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Full Length Research Paper

Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: An application of statistical optimization technique

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The applicability of the Taguchi DOE (design of experiment) methodology for optimization of medium composition for maximum antimicrobial protein (AMP) production by *Bacillus amyloliquefaciens* MBL27 has been demonstrated in the present study. The influence of individual factors and the relationships between the factors and their levels were established. Three factors viz, glucose, triammonium citrate and K_2HPO_4, each at three levels were selected and an orthogonal array (OA) layout of L_{27} containing 27 well-defined experiments were performed. Two response variables (bacterial growth and inhibitory activity of the AMP) were measured. Maximum AMP production was achieved at a concentration of 1.0% glucose, 0.25% triammonium citrate and 0.2% K_2HPO_4. The experiments conducted provided basic information to improve the efficiency of AMP production and supported the analysis of main effect of each constituent in the medium. This study is therefore another example of the application of the Taguchi methodology for improvement of biological processes.

Key words: Antimicrobial protein, *Bacillus amyloliquefaciens*, optimization, response surface methodology (RSM), Taguchi design.

INTRODUCTION

Bacteriocins are ribosomally synthesized, proteinaceous compounds, which are capable of inhibiting, both spoilage and pathogenic bacteria (Klaenhammer, 1988). In recent years, AMPs and bacteriocins are gaining lot of attention as alternative therapeutics against antibiotic resistant pathogens and spoilage bacteria (Hoskin and Ramamoorthy, 2008; Zhang et al., 2008; File, 2004). Production of AMPs is widespread among diverse bacteria. *Bacillus* is an interesting genus to be investigated for antimicrobial activity because *Bacillus sp*. produces a large number of peptides with biological activities. eg. cerecin 7 (Oscariz et al., 1999) produced by *Bacillus cereus* Bc7, tochicin (Paik et al., 1997) from *Bacillus thuringensis*, thuricin 7 (Cherif et al., 2003), subpeptin JM4-A and subpeptin JM4-B produced by *Bacillus subtilis* JM4 (Wu et al., 2005). The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas, especially the agri-food industry. AMP production is strongly influenced by many factors such as pH, temperature, incubation period, cell density and nutrient sources. It is always growth associated (Aasen et al., 2000; Mataragas et al., 2004). Therefore, the optimization of environmental conditions is very important for the enhancement of AMP production. Several studies have been conducted on optimization of the growth medium aiming to enhance AMP production (Li et al., 2002; Mizumoto and Shoda, 2007; Ogunbanwo et al., 2003).

Much of scientific research is empirical and makes extensive use of experimentation. However, statistical methods can greatly increase the efficiency of these experiments and often strengthen conclusions so obtained. Taguchi's method is based upon an approach, which is completely different from the conventional

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practices of quality engineering. Taguchi method employs a special design of orthogonal arrays to learn the whole parameter space with only a small number of experiments. By applying Taguchi method based on orthogonal arrays, the time and cost of experiments can be reduced (Oskouie et al., 2007; Im et al., 2009). Thus, optimizing process parameters by the Taguchi method is an attempt not only to bring the average quality near to the target value but also to simultaneously minimize the variation in quality.

In this investigation, we explored the power of Taguchi experimental design to optimize the production of AMP by *Bacillus amyloliquefaciens* MBL27 by testing the relative importance of medium composition. We evaluated and validated the effects of three factors, - glucose, triammonium citrate and K_2HPO_4 for maximum AMP production by *B. amyloliquefaciens* MBL27 using Taguchi's experimental design of DOE. In this method, linear or quadratic models of experimental variables generate contour plots and three dimensional response surface graphs and a model equation fitting experimental data.

