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IMPACT OF ENVIRONMENTAL VARIABLES ON ANTIMICROBIAL SUBSTANCE PRODUCTION BY LACTIC ACID BACTERIA: A BOX-BEHNKEN DESIGN APPROACH

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ABSTRACT

In this research, we employed a 3-factor Box-Behnken experimental design (BBD) to ascertain the optimal conditions for the production of antimicrobial substances by lactic acid bacteria (LAB). The experimental parameters encompassed three variables: temperature (30, 35 and 37°C), incubation time (24, 48 and 72 hours), and substrate concentration (1%, 2% and 3%). The production of antimicrobial substances by *Lactiplantibacillus plantarum* F2 isolate was influenced by the incubation period. The highest antimicrobial substance production (average inhibition zone diameter 12.00 mm for antimicrobial activity assay; 12.09 mm for Box-Behnken estimation) of *Pediococcus pentosaceus* 50 isolate was achieved at a temperature of 37°C, an incubation period of 24 hours, and a substrate concentration of 2% in the environment. These findings indicate that the antimicrobial activity of LAB typically intensifies toward the end of the logarithmic phase and into the stationary phase, likely due to the increased production of secondary metabolites.

Keywords: Lactic acid bacteria, antimicrobial activity, optimization, Box-Behnken design

ÇEVRESEL DEĞİŞKENLERİN LAKTİK ASİT BAKTERİLERİ TARAFINDAN ANTİMİKROBİYAL MADDE ÜRETİMİ ÜZERİNDEKİ ETKİSİ: BOX-BEHNKEN TASARIM YAKLAŞIMI

ÖZ

Bu araştırmada, laktik asit bakterileri (LAB) tarafından antimikrobiyal madde üretimi için optimum koşulları belirlemek amacıyla 3 faktörlü Box-Behnken deneysel tasarımı (BBD) kullanılmıştır. Deneysel parametreler üç değişkenden oluşmakta olup, bu değişkenler: sıcaklık (30, 35 ve 37°C), inkübasyon süresi (24, 48 ve 72 saat) ve substrat konsantrasyonu (%1, %2 ve %3) olarak

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belirlenmiştir. *Lactiplantibacillus plantarum* F2 izolatı tarafından antimikrobiyal madde üretiminin, değişen inkübasyon süresinden etkilendiği gözlenmiştir. *Pediococcus pentosaceus* 50 izolatının en yüksek antimikrobiyal madde üretimi (deneysel süreçte elde edilen ortalama inhibisyon zon çapı 12.00 mm; Box-Behnken tasarımında tahmin edilen değer 12.09 mm), inkübasyon sıcaklığı 37°C, inkübasyon süresi 24 saat ve ortamda %2'lik bir substrat konsantrasyonu olduğunda elde edilmiştir. Bu bulgular, LAB'nin antimikrobiyal aktivitesinin genellikle logaritmik fazın sonuna doğru ve durağan fazda arttığını, bunun da muhtemelen ikincil metabolitlerin artan üretiminden kaynaklandığını göstermektedir.
Anahtar kelimeler: Laktik asit bakterileri, antimikrobiyal aktivite, optimizasyon, Box-Behnken tasarımı

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria classified within the Firmicutes phylum, Bacilli class, and Lactobacillales order (Özoğlu et al., 2021; Wang et al., 2021; Ringø et al., 2018). Additionally, the majority of LAB are Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) status, making them frequently encountered in the food and health sectors (Zapaśnik et al., 2022; Chen and Wang, 2019; Alvarez-Sieiro et al., 2016). LAB play a significant role in the production of fermented foods, including dairy products (such as yogurt and cheese), olives, and pickles, which are part of daily nutrition of the individual (Özoğlu et al., 2021; Wang et al., 2019).

LAB are fermentative bacteria that can carry out the process through two different types of fermentation which are classified as homofermentative and heterofermentative (Zapaśnik et al., 2022; Abedi and Hashemi, 2020; Ringø et al., 2018). Homofermentative LAB utilize glucose in the environment as a source of carbohydrate and produce lactic acid, which defines their name (Abedi and Hashemi, 2020; Ringø et al., 2018). On the other hand, heterofermentative LAB can use carbohydrates in the environment as a carbon source and produce not only lactic acid but also acetic acid, alcohol, and carbon dioxide. In all these processes, LAB contribute to antimicrobial activity, preventing the proliferation of pathogens and, in turn, inhibiting the spoilage of food and producing various metabolites that serve as growth inhibitors. Among these metabolites, there are various compounds like bacteriocins, hydrogen peroxide, and diacetyl. Moreover, these metabolites can also inhibit the cellular adhesion and colonization of pathogenic groups within the gastrointestinal system (Zapaśnik et al., 2022; Ringø et al., 2018). Furthermore, LAB, known as probiotics and residing in the intestinal system, can utilize waste materials within the gastrointestinal tract as a carbon source for organic acid production (Wang et al., 2021; Neal-McKinney et al., 2012).

