Advanced Journal of Microbiology Research Vol. 2003
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1

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Accepted 9 July 2003

***Lactobacillus plantarum* F1 and *L. brevis* OG1 isolated from Nigerian fermented food products, produced bacteriocins that had broad spectrum of inhibition against both pathogenic, food spoilage organisms and various lactic acid bacteria. The test organisms exhibited activities of 6400 and 3200 AU/ml respectively against *Escherichia coli* NCTC10418 and *Enterococcus faecalis* EF1, but did not inhibit *Candida albicans* ATCC10231 and *Klebsiella* sp. UCH15. Comparison of the antimicrobial spectra and characterization of the two bacteriocins were not identical. Bacteriocin produced by *L. brevis* OG1 was the most heat stable at 121^{O}C for 60 min, while that of *L. plantarum* F1 was stable at 121^{O}C for 10 min. The bacteriocins produced by the test isolates maintained full stability after storage for 60 days at – 20^{0} C; partial stability after storage for 120 days at 4^{0}C; while activity was not detected after storage for 80 to 120 days at 37^{0}C. Bacteriocin produced by *L. brevis* OG1 was stable at pH range of 2.0 to 8.0 while, that of *L. plantarum* F1 was found to be stable at pH 2.0 to 6.0. Their active principle was proteinaceous in nature since the bacteriocins were inactivated by proteolytic enzymes, but not by other non–proteolytic enzymes. mitomycin C and uv light did not affect the activity of the bacteriocins, while chloroform extraction completely destroyed their activity. Exposure to surfactant resulted in an increase in the bacteriocin titre, except Nonidet P-40, which led to total loss of bacteriocin activity. The bacteriocins were able to pass through cellulose membranes with 100,000 KDa and 1,000,000 KDa but could not pass through one with a 10,000 KDa and 1,000 KDa molecular weight cut off. The paper concluded that the ability of bacteriocins produced by the test isolates in inhibiting a wide-range of bacteria, is of potential interest for food safety and may have future applications as food preservative.**

Key words: Bacteriocins, lactic acid bacteria, indicator organisms, fermented foods, antagonistic activity.

INTRODUCTION

Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliand, 1990). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990).

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Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strain (De Vugst and Vandamme, 1994) many bacteriocins are active against food-borne pathogens especially against *Listeria monocytogenes* (Vignolo et al., 1996; De Martins and Franco, 1998; Bredholt et al., 1999). *Leuconostoc mesenteroides* L124 and *L. curvatus* L442 isolated from dry fermented sausages, produce bacteriocin antagonistic towards closely related species and pathogens (Mataragas et al, 2002). An isolate of *L. mesenterioides* sub sp. cremoris

was also found to produce a bacteriocin- like inhibitory compound against the lactic acid bacteria of wines (Yurdugul and Bozoglu, 2002).

Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which the important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, and plantaricins (Nettles and Barefoot, 1993) Of these, bacteriocin and nisin produced by *Lactococcus lactis* ssp. lactis, has been the most extensively characterized (Buchman et al., 1988; Liu and Hansen, 1990). At present, nisin is the only bacteriocin commercially available and marketed (Balasubramanyam and Varadara, 1998). It has been reported that nisin is more active against Gram-positive bacteria, particularly the spore-formers (Delves-Broughton, 1990). Other bacteriocins of *Lactobacilli* have been reported to be effective against closely related species of mesophilic *Lactobacillus* and therefore considered as potential natural food preservatives (Daeschel 1993; De Vugst and Vandamme, 1994).

However, studies relating to the antibacterial properties of these organisms have been limited and not fully exploited for use (Reddy et al., 1984; Abdel-Bar et al., 1987). Three of the most important aspects in the study of bacteriocins are their production, characterization and purification. Therefore, this paper reports the bacteriocin of two lactic acid bacteria isolates from Nigeria fermented foods.

MATERIALS AND METHODS

Sample collection

Cassava (*Manihot esculenta* Crantz) tubers and maize (*Zea mays*) grains used in this study were obtained from retail market in South-Western Nigeria.

