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Optimized Production of Bacteriocin by *Lactobacillus plantarum* Isolated from Fermented Maize (Ogi)

***Aghemwenhio, I.S., & Omonigho, S.E.**
Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria

***Corresponding author email**: itohan.aghemwenhio@uniben.edu

Abstract
Bacteriocins, potent antimicrobial peptides synthesized by lactic acid bacteria (LAB), are primary metabolites with ribosomal origins. Their extensive inhibitory range indicates their role as food preservatives and their ability to fight human pathogens. This research focused on enhancing bacteriocin yield from genetically confirmed LAB strains derived from fermented maize (Ogi). The study investigated the influence of various nutrients and pH levels on bacteriocin activity. White and yellow maize samples (500g each) were sourced from Oba Market in Benin City and the International Institute of Tropical Agriculture (IITA) in Nigeria. Established microbiological techniques were used to isolate eight *Lactobacillus plantarum* strains from the maize fermented into ogi. These strains were further identified through molecular techniques, specifically the amplification of 16S rDNA genes Polymerase Chain Reaction (PCR). Bacteriocin presence was assessed via agar spot tests and well diffusion methods. The effect of enzymes, different growth media and vitamins were determined to optimize bacteriocin production. The study aimed to determine the optimal medium composition for bacteriocin production. Molecular identification through PCR amplification of 16S rDNA genes verified the LAB strains as *Lactobacillus plantarum* (TM 229), *Lactobacillus plantarum* (TM 224), and *Lactobacillus fermentum* (TM 217). These strains produced different levels of bacteriocin, 6500 AU/ml (TM 229), 3700 AU/ml (TM 224), and 3200 AU/ml (TM 217) at a pH of 6.5. TM 229 yielded 8800Au/ml when treated with maltose compared with control of 6500Au/ml The findings suggest further study on bacteriocin optimization is recommended.

Keywords: Bio-preservatives, Bacteriocin, Optimization, Enzymes, Glycerol, Vitamins

Introduction

Lactic acid bacteria (LAB) are non-spore-forming rods or cocci and are Gram-positive. They thrive in acidic environments, exhibit catalase-negative properties, and play a crucial role in food fermentation (Van Geel et al., 1998) LAB adds to the texture and flavour of fermented foods by primarily producing lactic acid from glucose. Additionally, they secrete substances that inhibit growth such as diacetyl and bacteriocins which suppress food spoilage organisms. The cell division of these microorganisms occurs in one plane and is non-motile. Their optimal growth ranges between pH 5.5 and 5.8. LAB have composite nutritional requirements, including minerals, nucleotide bases peptides, fatty acids, vitamins and amino acids. (Khalid et al., 2011) Based on carbohydrate fermentation, LAB can be classified as either homofermentative (predominantly producing lactic acid) or heterofermentative (producing a mix of alcohol, lactic acid, carbon dioxide and acetic acid) (Mokoena et al., 2016). One remarkable feature of these microorganisms is their ability to synthesize antimicrobial peptides called bacteriocins. These heat-stable molecules consist of 31–60 amino acids. Bacteriocins exhibit varying biochemical properties, action modes, molecular weights, genetic origins, and activity spectra. They primarily target gram-positive bacteria that are closely related. (Parada et al., 2007). LAB strains producing bacteriocins protect themselves from these toxins through specific immunity proteins coded within the operon of the bacteriocin. LAB derived from do-it-yourself fermented foods demonstrates broad inhibitory effects against significant gram-negative and gram-positive foodborne pathogens. Their prospects as natural biopreservatives in several food products offer a means of counteracting pathogens. Bacteriocins act on the cytoplasmic membrane of the bacteria thereby disrupting the proton motivating force targeting energized membrane vesicles (Parada et al., 2007).

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Materials and Methods

Local, yellow and white farmers' varieties of maize grains were bought from Oba market, Benin City, and taken to the Laboratory, and quality Protein Maize grains were purchased from the International Institute of Tropical Agriculture, Nigeria. The laboratory work was carried out at the Molecular Biology Laboratory of the Federal Institute of Industrial Research, Oshodi (FIIRO). Traditional fermentation of maize samples: During the fermentation; 300g of the different varieties of maize were weighed into tap water of 1 litre volume and fermented for 72 hours at 27±3ºC. The water was gradually poured and the grain was ground using a clean machine, the pastes were filtered with sterile lightweight cotton cloth with 300 µm pore size and the filtrates were put in a container that allowed for natural fermentation of the grain. 300g of different maize varieties were weighed and steeped in 1 litre of tap water for 72 hours at 30°C. After steeping, the water was decanted and wet-milled the grains using a properly washed grinding machine. The resulting pastes were sieved through sterile muslin cloths with a pore size of 300 µm. The filtrates were collected into sterile containers and allowed to settle for 76 hours. During this time, natural fermentation occurred due to the presence of Indigenous microflora (Kolawole et al ., 2007). Isolation of LAB: 10^6 cfu/g of each of the maize varieties were inoculated into MRS broth. The cultures were incubated microaerobically for 24 hours. Based on biochemical and physiological characteristics, we classified the isolates were classified as LAB. Fermentation of sugar was confirmed using the API 50 CHL system . Well-defined colonies were picked at proper dilutions and stored at -80°C in spent MRS broth with 15% glycerol. (De Man et al., 1960)

