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Optimization of Bacteriocin Production by *Lactobacillus fermentum* Strain COE20 from Fermenting *Pentaclethra macrophylla* Benth Using Response Surface Methodology

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Abstract This study evaluated the effect of varied culture conditions (Temperature, pH, and Sodium Chloride concentration) on bacteriocin production by *Lactobacillus fermentum* strain COE20 isolated from fermenting African oil bean seeds (*Pentaclethra macrophylla* Benth) using Response Surface Methodology (RSM). A Central Composite Design (CCD) was adopted with the interest of estimating the optimal conditions for its production using the response surface regression model, which estimated the linear, squared, and interactive relationship between the response variables. The Analysis of Variance (ANOVA) showed that the coefficient of determination in terms of predicted R^2 was 0.8697 which was in close agreement with an adjusted R^2 of 0.7393 and was accounted for by the predictors suggesting that the model was adequate. Optimal culture condition for bacteriocin production by *L. fermentum* strain COE20 was found at approximately 31 ℃, pH 5.9, 1.9% NaCl concentration at Y = 11.75mm. Y represents the response (zone of inhibition) against *Staphylococcus aureus* ATCC 19095 using the agar well diffusion assay method.

Keywords: *optimization, bacteriocin, Pentaclethra macrophylla, response surface methodology, Lactobacillus fermentum*

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1. Introduction

The consumption of food by animals and humans geared towards energy production can come in raw, processed, or fermented, promoting growth and maintaining excellent health [1]. Chemically, food products consist of carbohydrates, protein, fats, water, and other organic compounds and minerals that can encourage the growth of microorganisms as they serve as energy sources and nutrients [2].

Several methods of preservation to prevent microbial spoilage of food are proposed, either natural or synthetic. These preservatives are added to different food products to avoid their deterioration by microbial growth, leading to unwanted chemical changes, thus extending their shelf-life [3].

Classical agents applied as preservatives commonly used are weak organic acids such as lactic, acetic, sorbic, and benzoic acids. The growth of bacteria and fungi is inhibited by these molecules [4].

Bio-preservation is a concept employed in food preservation and shelf-life extension using naturally occurring microorganisms and metabolites. These Bio-preservative techniques utilized in food preservation relies mainly on the quality of microbial systems such as lactic acid bacteria (LAB) or their bacteriocins and bacteriophage-encoded enzymes. They are widely applied in food industries to achieve a typical flavour or texture of food products and help maintain the quality and safety of foods [5].

Fermentation is an example of processes where microbes are grown naturally or artificially. The process produces numerous beneficial products and further reduces spoilage and free from pathogens [6].

The most commonly applied microorganism in Bio-preservation is the lactic acid bacteria (LAB) and their metabolites. Functional foods fortified with LAB or their metabolites instead of synthetic additives have been aggressively commercialized by food industries [7].

Lactic acid bacteria are essential in bio-preservation because they possess antagonistic abilities against spoilage bacteria and some pathogens [6]. They produce metabolites,

including lactic acid, acetic acid, hydrogen peroxide, and bacteriocins. They achieve this by competing for nutrients while producing metabolites that are antimicrobial and act as preservatives for food [6,7]. Bacteriocins produced by LAB control the growth of spoilage bacteria in food products [6].

Bacteriocins are peptides or proteins synthesized in the ribosomes of bacteria, possessing antimicrobial activity. They are small, heat-stable peptides synthesized by some Gram-positive bacteria called LAB and maintain a broad spectrum of microbial inhibition [4]. They are generally regarded as safe for use in food preservation [5].

2. Materials and Methods

2.1. Isolation and Identification of LAB

Serial dilution of macerated fermenting *P. macrophylla* seeds was performed up to 10^{-5} concentrations. From these, 10^{-2}, $10^{-4,}$ and 10^{-5} were plated on MRS agar supplemented with 0.6% $CaCO_3$ and incubated anaerobically for 24-48 hours at 35°C. Colonies were sub-cultured on fresh MRS agar plates. Preliminary identification was made based on colony morphology, Gram reaction, catalase test, and ability to tolerate acidic pH and high bile concentrations.

