Optimization of Culture Conditions for Mass Production of the Probiotics *Pseudomonas* MCCB 102 and 103 Antagonistic to Pathogenic Vibrios in Aquaculture

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Abstract Rapid growth of shrimp farming industry is affected by the recurrence of diverse diseases, among which vibriosis is predominant. Eco-friendly disease management strategy by the application of antagonistic probiotics is widely accepted. In the present study, culture conditions of antagonistic probiotics, *Pseudomonas* MCCB 102 and 103, were optimized to enhance their biomass production and antagonistic activity against the shrimp pathogen *V. harveyi* MCCB 111. Primarily, one-dimensional screening was carried out to fix the optimum range of sodium chloride concentration, pH and temperature. The second step optimization was done using a full-factorial central composite design of response surface methodology. As per the model, 12.9 g/L sodium chloride and pH 6.5 for *Pseudomonas* MCCB 102, and 5 g/L sodium chloride and pH 7 for *Pseudomonas* MCCB 103 were found to be ideal to maximize antagonistic activity. However, optimum temperature was the same (25 °C) for both isolates. Finally, the models were experimentally validated for enhanced biomass production and antagonistic activity. The optima for biomass and antagonistic activity were more or less the same, suggesting the possible influence of biomass on antagonistic activity.

Keywords Probiotic · *Pseudomonas* · Antagonistic activity · Vibrios · Response surface methodology · Central composite design

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Introduction

Members of the genus *Pseudomonas* are common inhabitants of soil, fresh water and marine environments and are known to produce a wide range of secondary antagonistic metabolites [15, 18, and 19]. *P. fluorescens* and *P. aeruginosa* are widely used in agriculture as microbial control agents in lieu of chemicals for combating several plant diseases [11, 18]. Antibacterial and antifungal properties of phenazine antibiotics produced by *Pseudomonas* have been reported antagonistic to human, plant and aquaculture pathogens [1, 10 and 17]. Besides, they have been found to affect growth and viability of a wide range of microorganisms in nature [15] due to their broad-spectrum antibiotic activity [14].

Use of antagonistic probiotics has become popular in aquaculture in recent years. They can make alterations to microbial flora, control pathogens, and improve environmental conditions in the culture systems favorable to the species being cultured [13]. Under such category of

applications, *P. fluorescens* has been reported to inhibit *Saprolegnia* sp. and *A. salmonicida* in finfish culture [2, 20]. Gram et al. [6] described a strain of *P. fluorescens* AH2, which could reduce mortality in rainbow trout subsequent to challenge with *V. angulliarum.* Later, Chytanya et al. [4] reported *Pseudomonas* I-2, antagonistic to shrimp pathogenic vibrios, the antagonistic property mediated by a low molecular weight inhibitor. Precisely, *Pseudomonas* spp. as antagonists and their application as biological control agents have been well documented. However, for their commercial application, the production processes have to be optimized.

Jayaprakash [8] and Vijayan et al. [21] isolated *Pseudomonas* MCCB 103 and 102, respectively, from brackish water environments, antagonistic to a range of pathogenic vibrios such as *V. harveyi*, *V. alginolyticus*, *V. angulliarum*, *V. proteolyticus*, *V. fluviallis*, *V. parahaemolyticus*, *V. neris*, *V. vulnificus*, *V. mediterranei*, *V. cholerae* and *Aeromonas* spp. inhabiting shrimp culture systems. Considering the importance of these putative probiotic isolates in aquaculture, a comprehensive investigation was undertaken to maximize their biomass production and antagonistic activity by optimizing sodium chloride concentration, pH and temperature of incubation in the growth medium, ZoBell's broth, composed of 0.5 % peptone, 0.1 % yeast extract and 0.01 % ferric phosphate in distilled water. To accomplish this end, a full-factorial central composite design (CCD) of the response surface methodology (RSM) was employed.

Materials and Methods

Organisms and Culture Medium

The organisms used in this study were *Pseudomonas* MCCB 102 (GenBank Accession no. EF062514) and *Pseudomonas* MCCB103 (GenBank Accession no. EF053508) previously described by Jayaprakash [8] and Vijayan et al. [21]. These isolates formed part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India. The organisms were maintained at room temperature in ZoBell's marine agar 2216 E (Hi media, Mumbai) slants overlaid with liquid paraffin and at −80 °C as glycerol stocks and used for optimization of sodium chloride concentration and pH in the culture medium and temperature of incubation.