Molecular identification of isolated LAB: The molecular identification of the LAB was determined based on their 16s rRNA sequences. DNA extraction was performed using a DNA extraction solution. PCR samples were prepared, and sequencing was performed. Agarose gel electrophoresis confirmed the bands, BLAST search program was used to compare the sequences to the databases (Tamura et al. (2013). Bacteriocin bioassay: Agar-spot test and well diffusion were used to perform bacteriocin screening. LAB strains showing inhibitory effects against Escherichia coli and Listeria monocytogenes were further investigated. LAB strains were cultivated at 1.4 x 10^8 cfu/ml in 9.5 ml MRS broth at temperatures (20°C, 28°C, 37°C, and 42°C). After centrifugation, adjustment of the cell-free culture supernatant (CFCS) was done with 1 N NaOH to pH 7. Incubation of the inoculated broth lasted for 48 hours to assess bacteriocin activity. Serial dilutions of the bacteriocin were dappled on fresh indicator areas of *Listeria monocytogenes*. Reciprocal of the highest dilution was defined as the activity completely inhibiting the indicator. Arbitrary units (AU) per ml were used to express antibacterial activity. (De Vuyst et al., 1996). Determination of bacteriocin molecular weight: Strains were grown in MRS broth. 40% ammonium sulfate was used to precipitate bacteriocin from CFSF. After desalting, gel chromatography was used to separate peptides using molecular weight markers. *Staphylococcus aureus* served as the sensitive strain for determining the position of the active bacteriocin.

Optimization processes on bacteriocin production: Effect of enzymes on bacteriocin: LAB strains were incubated with various enzymes (e.g., Proteinase K, pronase, trypsin) to assess antimicrobial activity. (Elayaraja et al., 2014). Production of bacteriocin in different growth media: 18-h-old culture strains were inoculated into various media (BHI broth, M17 broth, MRS broth, molasses) and samples were taken every hour (OD at 600 nm), examined for bacterial growth, pH changes, and antimicrobial activity. Yang et al. (2018). Effect of different pH and temperature on bacteriocin production: The Volumes of 300 ml MRS broth were adjusted to different pHs 4.0,4.5, 5.0, 5.5, 6.0 and 6.5, with 6N Sodium hydroxide and then autoclaved. Inoculation of each flask was with 2% (v/v) of an 18-h-old culture of strain and incubation was 30°C and 37°C for 20 h, without shaking. Changes in the production of bacteriocin (AU/ml) and culture pH were determined every hour (Todorov and Dick, 2005). Effect of simple sugars on the production of bacteriocin: MRS broth was supplemented with 2% (v/v) fructose, lactose, sucrose, glucose, mannose and maltose. MRS broth was supplemented with 0.05 – 4.0% (w/v) Maltose. Malheiros et al. (2015). Effect of glycerol, potassium phosphate and vitamins on the production of bacteriocin: MRS broth was supplemented with 2 g/L KH_2PO_4 and 0 – 50 g/L glycerol. Filter-sterilized vitamins of L-ascorbic acid , cyanocobalamin, thiamine, and vitamin K1 were added to MRS broth at 1 mg/ml. 28°C for 20 h was used for incubation for all tests. Activity levels of bacteriocin were also determined. Todorov and Dick. (2005).

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Results

Table 1: Total Lactic acid bacteria counts

Code of samples	Cfu/g
TM -3	$1.71x\ 10^6$
TM-4	$2.4x\ 10^6$
TM-6	$1.81\ x\ 10^6$
YM-3	$1.19\ x\ 10^6$
YM-4	$1.14x10^5$
YM-6	$1.5x10^6$
WM-3	$1.8\ x\ 10^6$
WM-4	$1.4x10^6$
WM-6	$1.6x10^6$

