



Original Article

Optimization of Bacteriocin Production by Lactic Acid Bacteria

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ABSTRACT

Food spoilage and foodborne diseases are major challenges to food safety, creating a need for preservation strategies. Lactic acid bacteria (LAB) are promising sources of antimicrobial compounds, particularly bacteriocins, with potential applications in food preservation. The aim of this study was to optimize bacteriocin production by lactic acid bacteria under different fermentation conditions. Lactic acid bacteria were isolated from fufu and nono samples using de Man, Rogosa and Sharpe (MRS) agar. Eight LAB isolates were recovered, and the active isolate was identified by 16S rRNA gene sequencing as *Lactiplantibacillus pentosus* strain BSR3, showing 99.70% similarity. Bacteriocin-producing potential was assessed using the agar well diffusion method against *Escherichia coli* and *Salmonella* spp. Isolate F1 (*Lactiplantibacillus pentosus* strain BSR3) showed the highest antibacterial activity, with inhibition zones of 9.5 mm against *Escherichia coli* and 13.0 mm against *Salmonella* spp. Bacteriocin production was optimized using Box–Behnken response surface methodology, evaluating temperature, pH, carbon source concentration, fermentation time, and carbon source type. The regression model was significant, with temperature, pH, fermentation time, and carbon source type significantly affecting bacteriocin production, while carbon source concentration had no significant effect within the tested range. Optimal conditions were 37 °C, pH 6.5, 1.5% carbon source concentration, 24 hours fermentation, and glucose as the carbon source. The findings demonstrate that *Lactiplantibacillus pentosus* strain BSR3 is a promising bacteriocin producer. Optimization of production parameters provides useful information for improving bacteriocin synthesis and supports the potential application of LAB-derived bacteriocins as natural antimicrobial agents for biopreservation.

Keywords: Bacteriocin, biopreservation, isolate, lactic acid bacteria, optimization

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INTRODUCTION

Microbial spoilage and foodborne diseases remain major challenges to food safety and

contribute significantly to post-harvest losses, particularly in highly perishable foods such as tomatoes [1,2]. Concerns associated with the extensive use of

synthetic preservatives, including potential toxicity and consumer safety issues, have increased interest in natural antimicrobial compounds as safer alternatives for food preservation [3]. Lactic acid bacteria (LAB) are Gram-positive, acid-tolerant microorganisms widely recognized for their roles in food fermentation and biopreservation. They are generally regarded as safe (GRAS) and produce antimicrobial metabolites, including organic acids, hydrogen peroxide, and bacteriocins, which contribute to the inhibition of spoilage organisms and foodborne pathogens [1,4,5].

Among LAB-derived antimicrobial compounds, bacteriocins are ribosomally synthesized peptides with strong inhibitory activity against foodborne microorganisms. Their natural origin, antimicrobial effectiveness, stability, and potential application in food systems make them attractive alternatives to synthetic preservatives [6,7,8,9]. Bacteriocins exert antimicrobial effects mainly through membrane disruption, pore formation, leakage of cellular contents, and interference with essential cellular processes [10,11]. The potential of bacteriocins has led to increasing interest in their application as purified antimicrobial agents, bacteriocin-producing LAB cultures, and components of active food packaging systems [12]. Nisin remains one of the most extensively studied and commercially applied LAB bacteriocins due to its broad antimicrobial activity and acceptance for food applications [13,14]. Despite their potential, wider industrial application of bacteriocins is limited by variations in production yield and antimicrobial activity. Factors such as temperature, pH, carbon source, fermentation time, and

medium composition influence bacteriocin synthesis, making optimization of production conditions necessary for improved yield and commercial utilization [15,16,17]. Previous studies have demonstrated that fermented foods contain diverse bacteriocin-producing LAB strains with promising antimicrobial properties [18,19,20,21]. However, challenges related to low productivity, stability, and antimicrobial spectrum continue to limit their application [22,23]. Therefore, optimization of bacteriocin production from locally isolated LAB represents an important approach for developing sustainable food preservation technologies. The present study aimed to isolate and identify LAB from locally fermented foods, evaluate their bacteriocin-producing potential, and optimize production conditions to enhance bacteriocin yield for potential application as a natural biopreservative. This approach supports the development of safer food preservation systems and contributes to reducing post-harvest losses [24].

