OCCURRENCE OF AMYLOLYTIC AND/OR BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA IN OGI AND FUFU

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Abstract
Amylolytic and bacteriocin-producing properties by lactic acid bacteria have been reported to be important functional properties in food fermentation. Lactobacillus strains isolated from ogi and fufu were screened for amylolytic and bacteriocin-producing properties. Antagonistic activity against Listeria monocytogenes was determined by agar well diffusion test. Bacteriocin production was determined using the same method after eliminating the effects of organic acids and hydrogen peroxide. Sensitivity of bacteriocin to pH and temperature was determined. Amylolytic strains were detected by starch hydrolysis test. Optimal conditions for extracellular amylase activity were assayed at different pH (4-7; 0.1mol l^{-1} citrate phosphate buffer) and temperature (40-60°C) using the DNSA method. Lactic acid produced by the amylolytic strains was determined from starch fermentation studies. Antagonistic activity was observed in 21 strains while bacteriocin production was observed in 5 of these strains. Bacteriocins produced inhibited the growth of various indicator organisms with L. plantarum O9 having the widest inhibition spectrum. Bacillus subtilis and Proteus spp. were the most sensitive indicator organisms. pH changes and heat treatment up to 100°C had no effect on the activity of most of the bacteriocins produced. L. fermentum O3 and L. plantarum O14 showed relatively strong starch hydrolysis. Optimal extracellular amylase activities were obtained at pH of 5.0 and temperature of 50°C for L. fermentum O3 and a pH of 5.5 and temperature of 40°C for L. plantarum O14. This study complements reports on the occurrence of amylolytic lactic acid bacteria (ALAB) in starchy substrates and the preservative potentials of some strains.

Keywords: Bacteriocin, Amylolytic, Fermentation, *L. fermentum, L. plantarum*

1. INTRODUCTION

There is general agreement on the beneficial effects of lactic acid bacteria (LAB) in the fermentation processes of starchy food products in sub-Saharan Africa prepared either from cereals or cassava (Sanni, A.I. et al, 2002). The Generally Recognized As Safe (GRAS) status attributed to lactic acid bacteria has helped to attract great attention to them, leading to intensive research and extensive application of LAB strains and their metabolites. Leroy and DeVuyst (2004) reported that lactic acid bacteria are capable of producing antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins or useful enzymes. One of the most important contributions of lactic acid bacteria is the extended shelf life of fermented foods (Oliveira et al, 2008) and ensuring the microbiological safety of various foods by the production of bacteriocins and other antimicrobial substances (Ben Omar et al, 2008). Bacteriocins are small, ribosomally synthesized peptides or proteins which inhibits microorganisms that are usually closely related to producer strains (Oliveira et al, 2008). Increasing consumer demand for faster, healthier and ready to eat food products has relegated the use of synthetic chemical additives to the sidelines, stimulating heightened research interest in finding natural, but effective preservatives.

Starch fermenting capacity of lactic acid bacteria is due to amylase activity. The search for amylolytic lactic acid bacteria (ALAB) has been justified by the high starch content of raw materials. Amylolytic lactic acid bacteria have been reported from different tropical amylaceous fermented foods, prepared mainly from cassava and cereals (Diaz-Ruiz et al, 2003). They include strains of *Lactobacillus plantarum* from retted cassava (Giraud et al, 1991), *Lactobacillus plantarum* strains from Nigerian ogi (Johansson et al., 1995) and *Lactobacillus fermentum* from maize and sourdough in Benin (Agati et al, 1998). The use of pure starter culture of ALAB in fermentation

processes could be of economic interest in the production of lactic acid from the direct fermentation of starch products (Agati et al, 1998). They can couple the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of derived glucose in a single step for the production of lactic acid. Fermented foods such as *fufu, mawe, nham, ogi* and *uji* are considered as potential sources of new strains of lactic acid bacteria having original properties (Ben Omar et al, 2000).

There is no report of lactic acid bacteria strains possessing both amylolytic and bacteriocin producing properties from any environment or food substrate. The isolation of lactic acid bacteria strains with these properties is highly desirable as starter cultures, allowing for fermentation process optimization and microbiological safety of food products. This work reports some lactic acid bacteria strains showing relatively strong amylolytic and/or bacteriocin-producing properties during the fermentation of *ogi* and *fufu*.

