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Optimization, Partial Purification, and Characterization of Bioactive Peptides of Lactobacillus paracasei Isolated from Traditional Egyptian Cheese

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HIGHLIGHTS

- *Lactobacillus paracasei* isolated from traditional Egyptian cheese has great antimicrobial activity against pathogens.
- OFAT (One-Factor-at-a-Time) and RSM (Response Surface Methodology) were used to improve the yeild of bacteriocin.
- *L. paracasei* purified bacteriocin may be suitable for food applications as natural preservative.

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Acronyms and abbreviations
CCD=Central Composite Design
CFS=Cell Free Supernatant
DTT=Dithiothreitol
EDTA=Ethylene Diamine Tetraacetic Acid
LAB=Lactic Acid Bacteria
MRS=deMan Rogosa and Sharp
OFAT=One-Factor-at-a-Time
RSM=Response Surface Methodology

ABSTRACT

Background: Bacteriocins are small peptides which are ribosomally synthesized and have been shown to have wide range of antimicrobial activity. The aim of this study was to optimize the production of *L. paracasei* MG847589 bacteriocin. Furthermore, the potential antibacterial properties of the novel bacteriocins were characterized and evaluated against *Staphylococcus aureus*.

Methods: The present study optimized the growth media constituents of *Lactobacillus paracasei* MG847589 to improve bacteriocin yield by applying One-Factor-at-a-Time (OFAT) and Response Surface Methodology (RSM) methods.

Results: At OFAT, two-fold activity increased against *Staphylococcus aureus* in the presence of whey (22.5 g/L) as nitrogen source and sucrose (30 g/L) as carbon source. RSM tool was performed with media compounds using design expert 12.0.1.0. Whey (22.5 g/L), sucrose (30 g/L), temperature (30 ºC), and pH (6.5) condition yielded 25,600 AU/ml of bacteriocin against *S. aureus*. Bacteriocin was stable at pH range of 2.0 to 8.0 for one h and at 60 ºC for 15 min. The produced antimicrobial peptide is a novel bacteriocin with molecular mass of 2,611.122 Da.

Conclusion: Bacteriocin of *L. paracasei* MG847589 isolated from traditional Egyptian cheese (Kareish) showed great antimicrobial activity and could be applied as food preservative in food manufacturing.

Introduction

Food-borne pathogens cause many illnesses, with substantial damage to human health and economy. Lactic Acid Bacteria (LAB) have been used in food for a long time and generally recognized as safe by the scientific

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community. Through the production of various antimicrobial compounds, such as organic acid, hydrogen peroxide, ethanol, diacetyl, carbon dioxide, bacteriocin, or antibiotic-like substances, they have the ability to block or limit contamination by spoilage and/or disease germs (Bintsis, 2017).

In all of the major bacterial lineages and several of the Archaea, bacteriocins, a small family of inhibitory peptides, have been discovered (Gillor et al., 2008). Theses peptides are ribosomal synthesized inhibit the closely or non-closely related bacteria (Singh and Ghosh, 2012). Genes encoded bacteriocins held in chromosomes or cytoplasmic plasmids (Campelo et al., 2014). As they have ability to prevent microbial contamination throughout the vegetables, dairy, cheese, meats, and other food products, bacteriocins like nisin are deemed acceptable for use in the different food applications as a food preservative (Udhayashree et al., 2012). Although, the bacteriocins have a long history dating back to the early 1920s, the antimicrobial activity was discovered in 1928 as the colicin bacteriocin of *Escherichia coli* V shows inhibitory activity against *E. coli* S (Yang et al., 2014).

Before 1951, bacteriocins were not utilized in food items. By 1969, nisin was the first bacteriocin purified from *Lactococcus lactis* subsp. *Lactis* and used in food preservation (FAO/WHO, 2002). Then, in 1988, Food and Drug Administration (FDA) approved nisin for use in canned products in the United States to inhibit the growth of *Clostridium botulinum* (Shehata et al., 2019a). Different classes of bacteriocins have been identified, and when exposed to gastrointestinal enzymes in the stomach, they were proven to be inactive and safe for intake by humans (Khandelwal and Upendra, 2019).

Class I bacteriocins known as lantibiotics are linked to the type II lipid of the bacterial membrane, which acts as a transmitter of N-acetylglucosamine and N-acetylmuramic acid subunits of peptidoglycan layer from the bacterial cytoplasm to its cell wall. This activity encourages cell death and stops the formation of the bacterial cell wall (Pérez-Ramos et al., 2021). In case of bacteriocins class II also contain amphiphilic helical topologies that allow them to permeate the bacterial membrane, induce depolarization, and eventually kill the bacterial cell (Kaur and Kaur, 2015). Class III bacteriocins cause bacterial lysis, accelerate bacterial mortality, and hasten the disintegration of Gram-positive bacteria's cell walls (Shehata et al., 2019b). There are several layers in the human Gastrointestinal (GI) tract system, including the mucosa, submucosa, epithelial cell lining, mucus layer, and serosa (Jones et al., 2022). In the human gastrointestinal tract, probiotic microorganisms inhabit the gut and release bacteriocins to compete with the sensitive bacteria, thereby lowering the load of bacteriocin-sensitive bacteria there. The colonized probiotic bacteria may generate bacteriocins below the minimal inhibitory concentration levels because of the harsh natural conditions in the human gut; as a result, it suppresses bacterial development and is safe for humans (Dicks et al., 2018). For optimal commercial application, it will be critical to maximize bacteriocin production by modifying both genetic regulation and environmental growth variables.

Recent research has documented how some aspects of environmental growth conditions on the formation of bacteriocins (Bhattacharya and Das, 2010). Optimization of bacteriocin biosynthesis and enhancement of its activity may be of enormous economic relevance (Soltani et al., 2021). The growing environment and medium compositions have a significant impact on the development of bacteria and the accumulation of their metabolites.

It is challenging to identify the key parameters and optimize them for biotechnological procedures that involve multiple variables. In order to get high yields of the required metabolites, the typical "One-Factor-at-a-Time (OFAT) strategy" was utilized in medium optimization, but this method ignores the intricate relationships between numerous physicochemical factors (Wang and Liu, 2008). Factorial design and response surface analysis are examples of statistically based experimental designs that meet this requirement. It is more efficient to optimize microorganism metabolite synthesis utilizing Response Surface Methodology (RSM), an experimental strategy for determining optimal conditions in a multivariable system (He et al., 2004). A collection of statistical approaches for planning experiments, analysing the effects of factors, and looking for optimal settings of factors to achieve a desired goal are all included in RSM (Li et al., 2002). This approach has been used successfully to optimize medium composition, enzymatic hydrolysis conditions, and food preservation and fermentation process parameters (He et al., 2009).

The aim of this study was to optimize the production of *L. paracasei* MG847589 bacteriocin in simple growth media constituents instead of complex deMan Rogosa and Sharp (MRS) by RSM. Furthermore, the potential antibacterial properties of the novel bacteriocins were characterized and evaluated against *Staphylococcus aureus*.

Materials and methods

Isolation of LAB strain

Probiotic *L. paracasei* KC39, which was previously isolated from traditional Egyptian cheese (Kareish) and identified by 16S rDNA sequence analysis (GenBank accession number MG847589) and stored at -20 °C. Culturing of the isolated probiotic was in MRS broth

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with 1% bacterial supplement for 20 h at 30 °C in an anaerobic jar (Oxoid, Waltham, USA).