MATERIALS AND METHODS

Microorganisms, growth conditions and AMP production

B. amyloliquefaciens MBL27, exhibiting pronounced inhibitory activities, was isolated from dairy wastes. The flasks containing 25 ml of nutrient broth were inoculated with *B. amyloliquefaciens* MBL27 cells from glycerol stock and incubated at 30°C in an incubator shaker for 24 to 48 h. This was used as a preculture inoculum. For production studies the strain was grown in media containing varying composition of (g/L, w/v): K_2HPO_4, 1.0; $MgSO_4 .7H_2O$, 0.2; $MnSO_4$, 0.2; Tri ammonium citrate, 2.0; Glucose, 5.0. Glucose was filter sterilized separately and added to the medium. The pH of the medium was adjusted to 7.0. 50 ml of medium was inoculated with 1.0% (v/v) inoculum of *B. amyloliquefaciens* MBL27 containing 2.2 x 10^6 cells/ml and incubated at 30°C for 36 h at 200 rpm for AMP production studies. Bacterial growth was determined spectrophotometrically in a UV-Visible spectrophotometer (Shimadzu, Model UV- 2450) by measuring the OD at 600 nm. 1.0 OD is equivalent to 0.4 mg/ml dry cell weight (DCW).

Preparation of AMP

Antimicrobial protein (AMP) was recovered from culture supernatant by precipitation using 40% ammonium sulphate followed by centrifugation at 10,000 rpm using a refrigerated centrifuge (SIGMA, Model 3K30) at 4°C for 15 min. This crude antimicrobial protein was dialyzed, filter sterilized using sterile 0.22 µm syringe filter (Millipore, Bedford, MA, USA) and 0.02 ml of the supernatant was used to evaluate the activity.

AMP assay

The antimicrobial activity was detected by the agar well diffusion method (Schillinger and Lucke, 1989) using Mueller Hinton Agar (MHA). Indicator strains of ~10^5 cells/ml was added to MHA and poured onto sterile plates. After solidification, wells of ~0.5 cm diameter were created using a well borer. Crude AMP (0.02 ml) obtained after ammonium sulphate precipitation was loaded into the wells. After 24 h incubation, the plates were observed for zone of clearance. All the experiments were done in triplicates. The antagonistic activity was expressed in terms of arbitrary units (AU/ml). To determine AU/ml, filter sterilized AMP were serially diluted and their activities checked by well diffusion assay. One arbitrary unit (AU) against an individual indicator strain was defined as the reciprocal of the highest dilution that produced a minimum detectable zone of inhibition and expressed as AU/ml. The minimum detectable zone of diameter was 1.0 mm beyond well diameter. Zone diameter was measured using an antibiotic zone measuring scale (HIMEDIA).

Experimental design

A complete factorial design based on three levels and three variables was used to study the effect of three factors on AMP production by *B. amyloliquefaciens* MBL27. L_{27} orthogonal array design consisting of 27 experiments was used in this study. The optimal concentration of factors were obtained by a numerical optimization procedure and analyzing the response surface contour plots.

Two response variables were measured: bacterial growth and inhibitory activity of the AMP. The quality of the model obtained was measured using the co-efficient of determination (R^2), the significance of each parameter through an F-test (calculated p-value) and the lack of fit of the model. Co-efficients with a p-value lower than 0.01 were considered significant. Experimental designs were performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver 7.1.6). Three-dimensional surface plots were obtained to study the important and interactive effects of the independent variables on AMP production. ANOVA was used to estimate the statistical parameters for optimization of culture conditions. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be characterized.

RESULTS

The range and the levels of the factors investigated are shown in Table 1. Low and high factor settings were coded as -1 and +1 respectively, the centre points were coded as 0. Glucose was coded as A, K_2 HPO $_4$ as B and triammonium citrate as C. The individual and interactive effects of the variables were studied by performing the experimental runs at randomly selected and different levels for all 3 factors. The layout of the L_{27} Taguchi's OA, Orthogonal Array with the actual, predicted and residual values of 27 runs of the two responses are shown in Tables 2a and 2b. The responses were in terms of bacterial growth and inhibitory activity of AMP produced. Optimization of medium composition using Taguchi's experimental design resulted in the experimental value of 5.99 and 6800 AU/ml for maximum bacterial growth and AMP production respectively, while the predicted response based on Taguchi experimental design was found to be 5.33 and 7057.41 AU/ml for response 1 and 2, respectively. The closeness between the experimental and predicted data indicates the appropriateness of the experimental design.

Table 1. Factors and their levels employed in the Taguchi's experimental design for AMP production by *B. amyloliquefaciens* MBL27.

