

Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: Optimization of culture conditions using Taguchi's experimental design

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Abstract

We report the applicability of the Taguchi DOE methodology for optimization of culture conditions for maximum antimicrobial protein (AMP) production by *Bacillus amyloliquefaciens* MBL27. The influence of individual factors and the relationships between the factors and their levels were established. Three factors viz, pH, incubation temperature and incubation period, 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 of 6774.85 AU/ml against *S. aureus* was predicted at pH 7.0, incubation period 36 h and at temperature 30°C using response surface plots. This study serves as another example for the application of the Taguchi methodology for improvement of biological processes.

Keywords: Antimicrobial protein, antibiotic production, *B. amyloliquefaciens*, Taguchi design.

Introduction

Bacteriocins are ribosomally synthesized, proteinaceous compounds, which are capable of inhibiting both spoilage and pathogenic bacteria (Klaenhammer, 1988). In recent years, Antimicrobial proteins (AMPs) and bacteriocins are gaining lot of attention as an alternative therapeutics against antibiotic resistant pathogens and spoilage bacteria (Hoskin & Ramamoorthy, 2008; Zhang *et al.*, 2008; File, 2004; Feder *et al.*, 2001). Production of AMPs is widespread among diverse bacteria (Vandammae, 1994; Bizani & Brandelli, 2002; Duquesne *et al.*, 2007; Nes *et al.*, 2007).

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 *B. cereus* Bc7, tochicin (Paik *et al.*, 1997) from *B. thuringensis*, thuricin 7 (Cherif *et al.*, 2003), subpeptin JM4-A and subpeptin JM4-B produced by *B. 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.

Antimicrobial protein (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 components, composition and cultural conditions aiming to enhance AMP production (Sen & Swaminathan, 1997; Ogunbanwo *et al.*, 2003; Korenblum *et al.*, 2005).

Much of the scientific research is empirical and makes extensive use of experimentation. Statistical methods can however; greatly increase the efficiency of these experiments and often strengthen the conclusions

so obtained. Taguchi's method is based upon an approach, which is completely different from the conventional 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 method, linear or quadratic models of experimental variables generate contour plots and three dimensional response surface graphs and a model equation fitting experimental data.

In this investigation, we explore the valuable power of Taguchi experimental design to optimize the production of AMP by *B. amyloliquefaciens* MBL27 by testing the relative importance of environmental factors. In this study, we evaluated and validated the effects of three factors (temperature, pH and incubation period) for maximum AMP production by *B. amyloliquefaciens* MBL27 using Taguchi experimental design.

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 24-48 h. This was used as a preculture inoculum.

Initially, for production studies the strain was grown in media containing (g/l, w/v): K_2HPO_4 , 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4$, 0.2; tri ammonium citrate, 2.5; glucose, 10. Glucose was filter sterilized separately and added to the medium. The pH of the medium was adjusted to 6.5. 250 ml flasks containing 50 ml of production medium was

inoculated with 1.0% (v/v) inoculum of *B. amyloliquefaciens* MBL27 containing 2.2×10^6 cells/ml and incubated at 37°C for 24 h at 200 rpm for AMP production studies. Bacterial growth was determined spectrophotometrically using UV-Visible spectrophotometer (Shimadzu, Model UV-2450) by measuring the OD at 600 nm. pH was monitored throughout the cultivation period.

Preparation of Antimicrobial protein (AMP)

AMP was recovered from culture supernatant by precipitation method 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 dialysed (Dialysis membrane cut off is 10 kDa), 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 & Lucke, 1981) using Mueller Hinton Agar (MHA). Indicator strain (*S. aureus*) of $\sim 10^5$ 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.

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 still 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 obtained model 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 <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. All the experiments were done in triplicates.

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)
pH (Code A)	5	7	9
Temperature (°C) (Code B)	25	30	37
Incubation period (h) (Code C)	24	36	48

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. pH was coded as A, temperature as B and incubation period 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 are given in Tables 2a and 2b. The responses were in terms of bacterial growth and the inhibitory activity of AMP produced.

