Utilization of RSM for optimization of physical variables to improve bacteriocin production from *Lactobacillus plantarum* strain MS

Mahwish Salman [1], Anam Tariq[1*], Muhammad Shahid[2], Amna Rana[1] and Mehwish Naseer[1]

[1]Department of Biochemistry, Government College University Faisalabad, Faisalabad, Pakistan.
[2]Department of Biochemistry, University of Agriculture Faisalabad, University Road, Faisalabad, Pakistan.

Abstract

The utilization of natural antimicrobial substances has been increased in the pharmaceutical sector due to their stability and inhibition activity against pathogenic microorganisms. In the current study, the bacteriocin synthesis by locally isolated *Lactobacillus plantarum* MS from yogurt, has been optimized by using response surface methodology. Statistical optimization of physical variables by using Box-Behnken design, 3200 AU/mL bacteriocin activity was detected at 30 °C temperature, 18h incubation time, and pH 6 against *Staphylococcus aureus*. The resulting bacteriocin was found to be stable a broad range of pH (2-12) and temperature (37-100 °C) but the activity of bacteriocin was slightly disturbed at 121 °C. Besides, bacteriocin showed stability after treatment with various enzymes (catalase, amylase, and lipase), salt (NaCl), and surfactants (triton X-100, EDTA, and SDS) but found sensitive by treating with proteolytic enzymes (proteinase K and trypsin) in comparison with untreated bacteriocin. Because of such biochemical characteristics, these antibacterial peptides could be possibly incorporated in the field of pharmacy to fulfil consumer's demand for natural remedies.

Keywords: Lactic acid bacteria, bacteriocin, antibacterial activity, response surface methodology, Box-Behnken design.

1. INTRODUCTION

In relevance to the vast research studies, a quite crucial role of microbial secondary metabolites has been observed regarding nutrition, health, and economy including enzyme inhibitors, immune-modulating agents, pheromones, toxins, antibiotics, bacteriocins, antitumor agents, pigments, receptor agonists, and antagonist, and growth promoters in plants and animals. To activate the gene-induced synthesis of

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secondary metabolites, the stimulatory effect is brought about by nutritional as well as physical parameters e.g., incubation temperature and time, medium pH, aeration, etc[1]. Consequently, the resistance development by previously existing bacterial strains against antibiotics, there is a need to enhance the antimicrobial activity induced by these secondary metabolites, bacteriocins having low-molecular mass that impose antipathogenic impact even at low concentrations[2]. Bacteriocins are ribosomal-synthesized small proteinaceous compounds produced by lactic acid bacteria (LABs) exhibiting broad-spectrum antimicrobial action against *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus,* and many other related and even unrelated species through different bacteriostatic as well as bactericidal action mechanisms including permeabilization of the cell membrane, preventing cell wall formation, and ceasing of DNase or RNase activity[3]. Hence, it enables bacteriocin to be utilized in fermentation, biopreservation, extending shelf-life, and clinical antipathogenic action. Moreover, it is of great significance in the food industry as it gives rise to safer and minimally processed food products with enhanced nutritional and organoleptic characteristics in comparison to the use of chemical preservatives[4]. The categorization of bacteriocins leads to three classes as I, II, and III based on their physical and chemical properties along with the mode of action [5].

Various strategies have been employed to increase bacteriocin synthesis while keeping the cost low because of its widespread utilization. Of these, a systematic and effective statistical technique for optimizing various physical variables by employing certain statistical designing experiments, formulation models for evaluation of trouble-shooting of optimization process and analysis of individual as well as interaction effect of physical variables by response surface methodology (RSM)[6,7]. Of various experimental design of RSM, Box-Behnken (BBD) is an independent quadratic experimental design of 3-level applicable in many analytical techniques, but not suitable for extreme response conditions[8]. The purpose of current work is utilization of response surface methodology (RSM) for optimization of physical variables targeted to enhance the bacteriocin synthesis from *Lactobacillus plantarum* strain MS so that it would yield better prospects regarding its widespread applications as antibacterial peptide.