The diversity of metabolites produced by LAB depends on a multitude of factors, including environmental temperature, pH, composition of the growth medium, diversity of carbon and nitrogen sources, bacterial species and strains, as well as various other components (Managamuri et al., 2016; Singh et al., 2016). Optimal conditions can be determined through various growth medium optimizations conducted in a laboratory setting. Prior to experimental processes, statistical modeling for various components aims not only to achieve the most accurate and meaningful results in the optimization process but also to enhance process efficiency, thereby reducing the time and cost associated with manual optimization procedures (Managamuri et al., 2016; Elibol, 2004). Experimental designs often involve methods such as Plackett-Burman Design, Taguchi Design, Central Composite Design, Box-Behnken Design, or a combination of these techniques (Ahsan et al., 2022; Singh et al., 2016). Among these methods, Box-Behnken Design is a straightforward statistical technique, allowing for experimental modeling independently of complex factorial or fractional factorial calculations. In this design, modeling is carried out using three levels for each factor. It operates based on incomplete factorial designs, generating second- degree models, and typically focuses on three fundamental factors (Cardoso et

al., 2025; Singh et al., 2016; Ferreira et al., 2007). Box-Behnken Design is particularly preferred for optimization processes in fermentation-based studies.

This study aimed to optimize the antimicrobial activities of LAB (*Pediococcus pentosaceus* ATCC 43201 reference strain and *Lactiplantibacillus plantarum* F2, *Pediococcus acidilactici* 40, *Pediococcus pentosaceus* 50, *Lactobacillus plantarum* O2 isolated from various food sources) using the Box-Behnken Design method. Three factors (substrate content of the growth medium, temperature, and incubation time) were chosen for statistical modeling. In vitro experiments were conducted under the conditions derived from this model, and the antimicrobial activities of these LAB against *Escherichia coli* ATCC 25922, were assessed. As a result of all experiments, a condition was identified that was statistically significant and found to enhance the antimicrobial effects of the bacteria.

MATERIALS AND METHODS

Material

The lactic acid bacteria (*Lactiplantibacillus plantarum* F2, *Pediococcus acidilactici* 40, *Pediococcus pentosaceus* 50, *Lactobacillus plantarum* O2) which were isolated in the context of previous studies (Project No: 119O343, Çelik 2022), and found to have antimicrobial activity, were used as materials in the current study. De Man Rogosa Sharpe medium (MRS, Merck) was used to activate the isolates and Tryptic Soy Broth or Agar was used for the activation of pathogenic bacteria (*E. coli* ATCC 25922) used as indicator in testing antimicrobial activity *Pediococcus pentosaceus* ATCC 43201 was also used as a reference strain in the experiments.

Determination of the Antimicrobial Activity

In this assay, the well diffusion method proposed by Harris et al. (1989) was used with some modifications. The cultures activated in MRS broth medium inoculated with 1% inoculation rate were centrifuged at 6000 x g for 15 minutes at the end of the period. Following centrifugation, bacteria were separated from the medium with a 0.45 μm pore diameter filter (Sartorius Cellulose Acetate Membrane Filters) and the supernatant was obtained. The obtained supernatant was used in antimicrobial activity experiments after its pH was adjusted to 6.5. Following this step, the inhibitory effect of the bacterial supernatant on the pathogenic bacteria was observed. For this purpose, 100 μL from each sterile supernatant sample was transferred to wells on TSA (Tryptic Soy Agar), and the Petri dishes were kept at +4°C to allow the supernatant to diffuse. Then, 50 μL of *E. coli* ATCC 25922 were added to 8 mL of soft TSA (at 45°C), mixed, and subsequently transferred onto TSA. After the agar solidified, the Petri dishes were left for incubation for 24 hours. The diameters of the zones formed at the end of the incubation were measured.