MATERIALS AND METHODS

Study area and sample collection

The study was conducted in Minna, Niger State, Nigeria. Locally fermented food samples, including nono (fermented milk) and fufu (fermented cassava dough), were collected from Bosso and Kure markets. Minna is located at approximately 9°36'50"N and 6°33'25"E, with an elevation of about 229 m above sea level. Samples were aseptically collected into sterile, labelled containers and transported to the Microbiology Laboratory of the Federal University of

Technology, Minna, for analysis within 24 hours of collection.

Isolation of lactic acid bacteria

Isolation of lactic acid bacteria (LAB) was carried out using standard microbiological procedures. Samples were serially diluted by homogenizing 1 g of each sample in 9 mL sterile distilled water, followed by successive dilutions up to 10^{-4} . Aliquots (0.1 mL) from appropriate dilutions were spread onto de Man, Rogosa and Sharpe (MRS) agar plates and incubated at 37 °C for 24 hours. Colonies with typical LAB characteristics were selected, purified by repeated subculturing, and maintained on MRS agar slants at 4°C for further analysis [25,26].

Characterization and identification of lactic acid bacteria

The purified isolates were characterized based on colony morphology, Gram reaction, and biochemical properties. Tests performed included catalase, citrate utilization, urease, indole, motility, sugar fermentation, and gas production [27,28]. Carbohydrate fermentation ability was evaluated using lactose, glucose, mannitol, sucrose, sorbitol, and fructose as substrates [29].

Molecular Identification of Selected LAB Isolates

Genomic DNA was extracted from selected LAB isolates using the phenol-chloroform extraction method described by [30]. The extracted DNA was amplified through polymerase chain reaction (PCR) targeting the 16S rRNA gene using universal primers 27F and 1426R. PCR amplification was performed with an initial denaturation at

95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension was performed at 72 °C for 5 minutes.

Agarose Gel Electrophoresis and Sequencing

PCR products were separated using 1.5% agarose gel electrophoresis prepared in 1× TAE buffer and stained with ethidium bromide. Amplified DNA fragments were visualized under ultraviolet light to confirm successful amplification. The confirmed PCR products were subjected to Sanger sequencing using Primers 27F and 1426R at Inqaba Biotech. Obtained sequences were compared with available sequences in public databases for taxonomic identification.

Screening of LAB Isolates for Bacteriocin Production

Lactic acid bacteria isolates were screened for bacteriocin-producing ability using agar spot and agar well diffusion methods. Selected isolates were grown in MRS broth at 37 °C for 48 hours, and cultures were centrifuged at 8,000 rpm for 10 minutes at 4 °C to obtain cell-free supernatants (CFS). The CFS was adjusted to pH 7.0 using 1 N NaOH and treated with catalase (100 µg/mL) at 37 °C for 30 minutes to eliminate interference from organic acids and hydrogen peroxide. For preliminary screening, an agar spot (spot-on-lawn) assay was used. Briefly, 5 µL droplets of the treated CFS from each LAB isolate were spotted directly onto the surface of Mueller-Hinton agar plates previously seeded the test organisms (*Escherichia coli* and *Salmonella* spp.) standardized to 0.5 McFarland. The plates were allowed to stand at room temperature for 30 minutes

to permit complete absorption of the spots before overnight incubation. To quantitatively evaluate and confirm the antibacterial activity, the agar well diffusion method was subsequently performed on similarly inoculated Mueller-Hinton agar plates. Wells of 8 mm diameter were made and filled with 8 μ L of the prepared CFS, followed by incubation at 37 °C for 24 hours, the resulting zones of growth inhibition were measured using a digital caliper. To confirm the proteinaceous nature of the antimicrobial compounds, active CFS samples were treated with trypsin and proteinase K. Loss of antimicrobial activity after enzymatic treatment was considered confirmation of bacteriocin activity [31].

