

Production and Characterization of Antimicrobial agents by Lactic Acid Bacteria Isolated from Fermented Foods

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Abstract

Twenty-six lactic acid bacteria isolated from fermented foods and beverages consumed in Southwestern Nigeria were screened for antimicrobial agents production. Twenty-one isolates produced bacteriocin while 5 did not; hence the non-bacteriocin producing strains were used for antimicrobial assay. Hydrogen peroxide produced by *Leuconostoc mesenteroides* Ly had the highest inhibitory activities and a broad spectrum of inhibition, followed by lactic acid produced by *Lactobacillus plantarum* Ws, the least inhibition was observed in diacetyl produced by *Lactococcus lactis* Wa. The cultural conditions' alteration showed that larger amounts of antimicrobial agents were synthesized only when the medium was supplemented with glucose (1.0%), Tween 80 (0.5%), yeast extract (2-3.0%) and NaCl (1-2.0%), alteration of other components had no effect. The use of constituted medium at 300C incubation temperature, initial pH 5.5 and for 48 to 60 hours fostered the best production of antimicrobials by the test isolates.

INTRODUCTION

Lactic acid bacteria (LAB) are the most prominent non-pathogenic bacteria that play a vital role in our everyday life, from fermentation, preservation, and production of wholesome foods, and vitamins to prevention of certain diseases and cancer due to their antimicrobial action. Lactic acid fermentation is generally inexpensive often requiring little or no heat in the process, making them fuel-efficient as well (Keith, 1991). These microorganisms are one of the prominent bacteria inhabiting the gastrointestinal tract, and the importance of these non-pathogenic bacteria has recently been more noticed (Englund, 1992). A lot of *Lactobacilli* have been noted to have nutritional benefits, improved lactose utilization, have anti cholesterol, anti carcinogenic activities, and protection against other diseases (Englund, 1992; Reddy et al., 1984; Abdel-Bar et al., 1987).

Apart from the above-mentioned medicinal importance of *Lactobacilli*, it also helps in the control of intestinal pathogens. For instance *L. acidophilus* has been shown to be effective in the treatment of different types of diarrhea in human. It has been reported to have effect on diarrhea caused by *Salmonella* or *Shigella* (Zychowicz et al., 1974). While *L. casei* was reported to have curative effect on infections caused by *Salmonella typhimurium* and *E. coli* (Perdigon et al., 1991).

However, studies relating to the antibacterial properties of these organisms have been limited and not fully exploited for use. Two of the most important aspects in the study of antimicrobials are their production and characterization. Therefore, this paper reports the production and characteristics of antimicrobials produced by lactic acid bacteria isolated from fermented foods.

MATERIALS AND METHODS

Sample collection. The whole cereal grains yellow and white maize (*Zea mays*), red and white sorghum (*Sorghum bicolor*), millet (*Eleusine corocana*) seed, Cassava (*Manihot esculenta* Crantz) tubers, 'wara', fresh milk, and palmwine used in this study were obtained from retail market in South-Western Nigeria.

Traditional fermentation of cassava. Cassava tubers (1 kg) is peeled, cut into pieces and washed several times in water followed by soaking in water (submerged fermentation) for period of 3 days at ambient temperature (26oC \pm 1oC). The softened pulpy mass of the fermented cassava samples is disintegrated and passed through a clean coarse sieve to remove lumps and fibers after which the mass was allowed to sediment (Oyewole and Odunfa, 1990).

Traditional preparation of ogi. One kilogram of cereal grains (*Z. mays*, *Sorghum bicolor*, and *Eleusine corocana*) are

cleaned and steeped in water separately for 2 days in earthenware pot (or any suitable container). The water is decanted and the grains wet-milled before sieving with muslin cloth or fine wire-mesh. The pomace is discarded and the starch suspension is allowed to sediment during which fermentation is carried out for 2-3 days by the natural flora of the grains (Odunfa and Adeyele, 1985).

Bacterial strains and cultures. Lactic acid bacterial strains were isolated from cassava retting and traditional prepared *ogi*, 'wara', fresh milk and palmwine. For all samples, 10 g or 10 ml of the appropriate samples were added to 90 ml of sterile diluent's containing 0.1% peptone water and homogenized for 30s, from appropriate 10-fold dilutions; isolation of bacteria was carried out on MRS agar and incubated anaerobically at 30°C for 48 h. The cultures were purified by repeated streaking. Sugar fermentation patterns of the isolates were determined by API 50 CHL system (BioMérieux, France) at 30°C and analysed by APILAB PLUS software version 3.2.2. The production of gas from glucose was tested with MRS broth trapped in Durham tubes. Arginine hydrolase was measured with Nessler's reagent, and the patterns were compared with those of reference lactic acid bacteria described by Elliott et al. (1991).

