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Evaluation of Invertase and Amylase Activities of Latic Acid Bacteria Isolated from 'Pupuru' (An Indigenous African Fermented Cassava Staple Food)

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Authors' contributions

This work was carried out in collaboration among all authors. The work is a research project of author BTA under the supervision of author BCAO with the assistance of author IWO. All the authors read and approved the final manuscript.

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ABSTRACT

Lactic acid bacteria produce lactic acid as the major end product during sugar fermentation. This study was carried out to isolate lactic acid bacteria (LAB) from pupuru; a staple cassava food. The result of the bacterial count indicated that the count ranges from 3.1×10^4 CFU/g to 8.7×10^4 CFU/g. Base on the Gram reaction, microscopic morphology and biochemical characteristics, the isolated LAB were identified as *Pediococcus halophilus*, *Lactobacillus casei*, *Lactobacillus brevis*, and *Lactobacillus fermentum*. Analysis of the percentage of occurrence of the lactic acid bacteria indicated that *Lactobacillus casei* (50.0%) was the most dominant LAB, *P. halophilus, Lactobacillus brevis, L. fermentum* were least occurring (16.7%). The amylase enzyme index showed that *P. halophilus* had no enzyme index while *L. casei* and *L. fermentum* had enzyme index of 4.00 mm while *L. brevis* had an enzyme index of 3.00 mm. Of the LAB isolated, only *L. casei* (0.064 µg/mL) had invertase activity. *L. fermentum* (0.099 µg/mL) and *L. casei* (0.08 µg/mL) showed considerable effect in the production of amylase. The result of this study indicated that *Lactobacillus brevis, L. casei,* and *L. fermentum* will be a viable addition in the amylase production industries while *L. casei* could be investigated further for enhancement in enzyme production.

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1. INTRODUCTION

Lactic acid bacteria are Gram-positive, non-sporing, and catalase negative, devoid of cytochromes, anaerobic but aero-tolerant cocci or rods that are acid-tolerant and produce lactic acid as the major end product during sugar fermentation [1]. Lactic acid bacteria (LAB) have a long history of application in fermented foods because of their beneficial effects on the shelf-life, organoleptic and nutritional characteristics of food. They cause acidification of food through the production of organic acids [2]. Lactic acid bacteria play important role in the production of most staple foods in Nigeria and they are the major bacteria involved in fermentation of numerous carbohydrate based products [3]. They contribute to the preservation of foods by producing antimicrobial agents like bacteriocins which are considered as natural food preservatives [4].

Pupuru is a traditional fermented, smoked-dried cassava staple food which is believed to have originated from the riverine area (Ilaje, Ikale and Apoi) of Ondo State, South West Nigeria [5]. The traditional processing of *Manihot esculenta* (Cassava) into "pupuru" involves peeling of tubers, steeping of peeled tubers in stream water and fermentation of the tubers for 4-6 days. Water is then drained off the fermented marsh. The marsh is then pulverized, molded into balls and smoked dried [5,6].

Although most LAB are unable to degrade starch because of the lack of the amylolytic activity, a few have been reported to exhibit this activity and are qualified as amylolytic lactic acid bacteria (ALAB) which are able to decompose starchy material through the amylases production during the fermentation processes [1]. This study is aimed at evaluating the amylolytic and invertase activity of lactic acid bacteria isolated from Pupuru a staple cassava food.

2. MATERIALS AND METHODS

2.1 Isolation of Lactic Acid Bacteria

One gram (1 g) of the cassava product (Pupuru) was suspended in 9 mL of de Man-Rogosa-Sharpe (MRS) broth and incubated anaerobically at 30°C for 24 h. Then aliquots (0.1 mL) of the stock samples was spread plated on MRS agar plates and incubated anaerobically at 30°C for 48 h [3]. The resultant distinct colonies were purified by successive streaking on MRS agar plates to obtain pure colonies.

