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ORIGINAL ARTICLE

Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic (*Pseudomonas* MCCB 103) using response surface methodology

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antagonism, aquaculture, central composite design, media optimization, probiotic, *Pseudomonas*, response surface methodology, *Vibrio*.

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Abstract

Aim: To develop a new medium for enhanced production of biomass of an aquaculture probiotic *Pseudomonas* MCCB 103 and its antagonistic phenazine compound, pyocyanin.

Methods and Results: Carbon and nitrogen sources and growth factors, such as amino acids and vitamins, were screened initially in a mineral medium for the biomass and antagonistic compound of *Pseudomonas* MCCB 103. The selected ingredients were further optimized using a full-factorial central composite design of the response surface methodology. The medium optimized as per the model for biomass contained mannitol (20 g l^{-1}), glycerol (20 g l^{-1}), sodium chloride (5 g l^{-1}), urea (3·3 g l^{-1}) and mineral salts solution (20 ml l^{-1}), and the one optimized for the antagonistic compound contained mannitol (2 g l^{-1}), glycerol (20 g l^{-1}), sodium chloride (5·1 g l^{-1}), urea (3·6 g l^{-1}) and mineral salts solution (20 ml l^{-1}). Subsequently, the model was validated experimentally with a biomass increase by 19% and fivefold increase of the antagonistic compound.

Conclusion: Significant increase in the biomass and antagonistic compound production could be obtained in the new media.

Significance and Impact of the Study: Media formulation and optimization are the primary steps involved in bioprocess technology, an attempt not made so far in the production of aquaculture probiotics.

Introduction

The concept of application of biological control agents such as nonpathogenic strains of antagonistic probiotics and/or their products in aquaculture has received widespread attention (Gomez-Gil *et al.* 2000; Verschuere *et al.* 2000). Among the organisms of choice, pseudomonads have been listed, as they are common inhabitants of the aquatic environment including shrimp ponds (Otta *et al.* 1999), and several strains have showed antagonistic activity against aquaculture pathogens. Under this category of investigations, *Pseudomonas fluorescens* has been reported to inhibit *Saprolegnia* sp. and *Aeromonas salmonicida* in finfish culture and reduced mortality in rainbow trout after challenging with *Vibrio anguillarum* (Smith and Davey 1993; Bly *et al.* 1997; Gram *et al.* 1999). Later, Chythanya *et al.* (2002)) reported *Pseudomonas* I-2, which antagonized shrimp pathogenic vibrios by means of a low molecular weight inhibitor. Jayaprakash (2005) isolated and identified a probiotic strain of *Pseudomonas* MCCB 103 antagonistic to a range of vibrios as possible probiotic in shrimp larval rearing facility. However, to facilitate its application in commercial hatchery systems, mass production of the probiotic was required, warranting immediate development of an appropriate bioprocess technology. The first step in any such process is to design a suitable medium with optimum carbon, nitrogen and other growth factors. The present work was therefore undertaken with the

objective of designing an appropriate medium for the mass production of *Pseudomonas* MCCB 103 by employing the statistical design, response surface methodology (RSM).

RSM is a combination of statistical and mathematical techniques useful for optimization of bioprocesses, and it can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in limited number of experiments. The methodology has been utilized successfully to optimize composition of microbiological media (He *et al.* 2004), improve fermentation processes (Liong and Shah 2005) and for developing new products (Rodrigues *et al.* 2006). In the present study, different carbon and nitrogen sources and growth factors were screened by conventional 'one-variable-at-a-time method' and further optimized statistically by a full-factorial central composite design (CCD) of the RSM.

Materials and methods

Bacterial strain

The organism used in this study was *Pseudomonas* MCCB 103, described previously by Jayaprakash (2005). This isolate formed part of the culture collection of the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kerala, India.

Inoculum preparation

Pseudomonas MCCB 103, grown at 28°C for 24 h on ZoBell's marine agar 2216E (Oppenheimer and ZoBell 1952; Hi-Media, Mumbai, India), was harvested into sterile saline (8·5 g l^{-1} sodium chloride) and used as inoculum.

