African Journal of Biotechnology Vol. 11(65), pp. 12865-12877, 14 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.3672
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Isolation and screening of microorganisms from a gari fermentation process for starter culture development

Vinodh A. Edward[1,2,3,4*], Moutairou Egounlety[5], Melanie Huch[6], Petrus J. Van Zyl[1], Suren Singh[2], Naledzani D. Nesengani[1,2], Vetja M. Haakuria[1,7], Charles M.A.P. Franz[6]

[1]Council for Scientific and Industrial Research, Biosciences, Meiring Naude Road, Brummeria, 0184, Pretoria, South Africa.
[2]Durban University of Technology, Department of Biotechnology and Food Technology, P.O. Box 1334, Durban, 4000, South Africa.
[3]Wits Reproductive Health and HIV Institute, Faculty of Health Sciences, University of the Witwatersrand, P.O. Box 18512, Hillbrow, 2038, South Africa.
[4]The Aurum Institute, The Ridge, 29 Queens Road, Parktown, Johannesburg, 2193, South Africa.
[5]Université Nationale du Bénin, Abomey-Calavi, 01 Cotonou, Bénin.
[6]Max Rubner Institute, Haid-und-Neu-Str. 9, 76131, Karlsruhe, Germany.
[7]School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand, 1 Jan Smuts Avenue, Braamfontein, Johannesburg, 2000, South Africa.

Accepted 11 June, 2012

Cassava (*Manihot esculenta* Crantz), is used for the production of a variety of West African foods and ranks fourth in the list of major crops in developing countries after rice, wheat and maize. Gari is one of the most popular foods produced from cassava. Cassava may contain high levels of linamarin, a cyanogenic glucoside, which in its natural state is toxic to man. Therefore, some processing methods that can enhance the detoxification of cassava and lead to the improvement of the quality and hygienic safety of the food are vitally important for less toxic products to be obtained. Quality, safety and acceptability of traditional fermented foods may be improved through the use of starter cultures. There has been a trend recently to isolate wild-type strains from traditional products for use as starter cultures in food fermentation. A total of 74 bacterial strains and 21 yeast strains were isolated from a cassava mash fermentation process in a rural village in Benin, West Africa. These strains were assessed, together with 26 strains isolated at the Council for Scientific and Industrial Research (CSIR) from cassava samples sent from Benin previously, for phenotypic and technological properties. 24 presumptive lactic acid bacteria (LAB) were selected for further phenotypic, genotypic and technological characterization.

Key words: Lactic acid bacteria, gari, cassava, fermentation.

INTRODUCTION

Cassava is a staple food for more than 500 million people in the developing world (Cock, 1982, 1985). It ranks fourth after rice, wheat and maize on the list of major food crops in developing countries (Mlingi et al., 1992). Cassava has the ability to grow in poor and acidic soils, which are often not suitable for other crops, and yields a harvest in times of drought when all other crops have failed for lack of water (Mlingi, 1995). Despite these advantages, cassava has four major drawbacks which limit its utilisation as a food (Kimaryo et al., 2000). These are low energy density, low protein content, rapid postharvest deterioration and potential cyanide toxicity (Gidamis, 1988; Howlett et al., 1990; Mlingi et al., 1991; Oyewole and Aibor, 1992; Mlingi, 1995).

In cassava, cyanide occurs as cyanogenic glucosides,

*Corresponding author. E-mail: vedward@auruminstitute.org . Tel: +2710 590 1392, Fax +2786 295 6755.

Figure 1. A graphic representation of the gari production process. (1) Cassava tubers after harvesting, (2) tubers are peeled and washed, (3) mechanical grinding of the tubers, (4) collection of the ground tubers, (5) palm oil and soy bean added, (6) placed in buckets, sealed with lids, and left to ferment for 48 h, (7) and (8) mixture is placed in a large bag, (9) the dewatering process, (10) sieving to remove large particles, (11) area where mixture is fried/garified, (12) frying/garification, (13) fine sieving (14) comparison between traditional gari (left) and gari fortified with soybean and palm oil.

mostly linamarin (>80%) and to a lesser extent lotaustralin (Cereda and Mattos, 1996; Kimaryo et al., 2000). The cyanogenic glucosides are present in all parts of the plant, with possible exception of the seeds (Vasconcelos et al., 1990). Bitter varieties, which contain higher amounts of cyanogenic glucosides, have to be processed to remove the toxic compounds before consumption, whereas sweet varieties, which have low levels of cyanogenic glucosides, can be eaten fresh (Rosling, 1990). Despite this, populations which use cassava as main staple food, mainly grow the bitter varieties due to their higher yields (Mozambique Ministry of Health, 1984) as well as their resistance to insects, and therefore rely on processing methods for detoxification. Fermentation not only enhances detoxification, but may also improve the quality and hygienic safety of the food (Ogunsua, 1980). Gari, one of the most popular foods derived from cassava fermentation, is consumed by more than 200 million people across West Africa (Okafor and Ejiofor, 1990).

In order to gain a better understanding of the gari production process, a visit was made to a rural village in Benin, West Africa. Gari is traditionally processed by women in these villages, where they produce enough to sustain their household usage. The purpose of the visit was to document the gari process, as well as to take samples during the fermentation process for isolation of predominant bacteria which are typically associated with the fermentation. Commercial starter cultures generally originate from food substrates or from the processes in which they are applied (Holzapfel, 2002). There has also been a trend recently to isolate wild-type strains from traditional products for use as starter cultures in food fermentation (Beukes et al., 2001; De Vuyst et al., 2002; Leroy and De Vuyst, 2004). It was envisaged that the microorganisms isolated from the samples taken would be ideal for the development of a starter culture, as they would be typical of the fermentation and well adapted to the ecological conditions as experienced during the gari fermentation.

MATERIALS AND METHODS

Microbiological sampling during preparation and fermentation of cassava in a Benin village

Gari, fortified with soybean and palm oil, was prepared in a village near Cotonou, Benin. The process is illustrated in Figures 1 and 2. Samples were taken initially (T_0) just before the buckets were sealed with lids (Figure 1, step 6) and every six hours thereafter