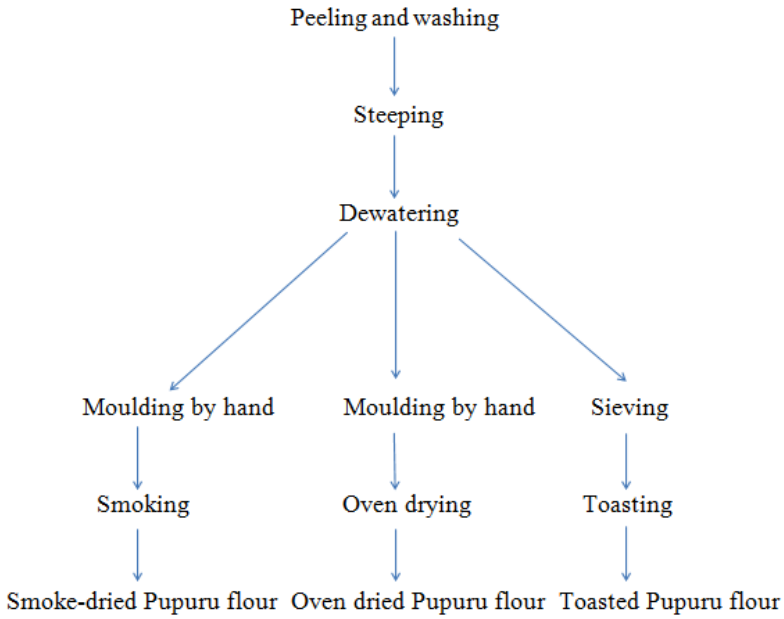

Fig. 1. Flow chart for the production of Pupuru using different drying methods [7]

2.2 Determination of Bacterial Load

The total bacterial load from each Pupuru sample was determined by carrying out tenfold serial dilution up to 10^9. Aliquot (0.1 mL) of 10^{-4} and 10^{-6} was dispensed on already solidified Nutrient agar and MRS agar and inoculated by spread plate technique of Isu and Onyeagba [8]. The plates were allowed undisturbed for 15 minutes and were incubated anaerobically on MRS agar plates at 37°C for 24 h. Resultant colonies were counted using the colony counter and expressed as CFU/gm.

2.3 Phenotypic and Biochemical Characteristics of LAB

Lactic acid bacteria were identified using their typical morphological, microscopic and physiological characteristics. The isolates were subjected to Gram reaction, catalase test, ability to growth at 30°C, 37°C, 40°C, 45°C and concentrations of 3%, 6%,10% NaCl.

2.4 Gram Staining

A smear of each isolate from 48 h old culture was made on slide and heat fixed. The slide was flooded with crystal violet for 60 s and then washed off in slow running tap water after which Lugol's iodine was added for another 30 s and washed off. The smear was decolorized rapidly with Gram's alcohol and then counter stained with 1% safranin for 60 s after which the stain was washed off in running water and air dried. Air dried smear was examined microscopically under the oil immersion objective (×100). The Gram reaction and morphology of the LAB was observed and recorded.

2.5 Catalase Test

This was used for detecting the presence of catalase enzyme in the isolates. The catalase enzyme catalyzes the breakdown of hydrogen peroxide to release free oxygen and water.

The reaction follows thus;

$$2H_2O_2\,(l) \rightarrow 2H_2O\,(l) + O_2\,(g)\ [9]$$

A few drop of hydrogen peroxide will be added to smear of 48 h old culture on a glass slide and observed for the presence or absence of white froth or bubbles. The production of white froth indicates a catalase positive reaction while absence of the white froth indicates a negative reaction.

2.6 Effect of Temperature on Growth

The isolated lactic acid bacteria will be tested for their ability to grow at 30°C, 37°C, 40°C and 45°C. The isolated bacteria will be sub-cultured on different MRS agar plates and incubated at 30°C, 37°C, 40°C and 45°C anaerobically for 48 h. After which the resulting growth will be observed.

2.7 Effect of NaCl on LAB Growth

The effect of 3%, 6% and 10% NaCl concentrations on LAB growth will be determined by supplementing MRS agar with these salt concentrations. The isolated LAB will be inoculated onto salt supplemented agar and incubated anaerobically for 48 h at 40°C. The effect of different NaCl concentration on the reduction of LAB growth will be recorded.