Quantification of antimicrobial activity

The LAB strain were cultivated in MRS broth medium for 16-18 h with inoculum 1%, cells were removed from MRS medium by centrifugation (6,500×g for 10 min, 4 °C) to obtain Cell Free Supernatant (CFS). Antibacterial and antifungal activity in the supernatant was determined by two-fold dilution method according to Shehata et al. (2016) against seven pathogens, six food-borne pathogens (*Salmonella senftenberg* ATCC 8400, *E. coli* BA 12296, *Bacillus cereus* ATCC 49064, *Listeria monocytogenes* ATCC 19116, and *S. aureus* NCTC 10788) and two fungi (*Penicillium chrysogenum* ATCC 10106 and *Aspergillus parasiticus* ITEM 11). Lawns of indicator strains were prepared by adding 0.125 ml (2×10^7) of 10× diluted overnight culture to 5 ml suitable soft agar (0.75%). The contents of culture tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Ten µl of each CFS were spotted on the surface of the soft agar plate. The plates were incubated at 30 °C for 48 h then examined for inhibition zones. Clear zones around the spots indicate the antibacterial activity of isolated bacteria. Antifungal activity, expressed as arbitrary units (AU/ml), was defined as the reciprocal of the highest dilution at which fungal growth was inhibited. The antifungal titre was calculated as (1000/d) D, where D is dilution factor and d is the dose (the number of antifungal samples pipetted on each spot). Moreover, antifungal activity also evaluated using well diffusion assay. The plates were incubated at 30 °C for 48 h and examined for inhibition zones (Shehata et al., 2016).

Initial stage of optimization

OFAT was used to select affordable carbon and nitrogen sources. To test the effect of carbon sources, each carbon source (molasses were obtained from The Egyptian starch, yeast and detergents Co., Alexandria, Egypt; sucrose, malt extract, lactose, fructose, and soluble starch, dextrin purchased from Fisher Scientific, USA) were added at 20 g/L level to MRS medium, by replacing 20 g/L glucose and the influence of nitrogen sources in a modified MRS medium was used by replacing protease peptone with each nitrogen source such as corn flour meat extract, soybean meal, casein, peptone, tryptonea, urea, and whey at 2% level. Cultivation was done for 24 h of incubation at 30 ºC with pH 6.5. Antimicrobial activity against *S. aureus* NCTC 10788 was also examined using spot-on-lawn method, as described previously. CFS was serially diluted two-fold. MRS broth was used as diluents and each diluted solution of 10 µl was loaded on solidified mannitol salt agar (Waltham, USA) lawn containing *S. aureus* NCTC 10788. The bacteriocin activity was expressed as activity unit (AU/ml).

RSM

To increase the production of bacteriocin, RSM was used to find the ideal amounts of the relevant factors from OFAT. The Central Composite Design (CCD) was used in this study to perform RSM. Each factor in CCD has a distinct number of levels (Shehata et al., 2021). Thirty trials were conducted with four independent variables, X_1 (whey as nitrogen source), X_2 (sucrose as carbon source), X_3 (temperature), X_4 (pH) at 3 coded levels (+1, 0, -1). A second order polynomial equation was used to explain the data (Wang and Liu, 2008). The relationship between the variables and the response was deduced using the equation. Polynomial equations of the second degree are as follows:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_{12}+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4 \quad (1)$$

Where, β13, β14, β23, β24, β34 the interaction co-efficients, β1, β2, β3, β4 the linear co-efficients, β11, β22, β33, β44 the squared co-efficients, and β12, β0 is the model constant, Y is the predicted response.

Design expert software was used to create all of the experimental trials and estimations (Version 12.0.1.0, State-Ease, Minneapolis, MN, USA). Analysis of variance (ANOVA) was used to find statistically significant factors in the data, which was then used to evaluate the impacts of each variable.

Bacteriocin stability

Bacteriocin resistance up on enzyme incubation according to Noonpakdee et al. (2003) method. Proteinase K, trypsin, chymotrypsin, amylase, and lipase were incubated with 200 µl of filter-sterilized CFS at a final concentration of 0.1 mg/ml. For 3 h, each sample was incubated at 37 °C. After 5 min of boiling, the reaction was brought to a halt. The pH tolerance of the bacteriocin was evaluated at pH values ranging from 2.0 to 10.0 for 2 h at 30 °C. To investigate the impact of pH on antimicrobial activity, each sample was returned to their original pH of 6.5 after treatment in several pH tests. Heat stability was determined after bacteriocin incubation at 40, 50, 60, 70, 80, 90, 100 °C for 15 min. CFS containing bacteriocin was incubated with the following detergents: Ethylene Diamine Tetraacetic Acid (EDTA) at final concentrations of 0.1, 0.3, and 0.5 mM; β-mercaptoethanol; Dithiothreitol (DTT); urea; Triton X-100; Triton X-114; Tween 20; and Tween 80, at final concentrations of 1%. There were two options for the control: CFS or detergent, both in pH 7.0 sodium phosphate buffers. At 30 °C for 5 h, all samples and controls were incubated (Chumchalová et al., 2004); *S. aureus* as an indication of residual bacteriocin

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antibacterial activity was determined using the spot-on-lawn method (Shehata et al., 2016).

Kinetics of bacteriocin production

Bacteria from *L. paracasei* MG847589 were added to the MRS broth overnight and incubated at 30 °C for 24 h under non-regulated pH conditions. The culture's pH and optical density (600 nm) were monitored every 2 h. This was conducted in conjunction with measuring antimicrobial activity in CFS every 2 h using the spot-on-lawn method (Barefoot and Klaenhammer, 1983). The experiment was repeated three times.

Preparation and partial purification of crude bacteriocin

CFS was collected in the same manner as detailed in the preceding section. Ammonium sulphate (80% saturate) was used to precipitate crude bacteriocin, and the precipitate was dialyzed with ultrapure water at 4 °C to desalt it, then, centrifuged at 10,000 g for 20 min at 4 °C for collection. After that, a small amount of a 10 mM ammonium acetate buffer (pH 6.0) was used to disperse the precipitated proteins, which were dialyzed for 16 h at 4 °C with a 1 kDa pore size dialysis membrane (spectrum labs, USA).

The dialyzed crude bacteriocin was filtered through a 0.45 micron filter membrane before being submitted to NGC chromatography (Bio-Rad, Hercules, CA, USA). Tris-HCl buffer was used to equilibrate the Enrich Q 5/50 mm anion exchange column (pH 8.8; 20 mM). The pre-equilibrated column was then loaded with 100 L of bacteriocin at a flow rate of 0.5 ml/min and washed for 10 min with Tris-HCl buffer. After then, a linear gradient of 1 M NaCl (0-100%) at a flow rate of 0.5 ml/min was used for elution. The 280 nm wavelength was used to monitor the fractions, which were then collected in 1 ml portions. Protein profiling by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and antimicrobial activity was conducted for each fraction. Antimicrobial activated fraction lyophilized, suspended in 200 L of 50 mM phosphate buffer (pH 6.5), then purified by size exclusion (ENrich SEC70, 10/300 mm) (Ge et al., 2016). Phosphate buffered saline was used for elution at a flow rate of 1 ml/min for 100 min. Fractions were obtained using fraction collection (NGCTM, Bio-Rad Laboratories, Inc., USA) and monitored at a wavelength of 280 nm. The fractions were lyophilized using a lyophilizer (0.04 Mbar; FTS Process, USA). The fractions were dissolved in a phosphate buffer (pH 6.0) and utilized in the activity assay of bacteriocins. The protein content was determined using the Bradford technique with bovine serum albumin as a reference (Altuntaş et al., 2014).

