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Growth Enhancement of Lactic Acid Bacteria for Production of Bacteriocin Using a Local Condiment Supplemented with Nitrogen Sources

Cyprian E. Oshoma*, Olaitan A. Allen, Priscilla O. Oyedoh

Department of Microbiology, Faculty of Life Sciences, University of Benin, P.M.B. 1154, Benin City, Nigeria

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ABSTRACT

Bacteriocin produced by lactic acid bacteria (LAB) inhibits the growth of pathogenic and spoilage microorganisms in foods. This metabolite can be produced using locally sourced condiment such as *Ogi*, a fermented cereal gruel. The study investigated the production of bacteriocin from nitrogen supplemented *Ogi* medium by the isolated *Lactobacillus* spp. LAB was isolated from *Ogi*, a locally fermented grain. The test isolate was selected after LAB isolates were screened for antibacterial activity against selected indicator microorganisms; *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*. The formulated media investigated were *Ogi* (O), O*gi* and peptone (OP), *Ogi* and yeast extract (OYE), and *Ogi*, peptone and yeast extract (OPY). The effect of substrate (*Ogi*) concentration and inoculum size on the bacteriocin production was also determined. Among the identified LAB, *Lactobacillus plantarum* had the highest antibacterial activity with a zone of inhibition of 22.33 ± 1.20 mm against *Escherichia coli*. For all the formulated growth media used and inoculated with *L. plantarum*, OPY medium had the highest bacteriocin concentration of 15.14 ± 0.44 mg/L whereas the least, 5.76 ± 0.96 mg/L, was from *Ogi* medium. Effect of substrate (*Ogi*) concentration and inoculum size on bacteriocin production revealed that 0.6% w/v and 0.9 x 10^6 cfu/mL recorded the highest concentration of 14.29 ± 0.05 and 16.28 ± 0.50 mg/L, respectively. Utilization of *Ogi* as a basal substrate for bacteriocin production may create an avenue for further research on the large scale production of this metabolite.

Keywords: Bacteriocin, Antibacterial activity, *Lactobacillus plantarum*; local condiment, "*Ogi*" substrate.

Introduction

The failure of antibiotics due to drug resistant pathogens and consumers' antipathy for chemical additives as preservatives has led researchers to seek alternative methods of biopreservation of food of natural origin.[1,2] The nutritional composition of food enhances the growth and survival of pathogenic and spoilage microorganisms in the absence of preservatives.[3] Hence, natural preservatives such as bacteriocins which are non-detrimental to human health are a good alternative to chemical preservatives in foods.[2] Bacteriocins are the low molecular mass of extracellularly released and ribosomally synthesized peptides or proteins. They functionally inhibit the growth of spoilage and pathogenic Gram-positive and negative bacteria in foods.[4] Many Gram positive and negative microorganisms can produce bacteriocins.[5] Bacteriocins derived from lactic acid bacteria (LAB) have excellent preservative properties mostly to the food industry.[3] Also, it does not alter the gastrointestinal tract (GIT) microflora as experienced with antibiotics[4] hence, is a generally-regarded–as-safe (GRAS) preservative.[3] Bacteriocin in the GIT successfully maintains microbial population and reduce members of competitors to establish a more nutrient and environmental-friendly living space while being harmless to consumers.[6] Asides Food Science application, bacteriocin can be applied in Veterinary medicine to hinder infectious pathogens in animal diseases, in pharmaceuticals and modern medicine to treat malignant cancer.[3]

