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Full Length Research Paper

Development of a new medium containing date syrup for production of bleomycin by *Streptomyces mobaraensis* ATCC 15003 using response surface methodology

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A combined statistical approach of orthogonal design and polynomial regression were applied to optimize the composition and concentration of a liquid fermentation medium for the production of bleomycin (BLM) by *Streptomyces mobaraensis*. Optimal conditions for maximal productivity were determined based on eight parameters at three different levels. The sources of carbon and nitrogen concentration and their interactions with other precursors were found to be statistically significant factors. When date syrup was used as an additional carbon source, higher BLM amount was obtained in comparison to glucose. It was found that the optimum nitrogen source was achieved with the use of soyabean meal. The combined orthogonal design and response surface methodology predicted optimal conditions for production of BLM to be 138 mg dl^{-1}. A confirmatory experiment of the optimal medium composition produced 142 mg dl^{-1} in the fifth day fermentation at 30°C. The complex medium containing 40 gml^{-1} date syrup as additional carbon source enhanced the production of BLM by 73%. The combined statistical approach enabled rapid identification and integration of key medium parameters for optimizing secondary metabolite production and could be very useful in pharma-ceutical screening programs.

Key words: Bleomycin, *Streptomyces mobaraensis*, orthogonal design, medium optimization, date syrup.

INTRODUCTION

Bleomycin (BLM) is a family of glycopeptide-derived antibiotics originally isolated from the fermentation broth of *Streptomyces verticillus* (Umezawa et al., 1966; Fujii et al., 1974; Troost et al., 2009). The naturally occurring BLM differs structurally and primarily at the C-terminus of the glycopeptide. BLM exhibits a strong antitumor activity and therefore, it has been widely employed for the treatment of several malignancies, including non-Hodgkin's lymphoma, squamous cell carcinoma and testicular tumors (Aras and Dilsizian, 2008; Evens et al., 2008).

In developing a biotechnology-based industrial process, designing the fermentation media is of critical importance since it affects the product yield and volumetric productivity

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Abbreviations: BLM, Bleomycin; **SS,** soluble starch (millet jelly); **GC,** glucose; **SM,** soybean meal; **CS,** corn steep liquor; **DS,** date Syrup; **Zn,** Zinc source ($ZnSO_4.7H_2O$); **Cu,** copper source ($CuSO_4.5H_2O$); **NO,** nitrogen source ($NaNO_3$).

(Grag and Neelakantan, 1981; Furuhashi and Takagi, 1984). Microorganisms require some specific nutrients for their growth and metabolic activities, which varies depending on the type of the organism as well as the nature of basal medium used (Raytapadar and Paul, 2001). The ability of certain microorganism cultures to form antibiotics, for example, is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation (Loun'es et al., 1996). Therefore, constituents of the medium and metabolic capacity of the producing organism greatly influence antibiotic biosynthesis. Changes in the nature and type of carbon, nitrogen or phosphate sources and trace elements have been reported to influence antibiotic biosynthesis in Streptomycetes (Loun'es et al., 1996).

Several approaches have been implemented to rapidly identify the variables which need to be controlled for optimizing the production of useful metabolites and/or antibiotics (Grothe et al., 1999; Wang et al., 2008a; Wang et al., 2008b). The effect of nitrogen sources on secondary metabolism is conditioned by several factors including type of metabolic pathway, producing organism, type and concentration of the nitrogen sources and whether cultures are stationary or submerged (Panda et al., 2004). Very often, secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth (Betina, 1994). Negative effects of ammonium salts have been reported in the production of cephalosporin and other metabolites/antibiotics (Panda et al., 2004). The earlier developed medium for production of BLM, contained complex constituent such as melt jelly, corn steep liquor and peptone. Date syrup (DS) was approved to contain adequate nutrients in a good amount to be suitable for growth of microorganisms (Khiyami et al., 2008). Date syrup is viscous liquid produced as a by-product of date industry; it is rich in carbohydrates (75% w/w) and small amount of protein and fat (1.1 and 2.9%, respectively) (Al-Farsi et al., 2007) in addition to many macro- and micro-elements (Al-Hooti et al., 2002). Kingdom of Saudi Arabia (KSA) is currently the third largest date producer over the world (Entezari et al., 2004). Date syrup is available over the year from different producers at cheap prices.