Primary screening of nutrients and their effect on biomass and antagonistic compound production

Twenty-four carbon sources (glucose, sucrose, galactose, maltose, fructose, lactose, arabinose, cellobiose, mannose, ribose, trihalose, xylose, rhamnose, mannitol, sorbitol, starch, succinic acid, dextrin, glycerol, potassium sodium tartarate, pyruvic acid, sodium acetate, sodium citrate and sodium gluconate; Hi-Media, Mumbai, India) were screened as described by Oliver (1982) as sole source of carbon for growth. Seven carbon sources (glucose, glycerol, mannitol, pyruvic acid, sodium acetate, sodium citrate and sodium gluconate) from the above list were selected based on their influence as sole carbon source for further screening at 10 g l^{-1} level in mineral medium (Chang and Blackwood 1969) supplemented with 10 g l^{-1} sodium chloride. Concentration of sodium chloride was obtained from a previous study (data not given). The composition of the mineral medium was $MgCl_2 \cdot 6H_2O$, 4·1 g l^{-1}; Na_2SO_4, 7·1 g l^{-1}; K_2HPO_4, 0·1 g l^{-1} and urea, 2 g l^{-1}. Ammonium chloride, ammonium nitrate, ammonium sulfate, calcium nitrate, potassium nitrate, sodium nitrate and urea were screened as sole nitrogen source at 2 g l^{-1} level in the same mineral-based media with 10 g l^{-1} glucose and without nitrogen source. Twenty-four amino acids (DL-Ala, DL-2-amino-*n*-butyric acid, L-Arg-monohydrochloride, DL-Asp, L-Cys-hydrochloride, L-Cys, 3-(3,4-dihydroxyphenyl) DL-Ala, L-Glu, Gly, L-His-monohydochloride, L-hydroxy-Pro, L-Lue, L-Ile, DL-nor-Lue, L-Lys-monohydrochloride, L-Met, DL-Orn-monohydrochloride, L-Pro, DL-β-Phe, D-Ser, DL-Tyr, L-Trp, L-Tyr and DL-Val; Hi-Media) and casamino acid (BD Biosciences, Sparks, MD, USA) were screened as growth factors at 0·2 g l^{-1} level and vitamins such as ascorbic acid, biotin, cyanocobalamine, folic acid, inositol, pantothenic acid, riboflavin and thiamine (Hi-Media) at 0·02 g l^{-1} level in a pattern one-at-a-time for biomass and antagonistic activity by antagonism assay as given below. All incubations were carried out for 96 h at 28°C.

Shake flask experiment

Primary screening of nutrients (one-at-a-time) and the final optimization of selected ingredients were carried out in Erlenmeyer flasks (250 ml capacity) with 100 ml mineral medium without ferric sulfate (Chang and Blackwood 1969). Sugars, amino acids and vitamins were filter-sterilized using cellulose acetate membrane (Millipore, Bangalore, India) having 0·22 μm porosity and added to sterile mineral medium. After adding the nutrients, pH was adjusted to 7·0 by the addition of sterile 1 mol l^{-1} NaOH and 1 mol l^{-1} HCl, employing narrow range pH paper. All flasks, throughout the study, were inoculated with the culture to a final concentration equivalent to 0·01 at optical density A_{600nm} (10^3 CFU ml^{-1}) in 100 ml aliquots. Incubations were carried out in a temperature controlled rotary shaker [Orbitek; Scigenics Biotech. (Pvt.) Ltd, Chennai, India] at 120 rev min^{-1}.

Analysis of the sample

Samples (5 ml) were aseptically withdrawn from the flasks at 24-h interval. The cells were removed by centrifugation at 10 000 ***g*** for 15 min and the supernatant was filter-sterilized using cellulose acetate membrane having 0·22 μm porosity and used for the determination of the antagonistic phenazine compound, pyocyanin. The cell-free supernatant (5 ml) was extracted into 3 ml chloroform and then re-extracted into 1 ml of 0·2 mol l^{-1} HCl to give a pink to deep red colouration. This was measured spectrophotometrically (UV-1601; Shimadzu Corporation, Tokyo, Japan) at 520 nm and the concentration of the antagonistic compound determined as described by

Essar *et al.* (1990). The pellets were repeatedly washed in sterile saline (8·5 g l^{-1} sodium chloride), resuspended in fresh aliquots and at A_{600nm} determined spectrophotometrically and converted to dry cell mass using a standard curve constructed as described by Guerra and Pastrana (2002). Filter-sterilized supernatant was used for the antagonism assay against the target pathogen, *Vibrio harveyi* MCCB 111 following Jayaprakash *et al.* (2005).

