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Characterization of *Pseudoalteromonas* sp. from Aquaculture Environment and Optimization of Fermentation Culture Parameters by RSM-Based Modeling

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Abstract

The aim of the study was to identify *Pseudoalteromonas* strain OS-9 and evaluate its anti-*Vibrio* potential. Based on the morphological, biochemical, and 16S rRNA sequence analysis, the strain was identified as *Pseudoalteromonas haloplanktis*. Experiments designed with Box Behnken showed that cell density and cell-free supernatant activity were simultaneously maximum at 30°C, pH 7, and 90 h. The coefficient values obtained from the response surface methodology regression equations determined that pH was more effective on responses than other physical variables. The cell-free supernatant of *P. haloplanktis* OS-9 extracted under optimized conditions was only effective on Gram-negative fish pathogenic bacteria and especially on *Vibrio*. Among *Vibrio* spp., the strongest inhibitory effect was observed against *V. rotiferianus*. At the maximum cell density (10.48 OD), the strain OS-9 showed the maximum inhibitory effect against *V. rotiferianus* (26.42 mm zone diameter). In co-culture assay, the growth of *V. rotiferianus* was inhibited after 96 h with an initial level of 1.0×10^6 CFU ml^{-1} by the strain OS-9. These results indicated that *P. haloplanktis* strain OS-9 could be considered as a potential *Vibrio* bio-controlling agent.

Introduction

The genus *Vibrio* are natural inhabitants of the normal microbiota of marine and estuarine ecosystems. Vibriosis caused by *Vibrio* species is an economically important disease that hinders the development of aquaculture (Ananda Raja et al., 2017). Vibriosis outbreaks have been reported in almost all cultured aquatic animals such as crustaceans, mollusks, and fish (Gómez-León et al., 2005; Bilen et al., 2013). *Vibrio* species associated with raw mollusks and shellfish or fetal contaminated water cause gastroenteritis, wound infections, diarrhea, and septicemia in humans (Levine & Griffin, 1993), and *Vibrio*-induced deaths were observed in patients with chronic conditions (Daniels et al., 2000). Antibiotics have been used for many years to control and manage bacterial diseases in aquaculture (Done et al., 2015). However, the excessive use of antibiotics causes antibiotic accumulation in organs, deterioration of the microbiota of the aquaculture systems, and development of antibiotic-resistant bacteria (Resende et al., 2012; Kavitha et al., 2018). It has been reported that *Vibrio* species develop resistance to antibiotics such as ampicillin, carbapenem, chloramphenicol, kanamycin, tetracycline, trimethoprim, and streptomycin, which are primarily used in the treatment of vibriosis (Zanetti et al., 2001; Lee et al., 2018). To reduce these risk factors, probiotic microorganisms as biocontrol agents can be an innovative and alternative approach to the control and treatment of vibriosis (Mohamad et al., 2019; Yaylacı, 2021).

Probiotics are defined as harmless live microorganisms that improve host health (Romero et al., 2012). Probiotics, taken as food additives or supplements, enhance digestion by improving the intestinal microbial balance, and providing protection against pathogens (Morya et al., 2014). Therefore, probiotic applications are considered an alternative method to antibiotics in managing bacterial infections in aquaculture (Tinh et al., 2008). In addition, the effective use of probiotics against diseases encourages researchers to search for new strains with different properties. Marine-derived bacteria, such as *Pseudoalteromonas*, are being investigated as probiotics as they are sources of various bioactive metabolites.

The yield is critical in processes where secondary metabolites are harvested. The low yield and high production costs encountered in the fermentation process are limiting factors in the development, commercialization, and application of bacteria-based products (Schisler et al., 2004). During the fermentation process, temperature, pH, incubation time, agitation, and nutrients affect the amount and yield of production. Therefore, developing a culture medium that optimizes the production of the microbial strain is the most crucial step in increasing the yield (Kamaran et al., 2015). Different methods have been applied to optimize the culture medium to increase microbial growth and production of metabolites (Masurekar, 2008). The classical optimization approach is one-factor-at-a-time, which is time-consuming and cannot fully demonstrate the combined interactions of independent variables in the production process (Venkateswarulu et al., 2017). These disadvantages of the one-factor-at-a-time method can be overcome by using alternative approaches such as response surface methodology (RSM) (Shafi et al., 2018). RMS is a multivariant statistical technique used to construct models, design experiments, evaluate the effects of factors, and find the optimal conditions to describe relationships between response and independent variables (Basri et al., 2007). This experimental methodology is frequently used to optimize the fermentation medium (Rao et al., 2000). Box-Behnken design of RSM is an efficient and economical method used in three-level-factor designs where the independent variables are more than two (Ferreira et al., 2007).

This study aimed to characterize and evaluate the anti-*Vibrio* potential of *Pseudoalteromonas* strain OS-9 isolated from sea bass (*Dicentrarchus labrax*) aquaculture cage surface water. To maximize cell density and cell-free supernatant activity simultaneously, the independent physical factors (temperature, pH, and incubation time) of the fermentation culture were optimized with the Box Behnken experimental design. In addition, a co-culture experiment was carried out under optimized conditions with the *Vibrio* strain in which the isolate had the most inhibitory effect.