2.8 Casein Hydrolysis Test

The isolates were streaked on casein containing media (1% casien and 2.8% nutrient agar) and incubated for 24 h. After incubation, trichloroacetic acid was added over the cultures to observe the clear hydrolysis zone formed by the colony if the organism is positive for casien hydrolysis [10].

2.9 Urease Test

The urease test was used to determine the ability of an organism to split urea, through the production of the enzyme urease.

$$(NH_2)_2CO + H_2O \xrightarrow{urease} CO_2 + 2NH_3$$
(ammonia)

Ammonia + Phenol red $\longrightarrow$ deep pink colour [11]

Units of any ammonia formed with resulting alkalinity in the presence of the enzyme and the increased pH was detected by a pH indicator. The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea agar containing the pH indicator phenol red. The bacteria were aseptically inoculated into urea agar using a sterile wire loop and incubated at 30°C for 24 h, after which observations on colour change were done.

2.10 Starch Hydrolysis Test

Starch hydrolysis was observed by using iodine solution. Iodine with starch gives blue colour. Clear zone around colony shows starch hydrolysis [12]. 1% starch was incorporated in nutrient agar. Plates were inoculated with the isolated bacteria and incubated at 37°C for 24 h. Colonies were exposed to 2% iodine solution and observed for the zone of hydrolysis around each colony. Enzymatic index was calculated as:

$$\text{Enzyme Index} = \frac{\text{Diameter of zone of degradation}}{\text{Diameter of colony}} \quad [12]$$

2.11 Screening of Amylase Producing Bacteria

The qualitative analysis of the amylolytic activity of the isolated strains will be performed by culturing the isolated bacteria in de Man Rogosa and Sharpe agar (MRS) supplemented with 10 g/L of starch as sole carbon source. The detection of the amylolytic activity of isolated lactic acid bacteria will be performed with a culture of 24 h on MRS agar/starch. After incubation at 30°C for 48 h, a solution of iodine and potassium iodine in w/v (0.15% I and 1.5% KI) will be spread on the plate surface forming a blue starch-iodine complex. A clear zone around a colony indicates a positive amylolytic activity [13]. The presence of a clear zone is due to the absence of starch consequently hydrolyzed by amylase.

2.12 Crude Enzyme Extraction

The bacterial isolate will be grown at 30°C for 70 h in a 50 mL basal medium containing (gram per liter): 10 g soluble starch, 5 g peptone, 5 g yeast extract, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g NaCl. After incubation, 6 ml of the basal medium will be pipetted into sterile test tubes and the test tube centrifuged at 4000 rpm for 30 min. after which the supernatant will be separated from the biomass and the supernatant will be used as crude enzyme.

2.13 Enzyme Activity

Reducing sugars will be determined by the 3,5-dinitrosalicylic acid (DNS) colorimetric method [3]. In the reaction mixture, 1 mL of crude enzyme will be taken and added in a mixture of 0.5 ml of 1% starch solution (1 g of starch in 100 mL of distilled water) and 0.1 mL of citrate phosphate buffer (Citrate buffer; 50 mL of 0.05 m/L citric acid in a volumetric flask and made up the volume by using 0.05 ml tri-sodium citrate to get citrate buffer with pH 6.0). The mixture will then be vortexed and incubated in a water bath at 60°C for 1 h. After incubation, the reaction will be stopped by keeping the reaction tubes in boiling water bath at 100°C for 1 min. The mixture will be brought to room temperature and 1 ml of DNS reagent will be added to it, the mixture will be vortexed and boiled for 5 minutes in a water bath followed by the addition of 0.4 mL distilled water to the reaction mixture in a test tube. The blank will contain 0.1 mL of 1M citrate phosphate buffer (pH 6.0), 0.5 ml of 1% starch solution, 0.4 mL of distilled water and 1 mL of DNS. The mixture will be cooled to room temperature, then absorbance taken at 540 nm.

