academicJournals

Vol. 8(46), pp. 3775-3781, 12 November, 2014
DOI: 10.5897/AJMR12.2226
Article Number: ED6DA9448846
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimization of fermentation conditions for the anti-cyanobacterial substances production by *Streptomyces* sp. HJC-D1 using response surface methodology

Yun Kong[1,3]*, Pei Zou[2], Jiaoqin Qi[1,3], Liming Song[3], Fei Shao[3], An Zheng[3], Chengcheng Xie[4], Jianzhong Zhou[4], Liang Zhu[1] and Xiangyang Xu[1]

[1]Department of Environmental Engineering, Zhejiang University, Zhejiang Hangzhou 310058, China
[2]Zhongyi Instrument Co., Ltd., Jiangsu Yixing 214205, China.
[3]Yixing Urban Supervision and Inspection Administration of Product Quality, National Supervision and Inspection Center of Environmental Protection Equipment Quality (Jiangsu, preparation), Jiangsu Yixing 214205, China.
[4]Yixing Industrial Park for Environmental Science and Technology, Jiangsu Yixing 214205, China.

Received 25 October, 2012; Accepted 11 November, 2014

To investigate the influence of fermentation conditions such as temperature, initial pH, volume and agitation rate on anti-cyanobacterial active substances production, response surface methodology (RSM) was carried out to optimize the fermentation conditions of an anti-cyanobacterium *Streptomyces* sp. HJC-D1, and the anti-cyanobacterial effect was evaluated. Most common and widespread bloom-forming cyanobacterium *Microcystis aeruginosa* that is associated with microcystic toxins secretion was used as indicator cyanobacterium. The central composite design (CCD) was applied to evaluate the combined effects of the four factors, that is, temperature, initial pH, volume and agitation rate. Based on the analysis of 30 performed experiments, the best optimum level of operating parameters was 33.1°C for temperature, 11.8 for initial pH, 91.2 mL for volume and 337.5 rpm for agitation rate. Additionally, the maximum removal efficiency of chlorophyll *a* under the optimized culture conditions in flask cultures was 93.7%. It is noteworthy that the yield of the anti-cyanobacterial active substances produced by *Streptomyces* sp. HJC-D1 was significantly improved using response surface methodology and suggested the potential to develop a commercial biological control agent against *M. aeruginosa*.

Key words: Response surface methodology, optimization, fermentation conditions, anti-cyanobacterial effect, eutrophication control.

INTRODUCTION

Eutrophication has caused a series of problems such as odor and microcystins (MC) pollution in recent years and damages to ecological systems and threats to human health (Davis and Koop, 2006; Hitzfeld et al., 2000; Qu

*Corresponding author. E-mail: ky020241@hotmail.com. Tel: +86-571-88982343. Fax: +86-571-88982343.

and Fan, 2010). Biological methods of eutrophication control such as anti-cyanobacterial compounds have received increased scientific and technological interest because the microbial-produced anti-cyanobacterial active substances are biodegradable and nontoxic and their degradation intermediates are not secondary pollutants (Qin et al., 2006; Qu and Fan, 2010). Microorganisms such as viruses (Yoshida et al., 2006), bacteria (Kim et al., 2008b; Lovejoy et al., 1998; Shi et al., 2006; Yoshida et al., 2006; Zhang et al., 2011) and golden alga (Zhang et al., 2009) are of particular interest for cyanobacteria control (Kim et al., 2008a; Qin et al., 2006). However, the anti-cyanobacterial bacteria are far from being applied for eutrophication control as the anti-cyanobacterial active substances are so limited in quantity, moreover, the aquatic environment conditions are not the optimal conditions for the growth of anti-cyanobacterial bacteria. Thus, we aim to improve the anti-cyanobacterial active substances production by optimizing the fermentation conditions of the anti-cyanobacterial bacteria.