Culture preservation. The isolated *Lactobacilli* were sub cultured onto maintenance medium consisting of MRS broth with 12 % (v/v) glycerol and incubated at 30°C until growth becomes visible. The stock cultures were stored at 4°C for subsequent use for a period of 2 to 4 weeks before sub culturing into fresh maintenance medium. When the preservation of the isolates was for more than one month, the broth cultures were centrifuged separately for 3 minutes at 6500 rpm (Beckman). The pellet was washed with 2 ml of sterile distilled water and the cells harvested by centrifugation. The latter was mixed with Hogness-Freezing Medium and kept frozen at -20°C.

Escherichia coli were grown in Luria-Bertani (Difco) medium at 37°C. The selective concentration of ampicillin for growing *E. coli* was 100 µg ml⁻¹. All other strains were grown in brain heart infusion (Difco) medium at 30 or 37°C. All cultures were maintained as frozen stocks held at -70 °C in appropriate broth containing 20% glycerol (w/v). Throughout the experiments, strains were sub-cultured every 2 weeks on agar plates and kept at 4°C. Before use in experiments, cultures were propagated twice in broth overnight.

Production of crude bacteriocin from isolates: *Lactobacillus* species were propagated in 1000 ml MRS broth (pH 7.0, glucose, 0.25% w/v, peptone, 0.5% w/v) for 72 h at 30°C anaerobically (Oxoid Gas Generating Kit) in triplicate. For extraction of bacteriocin, a cell-free solution was obtained by centrifuging (10,000 rpm for 20 min. at 40°C with Beckman L5050B), the culture was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatant through a 0.2 µm pore-size cellulose acetate filter. The supernatant was dialysed for 24 h at 40°C (Schillinger and Lucke, 1989). Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (C-100 bovine liver, Sigma) (Daba et al., 1991). The isolates that produced bacteriocin were not used for further investigations.

Production of other antimicrobial agents by the test isolates.

Quantitative estimation of lactic acid. The production of lactic acid was determined by transferring 25ml of broth cultures of test organisms into 100 ml flasks. This was titrated with 0.25M NaOH and 1 ml of phenolphthalein indicator (0.5 % in 5 % alcohol). The titratable acidity was calculated as lactic acid % w/v (Fortina et al., 1973). Each millilitre of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acidity was then calculated as stated in A.O.A.C (1980).

Quantitative estimation of diacetyl. Diacetyl production was determined by transferring 25ml of broth cultures of test organisms into 100 ml flasks. Hydroxylamine solution (7.5 ml) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1 M HCl to a greenish yellow end point using bromophenol blue as indicator (Sanni et al., 1995). The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated using the A.O.A.C. (1980).

Quantitative estimation of hydrogen peroxide. Hydrogen peroxide production was determined by measuring 25 ml of broth cultures of the test organisms into a 100 ml flask. To this was added 25 ml of dilute H₂SO₄. This was then titrated with 0.1M potassium permanganate (KMnO₄). Each millilitre of 0.1 N KMnO₄ is equivalent to 1.701 mg of H₂O₂. A de-colorization of the sample was regarded as the end point. The volume of H₂O₂ produced was then calculated (A.O.A.C; 1980).

Screening antimicrobial agents for inhibitory activity. Antimicrobial activity was screened by the agar-well diffusion method (Tagg and McGiven 1971). MRS broth (5 ml) inoculated with overnight culture (1% v/v) of an indicator strain was overlaid on an agar plate. After cooling, wells (3 mm diameter) were punched in the agar plate and filled with 100 µl of test samples (i.e. highest producing organism of the parameters above). After incubation overnight, the antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Zones of inhibition ≥ 3 mm were regarded as negative.

Influence of medium component on production of antimicrobials. The effect of medium ingredients on antimicrobials production was evaluated using composed media. The supplements studied were tryptone (0.0 – 3.0%), yeast extract (0.0 – 3.0%), beef extract (0.0 – 3.0%), NaCl (0.0 – 3.0%), glucose (0.0 – 3.0%), tween 80 (0.0 – 1.5%), triammonium citrate (0.0 – 0.3%) Sodium acetate (0.0 – 0.3%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0 – 0.3%), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.0 – 0.3%), and K_2HPO_4 (0.0 – 0.3%) the quantity of antimicrobials produced was monitored.