Molecular weight of bacteriocin

Molecular weight of bacteriocin was estimated by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF/TOF-MS). Bruker was used for antimicrobial activated fractions after lyophilization and dissolution in phosphate buffer (pH 6.0).

Results

Screening of LAB isolates for their antimicrobial activity

The agar spot assay was used to evaluate the antimicrobial activity of 76 LAB isolated from Domiatti and Kareish cheese and their CFSs. Practically, 12 out of the 76 tested strains showed a significant influence on reducing the proliferation of pathogens. The supernatant from these 12 strains was exhibited an antimicrobial effect against food pathogenic microbes as *L. monocytogenes* ATCC 19116, *S. senftenberg* ATCC 8400, *B. cereus* ATCC 49064, *E. coli* BA 12296, and *S. aureus* NCTC 10788; and two strains of toxigenic fungi, *A. parasiticus* ITEM 11 and *P. chrysogenum* ATCC 10106 which had the ability to produce mycotoxins. Strains with positive antimicrobial activity are presented in Table 1. They had distinct antimicrobial activity against *L. monocytogenes*, *S. senftenberg*, *E. coli*, *B. cereus*, *S. aureus*, *A. parasiticus* ITEM 11, and *P. chrysogenum*. Among the 12 isolates of LAB, only one isolate (KC39) showed a significant activity of 6,400 AU/ml against *S. aureus* and *A. parasiticus* ITEM 11 while showed moderate activity of 3,200 AU/ml against *L. monocytogenes*, *S. senftenberg*, and *E. coli*. On the other hand, five isolates exhibited inhibitory activity of 3,200 AU/ml against each of *E. coli* and *L. monocytogenes*.

Primary optimization of bacteriocin

At first, OFAT optimization was analysed the effect of nitrogen, carbon sources, temperatures, and pH on bacteriocin yield by *L. paracasei* MG847589 (Table 2). The maximum antimicrobial activity (12,800 AU/ml) against *S. aureus* NCTC 10788 was showed in whey protein media supplementation (Figure 1A). The maximum bacteriocin activity was 12,800 AU/ml, 6,400 AU/ml for sucrose and lactose, respectively. In case of other carbon sources, the antimicrobial activity was less than 12,800 AU/ml. The results showed that sucrose was the best carbon source for obtaining the maximum bacteriocin antibacterial activity when compared to fructose and other sugars (Figure 1B). After that, the temperature and starting pH of the medium were measured and analysed. *L. paracasei* MG847589 is a mesophilic bacterium that thrives at moderate temperatures, hence the

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ideal temperature for producing bacteriocin was between 30 and 35 °C (Figure 1C). In this study, maximum bacteriocin production was obtained at pH 6-7. However, gradual decrease in the activity was observed as pH increases (Figure 1D). After OFAT optimisation, several approaches have been implemented to further increase bacteriocin production for potential applications. This study is the first study on enhanced bacteriocin production using whey as simple source in modified MRS media. Hence, whey and sucrose were selected as the potential nitrogen and carbon source for production of bacteriocin from *L. paracasei* MG847589.

Experimental design of RSM

Whey protein, sucrose, pH, and temperature were selected for the optimization by RSM. Table 3 shows the impact of four independent variables on the yield of bacteriocin as assessed by CCD over the course of 30 trials. The maximum antimicrobial activity obtained from RSM was 25,600 AU/ml.

Equation 1 states that the entire actual model for bacteriocin production was derived from the second-order regression equation created by fitting the regression analysis data to a quadratic model.

$$Y=-2.57581E+05+2,682.22222\ A-268.33333\ B+10,806.66667\ C+27,914.81481\ D+24.00000\ AB+26.66667\ AC-17.77778\ AD+28.00000\ BC+13.33333\ BD-80.00000\ CD-83.70370\ A^2-17.08333\ B^2-188.33333\ C^2-2,159.25926\ D^2 \quad (1)$$

Where Y is bacteriocin activity (AU/ml), A is whey as nitrogen source (g/L), B is sucrose as carbon source (g/L), C is temperature (ºC), and D is pH. The optimum condition that produced the highest yield of bacteriocin was whey protein (22.5 g/L), sucrose (30 g/L), supplemented media, temperature 30 °C, pH 6.5, and incubation for 20 h (Run 30). The value of predicted R^2 (0.9701) and adjusted R^2 (0.941) are almost equal. Furthermore, a high value of the coefficient value (R^2=0.97) has shown that the mathematical model is adapted appropriately. From the ANOVA analysis for bacteriocin production, all linear terms A, B, C, D, and interaction terms AB, AC, and BC, and all quadratic terms were statistically significant (p=0.045). It is evident from the Table 4, all linear terms, interaction terms AB, AC, and BC; and all quadratic terms were statistically significant (p=0.048). The current research showed that nitrogen, carbon sources, temperature, and pH were the main contributors in bacteriocin production with 95% confidence level. Table 4 showed that all variables interaction effects significantly the bacteriocin production except AD, BD, and CD at 95% confidence level. Response surface plots with oval shape was used to compare between any two variables. Figure 2 explains the response surface plots which revealed that there was significant effect of nitrogen, carbon sources, incubation temperature, and pH on bacteriocin synthesis over a 20 h period. Incubation at 30-35 °C, a pH of initial (5.5-6.5), and a period of 20 h were also studied for increasing of bacteriocin synthesis. These results indicated that the increase whey protein as nitrogen source of the medium revealed an increasing the bacteriocin yield. So, the interaction of AB (whey, sucrose) and AC (whey, temperature) has a positive effect on activity of bacteriocin against *S. aureus* (Figure 2A, C). The 3D plots in Figure 2 illustrate the significant effects of whey and sucrose on bacteriocin yield and their correlation with temperature and pH. As seen in Figure 2B, the optimum activity was obtained at 22.5 g/L whey concentration with temperature (30 ºC) at the same range as mentioned earlier. Further, Figure 2C shows that sucrose concentration (30g/L), temperature at 30 ºC increased the bacteriocin production. In a Figure 3, normal probability versus residuals was plotted, showed that data was very similar to the straight line and positioned on both sides, suggesting that the model is reasonably good. The standard percent probability vs. studentized residuals showed that the precise values provide the model with a sufficient estimate. Moreover, the residual plot along a straightaway line was satisfied with a normality assumption and proved the accuracy of CCD. On experimentation, the observation of bacteriocin yield from the *L. Paracasei* MG847589 was 25,600 AU/ml prove that the model is suitable.

Stability of L. paracasei bacteriocin

Preliminary experiments showed that the treatment of CFS of *L. paracasei* MG847589 with catalase and adjustment of their pH to 6.5 had no effect the antibacterial activities against *S. aureus*. The CFS of *L. paracasei* MG847589 was treated by a variety of proteolytic enzymes to verify the protein nature of the antibacterial substance (Table 5). The antibacterial compound of the CFS of *L. paracasei* MG847589 was completely degraded after treatment with proteinase K and showed no antibacterial activity when *S. aureus* used as indicator strain. In addition, the treatment of this CFS with other proteolytic enzymes caused remarkable decrease in its antibacterial activity (50-75%). Similarly, the activity remained stable after the treatments with different enzymes, including lipase and α-amylase.