The optimization of antimicrobial substance production

There can be many factors that influence the production of antimicrobial substances. Among these, factors like incubation temperature and the type and quantity of the carbohydrate source in the medium are crucial parameters. In this study, the production of antimicrobial substances was attempted to be optimized based on these factors. For incubation temperature, values of 30, 35, and 37°C were selected, and for incubation time, values of 24, 48, and 72 hours were chosen. The concentration of the substrate (glucose present in the medium) was set to 1%, 2%, and 3%. The values included in the experiments were selected based on the literature information provided for the optimum growth conditions of LAB up today (Moradi et al., 2023; Mahato et al., 2021; Rohmatussolihat et al., 2018).

Response Surface Modeling (RSM): RSM is an approach that combines statistical and mathematical techniques to improve and optimize processes. The design space for this process is an n-dimensional space determined by "n independent variables", where each variable is defined within an acceptable range. RSM constructs empirical models to more precisely and accurately predict the possible values of response variables concerning the inputs. With the established model, the aim is to predict the necessary factor values to achieve the desired

response value and determine the most suitable operating range for the relevant product in the process (Demir et al., 2017).

In RSM, multivariate regression analysis methods are used. The multiple regression model, which includes k regression predictors believed to have an effect on the response variable Y, is represented as Equation 1.1:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots\ldots + \beta_k x_k + \varepsilon \quad (1.1)$$

For k = 2, the model is obtained as shown in Equation 1.2:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon \quad (1.2)$$

A first-degree response surface model with two predictors and an intercept term is obtained as follows:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \varepsilon \quad (1.3)$$

Equation 1.3 can be referred to as the standard regression model. Similarly, a second-degree response surface model is obtained as shown in Equation 1.4:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (1.4)$$

Here, Y is the dependent variable, β0 is the intercept, β1, β2, β12, β11, and β22 are regression coefficients, and X1 and X2 are independent variables (Montgomery, 2001; Box and Draper, 1987).

Box-Behnken Design: Box-Behnken is one of the types of experimental designs, which is a three-level design that does not include complete or fractional factorial designs. For the model evaluation, R2, R2 (adj) measurements and the "lack of fit" value for model fit were taken into account. In the Box-Behnken experimental arrangement, one of the factors is fixed at its center value while combinations of all levels of the other factors are applied. First, the level of factor X_1 is fixed, and combinations of levels of factors X_2 and X_3 are applied. Then, the same procedures are applied successively for the levels of factors X_2 and X_3. The central point values are located in the last rows of the design matrix (Tekindal and Kaymaz, 2018; Tekindal et al., 2012).

With this design, coefficients for the 1st and 2nd-degree models are estimated. In a three-factor Box-Behnken design, 15 experiments need to be conducted.

In this study, a 3-factor Box-Behnken experimental design (Box and Behnken, 1960) was employed to determine the effects of factors that affect antimicrobial production during media optimization and select the optimal conditions. The MINITAB 19 trial version software package (MINITAB Statistical Software, State College, Pennsylvania, USA) was used for the analysis. A significance level of $\alpha = 0.05$ was assumed for all analyses.

The independent variables that influence antimicrobial production, including the range and levels of incubation temperature, incubation time, and substrate concentration, are provided in Table 1.

Table 1 The experimental design levels of selected variables

Variable parameter	**Levels**		
	-1	**0**	**1**
Temperature (X_1)	30°C	35°C	37°C
Time (X_2)	24	48	72
Substrat (X_3)	%1	%2	%3

RESULTS

In the scope of the study, the bacteria investigated were previously isolated from fermented food products. The bacteria with codes of F2, O2, 40, and 50 were identified as *Lactiplantibacillus plantarum*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus*, respectively (Project No:119O343; Çelik, 2022). It was observed that these bacteria exhibited inhibitory effects on food pathogens, including *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 43300, *Salmonella* Enteritidis ATCC 13076, and *Escherichia coli* ATCC 25922.

The experiments required for the 3-factor Box-Behnken design, as stated in Table 1, were conducted in the study and the results of the experiments are recorded. In this study the inhibition zone diameters of the isolates on *E. coli* ATCC 25922 pathogen under different conditions are used to predict the Box-Behnken design the details of the results are presented as Supplementary Material.

The Box-Behnken Design Results of *Pediococcus pentosaceus* ATCC 43201

The Box-Cox transformation was applied to the antimicrobial substance production data because it did not satisfy the assumption of a normal distribution. However, when the variance analysis results were examined, it was seen that the variables in the model, such as temperature, time and substrate amount, which are thought to affect antimicrobial production, did not significantly explain the response variable ($p>0.05$).