Experimental design and optimization using response surface methodology

Carbon sources, nitrogen sources and growth factors for the production of the probiotic have been screened by conventional 'one-variable-at-a-time' approach (Elibol 2004). The minimum and maximum ranges of selected variables were investigated and a set of 50 experiments was carried out. The medium composition that resulted in the maximum biomass and antagonistic compound production was further optimized statistically by RSM using CCD. CCD has three groups of design points: two-level-factorial or fractional-factorial design points, axial points (sometimes called 'star' points) and centre points. CCDs are designed to estimate the coefficients of a quadratic model. The experiments were carried out using a software, Design Expert (version 6.0.9, Stat Ease; Minneapolis, MN, USA).

Results

Primary screening of nutrients

Variables such as mannitol, glycerol, urea and mineral salts solution were chosen for the study by a 'one-variable-at-a-time method', and the range of sodium chloride concentration was taken from the previous study (data not given). The minimum and maximum limits of the variables were mannitol: 2–20 g l^{-1}, glycerol: 2–20 g l^{-1}, sodium chloride: 5–15 g l^{-1}, urea: 0·1–4 g l^{-1} and mineral salts solution: 5–20 ml l^{-1}. Other growth factors did not have any effect on biomass and activity (see supplementary material).

Optimization of medium for enhanced biomass production and antagonistic activity

The most popularly used CCD of RSM was employed to maximize the biomass production and antagonistic activity. The interactive effect of nutritional factors on both biomass and activity was also investigated. The coded values of the independent variables (mannitol: *A*, glycerol: *B*, sodium chloride: *C*, urea: *D* and mineral salts solution: *E*) along with the experimental and predicted values of biomass and antagonistic activity are given in Table 1. The CCD matrix was analysed by standard analysis of variance (ANOVA). The ANOVA of the quadratic regression models demonstrated that the models were highly significant ($P < 0·0001$) for both biomass and activity. The model *F*-value was 390·48 for biomass and 27·99 for antagonistic compound production, which implied that the model was significant.

The 'lack-of-fit' value was insignificant for both biomass and activity and the goodness of fit of the model checked by coefficient of determination (R^2). R^2 was 0·9963 in the case of biomass and 0·9507 in the case of antagonistic compound. It could be expressed in percentage also, and it is interpreted as the percentage variability in the response in the given model. As per the model, sample variation of 99·63% for biomass and 95·07% for antagonistic compound was attributed to the independent variables and the model did not explain only 0·37% and 4·93%, respectively, of the total variation. A higher value of correlation coefficient (R) indicated an excellent correlation between independent variables. For biomass, *R*-value was 0·9981 and for antagonistic compound 0·9750. The purpose of statistical analysis was to determine which experimental factors generated signals, large in comparison to the noise. Adequate precision measures signal to noise ratio, and a ratio greater than four was desirable (Wang and Lu 2004). Accordingly an adequate precision was obtained, such as 90·378 and 21·306 for biomass and antagonistic compound, respectively. In the case of biomass, 'Pred R^2' 0·9845, was in reasonable agreement with the 'Adj R^2' 0·9937, and in the case of antagonistic compound production, the 'Pred R^2' 0·7944, was in reasonable agreement with the 'Adj R^2' 0·9168.

The RSM gave the following regression equations for the biomass and antagonistic compound as a function of *A*, *B*, *C*, *D* and mineral salts solution *E*.

