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Optimization of Growth Conditions for Enhanced Bacteriocin Production from *Lactobacillus brevis* UN by One Variable at a Time (OVAT) and Response Surface Methodology (RSM)

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***Abstract*:** Lactic acid bacteria have recently caught the attention of scientific researchers. These food associated organisms and transient inhabitant of human gut has potential to curb down the growth of spoilage and disease causing microbes due to its ability to produce bacteriocin, lactic acid, acetic acid, hydrogen peroxide etc. Bacteriocins are biologically derived primary or modified product of bacterial ribosomal synthesis, which have an antibacterial activity. Bacteriocin producing isolates were isolated from traditional and least explored food samples of Himachal Pradesh and North East India by standard microbiological techniques. Isolated strains screened for their bacteriocin production. Growth conditions for bacteriocin production have been optimized by statistical tools viz. One variable at a time method and Response Surface Methodology (RSM). In the present study bacteriocin producing isolate UN was identified and selected for further studies. Various growth conditions viz. growth cycle, effect of incubation temperature, pH, inoculum size were studied to monitor their effect on bacteriocin production. Bacteriocin production was optimized through Classical One Variable at a Time Method (OVAT) and Response Surface Methodology (RSM). Bacteriocin production was maximum at early stationary phase i.e. 34th h, at pH range 4.0-6.5, at temperature 30-35 ^{0}C with an inoculums size 1.0, 1.5, 2.0 OD at 10 %. The production of bacteriocin seems not to be inducible. The incubation conditions and perfect combination of these conditions particularly temperature, pH, inoculum size and time strongly influence the effective yield of active bacteriocin. One Variable at a Time Method and Response Surface Methodology proved to be powerful tools in optimization of bacteriocin production by *Lactobacillus brevis* UN.

***Keywords*:** Antagonism, Bacteriocin, Optimization, Stationary Phase

Diversity of Indian fermented foods is related to incomparable food culture of each community. India, being a huge country has been the home of innumerable religions, human population and diversity in climatic conditions has resulted in a large number of fermented food. Diverse type of ethnic fermented foods and alcoholic beverages are produced either naturally or by adding mixed starter cultures using indigenous and scientific knowledge of food fermentation (Tamang 2021). Food fermentation develops flavour and aroma in food and also detoxify the food and reduce cooking time and fuel requirements. Indigenous food fermentation is one of the oldest process that is dependent upon the microbial activity. Lactic acid bacteria play an important role in the traditional fermentation processes by their functional properties such as bio preservation, bio enrichment of nutritional value, bioavailability of minerals, production of antioxidants, antimicrobial activities and probiotic and postbiotic properties (Gautam and Sharma 2015, Zapasnik et al 2022). The use of lactic acid bacteria and antimicrobial compounds especially bacteriocins is a promising ongoing development in food preservation. Bacteriocins are ribosomal synthesized extracellularly released bioactive peptides or peptide complexes that vary in spectrum of activity, mode of action, molecular weight, genetic organization and considered to be safe bio preservatives since they are assumed to be degraded by proteases in gastrointestinal tract (Gautam and Sharma 2015). Bacteriocin production has been reported to be affected by several factors and fermentation conditions, such as pH, temperature, inoculum size. The optimization of bacteriocin production and enhancement of its activity are economically important to reduce the production cost. Thus, the objective of this paper is to statistically investigate the effect of growth conditions on the maximum bacteriocin production by *L. brevis* UN isolate obtained from Dhulliachar-Traditional Food product of North East India.

MATERIAL AND METHODS

Collection, Isolation and Screening of bacteriocin producing isolates: Diverse traditional least explored food samples of Himachal Pradesh and Sikkim States of India viz., sepu vari, dangal vari, goat meat, chur saag, salori,

gundruck, nimboosat, nashasta, chillipickel, dried shrimp, dried fish, rice beer, chaang (wheat fermented), chaang (rice fermented), sauerkraut and dhulliachar were explored for isolation of bacteriocin producing lactic acid bacteria. All samples were collected in clean and sterilized polythene bags or test tubes and stored in refrigerator until further use. In total 16 food samples were taken for isolation of bacteriocin producing lactic acid bacteria. The isolation was carried out by serial dilution method on de Man Ragosa sharpe (MRS) agar (Hi Media Laboratory Pvt. Ltd. Mumbai, India) under anaerobic conditions. The bacterial colonies obtained on MRS agar were further streaked and the pure lines were obtained. Pure cultures were further preserved on stabs and 40 % glycerol in deep freezer (-20^{0}C). Screening of isolates were performed on the basis of morphological, physiological, biochemical and antagonistic pattern. In total, 53 bacteria were isolated from diverse food sources and were differentiated and observed on the basis of morphological, cultural and biochemical characteristics.

