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Full Length Research Paper

Statistical experimental methods for optimizing the cultivating conditions for *Rhodococcus erythropolis*

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***Rhodococcus erythropolis* was found to effectively degrade aflatoxin B_l produced by *Aspergillus flavus* and *Aspergillus parasiticus*. However, one problem of concern was the slow growth of this strain. In this study, Plackett–Burman design was used to select the most important variables, namely, temperature, pH, inoculum size, liquid volume, agitation speed and culture time that affected the growth of *R. erythropolis*. Central composite experimental design and response surface analysis were adopted to derive a statistical model for optimizing the culture conditions. From the obtained results, it can be concluded that the optimum parameters were: temperature, 15.3°C; pH, 5.56; inoculum size, 4%; liquid volume, 70 ml in 250 ml flask; agitation speed, 180 rpm; and culture time, 58.2 h. At this optimum point, the populations of the viable organisms could reach 10^8 colony forming units (CFU)/ml, which was 100 times higher than that incubated under the initial conditions. After 58.2 h incubation in this optimum cultivating conditions, 53.9 ± 2.1% of aflatoxin B_1 was degraded, while only 20.6±1.4% of aflatoxin B_1 was degraded in the initial conditions.**

Key words: *Rhodococcus erythropolis*, culture condition, optimization, Plackett–Burman design, central composite design, response surface methodology.

INTRODUCTION

Aflatoxins (AFs) are a family of toxic secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Christensen, 1983). Aflatoxins have been found in some foods and feed, and their production is dependent on a wide range of biological, chemical and physical factors including substrates, temperature, pH, moisture content, atmosphere and competitive micro-organisms (Gourama and Bullerman, 1997). Temperature and relative humidity (r.h.) are considered to be the most critical factors. Studies performed on hazelnuts and pistachios suggested that optimum temperature and relative humidity for aflatoxin production is 25 to 30°C and 97 to 99%, respectively (Arrus et al., 2005). They are especially dangerous to human and animal health because of their high hepatotoxicity and nephrotoxicity, as well as their carcinogenic and genotoxic effects (Burkin et al., 2000).

AFs have at least 18 isomers including B_1, B_2, G_1, G_2, M_1 and M_2 (Figure 1). The aflatoxins are freely soluble in moderately polar solvents (example chloroform and methanol), especially in dimethylsulphoxide, and also have some water solubility. These compounds are very stable at high temperature, with little or no destruction occurring under ordinary cooking conditions or during pasteurization. The presence of the lactones ring in their structure makes the aflatoxin molecule susceptible to alkaline hydrolysis. Acid treatments (example propionic acid) are also used frequently for their detoxification (McLean and Dutton, 1995). Among them, aflatoxin B_1 is considered the strongest hepatocarcinogen agent so far. AFB_1, AFG_1, and AFM_1 have higher carcinogenic activity than AFB_2, AFG_2 and AFM_2, respectively (Hwang and Lee, 2006).

As a result of the high toxicity to both humans and

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Abbreviation: AFs, Aflatoxins.

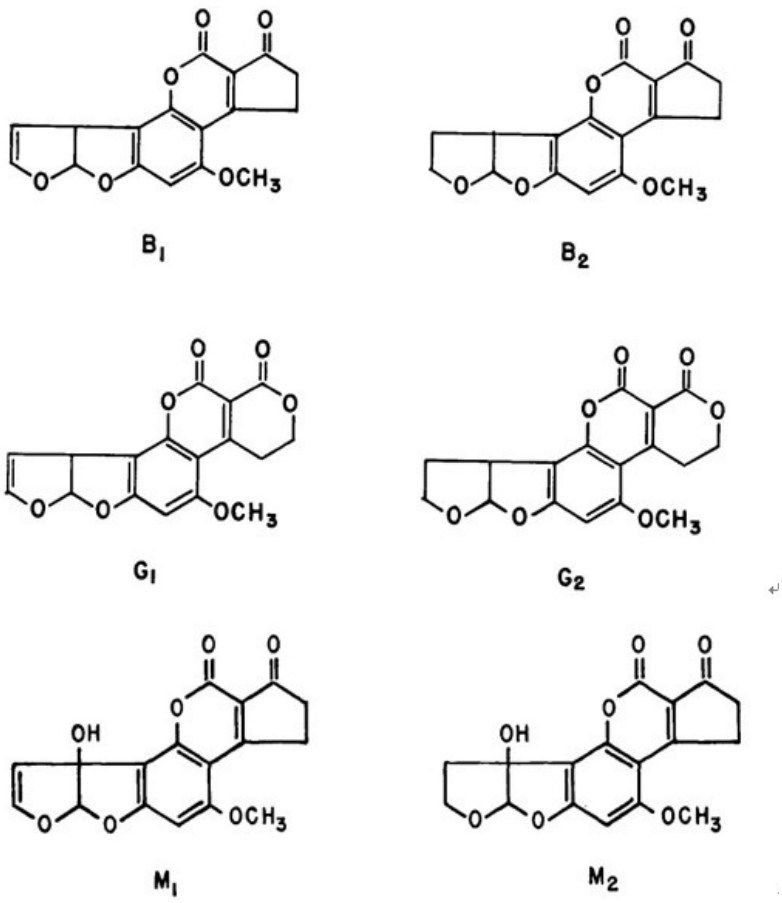

Figure 1. Structures of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2.