TM= Fermented treated maize YM= Yellow maize WM=White maize

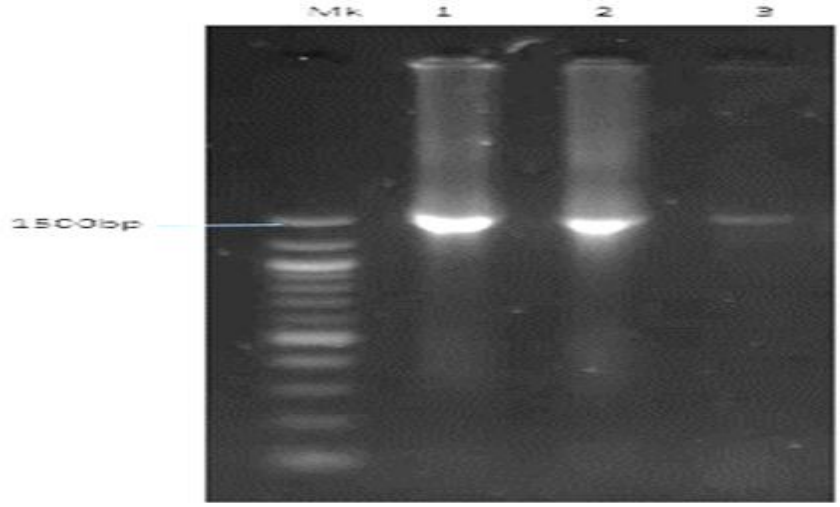

Figure 1: Agarose gel electrophoresis of the PCR products of LAB. Band 1; Isolate TM 229, Band 2; TM 224; Band 3: TM 217

The result showed Band 1 as *Lactobacillus plantarum*, Band 2 *Lactobacillus plantarum* and Band 3 as *Lactobacillus fermentum*

There was inactivation in antimicrobial activity after treatment of the CFCS with trypsin and proteinase K from 18mm±1 to <5mm±1. Catalase and amylase treatment resulted in no activity change.. Optimization of bacteriocin in different growth media resulted in very low levels of bacteriocin with TM 229 having less than 400 Au/ml for M17 and BHI broth, Molasses and Soy milk.

Table 2: Molecular identification of LAB

S/N	Codes of isolates	Bacterial Identity	% Similarity	Accession Number
1	TM 229	*Lactobacillus plantarum*	99.92	APO19815.1
2	TM 224	*Lactobacillus plantarum*	100	MT645511.1
3	TM 217	*Lactobacillus fermentum*	99.99	MT007524.1

Key: TM = standard maize (treated maize**)**

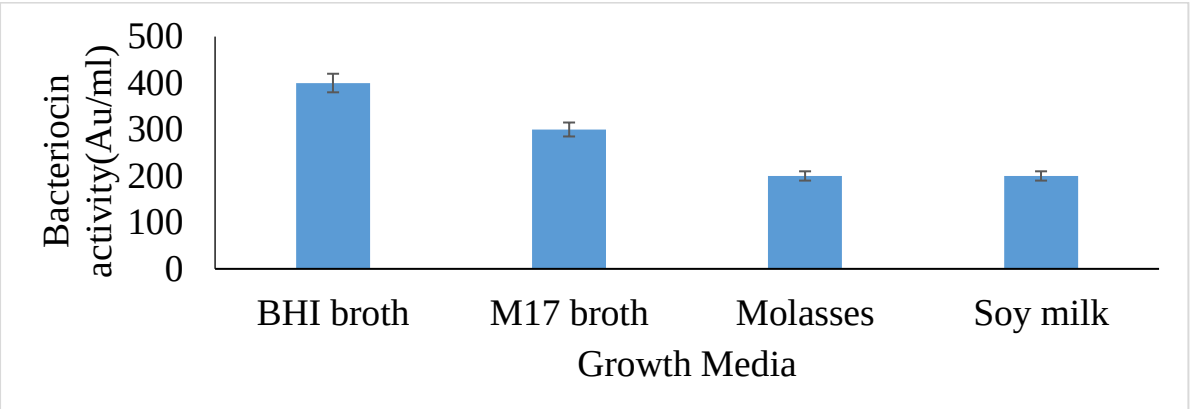

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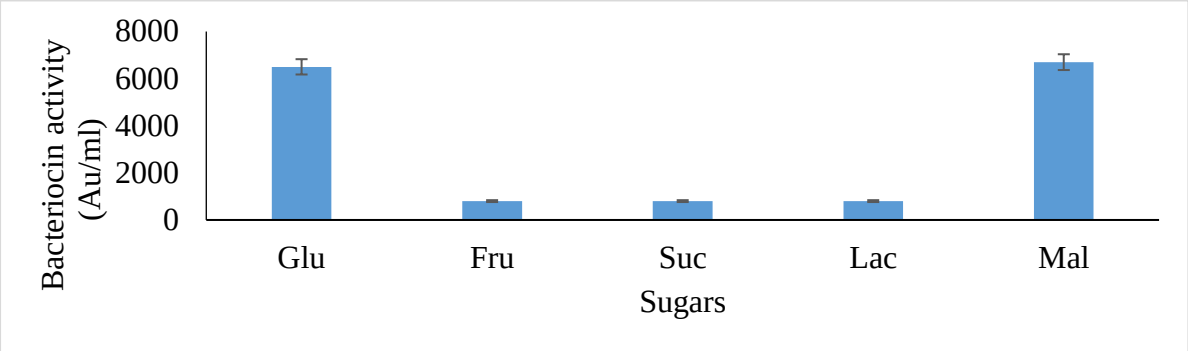
Figure 2: Optimization of TM 229 bacteriocin in different growth media.

