Natural Resources for Human Health

eISSN: 2583-1194

Original Research

Received 26 March 2026

Revised 29 April 2026

Accepted 20 May 2026

Natural Resources for Human Health 2026;

Vol. 6 (2s): 804–822

KEYWORDS:
Response Surface Methodology (RSM), Biomass, kinetics parameters, bacteriocin, Central Composite Design (CCD),

Stochiometric and Kinetic Parameters for Bacteriocin and Biomass Formation from Local Isolate Lactobacillus Fermentum by Response Surface Methodology

[1]Bayan Shakir Mahmood AL-Saeedi, [2]Hiba A. Jasim*, [3]Sahar I. Hussien, [4]Gasem Mohammed Breig ,[5]Sura Jasem Mohammed

[1,2,3]Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

[4]Anesthetic and intensive care techniques, Madenat Alelem university, Baghdad, Iraq
[5]Engineering department Baghdad health Alkarkh Directorate, Ministry of health- Iraq

*Corresponding Author: Hiba A. Jasim

E. mail hiba.jasim@sc.uobaghdad.edu.iq

ABSTRACT: Response surface methodology (RSM) based on a Central Composite Design (CCD) was applied to optimize both biomass and bacteriocin production by Lactobacillus fermentum through modification of MRS medium composition. Glucose concentration, yeast extract concentration, and inoculum size were selected as independent variables and optimized using Design-Expert software. The developed quadratic models showed excellent predictive performance with coefficients of determination (R^2) of 0.987 and 0.985 for biomass and bacteriocin production respectively. The optimized conditions for biomass production consisted of glucose at 39.9 g/L, yeast extract at 19.9 g/L, and an inoculum size of 2.0%, resulting in the highest biomass yield of 15 g/L. For the production of bacteriocin, the model predicted slightly varied different optimal values to be 40.1 g/L of glucose, 20.0 g/L yeast extract and the inoculum size of 2.0% which collectively produced a maximum activity of 89.8 AU/ml. The validation experiments confirmed that the reliability and accuracy of the developed models, producing 15.8 g/L biomass and approximately 90 AU/mL of bacteriocin under optimized conditions.The Growth-kinetic analysis demonstrated that the production of bacteriocin was growth associated and it reaches to the maximum level after 48 hrs of incubation. The partial purification of the bacteriocin was achieved through using ammonium sulfate precipitation, dialysis, sucrose concentration, and gel filtration chromatography using Sephadex G-150. The specific activity was increased from 166.7 to 3000 U/mg protein in final purification process, corresponding to an 18- purification fold. The chromatographic profile showed a distinct activity peak related with the active bacteriocin fractions. These findings revealed the effectiveness of CCD-RSM for enhancing and improving bacteriocin biosynthesis by L. fermentum.

1. INTRODUCTION

The Lactic acid bacteria (LAB) represent a various group of G +ve microorganisms that generally recognized for their long standing history of safety that use in food fermentation and their significant impact to human health (Vieira *et al.* 2023). Besides to their probiotic features, they produce numerous bioactive metabolites, involving organic acids, hydrogen peroxide, diacetyls and bacteriocins, which contribute to their antimicrobial potential (Silva *et al.*, 2024). The bacteriocins are antimicrobial peptides produced by several bacterial species (Al-Rubaye and Al-Rubaye, 2025). These compounds exhibit inhibitory activity against closely related bacteria and in some cases against a wide spectrum of microorganisms. Their antimicrobial mechanism generally involves interaction with the cell membrane of target organisms, leading to pore formation, disruption of membrane integrity

and eventual cell death (Cotter *et al*., 2013). Among LAB, *Limosilactobacillus fermentum* has emerged as an important probiotic microorganism because of its beneficial effects on host health and its ability to produce antimicrobial metabolites (Wang *et al*., 2023). Several strains of *L. fermentum* have demonstrated promising antibacterial activity through the production of bacteriocins capable of inhibiting spoilage microorganisms and foodborne pathogens (Sharma *et al*., 2023). Fermentation of *L. fermentum* strains to yield biomass and bacteriocins is an area of research interest due to their possible use in industry, preservation of food, and health (Gaber *et al*., 2021). Knowledge of kinetic and stoichiometric parameters necessary for their formation will facilitate maximum formation processes and yield improvement. biomass belonging to the genus *L. fermentum* are well recognized for their beneficial health effect, including the ability to produce bacteriocins—bactericidal peptides that pathogenic bacteria cannot use for growth (Costa *et al.,* 2020). Bacteriocins have been of specific interest as antioxidants and antimicrobials that are applied to enhance the quality and functionality of food (Wu *et al.,* 2020), since they are heat stable and can function in an exceedingly wide pH range, thus being effective in a wide variety of applications (Lingli *et al.,* 2021).

In an extensive study concerning the formation of bacteriocin and biomass, kinetic studies are inevitable. An accurate mathematical model is very valuable for predicting, evaluating, describing, and understanding the process of fermentation. Mathematical Models enable the description of cellular growth-substrate-product interactions, and may be applied in control system optimization and scaling up of the fermentation process. Mathematical models were categorized based on some criteria (Zacharof and Lovitt, 2012). Response surface methodology (RSM) is a statistical technique, which includes interaction and single impacts of variables for improving the formation and for building the models in order to appraise the influences of parameters for the needed responses. RSM was effectively applied in various zones of biotechnological field, encompassing certain recent works on biomass formation of many strains and optimization of bacteriocins (Sharma *et al*., 2023; Silva *et al*., 2024).

Although several studies have investigated bacteriocin production by LAB, limited information is available regarding the simultaneous optimization of biomass and bacteriocin production by *L. fermentum* using CCD-RSM combined with kinetic evaluation and purification analysis. Furthermore, studies integrating statistical optimization, kinetic characterization and downstream purification of bacteriocin-producing *L. fermentum* strains. Therefore, the present study aimed to optimize the production of biomass and bacteriocin by *L. fermentum* using response surface methodology (RSM) based on Central Composite Design (CCD), evaluate growth kinetic parameters under optimized conditions followed by partially purification of the produced bacteriocin.

2. MATERIALS AND METHODS

2.1 Isolation and screening of bacteriocin producing bacteria

A local isolate was obtained from buffalo milk samples collected from local markets in Baghdad. These samples were aseptically transferred to the laboratory in some ice-cooled containers to preserve microbial viability. A small amount of each sample was suspended in 10 ml of sterile distilled water and One milliliter from the suspension was added to 9 ml of sterilized De Man Rogosa-Sharp-broth (MRS broth) (HIMEDIA, INDIA) and incubated anaerobically (via candle jar) for 24hr at 37°C. After incubation, the suspension was serially diluted in a sterile normal saline (NaCl) 0.85% from 10^{-1} to 10^{-5} and streaked on MRS agar plates in duplicates and incubated anaerobically for 48hr at 37°C. Distinct colonies were selected and subjected to preliminary screening for bacteriocin production using agar well diffusion assay against indicator microorganism. The isolate exhibiting the highest antimicrobial activity was selected for further investigation.

2.2. Identification of the selected isolate

The selected isolate was identified based on morphological, physiological, biochemical and cultural characteristics as described by Bergey's manual for Systemic Bacteriology (results are not shown). Molecular identification was performed through amplification and sequencing using 16S ribosomal RNA gene sequencing analysis. Genomic DNA was extracted and the 16S rRNA genes was amplified by PCR using universal bacterial primers. The amplified PCR was sequenced and the obtained nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) available at the NCBI database. Sequence alignment was carried out to determine the taxonomic affiliation of the isolate with closely related bacterial strains

2.3. Detection of bacteriocin activity

Bacteriocin activity was evaluated using agar well diffusion assay. Cell free supernatant (CFS) was prepared by centrifugation of the culture broth, followed by filtration with 0.22µmMillipore filter paper under sterile conditions. The antimicrobial activity was assessed using a serial two-fold dilution assay. Briefly, CFS was subjected to successive two-fold dilution against indicator strains *Staphylococcus aureus* and *Pseudomonas aeruginosa* by measuring the diameter of the inhibition zone. The highest dilution producing an inhibition zone reflected the strength of bacteriocin activity. Production of biomasses was quantified applying approach of the dry cell weight (DCW) according to the Stanbury *et al.* (2016) procedure.

