Optimization of Medium for the Production of a Novel Aquaculture Probiotic, *Micrococcus* MCCB 104 Using Central Composite Design

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Abstract A marine isolate of *Micrococcus* MCCB 104 has been identified as an aquaculture probiotic antagonistic to *Vibrio*. In the present study different carbon and nitrogen sources and growth factors in a mineral base medium were optimized for enhanced biomass production and antagonistic activity against the target pathogen, *Vibrio harveyi*, following response surface methodology (RSM). Accordingly the minimum and maximum limits of the selected variables were determined and a set of fifty experiments programmed employing central composite design (CCD) of RSM for the final optimization. The response surface plots of biomass showed similar pattern with that of antagonistic activity, which indicated a strong correlation between the biomass and antagonism. The optimum concentration of the carbon sources, nitrogen sources, and growth factors for both biomass and antagonistic activity were glucose (17.4 g/L), lactose (17 g/L), sodium chloride (16.9 g/L), ammonium chloride (3.3 g/L), and mineral salts solution (18.3 mL/L). © KSBB

Keywords: probiotic, *antagonism*, Micrococcus, *response surface methodology*, *central composite design*, *aquaculture*

INTRODUCTION

Vibriosis has been recognized as the major systemic bacterial disease of shrimp and prawn larvae in hatcheries [1-3]. Administration of antibiotics, although recognized as one of the management measures, leads to multiple antibiotic resistance among aquaculture pathogens [1,4,5] and the same is likely to be transferred to human pathogens too. Therefore, as an alternative strategy in the management of vibrios, application of biological control agents, especially antagonistic probiotics has been suggested [6,7]. *Micrococcus* MCCB 104 isolated and identified in our previous work [8] as a novel antagonistic probiotic suitable for tropical culture systems was found to inhibit a range of vibrios. As the next step, optimization of carbon, nitrogen and other growth factors were to be accomplished for enhanced production of biomass and antagonistic activity under aerated conditions as the organism was a strict aerobe. This work was undertaken accordingly with the above objective by employing response surface methodology (RSM).

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. It is used for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments and their by it can improve product yield and reduce process variability, time, cost *etc*. This technique has been used for designing media for the production bacteriocins [9], enzymes [10], antibiotics [11], polysaccharides [12], and organic acids [13].

This paper deals with the outcome of an attempt to optimize carbon, nitrogen, and growth factors in a mineral based

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medium M-9 [14] for both biomass production and antagonistic activity employing central composite design of RSM.

MATERIALS AND METHODS

Bacterial Strain and Inoculum Preparation

The organism (*Micrococcus* MCCB 104), used in this study was previously described by Jayaprakash *et al*. [9] and formed part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kerala, India. The organism was grown on ZoBell's marine agar 2216E (HiMedia, India), harvested in sterile saline (0.85% NaCl) and used as the inoculum.

Screening and Selection of Nutrients

Different C-sources (glucose, sucrose, galactose, maltose, fructose, lactose, arabinose, cellobiose, mannose, ribose, trihalose, xylose, rhamnose, manitol, sorbitol, starch, succinic acid, dextrin, glycerol, potassium sodium tarterate, pyruvic acid, sodium acetate, sodium citrate, and sodium gluconate; HiMedia, India) were screened as described by Oliver [15] as the sole source of carbon based on biomass production and antagonistic activity. Among them five carbon sources such as glucose, fructose, lactose, maltose, and sucrose were selected for further screening (for both biomass production and antagonistic activity) at 1% (w/v) level in M-9 mineral medium [Na_2HPO_4: 0.68% (w/v), KH_2PO_4: 0.3% (w/v), NH_4Cl: 0.1% (w/v), $MgSO_4 \cdot 7H_2O$: 0.049% (w/v), $CaCl_2$: 0.001% (w/v)] supplemented with 2% (w/v) NaCl. Ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate, sodium nitrate, and urea were screened as sole N-source at 0.2% level in the same medium 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 (HiMedia, India) and Casamino acid (BD Biosciences, USA)] were screened as growth factors at 0.02% level and vitamins such as ascorbic acid, biotin, cyanocobalamine, folic acid, inositol, pantothenic acid, riboflavin, and thiamine (HiMedia, India) at 0.002% level in a pattern of one at a time for biomass and antagonistic activity. All incubations were done at 28°C at pH 7.0 for 96 h.

