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Optimization of Antagonistic Effect of *Bacillus* sp. with Response Surface Methodology

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ABSTRACT

The aim of the study was to optimize the culture conditions of the bacteria to increase the antagonist effect of *Bacillus pumilus* PJ_11 on *Vibrio vulnificus*. In the study, the antagonistic activity of the whole cell product of *Bacillus* PJ_11 was tested by the agar well diffusion method. Parameters (temperature, NaCl concentration, and incubation time) of the culture medium were optimized with response surface methodology and evaluated at three different levels. In the experiment designed with the Box Behnken method, it was observed that the inhibitory effect was highest (zone diameter 26.44 mm) at 30 °C, 2% NaCl concentration, and 18th hour. The coefficient values of the response surface regression equation showed that temperature was more effective on the response than other parameters. Additionally, the coefficient values indicated that the combined effect of temperature and NaCl concentration was more effective on the response than other combinations (temperature-incubation time and NaCl concentration-incubation time). The results showed that the Box-Behnken design in three-factor optimization of culture conditions can be used to determine the most effective antagonistic activity of inhibitor bacteria on pathogenic bacteria.

Keywords: *Bacillus pumilus*, *Vibrio vulnificus*, Box-Behnken design, Antagonistic effect.

Bacillus sp.'nin Antagonistik Etkisinin Tepki Yüzey Metodolojisi ile Optimizasyonu

ÖZ

Çalışmanın amacı, *Bacillus pumilus* PJ_11'in *Vibrio vulnificus* üzerindeki antagonist etkisinin artırılması için bakteriye ait kültür koşullarını optimize etmektir. Çalışmada, *Bacillus* PJ_11'in tüm hücre ürününün antagonistik aktivitesi agar kuyusu difüzyon yöntemiyle test edilmiştir. Kültür ortamına ait parametreler (sıcaklık, NaCl konsantrasyonu ve inkübasyon süresi), yanıt yüzey metodolojisi ile optimize edilmiş ve üç farklı seviyede değerlendirilmiştir. Box Behnken yöntemiyle tasarlanan deneyde, inhibitör etkinin 30°C'de, %2'lik NaCl konsantrasyonunda ve 18. saatte en yüksek (halka çapı 26,44 mm) olduğu gözlenmiştir. Tepki yüzeyi regresyon denkleminin katsayı değerleri, sıcaklığın yanıt üzerinde diğer parametrelere göre daha etkili olduğunu göstermiştir. Ayrıca katsayı değerleri, sıcaklık ve NaCl konsantrasyonunun birleşik etkisinin diğer kombinasyonlara (sıcaklık-inkübasyon süresi ve NaCl konsantrasyonu-inkübasyon süresi) göre yanıt üzerinde daha etkili olduğunu göstermiştir. Sonuçlar, kültür koşullarının üç faktörlü optimizasyonunda Box-Behnken tasarımının inhibitör bakterinin patojenik bakteri üzerindeki en etkin antagonistik aktivitesinin saptamasında kullanılabileceğini göstermiştir.

Anahtar Kelimeler: *Bacillus pumilus*, *Vibrio vulnificus*, Box-Behnken tasarımı, Antagonistik etki.

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1. Giriş

Vibrio species are part of the microbiome of tropical and subtropical marine ecosystems and the guts of many aquatic species (Egerton et al., 2018). Vibriosis caused by pathogenic *Vibrio* species is an important fish disease that affects many cultured and wild fish species thus can cause significant economic losses (Ananda Raja et al., 2017; Mohd Nor et al., 2019). Considering the financial losses, effective disease management in aquaculture is a crucial factor for sustainable production (Bentzon-Tilia 2016). Chemotherapeutics are widely used in disease prevention and control in aquaculture. However, their use has been restricted by many countries as they accumulate in animal tissues, cause the development of resistant bacteria, and pollute the environment (Sutili and Gresler, 2021). The use of probiotics in aquaculture is considered an alternative approach to sustainable production (Austin et al., 1995).

