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Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology

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ABSTRACT

Response surface methodology was employed to optimize the composition of medium for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A in shaker flask cultivation. Corn starch and yeast extract were found to have significant effects on avermectin B1a production by the Plackett–Burman design. The steepest ascent method was used to access the optimal region of the medium composition, followed by an application of response surface. The analysis revealed that the optimum values of the tested variables were 149.57 g/l corn starch and 8.92 g/l yeast extract. A production of 5128 mg/l, which was in agreement with the prediction, was observed in verification experiment. In comparison to the production of original level (3528 mg/l), 1.45-fold increase had been obtained.

1. Introduction

Avermectins produced by *Streptomyces avermitilis* are a class of widely used anathematic and insecticidal agents (Burg et al., 1979; Ikeda and Omura, 1995). They are a series of 16-membered pentacyclic compounds with a disaccharide of methylated deoxysugar L-oleandrose polyketides (Knight et al., 2003; Yoon et al., 2004). Avermectins exhibit excellent anthelmintic activity against a variety of nematode and arthropod parasites with a low level of side effects on the host organism. Eight major avermectin compounds result from structural differences at C5, C22–C23 and C26 (Ikeda and Omura, 1997). Of the eight compounds, avermectin B1a is the most effective components. The properties and applications of avermectins have promoted the commercial production of these compounds. Hence, the investigations on the improvement of the production of these products are of commercial importance in the biological pesticide market.

Designing an appropriate fermentation medium is of crucial importance because medium composition can significantly affect product yield. However, the conventional methods of optimizing medium composition via sequential manipulation of single parameter; these often fail to identify the optimal conditions for the bioprocess because interactions between different factors are neglected. Moreover, they require a considerable amount of work and time (Xiong et al., 2008). So, effective problem solving methods are preferred.

Response surface methodology has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production (Deepak et al., 2008; Liu and Wang, 2007; Sayyad et al., 2007). It can also be used to evaluate the relative significance of several variables simultaneously (Li et al., 2008).

The aim of this work was to apply the Plackett–Burman design, followed by the paths of steepest ascent and response surface methodology to optimize the culture medium composition for avermectin B1a production by *S. avermitilis* 14-12A. The major variables affecting the performance of the culture in terms of avermectin B1a production were also investigated.

2. Methods

2.1. Microorganism

The strain of *S. avermitilis* 14-12A used in the study was stored in 20% glycerol at −80 °C. It was a high producing mutant isolated and identified after *N*-methyl-*N*′-nitroso-*N*-nitrosoguanidine (NTG) treatment of an industrial strain 3–115 in our laboratory (unpublished data). The culture was maintained at 4 °C on yeast extract-malt extract-glucose (YMG) agar slants.

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2.2. Medium

Seed medium adjusted to pH 7.0 was composed of (g/l): corn starch 30; soya flour 8; peanut meal 10; yeast extract 4, $CoCl_2$ 0.03, and α-amylase 0.04.

Non-optimized fermentation medium adjusted to pH 7.5 was composed of (g/l): corn starch 140; α-amylase 0.1; soya flour 28; yeast extract 10; $Na_2MoO_4 \cdot 2H_2O$ 0.022; $MnSO_4 \cdot H_2O$ 0.0023; $(NH_4)_2SO_4$ 0.25; $CoCl_2$ 0.02; and $CaCO_3$ 0.8.

In all cases, media were sterilized by autoclaving for 25 min at 121 °C. After sterilization, the pH of seed and fermentation medium was 6.8 and 7.2, respectively.

2.3. Culture conditions

Fermentation was performed in two stages: seed growth and avermectin B1a production. For the seed growth stage, mycelium from a plate culture was inoculated into 40 ml of seed medium and grown at 28 °C with 220 rpm on a shaker incubator (5 cm shaking diameter, XWW 50/300, Long March, China) for 40 h. Then, 5% (v/v) seed cultures were inoculated into the fermentation medium. The strain was incubated at 28 °C with 220 rpm for 10 days.