The traditional "one-factor at a time" technique used for optimizing a multivariable system is not only time consuming but also misses alternative effects between components, ignores possible interactions and it often requires so many runs that they become impractical to carry out (Escamilla et al., 2000; Xu et al., 2003; Aras and Dilsizian, 2008). Response surface methodology (RSM) is a combination of mathematical and statistical techniques that is commonly employed for designing and evaluating the effects of experimental factors for optimizing the conditions of these factors for desirable responses (Box et al., 2005; Kammoun et al., 2008). The optimization process of this methodology involves studying the response of the statistically designed combination as well as estimating the coefficient to determine the best mathematical model that fits the experimental conditions with an appropriate adequacy (Kammoun et al., 2008). Recently, statistical experiment design methods have been employed in various bioprocess optimizations. Among them, Box-Behnken design, a spherical and revolving design, is widely applied for optimization of chemical and physical processes because of its suitability and excellent outcomes (Oscar et al., 1999; Zou et al., 2008).

To the best of the authors' knowledge, there are no reports of production of BML from *Streptomyces mobaraensis* by engineering the condition of fermentation using orthogonal design. The objective of this study was to formulate a suitable production medium containing date syrup using a combination of statistical strategies involving the use of Box-Behnken design (orthogonal design) in an attempt to optimize the production of BLM by *S. mobaraensis* in a submerged fermentation system.

MATERIALS AND METHODS

Microbial strains

Lyophilized spore suspension of the *S. mobaraensis* (DSM No. 40903 = ATCC 15003) was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *Bacillus subtilis* (ATCC 6633) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA).

Chemicals and Materials

Peptone and meat extract was purchased from Difco Ltd. (Difco Ltd., Wakefield, UK). Brain-heart infusion broth and Mueller-Hinton agar were gotten from Oxoid Ltd. (Oxoid Ltd., Hampshire, UK). Date Syrup was obtained from Albadia Company, Kingdom of Saudi Arabia batch No. 050501. Standard bleomycin (Lot CP3 # 28) was obtained directly from Nippon Kayaku (Nippon Kayaku Co. Ltd., Tokyo, Japan). All other chemicals were obtained from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA).

Culture and maintenance of the microorganisms

The spore suspension of the *S. mobaraensis* (ATCC 15003) was maintained in 0.5 ml cryogenic vials. Aliquots of the spore suspension (2.0×10^7 spores/ml ± 5%) were kept in glycerol broth (40%, v/v) at -20°C for 1 year (Yu et al., 2008). *B. subtilis* (ATCC 6633) was used for the assay of BLM. Lyophilized *B. subtilis* was cultured in brain-heart infusion broth for 16 - 18 h at 37°C. The microorganism was then transferred to Mueller-Hinton agar plates and incubated for 3 - 5 days. The bacterial colonies were transferred to 5 ml of sterile distilled water by means of a culture loop, and incubated at 60°C for 30 min to kill vegetative forms. They were then washed three times with sterile distilled water, reheated to 60°C for another 30 min, then stored at 4°C until use, under which conditions, the stock spore suspension was stable for several months.

Preparation of seed culture

The seed was prepared by shake-culturing *S. mobaraensis* at 27°C

Table 1. Experimental design combinations for the factors and levels used for the optimization of BLM production based on substrate and concentration reported.

Substrate	Levels			
	Orthogonal design	Low	Medium	High
	Response surfaces	-1	0	+1
Soluble starch (millet jelly)		32.0 gl^{-1}	80.0 gl^{-1}	128 gl^{-1}
Glucose		2.50 gl^{-1}	5.00 gl^{-1}	7.50 gl^{-1}
Soybean meal		1.75 gl^{-1}	4.375 gl^{-1}	7.00 gl^{-1}
Corn steep liquor		3.25 mll^{-1}	7.50 mll^{-1}	15.0 mll^{-1}
$ZnSO_4.7H_2O$		0.05 gl^{-1}	0.10 gl^{-1}	0.15 gl^{-1}
$CuSO_4.5H_2O$		1.00 gl^{-1}	2.50 gl^{-1}	4.00 gl^{-1}
$NaNO_3$		0.05 gl^{-1}	0.01 gl^{-1}	0.15 gl^{-1}
Date Syrup		10 gl^{-1}	25 gl^{-1}	40 gl^{-1}

for 5 days in 100 ml of the medium containing: 1.0% glucose, 1.0% starch, 0.75% peptone, 0.75% meat extract, 0.3% NaCl, 0.02% Pronal ST-1(antifoam agent) in 500 ml baffled Erlenmeyer flask and pH 7.2 before sterilization (Fujii et al., 1974).

Production media design and composition

The production medium contained: 6.4% soluble starch, 0.5% glucose, 3.5% soybean meal, 0.75% corn steep liquor, 0.3% NaCl, 0.1% K_2HPO_4, 0.05% $ZnSO_4.7H_2O$, 0.01% $CuSO_4.5H_2O$, 0.2% $NaNO_3$, 0.01% Pronal ST-1 and pH 6.5 before sterilization. Glucose and date syrup were dissolved in water and sterilized by filtration through millipore filter 0.2 micron.