Probiotics are live microorganisms that can alter the host's microbiota, inhibit the development of pathogens, or compete with them. *Lactobacillus, Lactococcus, Enterococcus, Bacillus, Streptococcus, Micrococcus, Carnobacterium* and *Weissella* genera are probiotics widely used in aquaculture. *Bacillus* are Gram-positive, aerobic, and rod-shaped bacteria. They can survive in many extreme environments. Therefore, *Bacillus* has the potential to be used in the control and treatment of pathogenic *Vibrio* species in aquaculture (Liu et al., 2015; Nayak et al., 2012; Gao et al., 2017; Yaylacı, 2022a).

Optimization of factors such as temperature, pH, incubation time, agitation, and nutrients increases microbial growth and metabolite production (Masurekar, 2008). The one-factor-at-a-time method, which is a classical approach, is time-consuming and cannot reflect the combined interactions of independent variables (Venkateswarulu et al., 2017). Response surface methodology (RSM) is a multivariate statistical technique in which the combined effect of independent variables can be analyzed (Basri et al., 2007). It is frequently used in optimizing bacterial culture conditions (Rao et al., 2000, Yaylacı, 2022b). Box-Behnken design is a response surface methodology and is an effective and economical method used in three-level factor designs (Ferreira et al., 2007).

The aim of the study was to optimize the culture conditions of the bacterium to increase the antagonist effect of *B. pumilus* PJ_11 on *V. vulnificus*. For this purpose, physical parameters of the culture medium such as temperature, NaCl concentration, and incubation time were assessed with RSM's Box-Behnken experimental design. The parameters that most affected the inhibitory activity, and their combined relationships were determined.

2. Materials and Methods

2.1. Bacterial samples

The inhibitory effect of *Bacillus pumilus* PJ_11 (MZ816925), isolated from sea cage water, on *Vibrio vulnificus* (KJ651247.1) was previously confirmed by Uzun Yaylacı (2022). Stock cultures were maintained at −70 °C with 10% (v/v) glycerol.

2.2. Antagonistic activity

The antagonistic activity of *Bacillus* PJ_11 cell culture was performed by agar well diffusion method with a minor modification following Zhang et al. (2017). With an apparatus, 6 mm diameter wells were made on tryptic soy agar (TSA) plates swabbed with *V. vulnificus* culture. 10^6 CFU mL^{-1} overnight culture (30 µl) of *Bacillus* PJ_11 was added to each well and incubated at optimized conditions. Wells filled with 30 µl of sterile PBS (pH 7.2) were used as negative controls. The experiments were triplicated, and the diameter of the inhibition zone was measured.

2.3. Experimental design

The levels and interactions of important parameters affecting the inhibition zone were optimized with the three-level three-factor Box-Behnken design of RSM (Box and Behnken, 1960). The independent variables temperature (A), NaCl concentration (B) and incubation time (C) were examined at three different levels (-1, 0, and +1). (Table 1). The design of 17 trials was performed with 12 random and 3 center points by MINITAB software (version 21, Minitab, State College, PA).

The effect of variables on response was predicted through multiple regression analysis using the second-order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \textbf{(1)}$$

where *Y* is predicted response, *A* is temperature, *B* is NaCl concentration, *C* is incubation time, β_0 is model constant, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13}, and β_{23} are interaction coefficients and β_{11}, β_{22}, and β_{33} are quadratic coefficients.

The quality of fit and competence of the polynomial model equation was tested by the coefficient of determination R^2, *adj* R^2, and *pred* R^2 values. The significance of the regression coefficients of the model was evaluated by *F*-test. The results were shown with contour plot graphics.