Influence of growth conditions on production of antimicrobials. The effect of incubation temperature and time on the antimicrobials production was carried out. Three portions of composed media were inoculated (1% v/v) with an overnight culture of test isolates; incubated at 25, 30, 37 45 and 55°C for 48h and the quantity of antimicrobials produced was estimated.

Determine the effect of initial pH on production of antimicrobials. 100ml of composed media were adjusted to initial pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, using 5 mMol l⁻¹ hydrochloric acid or 5mMol l⁻¹ NaOH. Each medium was inoculated (1% v/v) with an overnight culture of bacteriocin producing organism and incubated at 30°C for 48hr; the quantity of antimicrobials produced was estimated. (Yildirim and Johnson, 1998).

Effect of incubation period. This was studied in the same manner as described for pH. Active cultures of producer organism (1% v/v) were inoculated into 100ml aliquots of sterile composed media in Erlenmeyer conical flasks. Inoculated flasks were incubated at 37°C for periods of 12, 24, 36, 48, 60 and 72 h. Individual flasks were kept for each incubation period. At the end of each incubation period, the quantity of antimicrobials produced was estimated (Balasubramanyam and Varadaraj, 1998).

RESULTS AND DISCUSSION

Isolates and antimicrobial agents production. Twenty-six species of Lactic acid bacteria (LAB) were isolated from various substrates. Out of the 26 isolates, 21 produced bacteriocin, hence were not used for further investigation. The 5 non bacteriocin producing isolates were *Lactobacillus plantarum* Ws, *Pediococcus acidilactici* Ws, *Leuconostoc mesenteroides* Ly, *Lactococcus lactis* Wa, *Lactobacillus dextranicum* Cv. *Lactobacillus plantarum* Ws and *Pediococcus acidilactici* Ws were the highest lactic acid producers with values of 11.6 and 11.5 gram per litre respectively, while *Leuconostoc mesenteroides* Ly had the least value of 4.2 gram per litre (Table I). All the isolates produced H_2O_2 , however, *Lactobacillus plantarum* Ws produced the least (12.0g l⁻¹) and *Leuconostoc mesenteroides* Ly produced the highest H_2O_2 (28.0 g l⁻¹) (Table I).

Diacetyl was produced by all the isolates but the rate of production varied, *Lactococcus lactis* Wa had the highest value of 18.5g l⁻¹ while the least diacetyl producer was *Lactobacillus dextranicum* Cv with the values of 3.0 (Table I).

Figure 1

Table I
Antimicrobials production by the isolates

Isolates	Code	Yield of Antimicrobials (g l ⁻¹)		
		Lactic acid	Hydrogen peroxide	Diacetyl
<i>Lactobacillus plantarum</i>	Ws	11.6	12.0	10.4
<i>Pediococcus acidilactici</i>	Ws	11.5	18.7	14.6
<i>Leuconostoc mesenteroides</i>	Ly	4.2	28.0	4.1
<i>Lactococcus lactis</i>	Wa	8.7	15.0	18.5
<i>Lactobacillus dextranicum</i>	Cv	11.0	20.3	3.0

Antagonistic activities of the antimicrobial agents. Hydrogen peroxide produced by *Leuconostoc mesenteroides* Ly had the highest inhibitory activities 16mm and a broad spectrum of inhibition; it inhibited 29 out of 33 indicator organisms tested. This was followed by lactic acid produced by *Lactobacillus plantarum* Ws with inhibitory activity of 12mm and 26 out of 33 indicator organisms tested were inhibited. The least inhibition was observed from diacetyl

produced by *Lactococcus lactis* Wa, with the highest inhibitory activity of 7mm and the ability to antagonise 15 out of 33 indicator organisms tested (Table II). It was however interesting to note that none of the antimicrobial produced by the isolates have inhibitory activity against the organism producing it. However, the mode of production of the anti microbial is under investigation.

Figure 2

Table II: Inhibition of various organisms by antimicrobials produced by isolates.