2. MATERIALS AND METHODS

2.1 Microbial culture and medium

The isolation of *Lactobacillus plantarum* strain MS from yogurt followed by pour plate and streak plate method under aseptic conditions. *Lactobacillus plantarum* strain MS was grown in MRS agar/broth (Oxoid, England) whereas the indicator strain, *Staphylococcus aureus*, was grown on nutrient broth/agar medium (Oxoid, England). The sample was serially diluted by previously described method of Salman *et al*. (2020a) [9]. After dispensing off 0.1 mL diluents obtained from each dilution, all the MRS agar plates were incubated for 48 h at 37 °C under aerobic incubation. The well-defined colonies after incubation were purified by repetitive streak plate method and stored at 4 °C after being transferred into MRS broth. Meanwhile, a 15% glycerol (v/v) medium was prepared to preserve this culture at -80 °C [10,11] that was utilized for further experiments.

2.2 Growth characteristics of isolated strain

Following Bergey's manual, morphological as well as physiological traits provided a basis for the identification of microbial culture and its phenotypic characterization[12]. To do so, a catalase test was carried out by pouring 1 drop of 3% hydrogen peroxide solution on a microscopic glass slide. Bacterial cells from overnight cultures of the isolated colonies were transferred over that slide with the help of a sterilized nichrome loop to observe gas bubble formation [13]. The bacterial isolates having negative catalase test were employed for Gram staining by following Thairu *et al*. (2014) [14] to identify bacteria based on their physical and chemical properties of cell walls. Whereas to detect biochemically distinct strains, carbohydrate fermentation profile analysis was performed by using API 50 CHL (Bio Merieux, France).

2.3 Molecular characterization of isolated strain

After initial identification, bacterial culture was confirmed by 16S rDNA sequence analysis[15]. Genomic DNA was extracted by following CTAB (Cetyl Trimethyl ammonium bromide) method with slight modifications[16]. Polymerase chain reaction (PCR) for 16S rRNA gene amplification was conducted by utilizing universal primer pair, forward primer fD1: 5'-AGAGTTTGATCCTGGCTCAG-3'; 20 mer, GC% 50, Tm 55.4 °C and reverse primer rD1: 5'-AAGGAGGTGATCCAGCC-3'; GC% 58.8, Tm 52.7 °C[14]. The obtained amplicons were sequenced by availing commercial services of Macrogen, Seoul, South Korea.

2.4 Phylogenetic analysis

Using DNA Dragon software (1.6.0 version, SequentiXDigital DNA Processing, Germany), the resultant sequence of 16S rRNA gene was assembled into contig and compared with sequences in GenBank database using BLASTn (Basic Local Alignment Search Tool for nucleotides), NCBI (National Center for Biotechnology Information)[17]. Complete sequences of 16S rRNA genes of closely related species were retrieved from GenBank to perform multiple sequence alignment by ClustalW. By employing the Neighbor-joining method, a phylogenetic tree was reconstructed by MEGA software (6.0 version)[18]. By using bootstrap analysis, the relationship stability, and the level of confidence of each branch were tested. The analysis was done by performing 1000 re-samplings for the tree topology of the Neighbor-joining data[19]. The resultant 16S rRNA gene sequence of the isolate was deposited in the GenBank database.

2.5 Growth curve and bacteriocin synthesis

Culture (1.5%) was inoculated into 500 mL of growth culture medium that was kept in aerobic incubation at 37 °C for 24 h to hold a check over bacteriocin synthesis. After every 4 h, 10 mL of aliquots were pipetted out in a sterilized way followed by centrifugation at 8000×g for 10 min with the temperature maintained at 4 °C while their optical density was observed at 600 nm during 24 h. The supernatant obtained from the bacterial culture after centrifugation was followed by pH alteration to 6.0 and heating for half-hour at 80 °C. Whereas bacteriocin synthesis was verified by treatment with proteinase K[20]. Phosphate buffer (50 mM) of pH 7.0 was used to suspend precipitates resulting from saturation with ammonium sulfate (60%) at 4 °C for 4 h [21].