Final equations in terms of coded factors are

$$\begin{aligned}\text{Biomass} = {} & +0·75 + 0·034A - 0·006B - 0·041C + 0·073D \\ & + 0·40E + 0·061A^2 + 0·013B^2 - 0·0062C^2 \\ & - 0·086D^2 + 0·030E^2 - 0·0068AB - 0·029AC \\ & + 0·0068AD + 0·016AE - 0·014BC - 0·005BD \\ & + 0·015BE - 0·006CD - 0·033CE - 0·043 \quad (1)\end{aligned}$$

$$\begin{aligned}\text{Antagonistic compound} = {} & +65·53 - 1·69A + 2·69B \\ & - 9·77C + 9·15D + 22·93E \\ & - 0·66A^2 - 2·81B^2 - 4·01C^2 \\ & - 3·74D^2 - 2·64E^2 - 0·82AB \\ & - 2·86AC + 3·00AD - 3·81AE \\ & - 1·99BC + 0·57BD + 1·49BE \\ & - 0·11CD - 2·71CE - 4·47DE \\ & \quad (2)\end{aligned}$$

Table 1 Central composite design matrix of the variables (g l^{-1}) along with the experimental and predicted values of biomass and antagonistic activity

						Biomass (cell dry mass, mg l^{-1})		Antagonistic compound (mg l^{-1})	
Experiment	Mannitol	Glycerol	Sodium chloride	Urea	Mineral salts solution	Experimental	Predicted	Experimental	Predicted
1	2	2	5	1	5	0·24	0·21	20·16	16·66
2	20	2	5	1	5	0·3	0·3	22·16	22·26
3	2	2	5	1	5	0·24	0·22	27·03	23·53
4	20	2	5	1	5	0·29	0·29	28·93	25·84
5	2	2	15	1	5	0·28	0·29	14·87	12·47
6	20	2	15	1	5	0·29	0·27	10·01	6·61
7	2	20	15	1	5	0·26	0·24	14·83	11·4
8	20	20	15	1	5	0·2	0·19	5·07	2·26
9	2	2	5	4	5	0·44	0·45	33·6	36·97
10	20	2	5	4	5	0·56	0·57	51·32	54·58
11	2	20	5	4	5	0·43	0·44	40·06	46·12
12	20	20	5	4	5	0·5	0·53	56·3	60·45
13	2	2	15	4	5	0·5	0·5	28·18	32·33
14	20	2	15	4	5	0·5	0·51	34·36	38·49
15	2	20	15	4	5	0·42	0·44	31·4	33·54
16	20	20	15	4	5	0·42	0·42	30·83	36·41
17	2	2	5	1	20	1·09	1·09	86·45	81·5
18	20	2	5	1	20	1·28	1·25	76·23	71·86
19	2	20	5	1	20	1·18	1·16	95·59	94·34
20	20	20	5	1	20	1·3	1·29	85·6	81·42
21	2	2	15	1	20	1·08	1·04	68·86	66·48
22	20	2	15	1	20	1·1	1·08	50·36	45·38
23	2	20	15	1	20	1·08	1·05	78·65	71·37
24	20	20	15	1	20	1·08	1·07	47·94	47
25	2	2	5	4	20	1·16	1·16	80·65	83·94
26	20	2	5	4	20	1·34	1·34	80·52	86·32
27	2	20	5	4	20	1·2	1·21	98·4	99·06
28	20	20	5	4	20	1·38	1·36	94·6	98·15
29	2	2	15	4	20	1·1	1·08	67	68·47
30	20	2	15	4	20	1·13	1·15	55·86	59·39
31	2	20	15	4	20	1·08	1·08	70·61	75·65
32	20	20	15	4	20	1·11	1·12	62·07	63·28
33	0	11	10	2·5	12·5	0·98	1·01	64·19	65·85
34	32·4	11	10	2·5	12·5	1·18	1·18	60·35	57·8
35	11	0	10	2·5	12·5	0·82	0·84	43·91	43·21
36	11	32·4	10	2·5	12·5	0·8	0·81	56·2	56·01
37	11	11	0	2·5	12·5	0·8	0·81	67·74	66·08
38	11	11	21·9	2·5	12·5	0·6	0·62	18·85	19·62
39	11	11	10	0	12·5	0	0·091	0	22·62
40	11	11	10	6·1	12·5	0·5	0·44	89·66	66·14
41	11	11	10	2·5	0	0	−0·02	0	−3·94
42	11	11	10	2·5	30·3	1·81	1·86	102·08	105·13
43	11	11	10	2·5	12·5	0·74	0·75	61·25	65·53
44	11	11	10	2·5	12·5	0·75	0·75	63·58	65·53
45	11	11	10	2·5	12·5	0·73	0·75	62·21	65·53
46	11	11	10	2·5	12·5	0·78	0·75	74·09	65·53
47	11	11	10	2·5	12·5	0·74	0·75	75·87	65·53
48	11	11	10	2·5	12·5	0·76	0·75	63·37	65·53
49	11	11	10	2·5	12·5	0·72	0·75	63·64	65·53
50	11	11	10	2·5	12·5	0·76	0·75	60·84	65·53