2.4. Response Surface Methodology (RSM)

Three parameters (glucose concentration g/L, yeast extract g/L, and inoculum size %) were optimized according to central composite design (CCD) using RSM with 5 levels of every parameter, which were mentioned as (-2, -1, 0, 1, +2), independent parameters, for generation of bacteriocins and biomasses. **Table 1** evidenced uncoded and coded values of each independent parameter which mentioned via subsequent equation:

$xi = (Xi - Xo / \Delta X)$ …….. (1)

Where: ΔX: change of step

X_o: X_i at center point actual value

X_i: the parameter actual value

xi: the parameter coded value

Table 1: Independent levels and parameters in design of CCD.

Parameters	Unit	-2	-1	0	1	2
Glucose	g/L	23.1	30	40	50	4.681
Yeast extract	g/L	3.1	10	20	36.81	4.681
Inoculum size	%	0.318	1	2	3	3.681

Using Design-Expert software (version 7), a central composite design (CCD) matrix consisting of twenty experimental trials, including five replicated center points, was generated as represented in Table 2. For each run, agar plates were prepared and inoculated in triplicate to maintain experimental reliability. To investigate the kinetics of bacteriocin production the selected isolate was introduced at 1% (v/v) into 250 mL of MRS broth adjusted to pH 6.5, followed by incubation at 37 °C for 48 hours under a nitrogen atmosphere and without agitation. After incubation, both biomass and bacteriocin levels were measured, and the resulting information were processed using Design-Expert for assessing the influence of individual variables and their interactions. This test permitted the prediction of both outcomes of response according to the quadratic model of optimization.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_0 x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{i=2}^{k} \beta_{ij} x_i x_j \quad \text{.....2}$$

In this model, β_{ij} describes the interaction effects between variables X_i and X_j, β_{ii} captures the quadratic influence, β_i reflects the linear contribution of each factor, β_0 denotes the intercept, Y represents the predicted response, and which together shape the overall response.

Table 2: Central composite design and experimental design outcomes for concentration of bacteriocins and biomasses with predicted and actual values of responses.

Std	type of point	Run	Yeast extract (g/l)	glucose(g/l)	Size of inoculum (v/v)%	Biomass (g/l)		Bacteriocin (micrbial activity mm)	
						actual	predict	actual	predict
20	Center	1	20	40	2	14.8	14.71	90	87.65
19	Center	2	20	40	2	15	14.71	92	87.65
13	Axial	3	20	40	0.318207 169	6.4	7.30	30	35.88
10	Axial	4	20	56.81792 83	2	8	7.96	41	38.69
4	Fact	5	30	50	1	9.5	9.01	42	36.30
15	Center	6	20	40	2	14.5	14.71	86	87.65
1	Fact	7	10	30	1	6.1	5.37	18	13.14
3	Fact	8	30	30	1	5.22	5.11	12	9.67
17	Center	9	20	40	2	15.2	14.71	85	87.65
8	Fact	10	30	50	3	10	10.68	50	54.56
12	Axial	11	36.81792 831	40	2	7.5	7.33	19	22.74
18	Center	12	20	40	2	14.8	14.71	89	87.65
16	Center	13	20	40	2	14	14.71	84	87.65
2	Fact	14	10	50	1	3.7	3.48	11	13.76
5	Fact	15	10	30	3	8	8.44	20	25.40
7	Fact	16	30	30	3	7.6	7.77	25	21.94
6	Fact	17	10	50	3	5.5	5.56	30	32.02
9	Axial	18	20	23.18207 17	2	7	7.11	8	10.74
11	Axial	19	3.182071 695	40	2	3	3.25	10	6.69
14	Axial	20	20	40	3.681792 831	12.1	11.28	67	61.55

2.5. Validation of optimum condition

According to the model of generated regression, Design-Expert software was applied to construct an optimization plot that determines the ideal levels of glucose, yeast extract, and inoculum size accomplished of maximizing production of both biomass and bacteriocin production. Entire independent variables were set through their permissible ranges, while the two responses—

yield of both biomass and bacteriocin production—were assigned a "maximize" goal in the ramp of function. The predicted optimal conditions gained from the model were then validated through experiments of laboratory to verify the anticipated outcomes and ensure the model accuracy for both variables of response.

2.6. Growth kinetics Study

The growth pattern of the selected isolate, along with its substrate utilization and bacteriocin production, was examined under both optimized and non-optimized conditions. For this purpose, 50 mL of MRS broth was dispensed into 250 mL Erlenmeyer flasks and sterilized by autoclaving. Each flask was inoculated with the optimal inoculum size of the selected *Lactobacillus* isolate and incubated in anaerobically for 48 hrs at temp. of 37 °C. The samples were collected at the start of incubation (0 h) and at continuous intervals—2, 4, 6, 8, 18, 24, and 48 hours—to determine biomass accumulation and production of bacteriocin during the cultivation period. Based on the kienetic analysis, the incubation time corresponding to maximum bacteriocin production was selected and used for subsequent purification experiments.

2.7. Purification of bacteriocin

2.7.1. Precipitation by ammonium sulphate

Bacteriocin was first precipitated by ammonium sulfate at different saturation levels (20, 30, 40, 50, 60, 70, 80,90) % at 4°C. The precipitate was separated by centrifugation for 30 min at 10000 rpm and dissolved in an appropriate volume of phosphate buffer (0.1M, pH 7.2). Next, the dissolved precipitates were dialyzed in 0.5 liter of phosphate buffer overnight at 4°C using dialysis membrane tubes (1 kDa MW cutoff). The buffer was replaced four times. The antibacterial activity of the dialyzed protein was determined by agar well diffusion assay using indicator strains *S. aureus* and *P. aeruginosa* (Charles *et al.*, 2008).

2.7.2. Bacteriocin concentration by sucrose

The resulting bacteriocin solution was concentrated using dialysis against solid sucrose (Horowitz and Fling, 1962). The crude solution to be concentrated was packed in the dialysis tube (1 KDa MW cutoff), coiled up in a beaker and then covered with local sucrose. The liquid should be poured off as it accumulates outside the dialysis bag. At the end of process, a two-thirds reduction of volume was obtained taken to measure the activity.

2.7.3. Gel filtration chromatography using Sephadex G 150 gel

The resulting bacteriocin was loaded on a column (2.5 × 20 cm) of sephadex G-150 gel filtration. Elution step was performed with phosphate buffer saline (0.1 M, pH 7.2) with a flow rate of 20 ml/hr and fractions of 3 ml. The protein absorption was estimated at 280 nm wavelength. The fractions were tested for antibacterial activity against *S. aureus* and *P. aeruginosa* as an indicator strain by well agar diffusion assay. The purified bacteriocin was distributed in tubes, freezed and then archived for subsequent experiments (Hammami *et al.*, 2012).

2.8. Statistical analysis

Design-Expert software was employed as an effective platform for constructing experimental designs and analyzing the resulting response data. The optimization model underwent a comprehensive statistical evaluation through analysis of variance (ANOVA), and its performance was judged using several diagnostic indicators, including the coefficient of determination (R^2), adjusted R^2, predicted R^2, F-value, PRESS statistics, mean, coefficient of variation, standard deviation, adequate precision, lack-of-fit tests, and the associated probability ($P > F$), along with other relevant statistical parameters applied to ensure the strength and reliability of the model (Li *et al.*, 2001).

3. RESULTS AND DISCUSSION

The increase development of antibiotic-resistant pathogens has increased the global request for alternative bacterial agents. Amongst these promising alternatives, the bacteriocins produced by lactic acid bacteria have concerned a great attention because of their bacterial efficiency, safety, and possible applications in food preserving and biomedical areas.

The increasing developments of resistance to antibiotics have heightened the need for new strategies to compete pathogens. Researchers have shown an increased interest in bacteriocins as a possible alternative to antibiotics.

3.1. Isolation and Identification of *Lactobacillus fermentum*

Buffalo milk was used as a natural source for the isolation of bacteriocine producing lactic acid bacteria. Following preliminary screening, the isolate exhibited remarkable antagonistic activity against the indicator pathogenic bacteria and therefore selected for further investigation.