2.14 Preparation of Glucose Standard

Glucose standard will be prepared by weighing 0.1 g of glucose into a flask and diluted with 100 ml distilled water. Stock solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 mL will be transferred into test tubes and volume made up to 1 mL with distilled water. This will be followed by addition of 1.0 ml DNS, boiled for 5 min and absorbance of the cool mixture determined at 540 nm. A plot of glucose concentration against absorbance will be used to determine reducing sugar concentration and enzyme activity [3]. A unit of amylase activity (U) will be defined as the amount of enzyme able to hydrolyse a gram of soluble starch within 60 min under the experimental condition.

2.15 Screening for Invertase Producer

The bacterial isolates will be obtained by suspending the various samples in medium containing (g/l) sucrose 4.0, di-potassium phosphate 1.0, magnesium sulphate 1.0, and ammonium sulphate 3.0 (pH 7.0). All the isolates will be grown at 40°C for 48h with agitation. Efficient invertase producers will be screened out by estimating the enzyme activities and bacterial growth at 48 h [14].

2.16 Invertase Assay

Invertase activity will be assayed by measuring the amount of reducing sugars released from sucrose. The assay mixture for invertase will contain 0.1 mL of crude enzyme and sucrose (0.9 mL of 1.1% w/v) in 100 mM sodium acetate buffer (pH 7). The mixture will be incubated at 60°C for 1h, and then reaction will be stopped by

1 mL of dinitrosalicylic acid (DNS). Finally the absorbance will be read at 540 nm in spectrophotometer [12]. One unit of invertase (IU) will be defined as the amount of enzyme which liberates 1µ moles of glucose/minute/mL under the assay condition [14].

3. RESULTS

The result of the lactic acid bacteria counts are represented in Table 1. Sample E recorded the highest bacterial count while sample F recorded the lowest bacterial count.

Table 1. Total Lactic Acid Bacteria (LAB) count

S/N	Samples	Bacterial count (CFU/mL)
1.	A	8.3×10^4
2.	B	5.2×10^4
3.	C	3.9×10^4
4.	D	4.4×10^4
5.	E	8.7×10^4
6.	F	3.1×10^4

3.1 Biochemical and Morphological Characteristics of LAB

The biochemical and morphological characteristics of LAB isolated from Pupuru are shown in Table 2.

3.2 The Enzyme Index

The enzyme index of the isolated LAB was indicated in vitro by inoculating the bacteria on starch supplemented MRS agar. The result of the enzyme index is shown in Table 3.

3.3 Percentage of Occurrence

Analysis of the percentage of occurrence of the lactic acid bacteria indicated that *Lactobacillus casei* (50.0%) was the most dominant LAB, *P. Halophilus, Lactobacillus brevis, L. fermentum* were least occurring (16.7%).

3.4 Amylase and Invertase Activity

Enzyme Activity = µg of glucose produced X (1/180) µmole / Volume of enzyme solution) X (Incubation time) (1)

The enzyme activity was measured in units as shown in the formula (Eq. 1), where 1 unit of enzyme activity is equal to the amount of enzyme required to release 1µmol glucose equivalent per minute per ml of enzyme used under the assay conditions. It was observed that only *L. casei* (0.064 µg/mL) had invertase activity. *L. fermentum* and *L. casei* showed considerable effect in the production of amylase.

4. DISCUSSION

Lactic acid bacteria (LABs) are industrially important organisms used for the production of fermented food products like *Pupuru*, *Fufu* and *Garri*. The species used for these applications belong to the group of gram-positive bacteria including the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus*. They are recognized for their fermentative ability and thus enhancing food safety, improving organoleptic attributes, enriching nutrients and increasing health benefits [15,16].

This study was carried out to evaluate the presence of lactic acid bacteria in Pupuru; a staple cassava food mostly used by the people of Ondo State. The result of the total lactic acid bacteria load indicates that the count ranges from 3.1×10^4 CFU/g to 8.7×10^4 CFU/g.

Based on the gram reaction, microscopic morphology and biochemical characteristics, the isolated LAB were identified as *Pediococcus halophilus*, *Lactobacillus casei*, *Lactobacillus brevis*, and *Lactobacillus fermentum*. The result of the analysis of the percentage of occurrence of the lactic acid bacteria indicated that *Lactobacillus casei* (50.0%) was the most dominant LAB, *P. halophilus, Lactobacillus brevis, L. Fermentum* were least occurring (16.7%).