Traditional Fermentation of Cassava

Cassava tubers (1 kg) was 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 (26 C 1 C) . The softened pulpy mass of the fermented cassava samples was 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

The cereal grain (*Z. mays*) were cleaned and steeped in water 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 (Table 1) . For all samples, 10 g were added to 90 ml of sterile diluent's containing 0.1% peptone water and homogenized for 30 s. From appropriate 10-fold dilutions; isolation of bacteria was carried out on MRS agar and incubated anaerobically at 30^0C for 48 h. The cultures were purified by repeated streaking. Strains were characterized using the AP1 50CH strips and AP1 50 CHL medium (AP1 Systems, Biomerieux Sa, France). The food spoilage and pathogenic bacteria used as indicator organisms were obtained from the culture collection of Medical Microbiology, Laboratory, University College Hospital, Ibadan, Nigeria.

Table 1. Lactic acid bacteria isolated from fermented ogi and cassava retting.

Name of product	Associated lactic acid bacteria
Ogi	*L. reuteri, L. leichmani, L. plantarum, L. casei, L. fermentum, L. brevis, L. alimentarius, L. buchneri* and *L. jensenii.*
Cassava retting	*L. plantarum, L. brevis, L. fermentum, L. delbrueckii, L. jenseni, L. casei* and *L. mesenteroides.*

Production of crude bacteriocin samples

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 30^0C 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 4^0C with Beckman L5050B) the culture and 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 m pore- size cellulose acetate filter. The supernatant was dialysed for 24 h at 4^0C (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).

Purification of bacteriocin samples

Ammonium Sulphate Precipitation: The crude bacteriocin samples produced were treated with solid ammonium sulphate (Mallinckrodth Chemical, Inc., Paris, KY, USA) to 0, 30, 35, 40, 45, 50, 55 and 60% saturation. The mixtures were stirred for 2 h at 4ºC and later centrifuged at 20,000 rpm for 1 h (4ºC) . The precipitates were re-suspended in 25 ml of 0.05 M potassium phosphate buffer (pH 7.0). Dialysis was followed in a tubular cellulose membrane (Specrapor, 1000 dalton MWco, Fisher Scientific Pittsburgh, PA USA) against 2 litres of the same buffer for 18 h in spectrapor No. 4 dialysis tubing. Assay of the bacteriocin activity was carried out and titer was determined in both the precipitate and supernatant to know which one actually contain the bacteriocin (Fraction 1) (Jimenez-Diaz et al., 1993).

Trichloroacetic acid (TC) precipitation: Five percent (5%) equivalent of TC was added to 25 ml of Fraction 1 to precipitate the

protein (bacteriocin). The mixture was centrifuged at 13,000 rpm for 10 minutes after which the supernatant was decanted. The resulting pellet was dissolved in 2 ml of potassium phosphate buffer (Fraction 2).

Ultrafiltration studies: The Fraction 2 (Trichloroacetic acid precipitation) bacteriocin sample was resuspended to $^{1}/_{30}$ volume in potassium phosphate buffer (50mM, pH 7.0). Several aliquots (1ml) were ultrafiltered through various filtron membranes (Filtron Technology Corp; Northborough, Mass), including 1,000,000, 100,000, 10,000 and 1,000 KDa. molecular exclusion sizes. Bacteriocin activity was determined in retained and eluted fractions (Jimenez-Diaz et al., 1993) and protein concentration of the fractions were determined by the Bradford method (Bradford, 1976).

Determination of bacteriocin activity: A well diffusion assay procedure was used (Schillinger and Lucke, 1989; Takahiro et al., 1991). Aliquots of 50 l from each bacteriocin dilution (see determination of bacteriocin titire) were placed in wells in plates seeded with the bioassay strain. The plates were incubated overnight at 30ºC for lactic acid bacteria indicators (anaerobically) and at 37ºC for non- lactic acid bacteria indicators (aerobically), and the diameters of the inhibition zone were taken (Rammelsberg and Radler, 1990).

