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Bacteriocinogenic Activity of *Lysinibacillus fusiformis* NR_042072.1 Isolated from Cow Milk

Research Article

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Abstract

The prevailing increase in the search for bio-preservatives in the food industry has raised global concern. Biological substances with health benefits and no toxicity are considered as alternatives to the chemicals in food processing. Bacteriocins, which are proteinaceous substances with preservative properties, are now gaining attention in this regard and the search for new organisms for production is becoming global. This study focuses on the analysis of bacteriocinogenic activity of a local strain of Lysinibacillus fusiformis NR_042072.1 isolated from fresh cow milk obtained from Gaa Mobolohunduro, Tanke, Ilorin, Kwara State, Nigeria. Lactic acid bacteria were isolated from cow milk, characterized and identified using standard microbiological methods. Bacteriocin was extracted from the isolate, partially purified, and characterized using standard methods; and the antibacterial activity against some foodborne bacteria which are Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus subtilis was determined using agar-well diffusion method. The effect of certain physicochemical parameters on the antibacterial activity of the bacteriocin was also determined. The isolate was identified as Lysinibacillus fusiformis NR_042072.1. The bacteriocin showed antibacterial action against E. coli and P. aeruginosa with a zone of inhibition of 17.0±0.5 and 20.0±1.0 mm, respectively. The bacteriocin was active within temperatures of 4-50 °C, pH 5-7.5, bile salts concentration of 0.6-1.0 % and in the presence of the enzymes trypsin and pepsin. In conclusion, the bacteriocin produced by Lysinibacillus fusiformis NR_042072.1 could control food spoilage caused by the test organisms.

Keywords: Bacteriocin, Fresh Cow Milk, *Lysinibacillus Fusiformis*, Antimicrobial Action, Food Preservation.

1. INTRODUCTION

The menace of food spoilage and foodborne diseases with devastating effects on food production and consumers have become a global challenge. On the other hand, the method of food preservation with chemicals has been reported to be toxic and highly risky, hence, creating more problems than it solves. Research on food preservation has recently been focused on the search for alternative bio-preservatives to ameliorate the health challenges posed by chemicals used in food preservation.

Bacteriocins are protein substances with no toxic implications on humans but effective as an antibacterial agent. (Kaur-Sidhu and Nehra, 2021; O'Connor etal., 2020). They are reported to be selectively toxic on food spoilage microbes without self-destruction of the producing bacteria because they possess self-defensive proteins (Kaur-Sidhu and Nehra, 2021).

Bacteriocins produced by the lactic acid bacteria are generally considered as safe in food and have a high potential to inhibit proliferation and destroy other spoilage bacteria and pathogens in food and thus extend shelf life of food. They are also very stable in food (Johnson et al., 2018; Colombo et al., 2018; Kaur Sidhu and Nehra, 2021). Their bactericidal effect is through lysis of cell wall and membrane, destruction of DNA and prevention of protein synthesis (Meade et al., 2020; Yang et al., 2014). Bacteriocins have been naturally found and consumed in fermented foods throughout human history hence the renewed effort on their development and application in food (O'Connor et al., 2020). However, to date, there are few commercialized acceptable bacteriocins such as leucocin A, nisin, enterocins, pediocin and microgad (Kaur Sidhu and Nehra, 2021; Raj et al., 2021).

Lysinibacillus fusiformis is a member of the family Bacillaceae in the phylum Fermicutes. The bacterium is found in many habitats including soil, plants, and in animals (Hashmi et al., 2020). Species in the genus Lysinibacillus are notably recognized by the presence of a polyphasic cell wall peptidoglycan (Hashmi et al., 2020). Many Bacilli have been studied for their biotechnological potential; in the list are Pediococcus acidilactici, Lactococcus lactis, Streptococcus and Lactobacillus species have been employed in bacteriocin and dairy beverages production (Raj et al., 2021) but the antibacterial bacteriocin production potential of Lysinibacillus has received less attention. The current research is aimed at isolating and identifying bacteriocin-producing organisms for use as alternative to chemical in food bio-preservation.

2. MATERIALS AND METHODS

Source of BacteriaTest Strains

Four common foodborne bacteria strains, namely, *Staphylococcus aureus, Bacillus subtilis* (Gram positive) *Pseudomonas aeruginosa* and *Escherichia coli* (Gram negative) isolated from spoiled food were procured from the culture collection centre of the Microbiology Department, University of Ilorin, Ilorin, Kwara State, to study the antibacterial activity of the bacteriocin produced by the isolated strain.