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 (M-9). Sugars, amino acids, and vitamins were filter sterilized using cellulose acetate membrane (Sartorius, India) having 0.22 μm porosity and added to sterile mineral medium. Subsequently pH was adjusted to 7.0 by using sterile 1 M NaOH and 1 M HCl, employing narrow range pH paper (Qualigens, India). All flasks throughout the study were inoculated with the culture to a final concentration equivalent to optical density (OD) 0.01 at A_{600} (10^3 CFU/mL) in 100 mL aliquots. Incubations were done at 28°C in a temperature controlled rotary shaker at 120 rev/min. NaCl content (2%, w/v), pH (7.0), and temperature of incubation (28°C) employed here were the ones optimized following RSM using ZoBell's broth (data not shown).

Analysis of the Sample

The broth cultures (1 mL) were aseptically withdrawn from the flasks at 24 h intervals. The cells were removed by centrifugation at 10,000 × g for 15 min and the supernatant adjusted to pH 7.0 using 1 M HCl, filter sterilized using cellulose acetate membrane (Sartorius) having 0.22 μm porosity and used for antagonism assay towards *Vibrio harveyi*. *V. harveyi* grown on ZoBell's Marine Agar slants were harvested in saline and OD adjusted to 1.5 at A_{600} and 500 μL was plated on ZoBell's Marine agar 2216 E plates. Optical density and volume of cell suspension used for seeding the plates were kept constant throughout the experiment. Supernatant from the *Micrococcus* MCCB 104 culture was spotted in 20 μL aliquots and activity measured after 18 h of incubation (at 28°C) by measuring the inhibition zone as described earlier [9]. The pellets were repeatedly washed in sterile saline (0.8% NaCl) and OD at $A_{600\,nm}$ was determined spectrophotometrically (UV-1601, Shimadzu, Japan) from which the dry cell mass was determined based on a plot of OD versus dry cell mass as described by Guerra and Pastrana [16].

Experimental Design and Optimization

The medium for the production of probiotic was first optimized by 'one-variable-at-a-time' approach. The minimum and maximum limits of the variables were determined and a set of fifty experiments was programmed employing response surface methodology (RSM). Central composite design (CCD) of the RSM was used for the final optimization experiment. CCD has three groups of design points: two-level factorial or fractional factorial design points, axial points (some times called "star" points), and centre points. CCD's are designed to estimate the coefficients of a quadratic model. The experiments were done using a software Design Expert version 6.0.9 (StatEase, USA). Finally validation was carried out in shake flasks under conditions predicted by the model.

RESULTS AND DISCUSSION

Micrococcus spp. has been isolated from various environments and some strains produce bacteriocins, which display broad spectrum of inhibitory activity against variety of pathogenic and nonpathogenic microorganisms [17,18]. In our previous paper [9], we reported that *Micrococcus* MCCB

Table 1. Central composite design matrix of the variables (g/L) along with the experimental (n = 3) and predicted values of biomass and antagonistic activity