Factors	Level 1 (-1)	Level 2 (0)	Level 3 (+1)
Glucose (%) (Code A)	0.5	1.0	2.0
K_2HPO_4 (%) (Code B)	0.1	0.2	0.3
Triammonium citrate (%) (Code C)	0.1	0.25	0.3

Table 2a. L_{27} orthogonal array of Taguchi's experimental design with actual and predicted values for response 1.

Experimental run	Glucose	K_2HPO_4	Triammonium citrate	Bacterial growth (OD at 600 nm)		
				Actual	Predicted	Residual
1	-1	-1	-1	1.55	1.49	0.064
2	-1	-1	0	1.59	1.60	-0.014
3	-1	-1	+1	1.89	1.80	0.092
4	-1	0	-1	2.01	2.16	-0.15
5	-1	0	0	2.09	2.14	-0.046
6	-1	0	+1	2.14	2.19	-0.049
7	-1	+1	-1	1.76	1.76	2.222E-003
8	-1	+1	0	1.66	1.59	0.066
9	-1	+1	+1	1.54	1.51	0.033
10	0	-1	-1	2.43	2.20	0.23
11	0	-1	0	2.48	2.46	0.018
12	0	-1	+1	2.77	2.80	-0.031
13	0	0	-1	2.78	2.92	-0.14
14	0	0	0	2.92	3.04	-0.12
15	0	0	+1	2.98	3.24	-0.26
16	0	+1	-1	2.68	2.56	0.12
17	0	+1	0	2.54	2.54	-2.222E-003
18	0	+1	+1	2.78	2.60	0.18
19	+1	-1	-1	3.86	3.96	-0.10
20	+1	-1	0	4.33	4.37	-0.041
21	+1	-1	+1	4.64	4.85	-0.21
22	+1	0	-1	4.68	4.73	-0.047
23	+1	0	0	5.14	4.99	0.15
24	+1	0	+1	5.99	5.33	0.66
25	+1	+1	-1	4.44	4.42	0.024
26	+1	+1	0	4.53	4.54	-0.011
27	+1	+1	+1	4.33	4.74	-0.41

Model selection and fitting

It was observed that a quadratic model was suggested and a cubic model is aliased for both responses. The selected models had significant p-value (< 0.0001). For the other models p-value was found to be > 0.05, which indicates that the interaction among factors were not significant. Analysis of the data for the determination of significant parameters on AMP production and bacterial growth has been performed by ANOVA (Analysis of variance) (Tables 3a and 3b). Among the parameters, bacterial growth was significantly influenced by A, A^2 and B^2 whereas inhibitory activity by A and A^2. The regression equations obtained by multiple regression analysis on the experimental data are (coded factors);

Response = constant + coefficient (predictor) + . . . + coefficient (predictor)

$$Y1 = +3.04 +B1.43A +0.04B +0.16C +0.52A^2 -0.54B^2 + 0.038C^2 +0.045AB +0.14AC - 0.14BC \quad (1)$$

$$Y2 = +7057.41 +880.56A -55.56B +150C -813.89A^2 - 738.89B^2 -105.56C^2 -91.67AB -225.00AC -45.83BC \quad (2)$$

Where, A, B and C are the three variables in the model, A

Table 2b. L_{27} orthogonal array of Taguchi's experimental design with actual and predicted values for response 2.

Experimental run	Glucose	K_2HPO_4	Triammonium citrate	Inhibitory activity (AU/ml)		
				Actual	Predicted	Residual
1	-1	-1	-1	3800	4061.57	-261.57
2	-1	-1	0	4300	4587.96	-287.96
3	-1	-1	+1	4600	4903.24	-303.24
4	-1	0	-1	5100	4882.41	217.59
5	-1	0	0	6200	5362.96	837.04
6	-1	0	+1	6200	5632.96	567.59
7	-1	+1	-1	3900	4225.46	-325.46
8	-1	+1	0	4500	4660.19	-160.19
9	-1	+1	+1	4600	4883.80	-283.80
10	0	-1	-1	6400	6072.69	327.31
11	0	-1	0	6450	6374.07	75.93
12	0	-1	+1	6500	6464.35	35.65
13	0	0	-1	6750	6801.85	-51.85
14	0	0	0	6800	7057.41	-257.41
15	0	0	+1	6700	7101.85	-401.85
16	0	+1	-1	6200	6053.24	146.76
17	0	+1	0	6300	6262.96	37.04
18	0	+1	+1	6350	6261.57	88.43
19	+1	-1	-1	6500	6456.02	43.98
20	+1	-1	0	6600	6532.41	67.59
21	+1	-1	+1	6700	6397.69	302.31
22	+1	0	-1	6750	7093.52	-343.52
23	+1	0	0	6750	7124.07	-374.07
24	+1	0	+1	6750	6943.52	-193.52
25	+1	+1	-1	6500	6253.24	246.76
26	+1	+1	0	6300	6237.96	62.04
27	+1	+1	+1	6200	6011.57	188.43

