *Pseudomonas* are common soil organisms, they could be seen as merely contaminants from soil. However, their isolation from all the cassava varieties tested, and their ability to hydrolyze starch, could indicate their involvement in cassava fermentation. Differences in the isolated organisms might also be related to the microflora of the different soils from which the cassava tubers were harvested.

All organisms, isolated produce α-amylase at an optimum pH of about 5.0 (not shown). The succession of organisms during the fermentation process may depend, therefore, on the tolerance of the organisms to an acidic medium.

A major concern has been the discovery of antibiotic resistance among isolated organisms.Cassava processed for either 'garri' or 'fufu' production undergoes some temperature alterations, high enough to destroy the organisms. Some organisms, however, might survive due to improper processing.

Many of the genes coding for antibiotic resistance are borne on plasmids (Linton 1978; Smith 1979). The 31.50 kb plasmid, purified from the sole *Klebsiella* isolate CFOD11, carried genes for resistance to ampicillin, penicillin G, methicillin and sulfadiazine. This was ascertained from the resistance of *E. coli* RR1 cells to these antibiotics when plasmids from CFOD11 were used in transformation. We do not, however, fully understand the altered resistance pattern amongst the various transformants, even though all generated similar fragments on digestion with *Bam* H1. Mutations may however account for the altered antibiotic sensitivity. The genes for resistance to sulfamethoxazole/trimethoprim could reside on the smaller plasmid (2.3 kb) found in CFOD11. This class would not be recovered on the basis of resistance to ampicillin, except co-transformation with the 31.50-kb plasmid has occurred.

The α-amylase gene is not found on the 31.50-kb plasmid purified from *Klebsiella pneumoniae*. It could thus be located on the chromosome or the gene could not be expressed in *E. coli* if it is located on the 31.50-kb plasmid.

Restriction analysis of plasmids from all the isolates does not suggest that the plasmids have a common origin. They have in common, however, the inability to be maintained and/or expressed in *E. coli* RR1 cells.

A question that is still unaddressed is the role of cassava fermenting organisms, if any, in the degradation of the cyanogenic glucosides. Many species of bacteria are known to be capable of degrading cyanides (Castric 1975; Rogers & Knowles 1978). Ability of cassava fermenting organisms to degrade the poisonous cyanogenic glucosides needs further investigation.

Acknowledgement

We are grateful to New England Biolabs, Inc., Beverly (MA, USA) for supply of restriction enzymes and other materials without which this work would not have been undertaken. Our sincere thanks also go to the Agricultural services Division of the Ministry for Agriculture, Calabar for the supply of cassava tubers used in this study.

References

ABE, M. O. & LINDSAY, R. C. (1978) Evidence for a lactic streptococcal role in Nigerian acid cassava (*Manihot esculenta* Crantz) fermentations. *Journal of Food Protection* **41**, 781–784.

AKINRELE, I. A. (1964) Fermentation of cassava. *Journal of Science, Food and Agriculture* **15**, 589–594.

ANON. (1980) *11TA Research Highlights for 1979*. Ibadan. ISS No. 331–4340, pp. 32–33.

BIRNBOIM, H. C. & DOLY, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.

BOLIVAR, F. & BACKMAN, K. (1979) Plasmids of *Escherichia coli* as cloning vectors. In Wu, R. (ed.) *Methods in Enzymology*, Vol. 68, pp. 245–267. Academic Press, New York.

BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACK, M. C., HEYNECKER, H. L., BOYER, H. W., CROSA, J. H. & FALKOW, S. (1977) Construction and characterization of new cloning vehicles II. A multipurpose cloning system. *Gene* **2**, 95–113.

CASTRIC, P. A. (1975) Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Canadian Journal of Microbiology* **21**, 613–618.

COHEN, S. N., CHANG, A. C. Y. & HSU, L. (1972) Non chromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Science USA* **69**, 2110–2114.

COLLARD, P. & LEVI, S. (1959) A two stage fermentation of cassava. *Nature* **183**, 620–621.

COLMAN, A., BYERS, M. J., PRIMINOSE, S. B. & LYONS, A. (1978) Rapid purification of plasmid DNAs by hydroxyapatite chromatography. *European Journal of Biochemistry* **91**, 303–310.