Optimization of bacteriocin in the growth media resulted in very low levels of all bacteriocin with TM 229 having less than 400 ± xx Au/ml for BHI broth, M17 broth, Molasses and Soy milk as shown in Figures 5 and 18



Key: Glu (glucose), Fru (Fructose), Suc (Sucrose), Lac(Lactose), Mal(Maltose)

Figure 3: Optimization of TM 229 bacteriocin production with sugars

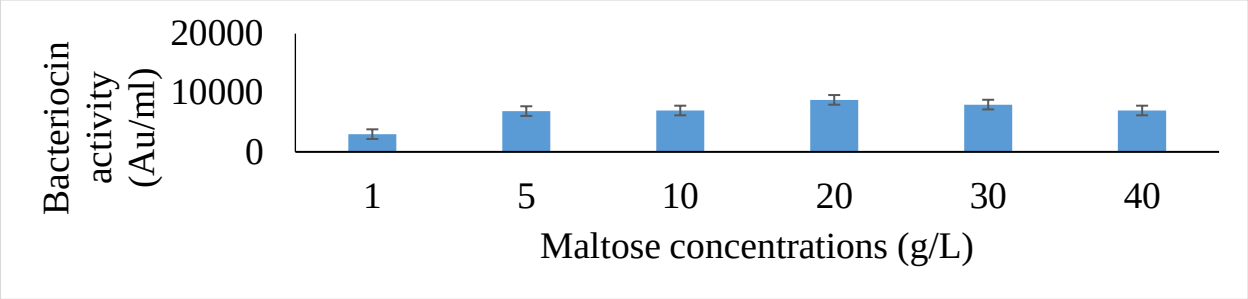


Figure 4: optimization of TM 229 bacteriocin with different concentrations of maltose(g/l)

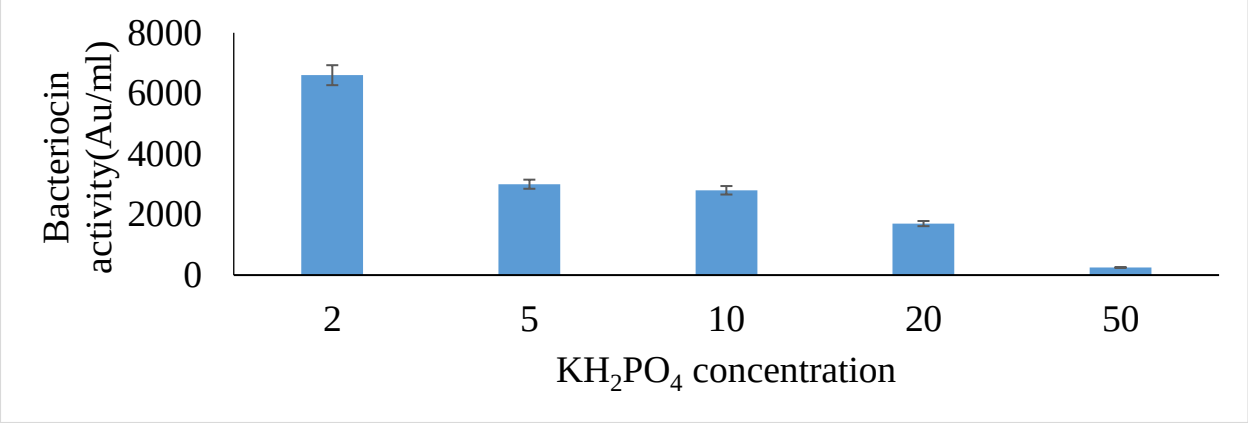


Figure 5: Optimization of TM 229 bacteriocin with different concentrations of potassium.