The selected isolate was initially characterized based on morphological and biochemical properties typical of lactic acid bacteria. Molecular identification using 16S rRNA gene sequencing was performed for the selected isolate. Successful amplification of the 16S rRNA gene was confirmed by agarose gel electrophoresis, which revealed a distinct PCR band of approximately 1400bp (figur1).

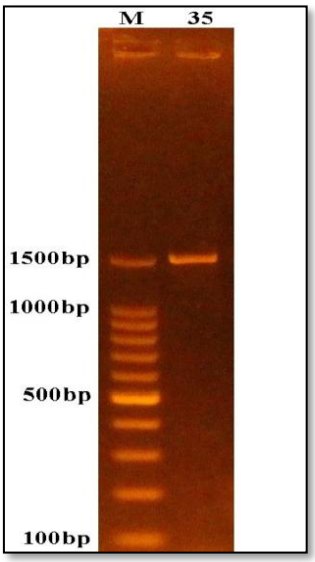


Figure 1: agarose gel agarose gel electrophoresis of PCR-amplified 16S rRNA gene of *L. fermentum*. Lane M: DNA marker; lane 1: amplified PCR product showing distinct band of approximately 1400 bp.

Sequence analysis using BLAST program available in the NCBI GeneBank database revealed 100% sequence similarity and complete query coverage with previously reported *Lactobacillus fermentum* strains (table 3). The obtained molecular identification results confirmed accurate taxonomic classification of the isolat as *Lactobacillus fermentum*.

Table 3: BLAST analysis of the amplified 16S rRNA gene sequence of the isolated *L. fermentum*

Closest related strain	Query cover(%)	Identity (%)	E-value	Accession No.
Lactobacillus fermentum strain 7456	100	100	0.0	MT516093.1
Lactobacillus fermentum strain 7245	100	100	0.0	MT516042.1
Lactobacillus fermentum strain 7234	100	100	0.0	MT516040.1
Lactobacillus fermentum strain 6472	100	100	0.0	MT515900.1
Lactobacillus fermentum strain 6425	100	100	0.0	MT518566.1
Lactobacillus fermentum strain 6424	100	100	0.0	MT515865.1

The high degree of identity and complete query coverage obtained in the present study further support the reliability of 16S rRNA gene sequencing as an accurate molecular tool for the identification of lactic acid bacteria isolated from dairy sources.

3.2 Detection of bacteriocin activity

The identified isolate, nominated as *L. fermentum* revealed the capability to produce an effective extracellular bacteriocin showing a broad spectrum of antimicrobial activity towards the tested pathogen indicators. Bacteriocin production was achieved by cultivating the isolate in MRS broth under optimized growth conditions at $37C^0$ for 24-48 hr under anaerobic conditions. Following incubation, the supernatant was separated by centrifugation and filtration to obtain a cell free supernatant containing the produced bacteriocin.

The antibacterial activity of the produced bacteriocin was assessed against indicator strains *Staphylococcus aureus* and *Pseudomonas aeruginosa* using agar well diffusion assay on Muller-Hinton agar. The results demonstrated the formation of distinct inhibition zone surrounding the wells containing the bacteriocin indicating strong antagonistic activity against the tested pathogens (figure 2)

Figure 2: Antimicrobial activity of bacteriocin produced by *L. fermentum* against indicator pathogenic bacteria

The observed antimicrobial activity may be attributed to the production of bacteriocins and other bioactive metabolites synthesized by lactic acid bacteria. The strong inhibitory effect exhibited by *L. fermentum* highlights its potential as a promising natural antimicrobial-producing strain with possible applications in food preservation and biotechnology.

3.3 Response surface methodology

Response surface methodology (RSM) based on Central Composite Design (CCD) was successfully applied to optimize biomass and bacteriocin production by *L. fermentum* under different cultivation conditions. The combined effects of glucose concentration, yeast extract concentration and inoculum size were evaluated to determine the optimal conditions for maximizing the biomass and bacteriocin production.

Table 4: Analysis of variance (ANOVA) for quadratic model generated by CCD modelling for biomass production from L. fermentum.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	316.717783	9	35.190865	88.3239	< 0.0001
A-glucose concentration	0.87750879	1	0.8775088	2.20242	0.1686
B- yeast extract concentration	20.1484168	1	20.148417	50.5696	< 0.0001
C-inoculum size	19.1366647	1	19.136665	48.0302	< 0.0001
AB	16.76205	1	16.76205	42.0703	< 0.0001
AC	0.49005	1	0.49005	1.22995	0.2934
	0.08405	1	0.08405	0.21095	0.6558

BC					
A^2	92.7804957	1	92.780496	232.865	< 0.0001
B^2	160.076602	1	160.0766	401.769	< 0.0001
C^2	53.0489589	1	53.048959	133.145	< 0.0001
Residual	3.98429723	10	0.3984297		
Lack of Fit	3.09596389	5	0.6191928	3.48514	0.0984
Pure Error	0.88833333	5	0.1776667		
Cor Total	320.70208	19			
Std. Dev.	0.6312				
Mean	9.396				
C.V. %	6.717				
PRESS	25.42				
R-Squared	0.987				
Adj R-Squared	0.976				
Pred R-Squared	0.920				
Adeq Precision	25.69				

The ANOVA results demonstrated that the developed quadratic model was highly significant with an F-value of 88.3 and a probability value ($P < 0.0001$) indicating the suitability and reliability of the model for predicting biomass production by *Lactobacillus fermentum* as outlined in table 4.

The coefficient of determination ($R^2 = 0.987\%$) indicated that 98.7% of the variability in biomass production could be explained by the developed model, while only 1.3% of the total variation remained unexplained. Similar observations were reported by Stanbury *et al.* (2016), who indicted that high R^2 values and significant ANOVA models reflect strong predictive capability and reliability of statistical optimization models in microbial metabolite production. Furthermore, the predicted and adjusted R^2 values (0.927 and 0.975, respectively) were in close agreement, confirming the adequacy and predictive accuracy of the statistical model. The adequate precision value of 21.4 demonstrated an acceptable signal-to –noise ratio and confirmed the applicability of the model for optimization purposes. Similar findings have also been recognized by Saxena et al. (2022) during the optimization studies that including microbial biomass and production of metabolites using response surface methodology (RSM).Furthermore, the interaction between glucose concentration and yeast extract concentration significantly affect on biomass production, indicating the important role of nutrient balancing during bacterial growth and cultivation. Similar observations were described by Myers *et al.* (2016), who highlights the importance of interaction effects among nutritious variables in response surface methodology.

For bacteriocin production, the ANOVA results displayed in Table 5 demonstrated that the developed quadratic model was also highly significant, with an F-value of 74.1 and a probability value ($P < 0.0001$) confirming the adequacy and reliability of the model for predicting bacteriocin production by *L. fermentum*. Similar optimization trends have been previously documented

for bacteriocin-producing lactic acid bacteria, where nutritional and environmental parameters markedly affected antimicrobial metabolite production (Melini *et al.*, 2023a; Veettil *et al.*, 2022a).

Table 5: Analysis of variance ANOVA based on CCD modelling for bacteriocin production by *Lactobacillus fermentum*.

Sources	Squares Sum	df	Square of Mean	F-Value	p-value Prob > F	
Model	18937.02	9	2104.113	74.10614	< 0.0001	Significant
A-glucose con.	943.267	1	943.267	33.22154	0.0002	
B- yeast extract con.	310.6657	1	310.6657	10.94154	0.0079	
C-inoculum size	795.4342	1	795.4342	28.01492	0.0004	
AB	338	1	338	11.90424	0.0062	
AC	18	1	18	0.633954	0.4444	
BC	0	1	0	0	1.0000	
A^2	7135.893	1	7135.893	251.3237	< 0.0001	
B^2	9583.603	1	9583.603	337.5312	< 0.0001	
C^2	2731.339	1	2731.339	96.19682	< 0.0001	
Residual	283.9324	10	28.39324			
Lack of Fit	234.599	5	46.91981	4.755386	0.0561	not significant
Pure Error	49.33333	5	9.866667			
Cor Total	19220.95	19				
Std. Dev.	5.32					
Mean	45.45					
C.V. %	11.72					
PRESS	1948.3					
R-Squared	0.9852					
Adj R-Squared	0.971					
Pred R-Squared	0.898					
Adeq Precision	21.4					

The coefficient of determination (R^2 = 0.987) further demonstrated that the majority (98.7%) of variability in bacteriocin production could be successfully explained by the developed statistical model, while only 1.3% of the total variation remained unexplained. Furthermore, the predicted and adjusted R^2 values (0.893 and 0.975, respectively) were in close agreement, confirming the predictive accuracy and adequacy of the statistical model.

