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Full Length Research Paper

# Optimization of fermentation medium for enhanced production of milbemycin by a mutant of *Streptomyces bingchenggensis* BC-X-1 using response surface methodology

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**A new strain *Streptomyces bingchenggensis* BC-X-1, in which one of the impute was disappeared had been screened by N-methyl-N'-nitroso-N-nitroso-guanidine (NTG) mutation. In order to improve the yield of milbemycin by the new strain, response surface methodology was employed to optimize the composition of fermentation medium. The Plackett-Burman design indicated that yeast extract, soybean flour,  $\text{KH}_2\text{PO}_4$ ,  $\text{FeSO}_4$  and  $\text{CaCO}_3$  had significant effects on milbemycin production. The concentrations of these five components were investigated using Box-Behnken design and a polynomial model related to medium components concentration effect on milbemycin yield had been established. Analysis of variance (ANOVA) showed a high coefficient of determination ( $R^2$ ) value of 0.9701, which indicated that good agreement between the experimental and predicted values of milbemycin yield. Student's T-test of each coefficient showed that all the linear and quadratic terms effect were significant. The optimum concentrations of components: yeast extract, soybean flour,  $\text{CaCO}_3$ ,  $\text{FeSO}_4$ ,  $\text{KH}_2\text{PO}_4$  were 2.58, 2.58, 0.40, 0.0058 and 0.088%, respectively. The yield of milbemycin reached  $1110 \pm 98 \mu\text{g/ml}$ , which was 2.61-fold of the initial yield.**

**Key words:** Milbemycin, *Streptomyces bingchenggensis*, medium optimization, response surface methodology.

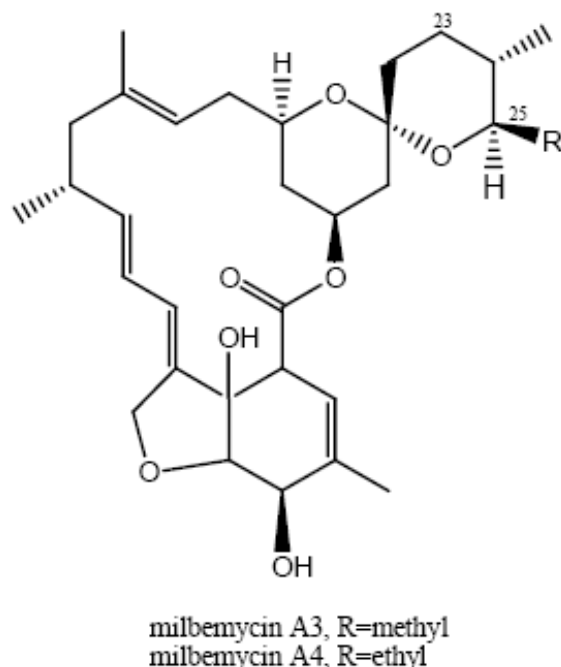
## INTRODUCTION

As macrolide antibiotics, milbemycins are a class of widely used anathematic and insecticidal agents. A mixture of milbemycin A<sub>3</sub> and A<sub>4</sub> (Figure 1) were marketed as an acaricide for the control of mites in 1990 (Ide et al., 1993). First reported to have been isolated from produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus* as part of an insecticidal screening program, further milbemycins have since been isolated from the original strain, its mutants and a number of other streptomycetes (Warr et al., 1994). The structures of the milbemycin A<sub>3</sub> and A<sub>4</sub> are similar to other 16-membered lactones, that is, avermectins, ivermectins, doramectin and moxidectin. But the difference among them is a bisoleandroxyloxy substituent found at 13-position of

milbemycins in the macrolide ring (Shoop et al., 1995). After the activity on nematodes was found, especially on *Dirofilaria*, demand for the milbemycin as in veterinary treatment and the production has been required (Okada and Iwamatu, 1997).

The optimization of fermentation medium is very important part of the development of fermentation processes (Krouse 1999). However, the conventional methods of optimizing medium composition "one-variable-at-a-time" was used more often than other approaches, but it is time-consuming and often fails to identify the optimal factors because interactions among different factors are neglected. In the process of screening the most suitable fermentation medium, some statistical techniques, such as Plackett-Burman design, fractional factorial designs (FFD) and Taguchi method which can evaluate the effects of many factors and determine the interaction of the factors are employed (Peter et al., 2006; Pio and Macedo, 2008; Teng and Xu, 2008), also the response

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**Figure 1.** Structures of milbemycin A3 and A4.

surface methodology (RSM) has some advantages, including less experiment numbers, suitability for multiple factor experiment numbers, search for relativity among factors and finding of the most suitable condition and forecasting response (Chang et al., 2006; Zhao et al., 2008; Alam et al., 2008; Chou et al., 2010). These statistical methods were proved to be useful for developing the medium to improve the production, optimizing processes and bioconversion which were extensively used in the industries (Singh et al., 2009; Deepak et al., 2008; Gao et al., 2009; Ghosh and Hallenbeck, 2010; Goswami et al., 2009).

