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Amylolytic Potential of Lactic Acid Bacteria Isolated from Wet Milled Cereals, Cassava Flour and Fruits

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Authors' contributions

This work was carried out in collaboration between all authors. Author TYA designed the study. Author HSD performed the statistical analysis. Authors TYA and HSD wrote the protocol and the first draft of the manuscript and managed literature searches. All the authors managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This research was conducted with the objective to evaluate amylolytic potential of lactic acid bacteria isolated from wet milled cereals, cassava flour and fruits.
Methodology: Strains of lactic acid bacteria were isolated from food samples and screened for amylolytic potential using starch hydrolysis technique. The resultant Amylolytic Lactic acid bacteria (ALAB) isolates were then characterized and identified based on their morphological and physiological properties using both conventional methods and API 50CHL kit (Biomerieux, France). These isolates were further evaluated for their amylase yield (U/ml) and amylase activity (mg/ml) using standard procedures.
Results: From this study, characteristics typical of the lactic acid bacteria showed that isolates were Gram positive rods, catalase negative, with growth occurring at 30, 37, 40, 45℃ and 3, 6, 10% (w/v)NaCl. On the basis of amylolytic potential, only 14 isolates exhibited amylolytic properties with halo diameter of between 25.00 to 68.00 mm. Comparatively, isolates (AMZ5) obtained from maize flour gave the highest amylase potential. Occurrence of the ALAB species

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identified showed that; *Lactobacillus plantarum* 8(57.14%), *Leuconostoc mesenteroides* 2(14.29%), *Lactobacillus fermentum* 1(7.14%), *Lactobacillus brevis* 1(7.14%), *Lactococcus lactis* 1(7.14%) and *Pediococcus acidolactici* (7.14%) were predominant. Quantitatively, maximum amylase yield of between 0.38 (U/ml) to 1.10 (U/ml) was observed for the isolates at 24 h of incubation. Amylolytic *Lactobacillus plantarum* (AMZ5) from maize dough flour however, gave the highest amylase concentration (1.10 U/ml). As a criterion of strain selection, hydrolytic action of AMZ5 gave the highest reducing sugar concentration of 0.55 (mg/ml) after 24 hr. Among the isolates, *Lactobacillus brevis* (ATt) isolated from tomato on the other hand, had lowest reducing sugar concentration (0.30 mg/ml) even after 48 hr of incubation.

Conclusion: Therefore, findings from this study indicate that cereal based products are rich sources of ALAB. Also the strain of *Lactobacillus plantarum* (AMZ5) isolated from maize flour possesses better starch degradation capability through production high extracellular amylase and reducing sugar yields.

Keywords: Amylolytic lactic acid bacteria; amylase; wet milled cereals; cassava flour; fruits.

1. INTRODUCTION

Lactic acid bacteria (LAB) are major bacteria involved in fermentation of numerous carbohydrate based products. In developing countries for example, Lactic acid bacteria play important role in the production of most staple foods commonly through spontaneous fermentation to improve acceptability, nutritional and functionality of the foods [1].

In spite of the ancient used of lactic acid bacteria (LAB) for production of fermented foods, their multi-potentials for amylase production has recently generated much research interest [2]. Therefore, amylase from microorganisms has found a broad spectrum of industrial applications in the starch, beverages, food, and textile industries [3,4]. Of particular importance, amylolytic Lactic acid bacteria found usefulness in modifying the structure and properties of starches for production of lactic acid and alpha-amylases extensively to improve bread making [5]. With the ban in usage of potassium bromate as bread improver in many countries, interest increased in finding an enzyme to replace this chemical oxidant. Therefore, supplementation of flour and dough with malt and microbial α-amylases as improvers became a usual practice widely used in the baking industry [6]. Therefore, selecting efficient amylase-producing strains, detailed investigations of biochemical and genetic basis of starch hydrolysis of a number of amylolytic LAB (ALAB) strains were generated [7].