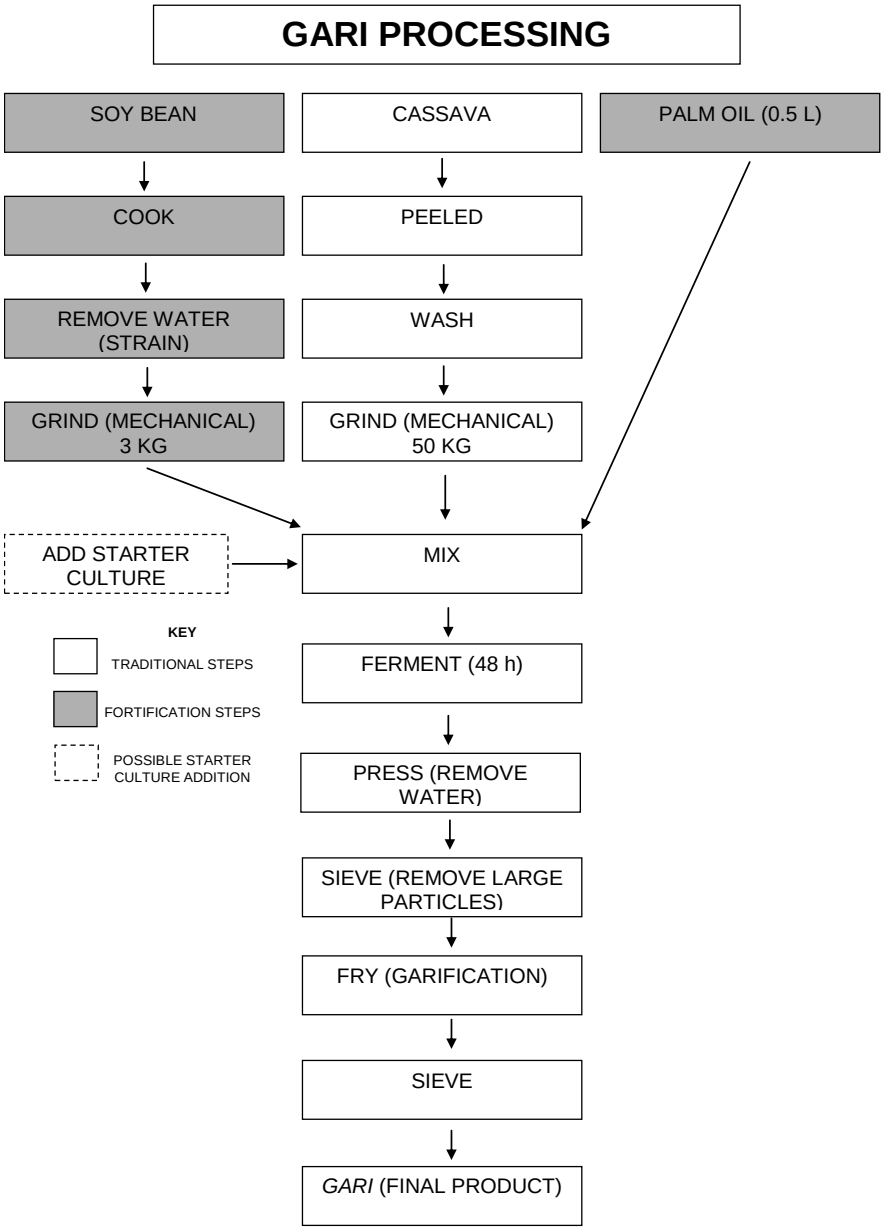

Figure 2. Diagramatic representation of the gari production process. Shaded areas show the fortification steps. Dashed box shows where potential starter culture can be added.

during the 48 h fermentation process of cassava for the preparation of gari in Benin. This was conducted in order to isolate the microbes involved in the fermentation. 10 g samples were diluted 1:10 using 90 ml Ringer's solution (Merck, Darmstadt, Germany) and homogenised for 1 min using a blender. Samples were diluted further in a ten-fold dilution series (10^{-2} to 10^{-9}) and spread plated onto different agar media: de Man, Rogosa and Sharpe (MRS) agar, M17 agar, Rogosa agar and malt extract agar (MEA) agar (all from Merck, Darmstadt, Germany). MRS, M17 and Rogosa media were suited for isolating different lactic acid bacteria (LAB) groups and were thus used to obtain the greatest diversity of LAB associated with the fermentation. MEA was used to isolate any yeast present during fermentation. The plates were incubated aerobically at 30°C for 48 h.

For isolation and identification of potential starter strains, colonies were randomly picked from the plates with the highest dilutions and purified by streaking onto agar. Picking the bacteria from plates of the highest dilution ensured that the most predominant bacteria associated with the fermentation, that is, those occurring in highest numbers, were isolated. For pH measurements during the fermentation, 10 g of samples were added to 20 ml of distilled water and homogenised. The pH was measured every 6 h using a portable pH meter (Jencons). For transport of the isolates to the Council for Scientific and Industrial Research (CSIR) in South Africa, the strains were streaked onto the selective media that they were isolated from, as descibed above. Samples were transported to South Africa within 48 h and then purified by streaking out repeatedly onto the appropriate growth media. The isolates were

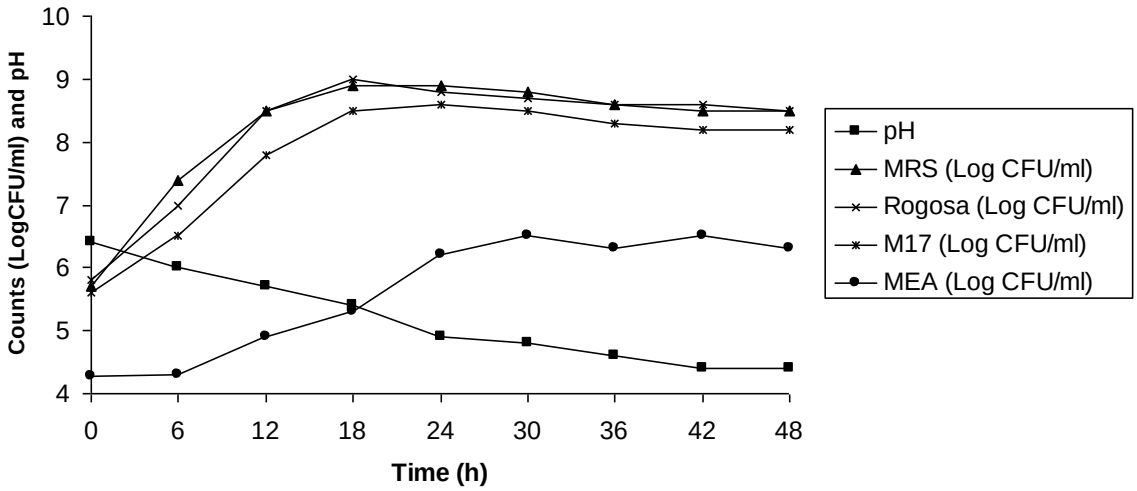

Figure 3. Growth of microorganisms and pH reduction in fermenting cassava mash.

kept in 20% (v/v) glycerol at -80°C and added to the culture collection already present at the CSIR. The microorganisms from Benin and LAB isolates from the CSIR culture collection, which were previously isolated from cassava (obtained from Benin), were revived on suitable agar media, and screened for the production of suitable compounds and enzymes as described below.