Production of Bacteriocin

The most active bacteriocin-producing LAB isolates were cultured in MRS broth, and bacteriocins were extracted from the resulting cell-free supernatant. The supernatant was neutralized to pH 7.0 using 1 M NaOH and treated with catalase to remove interference from organic acids and hydrogen peroxide, ensuring that antimicrobial activity was attributed to bacteriocin production [32].

Optimization of Bacteriocin Production

Optimization of bacteriocin production was performed by evaluating the effects of production parameters, including temperature, pH, carbon source, and fermentation conditions. Response Surface Methodology (RSM) was applied to determine the combined influence of these factors on bacteriocin yield. Bacteriocin activity was expressed as arbitrary units per millilitre (AU/mL), calculated based on inhibition zone measurements according to the method described by [33].

Data Analysis

All experiments were performed in triplicate, and results were expressed as mean $\pm$ standard error of mean (SEM). Statistical analysis was performed using SPSS version 25.0. Optimization data were analyzed using Response Surface Methodology (RSM) based on the Box-Behnken design. Analysis of variance (ANOVA) was used to evaluate the significance of the regression model and the effects of process variables on bacteriocin production. Statistical significance was considered at $p < 0.05$.

RESULTS

Identification of Lactic Acid Bacteria Isolates

Eight presumptive lactic acid bacteria (LAB) isolates were recovered from nono and fufu samples. Colonies grown on de Man, Rogosa and Sharpe (MRS) agar were circular, smooth, and creamy-white in appearance. Microscopic examination revealed that all isolates were Gram-positive and rod-shaped. Biochemical characterization showed that isolates from nono and fufu were negative for catalase, citrate utilization, urease, and indole tests, non-motile, and exhibited no gas production. Based on their morphological and biochemical characteristics, the isolates were tentatively identified as *Lactobacillus* species. Carbohydrate fermentation tests showed that all isolates fermented glucose, sucrose, and fructose, whereas lactose, mannitol, and sorbitol were not fermented.

Molecular Identification

Isolate F1, which exhibited the highest antibacterial activity was selected for molecular identification using 16S rRNA gene sequencing. Successful amplification of the 16S rRNA gene was confirmed by agarose gel electrophoresis (Plate I). Sequence analysis revealed 99.70% similarity with *Lactiplantibacillus pentosus* strain BSR3 strain BSR3, confirming the identity of isolate F1 (Table 1).



Plate I: Gel Electrophoresis of the Amplified 16S rRNA Genes

Table 1: GenBank accession numbers and molecular identification of LAB

Sample ID	Sequence bp	Identity of the closest homologs of the sequence	Identity (%)	Accession N° of the closest homolog
PB1 (F1)	662	<i>Lactiplantibacillus pentosus</i> strain BSR3	99.70%	KY203913.1

Screening of LAB Isolates for Bacteriocin-Producing Potential

The antibacterial activity of cell-free supernatants (CFS) obtained from selected LAB isolates evaluated against *Escherichia coli* and *Salmonella* spp showed inhibition zones that varied among the isolates across both assays (Table 1)." The inhibition zones varied among the isolates (Table 2). Isolate F1 (*Lactiplantibacillus pentosus* strain BSR3) exhibited the highest antibacterial activity, producing inhibition zones of 13.0 mm



against *Salmonella* spp. and 9.5 mm against *Escherichia coli*. A representative agar well diffusion plate showing the inhibitory activity of isolate F1 against *Salmonella* spp. is presented in Plate II. Isolate N1 also demonstrated appreciable antibacterial activity, whereas the remaining isolates showed comparatively lower inhibitory activity. One-way analysis of variance (ANOVA) showed significant differences in antibacterial activity among the isolates against both test organisms (p = 0.0001) (Table 3).