Inoculum Preparation

Pseudomonas cultures grown on ZoBell's marine agar 2216 E (HiMedia, Mumbai) slants were inoculated into 100 mL ZoBell's broth (0.5 % peptone, 0.1 % yeast extract and 0.01 % ferric phosphate prepared in distilled water supplemented with 3 % NaCl) and incubated at 28 °C for 24 h and used as the inoculum.

Shake Flask Experiments

Optimization was done in Erlenmeyer flasks (250 mL capacity) with 100 mL ZoBell's broth having the composition as mentioned above with NaCl content ranging from 0 to 35 g/L and pH 6.0–8.0 and temperature of incubation ranging from 25 to 40 ± 0.1 °C set in rotary shaker [Orbitek, Scigenics Biotech. (Pvt.) Ltd., India] at 100 rev/min. Flasks were inoculated with the cultures to attain initial count of 10^3 CFU/mL. pH was monitored using narrow range pH paper (Merck, Mumbai), and adjustments were done aseptically using 1 M NaOH and 1 M HCl.

Phenazine Antibiotic Production Versus Antagonistic Activity

Aliquots (6 mL) were aseptically withdrawn from the flasks at 24-h interval, and cells were removed by centrifugation at $10,000 \times g$ for 15 min. The supernatant was filtered through cellulose acetate membrane (Millipore, India) having 0.22-μm porosity and used for determining antagonistic phenazine compound, *N*-methyl-1-hydroxyphenazine. The cell-free supernatant (5 mL) was extracted with 3 mL chloroform and re-extracted with 1 mL 0.2 M HCl. In acidic solution, the compound gives a pink to deep red coloration. This was measured spectrophotometrically (UV-1601, Shimadzu Corporation, Japan) at 520 nm, and the concentration of the antagonistic compound was determined as described by Essar et al. [5]. Antagonistic activity of the same (cell-free supernatant) was determined against the target pathogen, *Vibrio harveyi* MCCB 111, by standard disc diffusion assay as described below [9]. The pathogen grown on ZoBell's Marine Agar slants was harvested and resuspended in saline (3.0 % sodium chloride, w/v). The absorbance of cell suspension was adjusted to 1.5 at Abs_{600} and 500 μL swabbed on to ZoBell's Marine agar 2216 E plates. Absorbance of *V. harveyi* cell suspension used for seeding the plates was kept constant throughout the experiment. Aliquots of supernatant from the culture (20 μL) were spotted on filter paper discs of 6 mm diameter, placed on seeded plates. The inhibition zone was measured after 18-h incubation (at 28 °C) using a Hi Antibiotic Zone Scale (HiMedia, Mumbai) [9]. Colorimetric quantitative determination of *N*-methyl-1-hydroxyphenazine and its antagonistic activity from direct inhibition through disc diffusion method were performed from fifty samples, and their correlation was determined by regression analysis. A strong positive

correlation was obtained between the quantity of phenazine compound produced and its antagonistic activity, and hence, the latter was used in the subsequent analysis as the response.

Cell Biomass Output and Antagonistic Activity

Aliquots of 1 mL were aseptically withdrawn from the flasks at 24-h intervals and centrifuged at $10,000 \times g$ for 15 min. The pellets obtained after centrifugation were repeatedly washed in sterile saline (0.85 % NaCl) and resuspended. The absorbance (at 600 nm) of the above cell suspensions was determined spectrophotometrically (UV-1601, Shimadzu Corporation, Japan) and converted to cell dry mass (CDM) using a standard curve constructed of dry cell biomass versus absorbance, as described by Guerra and Pastrana [7]. Supernatants obtained after centrifugation were filter-sterilized through cellulose acetate membrane (Millipore, India) having 0.22-μm porosity and used for antagonism assay as described above. Correlation (r) was worked out between cell biomass output and antagonistic activity.

First Step Optimization

One-dimensional screening was done initially to determine the range that has to be used for further optimization. Different concentrations of sodium chloride such as 0, 5, 10, 15, 20, 25, 30 and 35 g/L, pH 6, 6.5, 7, 7.5 and 8, and temperature 25, 30, 35 and 40 ± 0.1 °C were screened, one at a time maintaining the other parameters constant in the medium composed of 0.5 % peptone, 0.1 % yeast extract and 0.01 % ferric phosphate in distilled water. Biomass and antagonistic activity were determined daily for a week, and maximum biomass and antagonistic activity obtained under each combination were considered and subjected for regression analysis for the optimum range of each parameter.