The Box-Behnken Design Results of *Lactiplantibacillus plantarum* F2

Antimicrobial zone diameters of *L. plantarum* F2 on *E. coli* ATCC 25922 obtained at different incubation temperatures, incubation times and substrate concentrations are given in the Table 2.

Table 2 The antimicrobial activity zone diameters of *L. plantarum* F2 on *E.coli* pathogen at different conditions

Number	X_1	X_2	X_3	Zone (mm)
1	0	0	0	10.50
2	0	-1	-1	12.50
3	-1	0	1	11.00
4	-1	-1	0	14.00
5	0	-1	1	12.00
6	1	0	-1	11.00
7	0	1	-1	12.50
8	0	0	0	11.50
9	1	-1	0	13.00
10	1	1	0	14.50
11	0	1	1	12.75
12	1	0	1	11.00
13	0	0	0	13.00
14	-1	0	-1	10.50
15	-1	1	0	14.75

Since the antimicrobial production data did not meet the assumption of normality, Box-Cox transformation was applied. When the variance analysis results were examined, it was seen that the main and interaction effects of the variables such as temperature, time and substrate amount, which are thought to have an effect on antimicrobial production, were not significant ($p>0.05$). However, the quadratic term of incubation time was significant. This situation shows that antimicrobial production does not change linearly but curvilinearly depending on time. This situation suggests the existence of an optimum point where antimicrobial production is maximized at a certain level of time. The reduced model, obtained after eliminating the non-

significant variables, is shown in Table 3. The following regression model explained 67.11% of variability in the data (R2(adj)=61.63%), and showed an adequate fit (lack of fit,p=0.76).

The model based on incubation time obtained for *L. plantarum* F2 bacteria using the data obtained from the experiment is presented below:

Table 3 The results of transformed variance analysis of *L. plantarum* F2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	2	0.000785	0.000392	12.24	0.001
Linear	1	0.000082	0.000082	2.55	0.136
Time	1	0.000082	0.000082	2.55	0.136
Square	1	0.000766	0.000766	23.91	0.000
Time* Time	1	0.000766	0.000766	23.91	0.000
Error	12	0.000385	0.000032		
Lack-of-Fit	8	0.000207	0.000026	0.58	0.761
Pure Error	4	0.000178	0.000044		
Total	14	0.001170			

Model:

$$-Antimicrobial\ Production^{-1} = -0.0380 - 0.002287\,Time + 0.000025 Time * Time$$

Substrate and temperature variables were excluded from the Contour and Surface plots, as they were not found to be significant in the model. The graphs, therefore illustrate the relationship between incubation time and antimicrobial production only. Based on the plotted model, the maximum antimicrobial production is estimated to occur at approximately 72 hours. At this time point, the predicted antimicrobial activity zone diameter is 13.94 mm, with a 95% confidence interval of [12.68 mm; 15.48 mm] (Figure 1).

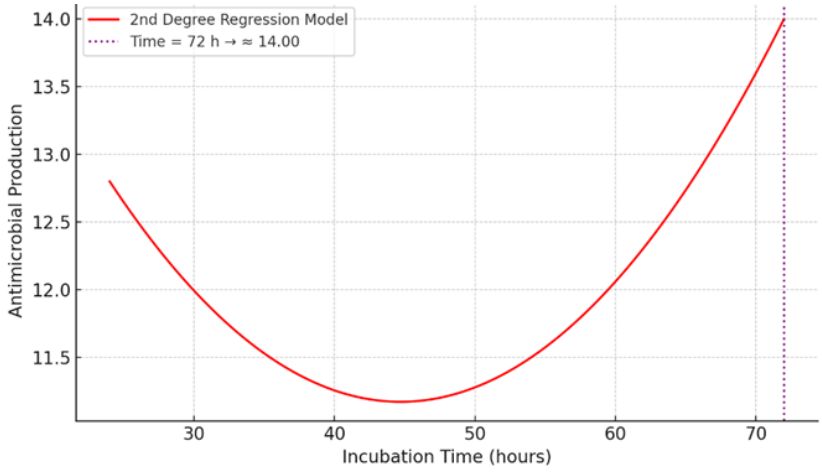

Figure 1 The incubation time related antimicrobial activity of *L. plantarum* F2 isolate according to the 2nd degree regression model.