Amylolytic lactic acid bacteria (ALAB) account for a substantial portion in different types of foods widespread among the dairy and non-dairy food [8]. In Nigeria, significant numbers of ALAB strains have been isolated from traditional cereal based fermented foods such as Fufu, 'Ogi', Burukutu, Ogi-baba and Kunu-zakki [9,10]. Predominantly, *L. amylovorus, L. plantarum, L. manihotivorans*, *L. brevis, L. delbrueckii* and *L. fermentum* were therefore reported to exhibit amylolytic activity [10,11,12]. Primarily among them however, *Lactobacillus plantarum* and *Lactobacillus fermentum* are the most efficient in synthesizing large amounts of extracellular α-amylase [1,10].

As a novelty, the addition of α-amylase to enhance rate of fermentation, reduction of dough viscosity with resultant improvements in the volume and texture of the bread have found great application [13]. Similarly, the potential to replace 10% of wheat flour with cassava flour [14] for baking of composite bread in Nigeria, the amylase- addition adopted bread technology could also be extended. Biotechnologically, this curiosity suggests the need to generate new ALAB with potentials to degrade cassava starch into fermentable compounds to enhance rate of fermentation. The resultant improvements in the taste, loaf volume and texture of wheat bread could also be transferred in the cassava-wheat composite bread. This study therefore reports on the characterization and taxonomic status of amylolytic lactic acid bacteria strains isolated from wet milled cereals.

2. MATERIALS AND METHODS

2.1 Sample Collection

Products of wet milled cereals (sorghum, millet, maize and acha) and cassava were purchased from Samaru and Ahmadu Bello University community markets in clean low density

polythene bags. These were taken to the Industrial Microbiology Laboratory, Department of Microbiology, Ahmadu Bello University Zaria for analysis.

2.2 Isolation of Lactic Acid bacteria (LAB)

Ten grams each of the cassava products and wet milled cereals products were suspended in 90 ml of de Man-Rogosa-Sharpe (MRS) broth and incubated anaerobically at 30℃ for 24 h. Then aliquots of 0.1 ml of the stock samples were spread plated on MRS agar plates and incubated anaerobically at 30℃ for 48 hours. The resultant distinct colonies were purified by successive streaking on MRS agar plates to obtain pure colonies.

2.3 Screening LAB for Amylase Production Potential

Determination of amylase production potentials of LAB isolates was carried out using starch hydrolysis test following procedures adapted by Sun et al. [15]. LAB isolates were spot inoculated onto sterile solidified MRS agar fortified with 2% soluble starch and incubated at 30℃ for 24 hours. The plates were then flooded with Gram's iodine (Gram's iodine; 0.15% iodine crystals added to 1.5% potassium iodide solution) to produce a deep blue colored starch-iodine complex. It was then observed for zone of decolorization which becomes visible within few seconds. Bacterial isolates surrounded by clearing zones were then sub-cultured on MRS agar to obtain pure cultures for further analysis.

2.4 Phenotypic and Biochemical Characteristics

LAB isolates were identified using their typical morphological, microscopic and physiological characteristics as adopted previously by Amapu et al. [16]. The isolates were first subjected to Gram reaction, catalase test, ability to growth at ambient, 30℃, 37℃, 40℃, 45℃ and concentrations of 3%, 6%,10% NaCl. Phenotypic relatedness of the isolates was then further determined through carbohydrate fermentation profiles using API 50CH strips and API CHL medium according to manufacturer's instructions (API system, Bio-Merieux, France).

2.5 Crude Enzyme Extraction

The bacterial isolate were grown at 40℃ for 70 hours in a 50 ml basal medium containing 1% (w/v) soluble starch and 0.5% (w/v) yeast extract. The flasks were incubated in a temperature regulated shaker. Sterile pipettes were used to aseptically transfer 5 ml into test tubes and thereafter centrifuged at 4000 rpm for 30 min.

2.6 Reducing Sugar Concentration and Enzyme Activity

Reducing sugars were determined by the 3, 5-dinitrosalicylic acid (DNS) colorimetric 115 method [17]. In the reaction mixture, 1 ml of crude enzyme was taken and added in a mixture of 0.5 ml of standard 1% starch solution (1% w/v starch solution- added 1g of starch in 100ml 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). This mixture was then vortexed and kept in a water bath at 60℃ for 1 h. After incubation, the reaction was stopped by keeping the reaction tubes in boiling water bath at 100℃ for 1 mins. The mixture was brought to the room temperature and 1 ml of DNS reagent was added to it, the mixture was vortexed and the mixture 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 contained 0.1 ml of 1 M 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 was cooled to room temperature, then absorbance taken at 540 nm. Glucose standard was prepared by weighing 0.1 g 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 were then transferred into test tubes and volume made up to 1 ml with distilled water. This was 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 was used to determine reducing sugar concentration and enzyme activity [17]. A unit of amylase activity (U) was defined as the amount of enzyme able to hydrolyse a gram of soluble starch within 60min under the experimental condition.