Isolation and Identification of Lactic Acid Bacteria

Bacteria were isolated from raw milk samples using De Man, Rogosa and Sharpe (MRS) agar (Merck-GranuCult-110660). Plates were incubated at 37^0C for 48 hours. The pure colonies of isolates obtained were maintained on agar slants at 4^0C for further research (Sidhu and Nehra, 2020). Lactic acid bacteria obtained were subjected to molecular identification using 16S rRNA gene amplification technique. DNA was extracted from the isolate using a genomic DNA extraction kit, and universal primers (27F and 1492R) were used to amplify the conserved region of 16S rRNA gene (Sidhu and Nehra, 2020). The pure PCR product was to obtain a consensus sequence using Bioedit software (Hall, 1999). The isolate was confirmed by comparing the homology of the resulting consensus sequence with a database of NCBI using BLAST (Altschul, 1997; Altschul*et al*., 1990), ascension number was obtained from NCBI for the isolate, and phylogenetic tree showing relatedness was also constructed using the BLAST results.

Extraction and Partial Purification of Bacteriocin

Crude bacteriocin was extracted from the isolate following the modified method of Yang *et al*. (2012). A 48-hour old culture of the isolate was subjected to centrifugation at 10, 000 rpm for 15 min at 4^0C to obtain cell-free supernatant (CFS). The pH of the CFS was adjusted to 6.0 using 1N NaOH. It was partially purified by precipitating crude bacteriocin-900 ml (prepared from 1 L MRS broth) with 429.96 g of ammonium sulphate up to 70% saturation levels overnight at 4°C. The mixture was centrifuged at 4000 rpm for 1 hour and the surface and bottom pellicles were harvested and resuspended in sodium phosphate buffer (50 mM, p H 6.5) (Goyal *et al.*, 2018). The solution was filtered using a Millipore filter (0.22 µm) paper.

Determination of Antibacterial Activity of Partially Purified Bacteriocin

The antibacterial screening of the partially purified bacteriocin produced by the isolate was carried out using Agar-well diffusion method (Chen *et al*., 2019), Exactly 0.1 ml of one day old culture of the test bacteria were seeded on to already solidify Mueller Hilton agar plates after the cultures have been adjusted to 0.5 % Mcfarland turbidity (containing approximately 1.5 x 10^8 CFU/ml). Wells bored in the plates were filled with 50 µl of the bacteriocin. The plates were incubated at 37 ^{0}C for 18 hours. Zones of inhibition around the test organisms were measured in millimetres using a scale rule. Bacteriocin activity was determined in terms of the diameters of the zones of inhibition around the wells against the test bacteria.

Effect of Enzymes on Stability of Bacteriocin

The stability and activity of bacteriocins in the gut are affected by the presence of digestive enzymes secreted into the environment for proper digestion of food. Hence, the stability of the partially purified bacteriocin in the presence of some enzymes was determined to mimic gut condition as described by Zhang *et al.* (2018). The bacteriocin was treated with 1 mg/ml of trypsin, pepsin and α-amylase. The mixtures were incubated at 37^0C for 2 hours and heated for 10 min at 95 ^{0}C. The treated bacteriocin was screened for antibacterial potency against the test organisms using agar-well diffusion method.

Thermal Stability of Bacteriocin

The thermal stability of the bacteriocin was determined by incubating the solution at temperatures of 4 – 80^0C for 15minutes (Elayaraja *et al.*, 2014). Effect of temperature on the antibacterial potency of the bacteriocin was determined against the test organisms using agar-well diffusion method.

pH Stability of Bacteriocin

To study the effect of pH on the bacteriocin, the pH of the bacteriocin was adjusted to between 3.0 and 11.0 using 1 N HCl and 1 N NaOH. The mixture was incubated for 30 minutes at 37 ^{0}C (Miao *et al*., 2014). The effect of varying pH values on the antibacterial activity of the bacteriocin was determined against the test organisms using agar-well diffusion method.