Expt. No.	Glucose	Lactose	Sodium chloride	Ammonium chloride	Mineral salts solution	Biomass (g/L)		Activity (Diameter of inhibition zone in mm)	
						Experimental	Predicted	Experimental	Predicted
1	2	2	15	0.4	5	0.6	0.56	12.33	10.75
2	20	2	15	0.4	5	0.8	0.91	15.33	15.29
3	2	20	15	0.4	5	0.56	0.64	10.67	11.79
4	20	20	15	0.4	5	0.5	0.40	10.88	7.928
5	2	2	25	0.4	5	0.5	0.44	11	8.357
6	20	2	25	0.4	5	0.8	0.89	15	15.63
7	2	20	25	0.4	5	0.84	0.78	16	14.51
8	20	20	25	0.4	5	0.7	0.65	14.67	13.38
9	2	2	15	4	5	0.6	0.51	12	10.39
10	20	2	15	4	5	1.12	1.14	16.33	17.43
11	2	20	15	4	5	0.88	0.84	14.33	13.98
12	20	20	15	4	5	1	0.89	15	12.62
13	2	2	25	4	5	0	-0.06	0	1.63
14	20	2	25	4	5	0.8	0.67	14.24	11.39
15	2	20	25	4	5	0.5	0.54	10.33	10.33
16	20	20	25	4	5	0.8	0.69	13.67	11.69
17	2	2	15	0.4	20	0	0.07	0	2.64
18	20	2	15	0.4	20	0.5	0.53	10	9.75
19	2	20	15	0.4	20	0.52	0.67	12	12.43
20	20	20	15	0.4	20	0.6	0.55	13	11.13
21	2	2	25	0.4	20	0	0.04	0	1.11
22	20	2	25	0.4	20	0.6	0.61	12	10.95
23	2	20	25	0.4	20	0.9	0.91	16.33	16.01
24	20	20	25	0.4	20	0.8	0.89	16.2	17.45
25	2	2	15	4	20	0.68	0.67	12.42	11.46
26	20	2	15	4	20	1.4	1.42	20	21.07
27	2	20	15	4	20	1.6	1.53	22.67	23.79
28	20	20	15	4	20	1.61	1.69	23.68	24.99
29	2	2	25	4	20	0	0.20	0	3.56
30	20	2	25	4	20	1.2	1.05	17.2	15.9
31	2	20	25	4	20	1.57	1.33	23.33	21.01
32	20	20	25	4	20	1.52	1.59	23.67	24.94
33	0	11	20	2.2	12.5	0.5	0.51	10	8.75
34	32.4	11	20	2.2	12.5	1.2	1.24	16	18.83
35	11	0	20	2.2	12.5	0.8	0.75	13	12.13
36	11	32.4	20	2.2	12.5	1.4	1.49	21.67	24.12
37	11	11	81	2.2	12.5	0.8	0.75	14	14.24
38	11	11	31.9	2.2	12.5	0.4	0.49	10	11.33
39	11	11	20	0	12.5	0	-0.16	0	1.54
40	11	11	20	6.5	12.5	0.4	0.61	10	10.03
41	11	11	20	2.2	0	0	0.18	0	5.07
42	11	11	20	2.2	30.3	0.8	0.66	14.67	11.18
43	11	11	20	2.2	12.5	1.46	1.57	23.33	22.55
44	11	11	20	2.2	12.5	1.62	1.57	22.33	22.55
45	11	11	20	2.2	12.5	1.45	1.57	23.33	22.55
46	11	11	20	2.2	12.5	1.6	1.57	23.67	22.55
47	11	11	20	2.2	12.5	1.56	1.57	23.67	22.55
48	11	11	20	2.2	12.5	1.6	1.57	23	22.55
49	11	11	20	2.2	12.5	1.67	1.57	20	22.55
50	11	11	20	2.2	12.5	1.6	1.57	20	22.55

Table 2. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	*F*-value	Probability *P* > F
Model	12.63	20	0.63	39.4	< 0.0001
Residual (error)	0.465	29	0.016		
Lack of fit	0.423	22	0.019	3.220	0.0586
Pure error	0.042	7	0.006		
Cor total	13.097	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV = 14.6%, R^2 = 0.9645, R = 0.9821.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of antagonistic activity

Source	SS	DF	MS	*F*-value	Probability *P* > F
Model	2201.366	20	110.068	19.404	< 0.0001
Residual (error)	164.5	29	5.672		
Lack of fit	147.66	22	6.712	2.790	0.0834
Pure error	16.838	7	2.406		
Cor total	2365.87	49			

SS, Sum of squares; DF, degree of freedom; MS, mean square, CV = 16.7%, R^2 = 0.9305, R = 0.9646.