3. RESULTS AND DISCUSSION

From this study, a total of 14 starch-hydrolyzing LAB isolates were found associated with wet milled cereals, fruits and cassava flour. Colonial morphology of the isolates ranged from whitish to cream colour and Gram positive rods; characteristics typical to lactic acid bacteria. Accordingly, biochemical characterisation confirmed by API 50CH kit bioMerieux system (Table1) revealed that isolates are catalase

negative, with thermal stability at 30, 37, 40, 45℃ and 3, 6, 10% NaCl. Typical fermentation profile base on gas production shows that four (4) of the resultant isolates are homofermentative however, heterofermentative bacteria formed an extensive group of the LAB population. This is in line with findings of Gobbetti et al. [18], where highest percentage of heterofermentative isolates (58%) had been reported on sourdough. According to the species description; *L. plantarum, L. Lactis, L. mesenteroides, L. Fermentum, P. acidolactici,* and *L. brevis* are predominant amylase producing LAB isolated from fruits, cassava and wet milled cereals (Table 2). The result showed that they occurred in the following order; *L. plantarum* 8(57.14%) followed by *L. mesenteroides* 2(14.29%), *L. fermentum* 1(7.14%), *L. brevis* 1(7.14%), *L. lactis* 1(7.14%) and *P. acidilactici* 1(7.14%). This study affirms that diversity of lactic acid bacteria occur in food-related ecosystems; however, strain of *L. plantarum* in the fermented substrates dominates the LAB flora. Similarly, *L. plantarum* are reported as one of the dominant species in cassava, Ogi and Fufu fermentations [19,8]. Importantly, Lactic acid bacteria constitute a group of organisms that have been associated with production of fermented foods for many centuries however; a key reason for the current widespread use is their amylase-producing potentials. Due to this, current research efforts have been redirected toward sourcing for amylolytic lactic acid bacteria (ALAB) from tropical starchy fermented foods owing to their relatively high starch content. Following this trend, capacity of LAB isolates to degrade starch is presented as average diameter (mm) in Table 2. Among the LAB isolated, 14 isolates exhibited amylolytic power with hydrolysis halo diameter of between 25.00 mm to 48.00 mm. Finding from this study also showed that isolates (AM_{Z5}) with the highest amylase producing potential (48.00 mm) were majorly obtained from maize flour. This result is however higher than mean value of 45±1.5 mm reported for LAB isolated from soils at the "Gari", corn and cassava mills submitted to natural fermentation [20]. This finding suggests that although amylolytic LAB species may be isolated from different carbohydrate sources, starchy flours from maize proved to be the best inducer of amylase production. Gelatinized starchy sources of corn and sorghum flour were reported most suitable for α-amylase and lactic acid production by *L. fermentum* 04BBA19 [21]. Similarly, Mugula et al. [22] affirmed that ALAB are dominant lactic acid bacteria of *togwa*, a Tanzanian food prepared from sorghum, maize, millet and maize-sorghum. Species of LAB are also microbial populations associated with cassava fermentations [19] and starter cultures for fufu and ogi production [8]. Amylolytic strains of *L. plantarum* and *L. fermentum* strains in various Nigerian traditional amylaceous fermented foods had been described [1]. Our data, suggest that *L. plantarum* is the most predominant amylolytic strains as previously highlighted in various Nigerian traditional amylaceous fermented foods [23,1,24]. High α-amylase production potential by lactic acid bacteria when starchy flours are used as substrate had been attributed to the proteins and vitamins contents of the flour which are required for their growth, enzymes and acids production [25].