*Streptomyces bingchenggensis* was isolated from a soil sample collected in Harbin, China. *S. bingchenggensis* produces at least two kinds of antibiotics, the nanchangmycin and the milbemycins including milbemycin A<sub>3</sub> and A<sub>4</sub>,  $\beta_{13}$ ,  $\beta_{14}$ ,  $\alpha_{28}$ ,  $\alpha_{29}$ ,  $\alpha_{30}$  and two new seco-milbemycins (Xiang et al., 2007a, b, 2008). *S. bingchenggensis* BC-X-1 was isolated from N-methyl-N'-nitroso-N-nitroso-guanidine (NTG) treated mutants. This strain did not produce nanchangmycin as that of the ancestral strain. But the yield of milbemycin was very low. This study aimed at optimizing the culture medium composition for milbemycin A<sub>3</sub> and A<sub>4</sub> production by using response surface methodology (RSM). In the first step, in order to evaluate the effect of nitrogen sources, trace element and CaCO<sub>3</sub> for the maximum production of milbemycin, Plackett-Burman design was applied to determine the most important factors. In the second step, a Box-Behnken design was used to optimize the levels of these controllable factors.

## MATERIALS AND METHODS

### Microorganism and cultural medium

*S. bingchenggensis* BC-X-1 was isolated from *S. bingchenggensis* by N-methyl-N'-nitroso-N-nitroso-guanidine (NTG) mutation. *S. bingchenggensis* has been deposited at the China General Microbiology Culture Collection Center (Accession No: CGMCC1734) and we have determined the 16S rDNA sequence (Accession No: DQ449953 in National Center for Biological Information). The BC-X-1 was maintained in the solid medium containing sucrose 1.0%, skim milk 0.2%, yeast extract 0.4%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, K<sub>2</sub>HPO<sub>4</sub> 0.05%, agar 2.0%, pH 7.2; the slants were subcultured every 12 days at 28°C and 40% relative humidity (Wang et al., 2009).

### Fermentation

The mycelium and spores on the slant were inoculated into a 250 ml Erlenmeyer flask (2~6×10<sup>7</sup> spores/ml) containing 25 ml of the seed medium (sucrose 1.0%, polypepton 0.35%, K<sub>2</sub>HPO<sub>4</sub> 0.05%, skim milk 0.2%, pH 7.2) and cultured in a rotary shaker (250 rpm) at 28°C for 44 h, then 1.5 ml of the seed culture was incubated into a 250 ml Erlenmeyer flask containing 25 ml of designed fermentation medium. Non-optimized fermentation medium was composed of: sucrose 16.0%, soybean flour 2.0%, yeast extract 0.5%, meat extract 0.5%, K<sub>2</sub>HPO<sub>4</sub> 0.05%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.005% and CaCO<sub>3</sub> 0.3% (Wang et al., 2009), the medium did not suit the new strain and the milbemycin production was only 425 µg/ml. The designed fermentation medium adjusted to pH= 7.2 were sterilized by autoclaving for 30 min at 121°C. And the fermentation culture was incubated for 10 days at 28°C in a rotary shaker (250 rpm).

### Extraction and HPLC analysis of the milbemycin

After incubated for 10 days, the maximum production of milbemycin was achieved. The samples were collected from fermentation flask and mixed one volume of the whole culture with four volumes of ethanol, and then sonicated at room temperature for 15 min. Samples were centrifuged at 3000 rpm for 15 min. Then the supernatant was filtered through glass fiber filter paper (Wang et al., 2009). The supernatant analysis was carried out by high-performance liquid chromatography (HPLC) using a reversed-phase column (Agilent Eclipse Plus C18, 1.8 µm, 3.0× 50 mm) eluted at 0.65 ml/min flow rate with a 15 min linear gradient from 75 to 90% (V/V) of Phase B. Phase A was ammonium formate solution (0.6 g/l) and Phase B was acetonitrile (90%, V/V). The chromatography was performed with an Agilent 1200 HPLC system and the elution was detected at 242 nm (detector type: SL G1314B Agilent 1200).