Optimization of culture conditions resulted in maximum experimental value of 3.0 for response 1 and 6700 AU/ml for response 2, while the predicted response based on Taguchi experimental design was found to be 2.76 and 6678.78 AU/ml for response 1 & 2, respectively. The closeness between the experimental and predicted data indicates the appropriateness of the experimental design.

Model selection and fitting: It was observed that a 2FI model was suggested for response 1 and a quadratic model for response 2. 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) (Table 3a and 3b). For culture conditions among the parameters considered growth was significantly influenced by C, AB and BC activity by A, B, A^2 , B^2 , C^2 , AB and AC.

The regression equations obtained by multiple regression analysis on the experimental data are (coded factors)

$$\text{Response} = \text{constant} + \text{coefficient (predictor)} + \dots + \text{coefficient (predictor)}$$

$$Y1 = +1.91 - 4.44E-003A + 0.1B + 0.39 + 0.21B + 0.12AC - 0.53C \quad (1)$$



$$Y_2 = +6677.78 + 408.33A + 363.89B + 197.22C + 629.17AB + 779.17AC - 154.17BC - 1258.33A^2 - 1375B^2 - 508.33C^2 \quad (2)$$

where Y1 is response 1 (bacterial growth, OD at 600 nm)
 Y2 is response 2 (inhibitory activity, AU/ml)
 A= pH, B= incubation temperature ($^{\circ}$ C) and C= incubation period (h)

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

Experimental Runs	pH	T ($^{\circ}$ C)	Incubation period (h)	Bacterial growth (OD at 600 nm)		
				Actual	Predicted	Residual
1	-1	-1	-1	1.26	1.23	0.03
2	-1	-1	0	1.95	2.03	-0.08
3	-1	-1	+1	2.58	2.83	-0.25
4	-1	0	-1	1.56	1.65	-0.09
5	-1	0	0	2.10	1.92	0.18
6	-1	0	+1	2.20	2.20	0.00
7	-1	+1	-1	1.90	2.08	-0.18
8	-1	+1	0	2.00	1.82	0.18
9	-1	+1	+1	1.51	1.56	-0.05
10	0	-1	-1	0.91	0.90	0.01
11	0	-1	0	1.89	1.82	0.07
12	0	-1	+1	3.00	2.73	0.27
13	0	0	-1	1.72	1.53	0.19
14	0	0	0	2.20	1.92	0.28
15	0	0	+1	2.13	2.31	-0.18
16	0	+1	-1	1.80	2.16	-0.36
17	0	+1	0	2.40	2.02	0.38
18	0	+1	+1	1.75	1.89	-0.14
19	+1	-1	-1	0.57	0.57	0.00
20	+1	-1	0	1.20	1.60	-0.40
21	+1	-1	+1	2.80	2.64	0.16
22	+1	0	-1	1.40	1.41	-0.01
23	+1	0	0	1.91	1.92	-0.01
24	+1	0	+1	2.42	2.42	0.00
25	+1	+1	-1	2.25	2.25	0.00
26	+1	+1	0	2.43	2.23	0.20
27	+1	+1	+1	2.00	2.21	-0.21

The goodness of fit of the model was proved 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 represents the ideal case. The value of R^2 for response 1 was 0.8786 indicating that only 12.14% of the total variation was not explained by the model. The value of the adjusted R^2 is also high and the predicted R^2 of 0.7808 is in reasonable agreement with the adjusted R^2 of 0.8422. This signifies a good correlation between factors. Also the F and P>F values for the model were 24.13 and <0.0001 respectively, which also implies that the estimated models adequately fit the experimental data. For response 2, R^2 was 0.9048, indicating that only 9.52% of the total variation was not explained by the model. The value of adjusted R^2 is high (0.8543) and this indicates a high significance of the model. Also the F and P>F values for the model were 17.94 and <0.0001 which also implies that the estimated models fit the experimental data adequately. Standard deviation, mean, CV and predicted residual sum of squares

(PRESS) values were 502.64, 4583.33, 10.97 and 1.243E + 007, respectively for response 2.