LAB are starter culture of controlled fermentation or natural flora of traditionally fermented food and produced antibacterial compounds which include: carbon dioxide, diacetyl, acetaldehyde, hydrogen peroxide, D-isomers of amino acids, reuterin and bacteriocin.[7] The commonest LAB utilized for bacteriocin production are of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Bifidobacteria* genera.[2,8] The selection of abundant complex media such as De Man Rogosa Sharpe (MRS), brain heart infusion (BHI), sodium lactate (NaLa) and Trypticase Soy Broth Yeast Extract (TSBYE) agar respectively are known to promote the growth of LAB and enhance bacteriocin production.[2] Consequently, the uneconomical nature and long incubation period involved in the aforementioned media cultivation stimulated the need for a cheaper LAB growth enhancer and bacteriocin producers.[9] Secondly, being that LAB are known to majorly utilize carbon sources in the midst of nitrogenous sources, a carbon-containing cheaper media is needed for their growth and bacteriocin production.[2,10] Therefore, a low-cost, but effective, growth media must be identified.[11] Generally, the cheap locally available materials such as cereals and protein source that contain considerable amount of protein and carbohydrates may serve as an alternative nutrient media to grow bacteria for bacteriocin production.[12] One of the locally available cereals that can be utilized for bacteriocin production is maize (*Zea mays*). It is rich in carbohydrate but less amount of protein and fat.[13,14] "*Ogi*" is a locally fermented gruel produced from maize (*Zea mays*), Sorghum (*Sorghum vulgare*) or millet (*Pennisetum typoideum*)[15] which contains consortium of yeast and LAB. These microorganisms use the rich starch content of this substrate to release antimicrobial compounds.[9] During "*Ogi*" fermentation, spoilage and pathogenic microorganisms are either removed or reduced due to the effect of antimicrobial producing LAB.[16] The major LAB implicated for fermentation and nutritional improvement of *Ogi* are *Lactobacillus lactis*, *L. fermentum*, *L. casei*

*Corresponding author. E mail: cyprian.oshoma@uniben.edu
Tel: +2349099727664

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and *L. plantarum*.[15,17] Furthermore, literatures have revealed the capacity of this fermented gruel to harbour and encourage *Lactobacillus* growth with bacteriocin production.[15,18]
The nutritional profile of Ogi,[18]offers a great potential to be partially or fully substituted as an inexpensive medium for bacteriocin production. Hence, the aim of the study was to produce bacteriocin from nitrogen supplemented *Ogi* by the isolated *Lactobacillus* spp.

Materials and Methods

Sample preparation
Maize (*Zea mays*) grains were randomly purchased at an open stall market at Ugbowo, Benin City and transported to the laboratory in sterile bottles. Stone and dirt were removed from the grains. From the cleaned grains, 500 g was soaked with 1 L of distilled water for two days, wet-milled, sieved and the filtrate was allowed to sediment for one day to obtain "*Ogi*" slurry.

Isolation of lactic acid bacteria
Ten (10) mL of the sample (*Ogi*) slurry from the medium of maize was serially diluted. The culture was carried out using pour plate method on De Mann Rogosa Sharpe (MRS) and the plates were incubated at 37°C for 48 h. After incubation, approximately five discrete colonies were selected using a flamed platinum wire loop, streak plated and sub-cultured on MRS agar plates to obtain pure colonies.[19]
Pure and discrete colonies were purified on nutrient agar. Pure isolates were identified based on morphology, Gram's reaction and biochemical test using the scheme in Bergey's manual of determinative bacteriology) after which they were selected and stored in slants for further use.[20,21]

Screening of lactic acid bacteria for antibacterial activity
The identified LAB isolates were sub-cultured on nutrient broth at 37°C for 24 hours. A cell pellet free of nutrient broth was obtained from each broth culture by centrifugation at 4,000 rpm for 15 min. The suspended culture was standardized using 0.5 MacFarland scale and dilute to a concentration of 1.0 x 10^6 cfu/mL. From the standardized culture, 1 mL was transferred into 100 mL Erlenmeyer flask containing 50 mL MRS broth and incubated at 37°C for 48 h. A cell-free solution of bacteriocin was obtained from each broth culture by centrifugation at 4,000 rpm for 15 min. The culture was adjusted to pH 7.0 using 1 M NaOH to exclude the antimicrobial effects of organic acid, followed by filtration of the supernatant to obtain crude bacteriocin for each sample. Inhibition activity from hydrogen peroxide (H_2O_2) was eliminated by the addition of 5 mg/mL catalase. [4]
The antibacterial activity of bacteriocin against the following indicator microorganisms - *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli* was determined using, agar well diffusion assay procedure. The indicator microorganisms were collected from the Department of Microbiology Laboratory, University of Benin, Benin City and confirmed using culture based method.[20,21] To determine the antibacterial activity, 0.2 mL of an overnight culture of the indicator organisms were inoculated in Petri plates containing 20 mL of Mueller Hinton agar. Wells were made with a sterile cork borer of 6 mm in diameter and the wells infused with 0.1 mL of each supernatant (or extract) of *Lactobacillus* isolates. The plates were then incubated overnight at 37°C. The inhibitory activity detected was expressed numerically in millimeters (mm).[22] Antibacterial activity for each organism were carried out in duplicate plates. *Lactobacillus* isolate that showed high inhibitory activity was selected for bacteriocin production.