Tolerance of acidic pH assay was carried out according to [8] by dissolving 26g MRS broth in 500 ml 0.3M phosphate buffer at pH 7. 1M HCl was added to lower the pH to 2, 3, 3.5, 4 and 6, mixed and sterilized at 121 ℃ for 15 mins. 1% fresh overnight culture was inoculated into the buffered MRS broth and incubated under anaerobic conditions at 35 ℃ for 24 h. Optical density at 600nm (OD_{600}) was obtained against a peptone water blank.

The same process was carried out for bile tolerance assay as bile concentrations were varied at 0.2%, 0.3%, 0.4%, 0.8%, 1.5%, 2% and 3.5% bile salts were prepared by mixing bile salts (1, 1.5, 2, 4, 7.5, 10 and 17.5g respectively).

The 16S rRNA sequence analysis was carried out for the ultimate identification of isolates. Genomic DNA isolation and purification was carried out according to the method of [9]. Estimating the concentration, purity, and yield of the DNA sample was accessed using the absorbance method (a measurement of absorbance) with the spectrophotometer [10].

The 16s region of the rRNA gene of the LAB isolates was amplified using the forward and reverse primers, 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 µl for 35 cycles. The PCR mix included: the X_2 Dream Taq Master mix by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.5 µM, and the extracted DNA as a template. The PCR conditions were as follows: Initial denaturation, 95 ℃ for 5 minutes; denaturation, 95 ℃ for 30 seconds; annealing, 52 ℃ for 30 seconds; extension, 72 ℃ for 30 seconds for 35 cycles and final extension, 72 ℃ for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator [11].

Sequencing was done using the BigDye Terminator Kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South Africa. The sequencing was done at a final volume of 10 µl; the components include 0.25 ul BigDye® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96 ℃ for 10s, 55 ℃ for 5s, and 60 ℃ for 4min [12].

Sequences were edited using the bioinformatics algorithm Trace Edit; similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were then aligned using MUSCLE. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X.

2.2. Indicator Strain and Culture Media

The indicator strain *Staphylococcus aureus* ATCC 19095 obtained from the Federal Institute of Industrial Research, Oshodi (FIRO), Lagos, Nigeria, was cultured on Mannitol salt agar (HiMedia) at 37 ℃. The strain was then sub-cultured on Nutrient broth and stored at -20 ℃ with 20% (v/v) glycerol.

2.3. Bacteriocin Assay

Assay against the indicator microorganisms was determined by the agar well diffusion method under aerobic conditions, as described by [13]. The surface of Mueller Hinton agar plates was inoculated with 100 mL of test microorganism after growing in a Nutrient broth and diluting appropriately. 100 mL of cell-free culture supernatant (neutralized with 0.1M NaOH, eluted using ammonium sulphate, washed and re-dissolved in sterile water) of the *L. fermentum* was placed into each well of plates. Plates were kept at ambient temperature for 2 hours and then incubated at 37 ℃ for 24 hours. The inhibition activities of the culture filtrates of *L. fermentum* on the indicator bacteria were indicated by a clear zone surrounding the agar wells.

2.4. Formulation of Optimum conditions and Experimental Design Using a Central Composite Design

A Central Composite Design with 3-factors, 1 block and 1 replication was designed to obtain the quadratic effect for the response variable. A 2-level, 3-factor (2^3) response surface methodology was adopted to estimate the optimal culture condition for bacteriocin production considering the factors. The factors considered include Temperature, pH and NaCl Concentration on bacteriocin production by isolates.

Given values of minimum and maximum level of Temperature, pH and Sodium Chloride concentration to be 28 ℃ and 32 ℃, 5.5 and 6.5, and 1.5% and 2.5% respectively, the uncoded and coded units for the design was obtained.

Bacteriocin production was estimated using a response surface regression model to estimate the linear, squared, and interactive relationship between the response variable and the predictors. Optimization comprises 20 experimental trials (8 factorial points with six axial and

six central points). Fermentations were in random order involving three variables (pH = X_1, temperature = X_2, and NaCl concentration = X_3) with five coded levels (-1.68, -α, 0, α and 1.68) to determine the effect of independent variables on response variable (antibacterial activity) according to [14].

2.5. Statistical and Mathematical Analysis

In developing the regression equation to determine the relationship between the independent and dependent variables for the RSM, MINITAB statistical software version 14.13 was used to determine the effects of linear, squared, and interactive terms of the independent variables.

