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Protein Improvement in Gari by the Use of Pure Cultures of Microorganisms Involved in the Natural Fermentation Process

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Abstract: The ability of microorganisms involved in cassava mash fermentation to produce and improve protein value by these microorganisms during fermentation was studied. Standard microbiological procedures were used to isolate, identify and determine the numbers of the organisms. *Alcaligenes faecalis*, *Lactobacillus plantarum*, *Bacillus subtilis, Leuconostoc cremoris, Aspergillus niger, A. tamari, Geotrichum candidum* and *Penicillium expansum* were isolated and identified from cassava waste water while standard analytical methods were used to determine the ability of the isolates to produce linamarase and the proximate composition, pH and titrable acidity of the fermenting mash. The linamarase activity of the isolates ranged from 0.0416 to 0.2618 μmol mL^{-1} $nmol^{-1}$. *Bacillus subtilis, A. niger, A. tamari and P. expansum* did not express any activity for the enzyme. Protein content of mash fermented with mixed fungal culture had the highest protein value (15.4 mg/g/dry matter) while the raw cassava had the least value (2.37 mg/g/dry matter). The naturally fermented sample had the least value for the fermented samples (3.2 mg/g/dry matter). Carbohydrate and fat contents of naturally fermented sample were higher than values obtained from the other fermented samples. Microbial numbers of the sample fermented with mixed bacterial culture was highest and got to their peak at 48 h (57×10^8 cfu g^{-1}). pH decreased with increase in fermentation time with the mash fermented by the mixed culture of fungi having the lowest pH of 4.05 at the end of fermentation. Titrable acidity increased with increase in fermentation time with the highest value of 1.32% at 96 h of fermentation produced by the mixed culture of fungi. Thus fermentation with the pure cultures significantly increased the protein content of mash.

Key words: Pure cultures, microorganisms, protein improvement, fermentation, gari, titrable acidity

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is African's second-most important food staple in terms of per capita calories consumed; and a source of calories for two out of every five Africans (Nweke *et al*., 2002). The tubers are usually processed into a variety of products such as gari which is a granulated product that has a long shelf life and is in a ready-to-eat form (Cardoso *et al*., 2005; Onabolu, 2001). Despite being a cheap source of food calories cassava is nutritionally deficient in protein and contains cyanogenic glucosides (linamarin) which upon hydrolysis by linamarase produces hydrogen cyanide (Sokari and Karibo, 1992; Adindu and Aprioku, 2006). The high consumption pattern of cassava diets as witnessed in developing countries without adequate protein supplementation exposes the vulnerable group (children, pregnant and lactating mothers) to incidence of protein-energy malnutrition (FAO, 1984). Cyanide is very poisonous because it binds to an enzyme cytochrome oxidase and stops its action in respiration which is a key energy conversion process in the body. There are some reported cases of death linked to consumption of cassava meals (Akintonwa *et al*., 1994). Smaller (non-fatal) amounts of cyanide cause acute intoxication with symptoms such as dizziness, headache, stomach pains, vomiting and diarrhea (Osuntokun, 1973). Several disease conditions have been reported to be associated with dietary cyanide: konzo (tied legs) (Ernesto *et al*., 2002), tropical ataxic neuropathy (Rosling, 1988) and goiter and cretinism (Ermans, 1980).

Grating cassava root is considered the most important stage in which the hydrolytic enzyme linamarase is brought to intimate contact with linamarin (Dixon *et al*., 1994). Some workers regard fermentation as being responsible for the softening of the grated pulp to liberate hydrogen cyanide and production of organic acids that impact sour taste to gari (Nwachukwu and Edwards, 1989; Vasconcelos *et al*., 1990). Some workers have attempted to reduce cyanide during ari fermentation

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by altering pH, temperature, duration of fermentation and use of preferment liquor (Owuamanam *et al*., 2010, 2011). However, some microorganisms have been reported to possess the enzyme linamarase (Kimaryo *et al*., 2000; Okafor and Ejiofor, 1985, 1986; Oyewole and Odunfa, 1990) and it is believed that these microorganisms also contribute to the liberation of cyanide from grated cassava during gari production. Elimination of cyanide from cassava products can be optimized by identification and use of microorganisms with linamarase activity in fermentation.

Research efforts in the area of protein enrichment of cassava are focused toward animal feed but little or no attempt to improve protein value of garri, fufu and commonly consumed traditional staples (Srinorakutara *et al*., 2006; Ubalua, 2007; Ezekiel *et al*., 2010). Proper identification, isolation and application of microorganisms that perform dual functions of improving protein content and also help to detoxify cyanogenic glucoside would be of immense benefit to consumers of cassava.