2. MATERIAL AND METHODS

2.1 Collection of samples
Ogi and *fufu* samples were obtained in sterile containers from local producers in Ibadan, Oyo State, South –West, Nigeria. They were transported to the laboratory for immediate analysis within 24 hours.

2.2 Isolation
Serial dilution was carried out on respective samples with distilled water. One ml of appropriate serial dilution was pour plated with molten MRS agar and incubated anaerobically at 30˚ C for 48 hours. Distinct colonies were randomly selected and repeatedly streaked until pure cultures were obtained. Pure cultures were maintained on MRS agar slants at 5˚ C.

2.3 Characterization of isolates
All LAB isolates were characterized using conventional biochemical and physiological tests. Gram's reaction and catalase activity were determined as described by Gregersen (1978). Gas production from glucose was determined in modified MRS broth containing inverted durham tubes (De Man et al, 1960). Growth in MRS broth at pH 4.5, adjusted with 1M HCL was assessed. Production of ammonia from

arginine was determined according to the method described by Harrigan and McCance (1966). LAB isolates were tested for the fermentation of sugars as described by De Man et al. (1960).

2.4 Bacteriocin production
The antimicrobial activity of LAB isolates against the indicator *Listeria monocytogenes* was detected by the agar well diffusion method as described by Schillinger and Lucke (1989). Diameter of clear zone of 5mm or more was considered a potential bacteriocin producer.

Cell free supernatants (CFSs) of the potential bacteriocin producers were obtained as described by Schillinger and Lucke (1989). The CFSs were concentrated 10 folds with a rotary evaporator. The inhibition spectrum of each CFS was determined using food spoilage and pathogenic bacteria as indicator organisms.

2.5 Sensitivity of CFS to heat, pH and protease
Culture supernatant of each producer LAB isolate was separately treated by heating to 50˚C and 100˚C for 20 min and at 121˚ for 15 min. They were also adjusted to acidic, neutral and basic pH values of 4, 7 and 10 respectively with 5M HCL or 5M NaOH. The residual bacteriocin activity for the respective temperature and pH ranges was assayed after 4 hr incubation period at room temperature. Sensitivity to protease was carried out by adding 1mg/ml of filter sterilized trypsin to respective culture supernatant (Sanni et al, 1999).

2.6 Extracellular amylase activity
Amylolytic lactic acid bacteria (ALAB) strains were initially detected by starch hydrolysis test as described by Sanni et al. (2002). Extracellular amylase activity was assayed in the supernatant fluid of the centrifuged fermentation broths using the DNSA method as described by Bernfeild (1951). The enzymatic activity was determined at different pH (4-7; $0.1 mol \ l^{-1}$ citrate phosphate buffer) and temperature values (40-60˚C).

2.7 Fermentation studies
Starch fermentation by selected ALAB strains was performed in duplicates without pH control as described by Calderon et al. (2001). Lactic acid produced in respective fermentation broth by the ALAB strains was determined at two

hourly intervals as described by A.O.A.C (1990).

3. RESULTS

3.1 Characterization of isolates
Characterization identified the lactic acid bacteria as *Lactobacillus plantarum, L. fermentum, L. delbrueckii, L. casei, L. brevis* and *L. coryniformis*.

3.2 Bacteriocin production
Six strains produced a diameter of inhibition zone equal or greater than 5mm against *Listeria monocytogenes* as indicator organism. The strains include *Lactobacillus fermentum* O3, *L. delbrueckii* O5, *L. plantarum* O9, *L. brevis* F7, *L. fermentum* F10 and *L. fermentum* F11. CFSs from *L. delbrueckii* O5, *L. plantarum* O9, *L. brevis* F7, *L. fermentum* F10 and *L. fermentum* F11 maintained antimicrobial activity against at least one indicator organism with diameter of inhibition zone ranging between 2 to 5mm. The CFSs of *L. brevis* F7, *L. fermentum* F10 and *L. plantarum* O9 showed broad spectrum of activity. *Bacillus subtilis* and *Proteus spp.* were observed to be the most sensitive indicator organisms (Table 1).