Indicator organisms	Strain No	Origin	Lactic acid (mm)	H ₂ O ₂ (mm)	Diacetyl (mm)
<i>Bacillus cereus</i>	ATCC3634	Reference strain	+10	+13	+7
<i>Bacillus stearothermophilus</i>	NCIB8222	---	+8	+16	+6
<i>Bacillus subtilis</i>	ATCC6633	---	+10	+13	+6
<i>Micrococcus luteus</i>	NCIB186	---	+12	+12	-
<i>Staphylococcus aureus</i>	ATCC14458	---	+8	+15	-
<i>Staphylococcus aureus</i>	NCTC4671	---	+8	+14	+4
<i>Staphylococcus epidermidis</i>	NCTC5413	---	+10	+10	-
<i>Staphylococcus faecalis</i>	ATCC19433	---	+6	+13	+6
<i>Staphylococcus pyogenes</i>	ATCC19615	---	+5	+10	+5
<i>Listeria dentrificans</i>	ATCC14870	---	+7	+10	+4
<i>Listeria monocytogenes</i>	S87CHRL	---	+7	+9	-
<i>Candida albicans</i>	ATCC10231	---	-	-	-
<i>Escherichia coli</i>	NCTC10418	---	+8	+8	-
<i>Escherichia coli</i>	K12	---	+7	+12	-
<i>Enterococcus faecalis</i>	EFI	---	+10	+12	-
<i>Aeromonas pobvia</i>	API5534	---	-	-	-
<i>Vibrio cholera</i>	AP23622	---	-	+8	-
<i>Shigella flexneri</i>	AP23498	---	+7	+5	+5
<i>Shigella dysentery</i>	AP2433	---	+6	+5	+4
<i>Salmonella typhimurium</i>	ATCC13311	---	+7	+6	+4
<i>Salmonella kentucky</i>	AT1	Reference strain	+8	+9	+6
<i>Klebsiella sp</i>	UCH15	Sputum	-	-	-
<i>Clostridium sporogenes</i>	NCIB532	Reference strain	+12	+8	-
<i>Serratia marcescens</i>	U15	Soil	+8	+4	-
<i>Helicobacter pylori</i>	NCTC11637	Reference strain	+10	+10	-
<i>Streptococcus thermophilus</i>	TW4	Iru	+5	+6	+5
<i>Lactobacillus acidophilus</i>	U1	Ugha	-	+13	+6
<i>Lactobacillus plantarum</i>	Ws	White sorghum	-	+14	-
<i>Pedococcus acidilactici</i>	Ws	White sorghum	+10	+9	-
<i>Leuconostoc mesenteroides</i>	Ly	Yoghurt	-	-	-
<i>Lactococcus lactis</i>	Wa	Cassava	+6	+11	-
<i>Lactobacillus delbrueckii</i>	Cv	Footoo	+10	+16	+6
<i>Lactobacillus reuteri</i>	PM3	Palm wine	+8	+12	+4

Key: No inhibition = - or <3mm, inhibition = + or > 3mm

Davidson et al., (1983) stated that H₂O₂ production is common in microorganism that do not produce catalase but possess flavoprotein oxidases that react with dioxide. Hydrogen peroxide could in some cases be a precursor for the production of other potent antimicrobial species such as super oxide (O₂-) and hydroxyl (OH-) radicals (Condon, 1987, Thomas and Pera, 1983).

Cultural conditions. The results showed the same trend with respect to the 3 antimicrobial agents considered, the antimicrobials were produced when nutrients were available for metabolic activity. Larger amounts of the antimicrobials were synthesized only when the medium was supplemented

with glucose (1.0%), Tween 80 (0.5%), yeast extract (2-3.0%) and NaCl (1-2.0%), while addition of tri-ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate and potassium phosphate had no effect on the antimicrobials production (Table III). Thus variation in the concentration of constituents / supplementation of cultivation media might have an influence on the amount of antimicrobials produced by microorganisms. Similar observations was previously by Daba et al; (1993) and Biswas et al. (1991) in the production of mensenterocin and pediocin ACH by *Pediococcus acidilactici* H cultivated in TGE broth and MRS broth with several modifications of the cultivation media.

Figure 3

Table III: Effect of nutrient components on antimicrobial production by the isolates.