Interactions among the factors on growth and AMP

The graphical representation provides a method for visualizing the relationship between the responses and the interactions among the variables to determine the optimum conditions. Fig. 1a, 1b & 1c show the contour and 3-D response surface graphs for the variation in the growth, as a function of pH, incubation temperature and incubation period. Figure 1a show the effect of pH and temperature on growth of the organism at an incubation period of 36 h. Bacterial Growth was found to be higher at a pH range of 7.0 - 9.0 at 30 - 37 $^{\circ}$ C and lower at a pH range of 5.0 - 6.0 at 25 - 30 $^{\circ}$ C. The F-value and p-value of AB interaction were 10.75 and 0.0038, respectively indicating that it was significant (p<0.05) in this model. Figure 1b shows that the optimum bacterial growth of 1.96 OD and high inhibitory activity (6733.54 AU/ml) was obtained at pH 7.0 and an incubation period of 36 h. But the F value and p-value of 3.54 and 0.0744, respectively show that the interaction between pH and incubation period was not significant, however incubation period was found to be a significant variable. Interactions between temperature and incubation period were highly significant (F-value = 69.45; P>F = <0.0001). From Figure 1c it was observed that for each rise in temperature and incubation period, a significant rise in bacterial growth was observed. However, inhibitory activity was found to be maximum (6774.85 AU/ml) at a temperature of 30 $^{\circ}$ C and an incubation period of 36 h for which the corresponding growth was 1.97 OD.

Figs. 2a, 2b & 2c show the contour and response surface graphs for the variation in inhibitory activity, as a function of pH, temperature and incubation period. The proposed quadratic model equation illustrates the interaction between 2 factors. The process parameters such as pH, temperature and incubation period were significant positive factor. pH interacted positively with temperature and incubation period. Temperature interacted positively with pH and negatively with incubation period. Incubation period interacted positively with pH and negatively with temperature.

Numerical optimization of factors: Based on the results obtained in run no.14 (Tables 2a & 2b), optimum growth (1.92) and maximum inhibitory activity (6677.78 AU/ml) were obtained when pH, temperature and incubation period were 7.0, 30 $^{\circ}$ C and 36 h respectively. 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 6677.78 AU/ml for which the corresponding growth would be 1.92 OD.

Validation and confirmation test

Experiments were carried out in optimum medium under optimal culture conditions to confirm the results obtained by Taguchi orthogonal array design. AMP

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

Experimental Runs	pH	T (°C)	Incubation period (h)	Inhibitory activity (AU/ml)		
				Actual	Predicted	Residual
1	-1	-1	-1	4500	3820.83	679.17
2	-1	-1	0	3700	3901.39	-201.39
3	-1	-1	+1	3000	2965.28	34.72
4	-1	0	-1	4900	5084.72	-184.72
5	-1	0	0	5200	5011.11	188.89
6	-1	0	+1	4000	3920.83	79.17
7	-1	+1	-1	3200	3598.61	-398.61
8	-1	+1	0	3500	3370.83	129.17
9	-1	+1	+1	1800	2126.39	-326.39
10	0	-1	-1	3700	4079.17	-379.17
11	0	-1	0	4400	4938.89	-538.89
12	0	-1	+1	4800	4781.94	18.06
13	0	0	-1	5800	5972.22	-172.22
14	0	0	0	6700	6677.78	22.22
15	0	0	+1	6100	6366.67	-266.67
16	0	+1	-1	5400	5115.28	284.72
17	0	+1	0	6100	5666.67	433.33
18	0	+1	+1	5800	5201.39	598.61
19	+1	-1	-1	1550	1820.83	-270.83
20	+1	-1	0	3400	3459.72	-59.72
21	+1	-1	+1	4800	4081.94	718.06
22	+1	0	-1	5200	4343.06	856.94
23	+1	0	0	6100	5827.78	272.22
24	+1	0	+1	5500	6295.83	-795.83
25	+1	+1	-1	3700	4115.28	-415.28
26	+1	+1	0	5200	5445.83	-245.83
27	+1	+1	+1	5700	5759.72	-59.72