Plate II: Zone of Inhibition of F1 against *Salmonella* spp

Table 2: Antibacterial Activity of CFS of *Lactobacillus* Species against Selected Pathogenic Bacteria

CFS samples	Zone of inhibition (mm)	
	<i>Escherichia coli</i>	<i>Salmonella</i> sp.
<i>Lactobacillus pentosus</i> strain BSR3 (F1)	9.5 ^b ±0.50	13.0 ^c ±0.00
F2	4.0 ^a ±0.00	4.0 ^{ab} ±1.00
F3	3.5 ^a ±0.50	4.5 ^{ab} ±0.50
F4	4.0 ^a ±1.00	4.0 ^{ab} ±0.00
N1	6.0 ^a ±1.00	7.0 ^b ±1.00
N2	3.0 ^a ±1.00	3.5 ^a ±0.50
N3	4.0 ^a ±1.00	3.5 ^a ±0.50
N4	4.0 ^a ±0.00	4.0 ^{ab} ±0.00
Control	12.0 ^b ±0.00	13.5 ^c ±0.50

Key: CFS: Cell free supernatant; Fufu (F) Nono (N). Values are presented as mean ± standard error of mean (SEM) of two replicates. Values with different superscripts in a column are significantly different at $p < 0.05$

Table 3: One-way ANOVA showing the effect of *Lactobacillus* spp CFS against selected pathogenic bacteria

Test organism (Source)	DF	SS	MS	F-value	P-value	Conclusion (Significant difference)
<i>Escherichia coli</i>	8	155.40	19.43	24.98	0.0001	Significant
<i>Salmonella</i> spp	8	264.00	33.00	49.50	0.0001	

Key: Degree of Freedom (DF), Sum of Squares (SS), Mean Square (MS)

Production and Partial Purification of Bacteriocin

Bacteriocin produced by *Lactiplantibacillus pentosus* strain BSR3 was recovered from the cell-free supernatant following neutralization and catalase treatment to eliminate the effects of organic acids and hydrogen peroxide. Partial purification was achieved by ammonium sulfate precipitation at 70% saturation. The precipitated protein fraction was resuspended in phosphate buffer (pH 6.5), yielding a crude bacteriocin preparation.

Optimization of Bacteriocin Production Using Box–Behnken Design

Regression model

The results for the regression model describing bacteriocin production are presented in Table 4. The regression model was statistically significant ($F = 3.23$, $p = 0.0006$), indicating that the selected process variables collectively influenced bacteriocin production. Among the main factors evaluated, temperature ($p = 0.0015$), pH ($p = 0.0002$), fermentation time ($p = 0.0207$), and carbon source type ($p = 0.0081$) significantly affected bacteriocin

production. Carbon source concentration did not significantly influence bacteriocin production within the experimental range investigated ($p = 0.4092$). Among the interaction terms, only the interaction

between fermentation time and carbon source type was statistically significant ($p = 0.0012$), whereas all other interaction effects were not significant ($p > 0.05$).

Table 4: Regression Model for Bacteriocin Production

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1.371E+08	44	3.116E+06	3.23	0.0006	Significant
A-Temperature	1.187E+07	1	1.187E+07	12.29	0.0015	
B-pH	1.758E+07	1	1.758E+07	18.21	0.0002	
C-Conc. of carbon source	6.765E+05	1	6.765E+05	0.7006	0.4092	
D-Fermentation time	5.759E+06	1	5.759E+06	5.96	0.0207	
E-Carbon source	1.097E+07	2	5.486E+06	5.68	0.0081	
AB	2.308E+06	1	2.308E+06	2.39	0.1326	
AC	2.840E+06	1	2.840E+06	2.94	0.0966	
AD	8924.11	1	8924.11	0.0092	0.9241	
AE	8.986E+05	2	4.493E+05	0.4653	0.6324	
BC	1.316E+05	1	1.316E+05	0.1363	0.7146	
BD	2.488E+05	1	2.488E+05	0.2577	0.6154	
BE	3.072E+06	2	1.536E+06	1.59	0.2205	
CD	2.160E+05	1	2.160E+05	0.2237	0.6396	
CE	5.094E+06	2	2.547E+06	2.64	0.0881	
DE	1.652E+07	2	8.258E+06	8.55	0.0012	
Residual	2.897E+07	30	9.656E+05			
Total	1.661E+08	74				