Phenotypic characterisation

LAB strains were characterised by determination of cell morphology using phase contrast microscopy, Gram staining, catalase test and gas (CO_2) production from glucose using the methods as described by Schillinger and Lücke (1987). Sugar fermentations patterns of LAB isolates were determined using the API 50 CHL system (bioMérieux, France) according to the manufacturer's instructions and the identification of LAB strains was performed using the computer program APILAB PLUS (Version 3.2.2., BioMérieux, France). Yeast strains were characterized by cell morphology. Sugar fermentation patterns were determined using the API 20 AUX system (bioMérieux, France) according to the manufacturer's instructions and the identification of yeast strains was also performed using the computer program APILAB PLUS (Version 3.2.2., BioMérieux, France).

Technological properties

Production of β-glucosidase

A medium for testing β-glucosidase was prepared by adding 0.1 g of 4-nitrophenyl-β-D-glucopyranoside (Merck, Darmstadt, Germany) to 100 ml 0.666 M NaH_2PO_4 (pH 6) (Merck, Darmstadt, Germany). The mixture was dissolved and filter-sterilized. The test culture was grown on MRS agar for 24 h at 30°C. Colonies were picked from the plates using a sterile loop and were emulsified in physiologic saline to McFarland Turbidity Standard No. 3. Thereafter, 0.75 ml of culture was added to 0.25 ml of the test medium. It was incubated at 30°C overnight. Positive isolates that produced β-glucosidase degraded the linamarin analogue and changed the colour of the mixture from colourless to a distinct yellow. For yeast isolates, the process above was repeated, except that MEA agar, containing 50 mg/L of the antibiotic kanamycin, was used instead of MRS agar.

Production of α-amylase

In order to detect α-amylase production, LAB strains were grown on modified MRS agar plates and yeast strains were grown on modified MEA plates (with 50 mg/L kanamycin) both containing 0.4% soluble starch as the sole carbon source. A cotton swab was used to streak out the LAB and yeast strains onto the starch MRS and MEA plates, respectively. The cultures were incubated at 30°C for 24 h, after which the plates were flooded with iodine. Production of amylase was evident by a zone of clearing surrounding the streak, indicating starch utilisation. *Lactobacillus amylovorus* DSM 20531 was used as a positive control for the LAB cultures and *Saccharomyces cerevisiae* SC 3 (CSIR culture collection) was used as a positive control for the yeast cultures.

Acid production

The LAB test strains (1% of an overnight culture) were inoculated into MRS broth (pH 6.2 after autoclaving) and grown aerobically at 30°C. Acid production was determined by measuring the pH of the culture after 24 and 48 h. MRS broth medium was prepared from a single batch which was pH adjusted and then dispensed into tubes of 10 ml each before autoclaving (Kostinek et al., 2005). Acid production was not assessed for the yeast strains.

RESULTS

Microbiological sampling from fermenting cassava mash

The growth of microorganisms in fermenting cassava mash and development of pH during the fermentation is shown in Figure 3. The numbers of bacteria were assessed during 48 h of cassava fermentation for the production of gari by plate counting. Bacterial strains were the most predominant microorganisms in the fermentation. Bacterial counts determined on Rogosa, MRS and M17 agar media started from an initial level of ca. 10^5 to 10^6 CFU/g and a rapid increase in their

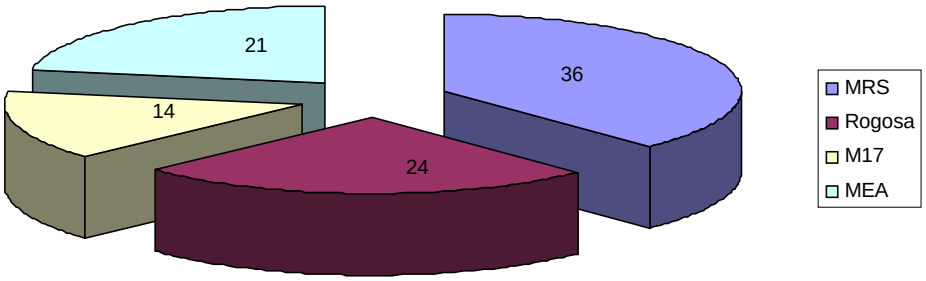

Figure 4. Number of bacterial strains (MRS, Rogosa, M17) and yeast isolates (MEA) isolated from the different media used in microbial count determinations of fermenting cassava mash.

numbers could be observed during the first 6 h of the fermentation (Figure 3) and continued to increase to a maximum of ca 10^8 CFU/g after 12 h. Yeasts were present at lower counts, their numbers increased little during the first 6 h but did increase from ca. 10^4 CFU/g to10^6 CFU/g over the 48 h fermentation period. During the 48 h of fermentation, pH was reduced from 6.4 to 4.4.

The majority of bacterial strains (36) were isolated from MRS agar plates, while 24 and 14 strains were isolated from Rogosa and M17 agar media, respectively. Thus a total of 74 bacterial strains were isolated. A total of 21 yeast isolates were also isolated from MEA plates (Figure 4). 56 of the bacterial strains were isolated during the first 18 h of fermentation, while 14 of the yeast isolates were isolated during the second half of the fermentation process (24 to 48 h). Counts from all fermentations timepoints generally ranged from between $1X10^5$ to $1X10^9$ log CFU/g (Figure 3). The microbial counts on MRS and Rogosa agar media compared well (Figure 3) showing that both these media are well suited for isolation of lactic acid bacteria from gari. Counts from M17 media, which is better suited for isolation of lactococci and streptococci as it contains lactose as sole carbon source, were generally slightly lower during the 48 hour fermentation process.

Characterisation of phenotypic and technological properties

The 74 bacterial strains and 21 yeast strains isolated from the fermenting cassava mash in Benin, as well as 26 bacterial strains isolated from cassava samples sent to the CSIR from Benin, were examined as shown in Tables 1, 2 and 3.

Isolates obtained from cassava samples sent from Benin

There were 23 (88.5%) rods and 3 (11.5%) cocci among the 26 bacterial isolates obtained from the cassava samples sent from Benin (Table 1). One (3.8%) strain (VE 18) was Gram positive and catalase positive. This meant that is was not a LAB, as LAB are typically Gram +ve and catalase negative. Three (11.5%) of the strains produced CO_2 from glucose, indicating that they are heterofermentative. All other strains were either facultatively heterofermentative or homofermentative. None of the isolates showed any α-amylase activity. Nine (34.6%) of the strains displayed *β*-glucosidase activity. The pH in MRS broth after growth for 24 h ranged from 3.91 and 5.23, with strain VE 26 demonstrating the best pH reduction. At 48 h, the pH ranged from 3.66 to 4.96, with strain VE 26 again demonstrating the best pH reduction. The API 50 CHL kit for identification of *Lactobacillus* and related species, was used to tentatively ranged from 3.53 to 5.37, with strain VE 82 demonstrating the best pH reduction. The API 50 CHL kit was again used to tentatively identify the isolates. The majority of strains (44.6%) were identified as *L. plantarum*. Other rod-shaped strains identified from the fermentation were *L. acidophilus* (16.2%), *L. fermentum* (9.5%), *L. buchneri* (4.1%) and *Lactobacillus pentosus* (2.7%). *Lactococcus lactis*4.1%), *Lactococcus* identify the isolates. The majority of strains (65.4%) were identified as *Lactobacillus plantarum*. Other rod-shaped strains identified from the fermentation were *Lactobacillus acidophilus* (3.8%), *Lactobacillus fermentum* (7.7%) and *Lactobacillus buchneri* (7.7%). The coccus-shaped isolates were all tentatively identified as *Lactococcus lactis* ssp. cremoris (11.5%) using the API kit. One strain (3.8%) was not identified because it was catalase positive.