Effect of Bile Salts on The Stability of The Bacteriocin

The effect of bile salts on the bacteriocin was studied by adding bile salts of concentrations of 0.1 to 0.6%. The solution was incubated for 30 minutes at 37°C. The effect of varying bile salts concentrations was determined against the test organisms using agar-well diffusion method. Bacteriocins without the bile salts were set as control (Tambekarand Bhutada, 2010).

Statistical Analysis

The data was shown as the mean ± standard deviation (SD, n = 5). The results obtained was analysed using SPSS 18.0 program for Windows (Munich, Germany).

3. RESULTS

Isolation and Identification of Bacteriocin Producing Strain

The isolate was a Gram-positive, catalase- negative, and oxidase-negative bacillus. Sequencing of the amplified 16S rRNA gene and its homology search using BLAST identified the isolate to be *L. fusiformis* (Figure 1). The ascension number NR_042072.1 was assigned. The agarose gel electrophoresis of 16SrRNA amplified genes from *L. fusiformis* NR_042072.1 was presented in Figure 2.

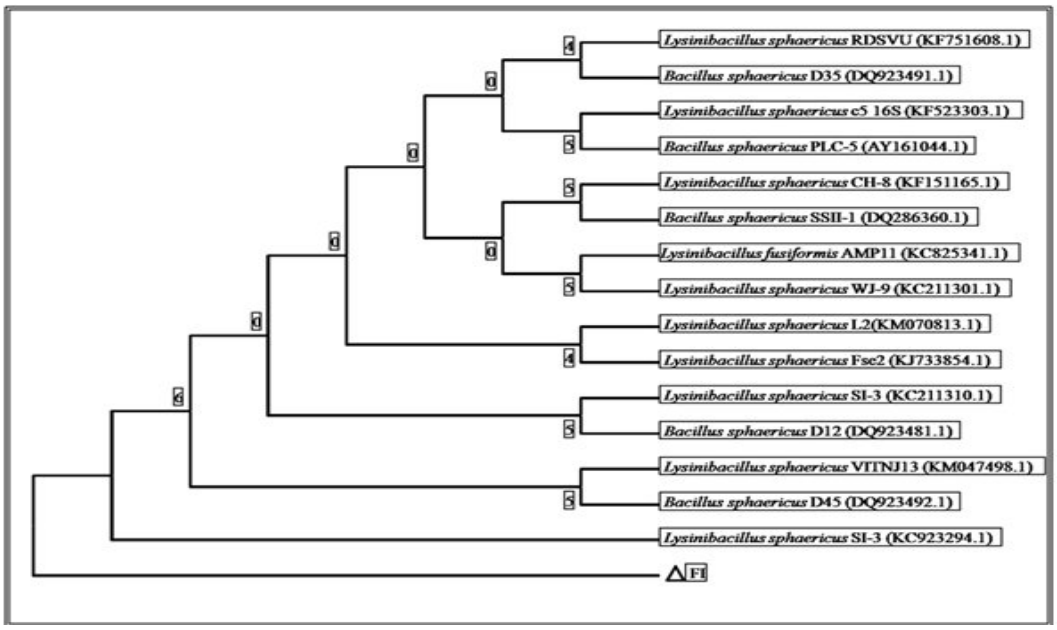

Figure 1. Phylogenetic tree of 16S rRNA gene *L. fusiformis* NR_042072.1 strain

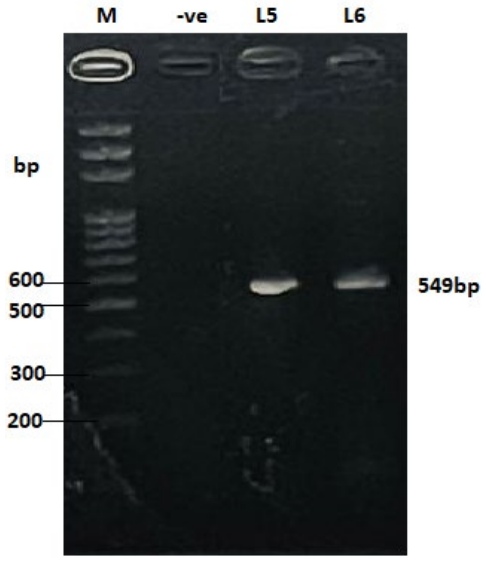

Figure 2. Agarose gel electrophoresis of 16SrRNA amplified genes from *L. fusiformis* NR_042072.1 (L5)

Antibacterial Activity of *L. fusiformis* NR_042072.1 on Test Organisms

The bacteriocin *L. fusiformis* NR_042072.1 was only potent against two of the test bacteria which were *E. coli* and *P. aeruginosa* (Table 1). A zone of inhibition of 20 mm was obtained against *P. aeruginosa* while 17 mm was recorded against *E. coli*.