The Box-Behnken Design Results of *Pediococcus pentosaceus* 50

Antimicrobial zone diameters obtained at different incubation temperatures, incubation times and substrate concentrations for *P. pentosaceus* 50 on *E. coli* ATCC 25922 are given in the Table 4.

Table 4 The antimicrobial activity zone diameters of *P. pentosaceus* 50 on *E. coli* at different conditions

Number	X_1	X_2	X_3	Zone (mm)
1	0	0	0	9.00
2	0	-1	-1	11.00
3	-1	0	1	9.50
4	-1	-1	0	12.00
5	0	-1	1	11.00
6	1	0	-1	11.00
7	0	1	-1	11.50
8	0	0	0	9.50
9	1	-1	0	12.00
10	1	1	0	12.00
11	0	1	1	9.50
12	1	0	1	10.00
13	0	0	0	11.00
14	-1	0	-1	10.00
15	-1	1	0	12.00

The Box-Cox transformation was applied as the antimicrobial production data violated the assumption of normality. When the variance analysis results were examined, it was seen that the main and interaction effects of the variables such as temperature, time and substrate amount, which are thought to have an effect on antimicrobial production, were not significant ($p>0.05$). However, the quadratic terms of the variables were significant. This situation shows that antimicrobial production does not change linearly but curvilinearly according to incubation temperature, substrate concentration and incubation time. In this case, the insignificant interaction terms were excluded from the model and the reduced model was obtained as follows. The second-order regression model was established for antimicrobial production. The model coefficients were estimated with a 95% confidence level and the model demonstrated an adequate fit (lack of fit,$p=0.69$), explaining 94.27% ($R^2(adj)=81.17\%$) of the variability in the data. While Table 5 displays the results of transformed variance analysis of *P. pentosaceus* 50, Figure 2 shows the Normal Probability Plot and Pareto Chart of *P. pentosaceus* 50.

The model based on incubation temperature, incubation time and substrate concentration obtained for *P. pentosaceus* ATCC 43201 bacteria using the data obtained from the experiment is presented below:

Model:

$$((Antimicrobial\ Production\text{^}(\lambda-1)))/(\lambda \times g\text{^}(\lambda-1)) = 162.1 - 9.53\,Temperature - 0.3559\,Time + 5.62\,Substrate + 0.1431\,Temperature * Temperature + 0.003626\,Time * Time - 1.491\,Substrate * Substrate$$

($\lambda = 14$, $g = 10.6830$ is the geometric mean of Antimicrobial Production)

Since time was identified as the least influential variable in the model, it was held constant in the contour and surface plots. Optimization analysis revealed that the maximum antimicrobial production occurs at an incubation temperature of 37°C, an incubation time of 24 hours, and a substrate concentration of approximately 2%. Under these conditions, the predicted antimicrobial activity zone diameter is 12.09 mm, with a 95% confidence interval of [11.91 mm; 12.24 mm]. These findings suggest that the specified conditions are favorable for optimal production. When the incubation time was fixed at "24 hours", the following plots were generated. Figure 3 presents the contour and surface plot analysis results, while Figure 4 illustrates the optimal antimicrobial production levels of each variable for *P. pentosaceus* 50.

Table 5 The results of transformed variance analysis of *P. pentosaceus* 50

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	32.0549	5.3425	21.92	0.000
Linear	3	1.3831	0.4610	1.89	0.209
Temperature	1	0.3263	0.3263	1.34	0.281
Time	1	0.2261	0.2261	0.93	0.364
Substrate	1	0.6873	0.6873	2.82	0.132
Square	3	31.6832	10.5611	43.33	0.000
Temperature*Temperature	1	6.5984	6.5984	27.07	0.001
Time*Time	1	14.6776	14.6776	60.22	0.000
Substrate*Substrate	1	7.7989	7.7989	32.00	0.000
Error	8	1.9500	0.2437		
Lack-of-Fit	4	0.7209	0.1802	0.59	0.691
Pure Error	4	1.2291	0.3073		
Total	14	34.0049			