The composition of induction medium was modified based on a combination of substrates reported in the literature (Fujii et al., 1974) and the ratio of the medium contents was adjusted according to the experimental design (Tables 1, 2a and b). The seed medium (15 ml) was inoculated into a 100 ml of the induction medium in a baffled Erlenmeyer flask of 500 ml grown at 27℃ on a rotary shaker (190 rpm).

Sample preparation and analysis

The samples were obtained from the culture filtrate after centrifugation at 17000xg followed by filtration through millipore filter 0.2 µ. One hundred microliters of samples of unknown concentration were placed into the wells in triplicate against known concentration of standard BLM. The plates were placed at 4℃ for 24 h to allow the diffusion of BLM, and then incubated at 37℃ for 18 h. A standard curve was made by plotting diameters of clear zones produced by BLM solutions against standard BLM concentration. BLM concentrations of unknown samples were measured using the standard curve.

Experimental design and data treatment

To investigate the relationship between substrate medium components and their concentrations in order to optimize the production of BLM by the *S. mobaraensis*, an orthogonal design was used. The medium compositions were selected according to the experimental design. The combinations of substrate and concentrations were selected using the design module of StatPlus 2008 Professional. The substrate levels were allocated into three categories: (1) low, (2) medium and (3) high. An additional dummy variable of glucose was included in the experimental design to measure the variability of the experimental design, which will give a direct estimate of the standard error of the effect of different factors. For the RSM and the polynomial regression, the coded values were changed as follows: -1 = low, 0 = medium and 1 = high and the analysis was performed with the experimental design module of Statgraphics plus Version 5.1 (Statpoint Technologies, Inc., War-renton, VA, USA).

Statistical optimization method for fermentation process could overcome the limitations of classic empirical methods and was proved to be a powerful tool for the optimization of the *S. mobaraensis*. In this study, RSM model (the orthogonal design) was proposed to study the combined effects of culture media compositions. Validation experiments were also carried out to verify the validity and the accuracy of the models.

RESULTS

Tables 2a and b summarize the mean value of triple trials for BLM obtained from the experiment in relation to the different treatment conditions. When date syrup was used as an additional carbon source, a higher BLM amount was obtained when compared to glucose (Tables 2a and b). The amount of BLM with added DS achieved 138 mg dl^{-1}. Table 3 shows the analysis of variance (ANOVA) of the results after the 5th day fermentation with an orthogonal design analysis for all medium elements. This indicated that BLM amount could be enhanced using a combination of solutes at different concentrations in the fermentation medium. The date syrup (DS), cupper (Cu), and soyabean (SM) were the most significant factors in the production of BLM followed by corn steep liquor (CS), nitrogen source (NO), zinc (Zn), soluble starch (SS) and finally glucose (GC). The data of most effective elements DS, Cu, SS, SM and CS were analyzed for the main, quadratic and interaction effects by standardized Pareto chart (a special type of bar chart where the values being plotted are arranged in descending order), the most effective elements were confirmed to be the DS and Cu followed by the SM. Table 4 shows the analysis of single and interaction of the five most effective culture medium elements. The following regression equation (Equation

Table 2a. Experimental conditions and mean amount of BLM (+SE, standard error) produced with the orthogonal Main-Effect design for trial ID (1 - 15).

Trial ID	SS	GC	SM	CS	Cu	DS	NO	Zn	BLM mg dl^{-1}	
									Experimental	SE
1	-1	0	0	0	-1	-1	0	-1	101	2.50
2	0	0	1	-1	0	0	1	1	118	1.91
3	0	0	0	0	0	0	1	0	112	2.95
4	-1	0	0	0	1	-1	1	1	128	3.41
5	0	0	-1	0	1	0	-1	-1	132	5.20
6	0	0	1	0	1	0	-1	-1	138	2.48
7	0	-1	0	0	-1	0	-1	1	112	0.00
8	0	0	0	-1	-1	0	-1	-1	96	2.89
9	0	0	1	0	-1	0	1	0	85	4.47
10	0	-1	0	0	1	0	-1	1	129	0.00
11	-1	0	1	0	0	-1	1	-1	100	2.89
12	1	0	1	0	0	1	-1	-1	112	3.64
13	0	0	0	0	0	0	0	-1	105	1.33
14	0	0	1	1	0	0	-1	0	90	3.02
15	0	-1	-1	0	0	0	1	-1	91	2.66

SS, Soluble starch (millet jelly); GC, glucose; SM, soybean meal; CS, corn steep liquor; Zn, $ZnSO_4.7H_2O$; Cu, $CuSO_4.5H_2O$; NO, $NaNO_3$; DS, date Syrup.

Table 2b. Experimental conditions and mean amount of BLM (+SE, standard error) produced with the orthogonal Main-Effect design for trial ID (16 - 46).