Determination of bacteriocin titre: The titres of bacteriocin produced were quantified by two fold serial dilutions of bacteriocin in saline solution and aliquots of 50 l from each dilution were placed in wells in plates seeded with the bioassay strain. These plates were incubated anaerobically at 30^{0}C for lactic acid bacteria indicators and aerobically at 37^{0}C for non-lactic acid bacteria indicators for 18-24 h and examined for the presence of 2 mm or larger clear zones of inhibition around the wells. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU ml-1) (Graciela et al., 1995)**.**

Characterization of bacteriocin

The purified bacteriocin samples (Fraction 2) were characterized with respect to thermal and pH stability, susceptibility to denaturation by enzymes, stability during storage, extraction with organic solvent, treatment with dissociating agents and mitomycin C and uv light induction.

Heat Resistance: Purified bacteriocin (400 l) was exposed to various heat treatments: 40, 60, 80, 100 and 121ºC. Aliquot volumes of each Fraction were then removed after 0, 30, 60 or 90 min (ten Brink et al., 1994) and assayed for bacteriocin.

pH Sensitivity: Purified bacteriocin (400 l) were adjusted to pH 2, 4, 6, 8, 10, and 12 with hydrochloric acid (HCl) and sodium hydroxide (NaOH), incubated for 4 h at room temperature (ten Brink et al., 1994) and similarly assayed.

Enzyme Treatments: Purified bacteriocin was assessed for its sensitivity to various enzymes. Enzymes (all obtained from Sigma) and their respective buffers were lipase (type 1, 8.6 U/mg) in 0.05 M Tris hydrochloride (pH 8.0), 0.01 M $CaCl_2$; -chymotrypsin (type 11, 47 U/mg) in 0.05 M Tris hydrochloride (pH 8.0) 0.01 M $CaCl_2$; pronase E (type xxvi, 4.1 U/mg) in 0.01m sodium borate, 0.05 M HCl, 5 mM $CaCl_2$, 1 mM $CaCl_2$ (pH 7.2); pepsin (3,2001 U/ml), in 0.2 M citrate (pH 6.0); catalase (2,000 U/mg), in 10 mM potassium phosphate (pH 7.0); phospholipase C (type 1, 10 U/mg), in 0.05 M Tris hydrochloride (pH 7.0), 0.01M $CaCl_2$; trypsin (type x, 15000 U/mg), in 0.05 M Tris hydrochloride (pH 8.0); lysozyme (Serva, 20600 U/mg), in 1N NaOH (pH 6.5); -amylase (type IX, 1000 U/mg), in 1N NaOH (pH 6.5); dextranase in 0.2 M citrate (pH 6.1) and proteinase K (11.5 U/mg) in 1N NaOH (pH 6.5). Samples of bacteriocin (500 l) were incubated with 500 g of each enzyme per ml for 60 min at 37ºC except for samples containing trypsin, -chymotrypsin and catalase, which were incubated at 25ºC. Prior to being assayed for bacteriocin activity, preparations containing papain were adjusted to pH 6.0 and those containing trypsin and chymotrypsin were treated with trypsin-chymotrypsin inhibitor (Sigma) according to the manufacturer's instructions (Wanda and Bonita, 1991).

Stability of bacteriocin during storage: Purified bacteriocin was stored at –20, 4 and 37ºC. At different time intervals, samples were taken from the stored material (ten Brink et al., 1994) to determine bacteriocin activity.

Extraction of bacteriocin with organic solvents: Various organic solvents including iso–amylalcohol, chloroform, n–propanol, hexane, Di – ethyl ether, petroleum ether were added to purified bacteriocin in 1:1 ratio. After thorough mixing, phase separation was achieved by centrifugation (10 min at 5000 rpm). When propanol was used for the extraction, 50 g/l NaCl was added to the mixture in order to obtain phase separation. The organic phase and the aqueous phase were collected and solvent removed by evaporation at 45ºC. The residue from the organic phase was resuspended in an amount of saline (8.5 g/l NaCl) equal to the starting volume of the original supernatant fluid (ten Brink et al., 1994). Bacteriocin activity of both preparations was then determined.

Effect of mitomycin C on bacteriocin activity: Mitomycin C was added at a final concentration of 1.0 g/ml to purified bacteriocin and incubation was carried out at 30ºC. Samples were removed at 60, 120, 240 and 360 min. and analysed by the well diffusion method (Wanda and Bonita, 1991).