The *P*-values are used as a tool to test significance of each coefficient and the pattern of interaction between the coefficient of both biomass and activity. The smaller the *P* value, the more significant is the corresponding coefficient (Rao *et al.* 2000). Linear coefficients such as *A*, *C*, *D* and *E*, quadratic coefficients A^2, B^2, D^2 and E^2 and interaction coefficients such as *AC*, *AE*, *BC*, *BE*, *CE* and *DE* were highly significant for biomass. In the case of antagonistic compound production, linear coefficients *B*, *C*, *D* and *E*, quadratic coefficients B^2, C^2, D^2 and E^2 and interaction coefficients such as *AC*, *AD*, *AE* and *DE* were the significant model terms. As it was a hierarchical model, the insignificant coefficients were notomitted from the eqns (1) and (2) (Wang and Lu 2004).

The response surface plots of the significant interactions of nutrients are given in Figs 1 and 2. Effect of interaction of varying concentrations of mannitol and sodium chloride, mannitol and mineral salts solution, glycerol and sodium chloride, glycerol and mineral salts solution, sodium chloride and mineral salts solution, urea and mineral salts solution on biomass production when all other parameters were at optimum is presented in (Fig. 1a–f). Based on the regression equation [eqn (1)], the optimum concentration of the ingredients of the medium for biomass production were mannitol, 20 g l^{-1}; glycerol, 20 g l^{-1}; sodium chloride, 5 g l^{-1}; urea, 3·3 g l^{-1} and mineral salts solution, 20 ml l^{-1}.

The interaction between nutrients and their effect on antagonistic compound production were also studied (Fig. 2). Effect of interaction of varying concentrations of ingredients, as listed above, on antagonistic compound production when all other parameters kept optimum is presented in (Fig. 2a–d). The optimum concentrations of the ingredients of the medium for antagonistic compound production from the regression equation [eqn (2)] were mannitol, 2 g l^{-1}; glycerol, 20 g l^{-1}; sodium chloride, 5·1 g l^{-1}; urea, 3·6 g l^{-1} and mineral salts solution, 20 ml l^{-1}.

Validation of the model

The validation was carried out in shake flasks under optimum conditions of the media predicted by the model. The experimental values for biomass (1·44 ± 0·05 g l^{-1}) and antagonistic compound (101·37 ± 2·76 mg l^{-1}) were closer to the predicted values (biomass: 1·4 g l^{-1} and antagonistic compound: 100·32 mg l^{-1}), validating the model. Moreover, at the above concentrations, biomass could be increased by 19% (1·21 ± 0·04 to 1·44 ± 0·05 g l^{-1}) with fivefold increase in antagonistic compound production (20 ± 2·23 to 101·37 ± 2·76 mg l^{-1}).

Discussion

In general, no defined medium has yet been established for mass production of any antagonistic probiotic used in aquaculture. As a general rule, each organism has its own requirement for maximum biomass and antagonistic compound production (Elibol 2004). This requirement necessitated the present study to optimize carbon and nitrogen sources and growth factors for *Pseudomonas* MCCB 103 so that a commercial production process could be evolved. Accordingly, new media were designed for both biomass and antagonistic compound production based on a full-factorial CCD of RSM. Composition of the medium obtained from the model for biomass production contained mannitol, 20 g l^{-1}; glycerol, 20 g l^{-1}; sodium chloride, 5 g l^{-1}; urea, 3·3 g l^{-1} and mineral salts solution, 20 ml l^{-1}, and the one for antagonistic compound production contained mannitol, 2 g l^{-1}; glycerol, 20 g l^{-1}; sodium chloride, 5·1 g l^{-1}; urea, 3·6 g l^{-1} and mineral salts solution, 20 ml l^{-1}. In the medium for biomass production, the linear and quadratic effects of mannitol, urea and mineral salts solution were more significant than the other factors. This suggested that mannitol, urea and mineral salts solution had direct relationship with biomass production. Liong and Shah (2005) reported better growth of a probiotic strain of *Lactobacillus* in the presence of mannitol and subsequent increase for cholesterol removal. Sodium chloride showed a linear effect on biomass production and this observation was in accordance with the previous study (Vijayan *et al.* 2006). Although the linear coefficient of glycerol in the medium for biomass production was not significant, it showed significant quadratic effect and interaction effect on sodium chloride concentration and mineral salts solution. This justifies inclusion of glycerol in the medium for biomass production.