The effect of pH on the activity and stability of bacteriocins (the CFSs) was evaluated (Table 5). CFS from *L. paracasei* MG847589 remained fully active against *S. aureus* at pH values ranging from 2 to 7. Reduction of activity was observed above pH 8. The activity was completely eliminated at pH 9 and 10.

The bacteriocin's heat stability was assessed by heating the CFS to 40, 60, 80, and 100 ^{o}C (Table 5). The heat treatment of bacteriocin produced by *L. paracasei*

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MG847589 after 15 min at 40 °C indicated no changes in inhibitory action. The antibacterial activity of CFS of *L. paracasei* MG847589 reduced up to 50% at 60 ºC and up to 75% at 80 ºC (Table 5).

Reduction of bacteriocin activity of *L. paracasei* MG847589 magainst *S. aureus* ranged from 50 to 93.5, 0 to 50, and 50 to 100% for anionic, non-ionic, and cationic surfactant, respectively (Table 5). Bacteriocin activity of *L. paracasei* MG847589 against *S. aureus* remained stable when treated with triton X-114 and tween 20. Other surfactants including EDTA (0.1 mM), EDTA (0.3 mM), Sodium Dodecyl Sulfate (SDS), Triton X-100, Tween 80, and urea reduced the bacteriocin activity by 50%. However, exposing to 0.5 mM EDTA and 1% DTT completely eliminated bacteriocin activity.

Kinetics of bacteriocin production

After 20 h of incubation, the bacteriocin inhibitory activity in the growth broth significantly increased (Figure 4), showing the highest level of inhibitory activity. After 20 h of incubation, this was the midpoint of the stationary phase, and the activity gradually decreased until completion of test at 24 h. *L. paracasei* MG847589 bacteriocin showed higher antimicrobial activity against *S. aureus* after 20 h of incubation (Figure 4).

Purification of bacteriocin from L. paracasei

The CFS of *L. paracasei* MG847589 containing bacteriocin with activity 25,600 AU/ml was recovered by ammonium sulphate precipitation. The precipitate obtained by 75% ammonium sulphate saturation with a 1.59-fold increase has a specific activity of 168.42 AU/mg (Table 6). The 14th to 27th fractions, which likewise displayed the highest protein concentration (A_{280}), were the only ones that exhibit bacteriocin activity (Figure 5). With a 7.40-fold purification, all of the identified fractions that were pooled produced a specific activity of 780.48 AU/mg.

The pooled fraction was subjected to the next step of purification by gel filtration chromatography. The sample was eluted in the form of one systematic peak as observed by A_{280} absorbance; also, the bacteriocin activity was found only in the first peak of A_{280} within the 8th to 15th fractions. With a 20.80-fold purification, the pooled fractions displayed a specific activity of 2,191.78 AU/mg (Table 6).

Molecular mass of bacteriocin

SDS-PAGE analysis of the various bacteriocin purification procedures revealed only one band, demonstrating the excellent purity of the bacteriocin obtained during gel filtration.

It was found that bacteriocin has a low molecular weight about 2.611 kDa (Figure 6). Additionally, the sample had a high-density peak at 2,611 Da, as shown in the MALDI-TOF chromatogram (Figure 7).

Table 1: Antimicrobial activities of Cell-Free Supernatants (CFSs) of 12 Lactic Acid Bacteria (LAB) isolates against food-borne pathogens

Isolate No.	Isolate code	Antimicrobial activity against indicator strain expressed in AU/ml						
		L. monocytogenes	*S. senftenberg*	*B. cereus*	*E. coli*	*S. aureus*	*A. parasiticus*	*P. chrysogenum*
1.	KD3	800	3,200	3,200	6,400	0	800	0
2.	KD8	1,600	400	400	3,200	0	800	0
3.	KD16	400	400	400	3,200	800	400	0
4.	KD23	400	800	800	3,200	1,600	400	0
5.	KD27	3,200	400	1600	6,400	800	800	400
6.	KC2	3,200	400	3,200	3,200	3,200	800	400
7.	KC11	800	1,600	400	1,600	1,600	400	400
8.	KC12	400	0	400	1,600	0	400	0
9.	KC30	3,200	0	1,600	1,600	1,600	400	800
10.	KC34	1,600	3,200	400	3,200	1,600	800	400
11.	KC39	6,400	6,400	3,200	6,400	6,400	12,600	3,200
12.	KC40	1,600	1,600	400	1,600	3,200	6,400	400

L. monocytogenes=Listeria monocytogenes ATCC 19116*; S. senftenberg=Salmonella senftenberg* ATCC 8400*; B. cereus=Bacillus cereus* ATCC 49064*;*
E. coli=Escherichia coli BA 12296*; S. aureus=Staphylococcus aureus* NCTC 10788; *A. parasiticus=Aspergillus parasiticus* ITEM 11;
P. chrysogenum=Penicillium chrysogenum ATCC 10106.
AU/ml=Activity Unit/ml calculated according to the following equation: 1000/d×D (D: dilution factor; d: amount of supernatant used).

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Table 2: The coded and actual levels of variables for bacteriocin production

Independent variables	Levels of variables		
A: Nitrogen source (g/L)	15	22.5	30
B: Carbon source (g/L)	20	30	40
C: Temperature (Cº)	25	30	35
D: pH	5	6.5	8

Table 3: Experimental design and results of Central Composite Design (CCD) for optimization of bacteriocin production

Run	Block	Std	Non coded levels				Bacteriocin activity (AU/ml)
			N source (g/L)	C source (g/L)	Temperature (Cº)	pH	
1	Block 1	17	22.5	30	30	6.5	25,600
2	Block 1	6	30	20	35	5	12,800
3	Block 1	9	15	20	25	8	3,200
4	Block 1	10	30	20	25	8	3,200
5	Block 1	20	22.5	30	30	6.5	25,600
6	Block 1	3	15	40	25	5	6,400
7	Block 1	12	30	40	25	8	6,400
8	Block 1	13	15	20	35	8	3,200
9	Block 1	16	30	40	35	8	12,800
10	Block 1	1	15	20	25	5	12,800
11	Block 1	7	15	40	35	5	12,800
12	Block 1	4	30	40	25	5	12,800
13	Block 1	19	22.5	30	30	6.5	25,600
14	Block 1	18	22.5	30	30	6.5	25,600
15	Block 1	11	15	40	25	8	3,200
16	Block 1	15	15	40	35	8	3,200
17	Block 1	8	30	40	35	5	25,600
18	Block 1	14	30	20	35	8	6,400
19	Block 1	5	15	20	35	5	12,800
20	Block 1	2	30	20	25	5	12,800
21	Block 2	26	22.5	30	40	6.5	12,800
22	Block 2	28	22.5	30	30	9.5	400
23	Block 2	30	22.5	30	30	6.5	25,600
24	Block 2	23	22.5	10	30	6.5	12,800
25	Block 2	27	22.5	30	30	3.5	12,800
26	Block 2	25	22.5	30	20	6.5	1,600
27	Block 2	22	37.5	30	30	6.5	12,800
28	Block 2	29	22.5	30	30	6.5	25,600
29	Block 2	24	22.5	50	30	6.5	25,600
30	Block 2	21	7.5	30	30	6.5	25,600

AU/ml=Activity Unit/ml calculated according to the following equation: 1000/d×D (D: dilution factor; d: amount of supernatant used).