All bacterial isolates which showed catalase test -ve were tested against indicators by Bit/Disc method (Gautam and Sharma 2015) and well diffusion method (Gautam and Sharma 2015). Serious food borne pathogens/food spoilage bacteria i.e. *Listeria monocytogenes* MTCC 839 and *Leuconostoc mesenteroides* MTCC 107, *Enterococcus faecalis* MTCC 2729, *Lactobacillus plantarum* CRI, *Bacillus cereus* CRI, *Clostridium perfringens* MTCC 1739, *Pectobacterium caratovorum* MTCC 1428, *Escherichia coli* IGMC and *Staphylococcus aureus* IGMC, *Aeromonas hydrophila* IGMC were used in screening of bacterial isolates for bacteriocin production. The test strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh, Central research Institute, Kasualli and Indira Gandhi Medical College (IGMC), Shimla. All these test strains were revived twice for 24 h at 37^{0}C before performing the experiments, as all these indicators were preserved in 40 % glycerol at -20°C. On the basis of bit disk method and well diffusion method isolate UN isolated from dhulliachar was finally selected for further study. On the basis of 16S rRNA gene technique UN identified as *Lactobacillus brevis.* The sequences so obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number. *L. brevis* UN registered under the accession no JX046150.

Estimation of activity units per ml (Arbitrary units - AU/ml) of culture supernatant of *L.brevis* UN: The activity units of culture supernatant of *L. brevis* UN was calculated by serial two fold dilution method. AU/ml was calculated as reciprocal of the highest dilution of bacteriocin containing sample that gave a minimal visible inhibition zone (Noorozi et al 2019)

Optimization of process parameters for bacteriocin production by using classical one variable at a time method (OVAT): Various growth conditions viz. incubation temperature, pH, inoculum size, incubation time were studied to monitor their effect on bacteriocin production.

Bacteriocin production during growth cycle of L. brevis UN: MRS broth (pH 6.5± 2) was seeded with active bacterial isolate *L. brevis* UN @10 % (1.0 OD). Bacterial isolate was incubated in orbital shaker at 35±2°C with a shaking speed of 120 rpm for 60 h on rotatory shaker. OD of isolate was noted down periodically after every 2 h at 540 nm. To detect bacteriocin production, the culture of *L. brevis* UN was centrifuged after every 2 h at 18,000 rpm at 4°C for 20 min. The cell free supernatant was neutralized to pH 7.0 (with sterilized 1N NaOH), catalase was added @ 10 mg in 100 ml and well diffusion method was repeated with these crude preparations of isolate against test indicators. The plates were kept for incubation at 35±2°C for 24 h and results were observed as clear halos of inhibition formed around the wells against three selected standard indicators i.e. *L. monocytogenes, S. aureus* and *C. perfringens.*

Effect of temperature: The optimum temperature for bacteriocin production for *L. brevis* UN was determined by inoculating 24 h old culture of each bacterial strain separately in Erlenmeyer flasks followed by their incubation at different temperature i.e. 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C, 45^{0}C, 50 ^{0}C. The inhibitory activity of *L. brevis* UN determined after 34th h of growth by well diffusion assay.

Effect of pH: The 24 h old culture of *L. brevis* UN was inoculated separately on MRS medium of varying pH in the range of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and effect of different pH on inhibitory activity of *L. brevis* UN was studied after 34h of growth against three standard indicators viz. *L. monocytogenes, S. aureus* and *C. perfringens* by well diffusion method.