In the medium designed for the antagonistic phenazine compound (pyocyanin) production, ingredients such as sodium chloride, urea and mineral salts solution were significant at their linear and quadratic levels. This implies that they can act as limiting factors and minor variations in their concentration may alter the rate of product formation. In the case of carbon sources, glycerol showed significant effect (both linear and quadratic) on the antagonistic compound production. Although mannitol failed to show any direct effect on the antagonistic compound production, its interaction effect with urea and mineral salts solution was significant. Moreover, it played an important role in biomass production and thereby enhanced antagonistic secondary metabolite production, as the latter was growth dependent (Marwick *et al.* 1999). These observations justified the inclusion of mannitol in the medium for the antagonistic compound production.

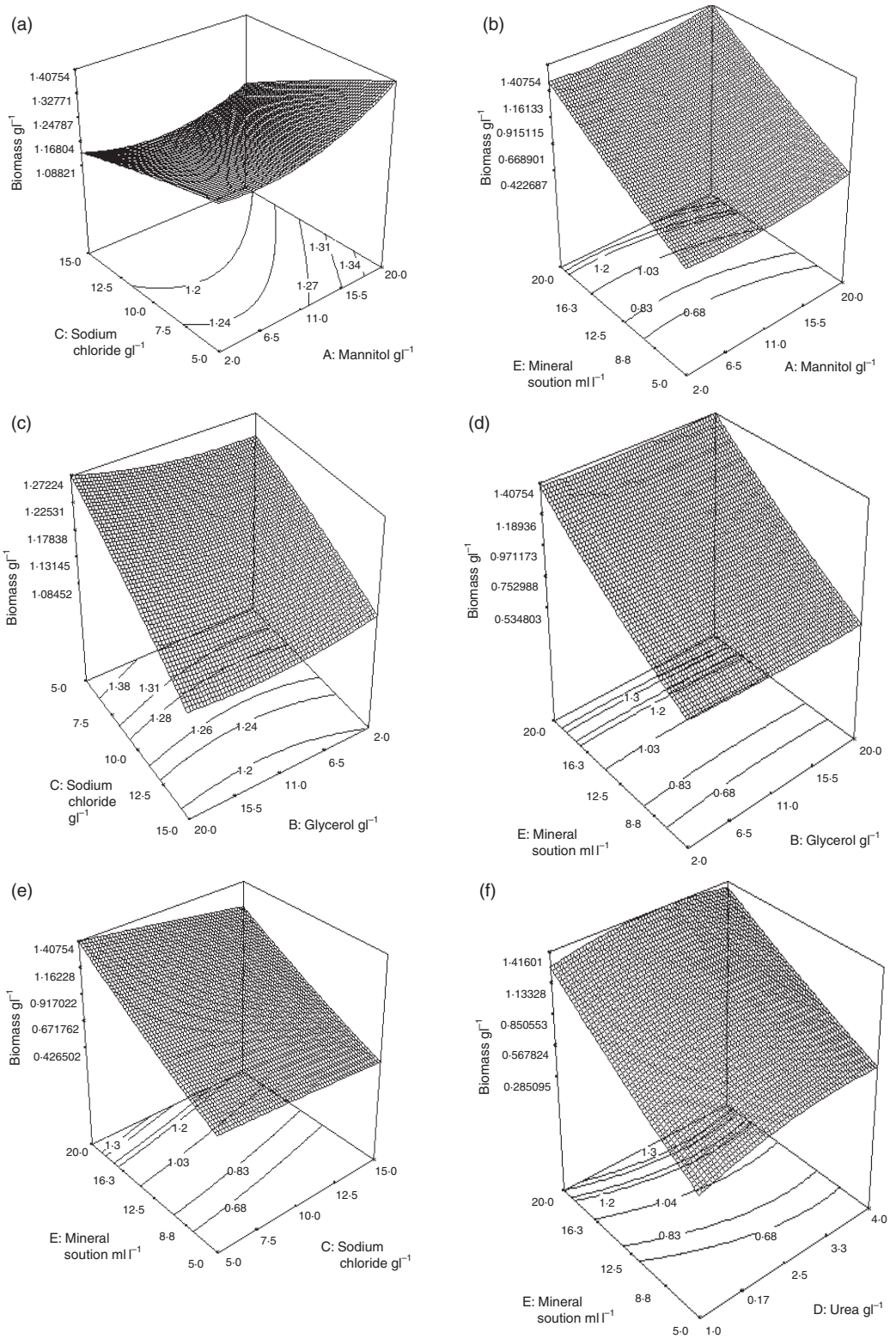

Figure 1 Interaction of nutrients on biomass production (g l^{-1}) of aquaculture probiotic *Pseudomonas* MCCB 103. Interaction of (a) mannitol and sodium chloride (b) mannitol and mineral salts solution (c) glycerol and sodium chloride (d) glycerol and mineral salts solution (e) sodium chloride and mineral salts solution (f) urea and mineral salts solution, when other factors are kept at their optimum.