In recent years, a lot of studies on the influencing factors and inhibiting mechanism by microbes have been published (Kim et al., 2008a; Lovejoy et al., 1998; Uribe and Espejo, 2003; Yoshida et al., 2006; Zhang et al., 2011). Previous studies indicated that the production of antimicrobial compounds by microbial cells were influenced by the composition of the medium, such as carbon sources, nitrogen sources and inorganic salts (Fu et al., 2009; Kong et al., 2014b; Mao et al., 2007; Purama and Goyal, 2008; Rao et al., 2007). In addition, the environmental conditions including temperature, initial pH, volume and agitation rate also had an effect on the production of antimicrobial compounds (Fu et al., 2009; Liu et al., 2011; Mao et al., 2007; Purama and Goyal, 2008; Rao et al., 2007; Sen et al., 2009; Song et al., 2007). Considering the significance of fermentation conditions and the interacting effects of the influencing factors, response surface methodology (RSM) is a useful mathematical and statistical technique for searching the optimal conditions as it could provide statistical models and help in designing experiments for revealing the interactions among the different factors (Bankar and Singhal, 2010; Gao et al., 2009; He et al., 2009), Furthermore, the optimal value of each parameter could be calculated according to the statistical models. Therefore, RSM has been widely used for improving the product yield, reducing the development time and the overall process costs of the fermentation.

It has been shown that microorganisms belonging to *Streptomyces* sp., which are common bacteria found in eutrophication ponds and soils, have been identified as producers of a wide range of anti-cyanobacterial active substances (Choi et al., 2005; Kong et al., 2013, 2014a; Luo et al., 2013; Zheng et al., 2013). In previous study, we isolated a strain of *Streptomyces* sp. HJC-D1 producing anti-cyanobacterial active substances which were efficient for inhibiting the growth of *Microcystis aeruginosa* (Kong et al., 2013, 2014a). The results obtained from preliminary research demonstrated that the optimal medium composition for the growth and anti-cyanobacterial substances production of strain HJC-D1 was 22.7 gL^{-1} sucrose, 0.96 gL^{-1} KNO_3 and at an initial pH of 8.8 (Kong et al., 2014b).

In the present paper, we tested the influence of other factors which may be taken into account to achieve a comprehensive optimization of the fermentation conditions. To optimize the fermentation conditions, the effects of four factors, including temperature, initial pH, volume and agitation rate on the production of anti-cyanobacterial compounds that inhibit the growth of *M. aeruginosa* were studied using RSM.

A full factorial design with relevant statistical analysis has also been investigated to predict the optimal operating parameters of the fermentation for attaining a higher anti-cyanobacterial activity.

MATERIALS AND METHODS

Microorganism

The strain *Streptomyces* sp. HJC-D1 used in this study was originally isolated from an eutrophication pond and was shown to have an anti-cyanobacterial effect on *M. aeruginosa* (Kong et al., 2013, 2014a). *M. aeruginosa* FACHB-905 was purchased from the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China).

Culture conditions

Preculturing of *Streptomyces* sp. HJC-D1 was carried out in a 250 mL Erlenmeyer flask, which contained 100 mL of preculture medium and inoculated with a loopful of the bacterium, and incubated at 30°C on a rotary shaker at 150 rpm for 72 h. The seed culture was then transferred to a 250 mL Erlenmeyer flask containing 100 mL Gause's synthetic medium (Kong et al., 2014b) using 5% inoculum, and incubated at 30°C on a rotary shaker at 150 rpm for 72 h.

M. aeruginosa FACHB-905 was cultured under standard conditions: sterilized BG11 medium (Rippka et al., 1979), 2000 lux white light, light:dark = 14:10 h, 25°C, for seven days to reach the log phase before using as inoculants (Kong et al., 2013).

Cyanobacterial inhibition bioassay

The cell-free filtrate of *Streptomyces* sp. HJC-D1 was obtained according to the method described in previous studies (Kong et al., 2013, 2014a, b). The anti-cyanobacterial effects were studied by adding 5 mL *Streptomyces* sp. HJC-D1 cell-free filtrate into 95 mL *M. aeruginosa* culture with the initial chlorophyll *a* (Chl *a*) concentration of 62.7 ± 7.4 μg L^{-1}. For the control group, the cell-free filtrate was the Gause's synthetic medium. Both control and treatment groups were replicated three times and incubated in 250 mL sterilized conical beaker at conditions described above.