Isolates obtained from fermenting cassava mash in Benin

There were 66 (89.2%) rods and 8 (10.8%) cocci among the 74 bacterial isolates obtained from fermenting cassava mash in Benin (Table 2). 10 (13.5%) strains were Gram positive and catalase positive. Three (4.1%)

Table 1. Phenotypic properties and tentative identification of strains isolated from cassava samples sent to CSIR from Benin.

Strain number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 1	rods	+	-	-	-	+	4.12	3.98	*L. plantarum*
VE 2	rods	+	-	-	-	-	4.27	4.10	*L. plantarum*
VE 3	rods	+	-	-	-	+	4.21	4.05	*L. plantarum*
VE 4	rods	+	-	-	-	-	4.15	4.02	*L. plantarum*
VE 5	rods	+	-	+	-	-	4.08	3.96	*L. fermentum*
VE 6	cocci	+	-	-	-	-	4.36	4.13	*Lc. lactis* ssp. *cremoris*
VE 7	rods	+	-	-	-	+	4.27	4.05	*L. plantarum*
VE 8	rods	+	-	-	-	-	4.02	3.92	*L. plantarum*
VE 9	rods	+	-	-	-	+	4.19	4.07	*L. plantarum*
VE 10	rods	+	-	+	-	-	4.26	4.10	*L. buchneri*
VE 11	rods	+	-	-	-	-	4.38	4.09	*L. fermentum*
VE 12	rods	+	-	-	-	-	4.22	3.99	*L. plantarum*
VE 13	rods	+	-	-	-	+	4.11	3.96	*L.plantarum*
VE 14	rods	+	-	-	-	+	3.92	3.78	*L. plantarum*
VE 15	cocci	+	-	-	-	-	4.15	4.01	*Lc. lactis* ssp. *cremoris*
VE 16	rods	+	-	-	-	-	4.31	4.06	*L. plantarum*
VE 17	rods	+	-	-	-	-	4.08	3.97	*L. plantarum*
VE 18	rods	+	+	-	-	-	5.23	4.96	N/D
VE 19	cocci	+	-	-	-	-	4.00	3.91	*Lc. lactis* ssp. *cremoris*
VE 20	rods	+	-	-	-	+	3.96	3.82	*L. plantarum*
VE 21	rods	+	-	-	-	+	4.18	4.04	*L. plantarum*
VE 22	rods	+	-	-	-	-	4.21	4.00	*L. acidophilus*
VE 23	rods	+	-	-	-	-	4.07	3.96	*L. planatrum*
VE 24	rods	+	-	-	-	-	4.13	4.01	*L. plantarum*
VE 25	rods	+	-	-	-	-	4.04	3.95	*L.plantarum*
VE 26	rods	+	-	+	-	+	3.91	3.66	*L. buchneri*

N/D, Not determined; strain catalase positive.

of the strains produced CO_2 from glucose, indicating that they are heterofermentative. All other strains were thus either facultatively heterofermentative or homofermentative. Three of the strains (4.1%) showed α-amylase activity. 38 (51.4%) of the strains displayed *β*-glucosidase activity. The pH of MRS broth after 24 h growth ranged from 3.92 and 5.56, with strain VE 98 showing the best pH reduction at 24 h. At 48 h, the pH (*lactis* ssp. *cremoris* (1.4%) and *Leuconostoc* (4.1%) were the cocci which could be identified with the aid of the kit. 10 (13.5%) strains were not identified using the API kit after they were found to be catalase positive. The majority of yeast strains (90.5%) were identified as *Candida* species (Table 3). Two (9.5%) yeast strains were identified as *Cryptococcus laurentii.* 14 strains (66.7%) showed α-amylase activity, and

Table 2. Phenotypic properties and tentative identification of strains isolated from fermenting cassava mash in Benin.

Strain number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 36	rods	+	-	-	-	+	4.01	3.70	*L. plantarum*
VE 37	rods	+	-	-	-	-	4.42	4.15	*L. plantarum*
VE 38	rods	+	-	-	-	-	4.36	4.09	*L. acidophilus*
VE 39	rods	+	-	-	-	-	4.19	4.03	*L. plantarum*
VE 40	cocci	+	-	-	-	-	4.20	4.11	*Lactococcus lactis*
VE 41	rods	+	+	-	-	-	5.36	5.12	N/D
VE 42	rods	+	-	-	-	-	4.18	4.07	*L. plantarum*
VE 43	rods	+	-	-	-	+	4.12	3.91	*L. fermentum*
VE 44a	rods	+	-	+	-	-	4.42	4.20	*L. fermentum*
VE 44b	rods	+	-	-	-	-	4.56	4.39	*L. plantarum*
VE 45	rods	+	-	-	-	+	4.38	4.27	*L. acidophilus*
VE 46	rods	+	-	-	-	-	4.31	4.16	*L. acidophilus*
VE 47	cocci	+	+	-	-	+	5.56	5.32	N/D
VE 48	rods	+	+	-	-	-	5.37	5.30	N/D
VE 49	rods	+	+	-	-	-	5.45	5.37	N/D
VE 50	rods	+	-	-	-	+	4.62	4.43	*L. fermentum*
VE 51	rods	+	-	-	-	-	4.55	4.32	*L. fermentum*
VE 52	cocci	+	-	-	-	-	4.38	4.24	*Lc. lactis* ssp. *cremoris*
VE 53	rods	+	-	-	-	+	4.61	4.46	*L. plantarum*
VE 54	rods	+	-	+	-	-	4.33	4.24	*L. buchneri*
VE 55	rods	+	-	-	-	-	4.45	4.36	*L. plantarum*
VE 56	rods	+	-	-	-	+	4.10	3.79	*L. plantarum*
VE 57	rods	+	-	-	-	+	4.38	4.10	*L. plantarum*
VE 58	rods	+	-	-	-	+	4.29	4.17	*L. plantarum*
VE 59	rods	+	-	-	-	+	4.05	3.89	*L. pentosus*
VE 60a	rods	+	-	-	-	+	4.28	3.90	*L. fermentum*
VE 60b	cocci	+	-	-	-	+	4.15	4.01	*Lactococcus lactis*
VE 61	rods	+	-	-	-	+	4.38	4.19	*L. plantarum*
VE 62	rods	+	-	-	-	+	4.28	4.08	*L. plantarum*
VE 63	rods	+	-	-	-	+	3.96	3.82	*L. buchneri*
VE 64	rods	+	-	-	-	-	4.30	4.14	*L. fermentum*
VE 65a	cocci	+	-	-	-	+	4.13	3.84	*Lactococcus lactis*
VE 65b	rods	+	-	-	-	+	4.02	3.76	*L. acidophilus*
VE 66	rods	+	-	-	-	+	4.56	4.37	*L. plantarum*
VE 67	rods	+	-	-	-	+	4.47	4.28	*L. acidophilus*
VE 68	rods	+	-	-	-	-	4.29	4.10	*L. plantarum*
VE 69	rods	+	-	-	-	-	4.38	4.12	*L. plantarum*
VE 70	rods	+	-	-	-	+	4.00	3.72	*L. plantarum*