In addition, the adequate precision value of 21.4 representing a strong signal-to-noise ratio and validated the applicability of the model for optimization purposes. The obtained results revealed that the selected cultivation parameters significantly influenced bacteriocin production and could be effectively optimized using response surface methodology. The enhancement in bacteriocin production under optimized conditions may be attributed to improve nutrient availability and stimulation of bacterial metabolic activity required for antimicrobial peptide biosynthesis.Furthermore, regression modelling was employed to evaluate the relationship between the dependent responses (biomass formation and bacteriocin production) and the independent cultivation parameters. The regression coefficient and ANOVA analysis confirmed that the Central Composite

Design (CCD) adequately fitted a second order polynomial model for predicting biomass and bacteriocin production across the investigated codded parameters, as presented in (table 6 and table 7).

Table 6: Regression Coefficient estimation for the quadratic model of biomass formation by *Lactobacillus fermentum*

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	14.714502	1	0.2574391	14.1409	15.2881	
A-glucose	0.25348391	1	0.170805	-0.1271	0.63406	1
B-extract of yeast	1.21463314	1	0.170805	0.83406	1.59521	1
C-inoculum size	1.18374399	1	0.170805	0.80317	1.56432	1
AB	1.4475	1	0.2231675	0.95025	1.94475	1
AC	-0.2475	1	0.2231675	-0.7447	0.24975	1
BC	-0.1025	1	0.2231675	-0.5997	0.39475	1
A^2	-2.5373297	1	0.166274	-2.9078	-2.1668	1.0182653
B^2	-3.3328248	1	0.166274	-3.7033	-2.9623	1.0182653
C^2	-1.9186113	1	0.166274	-2.2891	-1.5481	1.0182653

** Biomass = 14.71 +0.25 A +1.21B+1.18C+1.45AB-0.25AC-0.1BC-2.54AA-3.33BB-1.92CC*

Table 7: Regression Coefficient estimation for the quadratic model of bacteriocin production by *Lactobacillus fermentum*

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	87.65433388	1	2.173232	82.81207	92.4966	
A-glucose	8.310783826	1	1.44189	5.098053	11.52351	1
B-extract of yeast	4.769483095	1	1.44189	1.556752	7.982214	1
C-inoculum size	7.631796666	1	1.44189	4.419066	10.84453	1
AB	6.5	1	1.88392	2.302365	10.69764	1

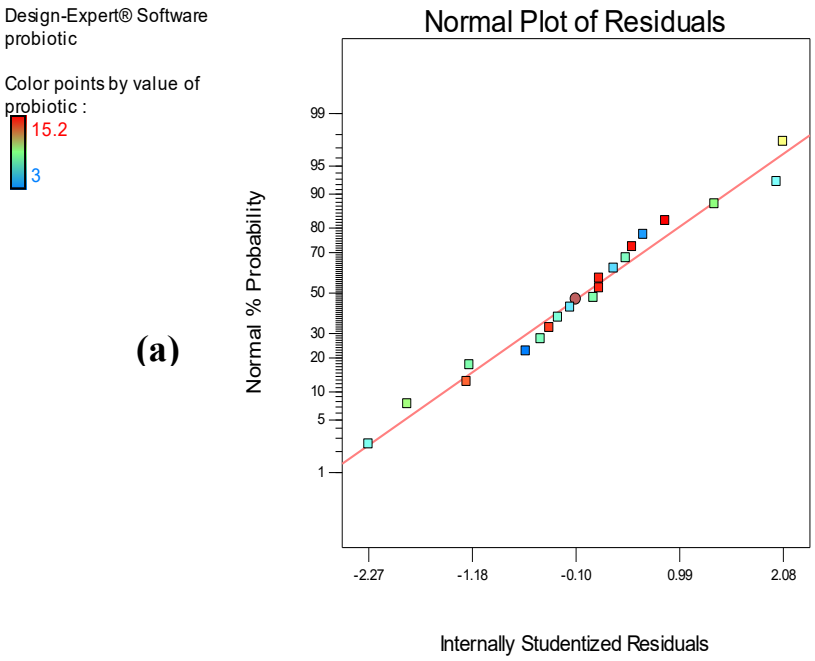
Natural Resources for Human Health
eISSN: 2583-1194

AC	1.5	1	1.88392	-2.69764	5.697635	1
BC	0	1	1.88392	-4.19764	4.197635	1
A^2	-22.25218782	1	1.403641	-25.3797	-19.1247	1.018265261
B^2	-25.78772172	1	1.403641	-28.9152	-22.6602	1.018265261
C^2	-13.76690644	1	1.403641	-16.8944	-10.6394	1.018265261

* *Bacteriocin = +87.65 +8.31A+4.77B+7.63C+6.5AB+1.5AC+0BC-22.2 AA-25.79BB-13.7CC*

The regression coefficient analysis demonstrated that glucose concentration (A), yeast extract concentration (B), and inoculum size (C) positively influenced bacteriocin production by *L. fermentum*. Among the tested variables, glucose concentration exhibited the highest positive linear effect, followed by inoculum size and yeast extract concentration.Furthermore, the interaction between glucose and yeast extract (AB) showed a positive influence on bacteriocin production, whereas the interaction between yeast extract and inoculum size (BC) exhibited negligible effect. The negative quadratic coefficients observed for A^2, B^2, and C^2 indicated that excessive levels of the investigated variables could reduce bacteriocin production beyond the optimal conditions.

These results confirmed the compatibility of the advanced quadratic polynomial model for describing and predicting production of bacteriocin under different culturing circumstances (Myers *et al.*, 2016). Besides to ANOVA test and regression analysis, the diagnostic residual plots were assessed to examine the adequacy and normal distribution of the developed quadratic models. The normal probability plots of the standardized residuals for biomass and bacteriocin production are presented in figure 3 (a) and (b).



(a)

Natural Resources for Human Health
eISSN: 2583-1194

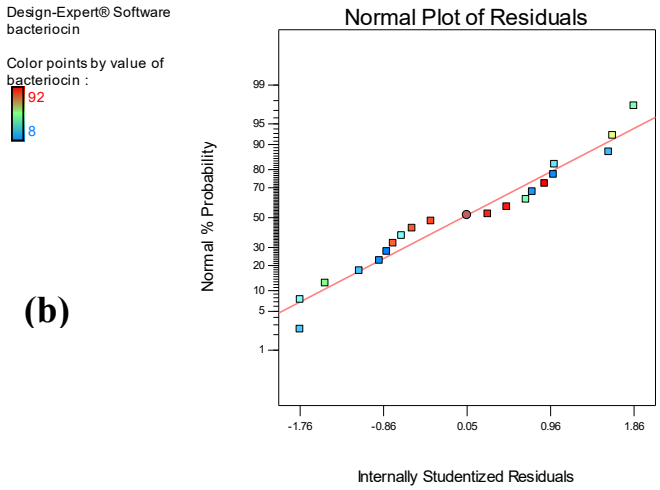
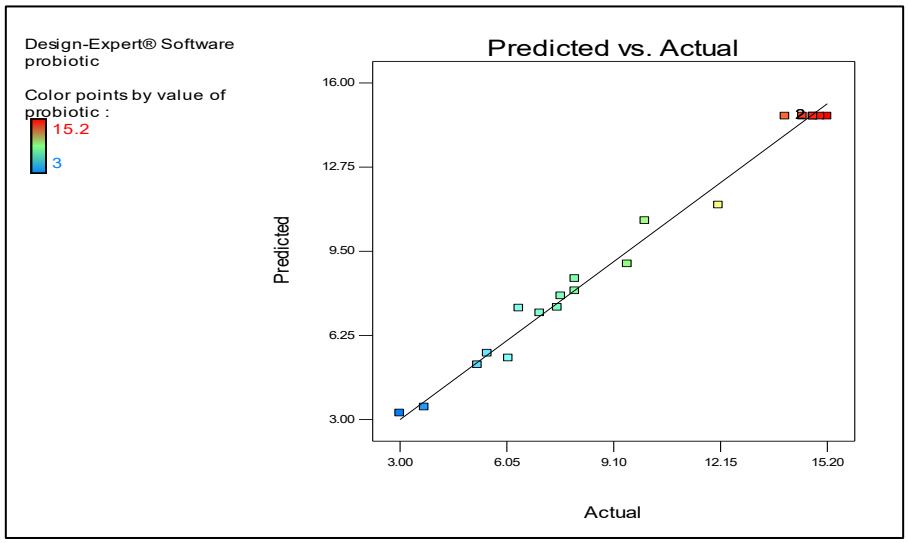