### Biomass assay

The packed biomass volume was used as a measure of biomass concentration. After the centrifugation of 10 ml of fermentation broth at 3000 g for 15 min, the packed mycelium volume (PMV) was obtained.

### Experimental design and data analysis

In order to obtain the most influential factors for milbemycin production, various carbon and nitrogen sources and elements trace were evaluated by 'one-at-a-time' approach. The best carbon source was sucrose and the concentration was 14% (data not

**Table 1.** Plackett–Burman design for screening variables in milbemycin production.

Factor (%)	Code	Low-level (-1)	High-level (+1)	Effect	t-value	p-value
Yeast extract	A	2	3	127.00	13.92	0.005
Meat extract	B	0.5	1.5	-13.80	-1.51	0.269
Soybean flour	C	1.5	3	168.20	18.44	0.003
CaCO <sub>3</sub>	D	0.25	0.35	314.80	34.51	0.001
MgSO <sub>4</sub>	E	0.03	0.07	-13.20	-1.45	0.285
FeSO <sub>4</sub>	F	0.003	0.007	78.20	8.57	0.013
ZnCl <sub>2</sub>	G	0.003	0.007	-23.80	-2.61	0.121
CuSO <sub>4</sub>	H	0.003	0.007	-25.20	-2.76	0.110
CoCl <sub>2</sub>	I	0.003	0.007	0.20	0.02	0.984
K <sub>2</sub> HPO <sub>4</sub>	J	0.05	0.1	-16.00	-1.75	0.222
KH <sub>2</sub> PO <sub>4</sub>	K	0.05	0.1	236.80	25.96	0.001
VB <sub>1</sub>	L	0.05	0.1	-20.80	-2.26	0.152
NaCl	M	0.2	0.8	-26.80	-2.94	0.099
NH <sub>4</sub> NO <sub>3</sub>	N	0.5	1	4.40	0.48	0.677
NaNO <sub>3</sub>	O	0.5	1	-12.80	-1.40	0.296
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	P	0.5	1	8.80	0.96	0.436
NH <sub>4</sub> Cl	Q	0.5	1	-31.60	-3.46	0.074

show); The preliminary experiments revealed that the nitrogen source including yeast extract, meat extract, soybean flour, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NaNO<sub>3</sub> and NH<sub>4</sub>NO<sub>3</sub> were suitable for the production of the new strain in some degree; the KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> were influencing the milbemycin production of milbemycin obviously; the element trace including MgSO<sub>4</sub>, CoCl<sub>2</sub>, FeSO<sub>4</sub>, CuSO<sub>4</sub>, ZnCl<sub>2</sub>, VB<sub>1</sub> and NaCl can also affect milbemycin production. The CaCO<sub>3</sub> used to adjust the fermentation pH was also concerned. These components were chosen for further optimization.

#### Plackett-Burman design

A Plackett-Burman design was used to screening the most significant fermentation parameters affecting the new strain production (Robert et al., 2006). The experimental design for screening of medium components is shown in Tables 1 and 2. The milbemycin production was carried out in triple times and the average value was taken as the response. The variables with confidence levels above 95% were considered to have significant effect on milbemycin production and thus, used for further optimization. The analysis of variance (ANOVA) for the data and the model coefficients were computed with Minitab 15.0 (Minitab Inc., Pennsylvania, USA) software.

#### Optimization by response surface methodology (RSM)

Further medium optimization by RSM was concerned with the five important components ( $P < 0.05$ ) and the negative factors were removed. Box-Behnken in Minitab 15.0 was used to optimize the concentration of the five factors. Each factor was tested at three levels (Table 3). All experiments (Table 4) were performed in triple times and the milbemycin production as the response variable was analyzed using Minitab 15.0.

#### Statistical analysis of data

The data of the response value (milbemycin production) were

subjected to analysis of variance (ANOVA) using Minitab 15.0 to estimate t-value, P-value and confidence levels. Optimal values of milbemycin production and concentration of important components were estimated using the solver of Minitab 15.0.