Given the values of the minimum and maximum level of temperature, pH, and NaCl concentration to be 28 and 32 ℃, 5.5 and 6.5, and 1.5 and 2.5%, respectively, we obtain the uncoded units for the design.

$$X = b(coded\ unit) + a$$

where: $a = \frac{X_{\max} + X_{\min}}{2}; b = \frac{X_{\max} - X_{\min}}{2}$

X is the absolute value, X_{min} = the minimum level value, X_{max} is the maximum level value.

The response factor (Y_i) was modelled into the regression analysis as a mathematical function of a few continuous factors. The response was then expressed using a second-order polynomial equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the predicted response (bacteriocin activity in terms of zone of inhibition), β_0 the intercept, β_1, β_2 and β_3 are the linear coefficients, β_{11} β_{11} β_{33} are the squared coefficients, β_{12} β_{13} β_{23} are interaction coefficients, X_1 the temperature, X_2 the pH and X_3 NaCl concentration.

A three-dimensional surface plot was drawn to show the interactive effect of the independent variables on bacteriocin production. The optimal values were obtained by solving the regression equation and analyzing the surface contour plots. The coefficient of determination (R^2) was employed for the goodness of fit of the model equation [15,16]. All experiments were conducted in triplicates. Obtained values were analyzed using Fisher's test, and differences were considered statistically significant at $p<0.05$.

3. Results and Discussion

3.1. Identification of LAB Isolates

From three (3) samples collected, ten (10) LAB isolates were recovered and purified. Isolates that were found to be Gram-positive rods were tested for the ability to survive low pH and high bile concentrations (Figure 1 and Figure 2). Isolates showed different growth responses as pH reduced and bile salt concentration increased.

The turbidity of the medium increased as pH reduced and reduced as pH increased towards alkalinity, as shown by their optical densities (OD). Different LAB species grew and responded differently when exposed to varying pH values [17,18].

This variation in growth could be linked to different physiological characteristics of LAB as many are known to thrive in neutral pH [19]. The same trend was seen in LAB responses to increasing bile salt concentrations. Isolate OD reduced as bile salt concentration increased and agrees with similar findings of [20,21]. This variation can be attributed to their ability to hydrolyze bile salts and their conjugates [22].

Using an initial grouping and selection using acidic pH (Figure 1) and bile salt tolerance (Figure 2) assays, isolate LB1 showed consistency in growth (at OD_{600}) and was selected and identified using the 16S rRNA gene method.

Figure 3 shows an evolutionary inferred phylogenetic tree, comparing the evolutionary relationship between related taxa from the NCBI database.