Figure 3: Normal probability plots of the standardized residuals for the quadratic models of (a): biomass formation (b): bacteriocin production by *Lactobacillus fermentum* based on CCD analysis

The normal probability the plots revealed that the experimental data points were distributed sufficiently along the straight reference line, signifying normal distribution of residuals and satisfactory arrangement between the predicted and the experimental values. The nonexistence of major deviations or abnormal scattering verified the adequacy and reliability of the developed quadratic models for predicting biomass and bacteriocin production by the isolated *L. fermentum*. Similar observations have been reported in previous response surface methodology studies, where normal distribution of residuals indicated good model fitness and acceptable predictive performance (Myers *et al.*, 2016; Montgomery, 2017).The relationship between the predicted and experimental values for bacteriocin production is presented in 4. The close distribution of the experimental data points around the diagonal line indicated good agreement between the predicted observed responses, confirming the adequacy and predictive accuracy of the developed quadratic model. These findings further demonstrated the reliability of the optimization model for predicting bacteriocin and biomass production by *L. fermentum* under the investigated cultivation conditions.



Natural Resources for Human Health
eISSN: 2583-1194

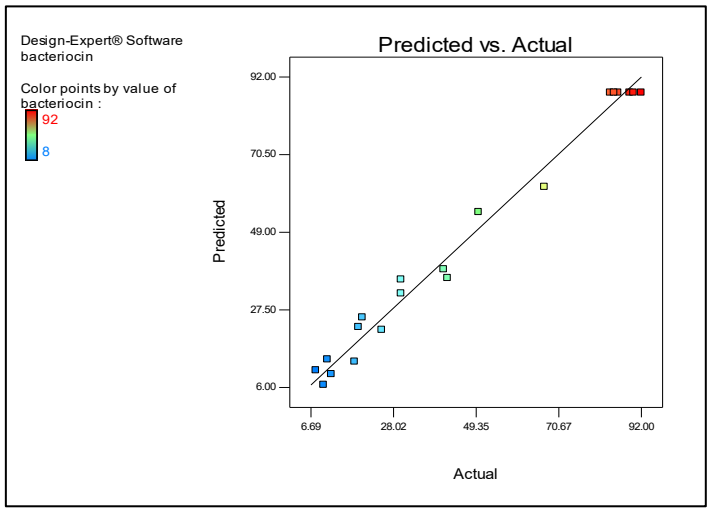
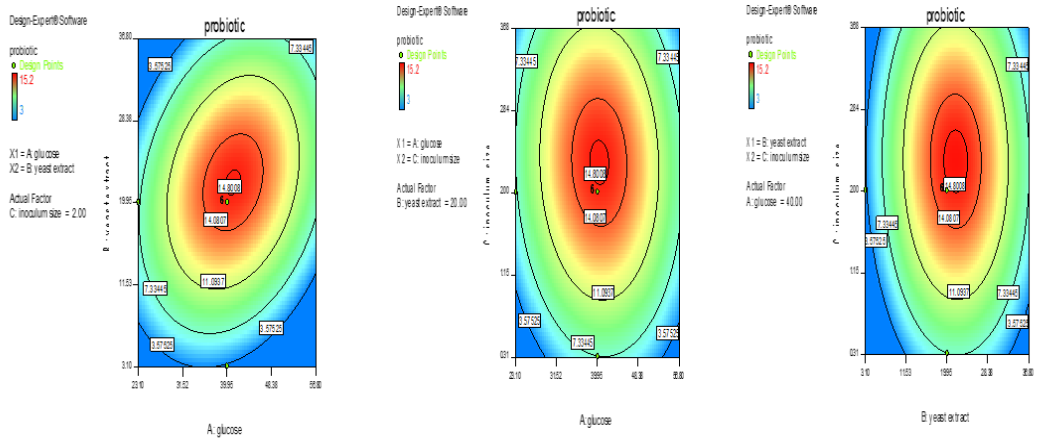


Figure 4: Correlation between predicted and experimental values for bacteriocin production by *L. fermentum* based on CCD optimization.

Contour plots (2-D response surface plots) were generated to evaluate the interactive effects of the investigated cultivation variables on biomass and bacteriocin production by *L. fermentum*.



(a) **(b)** **(c)**

Figure 5: Two-dimensional contour plots showing the effects of cultivation variables on biomass production by *L. fermentum* a: glucose concentrations and yeast extract, b:glucose concentrations and inoculum size , c: yeast extract concentrations and inoculum size.

The contour profiles provided a graphical interpretation of the relationships between independent variables and the corresponding experimental responses while maintaining the remaining variables at their coded central levels. The elliptical contour patterns observed in the plots indicated significant interactions among the studied variables and confirmed the adequacy of the developed quadratic optimization model. Similar observations have been reported in previous response surface optimization studies involving metabolite production (Reuben *et al.*, 2020; Meera and Charitha, 2021). As illustrated in figure 5(a), the interaction between glucose concentration and yeast extract concentration significantly enhanced biomass formation, where the maximum biomass yield (14.8 g/L) was obtained at approximately (39.9 g/L) glucose and (19.9 g/L) yeast extract.

Natural Resources for Human Health
eISSN: 2583-1194

Likewise, figure*Error! Reference source not found.* (b) demonstrated that the interaction between glucose concentration and inoculum size positively influenced biomass production, with the optimum biomass yield achieved at 39.9 g/L glucose and 2.0% inoculum size. Furthermore, figure 5(c) revealed that the combined effect of yeast extract concentration and inoculum size also contributed to biomass enhancement, confirming the importance of balanced nutritional and inoculum conditions for efficient bacterial growth. Overall, the contour plots demonstrated that moderate cultivation conditions were favorable for maximizing biomass (14.8 g /L) production by *L. fermentum*. Based on the obtained regression equations and ANOVA analysis, contour plots were generated to evaluate the interaction effects among the investigated cultivation variables bacteriocin and biomass production by *L. fermentum*.

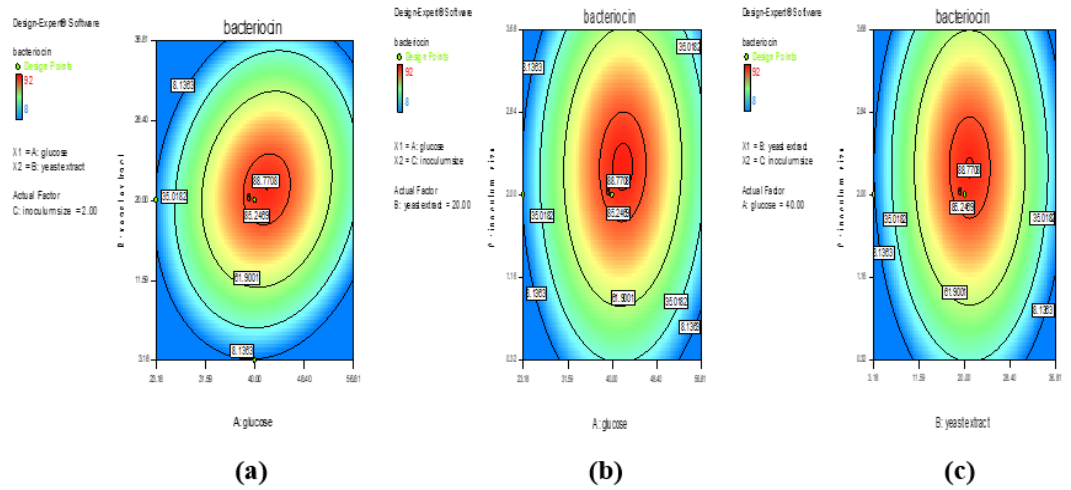


Figure 6: Two-dimensional contour plots showing the effects of cultivation variables on bacteriocin production by *L. fermentum* a: glucose concentrations and yeast extract, b:glucose concentrations and inoculum size , c: yeast extract concentrations and inoculum size.