Effect of inoculum size: Different inoculum size viz. 0.5, 1.0, 1.5 and 2.0 (O.D.) with 10 % of *L. brevis* UN was added in flask containing MRS broth having best studied pH. The growth of bacterial isolate was achieved by incubating them at best optimized temperature of previous experiments. The antagonistic pattern of *L. brevis* UN was determined after 34 h of growth by well diffusion assay against three reference targets.

Optimization of bacteriocin production by using Response Surface Methodology (RSM) Central Composite design: RSM was carried out using central composite design, optimize for further process to identify the interactions between the significant factors obtained from OVAT. The 4 variables chosen in this experiment were

temperature, pH, inoculum size and incubation time with 5 coded levels (−α,-1, 0, +1, + α) were used for their combined influence on bacteriocin production. Central composite design constituted of 30 experimental trials which were carried out with 16 factorial points, 8 axial points with α = 2 and 6 replication of central points.

In developing regression equation, the test factors were coded according to the Eq. (1)

$X_i = (X_i - X_0)/\delta X_i$

Where x_i is the dimensionless coded value of the i^{th} independent variable; X_i the natural value of the i^{th} independent variable; X_0 the natural value of the i^{th} independent variable at the centre point and δX_i is the difference in effect. The response data obtained from the design were fitted with a second order polynomial. The general polynomial equation is as follows in Eq. (2)

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+ \beta_{11}x_{12}+\beta_{22}x_2^2+\beta 33x_3^2 +\beta 44x_4^2+\beta 12x_1x_2+\beta_{13}x_1x_3+\beta_{14}x_1x_4+\beta_{23}x_2x_3+\beta_{24}x_2x_4+\beta_{34}x_3x_4$$

Where, Y is the predicted response i.e. bacteriocin produced (AU/ml), β_0 is the model constant, β_1, β_2, β_3, β_4 the linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} the squared coefficients and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} the interaction co-efficient.

Data analysis: Design expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA) was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2 and the significance of the model, an optimum value of parameters was assessed by the determination coefficient, correlation coefficient and statistical testing of the model was made by Fisher's test Myers et al (2016).

RESULTS AND DISCUSSION

Initial screening of 53 isolates was done on the basis of catalase test. Out of 53 isolates 41 isolates were short listed depending upon being catalase negative in nature (tentatively identified as *Lactobacilli*) and 12 isolates were discarded as these were catalase positive (tentatively identified as *Bacilli*). The above selected 41 catalase negative isolates were further screened for antagonistic activity by bit/disk method against test pathogens. The isolates which showed maximum percent inhibition against indicators were subjected to final screening by well diffusion method. Out of 41 isolates 8 isolates were selected for well diffusion assay as these suppressed maximum number of test pathogens. In well diffusion assay, UN exhibited highest percent inhibition i.e. 60% against highest number of test pathogens, thus finally screened for further characterization and preservation related experiments. The 16S rRNA sequences of the isolate have been submitted to GenBank databases. *L. brevis* UN is registered under the accession number JX046150.

Optimization of process parameters for bacteriocin production by using classical one variable at a time method (OVAT): Bacteriocin production is influenced by growth factors. Effect of incubation time, temperature, pH, inoculum size influence the yield of active bacteriocin significantly. Optimum conditions for bacteriocin production have been set by statistical tools i.e. one variable at a time approach and response surface model. Bacteriocin production is growth associated.

Production of bacteriocin during growth cycle: The growth curve of the isolates followed a sigmoid curve pattern based on measuring bacterial turbidity level OD_{540} nm (Fig. 1, 2). The bacterial cultures were incubated at 37°C in MRS broth (6.5 pH) for different interval of time (24 to 60 h). Optical density and inhibition zones of bacterial culture were measured after 2h of interval at 540 nm. *L. brevis* UN was quite effective against all the three tested indicators viz. *L. monocytogenes*, *S. aureus* and *C. perfringens* within 32nd to 62nd h of production time in the presence of organic acids but after ruling out the acidity of the supernatant it was observed