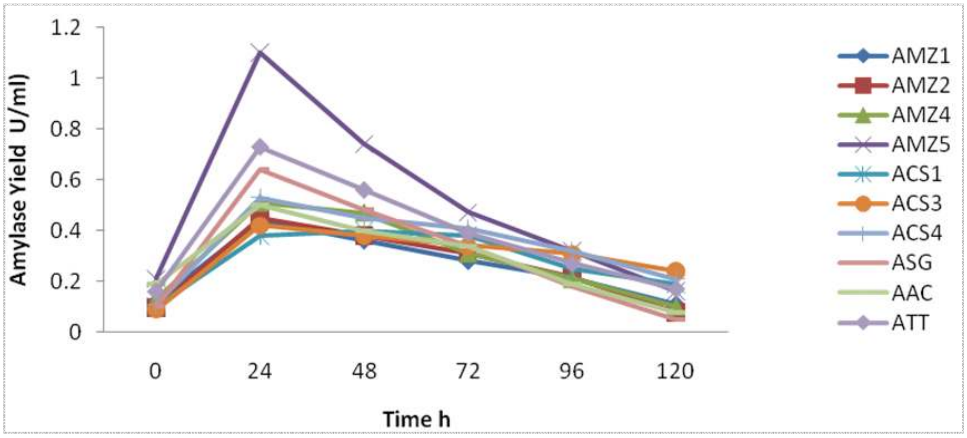

Fig. 1. Amylase yield of amylolytic lactic acid bacterial isolates
A= Amylolytic, MZ= Maize, CS= Cassava, SG= Sorghum, AC= Acha, TT= Tomato

Table 1. Morphological and physiological characteristics of amylolytic lactic acid bacterial isolates

Isolates code	Colonial morphology	Gram reaction	Temperature (℃)					Growth in NaCl (%)			Catalase test	Gas from glucose	Inference
			Ambient	30	37	40	45	3	6	10			
AMz_1	White	+rods	+	+	+	+	+	+	+		-	+	*Lactobacillus plantarum*
AMz_2	White	+rods	+	+	+	+	+	+	+	-	-	+	*Lactobacillus plantarum*
AMz_3	Creamy	+cocci	+	+	+	+	+	+	+	-	-	-	*Lactococcus Lactis*
AMz_4	White	+rods	+	+	+	+	-	+	+	-	-	+	*Lactobacillus plantarum*
AMz_5	White	+rods	+	+	+	+	+	+	+	+	-	+	*Lactobacillus plantarum*
ACs_1	Creamy	+rods	+	+	+	+	+	+	+	-	-	+	*Lactobacillus plantarum*
ACs_2	White	+cocci	+	+	+	+	+	+	+	-	-	-	*Pediococcus acidolactici*
ACs_3	White	+rods	+	+	+	+	-	+	+	-	-	+	*Lactobacillus plantarum*
ACs_4	Creamy	+cocci in chains	+	+	+	+	-	+	+	-	-	+	*Leuconostoc mesenteroides*
ACs_5	White	+rods	+	+	+	+	+	+	+	-	-	-	*Lactobacillus plantarum*
ACs_6	White	+rods	+	+	+	+	+	+	+	-	-	-	*Lactobacillus plantarum*
ASg	Creamy	+rods	+	+	+	+	+	+	+	+/-	-	+	*Lactobaicllus fermentum*
AAc	Creamy	+cocci in chains	+	+	+	+	-	+	+	-	-	+	*Leuconostoc mesenteroides*
ATt	White	+rods	+	+	+	+	+	+	+	-	-	+	*Lactobacillus brevis*

+/- Weak growth, + Positive, - Negative, A= Amylolytic, Mz= Maize, Cs= Cassava, Sg= Sorghum, Ac= Acha, Tt= Tomato

On the basis of the results obtained, a common feature of α-amylase formation could be observed in all cultures of *Lactobacillus spp* (Fig. 1) although, yield of amylase enzyme was observed to varied between the isolates. At peaks, all the ALAB strains exhibited amylase yields between 0.38 U/ml to 1.10 U/ml with maximum amylase concentrations achieved at 24 h of incubation. ALAB strain (AM_Z5) isolated from maize dough exhibited highest amylase yields (1.10 U/ml) followed by isolates (ATt) obtained from tomato fruits, while least yield (0.36 U/ml) was reported for isolates (AC_S1) from cassava flour. The amylase yields obtained in this study are relatively lower to the values of 0.444 (U/ml) to 2.813 (U/ml) lactic acid bacteria isolates from traditional fermented products in Benin at 30°C [26]. In general, the sugar fermentation patterns of the starch hydrolyzers were very similar however, amylase yield behavior of ALAB strain showed variation with natural medium where the LAB strain were isolated.