Table 1. Levels of variables used in experimental design

Symbol	Name of variables	Levels of variables		
		-1	0	+1
A	Temperature (°C)	24	30	36
B	NaCl (%)	0	2	4
C	Incubation time (h)	12	18	24

2.4. Statistical analysis

The data were expressed as means ± standard deviation of three replicates. The experimental data were analyzed using analysis of variance (ANOVA) was performed using MINITAB 21 software. $p<0.05$ was considered statistically significant.

3. Results and Discussion

A three-factor Box-Behnken experimental design was used to examine and optimize interactions between culture parameters. The design matrix and predicted values are shown in Table 2. The maximum inhibition zone (26.44 mm) was observed at 30°C, NaCl 2%, and at 18 hours. By multiple regression analyses of the responses, the following quadratic polynomial equations (2) were obtained:

$$\boldsymbol{R}_{\textbf{zone diameter}} = -201.38 + 11.939A + 11.704B + 3.298C - 0.19692A^2 - 2.1904B^2 - 0.10983C^2 + 0.1127AB + 0.03194AC + 0.0552BC \quad \textbf{(2)}$$

where *R* is response, *A* is temperature, *B* is NaCl, and *C* is incubation time.

The adequacy of the model was tested using analysis of variance (ANOVA) (Table 3). R^2 values explain the correlation between experimental results and predicted values. The R^2 coefficient value of the zone diameter (0.9975) can explain 99% of the change in the model's responses. The *pred* R^2 (0.9695) and *adj* R^2 (0.9942) values were also found to agree. The results confirmed the reliability of the model design. The statistical significance of the model was checked with the *F* test. The *F* value was 305.47 and $p < 0.05$, indicating that the model was significant. The independent variables used in the model were significant in terms of zone diameter. The "Lack of Fit *F* value" of 3.51 for the model indicates that the lack of fit is not significant compared to pure error. Since lack of fit is not part of the regression, a non-significant lack of fit is expected in RSM models (Kumar et al., 2019). The coefficients in the quadratic polynomial equations showed that the parameter with the greatest effect was temperature, followed by NaCl and incubation time.

Table 2. Box-Behnken design, experimental results, and RSM predicted values for zone diameter

Run	Temperature (°C)	NaCl (%)	Incubation time (h)	Zone diameter (mm)	
				Experimental	RSM-predicted
1	24	0	18	9.28	9.31
2	36	0	18	17.11	17.70
3	24	4	18	6.89	6.29
4	36	4	18	9.31	9.27
5	24	2	12	12.23	12.61
6	36	2	12	16.17	15.99
7	24	2	24	12.44	12.61
8	36	2	24	20.98	20.60
9	30	0	12	15.25	14.83
10	30	4	12	10.21	10.42
11	30	0	24	18.68	18.46
12	30	4	24	10.99	11.40
13	30	2	18	26.44	26.49
14	30	2	18	26.87	26.49
15	30	2	18	25.88	26.49
16	30	2	18	26.61	26.49
17	30	2	18	26.69	26.49

Table 3. ANOVA of quadric model for zone diameter

Source	Zone diameter				
	Sum of squares	*Df*	Mean square	*F* value	*P* value
Model	818.153	9	90.906	305.47	0.000
A-Temperature	64.582	1	64.582	217.01	0.000
B-NaCl	65.666	1	65.666	220.66	0.000
C-Incubation time	10.649	1	10.649	35.78	0.001
A^2	211.595	1	211.595	711.02	0.000
B^2	323.216	1	323.216	1086.10	0.000
C^2	65.828	1	65.828	221.20	0.000
AB	7.317	1	7.317	24.59	0.002
AC	5.290	1	5.290	17.78	0.004
BC	1.756	1	1.756	5.90	0.045
Residual	2.083	7	0.298		
Lack of fit	1.510	3	0.503	3.51	0.128
Pure error	0.573	4	0.143		
Total	820.237	16			
R^2		0.9975			
R^2 (adj)		0.9942			
R^2 (pred)		0.9695			

In this study, the interactions of independent variables with each other were explained using contour plots. While the effect of two variables was analyzed, the value of the other variable was fixed at the central point value. In the elliptical distribution in the graphs, the smallest ellipses showed the best prediction (Shafi et al., 2018; Salman et al., 2020). Figure 1a showed that AB (temperature - NaCl) had a significant effect on the zone diameter at the 18 h incubation time. AC (temperature - incubation time) and BC (NaCl - incubation time) had less influence on the zone diameter, expressed as an expanding elliptical shape in the contour plots (Figure 1b,c).