Trial ID	SS	GC	SM	CS	Cu	DS	NO	Zn	BLM mg dl^{-1}	
									Experimental	SE
16	0	1	0	1	0	0	1	-1	85	2.48
17	1	0	0	0	1	1	-1	1	124	2.18
18	-1	-1	0	0	0	-1	-1	-1	96	3.12
19	0	0	0	0	0	0	-1	0	85	0.00
20	-1	0	0	1	0	-1	1	1	96	5.00
21	1	0	0	-1	0	1	1	0	115	2.83
22	1	0	-1	0	0	1	1	1	113	2.48
23	0	0	0	1	-1	0	1	1	111	2.96
24	0	0	-1	-1	0	0	-1	1	114	2.18
25	1	1	0	0	0	1	1	0	116	1.52
26	0	0	-1	1	0	0	0	-1	120	2.89
27	0	-1	0	1	0	0	0	1	122	4.28
28	0	0	0	0	0	0	0	0	113	1.20
29	1	-1	0	0	0	1	0	0	120	2.55
30	0	1	0	0	-1	0	0	0	90	1.24
31	0	0	0	1	1	0	1	1	112	2.35
32	-1	0	0	-1	0	-1	1	1	112	2.22
33	1	0	0	1	0	1	-1	-1	115	2.60
34	-1	1	0	0	0	-1	0	0	111	0.00
35	-1	0	-1	0	0	-1	-1	-1	112	2.78
36	0	1	1	0	0	0	1	1	90	3.11
37	0	-1	0	-1	0	0	0	0	98	1.12
38	0	-1	1	0	0	0	-1	-1	99	1.22
39	0	0	0	0	0	0	-1	-1	112	0.95
40	0	0	0	-1	1	0	-1	-1	131	1.11

Table 2b. Contd.

41	0	0	0	0	0	0	-1	-1	100	0.00
42	0	1	-1	0	0	0	-1	-1	108	1.32
43	0	1	0	-1	0	0	0	0	99	2.54
44	0	1	0	0	1	0	0	0	130	1.01
45	0	0	-1	0	-1	0	-1	-1	112	1.00
46	1	0	0	0	-1	1	0	0	90	0.65

SS, Soluble starch (millet jelly); GC, glucose; SM, soybean meal; CS, corn steep liquor; Zn, $ZnSO_4.7H_2O$; Cu, $CuSO_4.5H_2O$; NO, $NaNO_3$; DS, date Syrup.

Table 3. The variance analysis of orthogonal test on optimization of culture medium in shake flask culture.

Substrate	SS*	df	F	P-value
SS	150.06	1	1.72	0.2022
GC	90.25	1	1.03	0.3195
SM	306.25	1	3.50	0.0731
CS	111.23	1	1.02	0.0954
Zn	53.26	1	1.76	0.3300
Cu	9.0	1	0.1	0.7511
NO	46.25	1	1.93	0.9630
DS	3813.06	1	43.58	0.0000
Residual	908.7148	9		

SS* = Sum squares; SS, soluble starch (millet jelly); GC, glucose; SM, soybean meal; CS, corn steep liquor; Zn, $ZnSO_4.7H_2O$; Cu, $CuSO_4.5H_2O$; NO, $NaNO_3$; DS, date Syrup.

Table 4. The analysis of single and interaction of the five most effective culture medium elements (A: SS, B: GS, C: SM, D: Cu AND E: DS) in shake flask culture.

Key	SS*	Df	F-Ratio	P-value
A:SS	150.063	1	1.72	0.2022
B:GC	90.25	1	1.03	0.3195
C:SM	306.25	1	3.50	0.0731
D:Cu	9.0	1	0.10	0.7511
E:DS	3813.06	1	43.58	0.0000
AA	77.4583	1	0.89	0.3557
AB	90.25	1	1.03	0.3195
AC	30.25	1	0.35	0.5618
AD	64.0	1	0.73	0.4005
AE	12.25	1	0.14	0.7114
BB	51.58	1	0.59	0.4486
BC	169.0	1	1.93	0.1768
BD	361.0	1	4.13	0.0530
BE	132.25	1	1.51	0.2303
CC	4.64	1	0.05	0.1897
CD	289.0	1	3.30	0.0811
CE	272.25	1	3.11	0.0899
DD	50.09	1	0.57	0.4563
DE	49.0	1	0.56	0.4612
EE	812.0	1	9.28	0.0054
Total error	2187.17	25		

R-squared = 76.01%; R-squared (adjusted for d.f.) = 56.819%; standard error of est. = 9.35343; mean absolute error = 518931; Durbin-Watson statistic = 1.16265; SS* = sum squares.