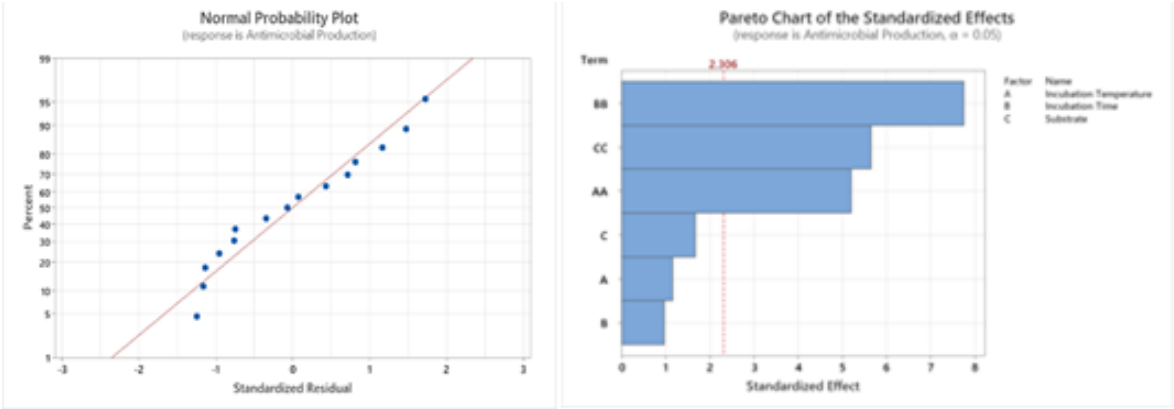

Figure 2 The normal probability plot and pareto chart of *P. pentosaceus* 50

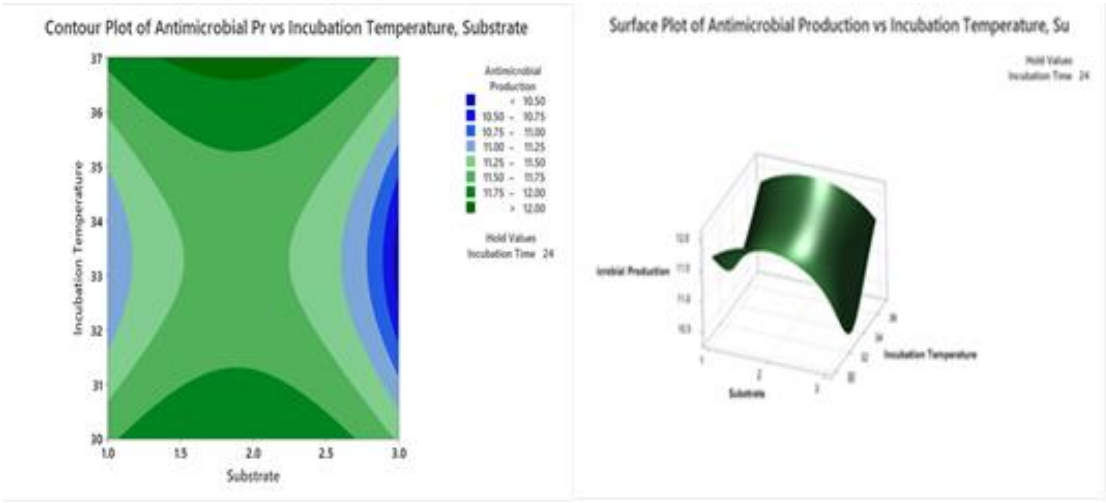

Figure 3 Contour plot and surface plot of variables for *P. pentosaceus* 50 isolate

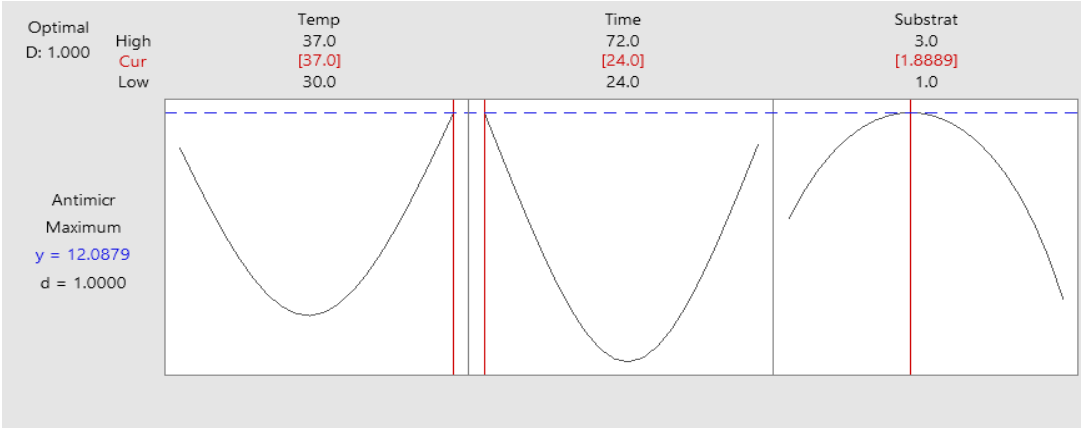

Figure 4 Changes in variables with antimicrobial production, showing the optimal antimicrobial production of each variable for *P. pentosaceus* 50.