The contour plots presented in figure 6 illustrated the interaction effects among glucose concentration, yeast extract concentration and inoculum size on bacteriocin production by *L. fermentum*.As shown in figure 6 (a), the combined interaction between glucose concentration and yeast extract concentration significantly enhanced bacteriocin production, where the maximum yield 88.7 AU/ml at around 40.1g/L glucose and 20.0 g/L yeast extract concentration. Similarly, figure 6(b) demonstrated that the interaction between glucose concentration and inoculum size positively influenced bacteriocin formation, with the optimum biomass yield achieved at 40.1 g/L glucose and 2.0% inoculum size. Furthermore, figure 6(c) revealed that the interaction between yeast extract concentration and inoculum size also contributed to increase bacteriocin production, confirming the importance of balanced nutritional and inoculum conditions for metabolite biosynthesis. The elliptical contour patterns detected in the plots showed significant interactions among the investigated variables and verified the appropriateness of the developed quadratic model for optimization purposes. Comparable findings regarding the influence of cultivation parameters on bacteriocin biosynthesis by lactic acid bacteria have been previously reported by Melini *et al.* (2023b) and Veettil *et al.* (2022b).

3.4 Validation of the optimum conditions

Numerical optimizations were subsequently made using Design-Expert software to determine the optimum cultivation conditions for the maximum bacteriocin and biomass production by the isolated *L. fermentum*. The optimization principles were established to maximize both responses at the same time within the examined experimental range. The generated desirability function identified that glucose concentration, yeast extract concentration and the inoculum size as the most effective factors that affecting bacteriocin and biomass production.

Natural Resources for Human Health
eISSN: 2583-1194

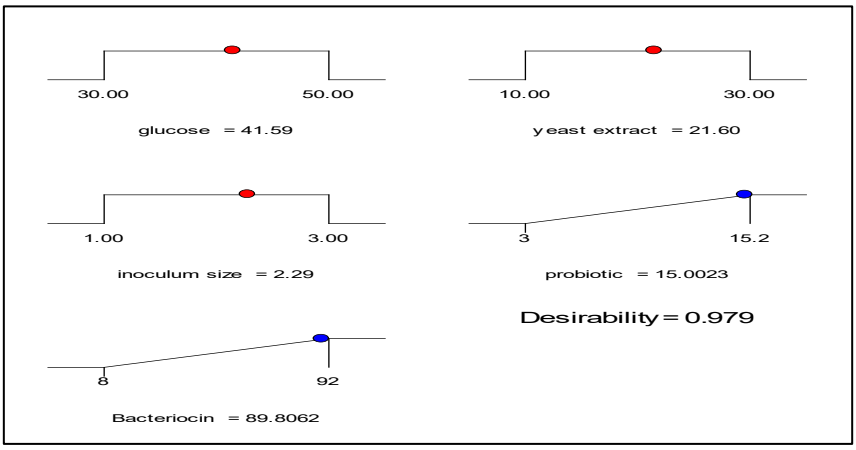


Figure 7: the numerical optimization ramp generated by the CCD-RSM showing the optimum cultivation conditions for maximum biomass and bacteriocin production by *L. fermentum*.

Figure 7 shows the numerical optimization ramp formed by the CCD-RSM model. The desirability function approach expected optimum cultivation conditions of about 41 g/L glucose, 21.6 g/L yeast extract, and 2.2% inoculum size resulting in the predicted biomass and bacteriocin values of 15.8g/L and 89 AU/ml respectively. The desirability value yielded 0.979, indicating excellent agreement between the selected factor levels and the optimization parameters. Experimental validation achieved under these conditions substantiated the reliability of the developed quadratic model, as the detected responses closely aligned with the predicted values.

3.5 The kinetic study under the optimization conditions

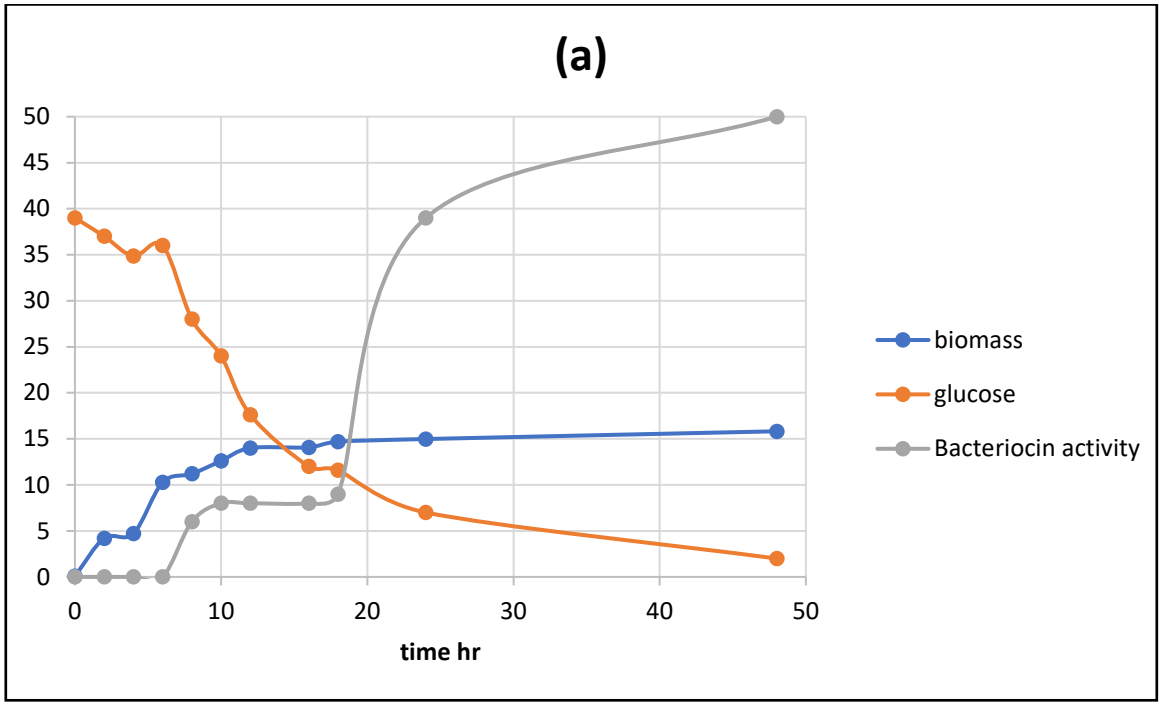
The kinetic behavior of the *L. fermentum* for the production of bacteriocin and biomass was examined under both optimized and non-optimized cultivation conditions. Under the non-optimized medium conditions, the obtained bacteriocin production was low, whereas the bacteriocin synthesis started after about 6 hrs. of incubation and attained to its maximum level after 48 hrs., cmnined by a biomass concentration of 11.3 g/L.

On the other hand, the cultivation under the optimized conditions significantly enhanced both of the biomass formation and bacteriocin production. The maximum biomass production reached approximately to15.8 g/L after 48 hrs. of fermentation, while the glucose concentration gradually decreased to nearly of 2 g/L, indicating effective substrate utilization. Therefore, the optimized medium composition improved the bacterial growth and metabolite biosynthesis efficiency compared with the non-optimized cultivation conditions. The kinetic and stoichiometric parameters correlated to the bacterial growth, bacteriocin formation, and substrate consumption under the optimized and non-optimized conditions are concluded in table 8.