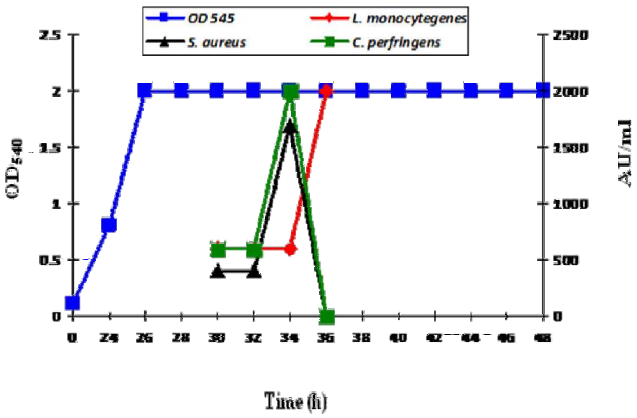


Fig. 1. Growth curve of *L. brevis* UN showing production pattern of bacteriocin

Fig. 2. Effect of different inoculum size on bacteriocin production by *L. brevis* UN

that *L. brevis* was effective against test indicators at 34thh of production time. Maximum bacteriocin production by isolate *L. brevis* UN was measured at 34th h having activity units of 6×10^2 AU/ml against *L. monocytogenes*, 1.7×10^3 against *S. aureus* and 2×10^3 AU/ml against *C. perfringens*. Bacteriocin is produced at a particular time of growth phase so it is necessary to find out that period of growth cycle where bacteriocin is produced. Maximum bacteriocin production by isolate *L. brevis* UN was measured at 34th h. After wards the decrease in bacteriocin production at the end of stationary phase may be due to protein aggregation, proteolytic degradation b**y** specific or nonspecific enzymes and re-adsorption of bacteriocin to the producer cell surface at low pH (Gupta et al 2010).

Effect of temperature: *L. brevis* UN showed best antagonism against all tested indicators at 35^{0}C (Table 1). At this temperature, *L. brevis* UN inhibited the activity of *L. monocytogenes*, *S. aureus* and *C. perfringens* by forming zones of 3mm, 10mm and 10mm with bacteriocin activity 6×10^2, 2×10^3 and 2×10^3 AU/ml. Bacteriocin of *L. brevis* UN failed to inhibit *L. monocytogenes* and *C. perfringens* at growth temperatures 25^{0}C, 45^{0}C and 50^{0}C. In contrast to these test indicators *S. aureus* was inhibited by the same bacteriocin produced at 25^{0}C, 45^{0}C and 50^{0}C. Temperature is a major factor that influences the antagonistic activity of bacterial strain. Growth at optimal temperatures usually results in optimal bacteriocin production but temperature stress and growth at suboptimal temperature may result in decrease in its yield.

Effect of pH: The maximum antagonism and bacteriocin activity by *L. brevis* UN was observed at pH 4.0 and 4.5 (Table 2). The inhibitory zone size and bacteriocin activity (AU/ml) at pH 4.0 and 4.5 against *L. monocytogenes*, *S. aureus* and *C. perfringens* were 3.0 mm (6×10^2 AU/ml), 10mm (2×10^3 AU/ml) and 10 mm (2×10^3 AU/ml), respectively. No inhibition/activity was observed below pH 4.0 and above 6.5 against *L. monocytogenes* and above 7.5 for *S. aureus*. The zone size of 10mm each with 2×10^3 AU/ml were observed against *S. aureus* and *C. perfringenes* while zone of inhibition of 8mm and bacteriocin activity 16×10^2 at pH 4.0 and 4.5

Table 1. Effect of temperature on bacteriocin production by *L. brevis* UN through classical one variable at a time method

Temperature (^{0}C)	*L. monocytogenes*		*S. aureus*		*C. perfringens*	
	Zone size* (mm)	AU/ml$^{\#}$	Zone size* (mm)	AU/ml $^{\#}$	Zone size * (mm)	AU/ml$^{\#}$
25	-	-	10	2×10^3	-	-
30	1	2×10^2	10	2×10^3	-	-
35	3	6×10^2	10	2×10^3	10	2×10^3
40	1	2×10^2	10	2×10^3	5	1×10^3
45	-	-	10	2×10^2	-	-
50	-	-	7	14×10^2	-	-

*Bacteriocin production in terms of inhibitory zone size
#AU/ml: Arbitrary unit (reciprocal of highest dilution forming detectable zone of inhibition) expressed in the form of AU