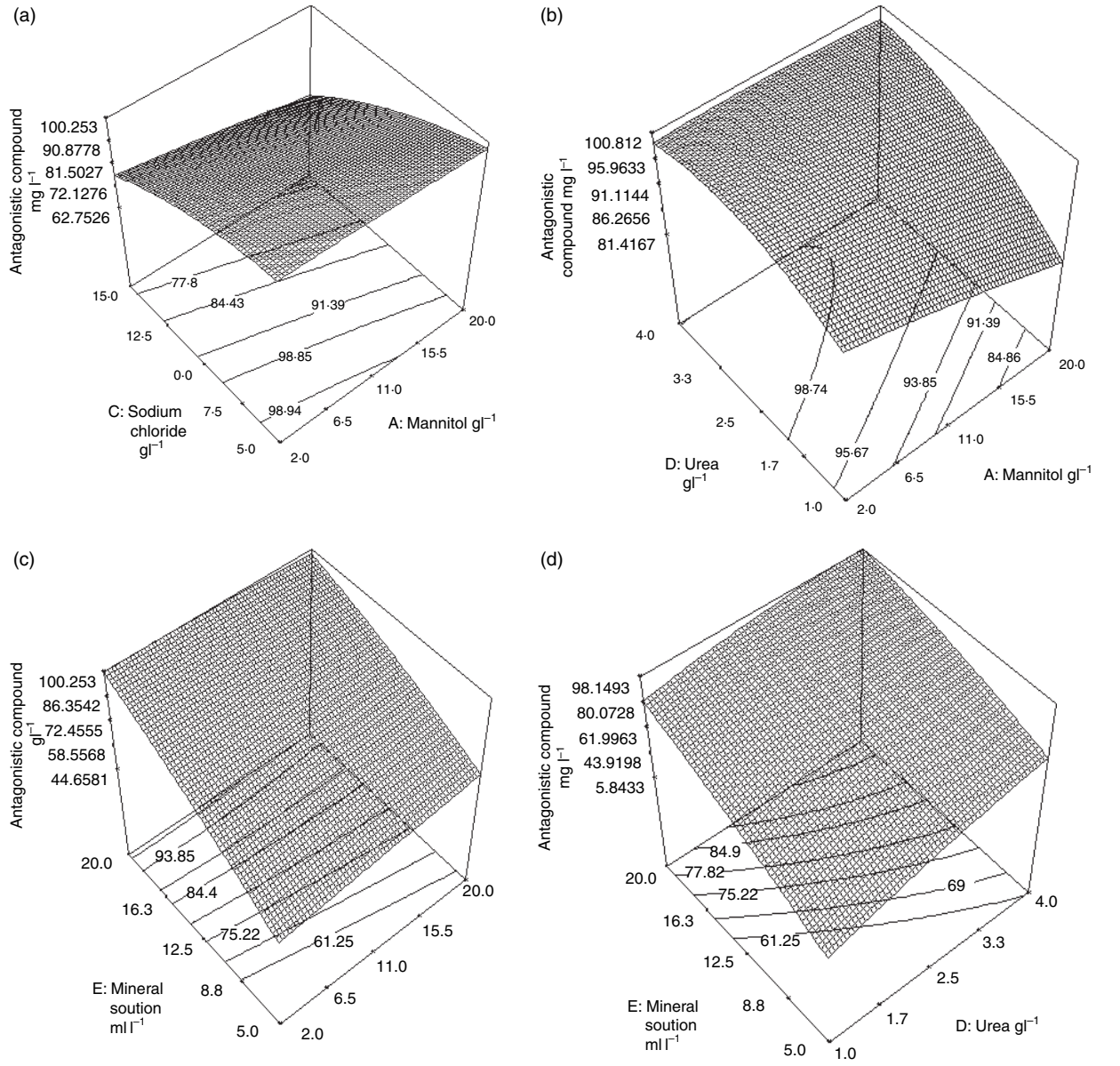

Figure 2 Interaction of nutrients on antagonistic compound production (mg l^{-1}) of aquaculture probiotic *Pseudomonas* MCCB 103. Interaction of (a) mannitol and sodium chloride (b) mannitol and urea (c) mannitol and mineral salts solution (d) urea and mineral salts solution, when other factors are kept at their optimum.

Both biomass and antagonistic compound production of *Pseudomonas* MCCB 103 was maximum at low concentration of sodium chloride (5 g l^{-1}), exhibiting a negative correlation. This observation was strengthened by the argument of Vijayan *et al.* (2006), who reported a brackish water isolate of *Pseudomonas* PS-102, showing maximum antagonistic activity in ZoBell's marine broth with 5 g l^{-1} sodium chloride.

Response surface plots are the graphical representations of regression equations (Wang and Lu 2004). Accordingly, response surface plots of the above models provided a method to visualize the interaction of nutrients and the optimum concentration of each nutrient required for maximum biomass and antagonistic compound production. In the present study, response surface of biomass showed similar pattern with the response surface of antagonistic compound indicating a strong correlation between the biomass and antagonistic compound production (Liong and Shah 2005).

In conclusion, the newly designed media for the aquaculture probiotic, *Pseudomonas* MCCB 103 provided 19% increase in its biomass and fivefold increase in the antag-

onistic phenazine compound pyocyanin production. In addition, validation of the model suggested, unequivocally, the reliability of RSM for optimization of media for antagonistic probiotics in aquaculture.