Effect of uv light on bacteriocin activity: A 10 ml aliquot of purified bacteriocin was placed in a sterile petri dish and exposed to short – wave uv light from a 15 – W General Electric germicidal bulb at a distance of 30 cm. Times of exposure ranged from 0 to 5 min. (Wanda and Bonita, 1991) . After each time interval, bacteriocin activity was analysed by the well diffusion method.

Effect of surfactant on bacteriocin activity: This was carried out by incorporating non-ionic (triton X100, tween 20, tween 80, nonidet P40), anionic (sodium dodecyl sulphate, deoxycholic acid) and dipolar ionic (N–hexadecyl–N,N–dimethyl-3-ammonio–1–propane sulphonate, N–lauroylsarcosine) surfactants. The surfactants were obtained from Sigma Chemical Co. and were added to purified bacteriocin at a concentration of 0.1 ml or 0.01 g of surfactant ml^{-1} of bacteriocin solutions. These preparations were incubated at 30ºC for 60 min. (Kelly et al., 1996) and assayed for bacteriocin activity against indicator organisms by using titre evaluation.

RESULTS

The selected *Lactobacillus* strains (*L. plantarum* F1 and *L. brevis* OG1) produced bacteriocin, which showed inhibitory activity against one or more of the Gram-positive and Gram-negative target strains (Table 2). The two species of *Lactobacillus* had different profiles of inhibition, and the profiles were strain specific. *L.*

Table 2. Inhibition of various indicator organisms by bacteriocin produced by lactic acid bacteria.

Indicator organisms	Strain No	Origin	*L. plantarum* (F1)	*L. brevis* (OG1)
Bacillus cereus	ATCC9634	Reference strain	+(10mm)	+(8mm)
Bacillus stearothermophilus	NCIB8222	‘’	+(8mm)	+(6mm)
Bacillus subtilis	ATCC6633	‘’	+(10mm)	+(7mm)
Micrococcus luteus	NCIB196	‘’	+(12mm)	+(10mm)
Staphylococcus aureus	ATCCI4458	‘’	+(8mm)	+(5mm)
Staphylococcus aureus	NCTC6571	‘’	+(8mm)	+(6mm)
Staphylococcus epidermidis	NCTC5413	‘’	+(10mm)	+(8mm)
Staphylococcus faecalis	ATCC19433	‘’	+(6mm)	-
Staphylococcus pyogenes	ATCC19615	‘’	+(5mm)	-
Listeria denitrificans	ATCC14870	‘’	+(7mm)	+(10mm)
Listeria monocytogenes	587CHRL	,,,	+(7mm)	+(9mm)
Candida albicans	ATCC10231	‘’	-	-
Escherichia coli	NCTC10418	‘’	+(8mm)	+(6mm)
Escherichia coli	K12	‘’	+(12mm)	+(8mm)
Enterococcus faecalis	EFI	‘’	+(10mm)	+(12mm)
Aeromonas pobvia	AP15534	‘’	-	-
Vibro cholerea	AP23622	‘’	-	+(8mm)
Shigella flexneri	AP23498	‘’	+(7mm)	+(5mm)
Shigella dysentry	AP22433	‘’	+(6mm)	+(5mm)
Salmonella typhimurium	ATCC13311	‘’	+(7mm)	+(6mm)
Salmonella kentucky	AT1	Reference strain	+(8mm)	+(9mm)
Klebsiella spp	UCH15	Sputum	-	-
Clostridium sporagenes	NCIB532	Reference strain	+(12mm)	+(8mm)
Serratia marcescens	UI5	Soil	+(8mm)	-
Helicobacter pylori	NCTC11637	Reference strain	+(10mm)	-
Streptococcus thermophilus	IW4	Iru	+(5mm)	-
Lactobacillus acidophilus	U1	Ugba	-	-
Lactobacillus brevis	OGW1	Ogi	-	-
Lactobacillus plantarum	F1	Foofoo	-	+(8mm)
Lactobacillus reuteri	PW1	Palm wine	-	+(6mm)
Lactobacillusdelbrueckii	PT6	Pito	+(8mm)	-
Leuconostoc mesentaroides	M8	Meat	+(6mm)	+(5mm)

Key: No inhibition = −; inhibition = +

plantarum F1 and *L. brevis* OG1 were characterized by the broad-spectrum inhibition of microorganisms. All the bacteriocin produced by the test isolates inhibited *E. coli* (NCTC 10418) and *Enterococcus faecalis* (EF1) but did not inhibit *Candida albicans*. (ATCC 10231) and *Klebsiella* spp (UCH15). The largest spectrum of inhibition was showed by *L. plantarum* F1, which inhibited 24 out of 32 indicator strains. However, it was generally observed that bacteriocin from the producer organism had no inhibitory effect on the organism producing it.