Table 2. Contd.

Strain number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	β-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 71	rods	+	-	-	-	-	4.26	4.12	*L. plantarum*
VE 72	rods	+	-	-	-	-	4.20	4.11	*L. plantarum*
VE 73	rods	+	-	+	-	-	4.35	4.28	*L. buchneri*
VE 74	rods	+	-	-	-	+	4.19	3.98	*L. fermentum*
VE 75	rods	+	-	-	-	-	4.44	4.26	*L. plantarum*
VE 76	rods	+	-	-	-	+	4.37	4.18	*L. plantarum*
VE 77	rods	+	-	-	-	+	3.99	3.67	*L. plantarum*
VE 78	rods	+	-	-	-	-	4.33	4.11	*L. acidophilus*
VE 79	rods	+	-	-	-	-	4.29	3.98	*L. acidophilus*
VE 80	rods	+	-	-	-	+	4.39	4.09	*L. plantarum*
VE 81	rods	+	-	-	-	-	4.36	4.25	*L. plantarum*
VE 82	rods	+	-	-	-	+	3.96	3.53	*L. plantarum*
VE 83	rods	+	-	-	-	-	4.23	3.95	*L. plantarum*
VE 84	rods	+	-	-	-	+	4.30	3.89	*L. plantarum*
VE 85a	rods	+	-	-	-	+	4.08	3.78	*L. plantarum*
VE 85b	rods	+	-	-	-	+	4.13	3.69	*L. acidophilus*
VE 86	rods	+	-	-	-	-	4.31	4.06	*L. plantarum*
VE 87	rods	+	+	-	-	-	5.35	5.00	N/D
VE 88	rods	+	-	-	-	+	4.32	4.11	*L. plantarum*
VE 89a	rods	+	+	-	-	+	4.39	4.07	N/D
VE 90	rods	+	-	-	-	+	4.22	3.95	*L. pentosus*
VE 91	cocci	+	-	-	+	-	4.26	3.97	*Leuconostoc* spp.
VE 93	rods	+	+	-	-	-	5.39	5.05	N/D
VE 95a	rods	+	+	-	-	+	5.28	5.02	N/D
VE 95b	rods	+	-	-	-	-	4.52	4.18	*L. acidophilus*
VE 97	cocci	+	-	-	+	-	3.99	3.71	*Leuconostoc* spp.
VE 98	rods	+	-	-	-	+	3.92	3.56	*L. acidophilus*
VE 99	rods	+	-	-	-	+	4.27	3.98	*L. plantarum*
VE 100	rods	+	-	-	-	+	4.19	3.90	*L. plantarum*
VE 101	rods	+	+	-	-	-	5.36	4.99	N/D
VE 102	rods	+	-	-	+	-	4.02	3.86	*L. acidophilus*
VE 103	rods	+	+	-	-	-	5.42	5.01	N/D
VE 104	cocci	+	-	-	-	+	4.37	4.02	*Leuconostoc* spp.
VE 105	rods	+	-	-	-	+	4.43	4.16	*L. plantarum*
VE 106	rods	+	-	-	-	+	4.33	4.12	*L. plantarum*
VE 107	rods	+	-	-	-	-	4.36	4.09	*L. acidophilus*

N/D, Not determined; strain catalase positive.

Table 3. Yeast isolates obtained from fermenting cassava mash in Benin.

Organism number	Description	α-amylase activity	*β*-glucosidase activity	Tentative ID
VE 89b	Yeast	+	-	*Candida tropicalis*
VE 92	Yeast	+	+	*Candida krusei*
VE 94	Yeast	+	+	*Cryptococcus laurentii*
VE 96	Yeast	-	-	*Candida inconspicua*
VE 108	Yeast	+	-	*Candida krusei*
VE 109	Yeast	+	+	*Candida famata*
VE 110	Yeast	-	-	*Candida rugopelliculosa*
VE 111	Yeast	+	-	*Candida maris*
VE 112	Yeast	+	-	*Candida inconspicua*
VE 113	Yeast	+	-	*Candida glabrata*
VE 114	Yeast	+	+	*Cryptococcus laurentii*
VE 115	Yeast	-	-	*Candida guilliermondii*
VE 116	Yeast	+	-	*Candida tropicalis*
VE 117	Yeast	+	-	*Candida famata*
VE 118	Yeast	-	+	*Candida rugopelliculosa*
VE 119	Yeast	-	-	*Candida tropicalis*
VE 120	Yeast	+	+	*Candida rugopelliculosa*
VE 121	Yeast	-	-	*Candida tropicalis*
VE 122	Yeast	-	+	*Candida rugopelliculosa*
VE 123	Yeast	+	+	*Candida tropicalis*
VE 124	Yeast	+	+	*Candida krusei*

nine strains (42.9%) showed *β*-glucosidase activity. Seven strains (33.3%) showed both α-amylase and *β*-glucosidase activities.

A selection was made from the bacterial strains that were isolated from the cassava samples sent from Benin, as well as the strains isolated from a cassava mash fermentation process in Benin during a research visit there. A comparison of these strains can be seen in Figure 5. Strains were selected for further characterisation and were further identified at the BFE in Germany during a research visit. The 24 strains selected for further characterisation (Table 4), were chosen on the basis of good acid production, that is, a good pH reduction, α-amylase activity or *β*-glucosidase activity. No yeast strains were selected for further characterisation.