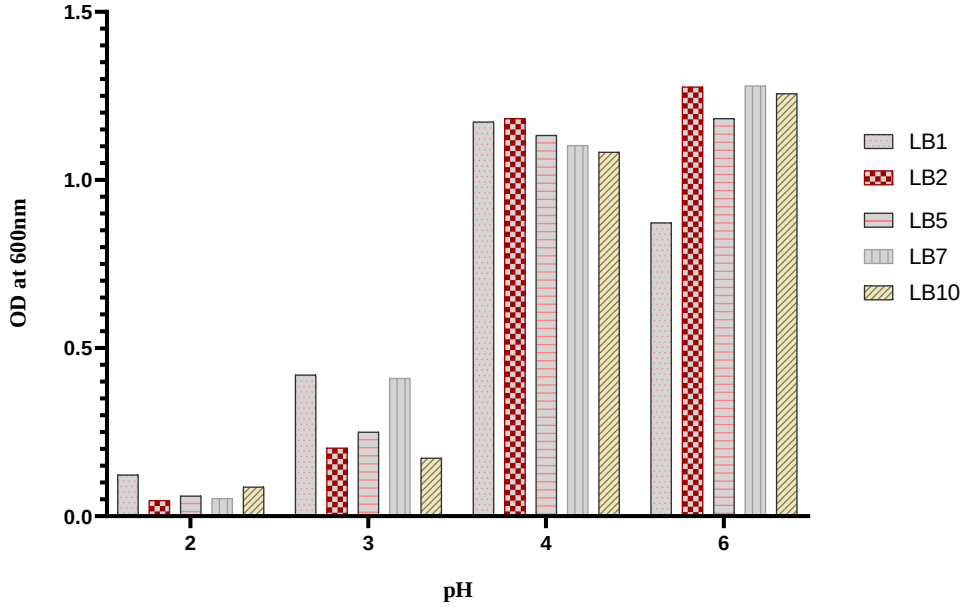

Figure 1. Growth (optical density) of LAB isolates at various pH after 24 h

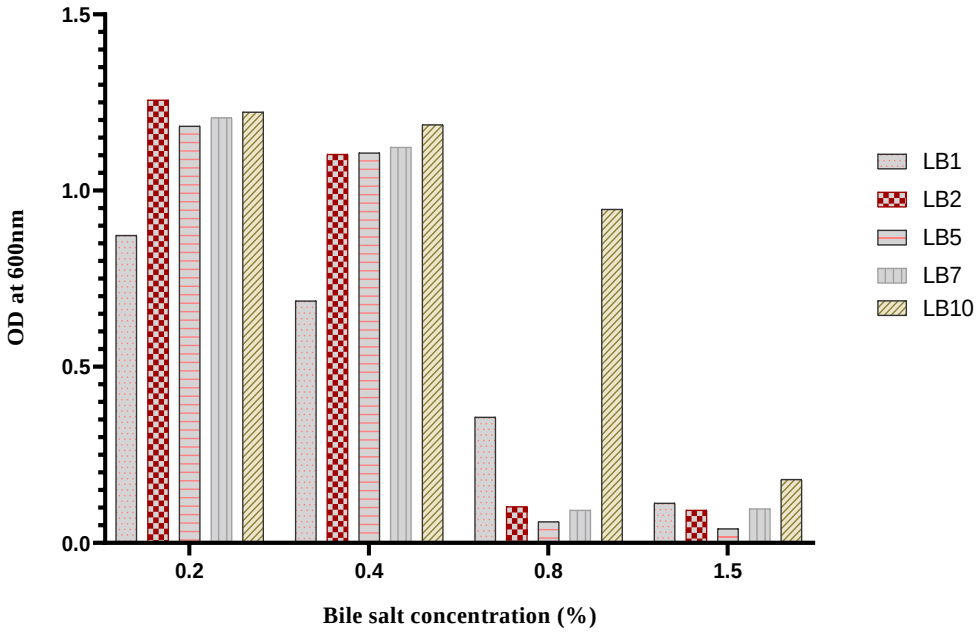

Figure 2. Growth (optical density) of LAB isolates at various Bile salt concentrations after 24 h

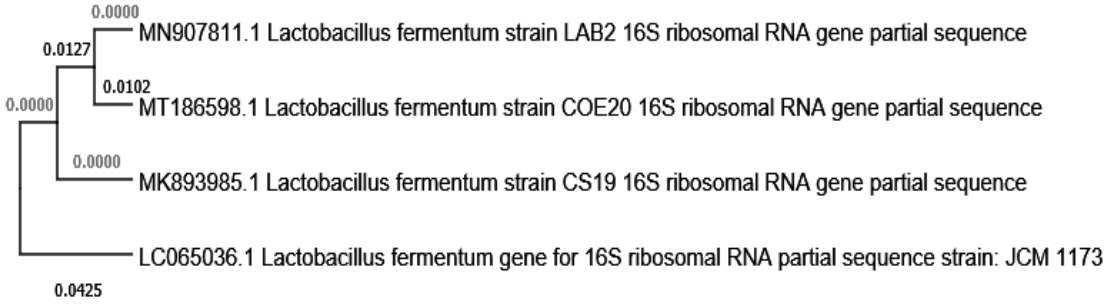

Figure 3. Inferred neighbour-joining tree showing evolutionary relationships of taxa

Amplified 16S rRNA region was sequenced using a 3510 ABI sequencer at Inqaba Biotech., South Africa. According to sequencing results, *Lactobacillus fermentum* strain COE20 showed 98.5% homology with *Lactobacillus fermentum* (Figure 3). NCBI GenBank assigned the obtained nucleotide sequence accession number MT186598.

The evolutionary history was inferred using the Neighbor-Joining method [23]. The optimal tree with the sum of branch length = 0.06539927 is shown. (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method [24] and are in the units of the number of base substitutions per site. This analysis involved four (4) nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1482 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [25]

3.2. Optimization

Table 1 shows the model obtained versus predicted values (inhibitory effect) of culture conditions on bacteriocin production and activity by *L. fermentum* against *Staphylococcus aureus* ATCC 19095.