2.4. Analytical method

For avermectins extraction, 9 ml methanol was added to 1 ml fermentation broth in a 20-ml tube and was treated in an ultrasonic cleaner for 20 min. After centrifuging (5000 rpm, 10 min), the supernatants were analyzed by high-performance liquid chromatography (HPLC, 1200, Agilent, USA) using a Waters C18 reverse phase column, methanol-water (85:15, v/v) as the mobile phase, 1 ml/min flow rate with detection at 245 nm (Curdova et al., 1989).

2.5. Experimental design and data analysis

In preliminary experiments, various carbon and nitrogen sources, and inorganic salts were evaluated for their suitability to sustain good avermectin B1a production by strain 14-12A (WH, 2008). The results revealed that the major variables affecting the performance of the culture in terms of avermectin B1a yield were corn starch, α-amylase, soya flour, yeast extract, $Na_2MoO_4{\cdot}2H_2O$, $MnSO_4{\cdot}H_2O$, $(NH_4)_2SO_4$, $CoCl_2$ and $CaCO_3$. These components were chosen for further optimization.

2.5.1. Plackett–Burman design (PBD)

PBD was employed for screening the most significant fermentation parameters affecting avermectin B1a production by *S. avermitilis* 14-12A (Sastry and Khan, 1998). Each independent variable was tested at two levels, high and low, which are denoted by (+) and (−), respectively. The experimental design with the name, symbol code, and actual level of the variables is shown in Table 1, whereas Table 2 shows the detail of the design.

Two dummy variables were studied in 12 experiments to calculate the standard error. Avermectin B1a production was carried out in duplication and the average value was taken as the response. The variables with confidence levels above 90% were considered to have significant effect on avermectin B1a production and thus used for further optimization.

2.5.2. Path of the steepest ascent experiment

To move rapidly towards the neighborhood of the optimum response, we used the method of steepest ascent. The experiments were adopted to determine a suitable direction by increasing or decreasing the concentrations of variables according to the results of PBD (Gheshlaghi et al., 2005).

Table 1
The Plackett–Burman design for screening variables in avermectin B1a production.

Factors (g/l)	Code	Low level (−1)	High level (+1)	Effect	Coef*	*t*-value	*P*-value
Corn starch	x_1	112	168	1378.8	689.4	4.42	0.048
α-Amylase	x_2	0.08	0.12	−49.4	−24.7	−0.16	0.889
Soya flour	x_3	22.4	33.6	−130.2	−65.1	−0.42	0.717
Yeast extract	x_4	8	12	−981.8	−490.9	−3.15	0.088
$Na_2MoO_4 \cdot 2H_2O$	x_5	0.0176	0.0264	−14.1	−7.1	−0.05	0.968
$MnSO_4 \cdot H_2O$	x_6	0.00184	0.00276	132.6	66.3	0.42	0.712
$(NH_4)_2SO_4$	x_7	0.2	0.3	−109.8	−54.9	−0.35	0.759
$CoCl_2$	x_8	0.016	0.024	−301.2	−150.6	−0.97	0.436
$CaCO_3$	x_9	0.64	0.96	−586.4	−293.2	−1.88	0.201

R^2 = 94.50%, R^2 (adj) = 69.75%.
* Coef: coefficient.

Table 2
The Placket–Burman design variables (in coded levels) with avermectin B1a as response.