2.6 Bacteriocin activity assay

Followed by saturation, the supernatant was filtered through 0.2 μm cellulose acetate membrane filters (Millex-GV filter, Millipore) for estimation of bacteriocin activity by using agar well diffusion method against *S. aureus* [22]. The bacteriocin activity titre was calculated in AU/mL where each AU (arbitrary unit) expressed the unit activity of bacteriocin and determined by unit area of inhibition zone per unit volume (mm^2/mL). Experiment was conducted in triplicate with a 5% mean standard error. Inhibitory potential was analysed via assessing the area of zone of inhibition (mm^2) by applying the formula mentioned as follows [23].

$$\text{Bacteriocin activity (mm}^2\text{/mL)} = \frac{Lz - Ls}{V}$$

Lz = clear zone area (mm^2); *Ls* = well area (mm^2); *V* = volume of sample (mL).

2.7 Biochemical characterization of bacteriocin under stress conditions

By treating with various enzymes, salt, surfactants, and varying range of temperature, and pH, the filtered supernatant was characterized biochemically. One milligram per milliliter of the solution was prepared with enzymes as follows: proteinase K, trypsin, α-amylase, and catalase (Sigma-Aldrich) in varying series of trials. The homogenates were incubated for 30 min at 30 °C and then heated at 95-97 °C for 5 min. To completely abolish possible inhibitory activity due to hydrogen peroxide, the cell-free supernatant was also treated with catalase. The untreated sample was used as a control. In distinct series of trials, the pH of culture supernatant was adjusted to 2, 4, 6, 8, 10, and 12 with 1M HCl/1M NaOH and incubated for 1 h at 30 °C. While the effect of temperature was determined by incubating at 37, 45, 60, 80, and 100 °C for 30 min. In another series of trials, cell-free supernatant was treated with NaCl, solutions (10 mg/mL) of Triton X-100, EDTA (Merck), and SDS (BioRad) and incubated for 30 min at 30 °C. Before measuring bacteriocin activity against *S. aureus* as an indicator strain, the pH of all treated samples was neutralized to 6.0 [24, 25].

2.8 Factorial optimization of physical variables

Box-Behnken design (BBD) was selected to optimize physical parameters as this design has been proved as appropriate for fitting the quadratic surface. The 3 significant experimental variables that opted for the concerned study included (A) temperature, (B) initial pH of the medium, and (C) incubation time with three levels -1, 0, +1. The total 17 experimental runs given in Table 1 were generated with the help of 12 design points as well as 5 replications of the centre points. These points were generated by using a formula $N=2k(k-1)+c_p$, whereas in this equation, N = the total number of experiments; c_p = the number of centre points; k = the number of factors.

2.9 Statistical data analysis

Design Expert-9 version 9.0.2 (Stat-Ease, Minneapolis, USA) was used to develop the experimental design, quadratic model, and data analysis. The predicted response (bacteriocin synthesis) was achieved by analysing the multiple linear regression to determine the set of coefficients. The significance of the model was depicted by the assessment of variance. Moreover, the p-values of individual linear, quadratic, and interaction coefficients also explain the significance of each term[26]. The value of R^2 (coefficient of determination) depicted the fitness of the quadratic polynomial model equation whereas F-value represented the significance of the statistical model while the t-test represented regression coefficients of the model system. The individual and interaction effects of independent variables for bacteriocin synthesis were assessed with response surface plots [27,28].

3. RESULTS

3.1 Morphological and biochemical Identification of isolated strain

The bacterial colonies isolated by serial dilution were examined morphologically. Of these, the colonies having catalase-negative and Gram-positive tests which is the characteristic of genus *Lactobacillus* were selected for further analysis. Subsequently, a biochemical profile was observed by using the API 50CHL system (Bio Merieux, France) and has a 99.9% similarity index with reported *L. plantarum* in apiweb database (V5.1). The growth characteristics of bacterial isolate was given in table 1.