## RESULTS AND DISCUSSION

### Screening of important factors by Plackett-Burman design

Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of factors in a few experiments; it also saves time and maintains convincing information on each component (Sharma and Satyanarayana, 2006; Li et al., 2007). The most important factor was determined by the  $P$ -value ( $P < 0.05$ ) and  $T$ -value ( $T > 0$ ) evaluation of each individual effect. As seen from Table 1, a wide variation of milbemycin concentrations from 96.9 to 703.0  $\mu\text{g/ml}$  reflected the importance of medium optimization to attain higher yields. Based on the statistical analysis, the effects of yeast extract, soybean flour, CaCO<sub>3</sub>, FeSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> on the milbemycin were identified as influencing the production significantly ( $P < 0.05$ ). The coefficient of determination  $R^2 = 0.9925$  in the experimental model means that the model could explain 99.25% of the total variations in the system.

All nitrogen sources can be divided into two groups; inorganic nitrogen sources are regarded as quick metabolized nitrogen sources, which are beneficial for fast microorganism growth relieving the need of long-time accumulation of product. Simultaneously, the organic nitrogen sources are sustainable nitrogen sources, which are beneficial for steady product accumulation. So the

**Table 2.** Plackett-Burman experimental design matrix for screening of medium components of milbemycin production.

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Production
1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	495.8
2	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	662.3
3	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	340.4
4	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	353.7
5	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	703.0
6	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	273.8
7	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	455.1
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	96.9
9	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	209.4
10	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	128.8
11	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	540.2
12	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	130.24
13	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	299.7
14	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	204.2
15	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	551.3
16	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	314.5
17	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	493.6
18	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	303.4
19	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	378.9
20	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	481.0

**Table 3.** Levels of response surface methodology.

Factor (%)	Code	Low-level (-1)	Center point (0)	High-level (+1)
Yeast extract	A	1	2.5	4
Soybean flour	C	1.5	2.25	3
CaCO <sub>3</sub>	D	0.15	0.3	0.45
FeSO <sub>4</sub>	F	0.003	0.005	0.007
KH <sub>2</sub> PO <sub>4</sub>	K	0.05	0.075	0.1

organic nitrogen sources in the medium (yeast extract and soybean flour) are very important for sustaining the levels of usable nitrogen sources in the long cultivation period (10 d) of milbemycin. Yeast extract is benefited for the production of antibiotics and enzymes (Gao et al., 2009; Maeda et al., 2010; Niladevi et al., 2009). There are very complicated molecular mechanisms and regulation in *Streptomyces* (Merrick and Edwards, 1995; Hodgson and Wellington, 1995; Amon et al., 2010).

The biosynthesis of many different type of antibiotics and other secondary metabolites is regulated by phosphate, the secondary metabolites occurs only under phosphate-limiting nutritional conditions (Martin, 2004). Extracellular phosphate is known to affect antibiotics production of several strains (Sarrà et al., 1999; Gonzalez et al., 1990) and it also control over the nitrogen metabolism in *Streptomyces coelicolor* (Rodriguez-Garcia et al., 2009). So phosphate play a key role in the fermentation of milbemycin, it is observed Pareto chart

(Figure 2) directly.

Fe<sup>2+</sup> is the important part of cytochrome P450 which is contacted with forming A3 and A4, the cytochrome P450 could close the furan ring at C8a by introducing an oxygen atom to the allylic methyl residue (Ikeda and Okazaki, 1997). The fermentation of milbemycin failed without Fe<sup>2+</sup> in our study. But high level concentration of Fe<sup>2+</sup> was also toxic to cell. The CaCO<sub>3</sub> had the most positive influence on the production from the Pareto chart by sustaining the fermentation broth pH. Actually, the broth pH was very stable in the process of fermentation using the CaCO<sub>3</sub>.