1) for the response BLM was obtained:

$$Y= 79.67 + 0.376X_1 + 5.253X_2 + 0.131X_3 - 15.803X_4 - 0.047X_1X_2 - 0.0677X_1X_3 - 0.038X_1X_4 + 0.663X_2X_3 - 0.687 X_2X_4 - 0.483 X_3X_4 \ 0.002X_1^2 - 0.173 X_2^2 + 0.51X_3^2 + 4.424X_4^2 \quad \ldots\ldots\ldots\ldots \quad (1)$$

Furthermore, Table 4 shows the analysis of variance (ANOVA) of the results with an orthogonal design analysis for only five most effective factors: DS, Cu, SS, SM and GC. Using the orthogonal design approach, the relationships between medium component variables and their concentrations on BLM amount could be calculated. The factors could be ranked in importance order as follows: DS, SM, CS, SS, Cu and GC. This pointed to DS and levels of soyabean meal being the most important factors in determining BLM amount. Table 5 presents the optimal combination and concentration of substrates required to achieve the highest BLM amount. A search was carried out to identify the optimum components for maximizing amount. The expected optimum BLM amount that this optimization could produce was 138.5 mg dl^{-1} (Table 6). Figure 1 presents a graphical analysis based on the effect of the mean BLM amount under the sets of conditions and treatment levels tested in the present study. This describes the most important factors which determine BLM production and shows the impact of changing treatment levels on production. The main effect of DS on the production of BLM was determined to be the highest effect among the tested medium constituents (Figure1). In order to find the optimum and statistically significant interactions between factors, factors were recoded to perform RSM. A first order polynomial model with interactions was selected. This confirmed the robustness and reproducibility of the experiment as confirmed by Equation (1).

The analysis of the model produced an R^2 of over 99%

Table 5. Optimal medium design for the production BLM and forecasted production.

Substrate	Level
Soluble starch (millet jelly)	80.0
Glucose	5.0
Soybean meal	1.75
Corn steep liquor	15.0
$ZnSO_4.7H_2O$	0.15
$CuSO_4.5H_2O$	2.5
$NaNO_3$	0.15
Date Syrup	40

and overall model significance of $P < 0.0001$. The model from the analysis consisted of an intercept, 5 main factor terms and 10 interception terms, thus including a total of 15 terms. The effect of factors, standard error, P-values and 95% confidence levels of the parameter variability (parameter certainty) are presented in Table 4. The effect are ranked by P-values from the most to the least significant. In the polynomial analysis, BLM yield was shown to be affected principally by the interaction between level of carbon sources (DS) and soybean meal. The yield of BLM (138.5 mg/ml) had a high correlation coefficient ($R^2 = 0.99$) with the predicted values (132.64) with a residual of 6.14. This showed that there were no significant violations of the model found in the analysis, with a good correlation of the model with the experimental data obtained. Illustrations of the critical effects and interactions between factors were identified by producing response surface plots. Such diagrams represented the production of BLM (mg dl^{-1}) as a function of substrate type or concentration of two nutrients, with the other factors kept at constant levels, which clearly demonstrated the reproducibility and robustness of the polynomial model based on the experimental data (Figure 2). To determine the most adequate operating conditions and analyze the process for yield, the response surface plots were plotted using the polynomial equation for all the combinations possible. Overall, date syrup was the best carbon source used (Figure 2). The optimum nitrogen source was soyabean meal (SM) which gave yields of 138 mg dl^{-1} of BLM (Figure 2I). Copper (Cu) has an intermediate level ranged from 128 to138 mg dl^{-1} BLM (Figure 2J). To confirm the optimal conditions (Table 6), a set of three replicate experiments with the optimal combination of substrates and concentrations were used as a confirmation of the forecasted production of BLM. The confirmatory experiments produced 138.5 mg dl^{-1} of BLM. The use of the modified medium enhanced the BLM productivity by 73.1% more than the highest amount reported of BLM in the literature of 80 mg dl^{-1} without addition of amines (Chen and Stubbe, 2004).