The effects of heat, storage time, pH, enzymes and surfactants on bacteriocin activity were determined using *E. faecalis* (EF1) as indicator organism. The inhibitory compound produced by the test isolates was considered to be heat stable. Bacteriocin produced by *L. brevis* OG1 was considered to be the most heat stable, as the activity (3200 AU/ml) remained constant after heating at 121ºC for 60 minutes, but declined thereafter (Figure 1), while bacteriocin produced by *L. plantarum* F1 activity (6400 AU/ml) remained constant after heating at 121ºC for 10 followed by subsequent decline. At 90 minutes there was no detectable bacteriocin activity in *L. plantarum* F1 (Figure 1).

Effect of time and temperature of storage on bacteriocin activity was also carried out. It was observed that all the bacteriocin produced by the test isolates maintained full stability after storage for 60 days at – 20ºC; partial stability after storage for 120 days at 4ºC, while no activity was detected after storage for 80 to 120 days at 37ºC (Figure 2). Effect of pH on activity of bacteriocin was carried out. It was observed that bacteriocin produced by *L. brevis* OG1was stable at pH 2 to 8, while for *L. plantarum* F1, it was found to be stable at pH 2 to 6 (Table 3).

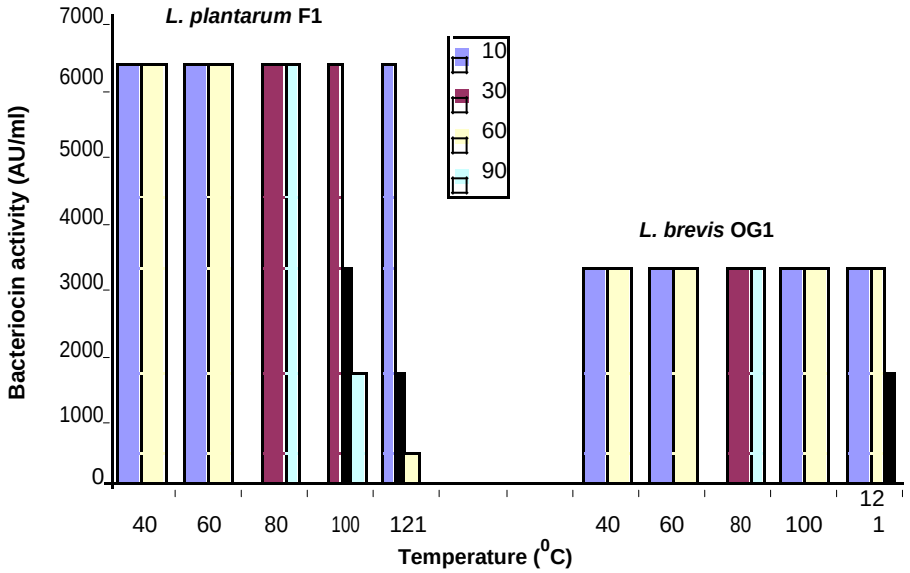

Figure 1. Effect of temperature on bacteriocin activity produced by the test isolates.