### Optimization by RSM

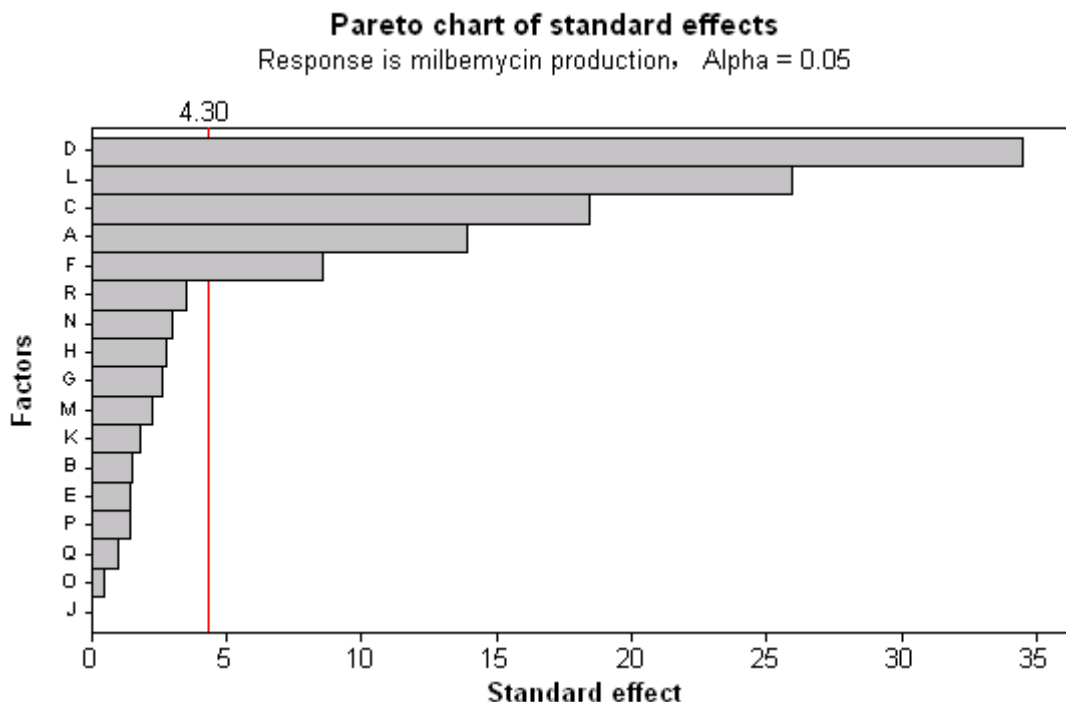
Based on the results of Plackett-Burman design, five components were selected for further optimization. Table 4 shows the experiments designed by RSM. The

**Table 4.** Box-Behnken experiments design matrix with experimental values of milbemycin production.

Run	A(yeast extract)	C(soybean flour)	D(CaCO <sub>3</sub> )	F(FeSO <sub>4</sub> )	K(KH <sub>2</sub> PO <sub>4</sub> )	Y(production)
1	0	0	0	0	0	1085
2	0	0	-1	0	-1	581
3	-1	0	0	1	0	890
4	1	0	1	0	0	1000
5	-1	0	1	0	0	1024
6	0	0	0	1	1	1084
7	0	0	1	0	-1	998
8	0	0	0	-1	-1	711
9	0	0	1	1	0	1124
10	0	0	1	0	1	1112
11	0	0	0	1	-1	807
12	0	-1	0	0	-1	666
13	0	0	0	0	0	1062
14	0	0	1	-1	0	1032
15	-1	0	0	0	1	980
16	0	1	0	0	1	1050
17	0	-1	0	1	0	854
18	0	1	-1	0	0	767
19	0	0	-1	1	0	765
20	0	-1	-1	0	0	627
21	-1	0	-1	0	0	664
22	-1	0	0	0	-1	706
23	-1	1	0	0	0	931
24	1	0	-1	0	0	825
25	-1	-1	0	0	0	757
26	1	0	0	1	0	950
27	0	-1	1	0	0	875
28	0	0	-1	-1	0	672
29	1	0	0	0	-1	816
30	0	1	1	0	0	1071
31	0	0	0	0	0	1083
32	0	0	0	0	0	1045
33	0	1	0	0	-1	855
34	0	-1	0	0	1	944
35	0	0	0	0	0	1045
36	-1	0	0	-1	0	797
37	0	0	0	0	0	1012
38	1	1	0	0	0	1028
39	0	1	0	1	0	1033
40	1	0	0	0	1	1090
41	1	-1	0	0	0	874
42	0	1	0	-1	0	944
43	1	0	0	-1	0	907
44	0	0	-1	0	1	857
45	0	0	0	-1	1	917
46	0	-1	0	-1	0	760

milbemycin production as the response variables was used to obtain the following experiential model through multiple regression analysis:

$$Y=1055.33 + 46.31A + 82.62C + 154.87D + 47.94F + 118.38K - 77.44A^2 - 94.35C^2 - 97.85D^2 - 76.77F^2 - 82.85K^2 - 5AC - 46.25AD - 12.5AF + 14CD - 1.25CF -$$



**Figure 2.** Pareto chart of 17-factors standard effects on milbemycin production.

**Table 5.** ANOVA results of the RSM experiential model.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	Pr >  T
Model	20	966285	966285	48324	47.67	0.000
Linear	5	788298	788298	157660	155.54	0.000
Quadratic	5	158371	158371	31674	31.25	0.000
Cross product	10	19615	19615	1962	1.94	0.088
Error	5	3781	3781	756		
Total	45	991625				

$$20.75CK - 0.25DF - 40.5DK + 17.75FK$$

Where, Y is the predicted milbemycin production; A, C, D, F and K is the yeast extract, soybean flour,  $\text{CaCO}_3$ ,  $\text{FeSO}_4$  and  $\text{KH}_2\text{PO}_4$ , respectively.