Fermentation process
Screened *Lactobacillus* isolate with high inhibitory activity was sub-cultured on nutrient agar at 37°C for 24 h and washed with sterile distilled water. The culture was compared with 0.5 MacFarland standard, which is equivalent to 1.5 ×10^8 cfu/mL.[5,23] Fermentation was carried out using formulated media comprising majorly of wet milled maize (*Zea mays*) and the screened LAB strain for bacteriocin production. One of the media designated as *Ogi* peptone (OP) medium had the composition of *Ogi* (0.5 g/L) and peptone (0.25 g/L). The second designated as *Ogi* Yeast extract (OYE) medium with composition *Ogi* (0.5 g/L) and yeast extract (0.25 g/L). The third designated as *Ogi* peptone yeast extract (OPY) medium with the composition of *Ogi* (0.5 g/L), peptone (0.25 g/L) and yeast extract (0.25 g/L). The fourth medium had only *Ogi* and served as the control medium. In all the media initial pH adjustment was done using 1 N NaOH and/or 1 N HCl. From the media 100 mL was transferred into 250 mL Erlenmeyer flask and sterilized at 121^{o}C for 15 min. The screened LAB strain of 1.5×10^6 cfu/mL was aseptically inoculated. Fermentation was at 37^{o}C on a shaker at 120 rpm for 72 h. At every 24 h interval for 72 h, samples were aseptically withdrawn from each fermentation flask to determine viable cell number, pH and bacteriocin production. Fermentations for each medium were carried out in triplicate. Medium with the highest yield of bacteriocin was further investigated for the effect of substrate concentration and inoculum size.

Effect of Ogi concentration on bacteriocin production
The effect of *Ogi* concentration on the production of bacteriocin was studied using the selected medium with the highest yield of bacteriocin. Several concentrations of *Ogi*, 0.2, 0.4, 0.6, 0.8 and 1.0% (w/v), were prepared. In all the media initial pH adjustment was done using 1 N NaOH and/or 1 N HCl. From the media 100 mL was transferred into 250 mL Erlenmeyer flask and sterilized at 121^{o}C for 15 min. The screened LAB strain (1.5×10^6 cfu/mL) was aseptically inoculated. Fermentation was at 37°C on a shaker at 120 rpm for 72 h followed by bacteriocin concentration determination. All fermentations were carried out in triplicate.

Effect of inoculum size on bacteriocin production
The effect of inoculum size on the production of bacteriocin was studied using the selected medium with the highest yield of bacteriocin. The different inoculum sizes used were 0.6×10^6, 0.9×10^6, 1.2×10^6, 1.5×10^6 and 1.8×10^6 cfu/mL. Fermentation was at 37°C on a shaker at 120 rpm for 72 h followed by bacteriocin concentration determination. All fermentations were carried out in triplicate.

pH analysis
The pH value was monitored using the pH meter.

Viable cell count determination
Viable cell count was determined by plating serial dilutions of the samples onto MRS agar using pour plate method and incubating at 37°C for 48 h according to Fernandez *et al.*.[24]

Determination of bacteriocin concentration
Bacteriocin concentration in the cultured samples was quantified using Lowry's method.[25,26] From the bacteriocin solution, free of cells, organic acids and hydrogen peroxide, 1.0 mL was mixed with 1.4 mL Lowry solution, vortexed and incubated in a dark at room temperature for 20 min. Then 1.3 mL of the suspension was taken out and 0.1 mL diluted Folin reagent was added, vortexed to mix and incubated under the same condition for 30 min. The absorbance reading was taken at 750 nm. From the standard curve prepared using bovine serum albumin, extract concentration was extrapolated.