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Supplementary material

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Table S1 Carbon utilization test of *Pseudomonas* MCCB 103

Table S2 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Table S3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic compound production

Table S4 Original ANOVA table for biomass

Table S5 Original ANOVA table for antagonistic compound (pyocyanin)

Figure S1. Screening of mineral media for biomass production of *Pseudomonas* MCCB 103.

Figure S2. Antagonistic activity in medium III (Chang and Blackwood 1969).

Figure S3. Screening of selected carbon sources for biomass production of *Pseudomonas* MCCB 103.

Figure S4. Screening of selected carbon sources for antagonistic compound production of *Pseudomonas* MCCB 103.

Figure S5. Screening of nitrogen sources for biomass production of *Pseudomonas* MCCB 103.

Figure S6. Screening of nitrogen sources for antagonistic compound production of *Pseudomonas* MCCB 103.

Figure S7. Screening of sodium chloride for biomass (a) and antagonistic activity (b).

Figure S8. Screening of amino acids as growth factors for biomass production of *Pseudomonas* MCCB 103.

Figure S9. Screening of amino acids as growth factors for antagonistic compound production of *Pseudomonas* MCCB 103.

Figure S10. Screening of vitamins as growth factors for biomass production of *Pseudomonas* MCCB 103.

Figure S11. Screening of vitamins as growth factors for antagonistic compound production of *Pseudomonas* MCCB 103.

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