Table 1. Biochemical and morphological properties of bacterial isolate.

| Properties	Bacterial isolate
Colony color	Whitish
Colony shape	Round
Colony margin	Regular
Catalase test	-
Gram staining	+ Rods
Lactose fermentation	+

3.2 Molecular identification of isolated strain

The resultant isolate was further identified and verified as *L. plantarum* after concluding the results of 16S rDNA sequence analysis (Fig. 1). After contig formation, the sequence was compared with LAB strains present in GenBank database. By using BLASTn, bacterial isolate was recognized as a strain of Lactobacilli. The nucleotide sequence of *L. plantarum* strain MS was assigned accession number KP874187 with 1441 bp and deposited in Genbank of NCBI. The phylogram has been classified into four distinct monophyletic groups (i.e., MPG-I, MPG-II, MPG-III, and MPG-IV) depending on common ancestors of the taxa appearing in

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these groups (Fig. 2). The *L. plantarum* strain isolated in this study (*Lactobacillus plantarum* strain MS) appeared in MPG-II and showed a close evolutionary relationship with *L. plantarum* strain JCM 1149 and *L. pentosus*. Other Lactobacillus species showing strong evolutionary relationships with *L. plantarum* strain MS included *L. herbarum*, *L. fabifermentans*, and *L. mudanjiangensis*. The Lactobacillus species (*L. plajomi*, *L. modestisalitolerans*, *L. garii*, and *L. xiangfangensis*) also joined this clade and exhibited similar patterns of evolution.
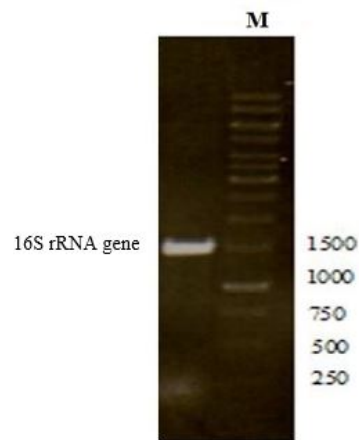

Fig. 1: PCR amplified 16S rRNA gene of bacterial isolate. M: 1kb DNA marker.