The statistical significance of this model was verified by variance analysis (ANOVA) using Minitab 15.0. As listed in Table 5, the high F-value and the low probability ( $P > |T| < 0.05$ ) indicated that the experimental model was in good agreement with the experimental results (Jo et al., 2008). The ANOVA showed that the linear, quadratic and cross product terms (between A and D; D and K) revealed that there were obvious interactions among the five factors. The coefficient of determination ( $R^2 = 97.44$ ) in the experimental model indicated a good agreement between experimental results and predictions (Guo et al., 2009). The adjusted determination coefficient ( $R^2 = 95.40\%$ ) was also satisfactory to confirm the significance

of the model.

The significance of each coefficient in the experimental model was determined by T-value and the probability of  $Pr > |T|$  using Minitab 15.0. The results are shown in Table 6. A high T-test value and a low probability indicated a high significance (Niladevi et al., 2009). Student's T test of each coefficient of the model showed all five linear and five quadratic terms have significant effects ( $P > |T| < 0.05$ ) on milbemycin production. The interactive effect of A and D; D and K was also significant, while the interactive effect of any other two factors was not significant.

These five important medium components were further analyzed using 3D response surface plots, which were the graphical representations of the regression model. The optimum values of the variables were obtained by simulating the experimental results using the empirical model. From the 3D response surface plots, the interactions between any two factors were convenient to

**Table 6.** Student's T-test of the experimental model.

Factor	Estimate	Standard error	T-value	Pr >  T
A	46.31	7.959	5.819	0.000
C	82.62	7.959	10.381	0.000
D	154.87	7.959	19.458	0.000
F	47.94	7.959	6.023	0.000
K	118.38	7.959	14.873	0.000
A×A	-77.44	10.777	-7.186	0.000
C×C	-94.35	10.777	-8.755	0.000
D×D	-97.85	10.777	-9.080	0.000
F×F	-76.77	10.777	-7.124	0.000
K×K	-82.85	10.777	-7.688	0.000
A×C	-5.00	15.919	-0.314	0.756
A×D	-46.25	15.919	-2.905	0.008
A×F	-12.50	15.919	-0.875	0.440
A×K	0.00	15.919	0.000	1.000
C×D	14.00	15.919	0.879	0.388
C×F	-1.25	15.919	-0.079	0.938
C×K	-20.75	15.919	-1.304	0.204
D×F	-0.25	15.919	-0.016	0.988
D×K	-40.50	15.919	-2.544	0.018
F×K	17.75	15.919	1.115	0.275

understand and their optimum levels could also been obtained.

When Y (milbemycin production) was observed as response variable to the interaction of A (yeast extract) and C (soybean flour), the rest of the parameters were 0 (at central points), it was observed that there was a maximum production at yeast extract and soybean flour concentrations between central and maximum levels (Figure 3a). So the maximum production of milbemycin was obtained by optimizing the value of the yeast extract and soybean flour. The rest of the medium components had the same course (Figure 3b to j). So the experimental model had a stationary point, the predictive milbemycin production was the maximal value in the stationary point.

### Validation of the optimized medium component

On the basis of medium optimization, the quadratic model predicted that the maximum production of milbemycin was 1168.4, when the code of A, C, D, F, K was 0.051, 0.434, 0.697, 0.374 and 0.535, respectively. After translating these coded values, the concentration of yeast extract, soybean flour,  $\text{CaCO}_3$ ,  $\text{FeSO}_4$  and  $\text{KH}_2\text{PO}_4$  was calculated as 2.58, 2.58, 0.40, 0.0058 and 0.088%, respectively. To verify the predicted results, validation experiment was performed in triple times. The observed experimental production of milbemycin was  $1110 \pm 98$   $\mu\text{g/ml}$  using the optimized component concentration. It suggested that experimental and predicted values were in good agreement.