Table 1. Actual and Predicted values of Response Variable for culture conditions

Order	Run	A	B	C	ZI (mm)	
					Actual	Predicted
5	1	28	5.5	2.5	6.82	6.6765
3	2	28	6.5	1.5	6.44	6.7359
20	3	30	6.0	2.0	11.17	11.3283
9	4	27	6.0	2.0	*	3.8947
1	5	28	5.5	1.5	6.11	6.2621
10	6	33	6.0	2.0	10.56	9.1037
7	7	28	6.5	2.5	6.48	7.0103
12	8	30	6.8	2.0	10.51	9.2957
15	9	30	6.0	2.0	11.33	11.3283
6	10	32	5.5	2.5	9.15	9.8838
16	11	30	6.0	2.0	11.62	11.3283
18	12	30	6.0	2.0	11.21	11.3283
2	13	32	5.5	1.5	10.25	10.7494
13	14	30	6.0	1.2	10.12	9.3554
17	15	30	6.0	2.0	11.41	11.3283
11	16	30	5.2	2.0	10.12	9.8780
14	17	30	6.0	2.8	9.55	8.8583
19	18	30	6.0	2.0	10.98	11.3283
4	19	32	6.5	1.5	8.55	9.7232
8	20	32	6.5	2.5	7.84	8.7176

Table 2. Analysis of Variance and Model Summary of Linear, Squared and 2-way Interaction of Predictors

Source	DF	Sum of squares	Mean squares	F-Value	Probability>F
Model	9	55.4913	6.1657	6.67	0.005
Linear	3	22.1515	7.3838	7.99	0.007
A	1	21.4439	21.4439	23.21	0.001
B	1	0.4092	0.4092	0.44	0.522
C	1	0.2984	0.2984	0.32	0.584
Square	3	39.9074	13.3025	14.40	0.001
A*A	1	24.5112	24.5112	26.53	0.001
B*B	1	5.1976	5.1976	5.63	0.042
C*C	1	8.4577	8.4577	9.15	0.014
2-way Interaction	3	1.9540	0.6513	0.70	0.573
A*B	1	1.1250	1.1250	1.22	0.298
A*C	1	0.8192	0.8192	0.89	0.371
B*C	1	0.0098	0.0098	0.01	0.920
Error	9	8.3160	0.9240		
Lack of fit	4	8.0743	2.0186	41.75	0.000
Pure error	5	0.2417	0.0483		
Total	18	63.8073			
Model summary					
S	R^2	R^2 (adj.)	R^2 (pred.)		
0.961250	86.97%	73.93%	0.00%		

3.2.1. Regression Equation, ANOVA and Model Summary

A regression equation developed from the RSM is shown in Equation 2. The ANOVA table is shown in Table 2.

$$\begin{aligned} &\textit{Bacteriocin} \\ &= -583 + 29.279A + 40.7B + 22.7C \\ &-0.426A*A - 2.46B*B - 3.14C*C \\ &-0.375A*B + 0.032A*C - 0.14B*C, \end{aligned} \quad (2)$$

where A is Temperature, B is pH, and C is Sodium chloride concentration.

Equation 2. Regression Equation for culture conditions for bacteriocin production by *L. fermentum* MT186598

The regression model estimated (linear effect) a +29.27 unit of temperature, a +22.7 unit of salt concentration, and +40.7 unit of pH. The model also estimated (squared effect) a -0.4268 of temperature, a -2.46 unit of pH and an average -3.14 unit of salt concentration. The interaction effect of temperature and pH have a +0.375 unit effect on the response. In comparison, the combined effect of temperature and NaCl concentration and pH and NaCl concentration were estimated to be a +0.320 unit and declined 0.14 unit, respectively. These interactive term coefficients denote the unit change in the response obtained from a unit change in the interaction term.

3.2.2. Regression Equation for Effect of Culture Conditions

After fitting the response surface regression model, the goodness of fit diagnostics was conducted to determine the adequacy of the fitted model. The analysis of variance suggests that the model was significant. At least one independent variable has a significant effect (greater than 0) on the response since the p-value was less than the significance level (α); therefore, the main, squared, or interactive effect was significant. The coefficient of determination (R^2) was employed to qualify the fit of the Quadratic model equation.