Run	Variable levels											B1a (mg/l)
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	x_{10}	x_{11}	
1	1	1	−1	−1	−1	1	−1	1	1	−1	1	4175
2	1	−1	−1	−1	1	−1	1	1	−1	1	1	4219
3	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	3888
4	−1	1	1	−1	1	1	1	−1	−1	−1	1	3430
5	1	1	−1	1	1	1	−1	−1	−1	1	−1	4019
6	1	1	1	−1	−1	−1	1	−1	1	1	−1	4056
7	−1	1	1	1	−1	−1	−1	1	−1	1	1	1803
8	−1	−1	−1	1	−1	1	1	−1	1	1	1	1720
9	1	−1	1	1	1	−1	−1	−1	1	−1	1	3267
10	1	−1	1	1	−1	1	1	1	−1	−1	−1	3877
11	−1	−1	1	−1	1	1	−1	1	1	1	−1	2654
12	−1	1	−1	1	1	−1	1	1	1	−1	−1	1845

2.5.3. Central composite designs (CCD) and response surface methodology

To describe the nature of the response surface in the optimum region, a central-composite design and response surface methodology was performed. The levels of each factor and the design matrix are given in Table 3. The low, middle, and high levels of each variable were designated as −1.41421, −1, 0, and 1, 1.41421, respectively.

2.5.4. Statistical analysis

Minitab 15.0 (Minitab Inc., Pennsylvania, USA) was used for the experimental designs and subsequent regressional analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test.

3. Results and discussion

3.1. Optimization by PBD

The importance of the nine components, namely, corn starch, α-amylase, soya flour, yeast extract, $Na_2MoO_4 \cdot 2H_2O$, $MnSO_4 \cdot H_2O$, $(NH_4)_2SO_4$, $CoCl_2$ and $CaCO_3$ for avermectin B1a production was investigated by PBD. Table 1 shows the effects of these components on the response and significant levels.

Based on the statistical analysis, the effects of corn starch and yeast extract were (+) 1378.3 and (−) 981.8, respectively, and both had confidence levels above 90%. So, they were identified as influ-

Table 3
The design and results for CCD.

Run	Corn starch		Yeast extract		
	Code X_1	X_1 (g/l)	Code X_2	X_2 (g/l)	B1a (mg/l)
1	1	198	1	10.7	4220
2	0	168	0	9.2	5130
3	0	168	−1.41421	7.1	4747
4	0	168	0	9.2	5227
5	−1	138	1	10.7	4800
6	0	168	0	9.2	5136
7	−1.41421	126	0	9.2	5131
8	1.41421	210	0	9.2	4095
9	0	168	0	9.2	5204
10	0	168	0	9.2	5127
11	−1	138	−1	7.7	5022
12	0	168	1.41421	11.3	4540
13	1	198	−1	7.7	4379

encing avermectin B1a production significantly. Others had no obvious effects and the low confidence level, so they were considered insignificant. In the results, R^2 was found to be 0.945, which means that model could explain 94.5% of the total variations in the system.

In the Pareto chart (Fig. 1), the maximal effect was presented in the upper portion and then progress down to the minimal effect. In addition, it directly shows that the most important factors determining avermectin B1a production were corn starch and yeast extract..

3.2. Optimization by the path of steepest ascent experiment

PBD results indicated that the corn starch effect was positive, whereas that of yeast extract was negative. Thus, increasing corn starch concentration and decreasing yeast extract concentration should result in a higher production of avermectin B1a. The center point of the PBD has been considered as the origin of the path. The titer of avermectin B1a production was obtained; these experiments (Table 4) showed the maximum production of avermectin B1a. This was obtained when the parameters were 168 g/l corn starch and 9.2 g/l yeast extract. It suggested that this point might be near the region of the maximum avermectin B1a response. This point was chosen for further optimization.

3.3. Optimization by response surface methodology

The data shown in Table 3 were analyzed using Minitab 15.0 software. The *t*-test and *P*-values were used to identify the effect of each factor on avermectin B1a production (Table 5). A *P*-value

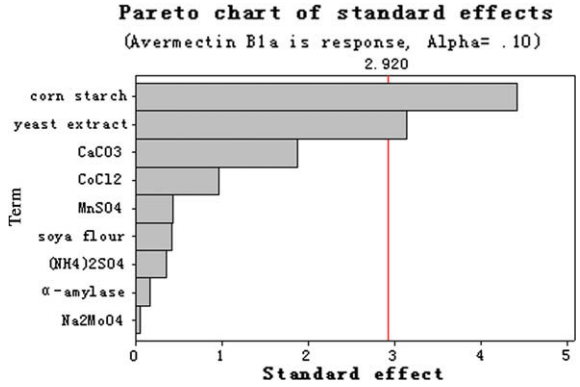

Fig. 1. Pareto chart of nine-factor standard effects on avermectin B1a production. The important terms were corn starch and yeast extract.