COWAN, S. T. & STEEL, K. J. (1974) *Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge. Cambridge University Press.

DELANGE, F., BOURDOUX, P., CAMUS, M., GERARD, M., MAFUTA, M., HANSON, A. & ERMANS, M. (1976) The toxic effect of cassava on human thyroid. In *Procedings of the 4th Symposium of the International Society for Tropical Root Crops*, 237–242.

HENNER, D. J. & HOCH, J. A. (1980) The *Bacillus subtilis* chromosome. *Microbiological Reviews* **44**, 57–82.

KETIKU, A. O. & OYENUGA, V. A. (1970). Preliminary report on the carbohydrate constituents of cassava root and yam tuber. *Nigerian Journal of Science* **4**, 25–30.

KNOWLES, C. J. (1976) Micro-organisms and cyanide. *Bacteriological Reviews* **40**, 652–680.

LINTON, A. H. (1978) Antibiotics, animals and man – an appraisal of a contentious subject. In Woodbine, W. (ed.) *Antibiotics and Antibiosis in Agriculture*, pp. 315–343. Butterworths, London.

MIELENZ, J. R. (1983) *Bacillus stearothermophilus* contains a plasmid borne gene for α-amylase. *Proceedings of the National Academy of Science USA* **80**, 5975–5979.

NGABA, P. R. & LEE, J. S. (1979) Fermentation of cassava (*Manihot esculenta* Crantz). *Journal of Food Science* **44**, 1570–1571.

OKAFOR, N. (1977) Micro-organisms associated with cassava fermentation for garri production. *Journal of Applied Bacteriology* **24**, 279–284.

OKAFOR, N. (1983) Indgenous fermented foods. *Nigerian Institute of Food Journal* **1**, 33–37.

ONWUEME, I. C. (1978) *The Tropical Tuber Crops*, pp. 109–148. John Wiley & Sons, London.

RODGERS, P. B. & KNOWLES, C. J. (1978) Cyanide production and degradation during growth of *Chromobacterium violaceum*. *Journal of General Microbiology* **108**, 261–267.

SMITH, H. W. (1978) Antibiotic resistance in bacteria and associated problems in farm animals before and after the 1969 Swann Report. In Woodbine, M. (ed.) *Antibiotics and Antibiosis in Agriculture*, pp. 345–357. Butterworths, London.

YAMAGUCHI, K., NAGATA, Y. & MARUO, B. (1974) Isolation of mutants defective in α-amylase from *Bacillus subtilis*: Genetic analyses. *Journal of Bacteriology* **119**, 416–424.

Summary

Organisms isolated during the fermentation of cassava tubers, as practised for 'fufu' production, included *Bacillus subtilis, Pseudomonas alcaligenes, Lactobacillus plantarum, Corynebacterium manihot, Leuconostoc mesenteroides* and *Pseudomonas aeruginosa*. All isolates displayed amylase activity. Optimum growth and amylase activities of isolated organisms was at 42°C and between pH values 5 and 6. Isolated organisms also displayed similar patterns of antibiotic resistance. Plasmids purified from isolates could not be transferred or maintained in *Escherichia coli* RR1 cells.

Résumé

Microorganismes fermentant le manioc

La fermentation des tubercules de manioc a été effectuée comme on le pratique traditionnellement pour la production de 'fufu'. On a utilisé quatre variétés différentes de tubercules de manioc. Au cours du processus de fermentation, un grand nombre d'espèces microbiennes agissent de concert pour dégrader l'amidon contenu dans les tubercules. Les glycosides cyanogènes, linamarine et lotaustraline sont également degradés. Les microorganismes isolés au cours de la fermentation des tubercules de manioc, comme on la pratique pour la production de 'fufu', comprennent *Bacillus subtilis, Pseudomonas alcaligenes, Lactobacillus plantarum, Corynebacterium manihot, Leuconostoc mesenteroides* and *Pseudomonas aeruginosa*. Tous les microorganismes isolés révèlent une activité amylolytique. Les croissances et activités amylolytiques des microorganismes isolés montrent un optimum à 42°C et un pH entre 5 et 6. Les organismes isolés exhibent aussi des profils semblables de résistance aux antibiotiques. Les plasmides purifiés des isolats n'ont pas pu être transférés ni maintenus dans des cellules d'*Escherichia coli* RR1.