Table 3a. ANOVA for response surface quadratic model for response 1.

Source	Sum of squares	DF	Mean square	F value	p-value prob > F	
Model	41.11	9	4.57	83.79	<0.0001	Significant
A-Glucose	36.72	1	36.72	673.59	<0.0001	Significant
B-K_2HPO_4	0.029	1	0.029	0.53	0.4772	
C-Triammonium citrate	0.46	1	0.46	8.39	0.0100	
A^2	1.65	1	1.65	30.33	<0.0001	Significant
B^2	1.73	1	1.73	31.70	<0.0001	Significant
C^2	8.817E-003	1	8.817E-003	0.16	0.6926	
AB	0.024	1	0.024	0.45	0.5133	
AC	0.25	1	0.25	4.57	0.0473	
BC	0.24	1	0.24	4.37	0.05200	
Residual	0.93	17	0.055			
Cor Total	42.04	26				

= Glucose, B = K_2HPO_4 and C = triammonium citrate, Y1 is response 1 (Bacterial growth, OD at 600 nm), Y2 is response 2 (Inhibitory activity, AU/ml), and this second-order equation was employed to fit the data individually for the responses Y1 and Y2.

The goodness of fit of the model was checked by the determination of co-efficient (R^2). The closeness of R^2 to 1.0 indicates a high significance of the model and this

Table 3b. ANOVA for response surface quadratic model for response 2.

Source	Sum of squares	DF	Mean square	F Value	p-value prob > F	
Model	2.247E+007	9	2.496E+006	17.72	<0.0001	Significant
A-Glucose	1.396E+007	1	1.396E+007	99.09	<0.0001	Significant
B-K_2HPO_4	55555.56	1	55555.56	0.39	0.5383	
C-Triammonium citrate	4.050E+005	1	4.050E+005	2.88	0.1082	
A^2	3.974E+006	1	3.974E+006	28.22	<0.0001	Significant
B^2	3.276E+006	1	3.276E+006	23.26	0.0002	
C^2	66851.85	1	66851.85	0.47	0.5002	
AB	1.088E+005	1	1.008E+005	0.72	0.4092	
AC	6.075E+005	1	6.075E+005	4.31	0.0533	
BC	25208.33	1	25208.33	0.18	0.6776	
Residual	2.394E+006	17	1.408E+005			
Cor Total	2.486E+007	26				

represents the ideal case. The value of R^2 for response 1 was 0.9780 indicating that only 2.2% of the total variation was not explained by the model. The value of adjusted R^2 is also high and the predicted R^2 of 0.9399 is in reasonable agreement with the adjusted R^2 of 0.9663. This signifies a good correlation between factors. Also, the F and P > F values for the model were 20.73 and < 0.0001 which also implies that, the estimated models fit the experimental data adequately. For response 2, R^2 was 0.9037, indicating that only 9.63% of the total variation was not explained by the model. The value of the adjusted R^2 is high (0.8527) and this indicates a high significance of the model. Also, the F and P > F values for the model were 17.32 and < 0.0001, respectively, which also implies that, the estimated models adequately fit the experimental data.

Interactions among the factors on growth and AMP production

The graphical representation provides a method for visualizing the relationship between the responses and the interactions among the variables to determine the optimum conditions. Figures 1a, 1b and 1c show the contour and 3-D response surface graphs for the variation in the bacterial growth. The effect of glucose and K_2HPO_4 on growth of the organism is depicted in Figure 1a. Figure 1b shows the contour and response surface graphs on interaction between glucose and triammonium citrate.