The Box-Behnken Design Results of *Pediococcus acidilactici* 40 and *Lactobacillus plantarum* O2 isolates

Antimicrobial production data of isolate *P. acidilactici* 40 and *L. plantarum* O2 was transformed using the Box-Cox transformation due to the lack of conformity with the normal distribution assumption. However, when examining the results of the variance analysis, it is observed that the variables, temperature, time, and substrate amount, which were thought to have an effect on antimicrobial production, do not significantly explain the response variable ($P > 0.05$).

DISCUSSION

The growth conditions are not only affect the survival of the microorganisms but also effect the metabolite production. It is a good way to use models to predict the optimum conditions for the growing and metabolite production of the organisms. Box-Behnken design is an experimental design and a nonlinear model of Response Surface Methodology developed by Box and Behnken in 1960 which is prefered to exhibit and predict the desired conditions for biolgical samples. Our study showed that Box-Behnken design may be a good vehicle for understanding the optimal conditions for most of the LAB. The most desirable results were obtained for isolate *P. pentosaceus* 50 in the current study. There are some literature declaring the successful Box-Behnken model for determining optimum conditions for LAB.

While our study focused on the production of antimicrobial substances by LAB, and Zhang et al. (2017) also used the Box-Behnken design for the optimization of culture medium for *Lactobacillus bulgaricus*. The study results indicated that when the glucose concentration is 9.5 $g \cdot L^{-1}$; caseinhydrolysate concentration is 15.5 $g \cdot L^{-1}$ and glutamate concentration is 7.0 $mg \cdot L^{-1}$ in the medium, the number of viable bacteria of *L. bulgaricus* could reach to $(2.95 \pm 0.07) \times 10^9$, which is very close to the predicted value of the model of 3.00×10^9 $cfu \cdot mL^{-1}$. The researchers declared that the optimized conditions and models used in the study were feasible and effective.

The lactic acid (which is the best-known antimicrobial substance produced by lactic acid bacteria) production of *Lactobacillus plantarum* JX183220 with cassava flour (Manihot esculenta Crantz) in semi-solid fermentation by Response Surface Methodology was searched by Sridevi et al. (2015). Box-Behnken design of Response Surface Methodology was used with different parameters such as substrate concentration, temperature and pH. Maximum production of lactic acid by *Lactobacillus plantarum* JX183220 was

observed on 4th day of incubation with 2% inoculum and 0.3% Calcium carbonate. Optimization using Box -Behnken design of RSM resulted in maximum lactic acid production of 18.3679 g/100 g of cassava at optimum conditions of substrate concentration, 1.225%; Temperature, 36.39°C and pH 6.43. The Box-Behnken design was reported as a convenient tool in optimization process of lactic acid production. The results obtained in the study, especially at the incubation temperature point, are similar to our study.

A study was conducted with an objective to develop a low cost growth medium for enhancing the biomass production of a bio-therapeutic bacterial strain *Lactobacillus plantarum* AS-14 (Manzoor et al., 2017). Cheese whey, corn steep liquor, yeast extract and two operating conditions (temperature and pH) were found to be the most significant parameters in the study. The best culture medium contained 60 g/L cheese whey, 15 g/L glucose and 15 g/L corn steep liquor in addition to other minor ingredients and it resulted in maximum dry cell mass (15.41 g/L) by using response surface methodology (RSM). The second-order polynomial regression model determined that the maximum cell mass production (16.02 g/L) would be obtained at temperature 40°C and pH 6.2. The cost effective medium developed in this research was offered for large-scale commercial application where economics is quite likely important.