Table 2. Effect of pH on bacteriocin production by *L. brevis* UN through classical one variable at a time method

pH	*L. monocytogenes*		*S. aureus*		*C. perfringens*	
	Zone size* (mm)	AU/ml$^{\#}$	Zone size* (mm)	AU/ml $^{\#}$	Zone size * (mm)	AU/ml$^{\#}$
3.0	-	-	1	2×10^2	-	-
3.5	-	-	7	14×10^2	-	-
4.0	3.0	6×10^2	10	2×10^3	10	2×10^3
4.5	3.0	6×10^2	10	2×10^3	10	2×10^3
5.0	3.0	6×10^2	8	16×10^2	7.0	14×10^2
5.5	3.0	6×10^2	8	16×10^2	7.0	14×10^2
6.0	3.0	6×10^2	5	1×10^3	5.0	1×10^3
6.5	3.0	6×10^2	5	1×10^3	3.0	6×10^2
7.0	-	-	5	1×10^2	3.0	6×10^2
7.5	-	-	5	1×10^2	-	-
8.0	-	-	-	-	-	-

* Same as in Table 1
# Same as in Table 1

AU/ml was observed against *S. aureus* and $14x10^2$ AU/ml against *C. perfringens* at pH 5.5. Every microorganism has a minimal, maximal and optimal pH for growth and metabolism. Microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH (Bhattacharya and Das (2010). Thus, studying the effect of pH on growth of isolate is an important criterion of the study. In the present investigation it is observed that maximum bacteriocin production by lactic acid bacteria took place at acidic pH. It is a well-known fact that lactic acid bacteria optimally grow under slightly acidic conditions, when pH is between 4.0 to 6.4. Genes and proteins involved in pH homeostasis and cell protection or repair play a role in acid adaptation mechanism of lactic acid bacteria (Yang et al. 2018). The three main systems involved in pH homeostasis in lactic acid bacteria i.e., the ADI system i.e. urease and arginine deiminase pathways, the H - ATPase proton pump, and the GAD i.e. the lysine, arginine, and glutamate decarboxylases (GAD). In addition to all these above mentioned mechanisms LABs are also capable of inducing an acid tolerant response to mild-acid treatment. The systems induced by this response include pH homeostatic, protection, and repair mechanisms (Cotter 2003).

Effect of inoculum size: Highest bacteriocin production by *L. brevis* UN was with an inoculum of 1.5 OD (Fig. 3). Bacteriocin of *L. brevis* UN with inoculum size of 1.5 OD inhibited *L. monocytogenes*, *S. aureus* and *C. perfringens* by forming zones of 5mm ($1×10^3$), 10 mm ($2×10^3$) and 10 ($2×10^3$) respectively. Though, bacteriocin of *L. brevis* UN was produced with an inoculum size of 2.0 OD inhibited all the three test indicators but potency of inhibition was less as it form zones of 3mm ($6×10^2$), 8mm ($16×10^2$) and 7.5 ($15×10^2$) against *L. monocytogenes*, *S. aureus* and *C. perfringens*. *L. brevis* UN did not inhibit *L. monocytogenes* and *C. perfringens* when seeded with inoculum of OD 0.5 and 1.0. Whereas *L. brevis* UN produced bacteriocin with each tested inoculum i.e. OD 0.5, 1.0, 1.5, 2.0 that was found active against *S. aureus* and formed zones of 3 , 7.5, 10 and 8 mm with bacteriocin activity of $6×10^2$, $15×10^2$, $2×10^3$ and $16×10^2$ AU/ml, respectively. Increase or decrease in bacteriocin production by changing the inoculum size may be explained on the basis of ratio of number of nutrient molecules to the number of microbial cells. At lower concentrations all the nutrients seemed to be occupied by the available microbial cells and some of the nutrients remained without access to the cells by increase in the inoculum size, thus the biosynthesis of bacteriocin is often an inducible trait that depends on the cell density of the cell culture Usmiati and Marwati (2009). Therefore, in the present study the improved