Analysis of the enzyme index for amylase activity showed that *P. halophilus* had no enzyme index while *L. casei* and *L. Fermentum* had enzyme index of 4.00 mm while *l. Brevis* had an enzyme index of 3.00 mm. The result of this study showed that *l. Casei* and *l. Fermentum* were potent amylase producers. This result is similar to study of Sanni et al. [17]. The authors described amylolytic strains of *L. plantarum* and *L. fermentum* strains in various nigerian traditional amylaceous fermented foods.

Amylolytic lactic acid bacteria have been reported from different tropical amylaceous fermented foods, prepared mainly from cassava and cereals. Amylolytic strains of *Lactobacillus plantarum* have been isolated from African cassava-based fermented products [18] and the new ALAB species *Lactobacillus manihotivorans*

Table 2. Biochemical and morphological characteristics of LAB

Isolate	Catalase	Morphology	Gram reaction	Methyl red	V.P	Indole	Oxidase	Nitrate red.	Starch hydrolysis	Gelatin	Casein	Citrate	Growth in 4% Nacl	Growth at 20ºC	Growth at 70ºC	Fructose	Glucose	Trehalose	Maltose	Sorbitol	Lactose	Sucrose	Mannitol	Probable Organism
LAB 1	-	Cocci	+	+	-	-	-	-	-	-		+	-	+	+	+	+	+	+	-	-	+	+	*P. halophilus*
LAB 2	-	Rod	+	+	-	-	-	-	-	-		-	-	+	+	+	+	+	+	+	-	+	+	*L. casei*
LAB 3	-	Rod	+	+	-	-	-	-	+	-		+	-	+	+	+	+	+	+	+	+	-	+	*L. fermentum*
LAB 4	-	Rod	+	+	-	-	-	-	-	-		-	-	+	+	+	+	+	+	-	+	+	+	*L. brevis*

[18] was isolated from cassava sour starch fermentations carried out in Colombia. ALAB have also been isolated from cereal-based fermented foods. Olympia et al. [19] characterized amylolytic strains of *L. Plantarum* isolated from burongisda, a fermented food made from fish and rice in Philippines. Amylolytic strains of *Lactobacillus fermentum* were isolated for the first time from Benin maize sourdough (Ogi and Mawè) by Agati et al. [20].

Table 3. Enzyme index of LAB

Bacteria	Starch utilization (mm)
P. halophilus	0.00 ± 0.00
L. casei	4.00 ± 0.01
L. brevis	3.00 ± 0.05
L. fermentum	4.00 ± 0.03

Table 4. Percentage of occurrence of LAB

Isolates	Frequency	Occurrence (%)
P. halophilus	1	16.7
L. casei	3	50.0
L. brevis	1	16.7
L. fermentum	1	16.7
Total	**6**	**100**

Table 5. Enzyme activity

Lactic acid bacteria	Amylase (µg/mL)	Invertase (µg/mL)
P. halophilus	0.00 ± 0.00	0.00 ± 0.00
L. casei	0.08 ± 0.00	0.064 ± 0.01
L. Bevis	0.03 ± 0.00	0.00 ± 0.00
L. fermentum	0.099 ± 0.00	0.00 ± 0.00

Values are means of Enzyme activity ± Standard Error of Mean (SEM)

5. CONCLUSION

This study was aimed at isolating lactic acid bacteria from Pupuru; a staple cassava food. The result of bacterial counts indicated that sample E recorded the highest bacterial count while sample F recorded the lowest bacterial count. Biochemical identification of lactic acid bacteria (LAB) indicated that *Lactobacillus casei* was the most dominant LAB while the least occurring were *P. halophilus, Lactobacillus brevis,* and *L. fermentum*. Result of the enzyme activity indicated that *Lactobacillus brevis, L. casei,* and *L. fermentum* produced considerable amount of amylase. However, *L. fermentum* was observed to produce the highest amylase activity. In the analysis of invertase activity, only *L. casei* exhibited invertase activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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