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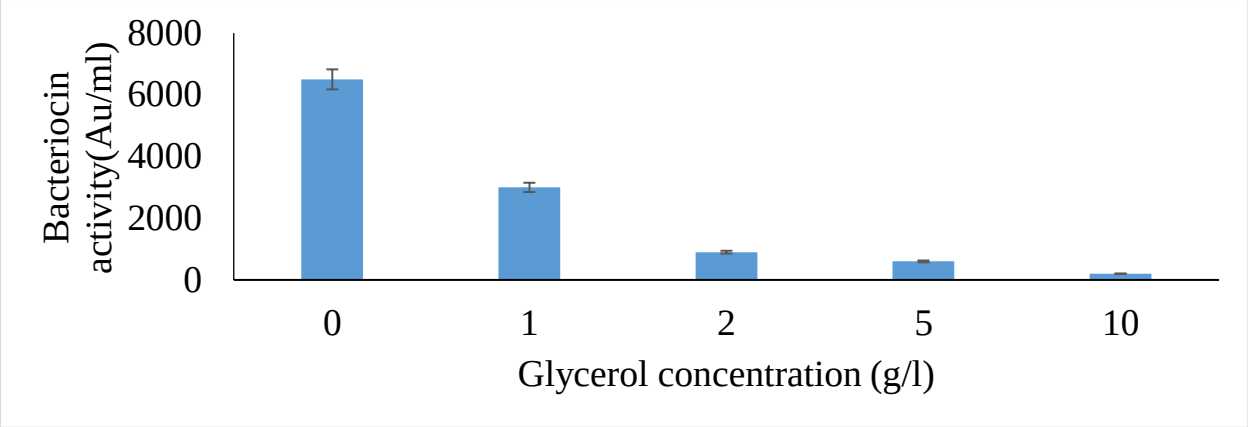
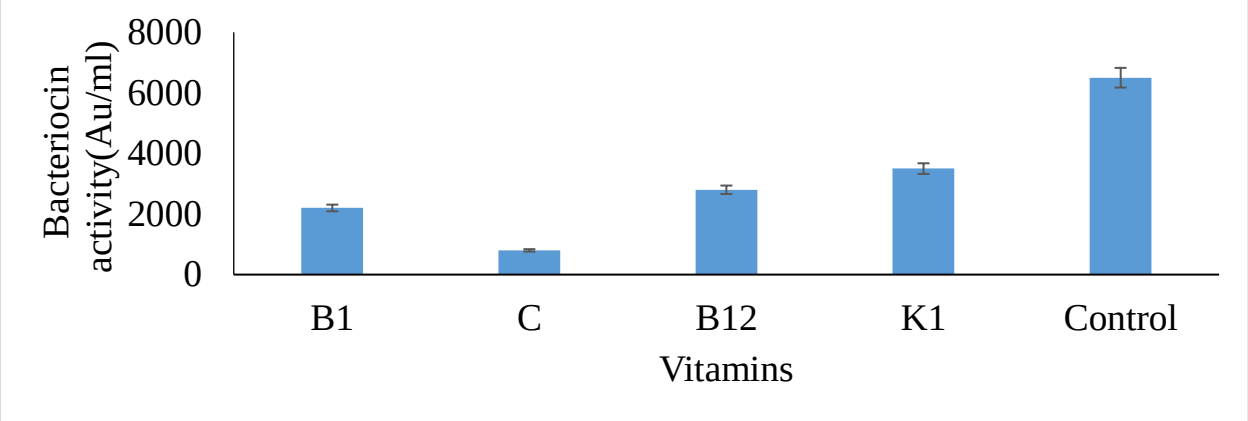
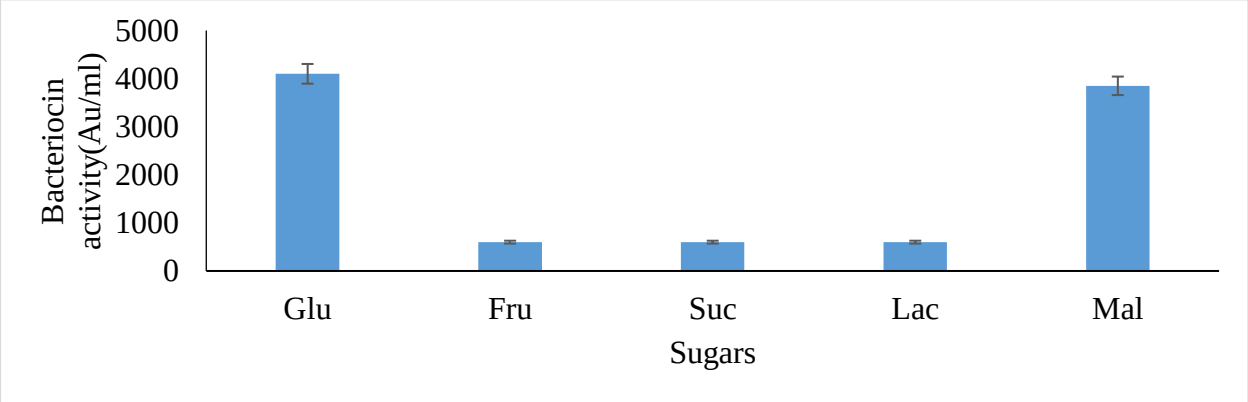