animals, many countries have developed the limit standard. The FDA has set limits of 20 ppb total aflatoxins for interstate commerce of food and feedstuff and 0.5 ppb aflatoxin M_1 in milk. The European Commission has set the limits on groundnuts which are subject to further processing at 15 ppb for total aflatoxins and 8 ppb for aflatoxin B_1, and nuts and dried fruits are subject to further processing at 10 ppb for total aflatoxins and 5 ppb for aflatoxin B_1. The aflatoxin standards for cereals, dried fruits and nuts intended for direct human consumption are even more stringent, and the limit for total aflatoxins is 4 and 2 ppb for aflatoxin B_1 (Chang, 2010). Trials to eliminate aflatoxin contamination from food and feed have been carried out (Motomura and Toyomasu, 2003) by numerous strategies, such as physical separation, thermal inactivation (Doyle, 1978), irradiation (Rustom, 1997), microbial degradation (Lillehoj et al., 1971; Kusumaningtyas et al., 2006; Alberts, 2009; Kong et al., 2010) and treatment with a variety of chemicals (Wheeler and Bhatnagar, 1995; Méndez-Albores et al., 2007). However, limitations such as losses of product nutritional and organoleptic qualities, undesirable health effects of such treatments and expensive equipment required for other degradation techniques has encouraged recent emphasis on biological methods (Teniola et al., 2005).

Rhodococcus erythropolis is an aerobic Gram-positive bacterium, it is between *Mycobacterium* and *Nocardia* and part of actinomycetales. It can grow under 4 to 35°C and can be isolated from the soil, rocks, groundwater, marine sediment, animal waste and insects. Currently, *R. erythropolis* is mainly used to desulfurize diesel fuels (Zhang, 2007). Previous studies have shown that *R. erythropolis* effectively degraded aflatoxin B_1 (Alberts et al., 2006). In our laboratory, we found the more viable *R. erythropolis* in the culture, and the less aflatoxin B_1 remained. So the problem of slow growth of *R. erythropolis* has markedly limited its application in degrading aflatoxin B_1.

Response surface methodology (RSM) is a powerful statistical analysis technique which is well suited to modeling complex multivariate processes, in applications where a response is influenced by several variables and the objective is to optimize this response (Cowpe et al., 2007). It is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and analyzing optimum conditions of factors for desirable responses (Li et al., 2007). In recent

Table 1. Range of values for Plackett-Burman (P-B)[a].

Code	Variable (unit)	Level[a]		
		-1	0	+1
X_1	Temperature (°C)	15	20	25
X_2	pH	5.5	6.0	6.5
X_3	Inoculum size (%)	2	4	6
X_4	Liquid volume (ml/250 ml)	50	70	90
X_5	Agitation speed (rpm)	160	180	200
X_6	Culture time (h)	60	72	84

[a] $x_1 = (X_1-20)/5$; $x_2 = (X_2-6.0)/0.5$; $x_3 = (X_3-4)/2$; $x_4 = (X_4-70)/20$; $x_5 = (X_5-180)/20$; $x_6 = (X_6-72)/12$

years, RSM has been used widely in the chemical (Song et al., 2011) and biological fields (Tabandeh et al., 2008), food science (Gan and Latiff, 2011), microbiology and enzyme applications (Levin et al., 2008; Li et al., 2009).

This study dealt with experiments designed to characterize the growth activity of *R. erythropolis*. The effects of culture conditions were evaluated using Plackett–Burman design and response surface methodology (RSM) based on central composite design (CCD) which was also applied to identify and optimize the conditions for the tested strain, *R. erythropolis*.

MATERIALS AND METHODS

Microorganism and culture medium

The bacterial strain (*R. erythropolis* 4.1491) used in this study was obtained from China General Microbiological Culture Collection Center (CGMCC), which was stored at 4°C and sub-cultured three generations at 30°C.

The initial medium to propagate *R. erythropolis* contained (g/L): KH_2PO_4, 1; $CaCl_2$, 0.1; $NaHCO_3$, 3; CH_3COONa, 1; $MgCl_2$, 0.5; NH_4Cl, 1; NaCl, 1; $C_4H_4Na_2O_4$, 0.5; yeast extract, 0.5; peptone, 0.5; 1.5 agar (if necessary). One milliliter trace element solution and one milliliter vitamin solution were also added to each one liter of the initial medium to maintain the necessary nutrient requirement of the cells. The trace element solution contained (g/L): $FeCl_2{\cdot}4H_2O$, 1.8; $CoCl_2{\cdot}6H_2O$, 0.25; $NiCl_2{\cdot}6H_2O$, 0.01; $CuCl_2{\cdot}2H_2O$, 0.01; $MnCl_2{\cdot}4H_2O$, 0.7; $ZnCl_2$, 0.1; H_3BO_3, 0.5; $Na_2MoO_4{\cdot}2H_2O$, 0.03; $Na_2SeO_3{\cdot}5H_2O$, 0.01. The composition of vitamin solution was (g/L): V_H, 0.1; nicotinic acid, 0.35; V_{B1}, 0.3; p-aminobenzoic acid, 0.2; $C_8H_{12}N_2O_2{\cdot}2HCl$, 0.1; calcium pantothenate, 0.1; V_{B12}, 0.05. For optimizing experiments, the seed of *R. erythropolis* was cultured in the initial medium and cultivated at 30°C at 160 rpm for 48 h.