Box-Behnken design was used to remove Aflatoxin M1 from the environment using microorganisms (Salem-Bekhit et al. 2023). When the parameters focused on in the study were evaluated, it was similar to the study we conducted in terms of incubation temperature and time. The researchers evaluated a biological method to remove Aflatoxin M1 (AFM1), a harmful toxin in milk derived from contaminated animal feed. Researchers used *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* to detoxify AFM1. A Box–Behnken design was employed to optimize the microbial ratio, incubation time, and temperature. Using ELISA for detection, the method achieved up to 98.4% AFM1 reduction in contaminated milk, offering a safe, low-cost, and efficient solution for milk detoxification.

A study performed by Rifa'i et al. (2025) aimed to develop a functional yogurt enriched with green tea (GT) and encapsulated *Lacticaseibacillus paracasei* E1 (LpE1) using a Box–Behnken Design (BBD). The experiment optimized three variables: GT concentration (2–4% w/v), encapsulated LpE1 (2–4% w/v), and incubation time (18–30 h). LpE1 beads, produced by extrusion and characterized via SEM and FT-IR, were spherical (~1.89 mm) and structurally stable. The optimal formulation—4% GT, 2% LpE1, 30 h incubation—achieved high antioxidant activity (89.84% DPPH), significant total phenolic (380.51 mg GAE/g) and flavonoid content (802.65 mg QE/g), good viability (9.55 log CFU/mL), and desirable physicochemical properties. Despite lower consumer acceptance than plain yogurt, the GT-enriched yogurt demonstrated improved functional and health-promoting characteristics, suggesting promise for future probiotic products. The study demonstrates the effective use of the Box-Behnken design in optimizing the experimental conditions.

Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum*) is known for producing antimicrobial peptides (AMPs), particularly bacteriocins, making it valuable in both food preservation and biomedical applications. Similar to our study, a study used Response Surface Methodology (RSM) with a Box–Behnken design to optimize conditions for maximum antibacterial production (Prema et al., 2024). The optimal parameters—35 °C, pH 6.5, and 48 h incubation—led to a more than 10-fold increase in antibacterial yield. Initial pH was the most significant factor influencing production ($p < 0.05$). The findings support the use of Box–Behnken design for efficient scale-up of AMP production from *L. plantarum*.

In our study, 5 bacteria were included to experiments. Among these bacteria one of them (*P. pentosaceus* 50) was more convenient for the modeling study. For *Lactiplantibacillus plantarum* F2

only incubation time could be used as a significant parameter in the modeling study. On the other hand this design could not be applied for some isolates (*P. pentosaceus* ATCC 43201, *P. acidilactici* 40 and *L. plantarum* O2). According to these results, Box-Behnken design can be accepted partially successful in the optimization process prediction. Mainly the growth conditions were take into consideration in this study but the design can be adapted to many other research area of microbiology. In the experiments carried out within the scope of this study, the Box-Behnken design was successfully applied for bacteria *L. plantarum* F2 and *P. pentosaceus* 50. The models developed for both bacterial isolates are presented in the relevant sections. For isolate F2, a model was constructed based solely on incubation time, while for isolate 50, a comprehensive model incorporating all experimental parameters—incubation temperature, incubation time, and substrate concentration—was designed.

CONCLUSION

Several parameters can affect bacterial growth environments. Evaluating these parameters individually may not yield accurate results, and it is necessary to consider the interactions of all these parameters within the matrix. Bacterial cells or bacterial metabolites may need to be produced as products on an industrial scale or in a laboratory setting. Optimal systems are preferred in all of these processes. In this regard, the Box-Behnken design can assist in predicting optimal conditions. In this study, we attempted to establish optimal conditions for the production of antimicrobial substances that are important in the environments where they are present, produced by LAB. The parameters considered in the experiments were incubation temperature, incubation time, and the concentration of substrate in the environment. When examining the results obtained, it is generally observed that longer incubation times lead to a simultaneous increase in antimicrobial production. Furthermore, it was determined that all three parameters were effective in the antimicrobial activity performance of the *P. pentosaceus* 50 which was included in the experiments. However, an optimal system proposal using the Box-Behnken design could not be made for the antimicrobial production of the three isolates (*P. pentosaceus* ATCC 43201 *P. acidilactici* 40 and *L. plantarum* O2). The model used in the study was found to be partially successful and applicable to microbial processes.

AUTHOR CONTRIBUTION

Conceptualization: EGA. Analysis and data curation: BS, AAC, OK, EGA. Funding acquisition: EGA. Methodology: OK, EGA. Investigation: BS, AAC, OK. Supervision: EGA. Writing – original draft - review & editing: EGA. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

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