104, isolated from shrimp hatchery sea water inhibited a wide range of pathogenic *Vibrio* and *Aeromonas* spp. and suggested it as novel 'antagonistic probiotic' for shrimp larval rearing systems. Antagonistic compound production in general is affected by composition of medium and therefore medium optimization has been found to be important for the enhancement of bacteriocin production [19]. Accordingly in the present study primary screening of nutrients was done by conventional screening method and the final optimization was conducted by means of central composite design of RSM.

Screening and Selection of Nutrients

Variables such as glucose, lactose, ammonium chloride, and mineral salts solution were chosen for the study by 'one variable-at-a-time method' [20], and the range of sodium chloride concentration was taken from the previous study (data not shown). The minimum and maximum limits of the variables were, glucose: 0.2~2%, lactose: 0.2~2%, sodium chloride: 1.5~2.5%, ammonium chloride: 0.04~0.4%, and mineral salts solution: 0.5~2% (v/v). Other growth factors did not have any effect on biomass and activity.

Medium Optimization and Empirical Modeling

Central composite design matrix of the variables (glucose: A, lactose: B, sodium chloride: C, ammonium chloride: D, and mineral salts solution: E) along with the experimental (n = 3) and predicted values of biomass and antagonistic activity are given in Table 1. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the model was highly significant ($p < 0.0001$) for both biomass and activity (Tables 2 and 3). The model *F*-value was 39.4 for biomass and 19.4 for antagonistic activity.

The 'lack of fit value' was insignificant for both biomass and activity and the goodness of fit of the model was checked by coefficient of determination (R^2). R^2 was equal to 0.9645 in the case of biomass and 0.9305 in the case of activity. It can be expressed in percentage also and interpreted as the percent variability in the response in the given model. As per the model, sample variation of 96.45% for biomass and 93.05% for antagonistic activity was attributed to the independent variables and the model did not explain only 3.55 and 6.95%, respectively, of the total variation. For biomass, correlation coefficient (R) was equal to 0.9821 and for activity 0.9646. A higher value of correlation coefficient (R) indicated a good agreement between experimental and predicted values of both biomass and antagonistic activity and thus suggesting a high significance of the model [21]. Adequate precision measures signal to noise ratio and, a ratio grater than 4 is desirable [12]. An adequate precision was obtained, 22.608 and 15.471 for biomass and activity respectively. In the case of biomass "Pred R-Squared" 0.8687 was in reasonable agreement with the "Adj R-Squared" of 0.94 and in the case of activity the "Pred R-Squared" of 0.7383 was in reasonable agreement with the "Adj R-Squared" of 0.8825.

The RSM gave the following regression equations for the biomass and antagonistic activity as a function of glucose (A), lactose (B), sodium chloride (C), ammonium chloride (D), and mineral salts solution (E).

Final equations in terms of coded factors are:

$$\text{Biomass} = +1.57+0.15A+0.16B-0.055C+0.16D+0.10E-0.12A^2-0.080B^2-0.17C^2-0.24D^2-0.20E^2-0.15AB+0.026AC+0.070AD+0.029AE+0.067BC+0.064BD+0.13BE-0.11CD+0.025CE+0.16DE \quad (1)$$

$$\text{Activity} = +22.55+2.12A+2.52B-0.61C+1.78D+1.28E-1.55A^2-0.78B^2-1.72C^2-2.96D^2-2.55E^2-2.10AB+0.68AC+0.62AD+0.64AE+1.28BC+0.64BD+2.19BE-1.59CD+0.22CE+2.29DE \quad (2)$$

Linear coefficients such as A (F-ratio 63.98 for biomass and 34.25 for activity), B (F-ratio 65.18 for biomass and 48.54 for activity), D (F-ratio 70.81 for biomass and 24.28 for activity), and E (F-ratio 27.92 for biomass and 12.59 for activity) and all quadratic coefficients [A^2 (F-ratio 53.45 for biomass and 23.47 for activity), B^2 (F-ratio 22.17 for biomass and 5.99 for activity), C^2 (F-ratio 98.26 for biomass and 29.14 for activity), D^2 (F-ratio 198.14 for biomass and 85.96 for activity), and E^2 (F-ratio 143.87 for biomass and 63.67 for activity)] were highly significant for both biomass and activity. This suggested that glucose (A), lactose (B), sodium chloride (C), and mineral salts solution (E) had direct influence on both biomass production and antagonistic activity. Moreover they can act as limiting factors and minor

