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Full Length Research Paper

Antibiotic sensitivity pattern of *Bacillus* species isolated from solid substrate fermentation of cassava for *gari* production

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In this study, the bacterial population dynamics during solid substrate fermentation of cassava at different time regimes was determined, and the *Bacillus* species isolated were identified phenotypically of probable identity as *Bacillus subtilis* (47%), *Bacillus pumilus* (26%) and *Bacillus mycoides* (27%). The bacterial population increased as fermentation progressed. 8.32 $\log_{10}$ cfu/g at 0 h increased subsequently to 8.90 $\log_{10}$ cfu/g at 72 h for Atiba sample, and 8.28 - 8.80 $\log_{10}$ cfu/g for Ijebu-Ode sample. The reduction in pH correlates with the increase in total titratable acidity (TTA) as being mediated by lactic acid bacteria (LAB). Diversity occurred in the antibiotic sensitivity pattern of the isolates. Nevertheless, all the isolates were resistant to gentamicin, ampiclox and zinnacef, and susceptible to ciprofloxacin. Caution should then be made not to include antibiotic resistance strains of *Bacillus* in the development of starter cultures or probiotic microorganisms for cassava fermentation as they may have the potential to transfer antibiotic resistance genes to intestinal pathogens.

Key words: *Gari*, solid substrate fermentation, *Bacillus* species, antibiotics, α-amylase.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important root crop in the tropics (De-Bruijn and Fresco, 1989). In West Africa, this crop is usually fermented using various methods before consumption (Hahn, 1989). One of the most popular foods derived from fermented cassava is *gari*, which is consumed by nearly 200 million people in West Africa (Okafor and Ejiofor, 1990). In Southern Nigeria, *gari* contributes about 60% of the total calorie intake (Akinrele et al., 1962). It is high in carbohydrates (>80% starch; Ahonkai and Koleoso, 1979) and very poor in protein, containing only about 1% (Sanni et al., 2002).

Gari production is by solid substrate natural fermentation in a process that involves dewatering after grating of the cassava pulp and fermentation period of 4 - 5 days in jute bags, followed by wet sieving, frying and dry sieving (Oguntoyinbo, 2008). Occasional addition of palm oil by some tribes in Nigeria gives a yellow coloured *gari* (Odunfa, 1985). During *gari* fermentation, the pH, linamarase activity and total cyanide (Ikediobi and Onyike, 1982). The major change during the fermentation is the increase in acidity (Odunfa and Oyewole, 1998).

Various studies have been conducted to evaluate the microorganisms involved during the fermentation of cassava (Ampe et al., 1999; Ben Omar and Ampe, 2000; Coulin et al., 2006). One of the notable organisms isolated at the beginning of fermentation of cassava is the *Bacillus* strains (Oyewole and Odunfa, 1988). These *Bacillus* strains are rod-shaped, Gram-positive and catalase positive bacteria. One important power possessed by *Bacillus* strains isolated from fermenting cassava is their ability to produce amylase enzyme (Amund and Ogunsua, 1987; Oyewole and Odunfa, 1992a, b). These amylases are involved in the initial breakdown of cassava starch into simple sugars, which are required by other organisms like lactic acid bacteria and yeast (Oyewole, 2002). While Oyewole and Odunfa (1992b) reported that in the submerged fermentation of cassava for *fufu* and *lafun* production, the *Bacillus* species that appear at the beginning of the fermentation became extinct towards the end of the process, Amoa-Awua and Jakobsen (1995) reported that *Bacillus* species

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occurred in high numbers and persisted throughout the solid substrate fermentation of cassava to *agbelima* (a product similar to *gari*).

Many investigators recently have speculated that commensal bacteria including lactic acid bacteria act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (Perreten et al., 1997a; Levy and Salyers, 2002). Such reservoir organisms could possibly be found in various foods and food products containing high densities of non-pathogenic bacteria as a result of their natural production process (Shalini and Singh, 2005). Lactic acid bacteria widely used as probiotics or starter cultures have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes to other organisms including pathogenic bacteria (Shalini and Singh, 2005). A closely related genus of lactic acid bacteria is *Bacillus*, which is becoming popular as probiotic cultures for use in various food products.