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Table 4: Analysis of variance for the quadratic polynomial model for bacteriocin production by *Lactobacillus paracasei* MG847589

Source	Sum of Squares	df	Mean Square	F-value	*p*-value
Block	1.803E+06	1	1.803E+06		
Model	2.228E+09	14	1.592E+08	32.40	<0.0001
A-N source	1.382E+08	1	1.382E+08	28.14	0.0001
B-C source	7.211E+07	1	7.211E+07	14.68	0.0018
C-Temp	1.092E+08	1	1.092E+08	22.24	0.0003
D-pH	3.527E+08	1	3.527E+08	71.79	<0.0001
AB	5.184E+07	1	5.184E+07	10.55	0.0058
AC	1.600E+07	1	1.600E+07	3.26	0.0927
AD	6.400E+05	1	6.400E+05	0.1303	0.7235
BC	3.136E+07	1	3.136E+07	6.38	0.0242
BD	6.400E+05	1	6.400E+05	0.1303	0.7235
CD	5.760E+06	1	5.760E+06	1.17	0.2972
A^2	6.080E+08	1	6.080E+08	123.78	<0.0001
B^2	8.005E+07	1	8.005E+07	16.30	0.0012
C^2	6.080E+08	1	6.080E+08	123.78	<0.0001
D^2	6.474E+08	1	6.474E+08	131.80	<0.0001
Residual	6.877E+07	14	4.912E+06		
Lack of Fit	6.877E+07	10	6.877E+06		
Pure Error	0.0000	4	0.0000		
Cor Total	2.299E+09	29			

Table 5: Effect of various proteolytic enzymes, catalase, α-amylase, lipase, pH, temperature, and surfactants on antimicrobial activity of the Cell Free Supernatant (CFS) of *Lactobacillus paracasei* MG847589 against *Staphylococcus aureus*

CFS treatment	Remaining activity (AU/ml)	Reduction (%)
Control	25,600	0
Enzyme (1 mg/ml)		
Catalase and pH 7.0	12,800	50
α-amylase	12,800	50
Lipase	12,800	50
Proteinase K	0	100
Trypsin	6,400	75
α-chymotrypsin	12,800	50
pH		
2	12,800	50
3	12,800	50
4	12,800	50
5	12,800	50
6	12,800	50
7	12,800	50
8	6,400	75
9	0	100
10	0	100
Temperatures (C°)		
40	25,600	0
60	12,800	50
80	6,400	75
100	0	100
Surfactants		
Anionic		
EDTA (0.1 mM)	12,800	50
EDTA (0.3 mM)	12,800	50
EDTA (0.5 mM)	0	100
SDS (At final concentration of 1%)	12,800	50
Non ionic (At final concentration of 1%)		
Triton X-100	12,800	50
Triton X- 114	25,600	0
Tween 20	25,600	0
Tween 80	12,800	50
Cation (At final concentration of 1%)		
β-Mercaptoethanol	800	96.87
DTT	0	100
Urea	12,800	50

AU: Arbitrary Units; DTT=Dithiothreitol; EDTA=Ethylene Diamine Tetraacetic Acid; SDS=Sodium Dodecyl Sulfate

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Table 6: Purification of antimicrobial compounds produced by *Lactobacillus paracasei* MG847589

Purification stage	Vol. (ml)	Bacteriocin activity (AU/ml)	Total protein (mg)	Specific activity (AU/mg)	Recovery (%)	Purification (fold)
Culture supernatant	1,400	25,600	243	105.34	100	1
Crude $(NH4)_2SO_4$ precipitate–conc.75	15	25,600	152	168.42	100	1.59
Ion exchange	10	6,400	8.2	780.48	25	7.40
Gel filtration	5	3,200	1.46	2,191.78	12.5	20.80

AU/ml=Activity unit/ml

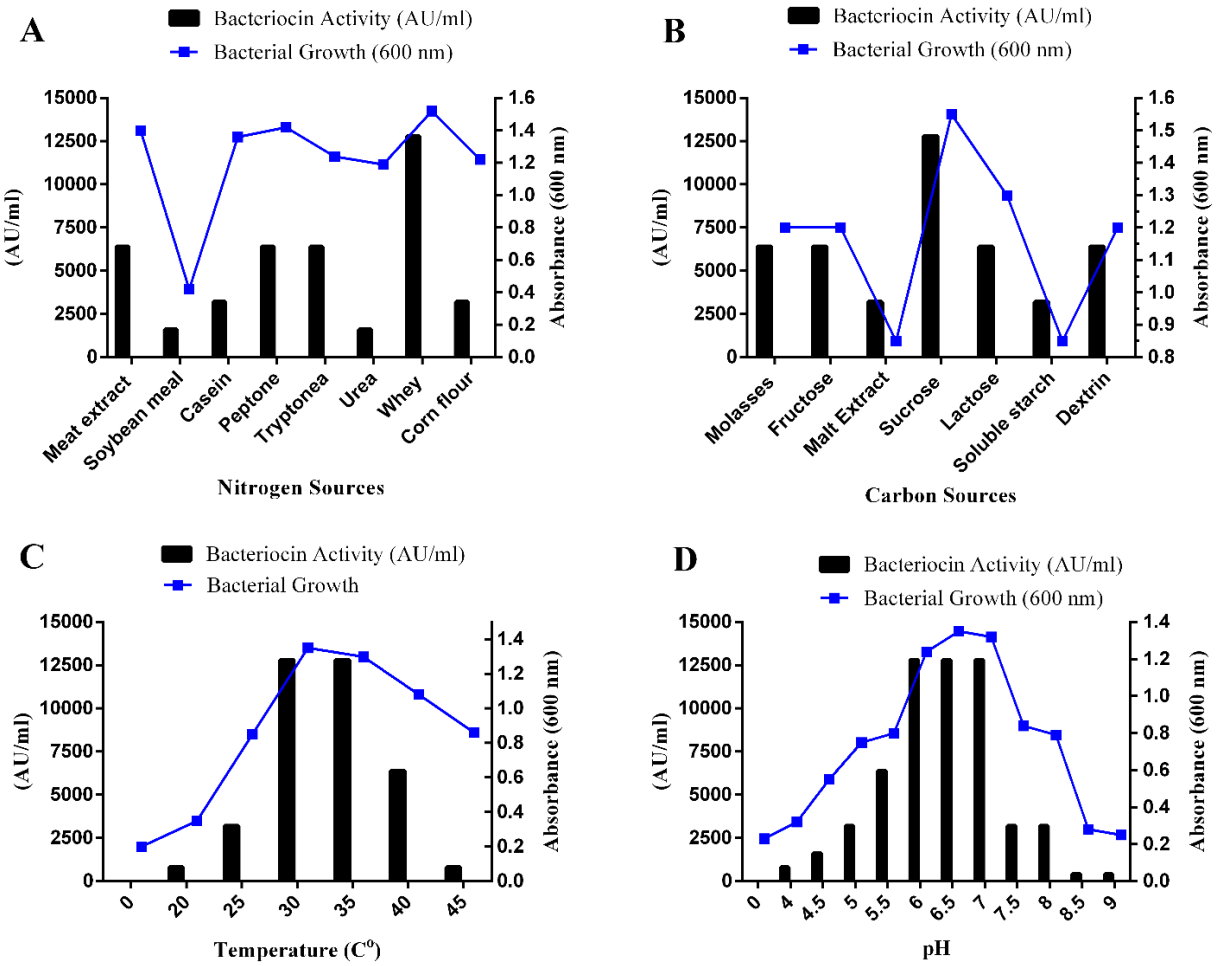

Figure 1: Optimization of bacteriocin production using One-Factor-at-a-Time (OFAT) approach. **A**: Effect of different nitrogen sources on the bacteriocin production; **B**: Effect of different carbon sources on the bacteriocin production; **C**: Effect of different temperatures; **D**: Effect of initial medium pH on the production of bacteriocin