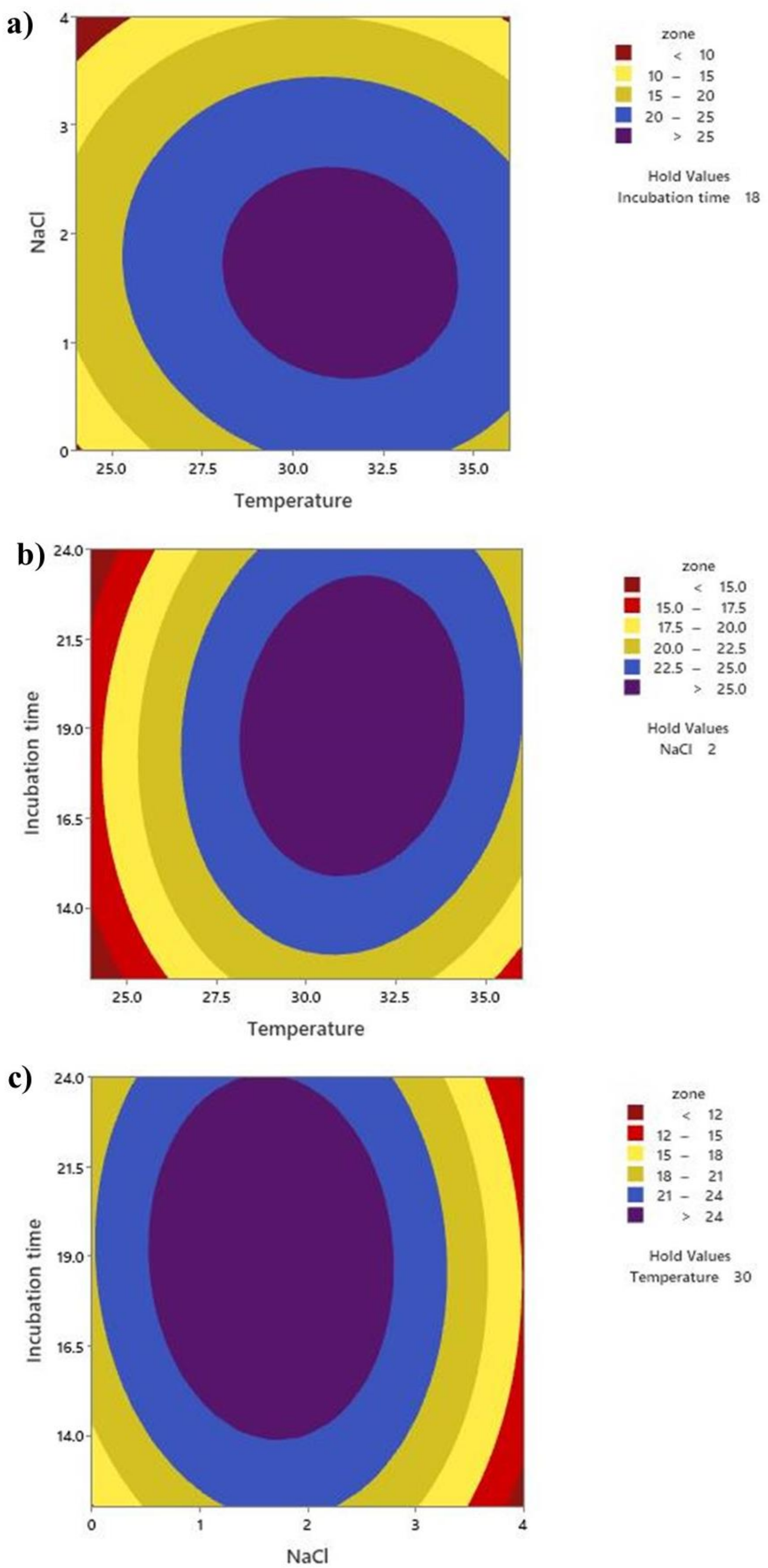

Figure 1. Contour plots showing the interaction of temperature-NaCl concentration (a), incubation time-temperature (b), and incubation time-NaCl concentration (c) on zone diameter.

4. Conclusions

In this study, important results were obtained for optimizing culture parameters to maximize the antagonistic effect of *B. pumilus* PJ_11 against *V. vulnificus* with the RSM approach. According to the coefficient values of the response surface methodology regression equations, temperature was more effective on the response than that of other parameters investigated. In addition, the results showed that the combined effect of two variables, temperature and NaCl concentration, was more effective than other combinations tested (temperature-incubation time and NaCl concentration-incubation time). In conclusion, the Box-Behnken design in three-factor optimization of culture conditions can be used to determine the most effective antagonistic activity of inhibitor bacteria on pathogenic bacteria.

Author Contributions

EUY: Conceptualization, Methodology, formal analysis, investigation, validation, writing, review, and editing.

Conflict of Interest

There is no conflict of interest between third parties.

References

Ananda Raja, R., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S., Nagarajan, K. (2017). Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific white shrimp, *Penaeus vannamei*. *Fish & Shellfish Immunology*, 67, 368–381. https://doi.org/10.1016/j.fsi.2017.06.020

Austin, B., Stuckey, L.E., Robertson, P.A.W., Effendi, I., Griffith, D.R.W. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing disease caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalli*. *Journal of Fish Disease*, 18, 93–96. https://doi.org/10.1111/j.1365-2761.1995.tb01271.x