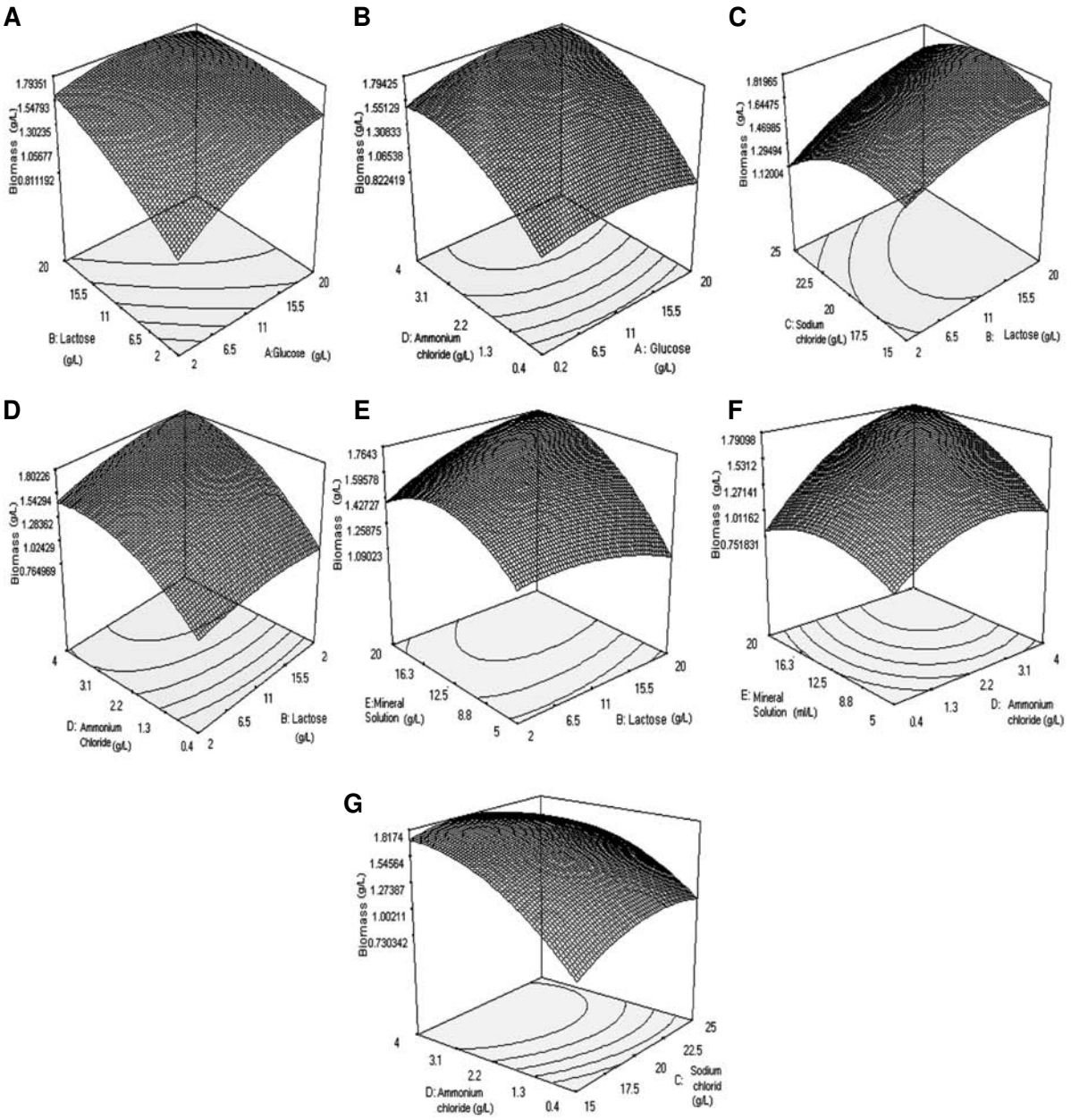

Fig. 1. Interaction of nutrients on biomass (g/L) of *Micrococcus* MCCB 104. Interaction of (A) glucose and lactose, (B) glucose and ammonium chloride, (C) lactose and sodium chloride, (D) lactose and ammonium chloride, (E) lactose and mineral salt solution, (F) ammonium chloride and mineral solution, and (G) sodium chloride and ammonium chloride, when other factors are kept at their optimum.

variations in their concentration may alter the biomass production and antagonistic activity as suggested by De Vuyst and Vandamme [22,23] with respect to nisin production which was affected by the source of carbon, nitrogen, and phosphorus. However, the linear coefficient C was found to be significant for biomass only (F-ratio 8.24 for biomass and 2.86 for activity).

The interaction coefficients such as AB (F-ratio 42.7), AD (F-ratio 9.78), BC (F-ratio 9.1), BE (F-ratio 34.06), CD (F-ratio 24.71), and DE (F-ratio 53.94) were significant model terms for biomass and in the case of activity AB (F-ratio 24.9), BC (F-ratio 9.23), BE (F-ratio 26.95), CD (F-ratio 14.32), and DE (F-ratio 29.7) were the significant model terms. This observation suggested that in the case of biomass production, lactose exhibited significant interaction with all other ingredients and for antagonistic activity it showed interaction with glucose, sodium chloride, and mineral salts solution. However, glucose showed interaction with only