The F value and p-value of 4.57 and 0.0473, respectively, show that the interaction between glucose and triammonium citrate was significant. Figure 1c shows the response surface graphs on interaction between triammonium citrate and K_2HPO_4. The F value and p-value of 0.18 and 0.6776, respectively, show that the interaction between triammonium citrate and K_2HPO_4 was not significant. Figures 2a, 2b and 2c show the contour and response surface graphs for the variation in inhibitory activity. The proposed quadratic model equation illustrates the interaction between the factors. The parameters such as A, A^2 and B^2 were significant positive factor.

Numerical optimization of factors

Based on the results obtained in run no.14 (Tables 2a and 2b), optimum growth (OD_{600} 2.92) and maximum inhi-bitory activity (6800 AU/ml) were obtained when glucose, K_2HPO_4 and triammonium citrate were added at a concentration of 1.0, 0.2 and 0.25% (w/v), respectively, in the culture medium. To obtain maximum optimum activity, the factor levels and response were set at the desired goal using Design Expert's Numerical optimization under desirability equal to one. Under optimal conditions the expected activity was 7057.41 AU/ml for which the corresponding bacterial growth would be OD_{600} 3.03.

Verification test

A verification experiment was conducted to compare the predicted values to near optimum levels of independent variables and the basal conditions settings. AMP production in the final optimized medium was 6800 AU/ml compared to 7057.41 AU/ml predicted using Taguchi design for the same composition. The closeness of the values shows that the model was adequate to predict the optimization of AMP production from *B. amyloliquefaciens* MBL27. Moreover, the final optimized culture condition produced 6800 AU/ml compared to 5100 AU/ml before optimization, which is almost 1.33 fold higher than that obtained from the non optimized condition. Bacterial growth corresponding to the same condition was found to have 1.45 fold increase.