Second Step Optimization by RSM

Response surface methodology (RSM) is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously [23]. The most popular RSM design is the central composite design (CCD) and has been used here to predict the maximum biomass and antagonistic activity, at different combinations of sodium chloride concentration, pH and temperature. CCD has three groups of design points: two-level factorial or fractional factorial design points, axial points (sometimes called "star" points) and center points all designed to estimate the coefficients of

a quadratic model. The experiments were done using the software Design Expert (version 6.0.9, State Ease, Minneapolis, M N).

Results

Phenazine Antibiotic Production Versus Antagonistic Activity

A strong positive correlation ($r = 0.76$, $P < 0.0001$) existed between the quantity of N-methyl-1-hydroxyphenazine produced (μg/mL) and the antagonistic activity expressed in terms of inhibition zone. As per the regression analysis, 58 % variability in the phenazine production was attributable to the antagonistic activity, and hence, for further analysis, antagonistic activity was used as the response.

First Step Optimization

One-dimensional screening was performed to determine the optimum level of each parameter. Regression analysis was done for pH, temperature and sodium chloride concentrations using X for biomass and Y for antagonistic activity (Table 1). Wherever regression was found highly significant, the corresponding factor levels (5–20 g/L sodium chloride for both *Pseudomonas* MCCB 102 and 103, pH 6–8 for *Pseudomonas* MCCB 102 and 6–7 for *Pseudomonas* MCCB103 and temperature 25–35 °C for both the cultures) were taken for further analysis. Accordingly, the antagonistic activity was negligible at 30 g/L sodium chloride, and no production recorded at 35 g/L. A strong positive correlation ($r = 0.944$) existed between cell biomass and antagonistic activity.

Second Step Optimization by RSM

The coded values of the independent variables such as sodium chloride concentration (X_1), pH (X_2) and temperature (X_3) are given in Tables 2 and 3 along with the experimental and predicted values of biomass and antagonistic activity. The CCD matrices were analyzed by standard analysis of variance (ANOVA). ANOVA of the quadratic regression model demonstrated that the model was highly significant ($P < 0.0001$) for both biomass and antagonistic activity of *Pseudomonas* MCCB 102 and 103 (Table 4) (Supplementary Tables S.1, S.2, S. 3 and S. 4). The model F value was 123.14 for biomass and 80.24 for antagonistic activity in the case of *Pseudomonas* MCCB 102. Meanwhile, for *Pseudomonas* MCCB 103, it was 22.34 for biomass and 33.78 for antagonistic activity. This

Table 1 Factor levels used for regression analysis and their significance in the screening experiment of *Pseudomonas* MCCB 102 and 103

Sodium chloride (g/L)	Significance F		pH	Significance F		Temperature (°C)	Significance F	
	Pseudomonas MCCB 102	*Pseudomonas* MCCB 103		*Pseudomonas* MCCB 102	*Pseudomonas* MCCB 103		*Pseudomonas* MCCB 102	*Pseudomonas* MCCB 103
0	0.933	0.238	6	0.018	0.029	25	0.0058	0.022
5	0.007	0.049	6.5	0.131	0.025	30	0.033	0.021
10	0.017	0.042	7	0.007	0.011	35	0.036	0.004
15	0.012	0.023	7.5	0.078	0.0094	40	0.935	
20	0.025	0.022	8	0.153	0.0058	25	0.0058	
25	0.0681	0.558						

Table 2 Central composite design matrix of the three variables along with the experimental and predicted values of biomass and antagonistic activity of *Pseudomonas* MCCB 102

	Sodium chloride (g/L)	pH	Temperature (°C)	Biomass (mg/L)		Activity (diameter of inhibition zone in mm)	
				Experimental[a]	Predicted	Experimental[a]	Predicted
1	5	6	25	1,122	1,109	12.66	13.03
2	20	6	25	1,100	1,101	15.33	15.07
3	5	8	25	1,024	1,019	16.00	16.55
4	20	8	25	1,020	1,016	10.00	8.92
5	5	6	35	1,000	1,003	0.00	1.46
6	20	6	35	1,031	1,031	0.00	0.17
7	5	8	35	1,120	1,118	10.66	11.30
8	20	8	35	1,144	1,150	0.00	0.0025
9	0	7	30	980	987	10.66	9.03
10	25.11	7	30	1,013	1,005	0.00	1.08
11	12.5	5.32	30	1,092	1,093	12.66	12.01
12	12.5	8.68	30	1,120	1,118	15.00	15.12
13	12.5	7	21.59	1,100	1,109	15.83	16.27
14	12.5	7	38.41	1,141	1,133	0.00	0.96
15	12.5	7	30	1,160	1,160	15.33	16.33
16	12.5	7	30	1,162	1,160	17.22	16.33
17	12.5	7	30	1,161	1,160	16.00	16.33
18	12.5	7	30	1,174	1,160	16.66	16.33
19	12.5	7	30	1,163	1,160	16.66	16.33
20	12.5	7	30	1,155	1,160	16.00	16.33