Table 3a. ANOVA for Response surface 2FI model for Response 1

Source	Sum of squares	DF	Mean square	F Value	p-value Prob>F	
Model	6.96	6	1.16	24.13	<0.0001	Significant
A-pH	3.556E+04	1	3.556E+04	7.396E+03	0.9323	
B-Temperature	0.20	1	0.20	4.08	0.0569	
C-Incubation period	2.74	1	2.74	56.95	<0.0001	
AB	0.52	1	0.52	10.75	0.0038	
AC	0.17	1	0.17	3.54	0.0744	
BC	3.34	1	3.34	69.45	<0.0001	
Residual	0.96	20	0.048			
Cor Total	7.92	26				

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	4.080E+07	9	4.533E+06	17.94	<0.0001	Significant
A-pH	3.001E+06	1	3.001E+06	11.88	0.0031	
B-Temperature	2.383E+06	1	2.383E+06	9.43	0.0069	
C-Incubation period	7.001E+05	1	7.001E+05	2.77	0.1143	
AB	4.750E+06	1	4.750E+06	18.80	0.0004	
AC	7.285E+06	1	7.285E+06	28.84	<0.0001	
BC	2.825E+05	1	2.825E+05	1.13	0.3029	
A ²	9.500E+06	1	9.500E+06	37.60	<0.0001	
B ²	1.134E+07	1	1.134E+07	44.90	<0.0001	
C ²	1.550E+06	1	1.550E+06	6.14	0.0240	
Residual	4.295E+06	17	2.526E+05			
Cor Total	4.510E+07	26				

production in the optimized culture conditions was 6700 AU/ml compared to 6677.78 AU/ml, predicted using Taguchi design for the same condition. The closeness of the values obtained shows that the model was adequate enough to predict the conditions required for the maximal production of AMP by *B. amyloliquefaciens* MBL27. Moreover, the final optimized culture conditions produced 6700 AU/ml compared to 5400 AU/ml before optimization (pH 6.5, temperature 37°C and incubation period 24 h), which is almost 1.24 fold of that obtained from the non optimized condition. Bacterial growth corresponding to the same condition was found to have a 1.22 fold increase.

From Taguchi experimental design optimum conditions for maximum production of AMP by *B. amyloliquefaciens* MBL27 was determined to be pH 7.0, temperature 30°C and incubation period 36 h.

Discussion

We optimized cultivation conditions for bacterial growth and inhibitory activity of AMP from *B. amyloliquefaciens* MBL27 by the Taguchi experimental design. Statistical methods have been applied for optimization of microbial production in many studies (Anthony *et al.*, 2008; Cladera-Olivera *et al.*, 2004; Ahmed *et al.*, 2009). pH, temperature and incubation period

were selected because these mostly influence bacterial growth and AMP production (Korenblum *et al.*, 2005; Sugita *et al.*, 1998). The effect of pH and temperature is very important for antimicrobial protein and bacteriocin production and this has also been reported for several bacteriocins produced by *Lactobacillus casei* (Vignolo *et al.*, 1995), *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1 (Ogunbanwo *et al.*, 2003) and *Leuconostoc mesenteroides* (Krier *et al.*, 1998). Leal-Sanchez (2002) reported that temperature was found to have positive significant effects ($p < 0.05$) on the production of the bioactive compounds which is in agreement with our study. Wefky *et al.* (2009), obtained maximal production of bioactive compounds by *E. faecium* after 48 h after which the activity decreased significantly in the culture medium using Plackett-Burman design. Like fermentation time increasing pH had a positive effect on the bioactive compounds production by *E. faecium* (Wefky *et*

Fig. 1. Response surface and contour plot of the combined effects of different process parameters on bacterial growth