Determination of anti-cyanobacterial activity

After incubating for 4 days, the Chl*a* concentrations of both control

Table 1. Values of experimental variables for the application of CCD.

X	Factor	Level				
		-2	-1	0	+1	+2
X_1	Temperature (°C)	20	25	30	35	40
X_2	Initial pH	4.0	6.0	8.0	10.0	12.0
X_3	Volume (mL)	40	80	120	160	200
X_4	Agitation rate(rpm)	0	75	150	225	300

Table 2. Experimental design and results of CCD.

Run	Code				Removal efficiency (%)	
	X_1	X_2	X_3	X_4	Actual value	Predicted value
1	-1	-1	-1	-1	78.4	78.1
2	+1	-1	-1	-1	88.1	86.6
3	-1	+1	-1	-1	80.8	79.9
4	+1	+1	-1	-1	91.3	89.5
5	-1	-1	+1	-1	89.3	86.1
6	+1	-1	+1	-1	82.0	81.9
7	-1	+1	+1	-1	83.5	82.9
8	+1	+1	+1	-1	81.0	79.8
9	-1	-1	-1	+1	79.7	78.9
10	+1	-1	-1	+1	87.4	86.6
11	-1	+1	-1	+1	87.4	86.1
12	+1	+1	-1	+1	93.7	94.9
13	-1	-1	+1	+1	85.2	85.5
14	+1	-1	+1	+1	81.7	80.6
15	-1	+1	+1	+1	88.2	87.7
16	+1	+1	+1	+1	85.2	84.0
17	-2	0	0	0	79.2	81.1
18	+2	0	0	0	84.2	85.8
19	0	-2	0	0	82.3	84.3
20	0	+2	0	0	88.1	89.5
21	0	0	-2	0	92.5	93.8
22	0	0	+2	0	88.6	90.7
23	0	0	0	-2	69.2	72.3
24	0	0	0	+2	76.8	77.1
25	0	0	0	0	90.5	90.3
26	0	0	0	0	90.2	90.3
27	0	0	0	0	90.4	90.3
28	0	0	0	0	90.2	90.3
29	0	0	0	0	90.2	90.3
30	0	0	0	0	90.1	90.3

and treatment groups were determined by spectrophotometric method (APHA, 1998). The removal efficiency of Chl *a* was calculated according to the following equation:

$$\text{Removal efficiency} = (1 - C_t / C_0) \times 100\% \quad (1)$$

Where, C_0 is the Chl*a* concentration at time *t* in the control group and C_t is the Chl *a* concentration at time *t* in the test group (Kong et al., 2014b).

Table 3. ANOVA for the response surface quadratic model.

Source	D.F.	S.S.	M.S.	*F*-value	*P>F*
Model	9	834.52	59.61	16.34	<0.0001
Residual (error)	15	54.70	3.65		
Lack of Fit	10	54.57	5.46	210.09	<0.0001
Pure Error	5	0.13	0.026		
Total	29	889.23			

D.F., degrees of freedom; S.S., sum of squares; M.S., mean square.Std. Dev. = 1.91; R^2 = 0.9385; C.V.= 2.23%; Adj. R^2 = 0.8811.