Literature search reveals that there is dearth of information on the antibiotic susceptibility profile of *Bacillus* species isolated from foods and food products; herein we present our findings on the investigation of the antibiotic sensitivity pattern of *Bacillus* species isolated from solid substrate fermentation of cassava for *gari* production.

MATERIALS AND METHODS

Sampling

Grated fermented cassava meant for *gari* production were obtained from local producers in Atiba and Ijebu-Ode, Ogun State, Nigeria at different fermentation time regimes of 0, 12, 24, 48 and 72 h. The samples were transported with the aid of ice pack into the laboratory for immediate analysis.

Determination of pH and total titratable acidity (TTA)

The pH and the total titratable acidity (TTA) were determined using 10 g of grated fermented cassava homogenized in 90ml sterile distilled water using a mortar and pestle in order to form a pulp. The pH was measured using digital pH metre (Model HI 255, Hanna Instrument Bedfordshire, UK), while the TTA was determined by titrating an aliquot of cassava filtrate with 0.1 M NaOH, using phenolphthalein as indicator. The titratable acidity (as lactic acid) was calculated using the below relationship:

$$\text{Titratable acidity \%} = \frac{Va \times Na \times 0.009 \times 100}{Ws}$$

Where Va = volume of base used; 0.009 acid milliequivalent factor for lactic acid; Na normality of base used; Ws = weight of sample.

Microbial enumeration

The grated fermented cassava at different time regimes was analyzed for population density. Ten grams each was weighed into 90 ml sterile diluents of 0.1% peptone water contained in a conical flask. This was homogenized to form a pulpy solution. The solution was filtered using Whatman No. 1 filter paper (Whatman International Limited, Maidstone, England), and boiled at 85°C for 3 - 5 min (to kill vegetative cells leaving the sporeformers). Serial dilutions were then made on Nutrient agar (Oxoid CM3, Basingstoke, Hampshire, England, UK) using the spread plate technique. These were done in triplicate and incubated at 37°C for 24 h. Results were calculated as means of three determinants.

Phenotypic characterization and identification

The phenotypic studies carried out on the representative isolates include: Gram and endospore staining, catalase test, starch and casein hydrolysis, growth at different pH values and NaCl concentration, and various sugar fermentation tests as described by Claus and Berkeley (1986).

Antibiotic sensitivity testing

Antibiotic sensitivity tests were carried out on the isolates using commercial antibiotic discs (Maxicare Medical Laboratory, Lagos, Nigeria). For each isolate, the antibiotics used were pefloxacin (10 µg/disc), gentamicin (10 µg/disc), ampiclox (30 µg/disc), zinnacef (20 µg/disc), amoxacillin (30 µg/disc), rocephin (25 µg/disc), streptomycin (30 µg/disc), septrin (30 µg/disc), erythromycin (10 µg/disc) and ciprofloxacin (10 µg/disc).

RESULTS AND DISCUSSION

The results of the parameters (that is pH and TTA) determined showed that the pH reduced for both samples (Atiba and Ijebu-Ode) as fermentation progressed, while the TTA increased (Figures 2 and 3). This confirmed the earlier report of Odunfa and Oyewole (1998) where increase in acidity was the major chemical change that took place during cassava fermentation. The results of the population dynamics obtained revealed that bacteria presumptively identified as *Bacillus* species were constantly isolated throughout the fermentation periods. Bacterial population of 8.32 $\log_{10}$ cfu/g at 0 h increased subsequently to 8.90 $\log_{10}$ cfu/g at 72 h for Atiba sample, and 8.28 $\log_{10}$ cfu/g to 8.80 $\log_{10}$ cfu/g for Ijebu-Ode sample (Figure 1). The high values in the population densities of the *Bacillus* species agreed with the findings of Amoa-Awua and Jakobsen (1995) who reported high numbers of *Bacillus* species and persistence throughout the solid substrate fermentation of cassava to *agbelima*, a similar product to *gari*. This is in contrast with the findings of Oyewole and Odunfa (1992b) with submerged fermentation of cassava for *fufu* and *lafun* production.