This study therefore aims at investigating the ability of the microorganisms involved in gari fermentation to produce linamarase and also the ability of the microorganisms to improve the protein base of the product.

MATERIALS AND METHODS

Sample collection: Cassava waste water (whey) was collected from a small-scale gari processing factory in Benin City, Nigeria. The samples were allowed to sediment for 12 h at ambient temperature (30±2°C). This was done to allow only microorganisms that can breakdown cyanogenic glucoside to survive. The supernatant was used immediately for isolation of the surviving microorganisms.

Cassava tubers (variety 98/0581) used for microbial cyanogens degradation were freshly harvested from Agricultural Development Programme (ADP) farm in Benin City, Nigeria 18 months after planting.

Isolation of microorganisms from cassava waste water: Bacteria were isolated using Nutrient agar, Tryptone Soy Agar, MRS Agar and Plate Count Agar while yeasts were isolated using Malt Extract agar and Potato Dextrose Agar. The samples were serially diluted and 0.1 mL was plated out on the surface of the solidified agar media. Plates for bacteria were incubated at 37°C for 24-48 h while those for yeasts were incubated at 30°C for 72 h. The microorganisms that were isolated were re-isolated into pure cultures, identified using various biochemical tests, cultural and morphological characteristics (Cowan, 1985) and stored on agar slants used for their isolation at 4°C until required.

Determination of linamarase activity of isolated microorganisms: The ability of the isolates to produce linamarase was assessed using the method described by Oyewole (2001) and O'Brien *et al*. (1991). Linamarase was quantitatively determined as β-glucosidase glycohydrolase. This was estimated by measuring spectrophotometrically the release of β-nitrophenol from para-nitrophenyl-β-D-glucoside.

A loop full of 18 h culture was added to 6 mL of 10 g L^{-1} of para-nitrophenyl-β-D-glucoside in 50 g L^{-1} sodium citrate (pH 6.0) and incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL of 0.1 M sodium carbonate (Na_2CO_3). The absorbance was then measured at 420 nm. A standard curve enabled the conversion of the absorbance obtained to the quantity of β-nitrophenol released from para-nitrophenyl-β-D-glucoside.

Fermentation of grated cassava pulp: Controlled fermentation by modifying the method of Okolie *et al*. (1992) was used. The cassava tubers were washed, drained and peeled. They were washed again and dipped in 96% ethanol for 5 min. for surface sterilization. Working in a sterile hood the tubers were rapidly grated into a sterile container with a sterile hand grater. Two hundred grams of the sample was weighed rapidly into a salt bag that has previously been bleached, washed and sterilized by autoclaving. This was then inoculated with a standard culture of the microorganism containing approximately 4.0×10^5 cells mL^{-1}. Each salt bag with the grated sample was inoculated with one isolate. A mixture of the various bacteria and fungi also containing approximately 4.0×10^5 cells mL^{-1} were used to ferment the mash. The control was allowed to ferment naturally as is obtainable during the indigenous production of gari. The salt bags were tied with fishing line thread and placed on a platform with sterile container to collect the waste water. The samples were left in the sterile hood to ferment at ambient temperature (30±2°C) for 96 h. Samples were collected on daily basis for analysis.

Determination of proximate composition:

Moisture content: Five grams of the raw cassava and fermenting samples were collected and used for the determination of the moisture content (AOAC, 1990).

Total carbohydrate: The Anthrone method (Plummer, 1971) was used. The results were expressed in mg/g/dry matter.

Total protein: This was determined by using the Buiret method as described by Cooper (1977). The results were expressed in mg/g/dry matter.

Fat content and crude fiber: Five grams of each of the samples was collected and used for the determination of fat by the solvent ether extraction method according to the method described in AOAC, (1990). They were expressed in mg/g/dry matter.

Determination of microbial count: One gram of sample was collected and added to 9.0 mL of sterile diluents. This was further serially diluted to the desired dilution and plated out on Nutrient agar and Plate Count Agar for bacteria and Malt Extract agar and Potato Dextrose agar for yeasts, using the spread plate method. Bacterial plates were incubated at 37°C for 24-48 h while yeast plates were incubated at 30°C for up to 72 h. At the end of incubation the cfu g^{-1} was calculated from the number of colonies formed.

Determination of pH: Fourty grams of sample was homogenized in a blender with 40 mL of distilled water and the pH measured with a Corning Pinnacle 530 pH meter (Obilie *et al*., 2004).