Experimental design and data analysis

On the basis of our previous studies, the fermentation condition for anti-cyanobacterial activity production by *Streptomyces* sp. HJC-D1 was optimized by central composite experimental design (CCD) (Kong et al., 2014b). The four factors (temperature, initial pH, volume and agitation rate) and respective code and actual levels are given in Table 1. A 30-run experiment generated by Design Expert 7.0 (Stat-Ease, Minneapolis, MN, USA) were carried out with 16 factorial points, 8 axial points and 6 trials at the center point (Table 2). In order to correlate the response variable to the independent variables, the removal efficiency of Chl *a* was fitted according to the following second-order polynomial model:

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} b_{ij} x_i x_j, \qquad i \neq j \quad (2)$$

Where, Y is the predicted response, X_i and X_j are the coded independent factors, b_0 is a constant; b_i, linear terms coefficients; b_{ii}, quadratic terms coefficients and b_{ij}, interaction coefficients.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability *p*(*F*), correlation coefficient R, determination coefficient R^2 which measured the reliability of the fit of the regression model. It also included the Student's *F*-value for the estimated coefficients and the associated probabilities *p*(*F*). For each variable, the quadratic models were represented as contour plots (3D).

RESULTS

Effects of the four variables, including temperature, initial pH, volume and agitation rate on the removal efficiency of Chl *a* were investigated. To examine the combined effects of these independent variables, thirty treatments were established using CCD. The results of the second-order response surface models for the Chl *a* removal efficiency in the form of ANOVA were given in Tables 3 and 4, respectively. Using the designed experimental data (Table 2), the following quadratic regression equation was obtained to describe the removal efficiency of Chl *a*:

$$Y = 90.26 + 1.16 X_1 + 1.29 X_2 - 0.76 X_3 + 1.21 X_4 + 0.29 X_1 \times X_2 - 3.16 X_1 \times X_3 - 0.18 X_1 \times X_4 - 1.24 X_2 \times X_3 + 1.35 X_2 \times X_4 - 0.33 X_3 \times X_4 - 1.71 X_1^2 - 0.84 X_2^2 + 0.50 X_3^2 - 3.89 X_4^2 \quad (3)$$

Table 4. Results of regression analysis of CCD.

Parameter	Estimate	Std. Error	*F*-Value	*P*-Value
Intercept	90.26	0.78	16.34	<0.0001
X_1	1.16	0.39	8.92	0.0092
X_2	1.29	0.39	10.98	0.0047
X_3	-0.76	0.39	3.81	0.0699
X_4	1.21	0.39	9.63	0.0073
X_1^2	-1.71	0.36	21.95	0.0003
X_2^2	-0.84	0.36	5.34	0.0355
X_3^2	0.50	0.36	1.86	0.1924
X_4^2	-3.89	0.36	113.87	<0.0001
$X_1 \times X_2$	0.29	0.48	0.37	0.5510
$X_1 \times X_3$	-0.36	0.48	43.88	<0.0001
$X_1 \times X_4$	-0.18	0.48	0.14	0.7114
$X_2 \times X_3$	-1.24	0.48	6.77	0.0200
$X_2 \times X_4$	1.35	0.48	8.00	0.0127
$X_3 \times X_4$	-0.33	0.48	0.47	0.5048

Where, *Y* is the predicted removal efficiency of Chl *a*, X_1, X_2, X_3 and X_4 are the coded values of temperature, initial pH, volume and agitation rate, respectively.

The actual and predicted values of Chl *a* removal efficiency based on CCD experimental design are shown in Table 2. By applying ANOVA (Table 3), the model was found to be significant ($P< 0.0001$), as is evident from the *F*-value (16.34) with a very low probability value [($P>F$)<0.0001]; likewise, the reliability of fit of the model was checked by determination coefficient (R^2), and the determination coefficient of the model was 0.9385, which indicated 93.85% of the variability in the response could be obtained by this model. The 0.8811 value of the adjusted R^2 was also sufficiently good. At the same time, the coefficient of variation (C.V. = 2.23%) demonstrated a good precision of the experiments. Nevertheless, the predicted R^2 value of 0.6463 was not as close to the adjusted R^2 value of 0.8811 as it was expected, this was probably due to a large block effect.