Materials and methods

Sample collection

Surface seawater samples were collected from an aquaculture cage of sea bass (*D. labrax*) farm located in Ordu province in the Black Sea Region of Turkey. 1 mL of sample was diluted tenfold serial dilutions in phosphate-buffered saline (PBS) (pH 7.2). 100 µl samples from each dilution were inoculated into Marine agar 2216 (MA) and incubated at 29°C for 48 h. Differently from the other colonies, one with brownish pigmentation was selected. The isolate labeled strain OS-9 was subcultured and stored at -70°C with 10% (v/v) glycerol.

Antagonistic Activity

The strain OS-9 was tested for antagonistic activity against *Vibrio vulnificus* (KF443056), *V. harveyi* (KF443058), *V. rotiferianus* (KF443057), and *Aeromonas veronii* (KF443053) were isolated from fish (*D. labrax*) with disease symptoms and confirmed previously by Uzun and Ogut (2015). The agar well diffusion method with minor modification after Zhang et al. (2017) was carried out to screen the antagonistic activity of the strain OS-9 cell culture. Using an apparatus, wells of 6 mm diameter were made on the tryptic soy agar (TSA) plates previously swabbed with indicator bacterial culture. 10^6 CFU ml^{-1} overnight culture (30 µl) of the strain OS-9 was added to each well and incubated at 29°C for 24 h. Wells filled with 30 µl of sterile PBS (pH7.2) were used as controls. The OS-9 strain was subjected to further analysis as it had antagonistic activity against fish pathogens.

Morphological and Biochemical Identification

To determine morphological and biochemical characteristics, the strain OS-9 was cultivated on MA plate at 29°C for 24 h. Cell morphology, Gram staining, catalase, and oxidase activities were examined by the methods of Lai et al. (2009). Hydrolysis of starch, casein, gelatine, H_2S, and indole production was carried out according to Ivanova et al. (2004a). API ZYM, API 20E, and API 20NE kits (BioMerieux, USA) were used for other biochemical tests.

Molecular Identification

Genomic DNA of OS-9 harvested with tri-reagent LS (Sigma) and Master Mix Kit (Qiagen) was used for molecular identification. PCR was performed with the universal primer set: 16S-27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1492R (5'-CGGTTACCTTGTTACGACTT -3') to amplify the 16s rRNA gene. The thermal profile consisted of initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. PCR

products were purified with QIAquick PCR Purification Kit (Qiagen Inc). The sequencing reaction was performed by Macrogen Inc. Comparison of the target sequence with other related taxa was analyzed using deposited sequences in the GenBank database (NCBI, http://www.ncbi.nlm.nih.gov) by basic local alignment (BLAST). A phylogenetic tree of 16S rRNA gene sequences was constructed using Mega X to determine the evolutionary relationship between strains using Neighbor-Joining method in ClustalW (Kumar et al., 2018).

Experimental Design

The overnight culture of the strain OS-9 was inoculated into Marine broth (MB) at an initial cell density of 10^5 CFU ml^{-1} and incubated in a shaker (120 rpm^{-1}). The culture conditions were optimized according to the Box-Behnken experimental design (Table 1). Uncultivated MB was used as a negative control and experiments were conducted in triplicate. The cell density of the strain OS-9 was monitored, and optical density (OD) was recorded at 620 nm.

A three-level three-factor Box-Behnken design of RSM was used to optimize the fermentation conditions of strain OS-9 (Box & Behnken, 1960). The factors included temperature (A), pH (B) and incubation time (C) with three coded levels (-1, 0 and +1) (Table 1). The design of 17 experimental runs was performed with 12 random points and 3 center points by MINITAB 19 software (version 19, Minitab, State College, PA).

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

$$Y=\beta_{0+}\ \beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\ \beta_{13}AC+\beta_{23}BC \quad (1)$$

where Y is predicted response, A is temperature, B is pH, 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, adjusted R^2, and predicted R^2 values. The significance of regression coefficients of the model was evaluated by *F*-test. The experimental data were analyzed using analysis of variance (ANOVA) to determine the statistical significance of the model. The effects of independent variables (temperature, pH, and incubation time) on the cell density of strain OS-9 and its cell-free supernatant inhibition activity against *V. rotiferianus* were evaluated by contour plots.

Antibacterial Activity

The cell-free supernatant (CFS) of the strain OS-9 was evaluated for the antibacterial activity to more fish pathogens (*V. anguillarum* (ON076422)*, V. furnissii* (ON076429), *Citrobacter freundii* (KR698931.1) and *Pseudomonas putida* (KY425616.1) confirmed previously by Uzun-Yaylacı (2019)), and clinical pathogens in addition to the above (*Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* (ATCC 43251), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Serratia marcescens* (ATCC 27117), *Salmonella enterica* (ATCC 13076) and *Pseudomonas aeruginosa* (ATCC 27853)). The CFS was extracted from the bacterial culture of strain OS-9 grown under optimized culture conditions. The culture was centrifuged at 10 000 × g for 15 min at 4°C and filtered through a 0.22 µm pore filter. The antibacterial activity was evaluated by the well diffusion method as mentioned above. The plates were incubated at 29°C for *Vibrio* spp. and 37°C for human pathogens. The experiments were repeated three times and the inhibition zone diameter was measured.