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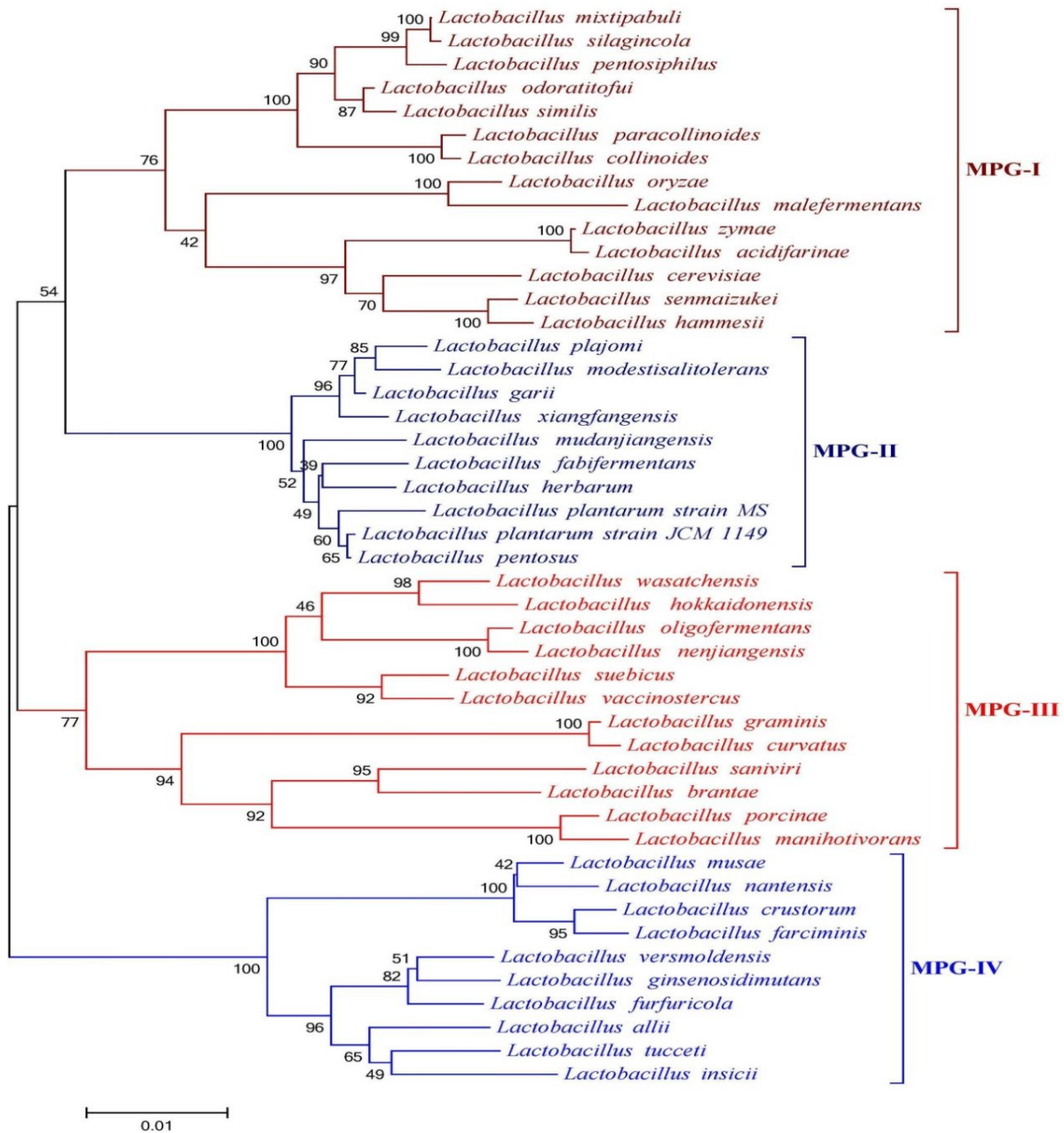
Fig. 2: Phylogenetic tree based on 16S rRNA sequences of isolated lactic acid-producing bacterium (*Lactobacillus plantarum* strain MS) with its selected homologs.

3.3 Bacteriocin Production

Maximum bacteriocin production was obtained after 18h of incubation and became steady upon 24h. The optical density and bacteriocin production were observed after successive growth intervals of 3h for 24h. The maximum antibacterial activity titre observed against *S. aureus* was 2040 AU/mL at 3.3 OD (600nm), as shown in fig. 3.
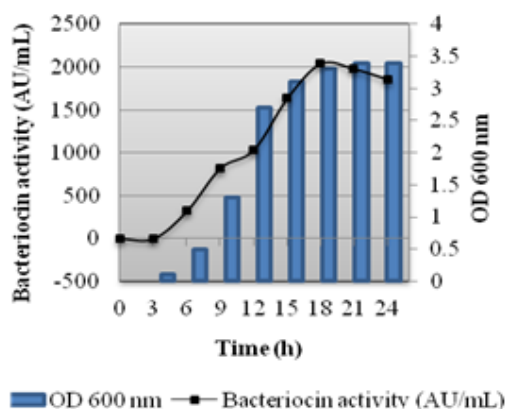
Fig. 3: Growth curve of *L. plantarum* MS bacterial isolate relative to optical density and bacteriocin production.

3.4 Effect of various enzymes, salt, surfactants, heat, and pH on bacteriocin activity

Being proteinaceous in nature, bacteriocin found sensitive after treating with proteinase K and trypsin. While its activity was not affected by amylase and lipase which confirmed that the obtained activity titre was independent of carbohydrate and lipid moieties. Beside this, antibacterial activity of bacteriocin was also not affected by catalase which excluded the effect of H_2O_2, and interpreted that activity was devoid of H_2O_2. Similarly, activity titre was not disturbed by treating with NaCl, SDS, EDTA, and Triton X-100. Moreover, the resultant bacteriocin was found resistant to heat and pH in the range of 2-12. The extremes of temperature and pH effects a little bit on residual activity of bacteriocin (table 2).