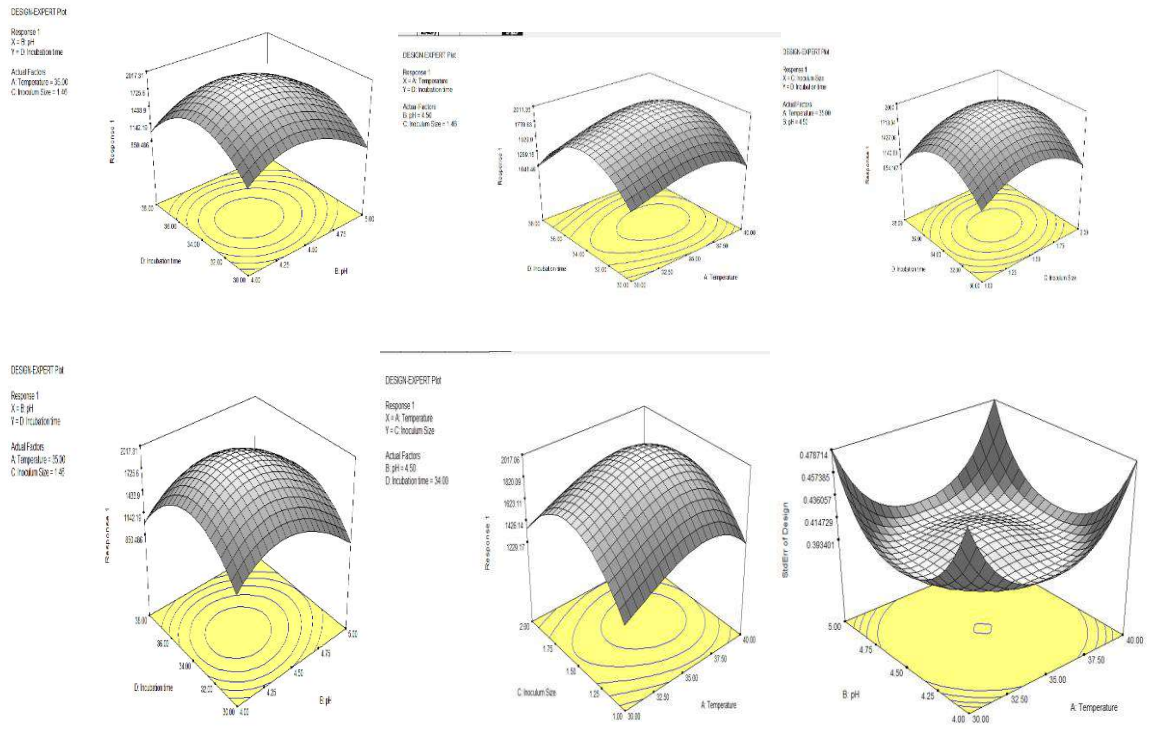


Fig. 3. Response surface curves for bacteriocin activity as influenced by the level of pH, incubation temperature, inoculum level and incubation time

bacteriocin production was achieved by optimizing the inoculum size of both the isolates.

Optimization for bacteriocin production by using Response Surface Methodology: The variables temperature, pH, inoculum size and incubation time were added for the optimization by RSM. The results of 30 run from CCD experiments for studying the effects of 4 independent variables on bacteriocin production are represented in Table 3. The maximum experimental value for bacteriocin production was 2000 AU/mL based on RSM. The regression analysis data were fitted to a quadratic model and the second order regression equation obtained was full actual model on bacteriocin production is shown in Eq. (3)

$$Y = +2000 + 141.67^{*}A - 208.33^{*}B + 41.67^{*}C - 58.33^{*}D - 257.29^{*}A^{2} - 432.29^{*}B^{2} - 432.29C^{2} - 582.29^{*}D^{2} - 43.75^{*}A^{*}B - 31.25^{*}A^{*}C + 43.75^{*}A^{*}D - 56.25^{*}B^{*}C + 18.75^{*}B^{*}D + 56.25^{*}C^{*}D$$