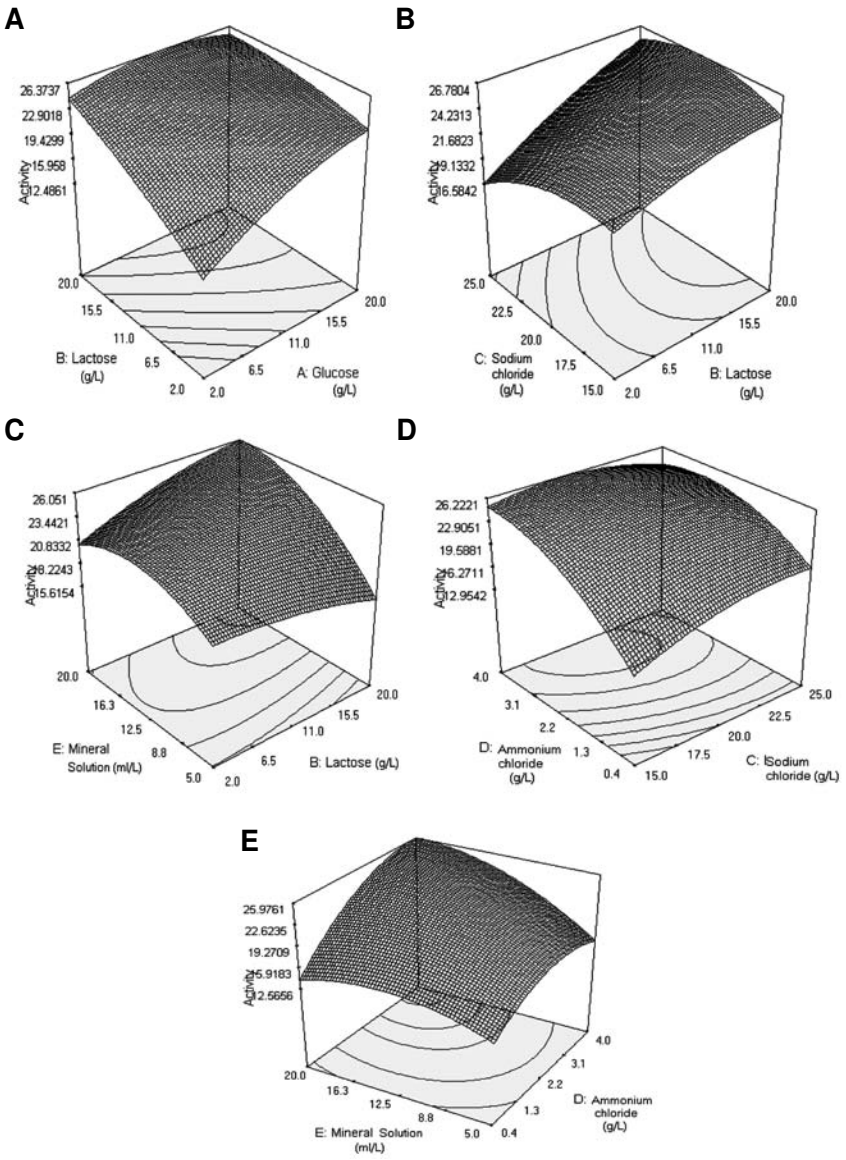

Fig. 2. Interaction of nutrients on antagonistic activity (g/L) of *Micrococcus* MCCB 104. Interaction of (A) glucose and lactose, (B) lactose and sodium chloride, (C) lactose and mineral salt solution, (D) sodium chloride and ammonium chloride, and (E) ammonium chloride and mineral salt solution, when other factors are kept at their optimum.

lactose for both biomass and antagonistic activity. This indicated that lactose played an important role for both biomass production and antagonistic activity compared to glucose. Kim *et al*. [19] reported that highest growth of a bacteriocin producing *Micrococcus* sp. was in a medium containing both lactose and glucose. However, they observed maximum bacteriocin (Micrococcocin) production in MRS medium supplemented with lactose.

Effect of interaction of varying concentrations of glucose and lactose, glucose and ammonium chloride, lactose and sodium chloride, lactose and ammonium chloride, lactose and mineral salts solution, sodium chloride and ammonium chloride on biomass production when all other parametres at optimum are presented in Figs. 1A~1G. The interaction between nutrients and their effect on antagonistic compound production were also studied (Figs. 2A~2E). The response surface plot of biomass showed similar pattern with that of antagonistic activity indicating a strong correlation between them [24]. Moreover, optimum concentration of the carbon, nitrogen sources and growth factors for both biomass and antagonistic activity, obtained from the regression equations, was glucose 17.4 g/L, lactose 17 g/L, sodium chloride 16.9 g/L, ammonium chloride 3.3 g/L, and mineral salt solution 18.3 g/L. This observation suggested that the antagonistic activity was growth depended as suggested by Kim *et al*. [19].