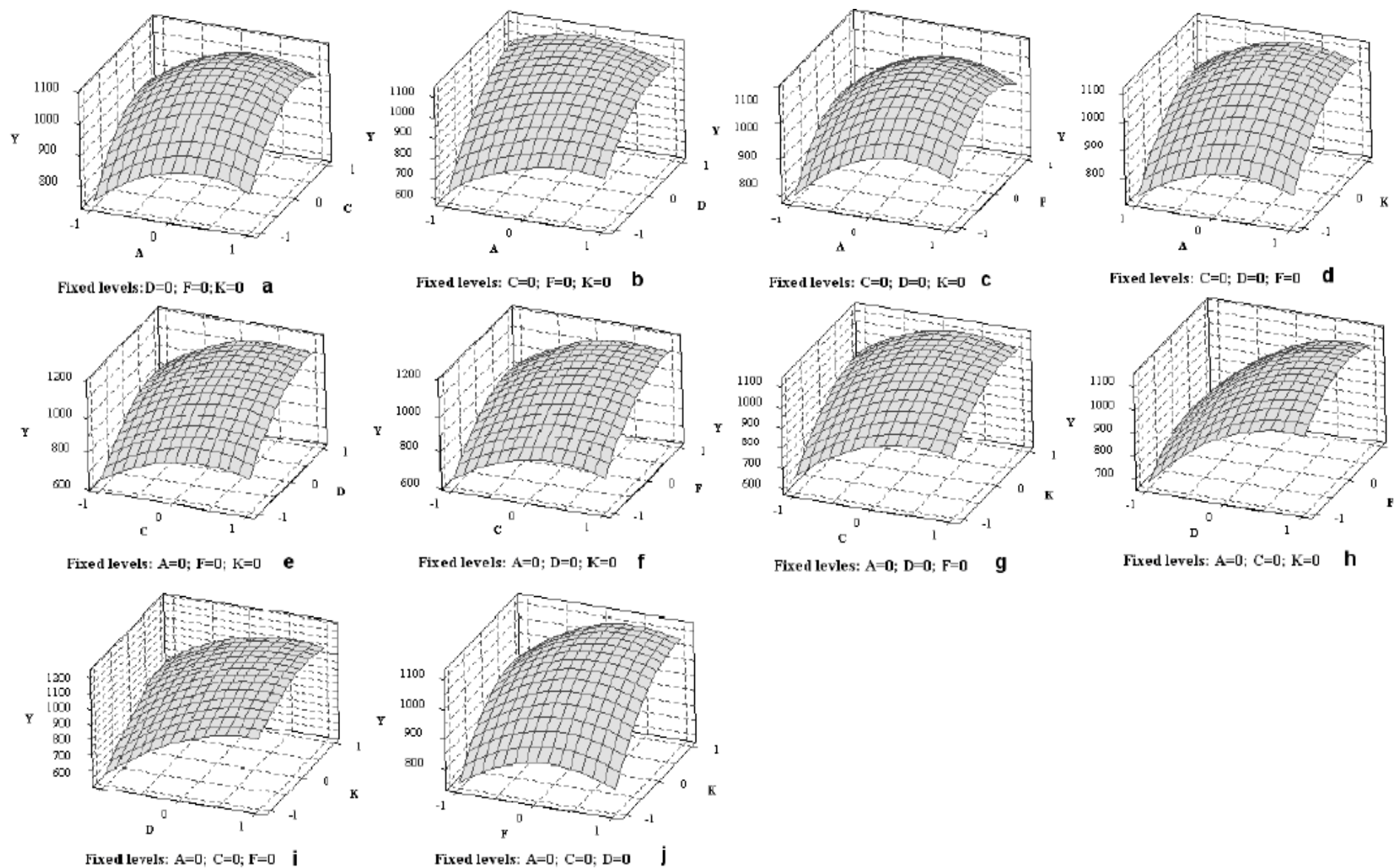
### Comparative analysis of optimized medium and non-optimized medium

The time courses of the production of milbemycin and biomass of *S. bingchenggensis* BC-X-1 were investigated under different cultures. Figure 4a and b shows the differences of biomass associated product forming in the two mediums. The mycelium growth increased in the first 4 days and sustained stable in the last 6 days with the milbemycin accumulation steadily in optimized medium. The fermentation was biphasic. In our previously studies, *S. bingchenggensis* produced large amount of milbemycin at the later stages of the exponential growth phase and stationary phase (Wang et al., 2009). In non-optimized medium, the biomass increased in the process of fermentation and the synthesis of the milbemycin appears to be limited to the mycelium growth.

Secondary metabolism occurs best at sub-maximal growth rates. In many cases, the distinction between the growth phase and production phase is clear (Demain et al., 1995). The timing between the two phases can be manipulated by medium optimized in order to improve the product yields. RSM has been extensively used in fermentation media optimization. The results of this study further supported that the statistical-based approach (RSM) was very effective.