3.5 Optimization of physical variables using BBD

Box-Behnken design, a rotatable or nearly rotatable experimental design implemented on critical variables of a study, was used to optimize three physical variables including incubation time, temperature, and pH to enhance the bacteriocin activity by *L. plantarum* strain MS using statistical means. The results depicted that the bacteriocin activity was significantly affected by physical variables. Predicted and experimental values of bacteriocin activity against *S. aureus* were given in table 3.

Table 2: Residual bacteriocin activity (%) after different stress treatments.

Treatments	Residual bacteriocin activity (%)	Treatments	Residual bacteriocin activity (%)
Enzymes		**Heat (°C)**	
Amylase	100	37 (30 min)	100
Catalase	100	45 (30 min)	100
Lipase	100	60 (30 min)	100
Proteinase K, trypsin	0	80 (30 min)	100
pH		100 (30 min)	100
2	93	121 (15 min)	87
4	100		
6	100	**NaCl**	100
8	100	**SDS**	100
10	100	**EDTA**	100
12	95	**Triton X-100**	100

Bacteriocin activity titre of untreated sample = 2040 AU/mL.

Table 3: Optimization of physical variables to improve inhibition activity of bacteriocin by using Box-Behnken design.

Runs	A	B	C	Response (Y1)	
	Temp.[a] (°C)	Initial pH	IT[b] (h)	Inhibition against *S. aureus* (AU/mL)	
				Observed	Predicted
1	15	6	32	2021.67	1981.9
2	45	9	18	356.87	321.17
3	30	6	18	3245.43	3285.56
4	45	6	32	598.23	604.63
5	15	9	18	732.45	742.92
6	15	6	4	160.65	154.25
7	30	9	32	1536.87	1566.17
8	30	6	18	3200	3285.56
9	30	9	4	1287.32	1283.25
10	30	3	32	2387.21	2391.28
11	15	3	18	943.14	978.84
12	45	6	4	721.57	761.34
13	30	3	4	1032.56	1003.26
14	45	3	18	640.87	630.4
15	30	6	18	3297.52	3285.56
16	30	6	18	3301.43	3285.56
17	30	6	18	3186.01	3285.56

[a]Temperature, [b]Incubation time

The full quadratic multiple regression equation for bacteriocin activity by *L. plantarum* MS was given in Eq. 1.

$$Y1 = -8149 + 264.9A + 1361B + 266.3C - 3.247A^2 - 106.5B^2 - 3.832C^2 - 0.2715AB - 1.575AC - 6.578BC$$

Equation 1

A maximum bacteriocin activity titre of 3200 AU/mL was achieved at temperature 30 °C, incubation time of 18h, and pH 6 (run 8) which was in close agreement with the predicted value of the model. The significance of the quadratic polynomial model was represented by ANOVA as it illustrated a quite low *p-value* (probability) < 0.0001 (Hwanhlem *et al.*, 2014) and value of $F_{(9,7)}$ = 857.53 > $Ft_{(9,7)}$ = 3.68. Adj R^2 (0.9980) and pred R^2 (0.9937) depicted a close relation between predicted and experimental model values. Moreover, coefficient of determination (R^2) for regression equation was found to be 0.9991 which accounted that 99.91% of total variations could be explained by model and 0.09% variations cannot be explained by the model in response to independent variables (Yu *et al.*, 2019). Moreover, the reliability of the conducted trials and the model significance was also depicted by the low value of C.V.% = 3.24 (coefficient of variation), shown in table 4.

Table 4: Analysis of variance (ANOVA) for quadratic polynomial model.