DISCUSSION

For development of starter cultures, it is important to isolate predominant strains from previous fermentation batches in order to achieve successful further fermentations. Screening of strains in this work showed that *L. plantarum* was predominant in the samples obtained from Benin, as well as the cassava mash fermentation process studied while in Benin. Oyewole and Odunfa (1990) showed that *L. plantarum* is a common occurrence in fermenting cassava. *L. plantarum* has been shown previously to be the predominant LAB species in sour cassava starch (Lacerda et al., 2005).

Bacterial counts compared well with MRS and Rogosa agar, and were lower on M17 agar. Lower counts on M17 agar, which is better suited for isolation of lactococci and streptococci, may be expected, as these bacteria would probably occur in numbers less than lactobacilli and leuconostocs.

Alternatively, this could also be attributed to the fact that the bacteria were less well adapted to utilise lactose, which appeared to be the case as we determined at a later stage that we could not identify any lactococci from the M17 agar plates. Thus, this media was not very selective for the growth of lactococci from gari, but instead allowed the growth of other LAB that were able to utilise lactose and which were associated with the gari fermentation. Nevertheless, as counts were generally lower on M17 media, it was determined that this media was not well suited for isolation of the majority of LAB from gari, probably a reflection of the fact that this is not a typical sugar present in the cassava root.

The majority of LAB strains isolated from both processes showed good pH reduction (Tables 1 and 2). Good pH reduction (a fast lowering of the pH to low levels) is important to reduce the levels of contaminating microorganisms present on the raw materials, utensils and the environment which can compete with the starters for nutrients (Holzapfel, 2002). According to Holzapfel (1997), starter cultures introduced in traditional small-scale fermentations should have attributes for improving

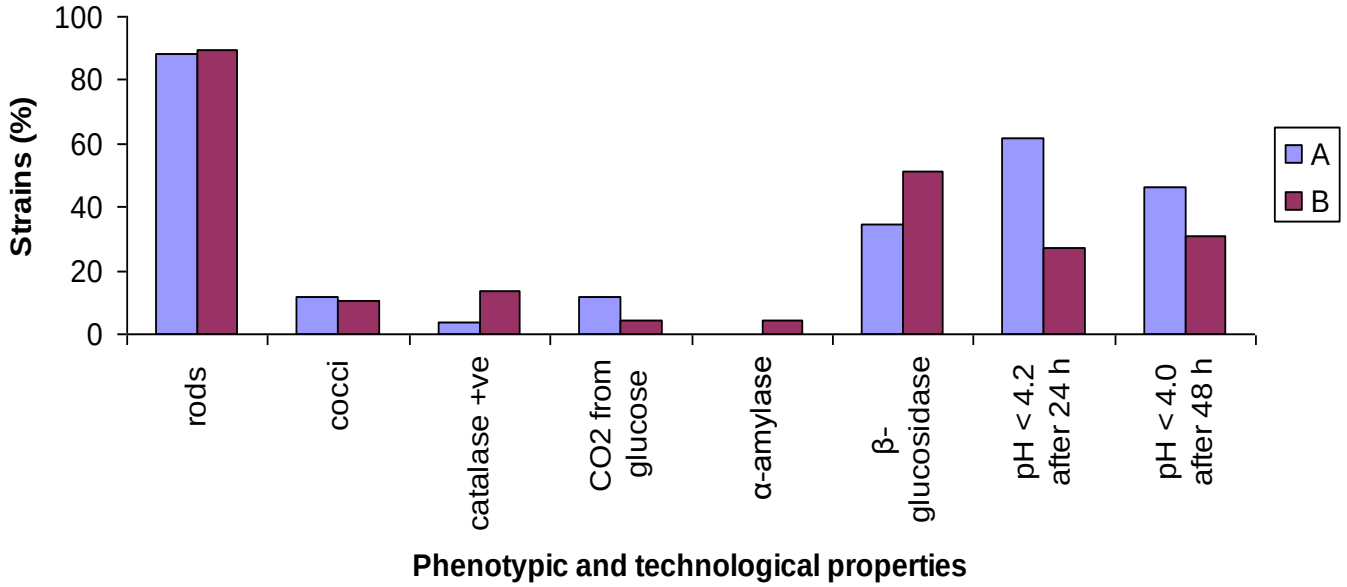

Figure 5. Comparison of phenotypic and technological properties of (A) strains isolated from cassava samples sent to CSIR from Benin and (B) strains isolated from fermenting cassava mash during a research visit to Benin.

processing conditions and product quality through: rapid accelerated metabolic activities (acidification or alcohol production); improved and more predictable fermentation processes; desirable sensory attributes; and improved safety and reduced hygienic and toxicological risks.

Giraud et al. (1993) showed that *L. plantarum* strain A6 isolated from cassava, cultured on cellobiose MRS medium, produced both an intracellular linamarase (76.4 U/g of biomass) and an extracellular amylase (36 U/ml) simultaneously. The use of this strain as a cassava fermentation starter for gari production caused a change from a heterofermentative pattern observed in natural fermentation, to a homofermentative one, a lower final pH, a faster pH decline rate and a greater production of lactic acid (50 g/kg) (Giraud et al., 1993). The ability of several yeast strains to produce *β*-glucosidase was shown by Fia et al. (2005). In addition, Freer (1993) demonstrated that *Candida wickerhamii* produced an extracytoplasmic, cell-bound beta-1,4-glucosidase, which is important to the gari fermentation. LAB and yeasts, which are responsible for the fermentation process, are also thought to contribute to linamarin degradation by *β*-glucosidase activity (Ikediobi and Onyike, 1982; Padmaja and Balagopal, 1985; Okafor and Ejiofor, 1990; Giraud et al., 1992; Okafor et al., 1998a; b).

Machado and Linardi (1990) studied a total of 105 yeast strains, belonging to the genera *Aureobasidium*, *Candida*, *Cryptococcus*, *Debaromyces*, *Rhodotorula* and *Trichosporum* for their ability to produce amylase and beta-galactosidase. They showed that some strains showed high enzymatic activity for amylase. Linardi and Machado (1990) screened 228 yeasts, isolated from natural habitats, for their ability to produce amylases in semisolid medium of wheat bran. Strains of *Aureobasidium pullulans*, *Candida famata*, and *Candida kefyr* showed high enzymatic activity for alpha -amylase, glucoamylase, and debranching enzyme. Azoulay et al., (1980) showed that *Candida tropicalis* possesses the enzyme needed to hydrolyze starch, namely, an α-amylase. *C. tropicalis* grows on soluble starch, corn, and cassava powders without requiring that these substrates be previously hydrolyzed. This property has been used to develop a fermentation process whereby *C. tropicalis* can be grown directly on corn or cassava powders so that the resultant mixture of biomass and residual corn or cassava contains about 20% protein, which represents a balanced diet for either animal fodder or human food (Azoulay et al., 1980).