Co-culture Assay

In the co-culture experiment, *V. rotiferianus* was inoculated into tryptic soy broth (TSB) at an initial cell density of 10^5 CFU ml^{-1}; the strain OS-9 was inoculated at initial densities of 10^4 CFU ml^{-1}, 10^5 CFU ml^{-1} and 10^6 CFU ml^{-1}. Control groups (the cultures of the strain OS-9 without *V. rotiferianus* and the culture of *V. rotiferianus* without the strain OS-9) were incubated under the same conditions (30°C and pH 7). All combinations were repeated in triplicate. Cultures were incubated in a shaker (120 rpm^{-1}) and samples (100 µl) were withdrawn at 0, 24, 48, 72, and 96 h. *V. rotiferianus* and strain OS-9 colonies were counted using the pour plate method on thiosulfate citrate bile salts sucrose agar (TCBSA) and MA 2216 respectively.

Antibiotic Sensitivity

The sensitivity of the strain OS-9 was tested using the disc diffusion method (Thankappan et al., 2015).

Table 1. Levels of factors used in experimental design

Symbol	Name of variables	Levels of variables		
		-1	0	+1
A	Temperature (°C)	15	30	45
B	pH	5	7	9
C	Incubation time (h)	30	90	150

Sensitivity to furazolidone (50 µg disc^{-1}), florfenicol (30 µg disc^{-1}), erythromycin (15 µg disc^{-1}), chloramphenicol (30 µg disc^{-1}), oxolinic acid (2 µg disc^{-1}), oxytetracycline (30 µg disc^{-1}), ampicillin (10 µg disc^{-1}) and streptomycin (10 µg disc^{-1}) was assessed with Oxoid disks.

Statistical Analysis

The data were expressed as means ± standard deviation of three replicates. The results were analyzed using one-way analysis of variance (ANOVA) was performed using MINITAB 19 software. p<0.05 was considered statistically significant.

Results and Discussion

Isolation, Characterization, and Phylogenetic Analysis of the Strain OS-9

The strain OS-9 was isolated from the aquaculture cage surface water of sea bass (*D. labrax*) farm located in Ordu province in the Black Sea Region of Turkey. In the preliminary screening for the antagonistic effect, it was determined that the OS-9 strain had antibacterial ability. The isolate appeared convex and 3 mm in diameter with brownish pigmentation on MA 2216 agar plate after incubation at 29°C for 48 h (Figure 1). Cells were Gram-negative, rode-shape, motile and non-spore-forming. The isolate was positive for catalase, oxidase, and urease but negative for H_2S, indole, and citrate utilization. The strain hydrolyzed starch and gelatin but not casein. Other biochemical characteristics are listed in Table S1. Morphological and physiological characteristics of the strain OS-9 matched with *Pseudoalteromonas* genus (Zhao et al., 2014). Molecular identification of the strain OS-9 was carried out by DNA-sequencing. The homology between phylogenetically related species was analyzed via sequence for the partial (1461 bp) 16S rRNA gene (GenBank Accession #: SUB11237414). The cladogram generated based on the 16S rRNA gene showed that the strain OS-9 exhibited 99.93% identical homology with *Pseudoalteromonas haloplanktis* strain ATCC 14393 (Figure 2).

Response Surface Model

A three-factor Box-Behnken experimental design with RSM was employed to optimize the fermentation parameters (temperature, pH, and incubation time) of *P. haloplanktis* strain OS-9. The design matrix and predicted values are presented in Table 2. The parameters included the cell density of *P. haloplanktis* strain OS-9 and the zone diameters of the most sensitive isolate, *V. rotiferianus*. The aim was to determine the values at which the highest cell density and CFS activity of *P. haloplanktis* strain OS-9 were observed. The maximum cell density (10.48 OD) and zone diameter (26.42 mm) were obtained at 30°C, pH 7, and 90 h. Regression analyses for the responses were performed via RSM with the quadratic polynomial equations (2, 3). The model equations obtained from responses are as follows:

$$\begin{aligned} R_{\text{cell density}} = {} & -51.25 + 0.8943A + 12.618B + 0.14185C \\ & - 0.018817A^2 - 1.0016B^2 - 0.000892C^2 \\ & + 0.02500AB - 0.000117AC - 0.001479BC \end{aligned} \quad (2)$$

$$\begin{aligned} R_{\text{zone diameter}} = {} & -101.46 + 1.702A + 25.91B + 0.3138C \\ & - 0.03965A^2 - 2.1204B^2 - 0.001978C^2 \\ & + 0.0753AB + 0.000244AC + 0.00367BC \end{aligned} \quad (3)$$

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

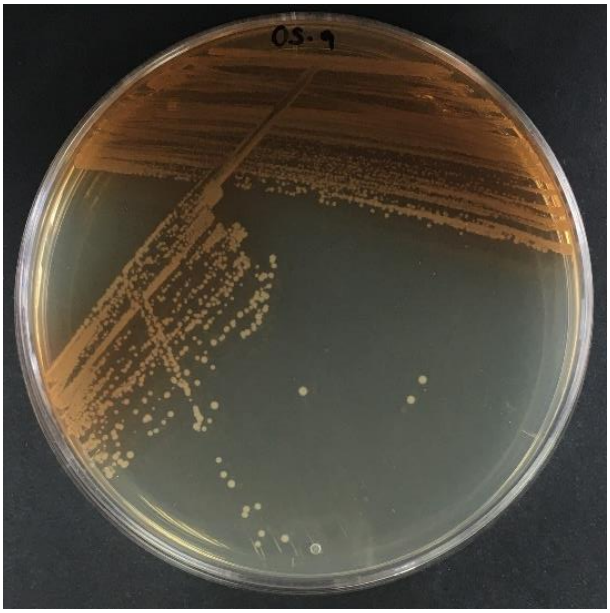

Figure 1. Brownish pigmentation of *P. haloplanktis* strain OS-9 on MA 2216 agar plate.