Determination of titratable acidity: Ten grams of sample was homogenized in 200 mL of distilled water and filtered using Whatman filter paper. Eight milliliter of filtrate was titrated with 0.1 M NaOH using 1% phenolphthalein as indicator (Obilie *et al*., 2004).

Analysis of data: The data obtained from the study were analyzed statistically using Analysis Of Variance and the means were separated using Tukey's Test (Wahua, 1999).

All experiments were carried out in triplicates.

RESULTS AND DISCUSSION

Table 1 shows the microbial isolates from cassava waste water (whey) and their linamarase activity. *Leuconostoc cremoris* had the highest value (0.2618 µmol/mL/nmol) while the least enzyme activity was expressed by *Geotrichum candidum* (0.0124 µmol/mL/nmol). *Bacillus Subtilis*, *Aspergillus niger*, *A. tamari* and *Penicillium expansum* did not express any enzyme activity. The various isolates obtained from the cassava waste water have also been isolated from fermenting cassava by various workers (Amoa-Awua *et al*., 1996; Essers *et al*., 1995; Kobawila *et al*., 2005; Okafor, 1977) with the lactic acid bacteria and yeasts being the major fermenting microorganisms. However, Ejiofor and Okafor (1981) have observed that the genera of microorganisms involved in cassava fermentation for gari production would depend on some factors which include how the cassava was processed before fermentation. It will also depend on the source of the cassava, the types and numbers of initial microorganisms on the pulp and the environment of processing.

Table 2 shows the proximate composition of the grated cassava pulp fermented by the various isolates. The results showed that the sample fermented with the mixed fungi had the highest amount of protein (15.4 mg/g/dry matter). This was followed by the sample fermented by the mixed bacteria (13.7 mg/g/dry matter). The values obtained from all the samples fermented by the various microorganisms were higher than the values

Table 1: Linamarase activity of the various bacteria

Microorganisms	β-glucosidase activity (µmol mL^{-1} $nmol^{-1}$)
Alcaligenes faecalis	0.0782
Lactobacillus plantarum	0.0416
Bacillus subtilis	-
Geotrichum candidum	0.0124
Aspergillus niger	-
Aspergillus tamari	-
Leuconostoc cremoris	0.2618
Penicillium expansum	-

Table 2: Proximate composition of grated cassava pulp fermented by various microorganisms

Sample type	Moisture content (%)	Carbohydrate mg/g/dry matter	Protein mg/g/dry matter	Fat mg/g/dry matter	Fibre mg/g/dry matter
Raw cassava	63.2	90.1	2.37	0.40	7.48
A. faecalis	42.8	78.1	5.90	0.14	10.40
L. plantarum	43.1	74.5	7.68	0.23	9.16
B. subtilis	42.9	77.6	9.06	0.17	5.78
L. cremoris	41.4	73.3	6.80	0.18	8.78
Mixed bacteria	41.9	74.4	13.70	0.21	4.37
A. niger	39.6	74.8	7.60	0.25	6.34
A. tamari	42.9	74.7	7.20	0.21	5.98
G. candidum	42.2	75.8	8.40	0.24	5.40
P. expansum	41.7	78.2	5.90	0.17	7.20
Mixed fungi	42.4	73.5	15.40	0.18	2.81
Control	43.1	80.5	3.20	0.32	12.63

Table 3: Microbial population during the period of fermentation

	Period of fermentation (h)				
Isolates	0	24	48	72	96
A. faecalis	23×10^4	27×10^7	39×10^7	27×10^6	14×10^5
L. plantarum	21×10^4	16×10^7	31×10^7	29×10^6	27×10^5
B. subtilis	25×10^4	29×10^7	34×10^7	30×10^6	28×10^5
L. cremoris	30×10^4	38×10^7	21×10^7	58×10^6	34×10^5
Mixed bacteria	27×10^4	46×10^7	57×10^8	38×10^7	12×10^7
A. niger	12×10^4	16×10^4	21×10^5	36×10^5	17×10^5
A. tamari	05×10^4	13×10^4	15×10^5	22×10^5	19×10^5
G. candidum	08×10^4	18×10^4	19×10^5	23×10^5	18×10^5
P. expansum	12×10^4	16×10^4	13×10^5	21×10^5	16×10^5
Mixed fungi	18×10^4	31×10^5	47×10^5	51×10^5	28×10^5
Control	NG	NG	NG	NG	NG