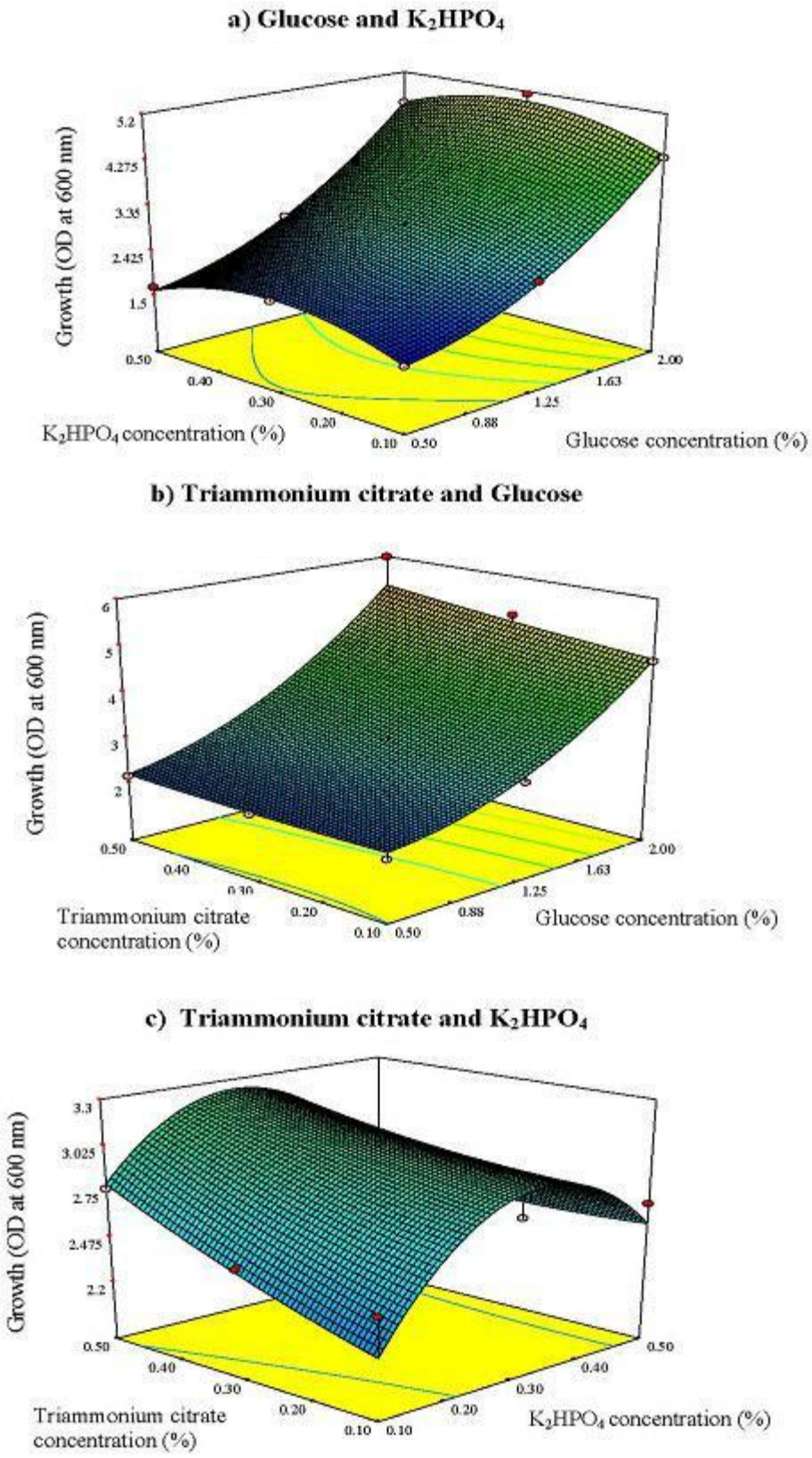

Figure 1. Response surface and contour plot of the combined effects of medium composition on bacterial growth. a) Glucose and K_2HPO_4. b) Triammonium citrate and Glucose. c) Triammonium citrate and K_2HPO_4.