Statistical analysis
All assays were carried out in triplicates. Descriptive statistics and analysis of variance were used to analyze the data obtained from the study using Statistical Package for the Social Sciences ® version 21 as well as Microsoft Excel version 2010.[27]

Results and Discussion

The lactic acid bacteria (LAB) isolates identified from *Ogi* were *Lactobacillus plantarum, L. fermentum, L. delbrueckii, L. acidophilus* and *L. casei* (Table 1). Of the five isolates, only *L. plantarum* had antibacterial activity against all indicator organisms used in the study,

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including *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*. Zone of inhibition using *L. plantarum* crude bacteriocin was highest against *E. coli* (22.33 ± 1.20 mm) and lowest against *P. aeruginosa* (9.33 ± 0.88 mm). The control showed no inhibitory signs against all indicator organisms.

The identified LAB have been implicated in food processing, such as in the fermentation of grains contribution to shelf life, texture, and organoleptic of the finished products.[4,16] These LAB produced antibacterial protein such as bacteriocins that kill or inhibit the growth of spoilage or pathogenic bacteria found in foods. The identified LAB isolates in this investigation were found to produce bacteriocin that inhibits the growth of the tested isolates. The antibacterial activity of *L. plantarum* against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* made this LAB strain a potent bacteriocin producer strain. This finding was in support of Mohammed *et al*.[22] who reported that LAB strains exhibited bacteriocin activity that is against *Staphylococcus* sp, *Bacillus* sp, *Pseudomonas* sp and *Salmonella* sp. Similar findings by Ogunbanwo *et al*.[28] revealed that *L. plantarum* and *L. brevis* isolated from *Ogi* had antibacterial activity against *E. coli* and *Enterococcus faecalis*. The result also confirms the report by Elyas *et al*.[4] that *Lactobacillus* spp isolated from Sudanese fermented foods produced antibacterial substance, bacteriocin, active against *S. aureus* and *E. coli*.

As shown in Figure 1, viable *Lactobacillus* cell count in all *Ogi* media supplemented with either peptone or yeast extract and without supplementation, progressively increased during the fermentation period. The *Ogi* peptone yeast extract (OPY) medium had the highest cell count of $3.66 \pm 0.10 \times 10^6$ cfu/mL after day 3 of fermentation, which is significantly different ($p < 0.05$) from *Ogi* peptone (OP) medium which reached a peak of $3.35 \pm 0.15 \times 10^6$ cfu/mL. The control medium had the least cell count of $2.10 \pm 0.10 \times 10^6$cfu/mL after day 3. This shows that nitrogen sources such as yeast extract and peptone support growth of *L. plantarum*.

Media supplementation was found to be important in enhancing bacteriocin production in the present study. Bacteriocin concentration in all supplemented media (Figure 2) was observed to be highest with OPY medium while the least was from the control medium with values 15.14 ± 0.44 mg/L and 5.76 ± 0.96 mg/L, respectively.

Several simple and cheaper culture media such as hydrolysed wheat flour, cheese whey and corn steep liquor have been formulated to enhance the growth and metabolic activities of LAB.[2] The composition of the culture media[14] has a number of factors claimed to affect the growth and functionality of LAB. Studies have shown the utilization of agricultural food as an inexpensive medium for the growth of *Lactobacillus* species based on its nutritional profile.[9] Similarly, '*Ogi* being a nutrient-rich inexpensive medium for LAB was harnessed and supplemented with peptone and yeast extract as nitrogen sources. Abbasiliasi *et al*.[2] reported that medium rich in peptone, yeast extract and protein hydrolysates are required for growth of LAB and good bacteriocin production. Hence, the supplementation of *Ogi* with yeast extract and peptone, as nitrogen sources, essential for metabolic activity and growth of *Lactobacillus* spp.[9,29] Yeast extract and peptone supplemented *Ogi* medium had the highest cell growth indicating that the medium supplied the required nutrient to support *L. plantarum* growth.