[a] Average of three experiments

implied that the model was significant for both the isolates of *Pseudomonas*. In the case of biomass, A, B, C, A^2, B^2, C^2, AC and BC were significant model terms for *Pseudomonas* MCCB 102 and 103. When we consider antagonistic activity, A, B, C, A^2, B^2, C^2, AB, AC and BC were significant model terms for *Pseudomonas* MCCB 102, and A, B, C, A^2, C^2, AB and AC for *Pseudomonas* MCCB 103. Here "A" stands for sodium chloride concentration, "B" for pH, and "C" for temperature.

The goodness of fit of the model was checked by coefficient of determination (R^2). $R^2 = 0.9911$ for biomass and 0.9863 for antagonistic activity of *Pseudomonas* MCCB 102, and 0.9524 for biomass and 0.9682 for antagonistic activity of *Pseudomonas* MCCB 103. These can be expressed in percentages and interpreted as percent variabilities in responses in the given model. As per the model, variations of 99.11 % for biomass and 98.63 % for antagonistic activity of *Pseudomonas* MCCB 102 were attributed to the independent variables, and the model did not explain 0.89 and 1.37 % of the total variations. In the case of *Pseudomonas* MCCB 103, variations of 95.24 % for biomass and 96.82 % for antagonistic activity were attributed to the independent variables, and the model did not explain 4.76 and 3.18 % of the total variation. The lack of fit was insignificant for both biomass and antagonistic activity of both the organisms.

Table 3 Central composite design matrixes of the three variables along with the experimental and predicted values of biomass and antagonistic activity of *Pseudomonas* MCCB 103

	Sodium chloride (g/L)	pH	Temperature (°C)	Biomass (mg/L)		Activity (diameter of inhibition zone in mm)	
				Experimental[a]	Predicted	Experimental[a]	Predicted
1	5	6	25	1,030	1,013	12.67	12.13
2	20	6	25	941	919	14.00	15.35
3	5	7	25	1,144	1,130	14.20	14.67
4	20	7	25	1,052	1,036	10.00	10.88
5	5	6	35	980	986	10.50	10.37
6	20	6	35	990	992	8.67	8.95
7	5	7	35	921	933	10.33	9.72
8	20	7	35	933	939	0.00	1.29
9	0	6.5	30	1,082	1,081	13.67	14.53
10	25.11	6.5	30	1,000	1,010	12.00	10.10
11	12.5	5.66	30	891	904	17.67	17.46
12	12.5	7.34	30	962	957	14.00	13.15
13	12.5	6.5	21.59	1,004	1,033	10.33	9.41
14	12.5	6.5	38.41	952	928	0.00	−0.134
15	12.5	6.5	30	844	865	16.00	16.08
16	12.5	6.5	30	852	865	17.67	16.08
17	12.5	6.5	30	889	865	16.00	16.08
18	12.5	6.5	30	902	865	15.67	16.08
19	12.5	6.5	30	864	865	15.67	16.08
20	12.5	6.5	30	850	865	15.33	16.08

[a] Average of three experiments

Table 4 Analysis of variance (ANOVA) for the fitted quadratic polynomial models of biomass and antagonistic activity of *Pseudomonas* MCCB 102 and 103

Strain/parameter	Model							Lack of fit probability $P > F$
	CV	R^2	SS	DF	MS	F value	Probability $P > F$	
Pseudomonas MCCB 102								
Biomass	0.75 %	0.9911	0.076	9	0.0084	123.14	<0.0001	0.1777
Antagonistic activity	10.04 %	0.9863	853.95	9	94.88	80.24	<0.0001	0.0673
Pseudomonas MCCB 103								
Biomass	2.59 %	0.9524	0.12	9	0.014	22.24	<0.0001	0.1472
Antagonistic activity	9.91 %	0.9682	445.7	9	49.52	33.78	<0.0001	0.1099

SS sum of squares, *DF* degree of freedom, *MS* mean square, *CV* coefficient of variation

In the case of *Pseudomonas* MCCB 102, correlation coefficient (R) of biomass production was 0.9955 and of antagonistic activity 0.9931. An adequate precision was observed 29.639 for biomass and 22.77 for antagonistic activity. In the case of biomass production, the "Pred R-Squared" 0.9459 was in reasonable agreement with the "Adj R-Squared" 0.9830, and in the case of antagonistic activity, the "Pred R-Squared" 0.9117 was in reasonable agreement with the "Adj R-Squared" 0.9740.