DISCUSSION

Date syrup was previously used to increase production of citric acid by fermentation (Roukas and Kotzekidou, 1997). The total sugar content of datessyrup exceeds 80%. The analysis of sugar contents of dates syrup showed fructose (41%), glucose (39%) and traces of sucrose (Khiyami et al., 2008). Furthermore, date syrup contains macro-elements: Ca, P, Na, K and Mg beside microelements Fe, Zn, Cu, and Mn (Al-Hooti et al., 2002). It is well known that the conventional optimization technique, e.g. one-factor-at-a-time method, is not only tedious and time-consuming, but also misleading of result interpretation, especially for the interactions among different factors which they are unable to detect. The orthogonal array method, coupled with variance analysis, has proved to be a cost-effective optimization strategy that can be used to assign experimental factors in a series of experimental trials (Doornbos and Haan, 1995; Ferreira et al., 2004; Imandi et al., 2007), but it cannot fit the results into a regression equation to locate the optimum level through the entire space of the tested independent variables. The response surface methodology is an efficient statistical technique for the optimization of multiple variables in order to predict the best conditions with a minimum number of experiments. In comparison with orthogonal design and variance analysis, Box-Behnken design, one of the designs of the RSM allows calculations to be made of the response at intermediate levels which were not experimentally studied. A three-level Box-Behnken design was employed in the present study and the optimal conditions were determined thro-ugh a minimal experiment number compared with other designs. To investigate the relationship between substrate medium components and their concentrations to optimize the production of BLM by the *S. mobaraensis*, an orthogonal design was used. To achieve the results obtained in this study using a full factorial design, it would have required 3^8 x 2 replicate (13122) experiments taking into account all the variables involved. However, by using the Box-Behnken designs, a significantly smaller combination of factors and levels could be used for effectively examining the effect of interacting factors on final yield. These were formed by combining 2^n factorials with balanced incomplete block designs, which reduces the number of experiments considerably. Thus, with only a limited number of experiments (46), an optimal medium com-position was found that represented a 73.1% increase in amount compared to the non-optimized medium. The BLM amount achieved in this work of 138.5 mg dl^{-1} represented a significant improvement when compared to the highest reported in the literature (Chen and Stubbe, 2004). Moreover, a considerable improvement in productivity (73.1%) was achieved because the new medium was designed to achieve a more rapid BLM production with suitability for industrial applications. These kinds of designs have been successfully applied to improving media formulation for

Table 6. Observed and predicted values of the response for the optimized medium.

Response	Observed value	Predicted Value	Residual
(Y) BLM production mg dl^{-1}	138.5	132.364	6.136

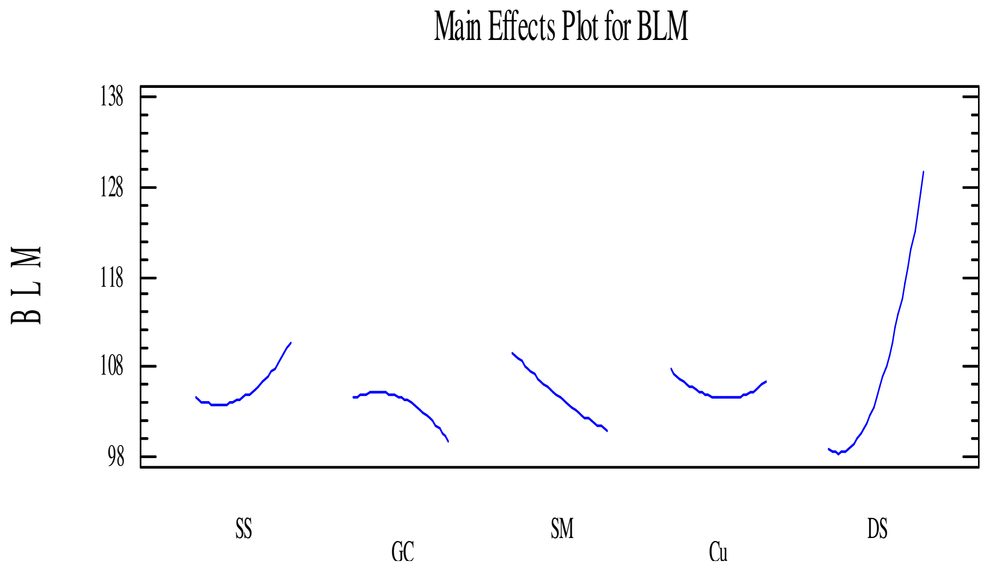

Figure 1. Graphical analysis of the relationship between media formulation and the amount of BLM. Each factor had three coded levels and the (○) represent mean BLM produced. The factors levels coded are: low (-1), medium (0) and high (+1) as presented in Table 1.

the production of primary and secondary metabolites in fermentation processes (Lee et al., 1997; Escamilla et al., 2000; Li et al., 2001; Xu et al., 2003). For example, yields of cholesterol- lowering drug, lovastatin, produced by *Aspergillus terreus,* were improved with Plackett-Burman screening factorial designs (Lai et al., 2003) and also monooxygenase production by *Escherichia coli* (Lu and Mei, 2007). Furthermore, a combined orthogonal design and contour surfaces methodology were used for optimization of cholesterol lowering drug sequalestin S1 (Parra et al., 2005). The combined statistical strategies of orthogonal design and surface response succeeded in predicting the optimal condition and interactions between substrate type and concentrations.