Effect of Process Variables on Bacteriocin Production

The effects of temperature, pH, carbon source concentration, fermentation time, and carbon source type on bacteriocin production are presented in Figure 4.1. Temperature exerted a significant positive effect on bacteriocin production ($p =$

0.0015). Carbon source concentration showed no significant effect ($p = 0.4092$). Increasing pH and fermentation time significantly reduced bacteriocin production ($p < 0.05$), while carbon source type significantly influenced bacteriocin yield.

Optimization of Process Variables for Enhanced Bacteriocin Activity

The optimization of process variables was performed using the Box–Behnken design to determine the combination of fermentation conditions that would enhance bacteriocin activity. Based on the developed regression model, the response optimizer identified the optimum levels of temperature, pH, carbon source concentration, fermentation time, and carbon source type for improved bacteriocin activity. The optimized

conditions for bacteriocin production by *Lactiplantibacillus pentosus* strain BSR3 were an incubation temperature of 37 °C, pH 6.5, 1.5% carbon source concentration, 24 hours fermentation, and glucose as the carbon source. The optimized conditions and the corresponding observed bacteriocin activity are presented in Table 5.

Figure 1: Effect of Temperature, pH, Concentration of Carbon Source, Fermentation Time, and Carbon Source on Bacteriocin Production

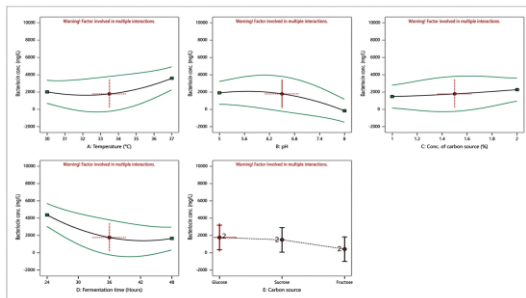


Table 5: Effect of Optimized Fermentation Conditions on Bacteriocin Activity

S/ N	Temperature (°C)	pH	Conc. of carbon source (%)	Fermentation time (hour)	Carbon source	Bacteriocin activity (AU/mL)	Standard error	Desirability	
1	37.000	6.500	1.500	24.000	Glucose	7260.568	620.430	1.000	Selected
2	33.500	8.000	1.500	24.000	Glucose	1862.594	750.501	1.000	
3	30.000	6.500	1.000	36.000	Sucrose	761.401	750.501	1.000	
4	33.500	8.000	2.000	36.000	Sucrose	189.807	750.501	1.000	
5	37.000	5.000	1.500	36.000	Glucose	4248.241	750.501	1.000	
6	33.500	8.000	1.500	48.000	Fructose	1055.381	750.501	1.000	
7	37.000	6.500	1.500	48.000	Glucose	2412.921	750.501	1.000	
8	37.000	8.000	1.500	36.000	Fructose	882.483	750.501	1.000	
9	37.000	6.500	1.000	36.000	Fructose	1814.597	750.501	1.000	
10	36.149	5.325	1.266	32.552	Fructose	1336.674	637.062	1.000	

11	30.000	8.000	1.500	36.000	Fructose	1000.295	750.501	1.000
12	33.500	6.500	1.000	48.000	Glucose	1398.994	750.501	1.000
13	33.500	6.500	1.500	36.000	Glucose	1793.330	982.636	1.000
14	33.500	6.500	2.000	24.000	Fructose	2514.371	750.501	1.000
15	37.000	8.000	1.500	36.000	Glucose	1108.832	750.501	1.000
16	30.000	5.000	1.500	36.000	Fructose	703.588	750.501	1.000
17	33.500	5.000	2.000	36.000	Sucrose	1435.535	750.501	1.000
18	30.000	6.500	2.000	36.000	Fructose	1790.601	750.501	1.000