Basri, M., Rahman, R.N., Ebrahimpour, A., Salleh, A.B, Gunawan, E.R., Rahman, M.B. (2007). Comparison of estimation capabilities of response surface methodology (RSM) with artificial neural network (ANN) in lipase-catalyzed synthesis of palm-based wax ester. *BMC Biotechnology*, 7, 53. https://doi:10.1186/1472-6750-7-53.

Bentzon-Tilia, M., Sonnenschein, E.C., Gram, L. (2016) Monitoring and managing microbes in aquaculture—towards a sustainable industry. *Microbial Biotechnology*, 9, 576–584. https://doi.org/10.1111/1751-7915.12392

Box, G., Behnken, D. (1960). Some new three level designs for the study of quantitative variables. *Technometrics*, 2(4), 455–475.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P. (2018). The gut microbiota of marine fish. *Frontiers in Microbiology*, 9, 873. https://doi.org/10.3389/fmicb.2018.00873.

Ferreira, S.L., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G., Portugal, L.A., dos Reis, P.S., Souza, A.S, dos Santos, W.N. (2007). Box-Behnken design: an alternative for the optimization of analytical methods. *Analytica Chimica Acta*, 597(2), 179–186. https://doi.org/10.1016/j.aca.2007.07.011

Gao, X.Y., Liu, Y., Miao, L.L., Li, E.W., Hou, T.T., Liu, Z.P. (2017). Mechanism of anti-*Vibrio* activity of marine probiotic strain *Bacillus pumilus* H2, and characterization of the active substance. *AMB Express*, 71, 23. https://doi.org/10.1186/s13568-017-0323-3

Kumar, N., Sinha, S., Mehrotra, T., Singh, R., Tandon, S., Thakur, I.S. (2019). Biodecolorization of azo dye Acid Black 24 by Bacillus pseudomycoides: Process optimization using Box Behnken design model and toxicity assessment. *Bioresource Technology Reports*, 8, 100311. https://doi.org/10.1016/j.biteb.2019.100311.