Table 4
Design and results of path of steepest ascent experiment.

Run	Factor		B1a (mg/l)
	X_1 (g/l)	X_2 (g/l)	
1	140	10.6	2956
2	154	9.9	3431
3	168	9.2	4151
4	182	8.5	3845
5	196	7.8	3511

of less than 0.05 indicates that the model terms are significant. In this case, corn starch and yeast extract had a significant effect on B1a yield ($P < 0.05$), as well as the quadratic terms of corn starch and yeast extract. The fitness of the model was examined by the coefficient of determination R^2, which was found to be 0.9904, indicating that the sample variation of 99.04% was attributed to the variables and only less than 1% of the total variance could not be explained by the model. A regression model having an R^2-value higher than 0.9 was considered as having a very high correlation (Chen et al., 2009). Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for avermectin B1a production in the present study. The adjusted determination coefficient (R–Sq = 98.35%) was also satisfactory to confirm the significance of the model. The model can be shown as follows:

$$Y = 5165.00 - 336.01X_1 - 84.19X_2 - 281.76X_1 * X_1 - 266.46X_2 * X_2 + 15.5522X_1 * X_2, \quad (1)$$

where Y is the predicted avermectin B1a yield, X_1 is corn starch, and X_2 is yeast extract.

Furthermore, an analysis of variance (ANOVA) for the response surface quadratic model is presented in Table 6, which also proves that this regression was statistically significant ($P < 0.0001$) at 95% of confidence level. The model also showed statistically insignificant lack of fit ($P = 0.358$), so the model was supposed to be ade-

Table 5
Regression coefficients and their significance for response surface model.

Term	Coef.	St. Dev. Coef.	T	P
Constant	5165.00	22.85	226.009	0.000
X_1	−336.01	18.07	−18.598	0.000
X_2	−84.19	18.07	−4.660	0.002
$X_1 * X_1$	−281.76	19.37	−14.543	0.000
$X_2 * X_2$	−266.46	19.37	−13.753	0.000
$X_1 * X_2$	15.55	25.55	0.609	0.562
S = 51.1010	PRESS = 81068.5			

R − Sq = 99.04%; R − Sq(adjust) = 98.35%.

Table 6
ANOVA of regression model.

Source	DF[a]	Seq. SS[b]	Adj. SS	Adj. MS[c]	F	P
Regression	5	18,86,601	18,86,601	377,320	144.49	0.000
Linear	2	959,934	959,934	479,967	183.80	0.000
Quadratic	2	925,700	925,700	462,850	177.25	0.000
Interactions	1	967	967	967	0.37	0.562
Residual error	7	18,279	18,279	2611		
Lack of fit	3	9463	9463	3154	1.43	0.358
Pure error	4	8816	8816	2204		
Total	12	19,04,880				

[a] DF, Degree of freedom;
[b] SS, sum of squares;
[c] MS, mean square.

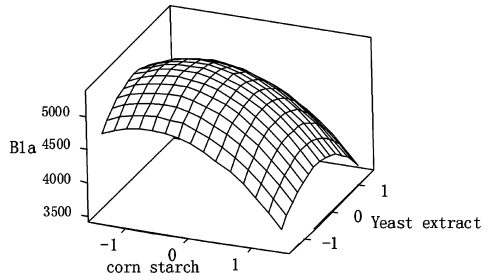

Fig. 2. 3D surface graph of corn starch vs. yeast extract for avermectin B1a (mg/l) production.

quate for prediction within the range of variables employed. In order to gain a better understanding of the effects of the variables on the production of avermectin B1a, the predicted model was presented as 3D response surface graphs (Fig. 2).