Table 8: Kinetic and stoichiometric parameters associated with biomass and bacteriocin production under optimized and non-optimized cultivation conditions

Kinetics parameters	Un-optimized medium	Optimized medium
xmax g/L	11.3	15.8
Δs	0.94	0.948
μmax	0.05	0.39
Yx/s	0.607	0.85
μ	0.1666	0.1666
Ks	1ave	2

The obtained results confirmed that statistical optimization through CCD-RSM markedly improved bacteriocin yield and biomass productivity by *L. fermentum*. Similar observations regarding the improved metabolite productivity following the culture medium optimization have been stated formerly (Soumya *et al.*, 2012).



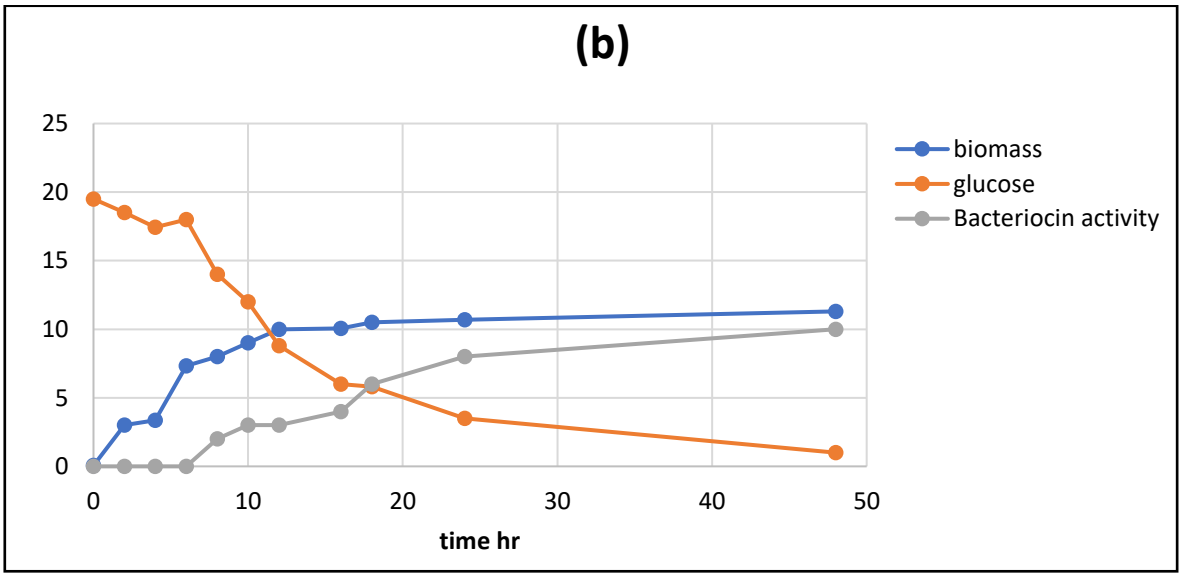


Figure 8: Growth kinetics of *L. fermentum* showing biomass production, bacteriocin formation, and glucose consumption under: a) optimized cultivation conditions; b) non-optimized cultivation conditions

Figure 8 illustrates the kinetic profiles of the biomass production, glucose consumption, and bacteriocin formation by *L. fermentum* under the optimized and non-optimized culture conditions. Under the optimized conditions, the production of bacteriocin increased noticeably and reached to its maximum activity up to 50 AU/ml after 48 hrs. of incubation, whereas noticeably lower production was detected under the non-optimized culture conditions. The accumulation of biomass was also

increased under the optimized conditions, reaching approximately to 15.8 g/L in comparison to 11.3 g/L for the non-optimized culture conditions. At the same time, glucose concentration decreased regularly throughout the fermentation time, demonstrating active substrate utilization related to bacterial growth and metabolites production. These results confirmed that the optimization of the culture composition using CCD-RSM considerably enhanced both the bacterial growth and bacteriocin biosynthesis efficacy.

3.6 Purification of the bacteriocin produced by *L. fermentum*

The purification profile of the bacteriocin produced by the isolated *L. fermentum* is presented in table 9, while the elution profile achieved by using Sephadex G-150 gel filtration chromatography is displayed in figure 9. The crude bacteriocin extract showed a specific activity of 166.7 U/mg protein. Following the precipitation by ammonium sulfate, the specific activity increased noticeably to 300U/mg, equivalent to a purification fold of 1.8. Thereafter dialysis efficiently removed the residual salts and other low-molecular weight impurities, leading to further increasing in the specific activity to 450 U/mg and purification fold of 2.7.

The active fractions were concentrated using sucrose to improve the efficiency of the purification, and yielding a specific activity of 2000 U/mg and a purification fold of 12. The final step of purification by using Sephadex G-150 gel filtration chromatography resulted in the maximum specific activity (3000 U/mg) and the overall purification fold of 18.0, approving a successful enrichment of the active bacteriocin fraction.

Table 9: Summary of the purification of bacteriocin from crude culture filtrate produced by *L. fermentum*

Sample	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude	50	20	0.12	166.7	1000	1.0	100
$(NH_4)_2SO_4$	10	75	0.25	300	750	1.8	75
Dialysis	15	67.5	0.15	450	1012.5	2.7	101
Sucrose	10	100	0.05	2000	1000	12.0	100
Gel Filtration (Sephadex G-150)	33	60	0.02	3000	1980	18.0	198

The profile of gel filtration chromatography in figure 9 showed two major protein peaks observed at 280 nm. However, the bacteriocin activity was detected only with the fractions 28-34, with a highest activity which observed around the fractions 31-32. These findings showed efficient separation of the active antimicrobial peptides from the majority of the extracellular proteins and impurities. The correlation of bacteriocin activity with a distinct chromatographic peaks indicated effective recovery of the active fraction after gel filtration. The continuous increase in the specific activity throughout the purification process demonstrated the effectiveness of the purification approach in concentrating the bioactive extracellular peptides while reducing non-active proteins. The selection of Sephadex G-150 was performed because it provides the efficient separation of proteins over a molecular weight range suitable for the most bacteriocins produced by the lactic acid bacteria. Similar purification methodologies have been extensively employed for the purification of bacteriocin due to their ability to preserve biological activity while enhancing the purity (Wang *et al.*, 2023; Li *et al.*, 2023). The purification fold obtained in the present study (18.0 fold) was higher than that reported by Rasheed et al. (2020), who achieved a 10-fold purification of bacteriocin produced by *L. acidophilus* using Sephadex G-50 chromatography. Likewise, Li et al. (2023) purified bacteriocin from *Enterococcus durans* YQ-6 and reported an 11.7-fold purification following chromatographic separation. More recently, Wang et al. (2023) successfully purified bacteriocin from *Lactiplantibacillus plantarum* using sequential Sephadex chromatography, confirming the effectiveness of gel filtration techniques for bacteriocin recovery.

Overall, the gradual increase in specific activity and purification fold throughout the purification process confirmed the efficiency of the adopted purification strategy and demonstrated that *L. fermentum* produces a highly active bacteriocin.