A detailed study of a typical starch (soluble) hydrolysis profile of the various ALAB isolates with time is presented in Fig. 2. Finding of this study further shows variation in reducing sugar yield with sources of ALAB isolates. Comparatively, maximum reducing sugar concentration (0.55 mg/ml) was observed by hydrolytic action of *L. plantarum* (AM_Z5) isolated from maize dough after 24 hr, followed 0.55 mg/ml by *L. fermentum* (ASg) isolated from sorghum. Hydrolytic action of *L. brevis* isolated from tomato gave the lowest reducing sugar concentration (0.30mg/ml) even after 48 hr of incubation. Trends of amylase activity with fermentation time showed initial progressive increased in reducing sugar concentration thereafter, gradual reduction was observed for majority of the isolates at 48hr of incubation. This affirm the fact that during the early stages of fermentation, ALAB breaks down starch into glucose which then afterward serves as carbon source for the yeast [10]. Although the yields of reducing sugar are not substantially concurrence with observations of other workers elsewhere, strain of *L. plantarum* isolated shows promise in production of amylase. This makes it possible to foresee numerous applications of *L. plantarum* (AM_Z5) as a starter in food processing especially where starch hydrolysis is required to obtain a high-quality product.

Table 2. Starch degrading potentials of lactic acid bacteria isolated from milled cereals, fruits and cassava flour

Lactic acid bacterial isolates	Zones of hydrolysis (mm)
AM_Z1	20.00
AM_Z2	20.00
AM_Z3	8.00
AM_Z4	30.00
AM_Z5	48.00
AC_S1	5.00
AC_S3	23.00
AC_S4	34.00
AC_S5	15.00
AC_S6	10.00
AS_g	40.00
AAC	28.00
ATt	42.00

A= Amylolytic, Mz= Maize, Cs= Cassava, Sg= Sorghum, Ac= Acha, Tt= Tomato

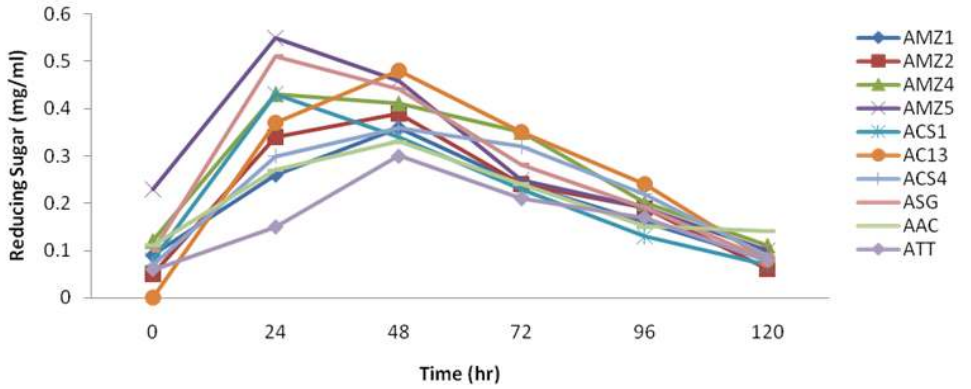

Fig. 2. Reducing sugar concentration (mg/ml) during starch hyrolysis by amylolytic lactic acid bacterial isolates

A= Amylolytic, MZ= Maize, CS= Cassava, SG= Sorghum, AC= Acha, TT= Tomato

4. CONCLUSION

Findings from this study showed that strains of; *L. plantarum, L. Lactis, L. mesenteroides, L. Fermentum, P. acidolactici* and *L. brevis* are predominant amylase producing lactic acid bacteria in wet milled cereals, cassava and fruits. On the basis of their technological potentials, the results suggest that strain of *Lactobacillus plantarum* (AMZ5) isolated from maize flour possesses better starch degradation capability and can be optimised as starters cultures in the manufacture of fermented foods such as yoghurt and bread.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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