3.3 Effect of heat, pH and Trypsin on the inhibitory activity of bacteriocin produced

There was complete loss of inhibitory activity of CFSs of all *Lactobacillus* strains on the addition of trypsin. All the CFSs were stable for 20 minutes at 50°C. *L. brevis* F7 and *L. plantarum* O9 maintained inhibitory activity after heat treatment for 20 minutes at 100°C while heat treatment at 121°C for 15 minutes led to complete loss of inhibitory activity for all CFSs (Table 2). CFSs from *L. plantarum* O9, *L. brevis* F7 and *L. fermentum* F10 were stable to pH changes (Table 3).
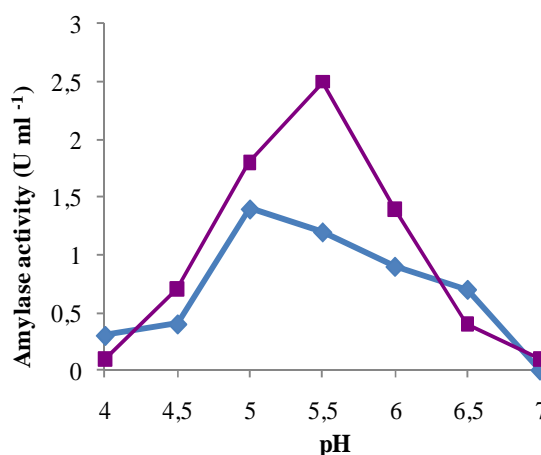

Figure 1: Amylase activity at different pH levels.
Symbols: *L. fermentum* O3 (◆),
L. plantarum O14 (■).

Table 1: Zones of inhibition of bacteriocin produced by *Lactobacillus* strains against indicator organisms.

Indicator Organisms	L. fermentum O3	L. delbrueckii O5	L. plantarum O9	L. brevis F7	L. fermentum F10	L. fermentum F11
Bacillus subtilis	-	+(5mm)	+(3mm)	+(5mm)	-	+(2mm)
Staphylococcus aureus	-	-	+(4mm)	-	-	-
Proteus spp.	-	+(3mm)	+(5mm)	+(5mm)	+(3mm)	-
Klebsiella spp.	-	+(3mm)	-	-	+(5mm)	-
Pseudomonas fluorescence	-	-	-	-	-	-
Escherichia coli	-	-		+(3mm)	+(4mm)	-
Salmonella spp.	-	+(4mm)	+(3mm)	-	+(4mm)	-
Pseudomonas aeruginosa	-	-	+(2mm)	-	+(2mm)	-
Haemophilus influenza	-	-	+(2mm)	+(4mm)	+(2mm)	-
Listeria spp.	-	-	+(2mm)	+(3mm)	-	-

+ = sensitive; - =resistance; 5-9mm= strong inhibition; 0.5-4mm= weak inhibition

Table 2: Effect of heat on the activity of bacteriocin produced by *Lactobacillus* strains against indicator organisms.

Indicator organism	Temperature (˚C)	Producer strains *L. delbrueckii* O5	*L. plantarum* O9	*L. brevis* F7	*L. fermentum* F10	*L. fermentum* F11
Proteus spp.	50	-	4mm	5mm	-	-
	100	-	3mm	4mm	-	-
	121	-	-	-	-	-
Escherichia coli	50	-	-	-	8mm	-
	100	-	-	-	-	-
	121	-	-	-	-	-
Bacillus subtilis	50	5mm	-	-	-	4mm
	100	-	-	-	-	-
	121	-	-	-	-	-

- =resistance; 5-9mm= strong inhibition; 0.5-4mm= weak inhibition

Table 3: Effect of pH on the activity of bacteriocin produced by *Lactobacillus* strains against indicator organisms.

Indicator organism	pH	Producer strains *L. delbrueckii* O5	*L. plantarum* O9	*L. brevis* F7	*L. fermentum* F10	*L. fermentum* F11
Proteus spp.	4	-	6mm	3mm	-	-
	7	-	4mm	3mm	-	-
	10	-	4mm	2mm	-	-
Escherichia coli	4	-	-	-	2mm	-
	7	-	-	-	3mm	-
	10	-	-	-	2mm	-
Bacillus subtilis	4	5mm	-	-	-	6mm
	7	5mm	-	-	-	2mm
	10	-	-	-	-	-

-=resistance; 5-9mm= strong inhibition; 0.5-4mm= weak inhibition

3.4 Effect of pH and temperature on amylase activity

As shown in figure 1, the optimum pH of *L. fermentum* O3 amylase for amylolytic activity was at pH 5.0 but at 7.0 the enzyme was almost completely inactivated. *L. plantarum* O14 amylase had an optimum pH of 5.5 for amylolytic activity and activity was observed from pH 4.5 to 6.5. The optimum temperature of *L. fermentum* O3 amylase under the condition of pH 6.5 was 50˚C and activity decreased rapidly. *L. plantarum* O14 amylase was stable from 30˚C to 55˚C with activity almost completely lost at 60˚C. The optimum temperature of the enzyme for amylolytic activity was 40˚C (Figure 2).