The interactions of the four factors on the Chl *removal* efficiency are illustrated in Figure 1. The Chl *a* removal efficiency exhibited a strong response surface depended on both temperature and initial pH (Figure 1a); the value of removal efficiency changed from about 79.7% (at the temperature of 25°C and pH 6.0) to about 93.7% (at 35°C and pH 10.0). Moreover, a good system behavior was consistent with the removal efficiency of 88%, which was obtained at 32°C and pH 6.0. The response surface versus temperature and volume is presented in Figure 1b. It is evident that a relatively weak effect of volume and a stronger effect of temperature could be noted, and the optimal temperature for the Chl *removal* efficiency was 35°C, while the worst conditions were achieved at 25°C with the volume of 100 mL.

Figure 1c shows the effects of temperature and agitation rate on the Chl *a* removal efficiency. It was obvious that the effect of temperature on Chl *a* removal efficiency became less significant as the agitation rate increased to nearly the middle range. Therefore, the maximum removal efficiency of Chl *a* is around the middle range of the corresponding variables. Figure 1d indicates that the removal efficiency is concerned with both initial pH and volume. At the same temperature of 30°C and stirring rate of 175 rpm, the removal efficiency of Chl *a* is dependent on initial pH, which is varied from about 86 to about 93% as the initial pH was increased from 6.0 to 10.0.

Figure 1e depicts the response surface of the effects of two factors, namely, initial pH and agitation rate. It is evident that the interaction between the two factors was significant ($P < 0.05$). The Chl *a* removal efficiency increases with the increase of agitation rate from 100 to 180 rpm, however, a further increase in the agitation rate leads to the decrease of removal efficiency. As agitation rate is fixed for the fermentation of microorganisms, volume becomes the important factor for microorganisms obtaining dissolved oxygen. Figure 1f shows the response surface of the effect of volume and agitation rate on the removal efficiency of Chl *a*. It is obvious that the Chl *a* removal efficiency was increased rapidly with the increase of agitation rate from 100 to 175 rpm.

After having accomplished the ANOVA test on the complete quadratic model, all the negligible effects were eliminated in order to improve the model predictive performance. The best optimum level of operating parameters to operate the fermenter was found to be 33.1°C, 11.8, 91.2 mL and 337.5 rpm for temperature, initial pH, volume and agitation rate, respectively. In order to check the agreement between the optimized fermentation conditions and the prediction by the present model (Equation 3), the predicted conditions were performed in triplicate with the batch cultivation. Under the suggested conditions, the mean value of the removal efficiency was 93.7%, which was in agreement with the optimum value predicted by the model. The good correlation between the experimental and predicted results demonstrated that the second-order model was accurate and reliable for predicting the removal efficiency of *M. aeruginosa* by strain *Streptomyces* sp. HJC-D1 (Table 2).

DISCUSSION

Fermentation conditions are one of the most important factors affecting biomass production (Song et al., 2007; Yin et al., 2010). Environmental conditions, including temperature, initial pH value, volume, agitation rate, and even medium composition such as carbon and nitrogen sources, could be optimized to increase the yield produced by microorganisms (Liu et al., 2011; Purama and Goyal, 2008; Queiroga et al., 2012; Song et al.,