Figure 6: Optimization of TM 229 bacteriocin with different levels of glycerol



Key: B1 = thiamine; C= L-ascorbic acid; B12= Cyanocobalamin

Figure 7: Optimization of TM 229 bacteriocin with vitamins 1mg/ml



Key: Glu (glucose), Fru (Fructose), Suc (Sucrose), Lac(Lactose), Mal(Maltose)

Figure 8: Optimization of TM 224 bacteriocin production with sugars

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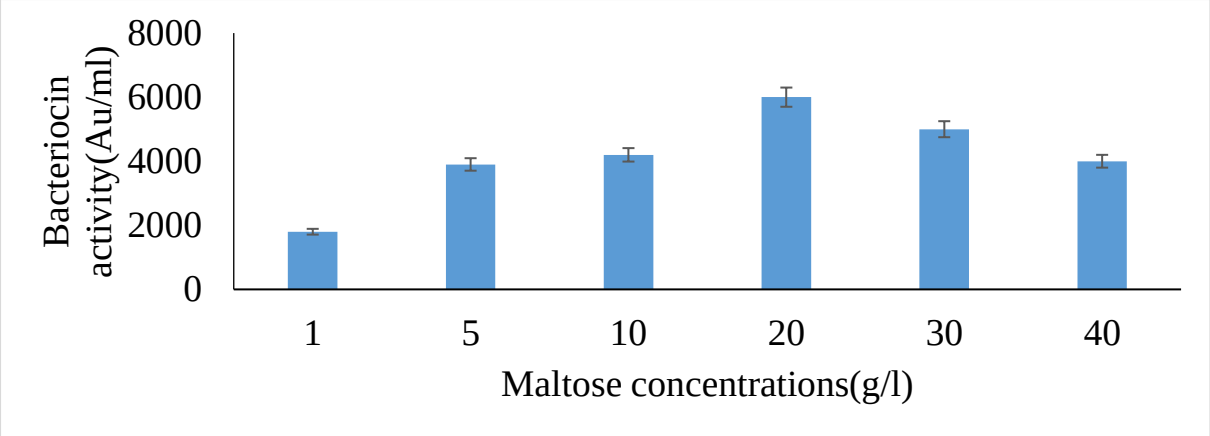


Figure 9: optimization of TM 224 bacteriocin with different concentrations of maltose(g/l)

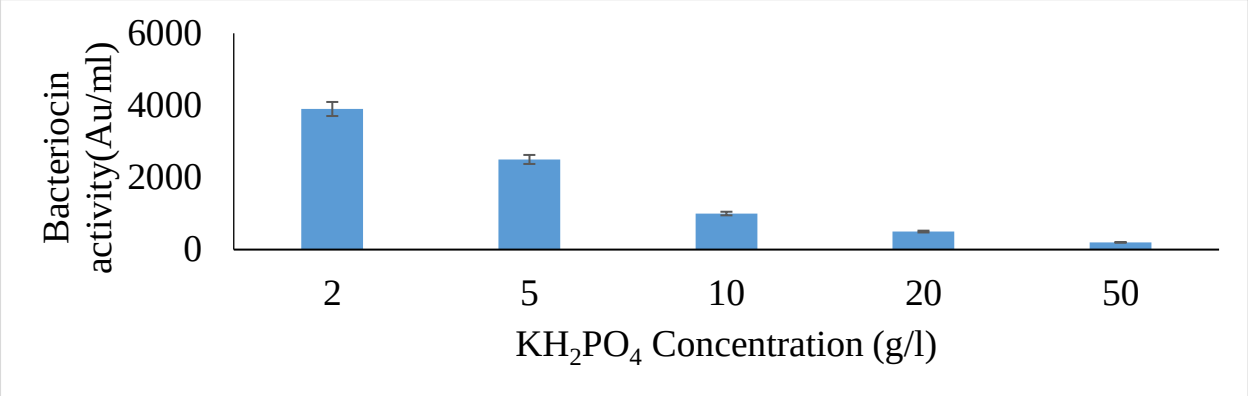


Figure 10: Optimization of TM 224 bacteriocin with different concentrations of potassium.