Optimal cell growth and bacteriocin production are complimentary to one another.[2,30] It has been accepted that bacteriocin production requires a complex medium, however, a relatively simple medium could also be used for high production of bacteriocin. The amino-acids or peptides in yeast extract could act as inducer or precursor for bacteriocin synthesis by LAB. An organic nitrogen source, especially peptone and yeast extract, is considered to be of vital importance by virtue of their stimulatory effect on microbial cell growth.

Mohammed *et al*. [31] reported that most bacteriocin producing lactic acid bacteria require an inducer or precursor for bacteriocin synthesis, hence, peptone was proposed as an agent for the protein production.[32] Nguyen and Tran[29] reported that the addition of peptone and yeast extract in enzyme production results in optimal performance in its activity. This further demonstrates their key importance as a supplement for the growth of Lactic Acid Bacteria.[33] Abbasiliasi *et al*.[2] also reported that organic sources of nitrogen such as peptone, yeast extract, meat extract and soy bean are crucial in stimulating bacteriocin production and are also used to support good growth of LAB.

Changes in pH during fermentation of *Ogi* media supplemented with peptone, yeast extract and without supplementation are presented in Table 2. There was a decrease from pH of 6.0 in all the media. OPY medium had the least pH of 5.16 ± 0.06 which was significantly lower than the pH of OP medium (5.20 ± 0.02). Decline in pH corresponded with an increase in viable cell count as the fermentation time increases.

The medium pH controls enzymatic reactions resulting to substrate degradation and bacterial growth to release bacteriocin/peptides.[2] It can be observed from the result that there was a decline in pH which corresponded with an increase in viable cell count as the fermentation time increased. In general, reduction of pH between the range of 5 to 6 increased nutrient efficiency consumption and *Lactobacillus spp* population hence, enhanced bacteriocin production.[2,9] There was a drop in the pH during fermentation of the various media due to the production of acidic metabolites.[16,34] Yang *et al*.[30] reported that bacteriocin production by LAB is influenced under acidic condition of pH 6.0 to 6.5. Changes in pH towards acidic condition observed during fermentation affects enzyme stability in the medium.[35]

Bacteriocin yield with varied substrate concentration using OPY medium is shown in Figure 3. Among the varied substrate concentration, 0.6% w/v gave the highest bacteriocin concentration of 14.29 ± 0.05 mg/L. The least bacteriocin concentration of 2.55 ± 0.26 mg/L was from substrate concentration of 0.2% w/v.

High production yields are achieved by the optimization of culture conditions or medium composition.[2] In the present study, different culture conditions were adjusted for bacteriocin production using *Lactobacillus* isolates from locally fermented maize (*Ogi*) to determine optimal fermentation conditions. Bacterial growth and metabolites accumulation are affected by culture condition and medium composition such as fermentable sugars.[2,8] Increase in substrate concentration increases the amount of sugar available for fermentation that will serve for both carbon and energy sources required for cell growth.[36]

Table 1: Antibacterial screening of *Lactobacillus spp* isolated from "*Ogi*" against Indicator organisms

Lactobacillus **Species**	**Indicator Organisms**			
	B. subtilis	***P. aeruginosa***	***S. aureus***	***E. coli***
L. plantarum	13.00 ± 1.15^{c}	9.33 ± 0.33^{b}	20.33 ± 0.88^{d}	22.33 ± 1.20^{d}
L. fermentum	0.00 ± 0.00^{a}	16.00 ± 0.19^{d}	0.00 ± 0.00^{a}	12.00 ± 0.00^{c}
L. delbrueckii	0.00 ± 0.00^{a}	11.00 ± 0.16^{c}	8.00 ± 0.10^{b}	0.00 ± 0.00^{a}
L. acidophilus	8.00 ± 0.20^{b}	18.00 ± 0.30^{e}	0.00 ± 0.00^{a}	12.00 ± 0.00^{c}
L. casei	0.00 ± 0.00^{a}	10.00 ± 0.13^{bc}	14.00 ± 0.00^{c}	4.00 ± 0.70^{b}
Control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

*Zone of inhibition (mm) are expressed as Mean ± S.D of duplicates, different superscripts letters ($^{a-e}$) in the same column indicate values significantly different ($p < 0.05$).