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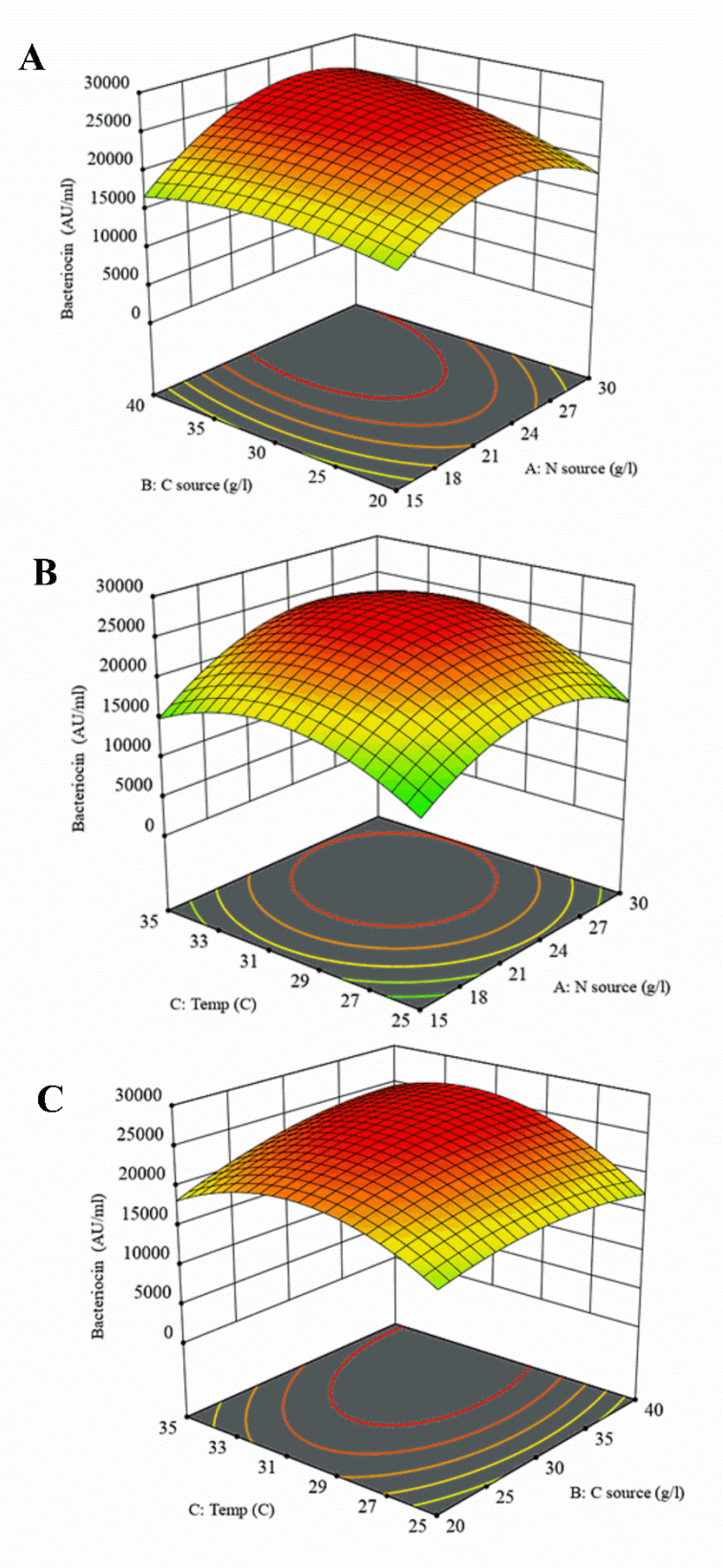

Figure 2: 3D response surface plot presenting the effect of interactions of independent variables on bacteriocin production. **A**: N source vs. C source, **B**: N source vs. Temperature; **C**: C source vs. Temperature

[DOI: 10.18502/jfqhc.9.4.11375]

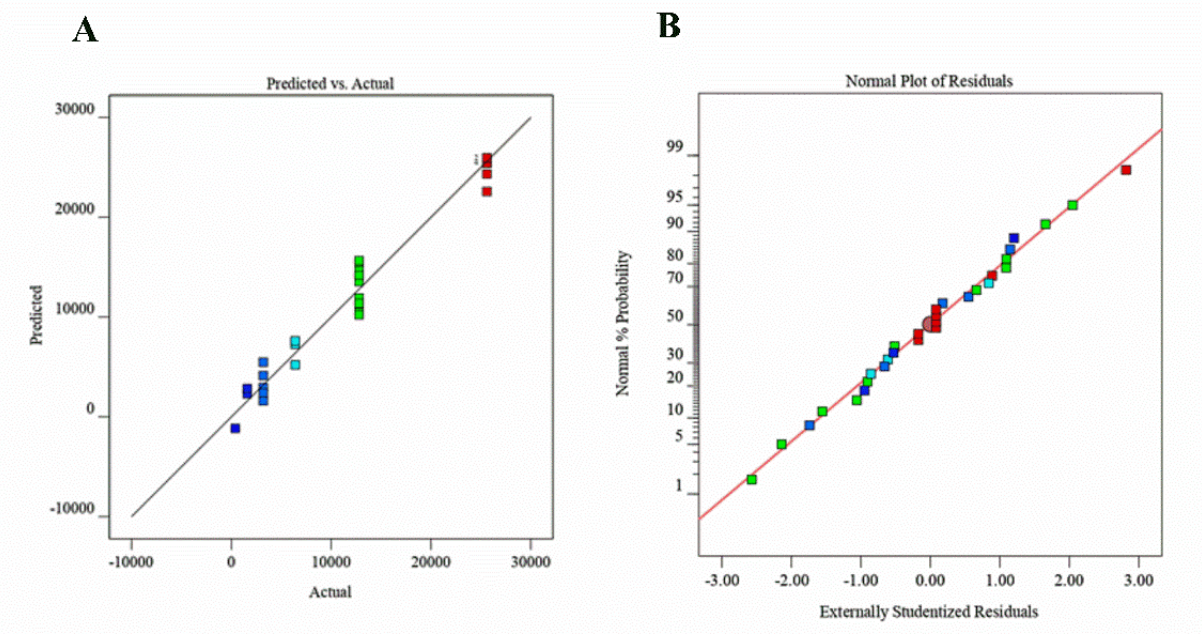

Figure 3: A: Correlation between experimental and predicted values of the quadratic polynomial model for bacteriocin production by *Lactobacillus paracasei* MG847589; B: Normal probability plot of the residuals of a quadratic polynomial model for bacteriocin production by *L. paracasei* MG847589