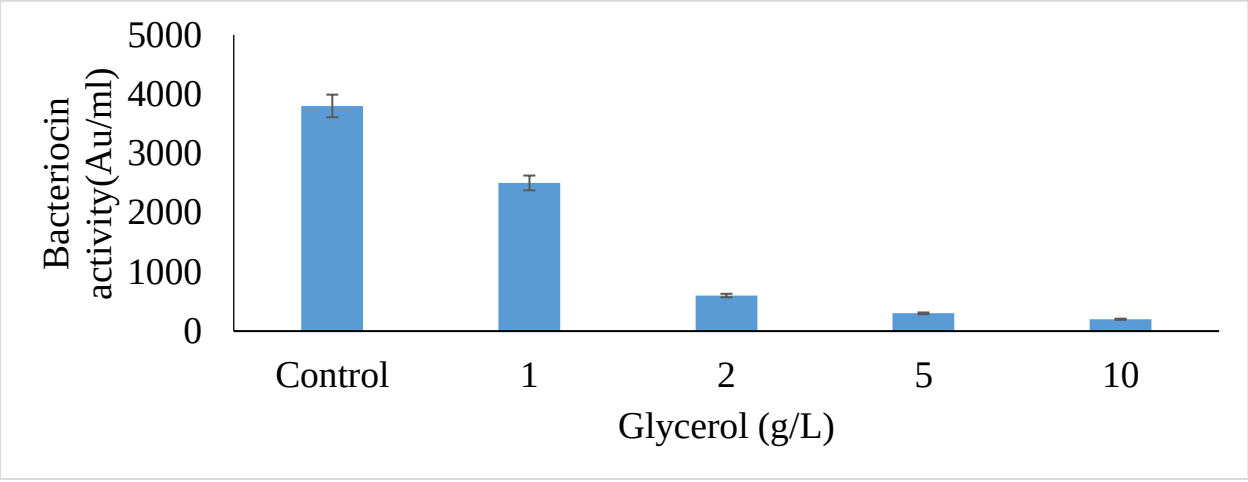
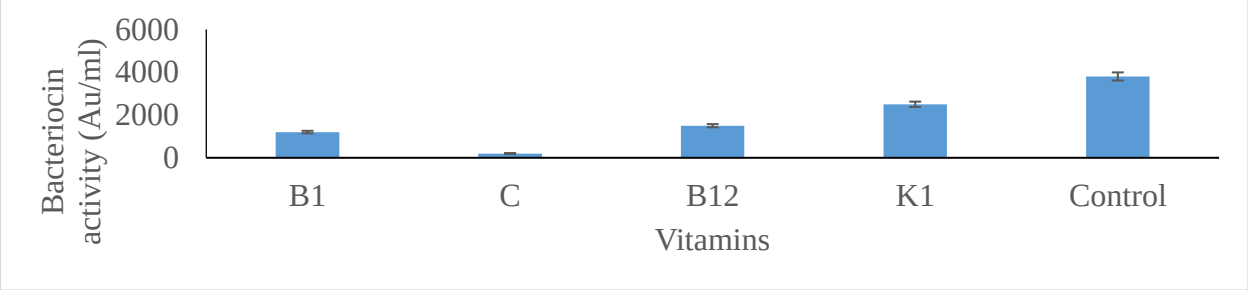


Figure 11: Optimization of TM 224 bacteriocin with different levels of glycerol



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Key: B1 = thiamine; C= L-ascorbic acid; B12= Cyanocobalamin

Figure 12: Optimization of TM 224 bacteriocin with vitamins 1mg/ml

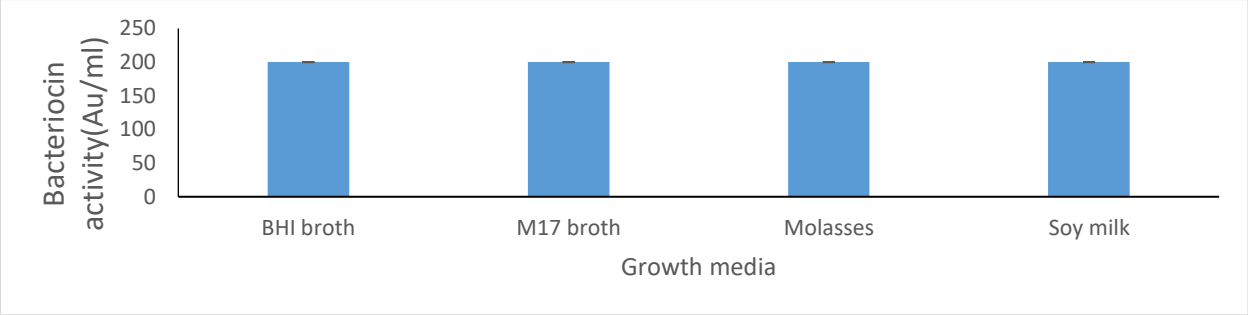
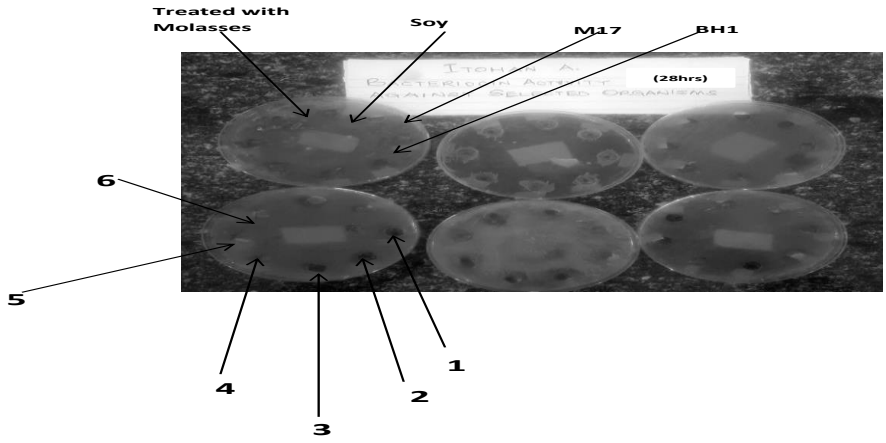