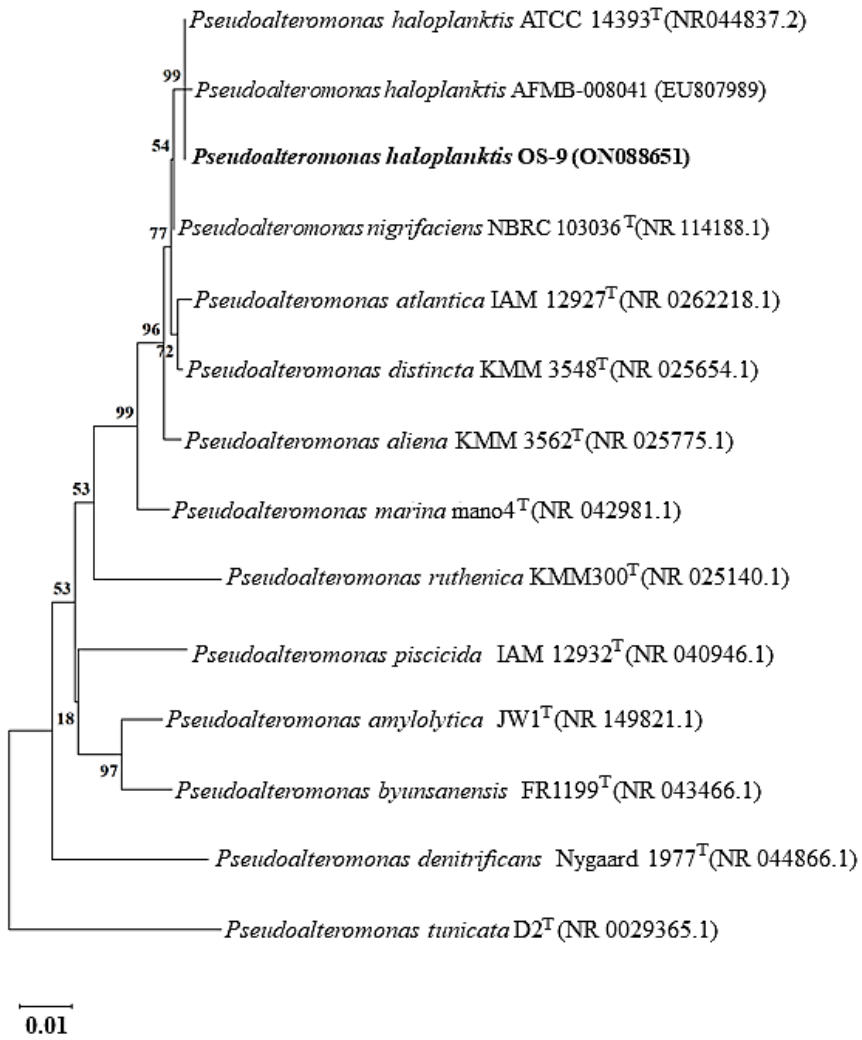

Figure 2. Evolutionary relationships were visualized as Neighbor-joining tree of 16S rRNA gene. Analysis was carried out based on the sequences compared using Mega X.

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

Run	Temperature (°C)	pH	Incubation time (h)	Zone diameter (mm)		Cell density (OD)	
				Experimental	RSM-predicted	Experimental	RSM-predicted
1	15	5	90	16.34	15.56	4.65	4.53
2	45	5	90	7.22	7.21	1.51	1.56
3	15	9	90	6.29	6.30	1.25	1.20
4	45	9	90	6.2	6.98	1.11	1.23
5	15	7	30	12.79	13.06	4.04	4.06
6	45	7	30	9.28	8.79	2.52	2.37
7	15	7	150	11.03	11.52	3.12	3.27
8	45	7	150	8.4	8.13	2.02	2.00
9	30	5	30	13.67	14.18	4.44	4.54
10	30	9	30	8.84	8.55	2.32	2.35
11	30	5	150	11.91	12.20	3.63	3.60
12	30	9	150	8.84	8.33	2.22	2.12
13	30	7	90	26.42	26.42	10.48	10.37
14	30	7	90	26.68	26.42	10.45	10.37
15	30	7	90	25.98	26.42	10.08	10.37
16	30	7	90	26.85	26.42	10.48	10.37
17	30	7	90	26.15	26.42	10.36	10.37

The ANOVA of the models is shown in Table 3. The correlation between the experimental results and the predicted values is represented by the R^2 values (Celik et al., 2018; Özbek Yazıcı & Özmen, 2020). The coefficient R^2 value for cell density and zone diameter (0.9991 and 0.9971, respectively) showed that the models could explain 99% response variations. In addition, for cell density and zone diameter, predicted R^2 (0.9923 and 0.9607, respectively) and adjusted R^2 (0.9978 and 0.9934, respectively) values were in good agreement with the value of R^2. The R^2 values obtained from the RSM models confirmed the reliability of the design.