This work demonstrated the efficacy of the combined methodology achieving the maximum BLM amount reported in liquid medium. It produced a rapid screening and reliable methodology for the optimization of medium design. The response surface plot was a good tool to separate the effect of interactions and to find the optimal conditions between two factors. Evaluating the response surface plot provided a better understanding of the interactions between the fermentation medium substrates and their effects on BLM amount. The R^2 values of all parameters gave a good fit between the model and the experimental data (Equation 1). The response surface plot facilitated easy location of optimal parameters. This combined approach for medium optimization of BLM production by the *Streptomyces species* has not been reported previously. In production of sequalestin S1 by *Phoma* sp., glucose was used as a dummy factor for an *in situ* evaluation of the reproducibility and robustness of the experiment (Parra et al., 2005). In our study, the use of glucose as a dummy factor did not succeed because DS contains sufficient amount of glucose that cover the growth of streptomycetes. Date syrup was also found to be a better substrate for the production of BLM and the productivity is proportional to the amount of date syrup (Figure 2D). Meanwhile, excess of Cu ion was found to reduce the production of BLM (Figures 2F and H). Date syrup contains 39% of glucose (Khiyami et al., 2008); therefore, glucose had no effect in the presence of DS on the production of BLM (Figure 2G). Moreover, the production of BLM started after three days and reached the maximum yield after seven days (data not shown), which suggests that catabolic repression of glucose did not occur (Pan and Xu, 2003). This leads to the conclusion that a combined strategy of orthogonal design and response surface plot is an excellent means for fermentation medium optimization in the production of pharmaceutical compounds and enzymes. The combined strategy demonstrated advantages in comparison with traditional methods. In trace element, however, the medium required the addition of some important new components