Validation of the Model

The predicted concentrations of ingredients of the medium from the regression equations (Eqs. 1 and 2) were same for both biomass and antagonistic activity *i.e.*, glucose 17.4 g/L, lactose 17 g/L, sodium chloride 16.9 g/L, ammonium chloride 3.3 g/L, and mineral salt solution 18.3 mL/L. At these conditions the predicted biomass was 1.7 g/L and activity in terms of halo zone was 24.5 mm. The experimental values were 1.83 ± 0.04 g/L for biomass production and 24.33 ± 0.6 mm diameters for halo zone. Moreover, in the above composition of the medium designed using response surface methodology biomass could be increased by 5.26% and antagonistic activity by 28.29% compared to the medium composition derived based on conventional 'one at a time' method.

CONCLUSION

The study suggested that central composite design of RSM was reliable for optimizing culture media for antagonistic probiotics used in aquaculture. Among the medium components tested glucose, lactose, sodium chloride, and mineral salts solution can act as limiting factors and minor variations in their concentration may alter the biomass production and antagonistic activity. The optimum concentration of ingredients for both biomass and antagonistic activity were glucose (17.4 g/L), lactose (17 g/L), sodium chloride (16.9 g/L), ammonium chloride (3.3 g/L) and mineral salts solution (18.3 mL/L). The growth medium designed for *Micrococcus* MCCB 104 shall find commercial application as it supports enhanced biomass production and antagonistic activity.

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