The statistical significance of the quadratic polynomial models was checked with the *F* test (ANOVA). The ANOVA of both RSM models was found highly significant for the fitted model with a high *F* value (819.43 and 267.41) and *p* value < 0.05. The terms with *p* value less than 0.05 indicate that models are significant. In this study, A, B, AB, CB, A^2, B^2, and C^2 were significant terms for cell density and zone diameter, but AC was insignificant. In addition, C was significant for cell density, but not for zone diameter, as shown in Table 3. "Lack of Fit *F* values" of 1.12 and 6.50 for both RSM models indicate that the lack of fit was not significant relative to pure error. A non-significant lack of fit was expected in the RSM models because it was not part of the regression (Kumar et al., 2019). Coefficient values indicated that pH was the most significant variable in both RSM models, followed by temperature and incubation time.

The contour plots describe the interactions of independent variables with each other (De Lima et al., 2010). In response plots, the effect of any two variables was analyzed while the value of the other variable was fixed at the central point value. The elliptical distribution of the contour plot showed a significant effect, and the smallest ellipses in the contour diagram represented the best-predicted values (Shafi et al., 2018; Salman et al., 2020). In this study, the contour plots of elliptical shape showed that AB (temperature - pH) had a significant effect on the cell density of *P. haloplanktis* strain OS-9 and zone diameter at the fixed incubation period of 90 h (Figure 3 a, d). It was observed in the graphs that the increase in pH from 5 to 7 and temperature from 15 to 30°C caused an increasing trend in the cell density and zone diameter. However, further increases in pH and temperature decreased the cell density and zone diameter. The mutual effects of AC (temperature - incubation time) and BC (pH - incubation time) on the cell density and zone diameter were non-significant, seen as a circular shape of contour plots (Figure 3b, e; Figure 3c, f).

Antibacterial Activity

The cell-free supernatant of *P. haloplanktis* was extracted under optimized conditions (30°C, pH 7, and 90 h) and investigated against seventeen pathogens (four Gram-positive and thirteen Gram-negative bacteria) using the agar well diffusion method. Similar to Handayani et al. (2022), it was determined that CFS produced by *P. haloplanktis* strain OS-9 inhibited only Gram-negative fish pathogenic bacteria and especially *Vibrio* (Table 4). In addition, as reported by Isnansetyo et al. (2009), the inhibitory effect of strain OS-9 showed heterogeneity among *Vibrio* species. This can be explained by the fact that the strain OS-9 produces narrow-spectrum antimicrobial compounds (Handayani et al., 2022). The cell-free supernatant of *P. haloplanktis* has inhibitory activity against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Bacillus subtilis* and *Micrococcus luteus* (Hayashida-Soiza et al., 2008). However, in this study, clinical pathogens including *S. epidermidis*, *P. aeruginosa, S. aureus, E.*

Table 3. ANOVA of quadric model for zone diameter and cell density

	Zone diameter					Cell density				
Source	Sum of squares	*Df*	Mean square	*F* value	*P* value	Sum of squares	*Df*	Mean square	*F* value	*P* value
Model	1048.59	9	116.510	267.41	0.000	222.168	9	24.6853	819.43	0.000
A-Temperature	29.45	1	29.453	67.60	0.000	4.351	1	4.3512	144.44	0.000
B-pH	44.98	1	44.983	103.24	0.000	6.716	1	6.716	6.7161	0.000
C-Incubation time	2.42	1	2.420	5.55	0.051	0.679	1	0.679	0.6786	0.002
A^2	335.15	1	335.148	769.21	0.000	75.472	1	75.4722	2301.54	0.000
B^2	302.91	1	302.906	695.21	0.000	67.579	1	67.5791	2243.29	0.000
C^2	213.41	1	213.405	489.79	0.000	43.419	1	43.4195	1441.31	0.000
AB	20.39	1	20.385	46.79	0.000	2.250	1	2.2500	74.69	0.000
AC	0.19	1	0.194	0.44	0.526	0.044	1	0.0441	1.46	0.266
BC		1			0.224	0.126	1	0.1260	4.18	0.080
Residual	3.05	7	0.436			0.211	7	0.0301		
Lack of fit	2.53	3	0.844	6.50	0.051	0.096	3	0.0320	1.12	0.441
Pure error	0.52	4	0.130			0.115	4	0.0287		
Total	1051.64	16				222.379	16			
R^2		0.9971				0.9991				
R^2 (adj)		0.9934				0.9978				
R^2 (pred)		0.9607				0.9923				