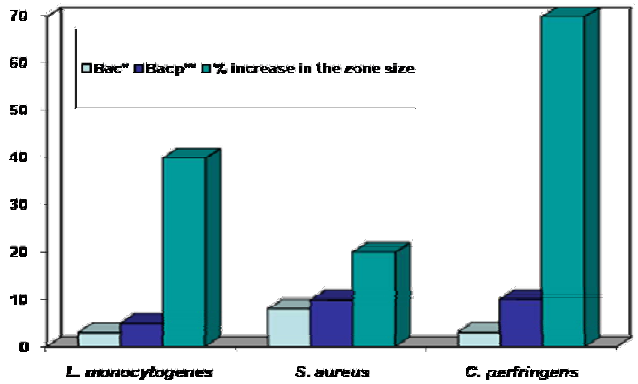
Where Y is bacteriocin activity AU/ml. A is temperature, B is pH, C is inoculum size and D incubation time. The statistical significance of equation 3 was checked by F-test implies model is highly significant. There is only a 4.20% chance that a "Model F-Value" this large could occur due to noise. The goodness of the model can be made by the determination coefficient (R^2) and the correlation coefficient (R). Values of "Prob > F" less than 0.0500 indicate model

Table 3. Bacteriocin activity of the culture *L. brevis* UN cultivated with different levels of pH, temperature, inoculum size and incubation time

Temperature	pH	Inoculum size	Incubation time	Bacteriocin activity
33	4.30	1.70	32	600
37	4.70	1.30	32	600
35	4.50	1.50	34	2000
31	4.50	1.50	34	800
35	4.50	1.90	34	600
35	4.50	1.50	34	2000
35	4.50	1.50	34	2000
35	4.50	1.10	34	600
33	4.30	1.30	32	600
37	4.30	1.70	32	600
33	4.30	1.30	36	400
35	4.50	1.50	38	0
35	4.50	1.50	34	2000
37	4.70	1.70	36	600
37	4.30	1.70	36	400
33	4.30	1.70	36	400
35	4.10	1.50	34	400
33	4.70	1.30	32	800
37	4.30	1.30	36	1000
39	4.50	1.50	34	0
35	4.50	1.50	34	2000
37	4.30	1.30	32	600
33	4.70	1.30	36	600
35	4.50	1.50	30	0
35	4.90	1.50	34	2000
37	4.70	1.50	36	1000
33	4.70	1.30	36	600
37	4.70	1.70	32	800
33	4.70	1.70	32	400
35	4.50	1.50	34	2000

Table 4. R -Squared, Adj R-Squared, Pred R-Squared and Adeq Precision value of the model

Std. Dev	466.88	R-Squared	0.9201
Mean	636.67	Adj R-Squared	0.6689
C.V. (%)	73.33	Pred R-Squared	-10.5099
Press	2.197E+008	Adeq Precision	5.698



Bac* = Initial production, Bacp** = Production after optimization

Fig. 4. Per cent increase in bacteriocin production of *L. brevis* UN after optimization incubation conditions through classic one variable at a time method

terms are significant. In this case A^2, B^2, C^2, D^2 are significant model terms. A negative "Pred R-Squared" implies that the overall mean is a better predictor of response than the current model."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here ratio of 5.698 indicates an adequate signal. This model can be used to navigate the design space (Table 4). Response surface representation provides a method to show relation between the response and experimental levels of each varabile. This technique has been widely adopted for optimizing the process of bacteriocins of *Pediococcus pentosaceous* Sanna (Upendra et al 2021). The increase in size of zones of inhibition after optimization is directly associated with the increase in bacteriocin production.

Per cent increase in bacteriocin production after optimization: The zone of inhibition of bacteriocin of *L. brevis* UN against test indicators increased after optimizing various parameters. The inhibitory zone size of bacteriocin of *L. brevis* UN increased 40% against *L. monocytogenes*, 20% against *S. aureus* and 70% against *C. perfringens* (Fig. 4).

CONCLUSIONS

The increase in bacteriocin production after optimization revealed that the conditions of incubation particularly incubation time, temperature, pH, inoculum size influence the yield of active bacteriocin significantly. Bacteriocin production was maximum at early stationary phase i.e. 34th h, at pH range 4.0-6.5, at temperature 30-35 ^{0}C with an inoculums size 1.0, 1.5, 2.0 OD at 10 %. It is necessary to optimize various process parameters of bacteriocin producing isolates to achieve maximum bacteriocin production. Response surface methodology (RSM) results indicated all the four factors studied have significant effect on bacteriocin production and proved to be a powerful tool in optimizing the culture conditions.

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