Table 1. Antibacterial Activity of Bacteriocin from *L. fusiformis* NR_042072.1 on Test Organisms Measured by the Diameter of Zones of Inhibition (mm)

Test Organisms	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
Zone of Inhibition	17±0.5	-	20.0±1.0	-

Key: -=Negative

Characterization of Partially Purified Bacteriocin

Effect of Enzymes on Bacteriocin Produced by *L. fusiformis* NR_042072.1 on Test Organisms

The bacteriocin became active against *S. aureus* (Table 2) after treatment with enzymes. The presence of the enzymes enhanced the bactericidal activity of the proteinaceous bacteriocin.

Table 2. Effect of Enzymes on the Antibacterial Activity of Bacteriocin from *Lysinibacillus fusiformis* NR_042072.1 on Test Organisms Measured by the Diameter of Zones of Inhibition (mm)

Test Organisms		*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
Enzymes	α –Amylase	21.5±0.5	-	19.0±1.0	-
	Pepsin	-	23.0±1.0	21.0±1.0	-
	Trypsin	-	22.5±0.5	-	-

Key: - =No activity

Effect of Temperature on Bacteriocin Produced by *L. fusiformis* NR_042072.1 on Test Organisms

The bacteriocin produced by *L. fusiformis* NR_042072.1 was stable over a wide range of temperature (Table 3). The bacteriocin was still potent against the test bacteria at a relatively high temperature of 50^0C; however, there was no activity at 80^0C.

Table 3. Effect of Temperature on the Antibacterial Activity of Bacteriocin from *L. fusiformis* NR_042072.1 on Test Organisms Measured by the Diameter of Zones of Inhibition (mm)

Test Organisms		*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
Temperature (^{0}C)	4	20.0±1.0	-	16.5±0.5	-
	20	-	-	18.5±0.5	-
	30	-	17.5±0.5	-	-
	50	20.0±0.0	21.5±0.5	-	20.5±1.5
	80	-	-	-	

Key: -=Negative

Effect of pH on Bacteriocin Produced by *L. fusiformis* NR_042072.1 on Test Organisms

The activity of the bacteriocin was better enhanced at pH 5 and 7.5. There was no effect at pH 9. The bacteriocinogenic activity was more pronounced at weakly acidic and near neutral pH (Table 4).

Table 4. Effect of pH on the Antibacterial Activity of Bacteriocin from *L. fusiformis* NR_042072.1 on Test Organisms Measured by the Diameter of Zones of Inhibition (mm)

Test Organisms		*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
pH	5.0	12.5±0.5	-	22.5±0.5	-
	7.5	18.0±0.0	-	25.0±1.0	-
	9.0	-	-	-	-

Key: - =No activity

Effect of Bile Salts on Bacteriocin Produced by *L. fusiformis* NR_042072.1 on Test Organisms

Antibacterial activity of bacteriocin produced by the *L. fusiformis* NR_042072.1 was stable after treatment with 0.6% and 1.0% of bile salts, though the activity was reduced (Table 5).

Table 5. Effect of Bile Salts on the Antibacterial Activity of Bacteriocin from *L. fusiformis* NR_042072.1 on Test Organisms Measured by the Diameter of Zones of Inhibition (mm)

Test Organisms		*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*
Bile salt (%)	0.6	10.5±0.5	-	11.5±0.5	-
	0.8	-	-		-
	1.0	12.0±0.0	11.0±1.0	12.0±0.0	-

Key: - =No activity

4. DISCUSSION

The present investigation highlights the production, characterization, partial purification and antibacterial potency of bacteriocin from *Lysinibacillus* NR_042072.1. The presence of pathogenic organisms in food and their toxic metabolites have been variously reported as a major cause of food related illnesses worldwide. Infections from Foodborne pathogens have become more prevalent in recent decades due to industrialization in general and dependence on ready to eat street-vended foods.