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Varying substrate concentration has been evaluated by numerous authors to improve bacteriocin production because LAB strains require an enriched medium to synthesize the protein.[37] From the study, substrate concentration of 0.6% w/v supplemented with peptone and yeast extract was an ideal medium for bacteriocin production by *L. plantarum*.

Bacteriocin yield with varied inoculum size of *L. plantarum* using OPY medium is shown in Figure 4. Among the varied inoculum size, 0.90×10^6 cfu/mL. gave the highest bacteriocin concentration of 16.28 ± 0.50 mg/L. The least bacteriocin concentration of 9.91 ± 0.55 mg/L was from inoculum size of 1.80×10^6 cfu/mL.

Inoculum concentration during fermentation process has been a significant factor in metabolites production.[38] In this study, there was an increase in bacteriocin production with an increased inoculum size. The optimal inoculum size will strike a balance with the cell biomass and accessible nutrient that will give a high yield of enzyme.[29,39] The inoculum size of 0.9×10^6 cfu/mL was optimal for production. Further increase in inoculum size had a detrimental effect due to limited nutrient availability for cell biomass and faster growth of the culture.[40] Also, multiplication of cells to sufficient number that will utilize the substrate for maximum production does not require lower inoculum size. Lower inoculum size may not rapidly initiate growth and increase metabolite production such as bacteriocin.[38,39] The work of Singh and Kaur[39] reported that the inoculum size 1×10^7 cells/mL was optimal for maximum enzyme production.

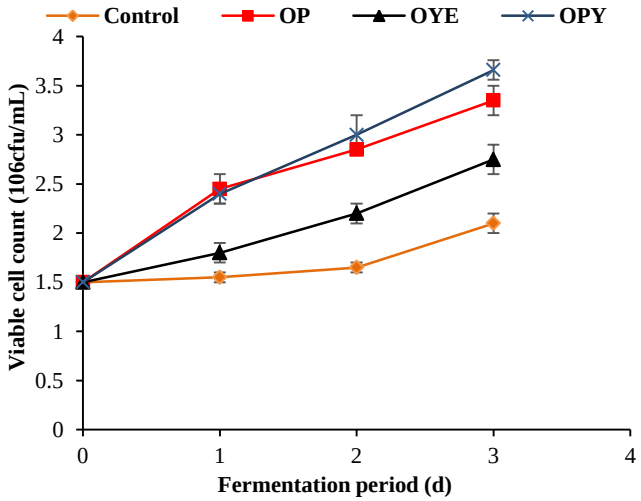

Figure 1: *L. plantarum* cell count in supplemented *Ogi* media during crude bacteriocin production.
Values are the mean of three experiments and error bars represent standard deviation. Key: Control (O): *Ogi*, OP: *Ogi* + Peptone, OY: *Ogi* + Yeast Extract, OPY: *Ogi* + Peptone + Yeast Extract.

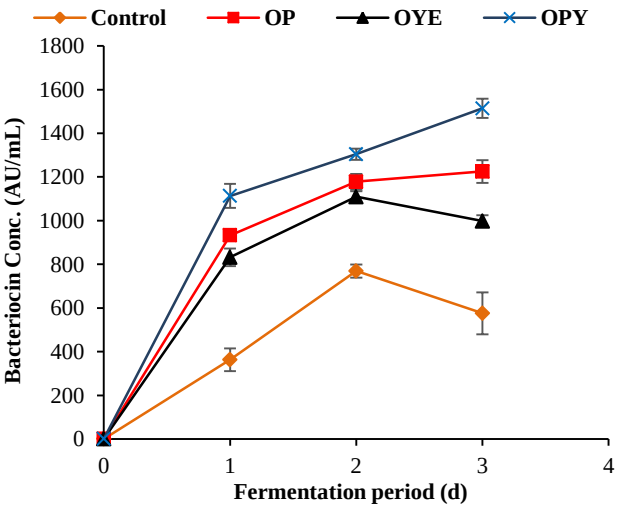

Figure 2: Concentration of crude bacteriocin extract in the supplemented *Ogi* media during *L. plantarum* fermentation.
Values are the mean of three experiments and error bars represent standard deviation. Key: Control (O): *Ogi*, OP: *Ogi* + Peptone, OY: *Ogi* + Yeast Extract, OPY: *Ogi* + Peptone + Yeast Extract