Table 4: pH values during the period of fermentation

	Period of fermentation (h)				
Type of sample	0	24	48	72	96
A. faecalis	6.72	5.79	4.72	4.42	4.22
L. plantarum	6.72	5.83	4.81	4.47	4.28
B. subtilis	6.72	5.72	4.76	4.49	4.35
L. cremoris	6.72	5.98	4.81	4.57	4.42
Mixed bacteria	6.72	5.73	4.63	4.27	4.19
A. niger	6.72	5.31	4.37	4.27	4.19
A. tamari	6.72	5.24	4.31	4.28	4.14
G. candidum	6.72	5.58	4.43	4.21	4.19
P. expansum	6.72	5.73	4.65	4.27	4.23
Mixed fungi	6.72	5.04	4.51	4.14	4.05
Control	6.72	6.13	5.67	5.02	4.97

obtained from the raw cassava (2.37 mg/g/dry matter) and the naturally fermented product (3.2 mg/g/dry matter). The high yield of crude protein is associated with the ability of the organisms to convert carbohydrate during the fermentation to protein (Gregory *et al*., 1976). However, the carbohydrate content of the raw cassava (90.1 mg/g/dry matter) and the naturally fermented product (80.5 mg/g/dry matter) were higher than the values obtained from the samples fermented by the isolates which validates the carbohydrate utilization. Ezekiel *et al*. (2010) obtained crude protein of 37.63 and 36.52% for enzyme and non-enzyme pre-treated fermented cassava waste from submerged fermentation using *Trichoderma viride* ATCC 36316. Also, Iyayi and Losel, (2001) obtained crude protein of 7.91 and 9.04%, respectively from solid state fermentation of cassava pulp with *Aspergillus niger* and *Saccharomyces cerevisiae*. The same trend was also observed for the fat content. The fibre content of the naturally fermented product (12.63 mg/g/dry matter) was the highest while the sample fermented by the mixed fungi had the least value (2.81 mg/g/dry matter) which reveals that soluble fibre is utilized by the organisms (Srinorakutara *et al*., 2006). The high protein values in the samples fermented by the isolates could be as a result of the growth and multiplication of the microorganisms. These isolates are known to contain high proteins and are used in the production of single cell proteins (Gregory, 1977).

Table 5: Total titratable acidity values during the period of fermentation

	Period of fermentation (h)				
Type of sample	0	24	48	72	96
A. faecalis	0.28	0.37	0.63	0.78	0.86
L. plantarum	0.28	0.34	0.61	0.80	0.91
B. subtilis	0.28	0.38	0.59	0.67	0.74
L. cremoris	0.28	0.30	0.53	0.60	0.63
Mixed bacteria	0.28	0.32	0.71	0.98	1.05
A. niger	0.28	0.41	0.80	1.01	1.07
A. tamari	0.28	0.44	0.80	1.12	1.26
G. candidum	0.28	0.39	0.76	0.97	1.06
P. expansum	0.28	0.39	0.72	0.88	0.98
Mixed fungi	0.28	0.49	0.79	1.10	1.32
Control	0.28	0.30	0.42	0.56	0.52

Microbial populations during the period of fermentation are shown in Table 3. The observed variations in the pH values which dropped systematically with the fermentation time are shown in Table 4. The decreasing values correspond with production of acids by the fermenting organisms (Table 5). The trends are comparable to that obtained from the work of Sokari and Karibo (1992).

The titrable acidity values obtained during fermentation of the samples are shown in Table 5. The values increased with increase in fermentation time. The percent titrable acidity differed among the groups and the highest value of 1.32 was obtained at 96 h of fermentation by mixed fungi which is followed by *A. tamari* (1.26) while the least (0.52) was obtained for the control. Thus, the microbial isolates were found to produce more organic acids than the control. These acids impact positively on the taste and the quality of the final product. Lactic acid content influences the taste and the consumer acceptability of gari. The sour taste has been taken as an index of gari quality (Achinewhu and Owuamanam, 2001). Thus the products with titrable acidity of 0.91 and above would give good quality gari.

CONCLUSION

Since gari is a major food in Nigeria and other African countries and the major constraint in its consumption is presence of linamarin which is converted to cyanide, efforts should be directed towards developing better processing methods that could help reduce or even completely eliminate the cyanide in the mash. It could also be possible to genetically modify the linamarase producing microorganisms in order to increase their ability to produce more linamarase to significantly increase rate of degradation of the cyanogenic glucosides. This may significantly reduce fermentation time and increase output of the product. The high yield of protein from the mixed bacterial and mixed fungal isolates is a vital outcome which should be exploited in order to improve the

nutritional well being of millions of cassava products consumers. Cassava requires mixed isolates for its fermentation probably because of varying pH of the ferment as fermentation progresses. Efforts should be expanded in the direction of exploiting preferably the mixed bacteria with high linamarase and ability to convert some portions of the carbohydrate and fibre to protein.

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