Lactic acid bacteria have been positively identified as a reliable source of bacteriocins to prevent pathogens from surviving and multiplying in food (Zoghi *et al.,* 2021). The obtained phylogenetic tree proved that the isolated bacterium was close to *L. fusiformis* (NR_042072.1). Bacteriocin produced by the *Lysinibacillus* NR_042072.1 was potent on two of the test organisms (*E. coli* and *P. aeruginosa*) but less active against *S. aureus* and *B. subtilis*.

Bacteriocins are generally known to be more effective against closely related Gram-positive species; however, the findings of this study were contrary. The work of Sidhu and Nehra (2020); De-Giani *et al.* (2019) reported the possibility of similar trend antibacterial activity of bacteriocin from bacteria. The activity of bacteriocin through mechanisms such as creating pores in cell envelope, inactivating anionic carrier and enzyme activity, and pore formation in cell membrane could have aided the activity against the Gram-negative bacteria whose outer cell wall is resistance to the action of bacteriocin.

The bacteriocin became more potent after it was exposed to proteolytic enzymes. It became active against *S. aureus* (Table 2) against the bacteriocin not exposed to the enzymes. The presence of the enzymes supposedly contributed to its antibacterial effect. The bacteriocins and enzymes are proteinaceous material and a level of synergy in action is advantageous to bacteriocin's potency. The enzyme amylase, pepsin and trypsin are secreted naturally in the gut to aid food digestion, the bacteriocin must be relatively stable in the presence of these enzymes if there will be any activity. Contrary to this finding was the report of Sidhu and

Nehra (2020); Zhang *et al*. (2018); Elayaraja *et al*. (2014) on partial and complete loss of antibacterial potency of bacteriocins after exposure to enzyme treatment. The enhanced ability of the bacteriocin against the test organisms after treatment with the enzymes confirms its proteolytic nature.

The antibacterial potency of the bacteriocin was most prominent between temperatures of 4-50^{0}C, with 50^{0}C as the optimum. Though it was still stable at 80^{0}C, there was no antibacterial potency at this very high temperature. Bacteriocins been protein are heat sensitive and are denatured at high temperature. The potency of the bacteriocin between 30-50^{0}C is desirous because this shows that it will be active in the human digestive system and in food processing. A similar submission was made by Sidhu and Nehra (2020) on loss of potency at high temperatures. On the contrary, Zhang *et al*. (2018) reported that bacteriocin Lac-B23 retained its antibacterial effect at an extremely high temperature of 121^{0}C. In another study, bacteriocin from species of *Lactobacillus* remained active after heat treatment (Moghaddam *et al.,* 2006). The variation in activity observed could be based on the fact that bacteriocins are unique in their behavior, being protein, they are sensitive to heat and the fact that the test organisms were not the same coupled with varied environmental questions. However, the bacteriocin obtained in this study was still effective at 50^{0}C and could therefore be used in food and dairy industry for food preservation at moderately high temperature. pH is notable among the factors that affect the antibacterial activity of bacteriocins. From the study, bacteriocin produced by *L.* NR_042072.1 was limited at high acidic and alkaline pH but more potent around pH 5 as the optimum and towards neutrality (Table 4). This observation is similar to the previous authors' report on the stability and activity of bacteriocin at pH around neutral (Sidhu and Nehra, 2020; Zhang *et al.*, 2018). In another report, antibacterial activity of bacteriocin was found to vary indirectly with the increase in pH until zero activity was detected (Wang *et al.*, 2018).

The gastrointestinal tract of man and other animals secretes bile salts; which is required for complete breakdown of lipids during digestion, bacteriocin must not be inhibited by the bile salts for effectiveness hence, the antibacterial activity of the synthesized bacteriocin was tested in the presence of bile salts at concentration similar to the gut; the bacteriocin however, was relatively stable and active against the test organisms in the presence of bile salts although zones of inhibition were reduced. This observation suggests that the bacteriocin could remain stable in the gut if consumed in food and be active against foodborne pathogens in the presence of bile salts secreted in the gut.

5. CONCLUSION

This study reports the extraction of bacteriocin from lactic acid bacteria *L. fusiformis* NR_042072.1 isolated from fresh cow milk. The bacteriocin displayed antibacterial activity against both Gram-positive and Gram-negative foodborne pathogens tested suggesting its potential for usability as a bio-preservative in food.

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