3.5 Starch fermentation

L. fermentum O3 and *L. plantarum* O14 showed stronger starch hydrolysis. Both strains grew over 24 h in the fermentation medium at a specific growth rate of 0.1204 and 0.1227 h^{-1}

for *L. fermentum* O3 and L. *plantarum* O14 respectively. Lactic acid was a major product of metabolism of liquefied starch by both strains (Figure 3).
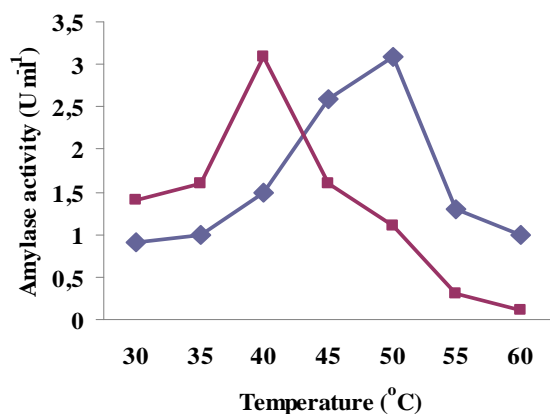

Figure 2: Amylase activity at different temperatures. Symbols: *L. fermentum* O3 (◆), *L. plantarum* O14 (■).
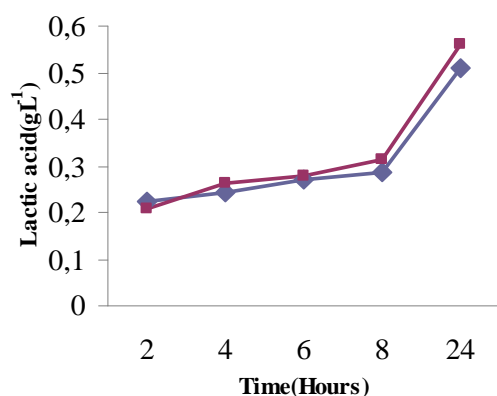

Figure 3: Lactic acid produced during starch fermentation. Symbols: *L. fermentum* O3 (◆), *L. plantarum* O14 (■).

4. DISCUSSION

In the last few decades, tremendous interest has swelled in the potential use of bacteriocins from lactic acid bacteria (LAB). The bacteriocins produced by this group of bacteria are considered potent bio-preservative agents and their application in food is currently the subject of extensive research (Mojgani et al., 2009). The antagonistic activity of 29 LAB isolates from *ogi* and *fufu* was investigated. A total of 21 strains exhibited antagonistic effects against

Listeria monocytogenes. Antagonistic activity by LAB strains have been reported in various studies (Oliveira et al., 2008; Todorov, 2008). They exert a strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens (Sanni et al., 1999). Lactic acid bacteria have the ability to produce a variety of antimicrobial substances as a natural competitive means to overcome other microorganisms sharing the same niche (Oliveira et al., 2008). Six strains exhibited antagonistic effects on solid agar medium under conditions that reduced the effect of organic acids and hydrogen peroxide. They include *L. fermentum* O3, *L. delbrueckii* O5, *L. plantarum* O9, *L. brevis* F7, *L. fermentum* F10 and *L. fermentum* F11 with varying spectra and zones of inhibition. The strain showing the largest spectra of inhibition is *L. plantarum* O9 which inhibited seven of the indicator organisms. Similar results have been obtained by Ben Omar et al. (2006) in which activity of plantaricins produced by *L. plantarum* strains isolated from *ben saalga* was described in several cases against Gram-negative bacteria. Bacteriocin for *L. plantarum* O9, *L. brevis* F7 and *L. fermentum* F10 showed significant activity over the acidic, neutral and alkaline pH ranges while heat resistance was observed for two bacteriocins. Like most LAB bacteriocins reported to date (Lade et al., 2006) the activity of the bacteriocin in the study appeared pH dependent. The bacteriocins from *L. delbrueckii* O5, *L. plantarum* O9, *L. brevis* F7 and *L. fermentum* F11 exhibited highest activities in acidic pH range, while some lost their activities in alkaline pH range. The broad spectra of bactericidal effect, pH stability and heat resistance are remarkable properties of bacteriocins, thus an indication of potential use as therapeutic agents for control of food borne illness (Ogunbanwo et al., 2004) and the biopreservation of foods (Oliveira et al., 2008). Amylase activity in lactic acid bacteria has been well reported (Agati et al., 1998; Giraud et al., 1991; Sanni et al., 2002). Frequently, ALAB isolated from foods belong to the genus *Lactobacillus* (Diaz-Ruiz et al., 2003). Amylase activity was exhibited by seven *Lactobacillus* strains in this study. They are