Meanwhile, R for biomass of *Pseudomonas* MCCB 103 was 0.9759 and the same for antagonistic activity 0.984.

An adequate precision of 15.196 for biomass and 20.549 for antagonistic activity was observed. The "Pred R-Squared" 0.7799 for biomass was in reasonable agreement with the "Adj R-Squared" 0.9096, and the "Pred R-Squared" 0.8003 for antagonistic activity was in reasonable agreement with the "Adj R-Squared" 0.9395.

The RSM gave the following regression equations for biomass (Y_1) and antagonistic activity (Y_2) as functions of salinity (X_1), pH (X_2) and temperature (X_3).

Final equations in terms of coded factors are:
For *Pseudomonas* MCCB 102,

$$Biomass = +1159.94 + 6.26\,X_1 + 7.36\,X_2$$
$$+ 7.12X_3 - 58.60\,X_1^2 - 19.19\,X_2^2 - 13.88\,X_3^2$$
$$+ 1.25X_1X_2 + 8.75\,X_1X_3 + 51.25\,X_2X_3$$

$$(1)$$

$$Antagonistic\ activity = +16.33 - 2.31\,X_1 + 0.92\,X_2$$
$$- 5.12\,X_3 - 4.02\,X_1^2 - 0.98\,X_2^2$$
$$- 3.07\,X_3^2 - 2.42\,X_1X_2$$
$$- 0.92X_1X_3 + 1.58\,X2\,X_3$$

$$(2)$$

For *Pseudomonas* MCCB 103,

$$Biomass = +864.68 - 22.01\,X_1 + 15.94\,X_2 - 31.05\,X_3$$
$$+ 64.60\,X_1^2 + 23.30\,X_2^2 + 40.98\,X_3^2$$
$$+ 0.000\,X_1X_2 + 25\,X_1X_3 - 42.50\,X_2X_3$$

$$(3)$$

$$Antagonistic\ activity = +16.08 - 1.30\,X_1 - 1.28\,X_2$$
$$- 2.84\,X_3 - 1.34\,X_1^2 - 0.28\,X_2^2$$
$$- 4.05\,X_3^2 - 1.75\,X_1X_2$$
$$- 1.16\,X_1X_3 - 0.80\,X_2X_3$$

$$(4)$$

The two-dimensional contour plots and their respective response surface plots of significant interactions were constructed. The interactive effect of sodium chloride concentration and temperature at the optimum pH (6), pH and temperature at the optimum concentration of sodium chloride (12.2 g/L) on biomass of *Pseudomonas* MCCB 102 are presented in Fig. 1a, b. Similar plots (Fig. 2) on antagonistic activity of *Pseudomonas* MCCB 102 revealed the interactive effect of sodium chloride concentration and temperature (Fig. 2a) at the optimum pH (6.5), pH and temperature (Fig. 2b) at the optimum concentration of sodium chloride (12.9 g/L), and sodium chloride concentration and pH (Fig. 2c) at the optimum temperature (25 °C). Based on the regression equations (Eqs. 1 and 2), predicted optima for biomass production of *Pseudomonas* MCCB 102 were sodium chloride 12.2 g/L, pH 6 and temperature of incubation 25 °C, and those for antagonistic activity were sodium chloride 12.9 g/L, pH 6.5 and temperature of incubation 25 °C.

The contour plots and their respective response surface plots of biomass production generated with respect to *Pseudomonas* MCCB 103 revealed interaction between sodium chloride concentration and temperature of incubation (Fig. 3a) at the optimum pH (7), pH and temperature (Fig. 3b) at the optimum sodium chloride concentration (5 g/L). Fig. 4 shows similar plots on antagonistic activity of *Pseudomonas* MCCB 103 at varying concentrations of sodium chloride and temperature of incubation at the optimum pH (7). The predicted optima derived from the models (Eqs. 3 and 4) for biomass and antagonistic

activities of *Pseudomonas* MCCB 103 were sodium chloride 5 g/L, pH 7 and temperature of incubation 25 °C.