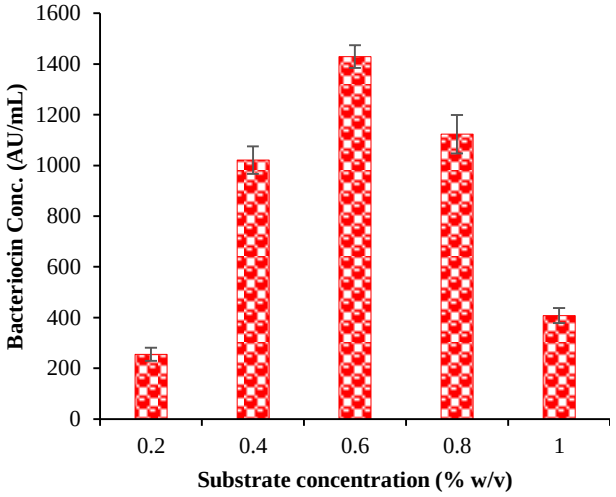

Figure 3: Effect of substrate concentration on bacteriocin activity from *L. plantarum*.
Values are the mean of three experiments and error bars represent standard deviation.

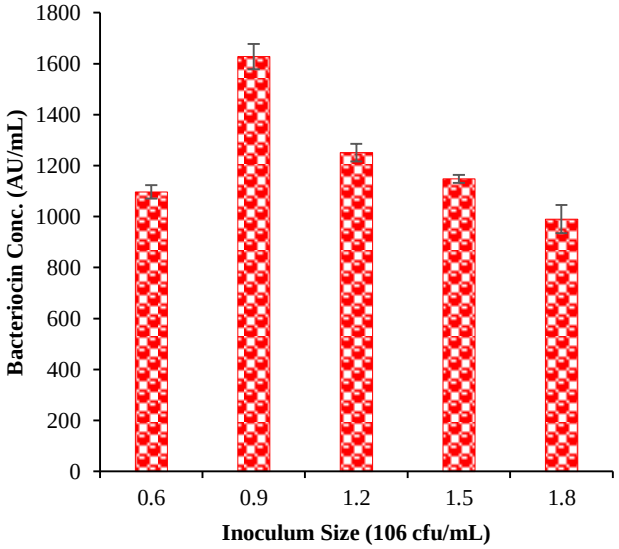

Figure 4: Effect of inoculum size on bacteriocin activity from *L. plantarum*.
Values are the mean of three experiments and error bars represent standard deviation.

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Table 2: pH changes of supplemented *Ogi* media during *L. plantarum* fermentation

Formulated Media	Incubation time (d)			
	0	**1**	**2**	**3**
Control	6.00 ± 0.00^{a}	5.85 ± 0.14^{a}	5.82 ± 0.08^{b}	5.60 ± 0.10^{a}
OP	6.00 ± 0.00^{a}	5.72 ± 0.15^{a}	5.70 ± 0.09^{b}	5.20 ± 0.03^{a}
OYE	6.00 ± 0.00^{a}	6.04 ± 0.13^{b}	5.91 ± 0.04^{b}	5.66 ± 0.45^{a}
OPY	6.00 ± 0.00^{a}	5.88 ± 0.02^{a}	5.41 ± 0.02^{a}	5.16 ± 0.15^{a}

Values are expressed as Mean ± SD of triplicates, different superscripts letters ($^{a-e}$) in the same column indicate values significantly different **(**$p < 0.05$**).**

Conclusion

This research work addresses the suitability of *Ogi* as a basal medium component in the production of crude bacteriocin. Conclusively, utilization of this cost effective source as a basal substrate for bacteriocin production may be researched further. Hence, this will create an avenue for large scale production of potential biopreservative and therapeutics applicable in food industry and pharmaceuticals.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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