p<0.05 are significant

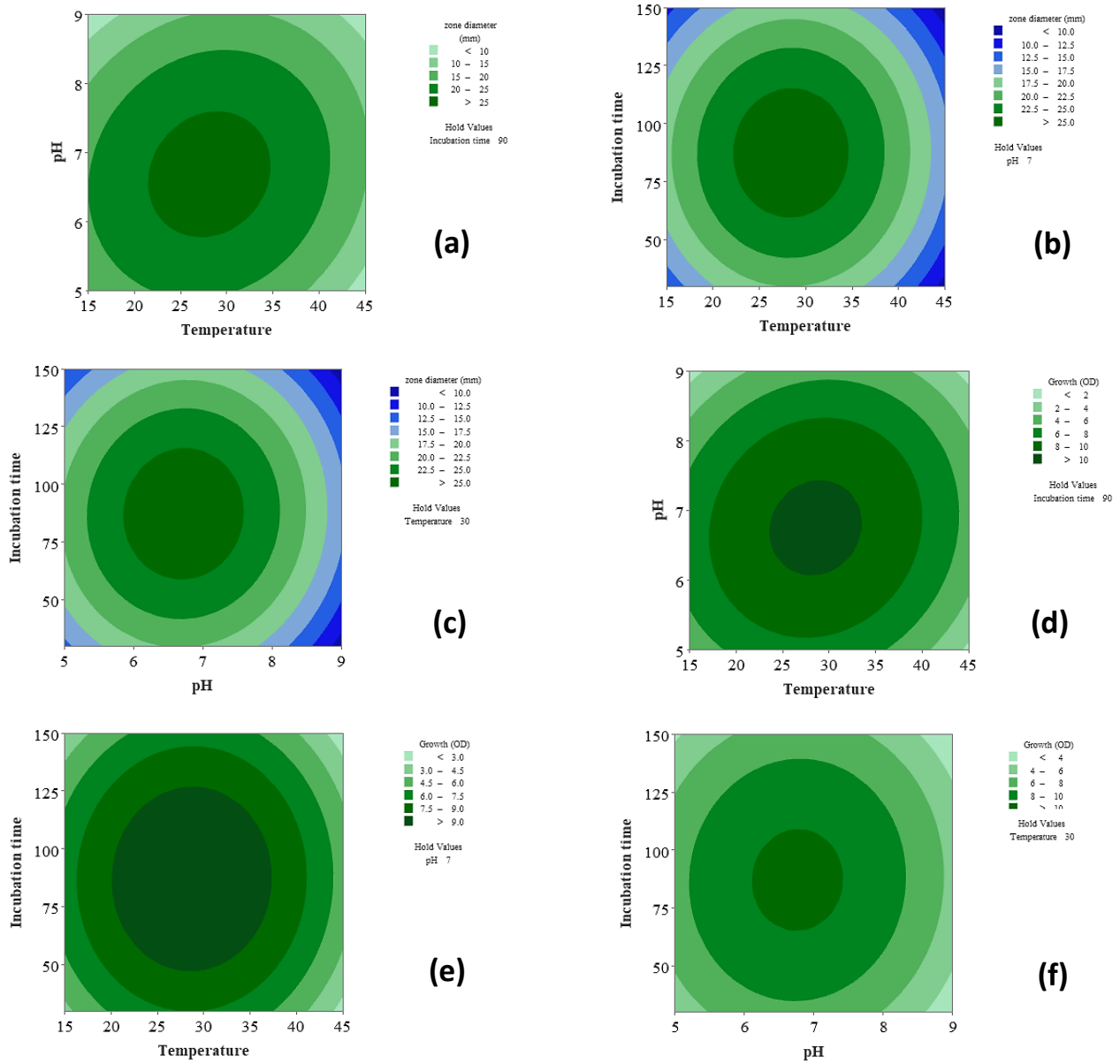

Figure 3. Contour plots showing combined effects of independent variables on zone diameter of the strain OS-9 against *V. rotiferianus* **(a, b, c)** and cell density **(d, e, f).**

Table 4. Antibacterial activity of cell-free supernatant from the strain OS-9

Bacterial strains	Inhibition zone (mm)
V. rotiferianus	26.42±0.22
V. harveyi	16.67±0.14
V. anguillarum	14.65±0.43
V. vulnificus	8.33±0.55
V. furnissii	7.85±0.11
A. veronii	-
C. freundii	-
P. putida	-
S. epidermis	-
P. aeruginosa	-
S. aureus	-
E. faecalis	-
S. marcescens	-
L. monocytogenes	-
S. enterica	-
E. coli	-
K. pneumoniae	-

[a] Values are the mean ± standard deviations of triplicate measurements.
- No inhibition zone

faecalis, S. marcescens, L. monocytogenes, S. enterica, E. coli, and *K. pneumoniae* were not inhibited by the CFS of *P. haloplanktis* OS-9. Antimicrobial properties of *Pseudoalteromonas* are associated with the host or ecological niche in which they are isolated (Offret et al., 2016). In this regard, the isolation of the strain OS-9 and the pathogens it inhibits from the same environment may have given the OS-9 strain an advantage in inhibiting these pathogens.

Pseudoalteromonas belonging to the class of Gammaproteobacteria are marine bacteria that can produce antibacterial compounds (Gauthier et al., 1995; Holmstrom & Kjelleberg, 1999; Desriac et al., 2014; Tang et al., 2020). *Pseudoalteromonas* spp. produce various extracellular products, such as violacein, indolmycin (Thøgersen et al., 2016), hexadecatetraenoic acid (Supardy et al., 2019), isovaleric and 2-methylbutyric acids (Hayashida-Soiza et al., 2008). It has been reported that extracellular serine proteases, metalloproteases, and other proteolytic substances produced by some *Pseudoalteromonas* species have antibacterial properties (Klein et al., 2011; Mitra et al., 2014). Some species of this genus also have antifouling (Yee et al., 2007) and antibiofilm (Dheilly et al., 2010) activities. *Pseudoalteromonas* genus includes pigmented and non-pigmented species (Gauthier et al., 1995). The ability to produce metabolites has often been associated with pigmentation, as most (>80%) of *Pseudoalteromonas* species that produce antimicrobial metabolites are pigmented (Huang et al., 2011). Non-pigmented *Pseudoalteromonas* strains can also be bioactive metabolite producers (Ivanova et al., 2014). It has been reported in many studies that *P. haloplanktis* produces melanin-like pigment, albeit weakly (Bowman, 1998; Matsuyama et al., 2014). Similarly, in this study, it was determined that the OS-9 is an isolate that produces melanin-like pigment. *Pseudoalteromonas haloplanktis* strain OS-9 demonstrated good antibacterial activity against *Vibrio* spp., especially *V. rotiferianus* (Figure 4). *V. rotiferianus* is common in the marine environment and is pathogenic in marine fish and crustaceans (Austin et al., 2005). Furthermore, *V. rotiferianus* is resistant to many antibiotics such as penicillin, streptomycin, kanamycin, and ampicillin (Zhang et al., 2019). In this study, the strain OS-9 was considered a potential probiotic as it showed an inhibitory effect against *Vibrio* species. *Pseudoalteromonas* species are non-pathogenic and considered safe (Ivanova et al., 2004b). Many studies have reported that *Pseudoalteromonas* species can be used as a potential *Vibrio*-biocontrol agent in aquaculture (Aranda et al., 2012; Morya et al., 2014).