The ANOVA table suggests a significant model because at least one independent variable had a greater than zero (0) effect on the response (significant effect) according to [26,27]. There was at least one significant effect in the linear and squared terms but not the interactive term. This outcome was in agreement with [27] which states that terms are significant when the p-value is less than the significance level (α). At $P < 0.05$, temperature had a significant effect at $P = 0.007$) but pH and NaCl concentration had no significant linear effect at $P = 0.552$ and 0.584 respectively. The squared effect was all significant at $P < 0.05$ whereas, the interactive effect were all insignificant at the P-value.

The model was adequate as the predictors showed an R-square (R^2) value of 86.97%. This variation showed that the response variables were accounted for by the predictors. This further strengthens the goodness of fit, according to [28,29]. The coefficient of determination in terms of predicted R^2 was 0.8697, which was in close agreement with an adjusted R^2 of 0.7393. This R-value validated the experimental and expected levels of Bacteriocin production shown by the closeness of the R-value to 1.0.

3.2.3. Regression Analysis and Residual Check

Residual plots, interactive contour plots of functional relationships between variables and optimal plots are shown in Figure 4, Figure 5 and Figure 6.

The contour plots depict the functional relationships between responses and variables at different operating conditions and find usefulness in determining the optimal conditions [30,31]. This technique has been utilized in various related optimization experiments carried out by [32,33].