Medium constituents	%	Lactic acid (g/L)	O ₂ (g/L)	Diacetyl (g/L)
MRS (Normal)	-	11.6	28.8	18.5
MRS + Tyrosine	0.0	8.8	11.8	18.0
	1.0	11.5	28.1	18.5
	2.0	6.7	18.8	9.9
	3.0	6.7	19.6	9.8
MRS + Yeast extract	0.0	10.5	12.9	14.4
	1.0	11.9	25.0	18.2
	2.0	20.0	29.7	26.4
	3.0	19.9	30.2	22.5
MRS + Beef Extract	0.0	9.9	12.7	16.9
	1.0	11.9	29.2	18.5
	2.0	10.6	26.2	18.0
	3.0	10.2	26.2	18.0
MRS + NaCl	0.0	11.4	28.4	17.2
	1.0	11.1	32.9	18.1
	2.0	11.1	33.0	17.9
	3.0	10.7	20.0	17.0
MRS + glucose	0.0	12.0	21.7	17.0
	1.0	20.4	32.2	18.6
	2.0	11.1	26.0	17.0
	3.0	10.0	22.1	17.6
MRS + Tween 80	0.0	10.1	19.9	16.9
	0.5	11.5	33.0	18.5
	1.0	9.7	24.0	17.8
	1.5	9.1	19.1	17.0
MRS + Tri-ammonium citrate	0.0	11.6	11.6	11.6
	0.1	11.5	11.6	11.6
	0.2	11.6	11.6	11.6
	0.3	11.5	11.6	11.6
MRS + Sodium acetate	0.0	11.6	11.6	11.6
	0.1	11.6	11.6	11.6
	0.2	11.6	11.6	11.6
	0.3	11.6	11.6	11.6
MRS + MgSO ₄ . 7H ₂ O	0.0	11.6	11.6	11.6
	0.1	11.5	11.6	11.6
	0.2	11.6	11.6	11.6
	0.3	11.6	11.6	11.6
MRS + K ₂ HPO ₄	0.0	11.6	11.6	11.6
	0.1	11.6	11.6	11.6
	0.2	11.6	11.5	11.6
	0.3	11.6	11.5	11.6

The question of production cost is an important issue to be taken into account when large-scale production of antimicrobials for use as a food preservative is considered. Our results showed that antimicrobials could be produced in a relatively inexpensive and readily available medium.

Effects of other parameters. As shown in figures 1-3, it was concluded that the use of constituted medium at 30oC incubation temperature, initial pH 5.5 and for 48 to 60 hours favored the best production of antimicrobials by isolates.

Figure 4

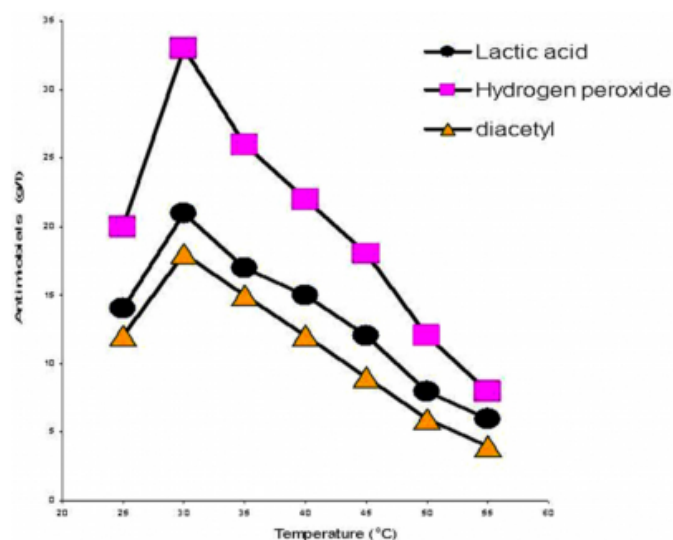


Fig.1. Effect of incubation temperature on antimicrobials production

Figure 5

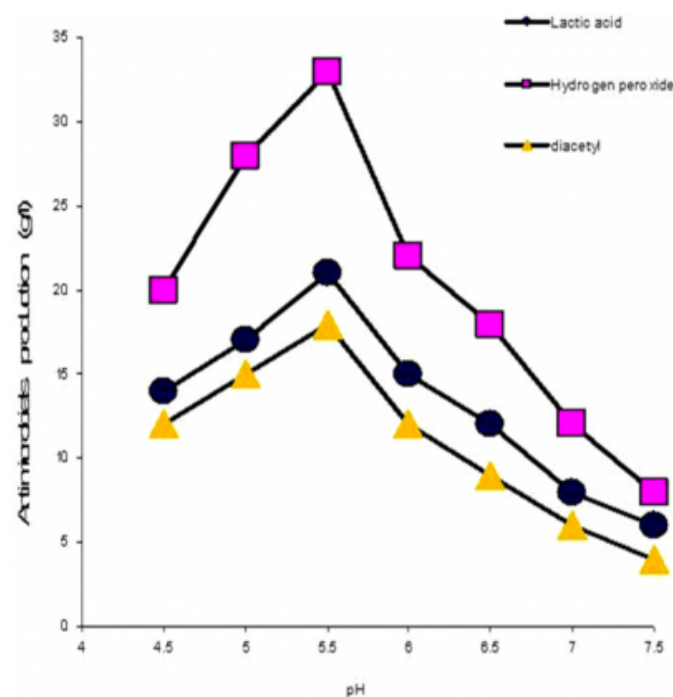


Fig2 Effect of initial pH on antimicrobials production by the isolates

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