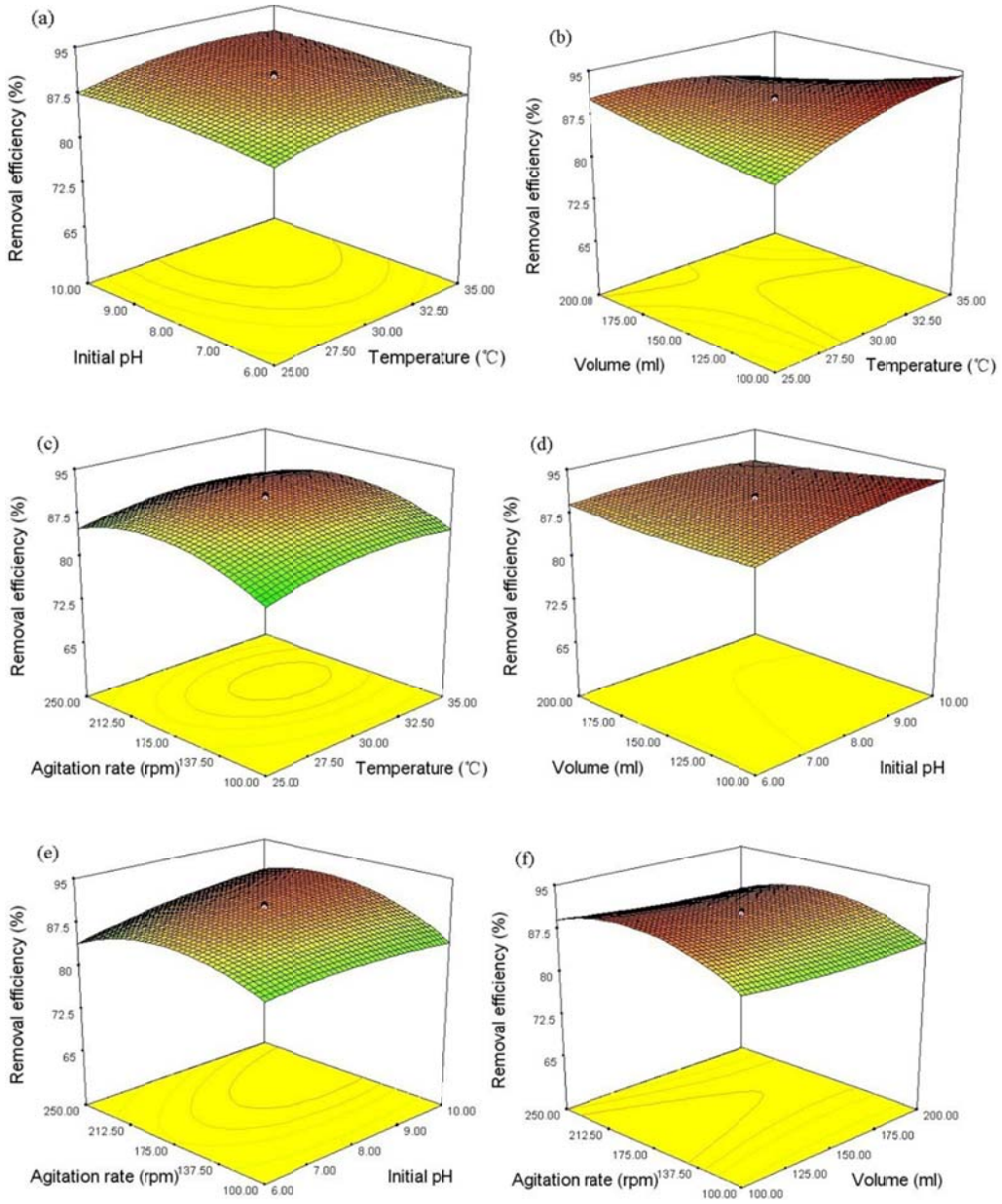

Figure 1. Effect of interaction between different factors on the Chl *a* removal efficiency: (a) initial pH and temperature, (b) volume and temperature, (c) agitation rate and temperature, (d) volume and initial pH, (e) agitation rate and initial pH and (f) agitation rate and volume.

2007). Compared with the traditional method, statistically based experimental design is a much efficient approach to deal with a great number of variables. As a useful statistical technique, RSM has been widely and successfully applied to the optimization of the medium components and culture conditions (Gao et al., 2009; He et al., 2009; Liu et al., 2011).

A previous study demonstrated the influence of the culture conditions on exopolysaccharides (EPS) production from *Zunongwangia profunda* SM-A87, and the optimum incubation temperature of 9.8°C was achieved by RSM (Liu et al., 2011). The reason for the low temperature for EPS production could be that *Z. profunda* was isolated from deep-sea sediment, which was regarded as extreme environments with low nutrient concentration, low temperature and high pressure. With the exception of the influence of a single factor, interactions between the factors should also be considered. It was reported that protease synthesis depended chiefly on temperature and peptone level (Queiroga et al., 2012), and a temperature of 43°C was considered to be the most favorable for protease synthesis