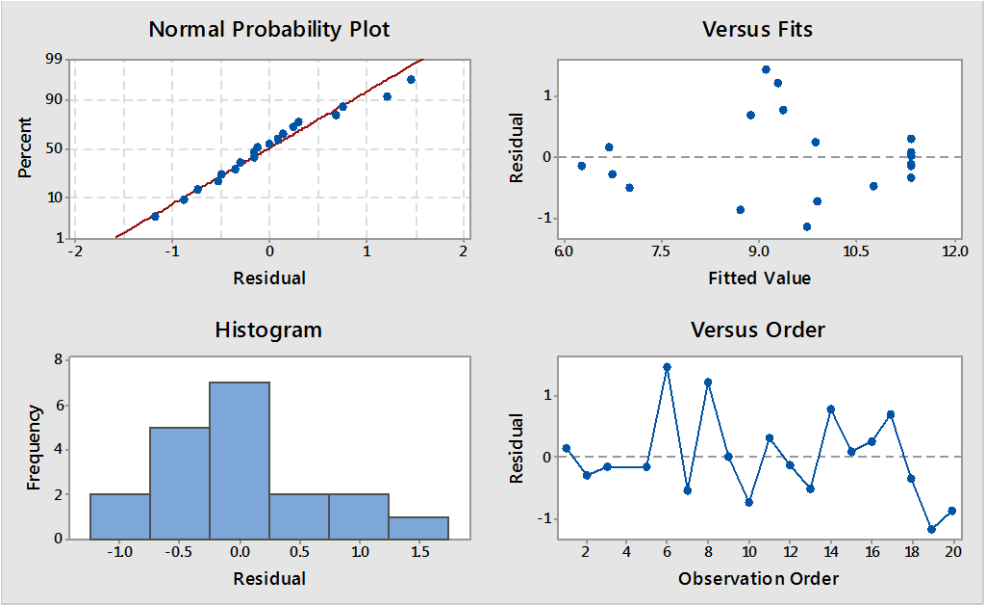

Figure 4. Residual plots for bacteriocin activity from *L. fermentum* MT186598

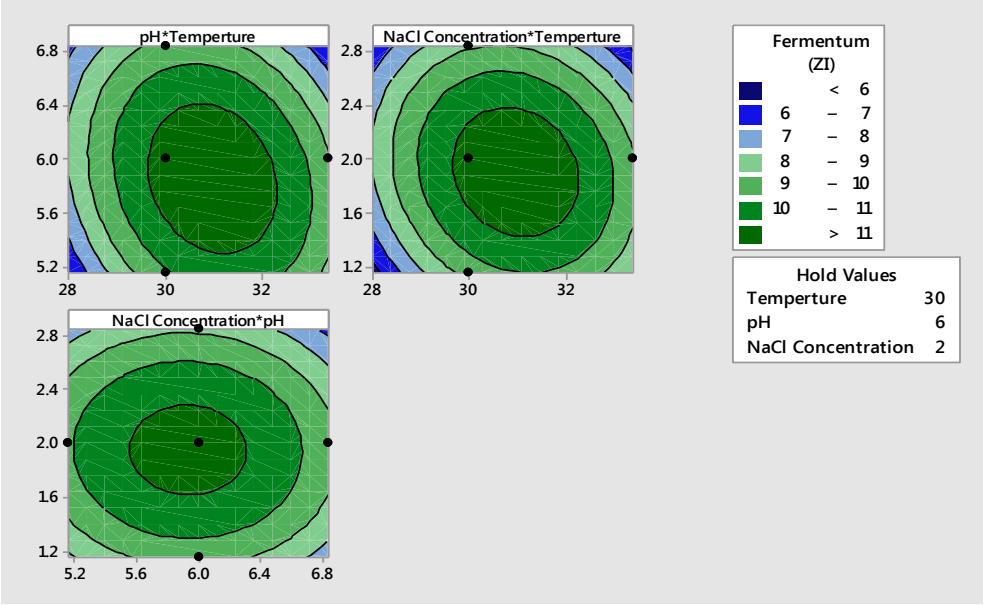

Figure 5. Interactive contour plots of functional relationships between variables

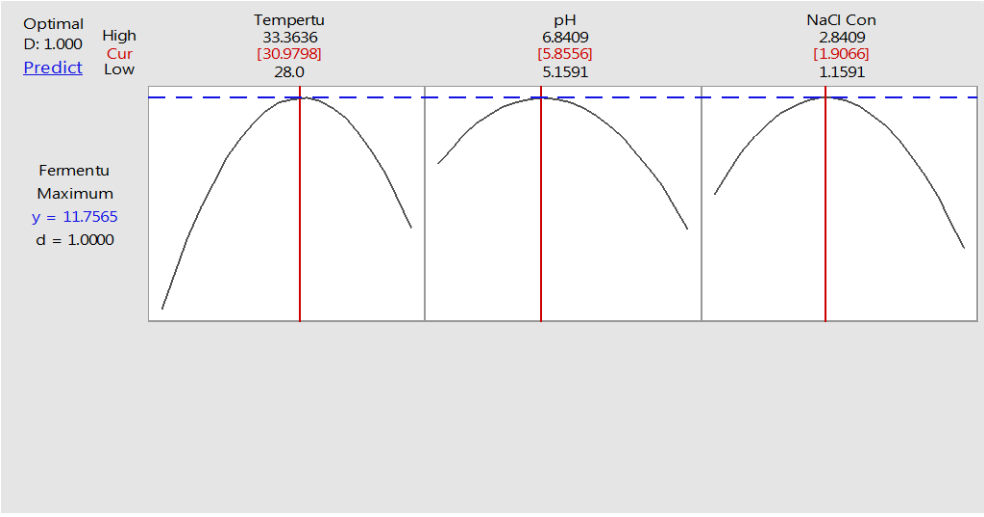

Figure 6. Optimal plots of culture conditions for bacteriocin production

The regression model results are shown in the optimal plots and show a maximum optimal value for the response variable at 11.75mm (zone of inhibition). This effect was achieved at temperature (31℃), pH (5.9) and NaCl concentration (1.9%).

The results showed considerable contribution by the variables on bacteriocin production. There was a significant quadratic effect of pH, followed by temperature and NaCl concentration. Minor adjustments in pH and temperature greatly influenced bacteriocin production and activity. They agreed with [34,35,36] that showed the influence of temperature and pH changes in bacteriocin production by LAB. This could be attributed to the strong influence pH, and temperature changes have on bacteriocin-producing genes [37,38].

3.3. Conclusion

L. fermentum MT186598 showed good acid and bile tolerance and survived human gastrointestinal bile concentration (0.3% w/v) and pH of 2.5; therefore, it could serve and function as probiotics. Significant antibacterial activity against the test organism indicates its ability to control bacterial pathogens. Thus, a bacteriocin produced when optimal conditions are employed may be helpful as a bio-preservative in the shelf-life extension of *Ugba*.

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