The phenotypic characteristics of the 15 probable identified species of *Bacillus* are Gram-positive rods, catalase positive, endosporeformers, ability to hydrolyse starch and casein, growth at pH 4.5 and 9.6, and growth in 4 and 6.5% NaCl. Out of these the dominating species was *B. subtilis* while the other species found were *B. pumilus* and *B. mycoides*. The sugar fermentation profiles of the organisms showed that all were able to ferment different sugars. However, *B. subtilis* was the major fer-

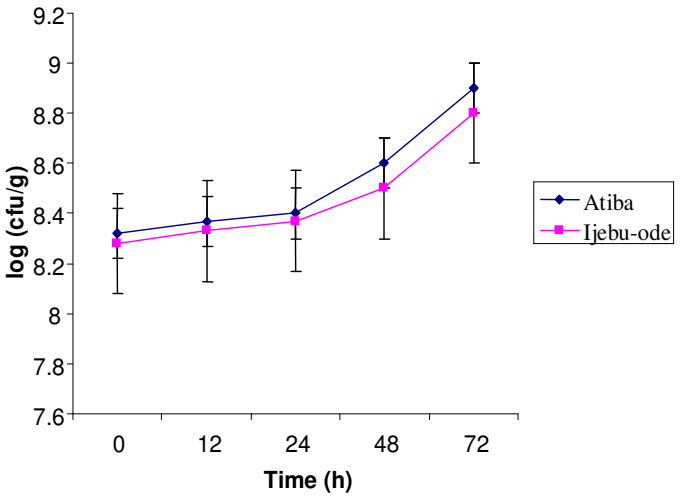

Figure 1. Total viable counts of bacteria isolated at different time regimes from fermented cassava. Error bars represent standard deviation of mean of three measurements.

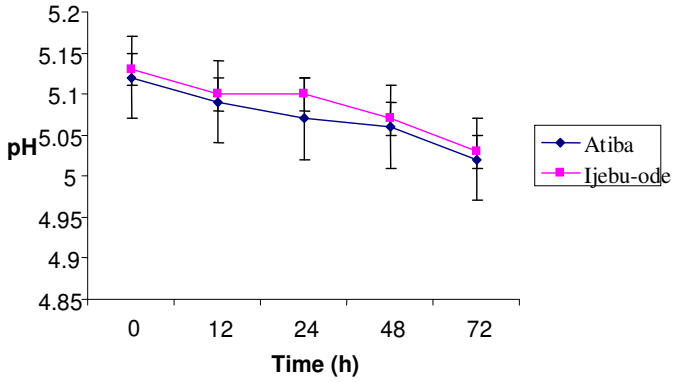

Figure 2. pH changes during solid substrate fermentation of cassava for *gari* production. Error bars represent standard deviation of mean of three measurements.

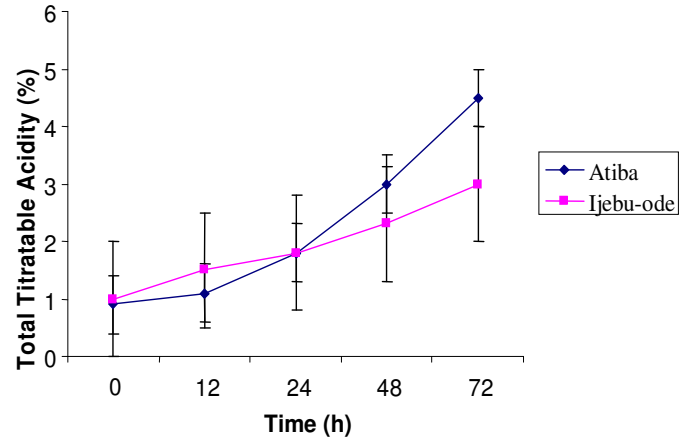

Figure 3. Total titratable acidity (%) during solid substrate fermentation of cassava for *gari* production. error bars represent standard deviation of mean of three measurements.