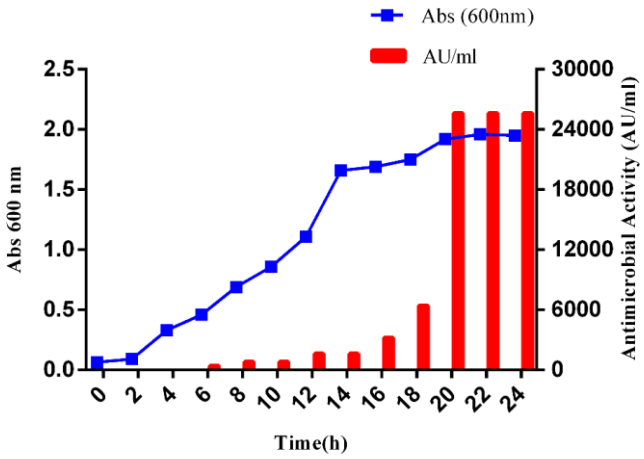

Figure 4: Kinetics of growth (■) and bacteriocin production (Red bar) during the growth of *Lactobacillus paracasei* MG847589 in deMan Rogosa and Sharp (MRS) media at 30 °C

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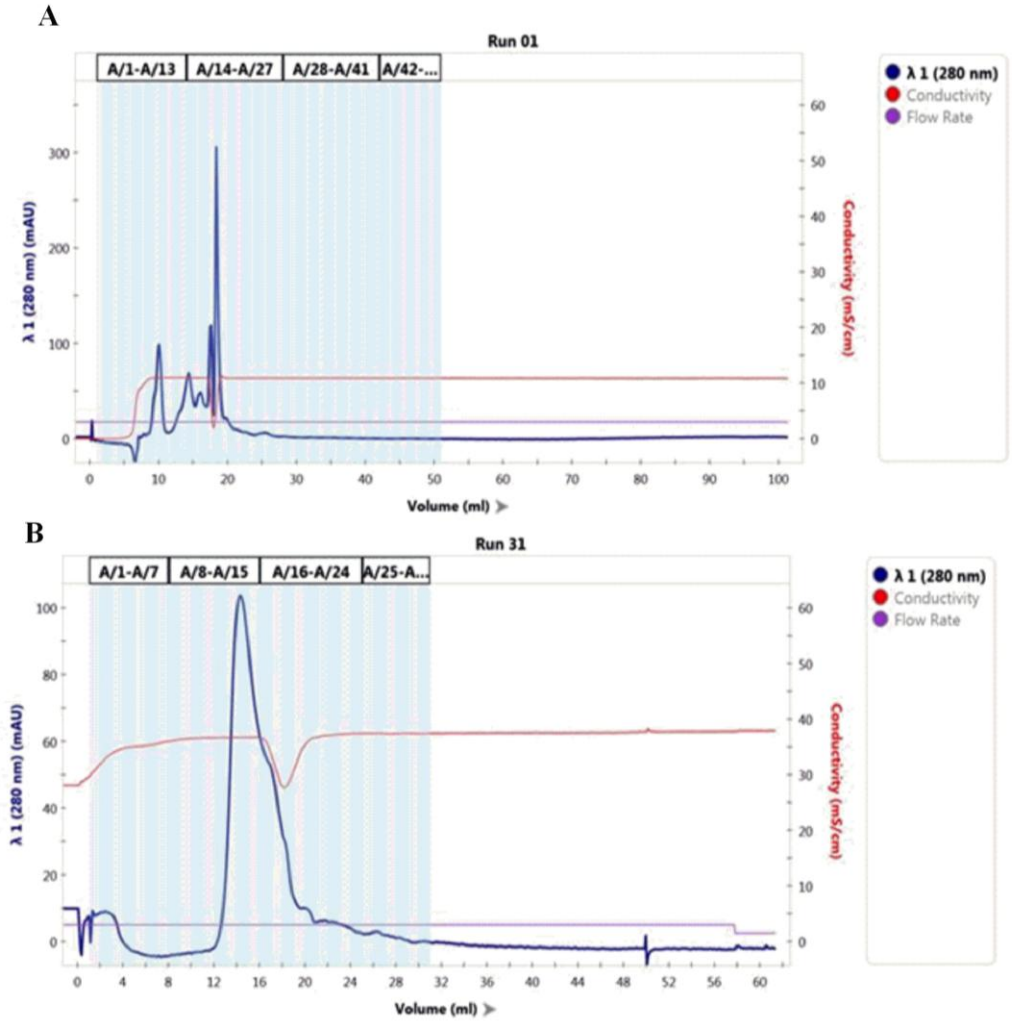

Figure 5: Purification of bacteriocin from *Lactobacillus paracasei* MG847589 culture supernatant by ion exchange chromatography (A) and gel filtration chromatography (B)

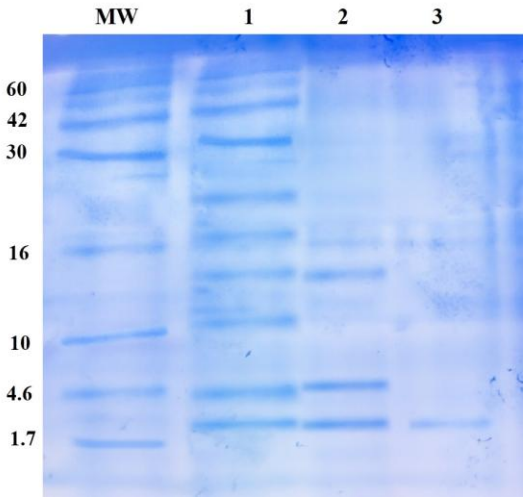

Figure 6: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of bacteriocin MG847589 purified by NGC protein purification system. MW=Molecular Weight markers; 1=Crude $(NH_4)_2SO_4$ precipitate; 2=Ion exchange; 3=Gel filtration

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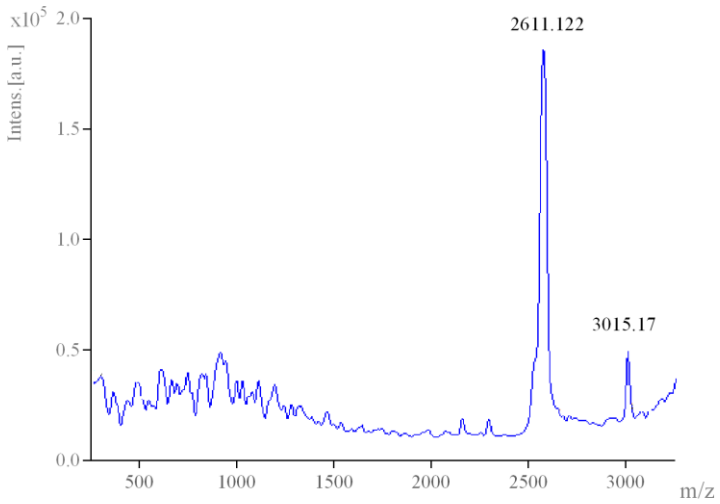

Figure 7: Mass-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) of the purified bacteriocin revealed a molecular mass of 2.611 KDa

Discussion

The antagonism behaviour for LAB might be attributed to the production of different secondary metabolites like acids, hydrogen peroxides, and mainly bacteriocin-like peptides (Lau and Liong, 2014; Todorov et al., 2022). Several innovative studies have unlocked the antimicrobial potentiality of bacteriocin against unpleasant microorganisms. Microorganisms are generally considered as safe for use in food preservation when they have been isolated from natural sources in order to produce bacteriocin. In the last decade, bacteriocins produced by LAB have been used in numerous food applications (Daba and Elkhateeb, 2020; Todorov et al., 2022; Yang et al., 2014).