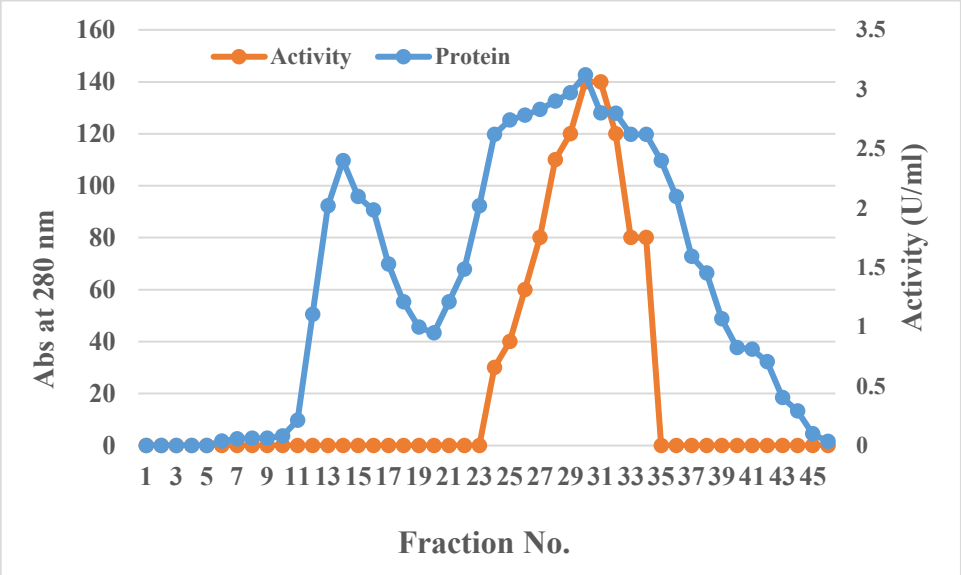


Figure 9. Purification profile of bacteriocin produced by *L. fermentum* by Sephadex G-150 column (2.5 × 20 cm). Column was equilibrated and eluted with sodium phosphate buffer, (pH 7, 0.2 M), in rate of flow 20ml/hr. 3ml for every Fraction

CONCLUSION

In the current research, Response Surface Methodology (RSM) depending on central composite design CCD was efficiently applied to enhance culture medium components for improved biomass and bacteriocin production by the isolated *L. fermentum*. The optimum culture composition composting of about 40 g/L glucose, 20 g/L yeast extract, and 2% inoculum size that significantly improve the biomass yield and bacteriocin production compared with the non-optimized culture conditions. The statistical analysis revealed high model adequacy and predictive reliability, as evidenced by significant ANOVA results and elevated coefficients of determination R^2, for both biomass and bacteriocin production models. The validation experiments verifieded the accuracy of the predicted optimum culture conditions, while kinetic analysis revealed that the highest bacteriocin production was obtained after 48 hrs of incubation, demonstrating a growth associated metabolite production.The sequential purification steps involving ammonium sulfate precipitation, dialysis and gel filtration chromatography using Sephadex G-150 was successfully enhanced the purity of bacteriocin. In the final purification step resulted in a specific activity of 3000 U/mg protein and an 18-purification fold, demonstrating effective recovery of the active antimicrobial extracellular peptides.

In general, the obtained outcomes highlight the efficiency of CCD-RSM as a reliable protocol for improving bacteriocin biosynthesis and purification by the selected *L. fermentum*. The purified bacteriocin revealed a promising antimicrobial potential, proposing its possible application as a natural bio-preservative and antimicrobial agent in the food and biotechnology industries.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest concerning the publication of this research

REFERENCES

[1] Al-Rubaye, D.S., and Al-Rubaye, T.S. (2025). Bioactivity of Streptomyces against colistin resistant K. pneumoniae isolated from different clinical samples. Iraqi Journal of Agricultural Sciences, 56(2), pp. 767–775.

[2] Charles, P.; V. Devanathan; P. Anbu; M. N. Ponnuswamy; P. T.Kalaichelvan and B. K. Hur, 2008. Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. J. of bas. Microbial., 48(5): 347-352

[3] Costa-Trigo, A.P., Patricia, O., David, O., Ricardo, P.O. and José, M.D. (2020). Detoxification of *chestnut burrs* hydrolysates to produce biomolecules. Biochemical Engineering Journal.
[4] Cotter, P. D., Ross, R. P., & Hill, C. (2013). Bacteriocins — a viable alternative to antibiotics? Nature Reviews Microbiology, 11, 95–105.
[5] Gaber, E., Diaa, E.H., Abdelazeem, M., Toyosi, T.G., Philippe, J., Ali, E., Achyut, T., Jorge, P.P., Clara, M.L., Nanasaheb, D.T., Muhammad, Z., Mohamed, E., Abhijit, D., Saad, A., Helal, F.H. and Natália, C. (2021). Application of natural antimicrobials in food preservation: recent views. Food Control.
[6] Hammami, I.; Jaouadi, B.; Ben Bacha, A.; Rebai, A.; Bejar, S.; Nesme, X. and Ali, R. (2012). *Bacillus subtilis* bacteriocin Bac 14B with a broad inhibitory spectrum: purification, amino acid sequence analysis, and physicochemical characterization. Biotechnol. and Bio,Eng, 17: 41-49.
[7] Horowitz, N. H., & Fling, M. (1962). A method for concentrating dilute protein solutions. Fungal Genetics Reports, 2 (1),19.
[8] Li, Y., Zhang, X., Wang, J., et al. (2023). Purification and characterization of a novel bacteriocin produced by *Enterococcus durans* YQ-6. Food Bioscience, 53, 102662.
[9] Lingli, L., Lanhua, Y., Jiaxin, C., Bianfang, L. and Xin, L. (2021). Antibacterial mechanisms of bacteriocin BM1157 against *Escherichia coli* and *Cronobacter sakazakii*. Food Control. 123.
[10] Meera, N.S. and Charitha Devi, M. (2021). Detection, optimization and characterization of bacteriocin produced by *Lactobacillus fermentum* isolated from homemade curd (Indian traditional food). Paripex – Indian Journal of Research, 10(10).
[11] Melini, F., Melini, V., Luziatelli, F., & Ruzzi, M. (2023a). Bacteriocins from lactic acid bacteria: Production, applications and future perspectives. Foods, 12, 2148.
[12] Melini, F., Luziatelli, F., Bonini, P., Ficca, A.G., Melini, V. and Ruzzi, M. (2023b). Optimization of growth conditions through response surface methodology and metabolomics for maximizing auxin formation by *Pantoea agglomerans* C1. Frontiers in Microbiology, 14, 1022248.
[13] Montgomery, D. C. (2017). Design and Analysis of Experiments (9th ed.). John Wiley & Sons, Hoboken, NJ, USA.
[14] Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2016). Response Surface Methodology: Process and Product Optimization Using Designed Experiments (4th ed.). John Wiley & Sons.
[15] Reuben, R.C., Roy, P.C., Sarkar, S.L., Rubayet UI Alam, A.S.M. and Jahid, I.K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. Journal of Dairy Science, 103, pp.1223–1237.
[16] Saxena, S., Singh, P., Kumari, A., (2022). A statistical optimization by response surface methodology for the enhanced production of riboflavin from *Lactobacillus plantarum* HDS27. *Frontiers in Microbiology, 13*, 982260
[17] Sharma, P., Sharma, N., & Singh, J. (2023). Response surface methodology for optimization of microbial metabolite production: Recent applications. Bioprocess and Biosystems Engineering, 46, 1235–1249.
[18] Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2024). Advances in bacteriocin production and optimization strategies. Microorganisms, 12, 456.
[19] Soumya, T.V., Reshma, John and Surya, Jose (2012). Characterization of bacteriocin produced by *Lactobacillus sp.* and optimization of cultural conditions. International Journal of Scientific and Research Publications, 2, pp.2250–3153.
[20] Stanbury, P.F., Whitaker, A. and Hall, S.J. (2016). Principles of Fermentation Technology. 3rd ed. Elsevier.
[21] Veettil, V.N. and Vijaya, C. (2022a). Optimization of bacteriocin formation by *Lactobacillus plantarum* using response surface methodology. Cellular and Molecular Biology, 68(4), pp.105–110.
[22] Veettil, V. N., Prasad, M. M., & Pandey, A. (2022b). Optimization of bacteriocin production from lactic acid bacteria through statistical and response surface approaches. Biocatalysis and Agricultural Biotechnology, 42, 102353.
[23] Vieira, A. D. S., Costa, R. J., & Souza, M. R. (2023). Lactic acid bacteria bacteriocins and their role in food preservation. Food Bioscience, 53, 102652.
[24] Wang, H., Liu, Y., Chen, X., et al. (2023). Purification and characterization of a novel bacteriocin produced by *Lactiplantibacillus plantarum* W3-2. LWT, 184, 114998.
[25] Wu, W., Pang, B., Yang, R. R., Liu, G., Ai, C., Jiang, C. and Shi, J. (2020). Improvement of the probiotic potential and yield of *Lactobacillus rhamnosus* cells using corn steep liquor. LWT, 131, 109862.
[26] Zacharof, M.P. and Lovitt, R.W. (2012). Bacteriocins produced by lactic acid bacteria. A review Article. APCBEE Procedia, 2, 50-56.