DISCUSSION

Lactic acid bacteria were isolated from fufu and nono, confirming fermented foods as valuable sources of beneficial microorganisms with biotechnological potential. The morphological and biochemical characteristics of the isolates were consistent with *Lactobacillus* species, while molecular identification using 16S rRNA sequencing confirmed the most active isolate (F1) as *Lactiplantibacillus pentosus* with 99.70% similarity. This supports the importance of traditional fermented foods as reservoirs of antimicrobial-producing LAB [34]. Screening of LAB isolates revealed variations in antibacterial activity, demonstrating that bacteriocin production is strain dependent. *Lactiplantibacillus pentosus* strain BSR3 (F1) showed the highest inhibitory activity against *Salmonella* spp and *Escherichia coli*, with inhibition zones of 13.0 mm and 9.5 mm, respectively. The significant differences among isolates confirmed variation in antimicrobial production. Neutralization and catalase treatment ensured that inhibition was mainly due to bacteriocin activity rather

than organic acids or hydrogen peroxide. The crude bacteriocin recovered through ammonium sulfate precipitation showed potential as a proteinaceous antimicrobial compound, consistent with reports of class II bacteriocin production by *Lactiplantibacillus pentosus* strains [12].

Optimization using Box–Behnken response surface methodology demonstrated that temperature, pH, fermentation time, and carbon source type significantly influenced bacteriocin production, while carbon source concentration had no significant effect within the tested range. The optimized conditions for bacteriocin production were an incubation temperature of 37 °C, pH 6.5, 1.5% carbon source concentration, 24 hours fermentation, and glucose as the carbon source. These conditions favour bacteriocin synthesis by providing suitable environmental conditions for bacterial growth and metabolic activity. Temperature influenced bacteriocin production by affecting cellular metabolism, enzyme activity, and peptide synthesis, with the optimum temperature agreeing with previous reports of maximum LAB bacteriocin production

around 36–37°C [35,36]. The slightly acidic optimum pH supported bacteriocin synthesis, while the fermentation period suggested production during active growth and early stationary phases. The influence of carbon source type indicates that nutrient metabolism plays an important role in regulating bacteriocin biosynthesis. Overall, the study demonstrates that response surface methodology is an effective approach for improving bacteriocin production by *Lactiplantibacillus pentosus* strain BSR3 and highlights its potential as a source of natural antimicrobial compounds.

CONCLUSION

The findings of this study demonstrate that traditional fermented foods are valuable sources of beneficial microorganisms with potential applications in food preservation. The successful identification of a bacteriocin-producing lactic acid bacterium as *Lactiplantibacillus pentosus* confirms that indigenous fermented food products can serve as reservoirs of microorganisms with important biotechnological properties. This study further shows that effective control and optimization of fermentation conditions are essential for improving bacteriocin production. The influence of production parameters and the application of response surface methodology provide valuable insight into enhancing microbial metabolite production and improving the potential utilization of LAB-derived bacteriocins as natural antimicrobial agents.

DECLARATION SECTION

Ethics Approval and Informed Consent.

This study did not use human or animal subjects. Therefore, ethical consideration was not applicable.

Authors' Contribution

Conceptualization: Bala Jeremiah David and Abubakar Asmau Niwoye; **Supervision:** Bala Jeremiah David and James Samaila; **Investigation:** Bala Jeremiah David and John Precious Audu; Writing the first draft of the article: John Precious Audu; **Editing:** Ummulkhair Salamah Ilyasu, John Precious Audu and Enejiyon Ozavize Sherifat. All authors contributed to the development of the final manuscript and approved its submission.

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Disclosure of Conflict of interest.

None

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