Co-culture Assay

In broth co-culture assay, the strain OS-9 was evaluated for inhibitory activity against *V. rotiferianus*. After 96 h, the growth of *V. rotiferianus* was inhibited 39% by *P. haloplanktis* strain OS-9 culture inoculated at an initial level of 10^4 CFU ml^{-1} and inhibition increased to 62.9% when the concentration of the strain OS-9 was 10^6 CFU ml^{-1} (Figure 5). Co-culture experiments showed that the inhibitory activity of the *P. haloplanktis* OS-9 increased when the concentration of inhibitor bacteria and the incubation period were increased, which agrees with the reports of Handayani et al. (2022).

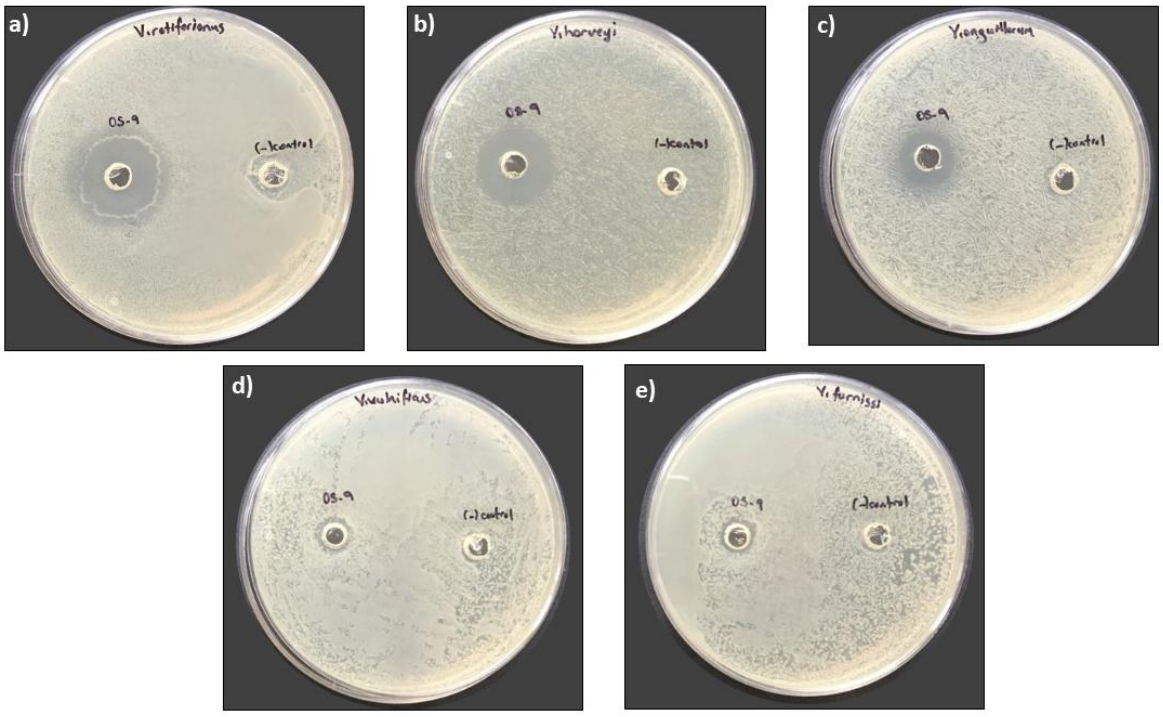

Figure 4. Inhibition zone against *V. rotiferianus* **(a)**, *V. harveyi* **(b)**, *V. anguillarum* **(c)**, *V. vulnificus* **(d)**, and *V. furnissii* **(e)**.