API kits are known to be inaccurate when it comes to identification of Gram-positive bacteria. A study conducted by Boyd et al. (2005) showed that the API 50 CH system misidentified 59% of the *Lactobacillus jensenii* and *Lactobacillus gasseri* isolates as *L. acidophilus* and the system also misidentified seven out of 20 (35%) *Lactobacillus vaginalis* isolates as *L. fermentum*. Over half of the 97 isolates yielded an uninterpretable or doubtful API profile (Boyd et al., 2005). For yeasts, similar problems with identification based on API profiles have been reported. Oguntoyinbo (2008), for example, showed that the results obtained using the API 20 AUX kit for *Candida* strains was is in agreement with that of the 18S recombinant deoxyribonucleic acid (rDNA) gene sequence identification analysis at the genus level only, and often did notprovide satisfactory identification.

Strains of *Candida krusei* have been consistently isolated from cassava and cereal fermentation in West

Table 4. Strains selected for further phenotypic, genotypic and technological characterisation at the BFE, Germany.

Organism number	Morphology	Gram reaction	Catalase	CO_2 from glucose	α-amylase activity	*β*-glucosidase activity	pH after 24 h	pH after 48 h	Tentative ID
VE 14	rods	+	-	-	-	+	3.92	3.78	*L. plantarum*
VE 20	rods	+	-	-	-	+	3.96	3.82	*L. plantarum*
VE 26	rods	+	-	+	-	-	3.91	3.66	*L. buchneri*
VE 36	rods	+	-	-	-	+	4.01	3.70	*L. plantarum*
VE 43	rods	+	-	-	-	+	4.12	3.91	*L. fermentum*
VE 56	rods	+	-	-	-	+	4.10	3.79	*L. plantarum*
VE 59	rods	+	-	-	-	+	4.05	3.89	*L. pentosus*
VE 60a	rods	+	-	-	-	+	4.28	3.90	*L. fermentum*
VE 60b	cocci	+	-	-	-	+	4.15	4.01	*Lactococcus lactis*
VE 63	rods	+	-	-	-	+	3.96	3.82	*L. buchneri*
VE 65a	cocci	+	-	-	-	+	4.13	3.84	*Lactococcus lactis*
VE 65b	rods	+	-	-	-	+	4.02	3.76	*L. acidophilus*
VE 70	rods	+	-	-	-	+	4.00	3.72	*L. plantarum*
VE 77	rods	+	-	-	-	+	3.99	3.67	*L. plantarum*
VE 82	rods	+	-	-	-	+	3.96	3.53	*L. plantarum*
VE 85a	rods	+	-	-	-	+	4.08	3.78	*L. plantarum*
VE 85b	rods	+	-	-	-	+	4.13	3.69	*L. acidophilus*
VE 90	rods	+	-	-	-	+	4.22	3.95	*L. pentosus*
VE 91	cocci	+	-	-	+	-	4.26	3.97	*Leuconostoc* spp.
VE 97	cocci	+	-	-	+	-	3.99	3.71	*Leuconostoc* spp.
VE 98	rods	+	-	-	-	+	3.92	3.56	*L. acidophilus*
VE 99	rods	+	-	-	-	+	4.27	3.98	*L. plantarum*
VE 100	rods	+	-	-	-	+	4.19	3.90	*L. plantarum*
VE 102	rods	+	-	-	+	-	4.02	3.86	*L. acidophilus*

West Africa (Hayford and Jakobsen, 1999). Oguntoyinbo (2008) however stated that the inadequacy of the use of phenotypic methods to differentiate *Candida inconspicua*, *C. krusei* and *Candida rugopelliculosa* in the previous studies have been responsible for the inability to determine the roles (functional and virulence) of these strains during cassava fermentation.

Yeasts, especially *Candida* species, may be important because of their ability for co-metabolism with lactic acid bacteria, a parameter reported as desirable for adequate fermentation of cassava (Amoa et al., 1996; Oyewole, 2001). On the other hand, the fermentation is rather quick and mainly dominated by lactic acid bacteria, especially in the latter parts of the fermentation, indicating that the yeasts may only play a minor role at the beginning of the fermentation. For purposes of this study, no further work was carried out with the yeast strains.

Research on yeast strains would be conducted by other members of the project consortium. Our aims were to more precisely identify the predominant lactic acid bacteria involved with the gari fermentation.

Results so far allowed a selection of a representative consortium of predominant lactic acid

bacteria that also displayed useful technological characteristics, such as production of α-amylase or *β*-glucosidase. These preliminary characterisation results also allowed a coarse grouping of the strains and it was determined that the majority of strains belonged to the *L. plantarum* group, while presumptive *L. fermentum, L. buchneri and L. acidophilus strains*, as well as heterofermentative cocci, that is, presumptive *Leuconostoc* spp., could also be identified. This work thus provided some of the foundation for the research conducted thereafter, where we aimed to more accurately identify these bacteria in a polyphasic taxonomical approach (Kostinek et al., 2005, 2007) and test them in small scale starter culture fermentations (Edward et al., 2011; Yao et al., 2009).

ACKNOWLEDGEMENT

Financial support from the European (EU) Commission within the framework of the INCO RTD programme is gratefully acknowledged. This study was partly carried out under the project 'Improving the quality and nutritional status of gari through the use of starter cultures and fortification with soybean, palm oil and coconut milk' (ICA4-CT-2002-10058). The work does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

REFERENCES

Amoa A, Frisvad LM, Sefa-Dedeh S, Jakobsen M (1996). Contribution of moulds and yeasts to the fermentation of cassava dough. J. Appl. Microbiol. 83:288-296.

Azoulay E, Jouanneau F, Bertrand JC, Raphael A, Janssens, J Lebeault, JM (1980). Fermentation methods for protein enrichment of cassava and corn with *Candida tropicalis*. Appl. Environ. Microbiol., 39: 41-47.

Beukes EM, Bester BH, Mostert JF (2001). The microbiology of South African traditional fermented milks. Int. J. Food Microbiol., 63: 189-197.

Boyd MA, Antonio MAD, Hillier SL (2005). Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of *Lactobacillus* species. J. Clin. Microbiol. 43:5309-5311

Cereda MP, Mattos MCY (1996). Linamarin-the toxic compound of cassava. J. Venomous Anim. Toxins 2:612.

Cock JH (1982). Cassava: a basic energy source in the tropics. Science 218:755-762.

Cock JH (1985). Cassava: New Potential for a Neglected Crop. Westview Press, Boulder and London p.191.

De Vuyst L, Schrijvers V, Paramithiotis S, Hoste B, Vancanneyt M, Swings J, Kalantzopoulos G, Tsakalidou E, Messens W (2002). The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. Appl. Environ. Microbiol. 68:6059-6069.

Edward VA, Huch M, Dortu C, Thonart P, Egounlety M, Van Zyl PJ, Singh S, Holzapfel WH, Franz CMAP (2011). Biomass production and small-scale testing of freeze-dried lactic acid bacteria starter strains for cassava fermentations. Food Control 22:389-395.