either homofermenters or heterofermenters. They all produced acid from glucose, sucrose and maltose. Strains with relatively stronger amylase activity were *L. fermentum* O3 and *L. plantarum* O14. Both strains were able to ferment raffinose. Raffinose is a α-galactoside contained in cereals and legumes that is responsible for digestive disorders (Diaz-Ruiz et al., 2003). The first report of the presence of amylolytic *L. fermentum* strains was in *mawe* and *ogi* from Benin (Agati et al., 1998). Natural amylolytic strain of *L. plantarum* was first isolated from cassava roots (Giraud et al., 1991). It is interesting to note that among 17 ALAB randomly isolated (without selection pressure) from Nigerian *ogi* all belonged to four clusters of *L. plantarum* species, with the exception of a strain of *Leuconostoc mesenteroides* (Johansson et al., 1995).

The acidotolerant and extracellular characteristics of the amylases produced were revealed in this study. Both amylases exhibited optimum activity between pH 5.0 and 6.0. The optimum pH obtained with *L. fermentum* O3 amylase was 5.0 while for *L. plantarum* O14 amylase it was 5.5. The optimum temperature of amylase activity for *L. fermentum* O3 and *L. plantarum* O14 was 50°C and 40°C respectively. *L. amylophilus* (Pompeyo et al., 1993), and *L. plantarum* A6 (Giraud et al., 1991), had amylase optimal pH and temperature between the range of 5.0–6.0 and 40–50°C, respectively. Both amylases maintained activity over a wide pH range. *L. fermentum* O3 amylase showed more thermotolerant characteristic compared to *L. plantarum* O14 amylase by remaining stable at 60°C. Similar thermotolerant amylase from lactic acid bacteria has been reported in *L. amylovorus* (Pompeyo et al., 1993).

Lactic acid production, causing acidification of the medium is as a result of the conversion by amylolytic lactobacillus strains of low-molecular-weight sugars from the hydrolysis of starch granules (Giraud et al., 1994). Lactic acid has various applications in food, pharmaceutical, leather, and textile industries (Oh et al., 2005). Lactic acid can be used in food technology as preservative or taste enhancing additive (Yumoto and Ikeda, 1995). Continuous effort to isolate and characterize non-dairy lactic acid bacteria, such as amylolytic lactic acid bacteria (ALAB), would bring increasing opportunities for selecting and adapting specific starters for non-dairy food applications (Sanni et al., 1993). The results make it possible to envisage the numerous application of *L. fermentum* O3 and *L. plantarum* O14 as starters in some traditional food fermentation processes where the quantity of lactic acid produced is sometimes too small to obtain a high-quality product. In addition, the capacity to convert starch into lactic acid might be of considerable interest for industrial production of the acid directly from raw starch.

5. CONCLUSION

This study has been able to further complement studies on the occurrence of amylolytic lactic acid bacteria (ALAB) in starchy substrates and the preservative potentials of some lactic acid bacteria (LAB) strains due to their bacteriocin-producing properties. These are highly desirable traits in LAB starter cultures for food and industrial application, also for immense economic and health benefits. However, none of the LAB strains isolated was found to possess both properties. An option worth exploring is the applicability of employing co-culture of LAB strains possessing either property as starter for food fermentation processes with products characterized by hygienic safety, high energy density, attractive sensory properties and storage stability.

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