Source	Sum of square	Df	Mean square	*F*-value	*p*-value
Model	2.28E+07	9	2.28E+07	857.53	<0.0001*
Residual	20639.32	7	2948.47		
Lack of Fit	7762.34	3	2587.45	0.8	0.5535 n.s
Pure Error	12876.98	4	3219.25		
Cor Total	2.28E+07	16			
$R^2 = 0.9991$, adj $R^2 = 0.9980$, pred $R^2 = 0.9937$, C.V. % = 3.14					

*Significant at 95% confidence level, ns., not significant at 95% confidence level, $p < 0.05$ indicates that the model terms are significant, $p < 0.01$ indicates that the model terms are highly significant.

Also evident by the research work of Jaswir *et al.* (2019) significant coefficients were estimated from the regression equation (Eq. 1) using the student's *t*-distribution and *p*-values (probability) as fewer *p*-values and high *t*-test values were indicative of coefficient significance. Since the *p*-value of interaction coefficient (AB) was greater than 0.05, it was, therefore, considered non-significant while the other interaction coefficients along with all quadratic and linear coefficients were significant at 5% of confidence level (table 5).

Table 5: Estimated regression coefficients for physical variables.

Factors	Coef.[a]	SE coef.[b]	Computed *t*-value	*p*-value
Intercept	-8238.1	168.8	-49.581	<0.0001*
Temperature (A)	372.7	5.743	61.917	<0.0001*
Initial pH (B)	1452	38.89	43.123	<0.0001*
Incubation time (C)	335.4	7.885	47.159	<0.0001*
Temperature × Initial pH (AB)	-0.3824	0.2852	-0.798	0.513
Temperature × Incubation time (AC)	-2.466	0.09333	-20.542	<0.0001*
pH × Incubation time (BC)	-7.689	0.5444	-12.492	<0.0001*
Temperature × Temperature (A^2)	-4.136	0.06212	-65.517	<0.0001*
pH × pH (B^2)	-217.6	3.972	-39.631	<0.0001*
Incubation time × Incubation time (C^2)	-4.479	0.2234	-30.736	<0.0001*

[a]Coefficient, [b]standard error coefficient, *statistically significant at 95% of confidence level.

3.6 Interaction of physical variables for bacteriocin production

The graphical representation of the regression equation was elucidated by response surface plots (3-D) to analyse the relationship between physical variables of the experiment and their resulting combined impact along with maximum bacteriocin yield (Salman *et al.*, 2020a). The response surfaces of the interaction effects of AC (temperature × incubation time) and BC (initial pH × incubation time) exhibited maximum bacteriocin yield by *L. plantarum* against *S. aureus* (fig. 4A and 4B). While the interaction of AB (temperature × initial pH) was found non-significant statistically at 95% of confidence level. Although the linear effects of both variables were significant.
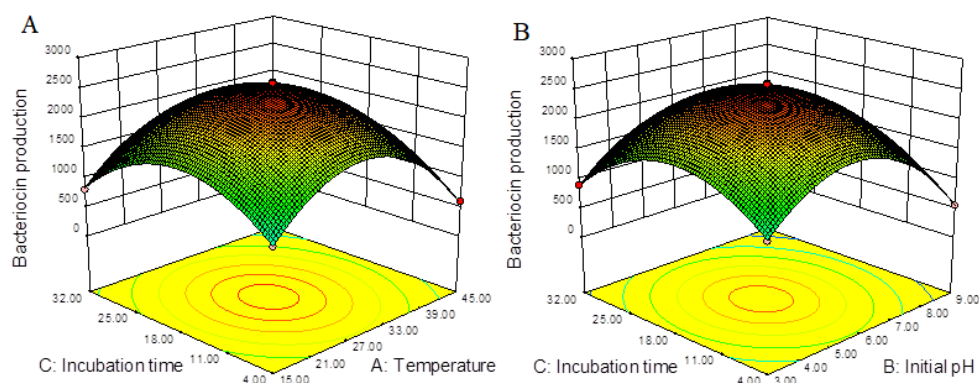
Fig. 4: Response surface plots of significant interactions of physical variables. (A) Temperature and incubation time at fixed pH 6 (B) Initial pH and incubation time at fixed temperature 30°C.