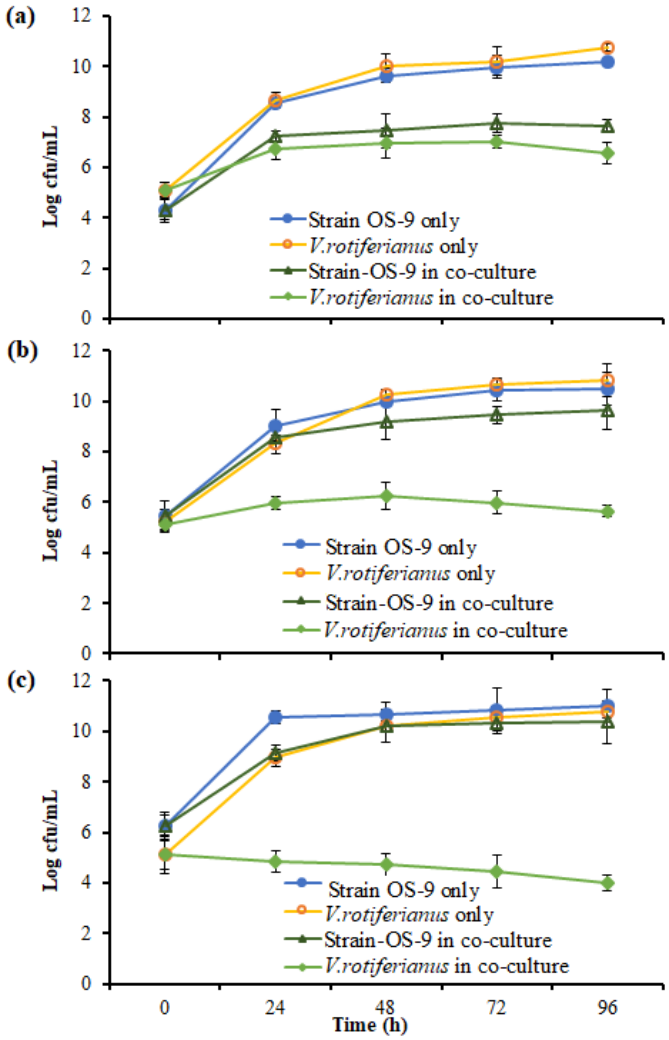

Figure 5. Growth inhibition activity of the strain OS-9 against *V. rotiferianus* (10^5 CFU ml^{-1}) at an initial cell density of 10^4 CFU ml^{-1}**(a)**, 10^5 CFU ml^{-1}**(b)**, and 10^6 CFU ml^{-1}**(c)**.

Table 5. Antibiotic susceptibility of the strain OS-9.

Antibiotics	Inhibition zone (mm)
Furazolidone	23.00±0.26[a]
Florfenicol	14.11±0.55
Erythromycin	26.22±0.33
Chloramphenicol	15.00±0.12
Oxolinic acid	27.00±0.30
Oxytetracycline	31.60±0.44
Ampicillin	26.10±0.67
Streptomycin	13.53±0.45

[a] Values are the mean ± standard deviations of triplicate measurements.

Antibiotic Susceptibility

Antibiotic sensitivity is one of the most important characteristics of potential probiotics (Patel et al., 2009). In the present study, *P. haloplanktis* OS-9 was susceptible to all tested antibiotics (Table 5). The susceptibility to many antibiotics suggests that *P. haloplanktis* OS-9 does not have antibiotic-resistant genes that can be transferred to pathogenic bacteria.

Conclusion

The strain OS-9 was isolated from the aquaculture cage water and identified as *P. haloplanktis* based on morphological and biochemical properties with a phylogenetic analysis using 16S rRNA gene sequence. The results showed that the Box-Behnken design of RSM is a suitable method when three factors are desired to be optimized in the fermentation culture. With the RSM

method, parameters that maximize both responses simultaneously were determined. Thus, it seems possible to decide on the appropriate time to harvest the culture in large-scale commercial production of the strain. *P. haloplanktis* strain OS-9 showed good antibacterial activity against *Vibrio* spp. Being susceptible to many antibiotics commonly used in aquaculture suggests that the strain OS-9 may not carry antibiotic-resistant genes. In conclusion, *P. haloplanktis* strain OS-9 can be considered a potential *Vibrio* bio-controlling agent. However, the strain OS-9 needs further research to elucidate its molecular mechanisms and determine its practical applicability under aquaculture conditions.

Ethical Statement

This article does not contain any studies with human or animal subjects.

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Author Contribution

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

Conflict of Interest

There is no conflict of interest between third parties.

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Table S1. Biochemical characteristics of OS-9 according to API ZYM, API 20E, and API 20NE kits

Characteristics	The strain OS-9	Characteristics	The strain OS-9
API ZYM		Gelatinase	+
Phosphatase alkaline	+	D-glucose	-
Esterase (C4)	+	D-mannitol	-
Esterase lipase (C8)	+	Fermentation of Inositol	-
Lipase (C14)	-	D- sorbitol	-
Leucine arylamidase	+	L- rhamnose	-
Valine arylamidase	w	D-sucrose	-
Cystine arylamidase	w	D-melibiose	-
Trypsin	+	Fermentation of amygdalin	-
α -chymotrypsin	w	**API 20NE**	
Acid phosphatase	+	Nitrate reduction	-
Naphthol-AS-BI- phosphohydrolase	+	Tryptophan deaminase	-
α-galactosidase	-	Glucose acidification	+
β-galactosidase	-	Arginine dihydrolase	+
β-glucuronidase	-	Urease	+
α-glucosidase	+	Esculin ferric citrate	+
β-glucosidase	-	Gelatinase	+
N-acetyl- β-glucosaminidase	-	β-galactosidase	-
α-mannosidase	-	Utilization;	
α-fucosidase	-	D-glucose	+
API 20E		L-arabinose	-
β-galactosidase	-	D-mannose	-
arginine dihydrolase	w	D-mannitol	-
Lysine decarboxylase	+	*N*-Acetyl-glucosamine	-
Ornithine decarboxylase	+	D-Maltose	-
Utilization of citrate	-	Gluconic acid	-
Production of H_2S	-	Capric acid	-
Urease	+	Adipic acid	-
Tryptophan deaminase	+	Malic acid	
Production of indole	-	Citric acid	-
Production acetoin (Voges-proskauer)	-	Phenylacetic acid	-