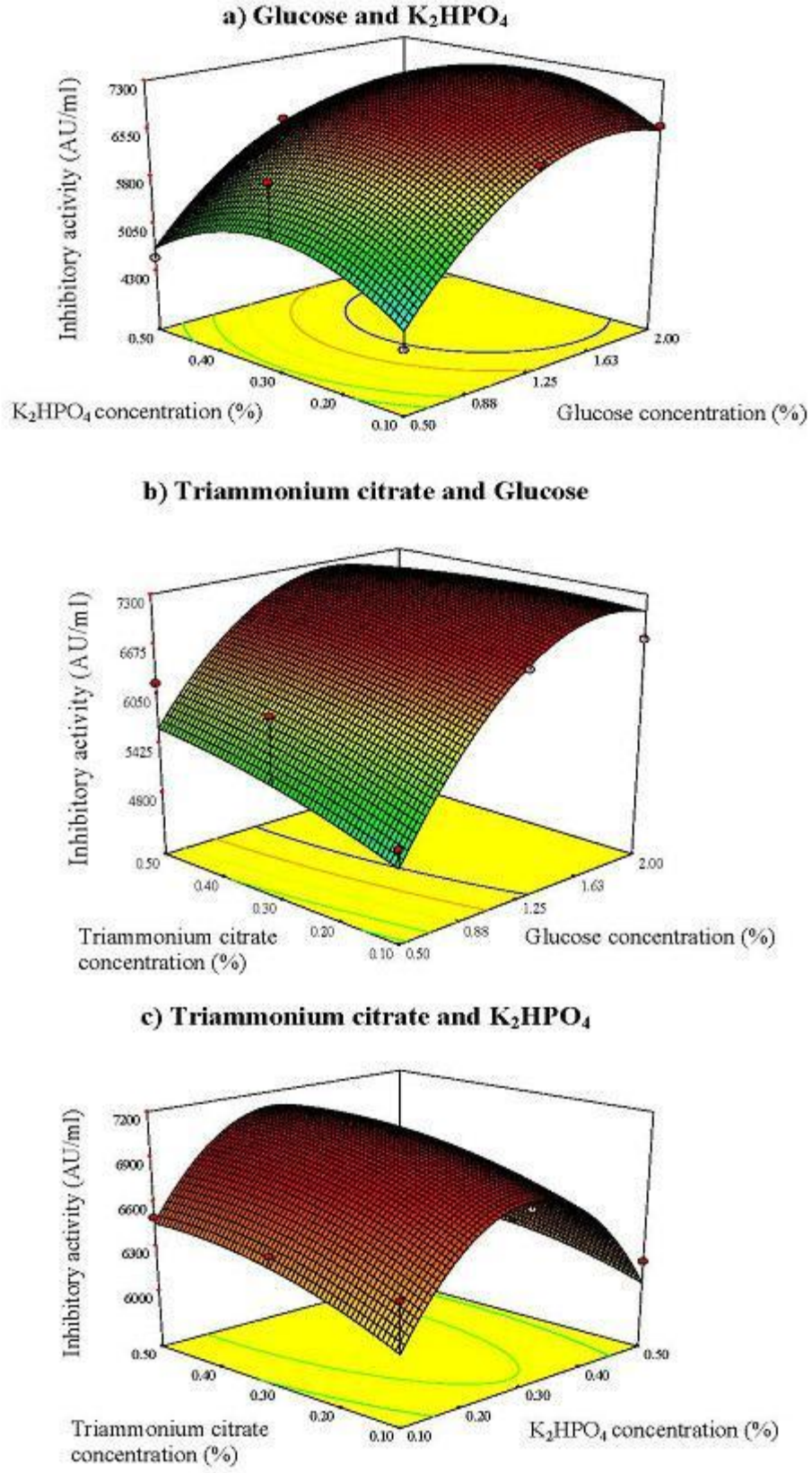

Figure 2. Response surface and contour plot of the combined effects of medium composition on AMP production by *B. amyloliquefaciens* MBL27. a) Glucose and K_2HPO_4. b) Triammonium citrate and Glucose. c) Triammonium citrate and K_2HPO_4.

DISCUSSION

Statistical methods have been applied for optimization of microbial production in many studies (Anthony et al., 2008; Cladera-Olivera et al., 2004; Nagarjun et al., 2005). In the present study, optimization of medium composition for bacterial growth and maximum production of AMP from *B. amyloliquefaciens* MBL27 by the Taguchi experimental method was studied. The medium composition has profound effects on AMP and bacteriocin production and this has also been reported for several bacteriocins produced by *Lactobacillus plantarum* LR/14 (Tiwari and Srivastava, 2008), *B. subtilis* (Ku et al., 2009) and *Lactobacillus plantarum* LPCO10 (Leal-Sanchez et al., 2002) . Xiong et al. (2008) reported that medium composition have significant effects on the production of the bioactive compound actinomycin X2 produced by *Streptomyces spp* JAU4234. The yield of actinomycin X2 was increased by 36.9% by culturing the strain *Streptomyces spp* JAU4234 in the nutritionally optimized fermentation medium. Similarly, statistical optimization led to a 2 fold improvement in surfactin production by *B. subtilis* ATCC 21332 compared to the control MMS medium (Wei et al., 2007).

Ku et al. (2009) optimized the glucose concentration by conducting experiments with its various levels by Box-Behnken's design and observed maximum activity with 1.0% (w/v) glucose. Im et al. (2009) also found glucose to be the best carbon source for hyaluronic acid (HA) fermentation by *Streptococcus* sp. ID9102 (KCTC 11935 BP) via a statistical approach using Taguchi L_{16} OA. Similarly, Drosinos et al. (2005) reported that glucose was a better carbon source for the maximum bacteriocin activity produced by *Leuconostoc mesenteroides* E131. Guerra et al. (2001) reported that KH_2PO_4 as a better source for nisin production. Cai and Zheng (2009) reported that K_2HPO_4 (2.38 g/l) as one of the most significant factors using statistically based experimental designs to optimize medium composition for keratinase production by *B. subtilis* KD-N2.

CONCLUSIONS

Each organism has its own special conditions for maximum AMP production. This investigation clearly establishes the effect of medium composition on AMP production by *B. amyloliquefaciens* MBL27. Taguchi's design proved to be powerful tool in optimizing AMP production in our study. Optimization of medium composition using Taguchi's experimental design has resulted in a medium containing glucose, triammonium citrate and K_2HPO_4 at concentrations 1.0, 0.25 and 0.2% w/v, respectively. The closeness of the actual and predicted values determined the validity of the model. The experiments conducted provided basic information to improve the efficiency of AMP production and supported the analysis of the main effect of each constituent of the medium. Therefore, the medium optimization strategy utilized in this work appears to elevate the AMP production yield to a substantially higher level.

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