Experimental Verification of Biomass and Antagonistic Activity at the Predicted Optimum Conditions for *Pseudomonas* MCCB 102 and 103

The optimum sodium chloride concentration and pH of the medium and temperature of incubation, for biomass production and antagonistic activity of *Pseudomonas* MCCB 102 and 103, derived from the response surface plots and regression equations (Eqs. 1, 2, 3 and 4) were experimentally verified and found significant. In the case of *Pseudomonas* MCCB 102, the biomass was found increased by 8.4 % (1,070 ± 0.04–1,160 ± 0.01 mg/L dry weight) and antagonistic activity by 25.6 % (13.25 ± 1–16.64 ± 0.5 mm diameter of the halo zone). Meanwhile, biomass of *Pseudomonas* MCCB 103 could be increased by 13.26 % (1,150 ± 0.17–1,320 ± 0.06 mg/L dry weight) and antagonistic activity by 31.75 % (9.33 ± 0.58–13.67 ± 0.58 mm diameter of the halo zone).

Discussion

The environment-friendly disease management strategy through the application of antagonistic probiotics is widely accepted in aquaculture. However, to bring forth the probiotics to an application mode at commercial level, an appropriate bioprocess is imperative. The prime requirement for developing such a technology is optimization of medium composition and growth conditions [16]. In the present study, antagonistic activity of probiotics such as *Pseudomonas* MCCB 102 and 103 could be increased after optimization and was found to be growth associated. In the case of *Pseudomonas* MCCB 102 and 103, linear, quadratic and all interaction effects of temperature were significant for biomass production. The linear and quadratic effects of sodium chloride and pH were significant; however, the interactive effect between them was not significant. This indicated that temperature played an important role in biomass output compared with the other factors. Meanwhile, models for antagonistic activity of *Pseudomonas* MCCB 102 and 103 implied that the linear, quadratic and all interaction effects of sodium chloride, pH and temperature had significant effects on antagonistic activity, and they acted as limiting factors. It was observed that even minor variations could alter the extent of product formation. The importance of pH, temperature and sodium chloride concentration for enhanced biomass production and antagonistic activity has been observed in the case of the marine isolate *Pseudomonas* I-2 [4]. However,