4. DISCUSSION

Lactobacillus plantarum MS isolated from yogurt was found to be lactose fermenting, catalase negative, Gram-positive rods (table 1). The isolated bacterial strain was initially identified by morphological and biochemical characterization and confirmed by 16S rRNA gene sequence analysis (fig. 1 and 2), which is significant for identifying bacteria at genus and species level [29].

The bacteriocin isolated from *L. plantarum* MS was proteinaceous in nature (table 2). Likewise, Zhang *et al.* (2018) [30] reported for bacteriocin Lac-B23, its activity was completely devalued after treating with proteinase K and trypsin, evaluated that these enzymes totally remove antibacterial effect of bacteriocin. However, activity was maintained at 25-100°C and pH 2-10 while affected at extremes of temperature and pH [31]. Similar findings have been reported for bacteriocins synthesized by *L. plantarum* LE7, *L. plantarum* LE5, *L. plantarum* ST71KS, *L. sake* C2, and *Bacillus subtillus* R75 [21,31, 32,33]. The heat stability of bacteriocin was due to the presence of a complex pattern of disulfide intramolecular bonds, hydrophobic interactions, and stable cross-linkages, high glycine contents, which overall stabilizes secondary and small globular structures of bacteriocins [24]. Whereas an adequate rise in temperature results in the loss of secondary and tertiary structure of proteins which causes protein denaturation leading to its impairment and almost entire loss of confirmation. Bacteriocin synthesized in current study was found to be stable at neutral pH whereas extreme acidic and basic conditions slightly disturbed its antibacterial activity. Resistance to high temperature opens a path for use of such bacteriocins in pharmaceutical sector which requires processing at high temperatures. Such low molecular mass bacteriocins were also effective against food pathogens which are resistant to heat and thus could be utilized as an influential source of antibacterial peptides.

The use of a regression model for enhanced bacteriocin production by optimizing physical variables was found to be more effective and economic in comparison with following one factor at a time method. BBD was primarily employed in combinations of the experimental variables that were considered sufficient for assessment of the potentially complex response function [22]. The experimental run 8 indicated maximum bacteriocin activity titre of 3200 AU/mL was in close agreement with the predicted value (3285.56 AU/mL) achieved at temperature 30°C, incubation time of 18h, and pH 6 (table 3). These findings were also verified by Martinez *et al.* (2013) [21] where physical parameters were similarly optimized at 30 °C for an incubation span of 18h in case of bacteriocin production by *L. plantarum* ST71KS. However, in the case of *L. salivarius* CRL 1328, the optimum temperature was found to be 37 °C while the optimum pH was ranging from 6.5-8. The bacteriocin production from *L. lactis* was enhanced by optimizing the physical variables as pH was adjusted to 6.0 at 30 °C of incubating temperature [34].

Naturally, bacteriocin production has been affected by different environmental factors including incubation time, temperature and pH of the medium which may vary from optimum growth conditions of the

bacterium[25]. Additionally, the transcriptional and translational modulators activated by certain physiological parameters that are controlled by pH determine their impact on gene product expression including bacteriocin synthesis. Also, pH is greatly involved in increasing medium acidification that ultimately contributes to stimulating bacteriocin synthesis as well as its adsorption on synthetic strains. Similarly, temperature also plays a crucial role in accelerating cell growth as well as the regulation of microbial enzymes.

5. CONCLUSIONS

Conclusively, a wide range of bacteriocins extracted from *Lactobacilli* are low molecular mass antimicrobial peptides. Efficiency, cost-effectiveness, and yield of bacterial metabolites including bacteriocin can be improved through optimisation of physical variables of the process preferably through statistical methods rather than the conventional one-factor-at-a-time approach as it is quite extensive and costly. Hence, the bacteriocins of *Lactobacilli* after biosafety assay can be employed in food industries as preservatives or in medical sectors as a natural proteinaceous substance to combat against causative microbes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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