Figure 13: Optimization of TM 224 bacteriocin in different growth media.

Table 3: Antibacterial activity in CFSF from *L. plantarum*

Indicator strains	***Lactobaccillus plantarum***
Escherichia coli	+++
Listeria monocytogenes	+++
Staphylococcus aureus	+++
Enterococcus faecalis	++

Key: +++, zone>15mm±1; ++, zone =15mm±0.5



1. Cell-free Supernatant
2. Cell-free Supernatant treated with α- analysis
3. Cell-free Supernatant treated with catalase
4. Cell-free Supernatant treated with pronase
5. Cell-free Supernatant treated with protenase K

Plate 1: Effect of enzymes on bacteriocin activity against selected organisms.

Discussion

Optimization of bacteriocin production under different conditions is in order with research that focuses on yield maximization while comprehending the biological mechanisms. Optimization with simple sugars of the bacteriocins resulted in increased yield compared with control for maltose, TM 229 had a yield of 8800Au/ml when treated with maltose compared with control of 6500Au/ml but less than 800 Au/ml for sucrose, glucose, fructose and lactose and this was also the pattern for the other bacteriocins. Optimization of bacteriocins with different concentrations of maltose(g/l) had the optimum production level at 20g/L and a reduction of production level at 30-50g/L

The Optimization of bacteriocin with different growth media resulted in very low levels of bacteriocin with TM 229 having less than 400 Au/ml for BHI broth, M17 broth, Molasses and Soy milk as shown in figure 5. The methodology employed in optimizing bacteriocin production was robust yet the variations in yields across different sugars and vitamins indicate that the metabolic pathways involved may respond differently to specific growth conditions.. Optimization of bacteriocins with different concentrations of potassium and different levels of glycerol resulted in decreased production levels compared with control for all bacteriocins. Optimization of

bacteriocins with vitamins B1, C, B12 and K 1mg/ml resulted in a decreased compared with control for all bacteriocins. Low activity of bacteriocin was recorded when strains were grown in BH1 and M17 broth despite good growth of LAB suggesting that some nutrient compositions may not provide the optimal conditions for bacteriocin production, in other words, bacteriocin requires specific nutrients for their synthesis and milk, molasses, BHI and M17 may not meet these requirements. Bacteriocin produced by *Lactobacillus plantarum* (TM 229) had a molecular weight of 11kDa and *L. plantarum* (TM 224) had a molecular weight of 9.5kDa. 250Au/ml bacteriocin activity was recorded after growth in MRS broth, which suggests peptide as a primary metabolite. Parente et al., (1997) reported similar results for enterocin 1146. Bacteriocin TM 229 production was highest (6500Au/ml) in the absence of glycerol, concentrations of 1 to 10g/L glycerol repressed the production of bacteriocin progressively. Adding vitamins B1, C, B12 and K suppressed production level/potential to 2200 Au/ml (vitamin B1) and 800Au/ml (vitamin C). Maximum activity 6500Au/ml by TM229 was recorded without added vitamins. Treatment with amylase did not affect the antimicrobial activity which suggests that bacteriocin is not glycosylated. The production of bacteriocin TM 229 through optimization processes has implications for developing new bio-preservatives in the food industry, which leads to natural alternatives in place of chemical additives. The variableness in bacteriocin activity across different media suggests that further research into the nutritional requirements for optimal production is required.

Conclusion

This study showed that maltose at 20g/L was a key source of optimized level of bacteriocin also establishing bacteriocin production by LAB species as primary metabolites. It also established that the bacteriocin from LAB strains from fermented maize have a broad spectrum activity against human pathogenic microorganisms which holds promise in control of human pathogens and food preservation. The study advocates for further research in bacteriocin optimization produced by Lactic acid bacteria, which can result in the development of more potent natural preservatives for food industries. The promising outcome also encourages the investigation of the medicinal prospects of bacteriocin against human pathogens with resistant strains.

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