Fig. 1 Contour plot of biomass of *Pseudomonas* MCCB 102. Interaction of **a** sodium chloride (g/L) and temperature (°C) at optimum pH, **b** pH and temperature (°C) at optimum sodium chloride concentration
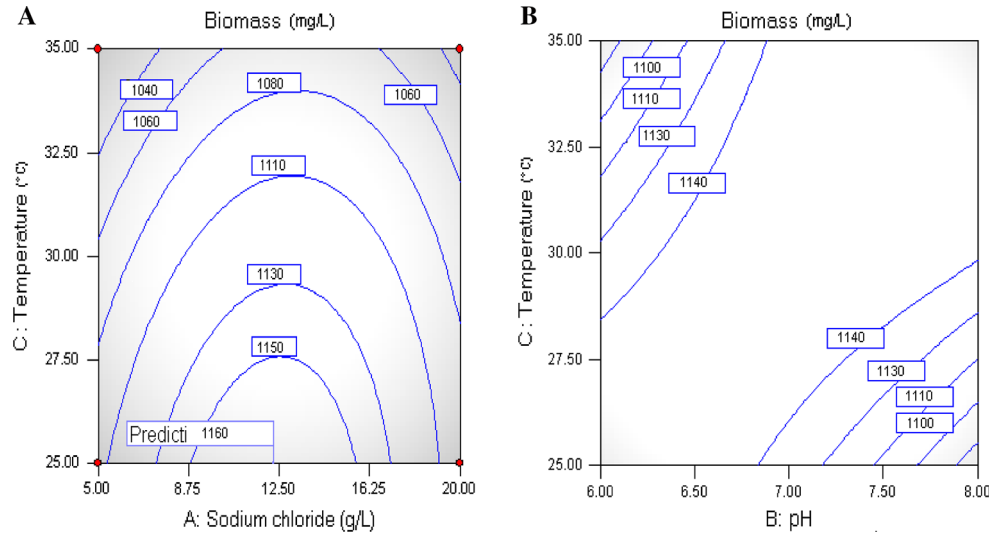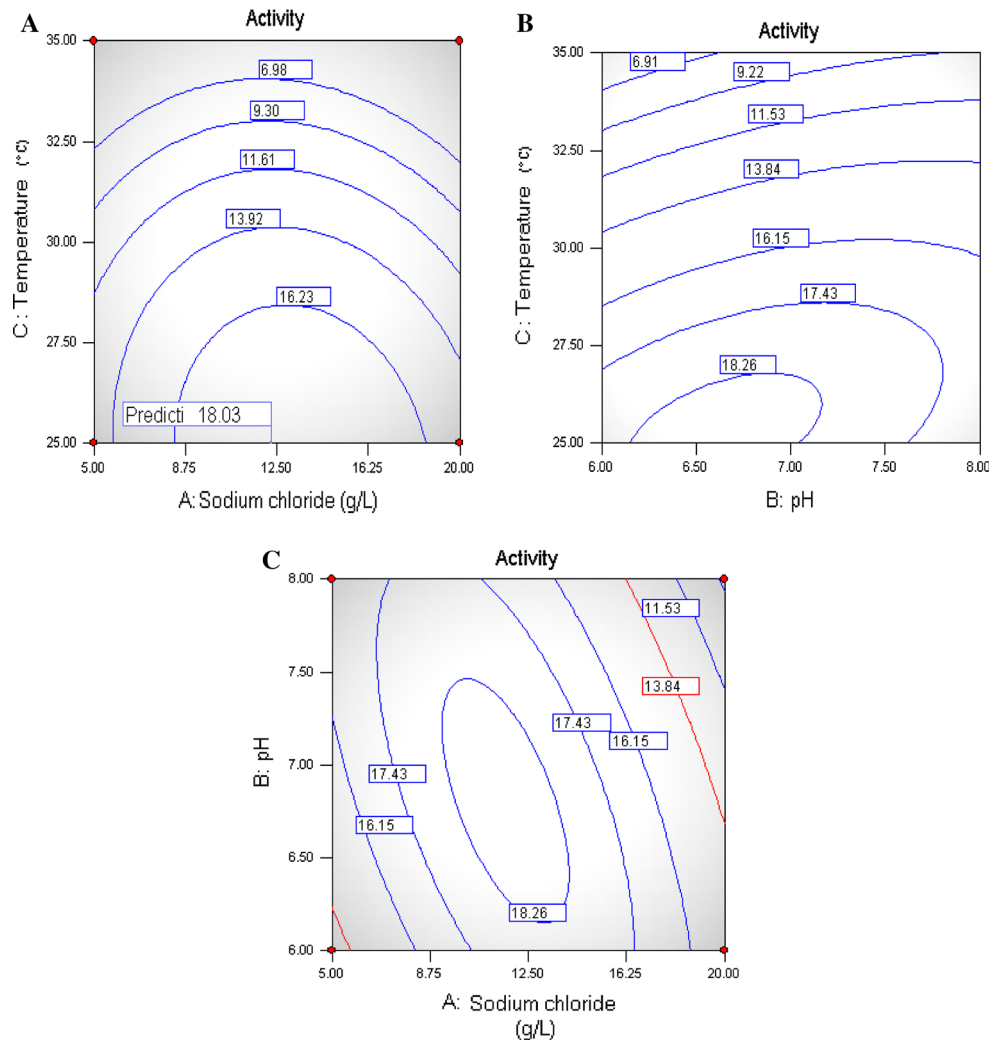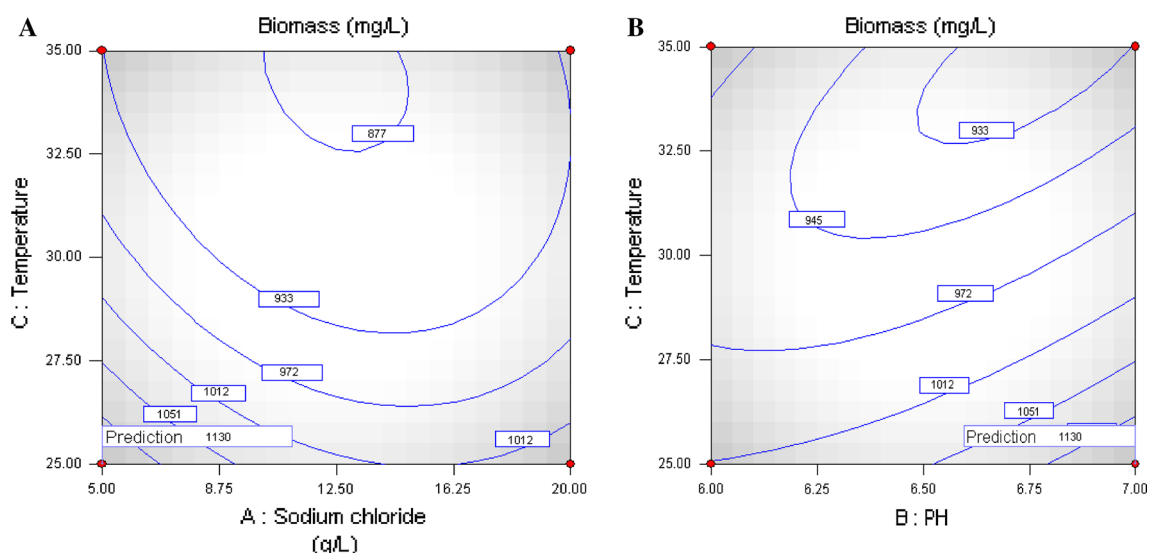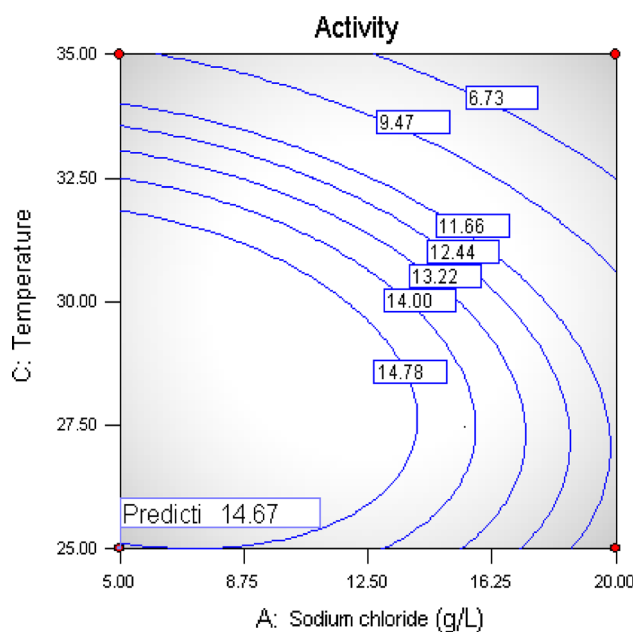