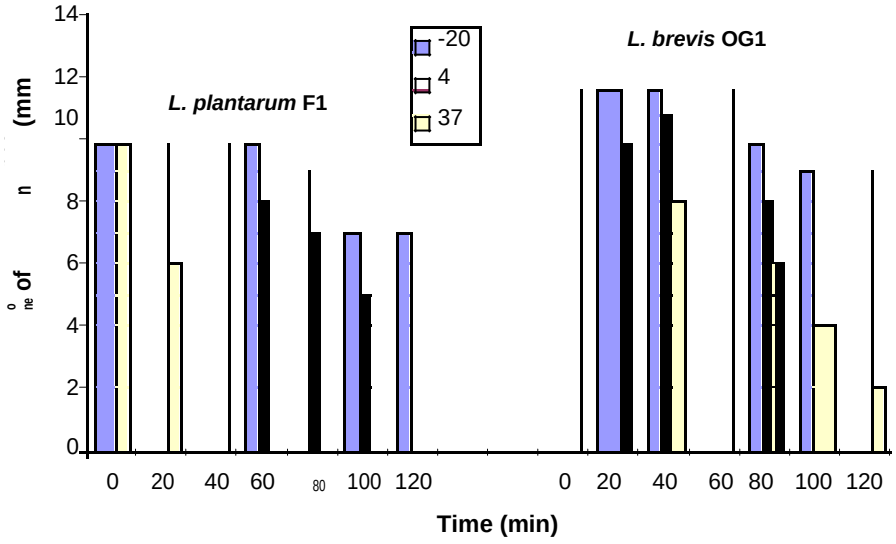

Figure 2. Effect of time and temperature of Storage on bacteriocin activity produced by the test isolates.

Table 3. Effect of pH treatment on bacteriocin activity (AU/ml) Produced by the test isolates.

PH	*L. plantarum* F1	*L. brevis* OG1
2	6400	3200
4	6400	3200
6	6400	3200
8	3200	3200
10	1600	1600
12	400	400

Bacteriocin produced by the test isolates was tested for their sensitivity (loss of activity) to various enzymes. The antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes, whereas treatment with lipase, catalase, phospholipase C, lysozyme, -amylase, dextranase, mitomycin and uv-light did not affect the activity of bacteriocin produced by the test isolates. Bacteriocin produced by *L. plantarum* F1 showed decreased in activity when treated with pronase E (Table 4).

Various organic solvents were tested for the extraction of bacteriocin produced by the test isolates. It was

Table 4. Effect of enzymes, mitomycins and uv light treatments on activity (AU/ml) of bacteriocin produced by the test isolates.

Treatments	***L. plantarum* F1**	***L. brevis* OG1**
Control	6400	3200
Lipase	6400	3200
-chymotrypsin	nd	nd
Pronase E	1600	nd
Pepsin	nd	nd
Catalase	6400	3200
Phospholipase C	6400	3200
Trypsin	nd	nd
Lysozyme	6400	3200
- Amylase	6400	3200
Dextranase	6400	3200
Proteinase K	nd	nd
Mitomycin C	6400	3200
UV light	6400	3200

nd = non-detectable

Table 5. Influence of organic solvents in the extraction of bacteriocin produced by the test isolates.

Organic solvent	*L. plantarum* F1		*L. brevis* OG1	
	Organic phase	Aqueous phase	Organic phase	Aqueous phase
Control	-	10	-	12
I – amylalcohol	6	6	8	8
Choloform	-	-	-	-
N – propanol	8	5	8	4
Hexane	-	8	-	6
Di – ethylether	-	10	-	10
Petroleum ether	-	7	-	5

Zone of inhibition (mm)

Control = bacteriocin without addition of organic solvent

observed that extraction with polar solvents such as hexane, di-ethyl ether, and petroleum ether did not result in the removal bacteriocin produced at the aqueous phase to the organic phase, while chloroform extraction completely destroyed the bacteriocins activity. However, when different alcohols such as n-propanol and Iso-amyl alcohol were used in the extraction procedure, bacteriocin was removed from the aqueous phase and recovered from the organic phase (Table 5).

Table 6 showed the effect of dissociating agents on bacteriocin activity. Exposure to surfactants resulted in an increase in the bacteriocin titre (by at least one to two fold dilutions) rather than any decrease in activity with the

Table 6. Effect of surfactants on activity of bacteriocin (AU/ml) produced by the test isolates.