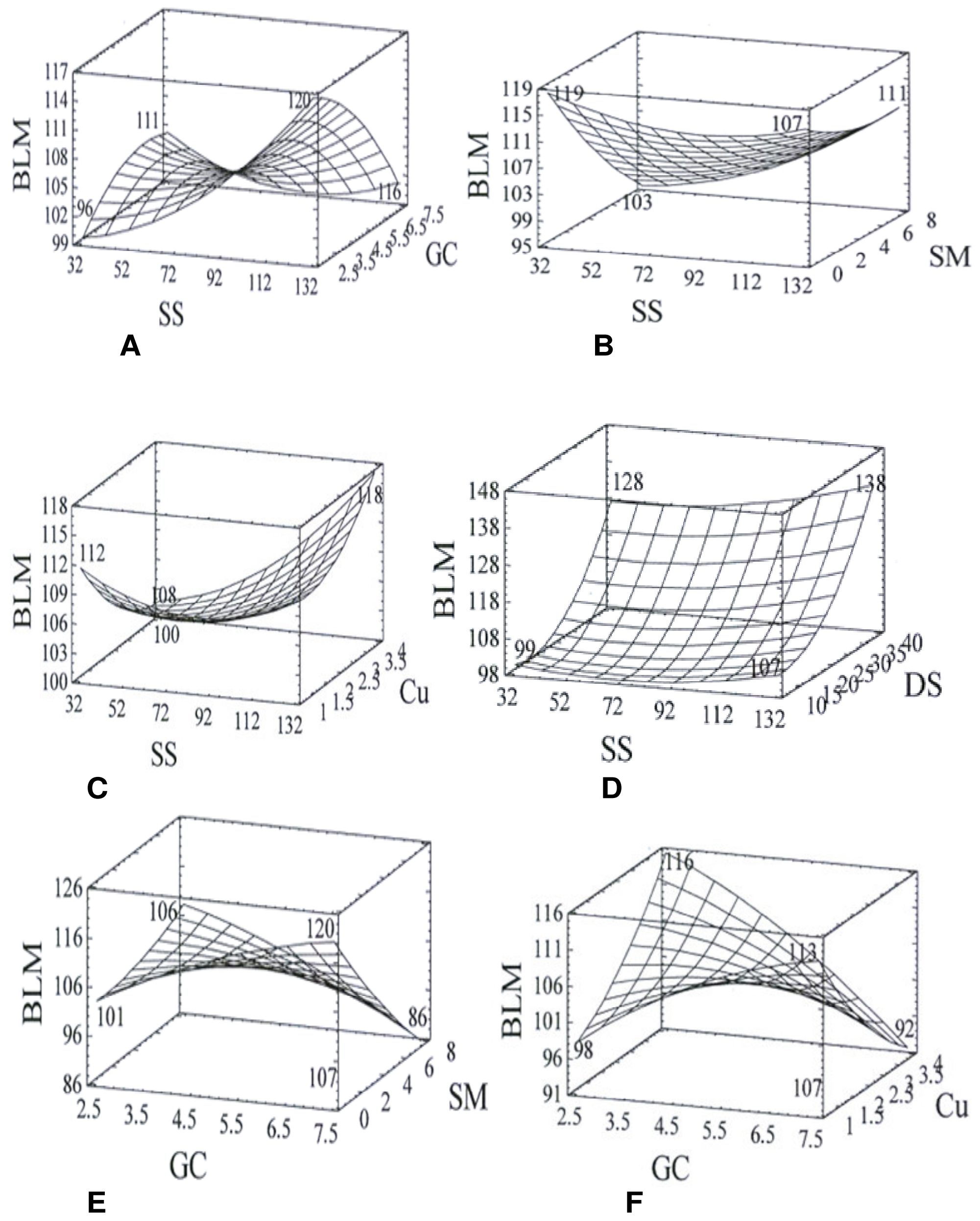

Figure 2. Response surface plot showing the effect of major factors on BLM production. (A) SS and GC; (B) SS and SM; (C) SS and Cu; (D) SS and DS; (E) GC and SM; (F) GC and Cu; (G); GC and DS; (H) SM and Cu; (I) SM and DS, and (J) CU and DS.

such as nitrogen, magnesium and iron as part of the improved medium for enhancing BLM amount (Xu et al., 2003; Lee et al., 1997).

Conclusions

In addition to establishing optimal fermentation medium composition for scale up, the present work makes it possible to predict both amount and productivity under different conditions by means of the contour surfaces and the polynomial model. This is useful not only for the additional knowledge supplied about the process, but also for the potential in medium engineering and evaluation under economic constrains of medium composition, yield and productivity. The results strongly support the use of RSM for optimization of fermentation conditions. The chosen method of optimization of fermentation condition was efficient, relatively simple, time and material saving. This work should help to build more rational control strategy, possibly involving scale-up of production of antibiotics by *S. mobaraensis*. Date syrup was approved

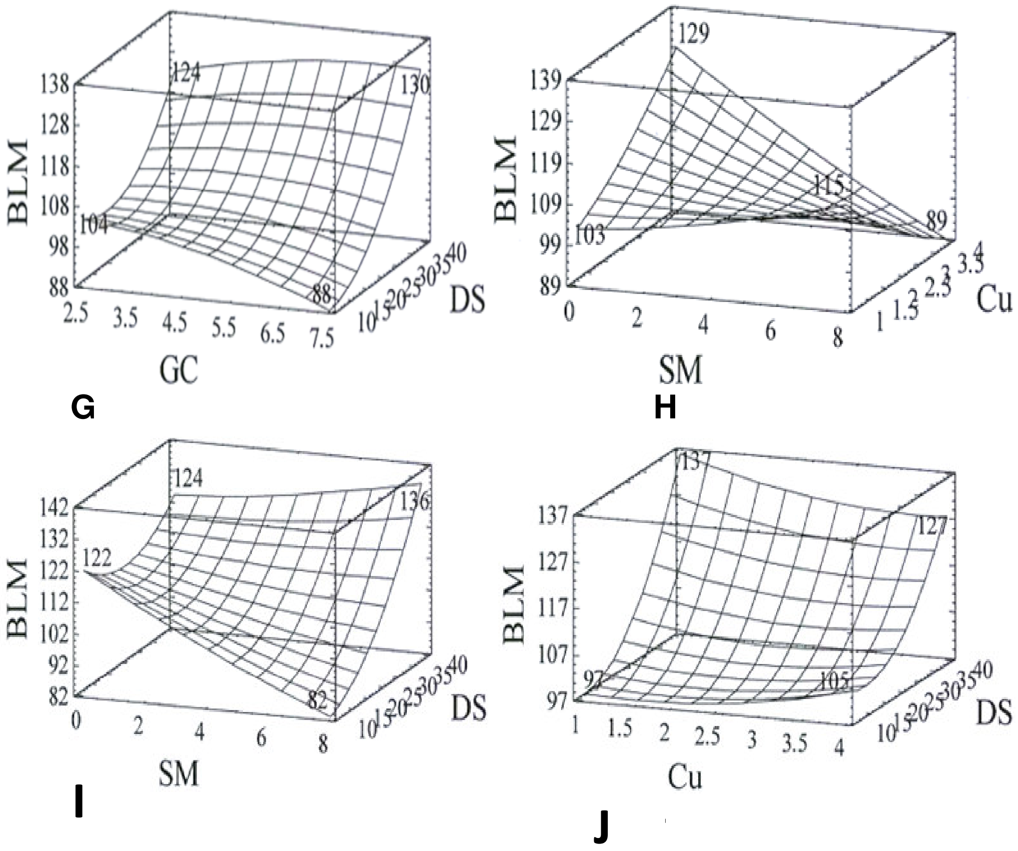

Figure 2. Continued.

to be a good carbon source and is sufficient to support microbial growth.

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