Fig. 2 Contour plot of antagonistic activity of *Pseudomonas* MCCB 102 (diameter of halo zone in mm). Interaction of **a** sodium chloride (g/L) and temperature (°C) at optimum pH, **b** pH and temperature (°C) at optimum sodium chloride concentration (g/L) and **c** sodium chloride and pH at optimum temperature

Fig. 3 Contour plot of biomass of *Pseudomonas* MCCB 103. Interaction of **a** sodium chloride (g/L) and temperature (°C) and **b** pH and temperature (°C)

Fig. 4 Contour plot of antagonistic activity of *Pseudomonas* MCCB 103 (diameter of the halozone in mm). Interaction of sodium chloride (g/L) and temperature (°C)

statistical optimization tools have not been used so far in optimizing aquaculture probiotics production. One of the important observations in the present study is the influence of sodium chloride on antagonistic activity. The optimum concentration varied with the isolate, 12.9 g/L for *Pseudomonas* MCCB 102 and 5 g/L for *Pseudomonas* MCCB 103. At the same time, antagonistic activity was negligible at 30 g/L sodium chloride and no production observed at 35 g/L. The optimum incubation temperature for biomass and antagonistic activity was the same (25 °C) for both the

isolates. Slightly acidic to neutral pH (6, 7) was of preference for optimum biomass production and antagonistic activity. Optimum requirements for biomass and antagonistic activity were more or less similar, and that might be due to the possible influence of biomass on the antagonistic activity [3].

Contour plots of models for biomass and antagonistic activity provided a method to visualize the relation between the response and experimental levels of each variable and the type of interactions between the test variables [19]. If the shape of the contour plot is circular, the interaction between the variables is negligible, and if it is elliptical, the interaction between the variables is significant [22]. In this study, the response surface plots of growth (biomass) showed similar pattern with that of antagonistic activity, indicating a strong correlation between the two [12]. Three-dimensional contour plots were avoided due to lack of clarity in the interaction of factors.

In conclusion, using central composite design of response surface methodology, it was possible to determine the optimum sodium chloride concentration and pH of the medium and temperature of incubation to enhance biomass production, and antagonistic activity of the aquaculture probiotics *Pseudomonas* MCCB 102 & 103. Subsequently, validity of the model was proven experimentally, and after optimization, antagonistic activity of *Pseudomonas* MCCB 102 and 103 could be increased by 25–31 %. As the antagonistic assay was based on disc diffusion, it had limitations in determining the extent of activity, as the antimicrobial compound might not diffuse beyond a particular distance. That could be cited as the reason for recording only 25–31 % increase in antagonistic activity in spite of optimization of culture conditions.

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