Surfactants	*L. plantarum* F1	*L. brevis* OG1
Control	6400	3200
Nonidet P – 40	0	0
N – lauroyl sarcosine	12800	3200
N-dimethyl-3-ammonio-1-propane sulphonate	6400	6400
Hexadeyltrimethyl ammonium bromide	12800	6400
Tritox X – 100	12800	6400
Tween 20	25600	6400
Tween 80	25600	6400
Deoxycholic acid	12800	3200
Sodium dodeyl sulphate	12800	6400

exception of nonidet P-40, which led to total loss of bacteriocin activity. N-lauroyl sarcosine had little or no effect on bacteriocin activity produced by the test isolates.

Purification steps of the bacteriocin are summarized in Table 7. The bacteriocin of *L. plantarum* F1 and *L. brevis* OG1 were recovered following the 60% saturation of the culture broths with ammonium sulphate with an increase to specific activity of 9.4 and 5.2 AU/ g protein respectively (Fraction 1). The second step in the purification protocol was Trichloroacetic acid precipitation of Fraction 1. At this stage of purification, the recovery was 18.3% for both isolates. The specific activity increased to 85.3 and 44.4 AU/ g protein for *L. plantarum* F1 and *L. brevis* OG1 respectively (Fraction 2). Finally, these fractions were subjected to ultrafiltration using various filtron membranes. The eluted and retained fractions were collected and assayed for bacteriocin activity. At this stage, when filtered through membrane with 1,000,000 KDa molecular weight cut off, the purification factor was 7.5 and the recovery was 1.2% in *L. plantarum* F1 while in *L. brevis* OG1, purification factor reached 9.0 and the recovery was 1.0%. The bacteriocins were able to pass through cellulose membranes with 100,000 KDa and 1,000,000 KDa, but filtration was not achieved with 10,000 KDa and 1,000 KDa molecular weight cut off. However, partial loss of bacteriocin activity was observed during ultrafiltration (Table 8).

DISCUSSION

Bacteriocins produced by the test organisms have some interesting characteristics that justify their study. The most striking is that none of these bacteriocins is limited by the extremely narrow antibacterial spectrum reported for some bacteriocins of some lactic acid bacteria, for example lactococcin A (Holo et al., 1991) and lactacin B (Barefoot and Klaenhammer, 1983). The largest

Table 7. Purification of bacteriocin produced by *L. plantarum* F1 and *L. brevis* OG1.

Organisms	Purification Stages	Volume (ml)	Activity (Auml^{-1})	Total activity (AV)[a]	Protein (gml^{-1})[b]	Specific activity[c]	Purification factor[d]	Recovery (%)[e]
L. plantarum	Culture supernatant	1000	1600	1600000	410	3.9	1	100
F1	Ammonium sulphate precipitation (Fraction 1)	25	3200	80000	340	9.4	2.4	82.9
	Tricholoroacetic acid Precipitation (Fraction 2)	2	6400	12800	75	85.3	9.1	18.3
	Ultrafiltration (1,000,000 KDa cut off) (Fraction 3)	5	3200	16000	5	640	7.5	1.2
L. brevis	Culture supernatant	1000	800	800000	394	2.0	1	100
OG1	Ammonium sulphate precipitation (Fraction 1)	25	1600	40000	310	5.2	2.6	78.7
	Tricholoroacetic acid Precipitation (Fraction 2)	2	3200	6400	72	44.4	8.5	18.3
	Ultrafiltration (1,000,000 KDa cut off) (Fraction 3)	6	1600	9600	4	400	9.0	1.0

[a]Total activity was determined by the multiplication of volume by activity
[b]Protein concentration was determined by the Bradford method
[c]Specific activity is the activity units divided by the protein concentration (AU g^{-1})
[d]Purification faction is the increase in the initial specific activity
[e]Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration

Table 8. Ultra filtration study of bacteriocins.