3.4. Validation of the optimized condition

On the basis of medium optimization, the quadratic model predicted that the maximum production of avermectin B1a was 5273 mg/l, when the X_1 code level was −0.614 and that of X_2 was −0.186, which were 149.57 g/l corn starch and 8.92 g/l yeast extract, respectively. To verify the predicted results, validation experiment was performed in triplicate tests. Under the optimized condition, the observed experimental titer of average B1a was 5128 ± 144 mg/l, suggesting that experimental and predicted values (5273 mg/l) of avermectin B1a yield were in good agreement. And the titer was 3528 mg/l in non-optimized media; 1.45-fold increase had been obtained, while the growth of the strain in the two medium was comparable (data not shown). This result therefore corroborated the predicted values and the effectiveness of the model, indicating that the optimized medium favors the production of avermectin B1a.

Nutritional requirements of *Streptomyces* play an important role during metabolite synthesis process (Yu et al., 2008). Amongst various nutritional requirements, carbon source and nitrogen source are generally regarded as important factors of metabolism, and several examples of the production of metabolites in media with optimized contents of these components are also described in the literature (Purama and Goyal, 2008; Yuan et al., 2008).

When provided a mixture of carbon energy sources, microorganisms preferentially utilize those they can metabolize most effectively. As many as 21 examples of secondary metabolites have been reported to be repressed by the presence of glucose during their production. Other carbohydrates such as glycerol, maltose, mannose, sucrose and xylose have also been reported to influence the production of secondary metabolites negatively (Jonsbu et al., 2002). The mechanism for catabolite repression in *Streptomyces* has not been unraveled, but appears to be unique in the way it regulates the rate of carbon consumption (Paulsen, 1996). For the majority of *Streptomyces*, the preferred carbon source is starch, especially for the production of secondary metabolites (Gupte and Naik, 1998; Jia et al., 2008; Syed et al., 2009). Thus, it is not surprising that corn starch is a significant factor, as shown by PBD results.

Just as microorganisms have preferred carbon energy sources, they also have preferred sources of nitrogen. It was reported that complex nitrogen sources could increase the production of antibiotic by *Streptomyces*. These sources could sustain high antibiotic titer and this property is supposed to be linked to the slow release of nitrogenous components during the course of the fermentation. More generally, several studies have shown that nitrogen assimilation is crucial for the regulation of antibiotic production but the mechanisms involved is not clearly understood (Voelker and Altaba, 2001).

In the complex nitrogen sources, yeast extract has been proved to support the greatest mycelium biosynthesis and favor a rapid growth (Suutari et al., 2002). In previous experiments, it was found that yeast extract is the preferred nitrogen source of *Streptomyces* strains (Gill et al., 2003; Lazim et al., 2009; Tuncer et al., 2004). However, every coin has two sides; it is also true for yeast extract in some *Streptomyces* strains (Niladevi et al., 2009). In this study, as shown by PBD and RSM results, yeast extract as an important factor has a negative effect, and redundant yeast extract may block avermectin synthesis. On the basis of data analysis, we appropriately decreased the amount of yeast extract and finally obtained a satisfactory result.

The RSM designs applied in the present investigation have been successfully applied in many recent biotechnological researches (Guo et al., 2009; Jo et al., 2008; Lotfy, 2007; Mu et al., 2009). However, to the best of our knowledge, no single report was obtained on avermectins or avermectin B1a production optimization using the RSM designs.

4. Conclusion

This study proved that statistical experimental designs offer an efficient and feasible approach for avermectin B1a fermentation medium optimization. A maximum avermectin B1a production of 5316 mg/l was achieved with the following optimized factors: 149.57 g/l corn starch, and 8.92 g/l yeast extract. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and results showed that the predicted value agreed with the experimental values well, and 1.45-fold increase compared to the original medium was obtained. The results also give a basis for further study with large scale fermentation for production of avermectin B1a.

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