Liu, X.F., Li, Y., Li, J.R., Cai, L.Y., Li, X.X., Chen, J.R., Lyu, S.X. (2015). Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahaemolyticus* for use as probiotics in aquaculture. *World Journal of Microbiology and Biotechnology*, 31(5), 795–803. https://doi.org/10.1007/s11274-015-1833-2

Masurekar, P.S. (2008). Nutritional and engineering aspects of microbial process development. *Progress in drug research. Fortschritte der Arzneimittelforschung. Progres des Recherches Pharmaceutiques*, 65, 291–328. https://doi.org/10.1007/978-3-7643-8117-2_8.

Mohd Nor, N., Yazid, S.H.M., Daud, H.M., Amal, M.N.A., Mohamad, N. (2019). Costs of management practices of Asian seabass (*Lates calcarifer* Bloch, 1790) cage culture in Malaysia using stochastic model that includes uncertainty in mortality. *Aquaculture*, 510, 347–352. https://doi.org/10.1016/j.aquaculture.2019.04.042

Nayak, S., Limsuwan, C., Chuchird, N., Pungpang, S. (2012). A study on the effect of *Bacillus* spp. to control the pathogenic bacteria in aquaculture. *Kasetsart University Fisheries Research Bulletin*, 36(2), 1–12.

Rao, K.J., Kim, C.H., Rhee, S.K. (2000). Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. *Process Biochemistry*, 35, 639–647. https://doi:10.1016/S0032-9592(99)00129-6

Salman, M., Shahid, M., Sahar, T., Naheed, S., Mahmood-ur-Rahman, Arif, M., Iqbal, M., Nazir, A. (2020). Development of regression model for bacteriocin production from local isolate of *Lactobacillus acidophilus* MS1 using Box-Behnken design. *Biocatalysis and Agricultural Biotechnology*, 24, 101542. https://doi.org/10.1016/j.bcab.2020.101542

Shafi, J., Sun, Z., Ji, M., Gu, Z., Ahmad, W. (2018). ANN and RSM based modelling for optimization of cell dry mass of *Bacillus* sp. strain B67 and its antifungal activity against *Botrytis cinerea*. *Biotechnology & Biotechnological Equipment*, 32, 58–68. https://doi org/10.1080/13102818.2017.1379359.

Sutili, F.J., Gressler, L.G. (2021). Antimicrobial agents. In Aquaculture Pharmacology (pp. 131–168). Academic Press.

Venkateswarulu, T.C., Prabhakar, K.V., Kumar, R.B., Krupanidhi, S. (2017). Modeling and optimization of fermentation variables for enhanced production of lactase by isolated *Bacillus subtilis* strain VUVD001 using artificial neural networking and response surface methodology. *3 Biotech*, 7(3), 186. https://doi.org/10.1007/s13205-017-0802-x

Yaylacı, E.U. (2022a). Isolation and characterization of *Bacillus* spp. from aquaculture cage water and its inhibitory effect against selected *Vibrio* spp. *Archives of Microbiology*, 204, 26. https://doi.org/10.1007/s00203-021-02657-0

Yaylaci, E.U. (2022b). Characterization of *Pseudoalteromonas* sp. from aquaculture environment and optimization of fermentation culture parameters by RSM-based modeling. *Turkish Journal of Fisheries And Aquatic Sciences*, 22(11), TRJFAS21726. https://doi.org/10.4194/TRJFAS21726

Zhang, L., Tian, X., Kuang, S., Liu, G., Zhang, C., Sun, C. (2017). Antagonistic activity and mode of action of phenazine-1-carboxylic acid, produced by marine bacterium *Pseudomonas aeruginosa* PA31x, against *Vibrio anguillarum in vitro* and in a zebrafish *in vivo* model. *Frontiers in Microbiology*, 8, 289. https://doi.org/10.3389/fmicb.2017.00289.