[a]Membrane molecular weight cut off (KDa)	AU (%Initial Bacteriocin activity)[b]			
	L. plantarum F1		*L. brevis* OG1	
	Retentive (%)	Eluted fraction (%)	Retentive (%)	Eluted fraction (%)
1,000,000	400(6.25)	3200(50.0)	400(12.5)	1600(50.0)
100,000	3200(50.0)	1600(25.0)	1600(50.0)	800(25.0)
10,000	6400(100.0)	0(0.0)	3200(100.0)	0(0.0)
1,000	6400(100.0)	0(0.0)	3200(100.0)	0(0.0)

[a]Filtron membrane were used.
[b]Initial bacteriocin activity from:
L. plantarum F1 = 6400 AU/ml
L. brevis OG1 = 3200 AU/ml

spectrum of inhibition was exhibited by *L. plantarum* F1, which inhibited 24 out of 32 indicator strains. Earlier reports (Tagg et. al., 1976; Daeschel et al., 1985; Sanni et al., 1999) have shown that some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity. However, it was generally observed that bacteriocin from the producer organism had no inhibitory effect on the organism producing it.

The inhibitory compound (bacteriocins) produced by the test isolates was heat stable. The bacteriocin produced by *L. brevis* OG1 was considered to be the most heat stable as there was no reduction in activity after heating at 121ºC for 60 min, while that produced by *L. plantarum* F1 was able to exhibit full activity after heating at 121ºC for only 10 min. Andersson (1986) also reported loss of activity after heat treatment at 121ºC for 15 min. Although heat stability of antibacterial substances produced by *Lactobacillus* spp. has been well established (Nettles and Barefoot, 1993), heat stability of *L. brevis* OG1 at 121ºC for 60 min is novel. Temperature stability is important if the bacteriocins are to be used as a food preservative, because many procedures of food preparation involve a heating step.

The activity of bacteriocin elaborated by the test isolates was also pH dependent. The highest antibacterial activity was exhibited in an acidic pH range of 2 to 6, while inactivation occurred at pH 8 to 12. Two bacteriocins, namely bulgarican and lactobulgarican, isolated from *L. bulgaricus*, were shown to have the highest activity and stability at pH 2.2 and 4.0 respectively, against a range of pathogenic and spoilage bacteria (Reddy et. al., 1984; Abdel–Bar et. al., 1987). Bacteriocins produced by the test isolates remained fully stable after storage for 60 days at -20 C, but declined or became non-detectable after storage for 80 to 120 days at 37 C, indicating that cold temperature may be the most

appropriate preservation technique. Results from enzyme inactivation studies demonstrated that antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes, whereas treatment with lipase, catalase, phospholipase C, lysozyme, -amylase, dextranase, mitomycin and uv light did not affect the activity of bacteriocin produced by the test isolates, confirming the protein status of bacteriocins. Antimicrobial activity of the bacteriocins produced by the organisms in this study was not due to hydrogen peroxide or acidity, as activity was not lost after treatment with catalase or peroxidase or adjustment of pH to 7.0. According to Fricourt et. al. (1994), lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity. Many of these agents are bacteriocins with a proteinaceous active moiety, while others are non-protein agents (Piard and Desmazeaud, 1991, 1992).

Extraction of bacteriocin in this study using organic solvents indicated that bacteriocin was removed from the aqueous phase and could be recovered from the organic phase. This suggests that at least part of the bacteriocin molecule has a hydrophobic character, and shares this property with most other bacteriocins (Klaenhammer, 1993). In addition, exposure of the bacteriocin samples to surfactants resulted in an increase in the bacteriocin titre. This increase might be due to the effect of surfactant on the permeability of the cell membrane (Graciella et. al., 1995).

During the purification procedures, each step resulted in a considerable loss of protein concentration while specific activity increases. The optimal bacteriocin recovery was achieved by including ammonium sulphate precipitation and Trichloroacetic acid precipitation. This agreed with the findings of Ivanova et al. (2000). Ultrafiltration experiments showed that bacteriocins were unable to pass through 1,000 and 10,000 KDa molecular weight cut-off membrane. A tendency to aggregate with other proteins has been reported in bacteriocins produced by other lactic acid bacteria (Bhunia et al., 1988; Klaenhammer 1988, Toba et al., 1991), and might have contributed to the reason why the bacteriocins could not pass through the membrane with low molecular weight cut-off. In conclusion therefore, the peculiar antimicrobial characteristics of *L. plantarum* F1 and *L. brevis* OGI, can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of the food products so produced.

ACKNOWLEDGEMENT

The authors thank Dr S. I. Smith of Genetics Laboratory, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria, for technical assistance.

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