Fia G, Giovani G, Rosi I (2005). Study of beta-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. J. Appl. Microbiol., 99: 509-517.

Freer SN (1993). Kinetic characterization of a beta-glucosidase from a yeast, *Candida wickerhamii*. J. Biol. Chem. 268:9337-9342.

Gidamis, A (1988). Cyanide detoxification of traditionally fermented, dried and prepared cassava food in Tanzania. M. Sc. Thesis, University of Reading, UK.

Giraud E, Gosselin L, Raimbault M (1992). Degradation of cassava linamarin by lactic acid bacteria. Biotechnol. Lett. 14:593-598.

Giraud E, Gosselin L, Raimbault M (1993). Production of *Lactobacillus plantrum* starter with linamarase and amylase activities for Cassava fermentation. J. Sci. Food Agric. 62:77-82.

Hayford AE, Jakobsen M (1999). Characterization of *Candida krusei* strains from spontaneously fermented maize dough by profiles of assimilation, chromosomal profile, polymerase chain reaction and restriction endonuclease analysis. J. Appl. Microbiol. 87:29-40.

Holzapfel WH (1997). Use of starter cultures in fermentation on a household scale. Food Control 8:241-258.

Holzapfel WH (2002). Appropriate starter culture technologies for small-scale fermentation in developing contries. Int. J. Food Microbiol. 75:197-212.

Howlett P, Brubaker R, Mlingi N, Roling H (1990). Konzo, an epidemic upper motor neuron disease studied in Tanzania. Brain 113:223-235.

Ikediobi CO, Onyike E (1982). The use of linamarase in *Gari* production. Process Biochem. 17:2-5.

Kimaryo VM, Massawi GA, Olasupo NA, Holzapfel WH (2000). The use of a starter culture in the fermentation of cassava for the production of 'Kivunde', a traditional Tanzanian food product. Int. J. Food Microbiol. 56: 179-190.

Kostinek M, Specht I, Edward VA, Pinto C, Egounlety M, Sossa C, Mbugua S, Dortu C, Thonart P, Taljaard L, Mengu M, Franz CMAP, Holzapfel WH (2007). Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. Int. J. Food Microbiol. 114:342-351.

Kostinek M, Specht I, Edward VA, Schillinger U, Hertel C, Holzapfel WH, Franz CMAP (2005). Diversity of predominant lactic acid bacteria from fermented cassava used for the preparation of *Gari*, a traditional African food. Syst. Appl. Microbiol. 28:527-540.

Lacerda ICA, Miranda RL, Borelli BM, Nunes AC, Nardi RMD, Lachance, MA, Rosa CA (2005). Lactic acid bacteria and yeasts associated with spontaneous fermentations during the production of sour cassava starch in Brazil. Int. J. Food Microbiol. 105:213-219.

Leroy F, De Vuyst L (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci.Technol. 15: 67-78.

Linardi VR, Machado KMG (1990). Production of amylases by yeasts. Can. J. Microbiol. 36:751-753.

Machado KMG, Linardi VR (1990). Production of amylase and beta-galactosidase by yeasts. Revista Latinoamericana de Microbiol. 32:177-180.

Mlingi NLV (1995). Cassava processing and dietary cyanide exposure in Tanzania. Doctorate Thesis, Uppsala University, Sweden pp.9-69.

Mlingi NLV, Poulter NH, Rosling H (1992). An outbreak of acute intoxification from consumption of insufficiently processed cassava in Tanzania. Nutr. Res. 12:677-687.

Mlingi NVL, Assey V, Poulter N, Rosling H (1991). Cyanohydrins from insufficiently processed cassava induces 'konzo', a newly identified paralytic disease in man. In: Westby A, Reilly PJA (Eds.), Proceedings of a Regional Workshop on Traditonal African Foods: Quality and Nutrition. Dar-es-Salaam, 25-29.11.1991. International Foundation for Science, Stockholm pp.171-177.

Mozambique Ministry of Health, Mozambique (1984). Mantakassa: an epidemic of spastic paraparesis associated with chronic cyanide intoxication in a cassava staple area in Mozambique. 1. Epidemiology and clinical and laboratory findings in patients, 2. Nutritional factors and hydrocyanic content of cassava products. Bull. World Health Org. 62:477-492.

Ogunsua AO (1980). Changes in some chemical constituents during the fermentation of cassava tubers (*Manihot esculenta* Crantz). Food Chem. 5:249-255.

Oguntoyinbo FA (2008). Evaluation of diversity of *Candida* species isolated from fermented cassava during traditional small scale *gari* production in Nigeria. Food Control 19:465-469.

Okafor N, Ejiofor AO (1990). Rapid detoxification of cassava mash by a yeast simultaneously producing linamarase and amylase. Process Biochem. Int. 25:82-86.
Okafor N, Umeh C, Ibenegbu C (1998a). Amelioration of garri, a fermented food derived from cassava, *Manihot esculenta* Crantz, by microbial inoculation. World J. Microbiol. Biotechnol. 14:835-838.
Okafor N, Umeh C, Ibenegbu C, Obizoba I, Nnam N (1998b). Improvement of garri quality by the inoculation of microorganisms into cassava mash. Int. J. Food Microbiol. 40:43-49.
Oyewole O, Aibor A (1992). Fermentation of cassava with cowpea and soya bean for enriched fufu. Trop. Sci. 33:129-133.
Oyewole OB (2001). Characterization and significance of yeast involvement in cassava fermentation for fufu production. Int. J. Food Microbiol. 65:213-218.
Oyewole OB, Odunfa SA (1990). Characterization and distribution of lactic acid bacteria in cassava fermentation during fufu production. J. Appl. Bacteriol. 68:145-152.
Padmaja G, Balagopal C (1985). Cyanide degradation by *Rhizopus oryzae*. Can. J. Microbiol. 31:663-669.
Rosling H (1990). Cassava associated neurotoxicity in Africa. Proceedings of the 5th International Congress of Toxicology, Brighton, UK, July 16-25, 1989.
Schillinger U, Lücke FK (1987). Identification of lactobacilli from meat and meat products. Food Microbiol. 4: 199-208.
Vasconcelos AT, Twiddy DR, Westby A, Reilly PJA (1990). Detoxification of cassava during *Gari* preparation. International J. Food Sci. Technol. 25:198-203.
Yao AA, Dortu C, Egounlety M, Pinto C, Edward VA, Huch (née Kostinek), M, Franz, CMAP, Holzapfel, W, Mbugua, S, Mengu, M and Thonart, P (2009). Production of freeze-dried lactic acid bacteria starter culture for cassava fermentation into *gari.* Afr. J. Biotechnol. 8:4996-5004.