menter. Apart from being able to ferment majority of the sugars with the exemption of xylose and dulcitol, it was isolated seven times during the fermentation process thus representing 47%. *B. pumilus* was isolated four times representing 26.5%, and fermented majority of the sugars except dulcitol and lactose. The remaining four isolates were *B. mycoides* representing 26.5%, and were able to ferment the sugars except xylose, mannitol, dulcitol and arabinose. All the 15 isolates were found to be amylolytic with the release of α-amylase enzyme for starch hydrolysis. This confirmed the important power possessed by *Bacillus* strains isolated from fermented cassava by their ability to produce amylase enzyme (Amund and Ogunsua, 1987; Oyewole and Odunfa, 1992a, b). These amylases are involved in the initial breakdown of cassava starch into simple sugars, which are required by other microorganisms (Oyewole, 2002).

The results of the antibiotic sensitivity tests as summarized in Table 1 showed that all the isolates displayed diversity in their ability to resist and be susceptible to the ten antibiotics used. However, all the isolates were resistant to gentamicin, ampiclox and zinnacef, and susceptible to ciprofloxacin. Gentamicin is cidal and narrow against Gram-negative organisms only. Ciprofloxacin is also cidal but broad against both Gram-positive and –negative organisms.

Conclusion

In the development of starter cultures or probiotic microorganisms for cassava fermentation, the presence of antibiotic resistance strains should be discouraged as they may have negative consequence of horizontal transfer of antibiotic resistance genes to intestinal pathogens.

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Table 1. Antibiotic sensitivity pattern of *Bacillus* species isolated from fermented cassava for *gari* production.

	Antibiotics									
Isolates	**R**	**CPX**	**S**	**SXT**	**E**	**PEF**	**GEN**	**APX**	**Z**	**AM**
AT1	S^{+++}	S^{+}	S^{+}	R	R	R	R	R	R	R
AT7	S^{++}	S^{+}	R	R	R	R	R	R	R	R
IJ9	S^{++}	S^{+}	R	R	S^{+}	R	R	R	R	R
AT10	S^{++}	S^{++}	S^{+}	R	R	R	R	R	R	R
IJ17	R	S^{+++}	S^{++}	R	R	R	R	R	R	R
AT3	S^{++}	S^{++}	R	R	R	R	R	R	R	R
IJ6	R	S^{+}	R	R	R	R	R	R	R	R
IJ13	R	S^{+}	S^{+}	R	R	R	R	R	R	R
AT2	S^{+}	S^{++}	S^{++}	S^{++}	S^{++}	R	R	R	R	R
IJ18	S^{++}	S^{++}	S^{++}	R	S^{++}	S^{+}	R	R	R	S^{+++}
AT12	S^{+}	S^{+}	R	R	R	R	R	R	R	R
IJ14	S^{++}	S^{+}	S^{+}	S^{+++}	S^{++}	S^{+}	R	R	R	R
IJ11	S^{+}	S^{++}	R	R	R	S^{++}	R	R	R	R
AT11	S^{++}	S^{+}	R	R	R	R	R	R	R	R
AT14	S^{+++}	S^{++}	S^{+}	R	R	R	R	R	R	R

Key: *Bacillus subtilis* - AT1, AT10, IJ6, IJ13, IJ18, IJ14 and IJ11; *Bacillus pumilus* - IJ9, AT2, AT3 and AT11; *Bacillus mycoides* - AT7, AT12, AT14 and IJ17.
Antibiotics: R – Rocephin, CPX – Ciprofloxacin, S – Streptomycin, SXT – Septrin, E – Erythromycin, PEF – Pefloxacin, GEN – Gentamicin, APX – Ampiclox, Z – Zinnacef and AM – Amoxacillin 0 – 5 mm Resistance (R) 6 – 15 mm Sensitive (S^{+}) 16 – 25 mm Sensitive (S^{++}) 26 – 35 mm Sensitive (S^{+++}).

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