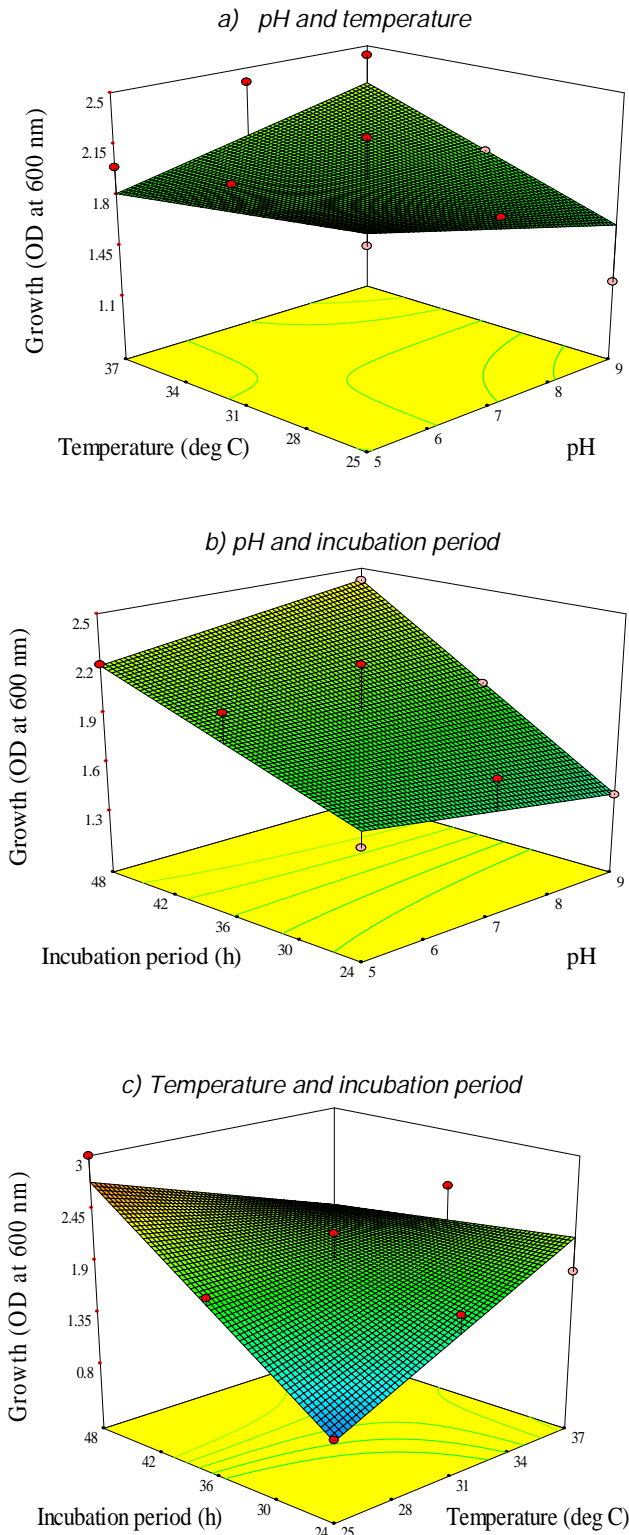
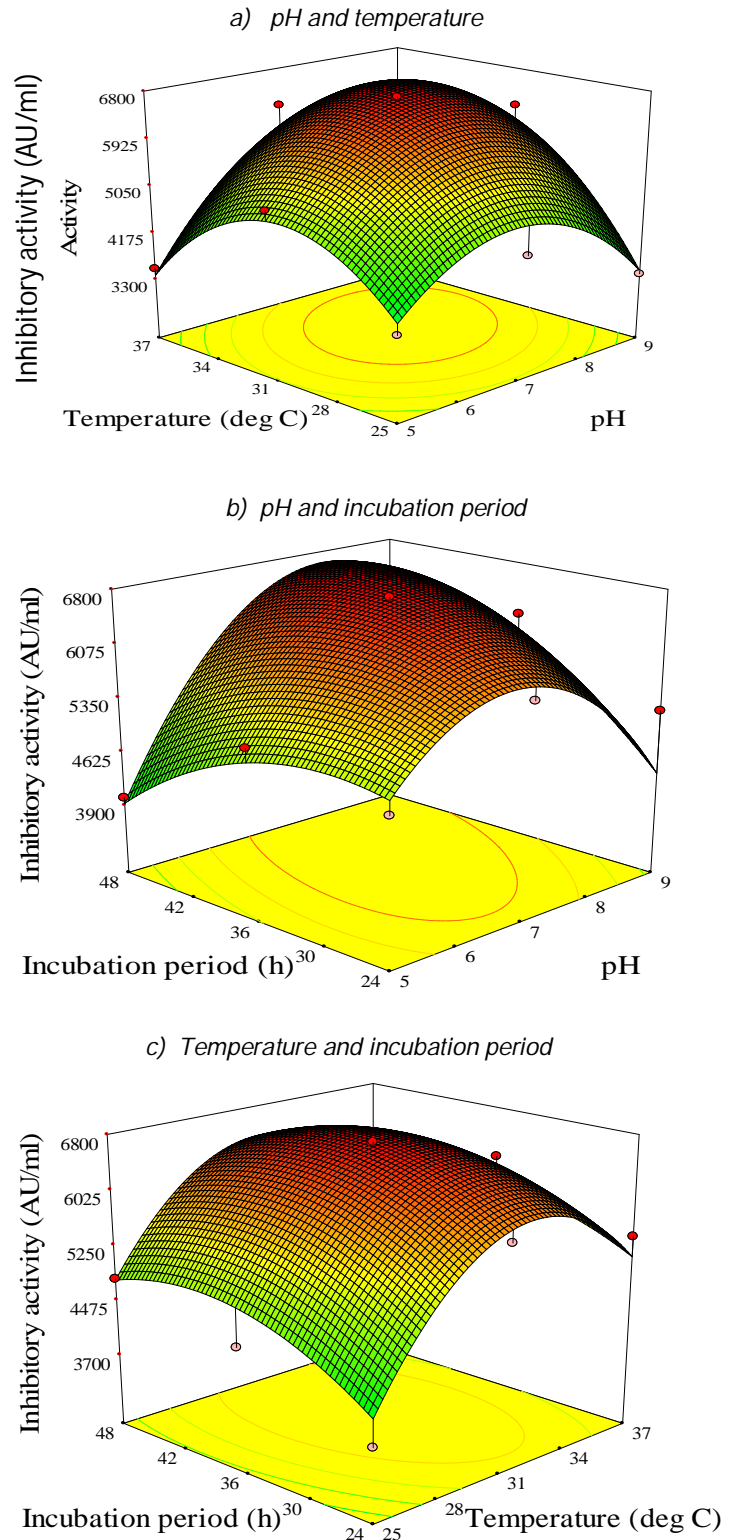


Fig. 2. Response surface and contour plot of the combined effects of different process parameters on AMP production by *B. amyloliquefaciens* MBL27



al., 2009). Initial pH, one of the parameters chosen for optimization for cyclodextrin glucanotransferase from *B. stearothermophilus* HR1 was optimized to 7.54 using response surface methodology (predicted value of 14.8 U/ml which is close to the experimental value 14.2 U/ml) (Rahman *et al.*, 2004). El-Sersy & Abu-Elela (2006) optimized the pH for maximum production of bioactive compounds from *Nocardia brasiliensis* using Plackett-Burman experimental design.

Conclusions

Each organism has its own special conditions for maximum AMP production. Taguchi's design proved to be powerful tool in optimizing AMP production in our study. This investigation clearly establishes the effect of culture conditions on AMP production by *B. amyloliquefaciens* MBL27. Quadratic effect of AMP activity was more significant followed by linear 2FI effect of bacterial growth. Results obtained showed that, maximum AMP production was achieved at pH 7.0, temperature 30°C and incubation period 36 h. The closeness of actual and predicted values determined the validity of the model. Therefore, the culture conditions optimization strategy utilized in this work appears to elevate the AMP production yield to a substantially higher level.

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