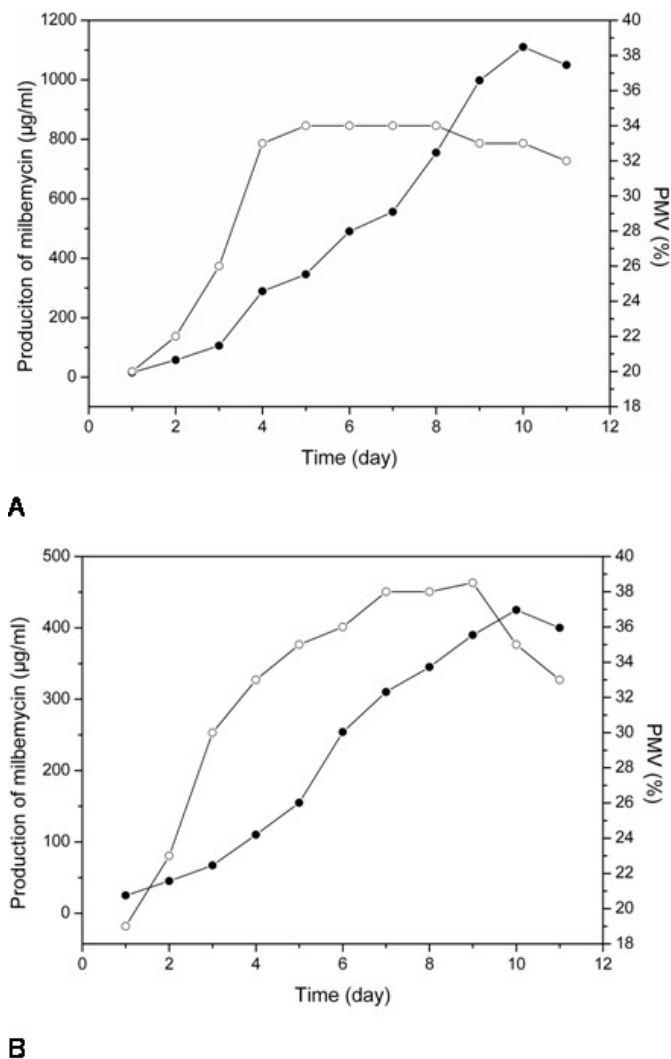
### Conclusion

In this study, we successfully established a model using



**Figure 3.** Response surface plots for the combinatory effects. (a) A (Yeast extract) and C (soybean flour); (b) A (yeast extract) and D (CaCO<sub>3</sub>); (c) A (yeast extract) and F (FeSO<sub>4</sub>); (d) A (yeast extract) and K (KH<sub>2</sub>PO<sub>4</sub>); (e) C (soybean flour) and D (CaCO<sub>3</sub>); (f) C (soybean flour) and F (FeSO<sub>4</sub>); (g) C (soybean flour) and K (KH<sub>2</sub>PO<sub>4</sub>); (h) D (CaCO<sub>3</sub>) and F (FeSO<sub>4</sub>); (i) D (CaCO<sub>3</sub>) and K (KH<sub>2</sub>PO<sub>4</sub>); (j) F (FeSO<sub>4</sub>) and K (KH<sub>2</sub>PO<sub>4</sub>).





**Figure 4.** (a) Time course of milbemycin fermentation in optimized media; (b) time course of milbemycin fermentation in non-optimized media.

RSM for medium optimization of milbemycin production from *S. bingchenggensis* BC-X-1. The important factors (yeast extract, soybean flour,  $\text{CaCO}_3$ ,  $\text{FeSO}_4$ ,  $\text{KH}_2\text{PO}_4$ ) influenced the production of milbemycin production obviously were attained by Plackett-Burman experimental design. Then, Minitab 15.0 Box-Behnken design was applied to optimize the medium composition. The optimal concentration of yeast extract, soybean flour,  $\text{CaCO}_3$ ,  $\text{FeSO}_4$ ,  $\text{KH}_2\text{PO}_4$  was 2.58, 2.58, 0.40, 0.0058 and 0.088%, respectively, when the maximum production of milbemycin was achieved. An empirical model was established for the description of the relationship between the medium components and milbemycin production using statistical analysis system. Validation experiments were carried out to verify the adequacy and the accuracy of the model, the results indicated that the predicted value agreed with the experimental values very well.

Using this optimization strategy, significant enhancement of milbemycin production from 425 to  $1110 \pm 98$  µg/ml was achieved.

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