In the current study, *L. paracasei* MG847589 has been improved with the combinatorial approach for the enhanced production of bacteriocin. The development of microbial strains makes upstream bioprocessing for industrial applications more affordable. The produced bacteriocin exhibited antimicrobial potential against *S. aureus* NCTC 10788 with 12,800 (AU/ml). Consuming food contaminated with *S. aureus* might result in food poisoning and gastrointestinal tract inflammation (Tong et al., 2015). Therefore, *S. aureus* NCTC 10788 was targeted for inhibition in this study. Now, isolated *L. paracasei* MG847589 will be used in further bacteriocin optimization. The development of LAB bacteriocins is highly influenced by medium and cultural formulation conditions, so optimising the production of bacteriocins is important in reducing production costs by increasing the productivity and efficiency (Soltani et al., 2021).

Optimization results revealed that sucrose was ideal carbon source to obtain highest bacteriocin antimicrobial activity comparing to fructose and other sugars effect. The same observations have been recorded by Biswas et al. (1991) and Ray (1995) as in liquid conditions with a pH lower than 4; sucrose has been found to be an effective carbon source for the pediocin AcH formation. Besides, bacteriocin of *Lactobacillus* strains produced in the range of 30-37 °C according to Gautam and Sharma (2009). In addition, bacteriocin production influenced by initial medium pH, because pH of the medium decreases the bacterial growth or inactivate the produced bacteriocin (Benjamín et al., 2022).

The optimal levels and interactions of the elements were determined using CCD according to Sadhukhan et al. (2016) protocol. Whey protein, sucrose, pH, and temperature were selected for the optimization by RSM. The optimum condition that produced the highest yield of bacteriocin was whey protein (22.5 g/L), sucrose (30 g/L), supplemented media, temperature 30 °C, pH 6.5, and incubation for 20 h (Run 30). The model's R^2 score indicates that it is able to explain 99.92% of the variance in the independent variables (Bezerra et al., 2008; Borah et al., 2019). Using the quadratic polynomial model, the ANOVA indicated that the fitted model had a high F (32.40), and $p<0.0001$ significance level. The t-tests and p values of the students were used to determine their significance (Cladera-Olivera et al., 2004). These results indicated that the increase whey protein as nitrogen source of the medium revealed an increasing the bacteriocin yield.

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Das and Goyal (2014) found that the activity of the bacteriocin produced by *L. plantarum* DM5 was completely lost after treatment with proteinase K, trypsin, and pepsin, the DM5 bacteriocin not exhibited activity after treatment with those enzymes. The data of the present study revealed the proteinaceous nature of the antimicrobial substances produced by *L. paracasei* MG847589 which might contain a compatible binding-site for proteases. The findings of the current study about the proteinaceous nature of the antimicrobial substances produced by *L. paracasei* MG847589 are confirmed by other investigations (Ge et al., 2016; Goh and Philip, 2015).

Accordingly, it can be said that the stability and activity of the bacteriocin produced by *L. paracasei* MG847589 showed an inverse relationship with the temperature and time. The reducing of activity of bacteriocin upon high temperature for a long time might be due to its proteinaceous nature, which is denatured and misfolded at a high temperature for a long time, while it will be stable and remained active when exposed to high temperature for a short time (15-20 min.). This property of bacteriocin is a limiting factor for using it in the food industry as a food preservative. Some previous investigations emphasized that, small hydrophobic proteins with little tertiary structure are defined as bacteriocins of class I and class II, with the former being particularly stable in the presence of high temperatures (Campelo et al., 2014; Daba and Elkhateeb, 2020; Todorov et al., 2022). The bacteriocin's capacity to withstand heat is facilitated by stable cross-linkages, a high glycine content, and the presence of very hydrophobic regions (Darbandi et al., 2022; Dini et al., 2022). Many bacteriocins and bacteriocin-like compounds made by LAB have a high heat resistance, which can range from 60 to 100 ^{o}C for 10 to 15 min (helveticin J) to autoclaving at 121 ^{o}C for 10 min (pediocine and nisin) (Bennett et al., 2021; Khorshidian et al., 2021).

Observing how detergents affect the structure of bacteriocins provides insight into the active molecules (Chumchalová et al., 2004). By interacting with the hydrophobic core of the protein's native structure, anionic detergents may change the protein's three-dimensional structure (Wang et al., 2012). Either partial denaturation of the enzyme or disruption of its interaction with other molecules that stabilize it may be responsible for its reduced or complete loss of activity following treatment with DTT and EDTA (0.5 mM) (Du et al., 2022). In this study, β-mercaptoethanol (1%) reduced bacteriocin activity. This finding found to be in agree with other reported results that β-mercaptoethanol has a negative impact on the antimicrobial activity of *L. paracasei* bacteriocin by destroying disulfide bonds existed in its structure (Wang et al., 2012). The characterized bacteriocins of *L. paracasei* are mostly belong to class II bacteriocins that have at least one disulfide bond which is necessary for bactericidal activity (Ge et al., 2016).

Accordingly, the purified bacteriocin produced by *L. paracasei* MG847589 in the current study is a low molecular weight peptide around 2.611 kDa. The size was different from bacteriocin BGUB9 which was a 3,500 Da class IIa bacteriocin purified from *L. paracasei* subsp. *paracasei* (Tolinački et al., 2010) and bacteriocin BMA which was of 1,770 Da size purified from *L. crustorum* MN04 (Yi et al., 2016). In contrast, the previously studied bacteriocins showed different molecular weights (4,890, 6,313, 5,383.2, and 1,770 Da; Perumal et al., 2016; Ullah et al., 2017). Since no earlier investigations have revealed an active bacteriocin with molecular weight of 2.611 kDa; the bacteriocin called MG847589 which purified during the current study is considered a novel bacteriocin. The antimicrobial activity of purified bacteriocin MG847589 from *L. paracasei* opens new possibilities for the control of food-borne pathogens in food manufacturing.

Conclusion

L. paracasei MG847589 is a LAB bacteriocin-producing strain isolated from traditional Egyptian cheese (Kareish) and exhibited antibacterial and antifungal activity and synthesizes toxins by its purified new bacteriocin. Optimization of this bacteriocin production conditions for maximum yield was adopted in the current study followed by purification and characterization of the produced bacteriocin. The produced bacteriocin showed a significant stability and activity at acidic pH values up to 8 and heat resistance for up to 15 min. The purified bacteriocin had a proteinaceous nature with low molecular weight of 2.611 kDa which is considered a novel antimicrobial peptide. The current work is a first step for future application of this bacteriocin in the food processing and manufacturing.

Author contributions

M.G.S., A.N.B., N.M.A.E.-A., H.S.A.-R., and S.A.E.-S.: Conceptualization; M.G.S., A.N.B., N.M.A.E.-A., H.S.A.-R., and S.A.E.-S.: Formal analysis; M.G.S., A.N.B., N.M.A.E.-A., H.S.A.-R.: Methodology; M.G.S., A.N.B., N.M.A.E.-A., H.S.A.-R., and S.A.E.-S.: Writing original draft and Writing review and editing. All authors read and approved the final manuscript.

Conflicts of interest

All the authors declared that this is no conflict of interest in the study.

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