by *Bacillus* sp. HTS102. A previous study also showed that the optimum fermentation conditions for fructo-oligosaccharides production by *Aureobasidium pullulans* were 32°C and 385 rpm (Dominguez et al., 2012), which suggested that temperature and agitation rate were the most significant parameters. In the present study, it was found that the influence of initial pH was greater than the other three variables (Table 4). The temperature and agitation rate optima were 33.1°C and 337.5 rpm for each as expected, moreover, the results were in agreement with another report (Dominguez et al., 2012). On the other hand, the factors such as temperature and volume level were found to be most significant upon Chl *a* removal efficiency ($P < 0.0001$), therefore, they were considered as the main factors which had a significant impact on the production of anti-cyanobacterial active substances (Table 4); surprisingly, the favourable effects of temperature towards volume were too marginal to be classified as statistically significant (Figure 1b), and the highest removal efficiency was obtained at a high level temperature.

In the natural environment, anti-cyanobacterial bacteria play an important role in regulating harmful cyanobacterial biomass (Davis and Koop, 2006; Qin et al., 2006). Previous studies revealed that anti-cyanobacterial bacteria had the ability to biodegrade cyanobacteria (Choi et al., 2005; Kim et al., 2008b; Shi et al., 2006; Yoshida et al., 2006; Zhang et al., 2011; Kong et al., 2013), suggesting that anti-cyanobacterial agents produced by these bacteria were a promising and environment-friendly way for eutrophication control (Qu and Fan, 2010; Luo et al., 2013; Zheng et al., 2013). By now, the harmful cyanobacteria are hard to be controlled by anti-cyanobacterial bacteria as these anti-cyanobacterial bacteria in natural environments were so limited. Therefore, it is particularly important to provide a suitable growing environment for the growth of anti-cyanobacterial bacteria. Although the anti-cyanobacterial bacterium strain *Streptomyces* sp. HJC-D1 selected for this study was isolated from a weak alkaline environment (pH from 9.2 to 10.6), the optimum levels of its anti-cyanobacterial effect were found to be at a higher pH (pH=11.8); in addition, the best optimum levels of temperature was 33.1°C, which was much higher than that in natural environment. It is common that, for a given micro-organism, the optimum culture conditions for growth are different from those required or specific metabolite production (Queiroga et al. 2012). It is noteworthy that strain *Streptomyces* sp. HJC-D1 could produce anti-cyanobacterial active substances with a higher activity after optimization of the fermentation conditions. Given the optimized fermentation conditions, the removal efficiency of Chl *a* was increased to 93.7%. In view of the results above, we consider this study useful for the highly efficient production of anti-cyanobacterial active substances that inhibit the growth of *M. aeruginosa* on a bioreactor scale.

In conclusion, the best optimum level of operating parameters for anti-cyanobacterial active substances was 33.1°C for temperature, 11.8 for initial pH, 91.2 mL for volume and 337.5 rpm for agitation rate, respectively. Furthermore, the maximal removal efficiency of Chl *a* under the optimized culture conditions was 93.7%. It should be noted that this study focused on laboratory research and examined the increase of anti-cyanobacterial active substances production on this scale. We have to point out that we did not test the characteristics (especially ecological safety) of the anti-cyanobacterial substances so they could not be used in nature. Another limitation of this study is that the biodegradation of cyanobacterium *M. aeruginosa* would result in the release of microcystin (Hitzfeld et al., 2000). Notwithstanding the limitations, this study clearly indicates the yield of anti-cyanobacterial active substances was significantly improved using response surface methodology and does suggest the potential to develop a commercial biological control agent against *M. aeruginosa*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was financially supported by the National Key Technology R&D Program (No. 2012BAJ25B07), the National Key Science and Technology Project: Water Pollution Control and Treatment (Nos. 2012ZX07101-012/2013ZX07504-004-03/2013ZX07314-004-08), and the Research Projects of Department of Education, Zhejiang Province, China (No. Y200909172).

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