Assay of biomass density and aflatoxin B1 concentration

Optical density at 600 nm (OD_{600}) was adopted to reflect the biomass density quantitatively using a spectrophotometer after incubation. The number of viable cells was determined by plate colony count method. The amount of aflatoxin B_1 was detected by aflatoxin plate kit (Beacon Analytical Systems Inc., Portland, Maine, USA).

Experimental design

Plackett-Burman design

Plackett-Burman (P-B) design as a two-level experimental design, requires fewer runs than a comparable fractional design and can be used to identify the more important independent variables from a long list of candidate factors and select them to realize a complete factorial design (He and Tan, 2006). The variables of the culture conditions were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i \quad (1)$$

Where, x_i is the coded value of an independent variable; X_i is the real value of an independent variable; X_0 is the real value of an independent variable at the center point; and ΔX_i is the step change value. The range and the levels of the variables with both coded values and natural values investigated in this study are given in Table 1. The biomass density (OD_{600}) was considered as the dependent variable or response (Yi). The calculation software SAS 8.0 (SAS Inst. Inc, Cary, N.C., U.S.A.) was used for the regression analysis of the experimental data obtained.

Path of steepest accent

After the P-B design, in the next experiment, if the optimal condition is not in the domain of our expected experimental scope, it will then be carried out along the path of steepest ascent (Kong et al., 2004). From the regression analysis, we could determine the more significant factors affecting biomass density from the *t*-values and design the new units as the stepwise increment for various factors. For every point in the path of steepest ascent, we perform an experimental run to attain a maximum increase of responses (Su et al., 2011).

Central composite design (CCD) and response surface analysis

A CCD with five coded levels was used for exploring the nature of the response surface in the optimum region. The quadratic model for predicting the optimal point was expressed according to following equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^{\,2} + \sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

Where, y is the response variable; β_0 is the constant; β_i is the linear coefficient; β_{ii} is the quadratic coefficients; β_{ij} is the second-order interaction. x_i and x_j are independent variables.

Data were analyzed using the response surface regression procedure (SAS 8.0) where x is the coded level of the independent variable.

RESULTS AND DISCUSSION

Plackett-Burman design

The design matrix selected for the screening of significant variables for the biomass density (OD_{600}) and the corresponding responses are shown in Table 2. The adequacy of the model was calculated, and the variables

Table 2. Experimental designs and the results of the P-B design.

Run	x_1	x_2	x_3	x_4	x_5	x_6	OD_{600}
1	-1	-1	-1	-1	-1	-1	0.898
2	1	-1	-1	-1	-1	1	0.797
3	-1	1	-1	-1	-1	1	0.859
4	1	1	-1	-1	-1	-1	0.772
5	-1	-1	1	-1	-1	1	0.897
6	1	-1	1	-1	-1	-1	0.807
7	-1	1	1	-1	-1	-1	0.876
8	1	1	1	-1	-1	1	0.756
9	-1	-1	-1	1	-1	1	0.916
10	1	-1	-1	1	-1	-1	0.860
11	-1	1	-1	1	-1	-1	0.809
12	1	1	-1	1	-1	1	0.748
13	-1	-1	1	1	-1	-1	0.925
14	1	-1	1	1	-1	1	0.797
15	-1	1	1	1	-1	1	0.802
16	1	1	1	1	-1	-1	0.776
17	-1	-1	-1	-1	1	1	0.925
18	1	-1	-1	-1	1	-1	0.833
19	-1	1	-1	-1	1	-1	0.884
20	1	1	-1	-1	1	1	0.770
21	-1	-1	1	-1	1	-1	0.891
22	1	-1	1	-1	1	1	0.790
23	-1	1	1	-1	1	1	0.865
24	1	1	1	-1	1	-1	0.779
25	-1	-1	-1	1	1	-1	0.945
26	1	-1	-1	1	1	1	0.819
27	-1	1	-1	1	1	1	0.864
28	1	1	-1	1	1	-1	0.768
29	-1	-1	1	1	1	1	0.900
30	1	-1	1	1	1	-1	0.832
31	-1	1	1	1	1	-1	0.828
32	1	1	1	1	1	1	0.743
33	0	0	0	0	0	0	0.866
34	0	0	0	0	0	0	0.822
35	0	0	0	0	0	0	0.844
36	0	0	0	0	0	0	0.828

showing statistically significant effects were screened via *t*-test for ANOVA. It indicated that temperature, pH and culture time were the most important factors (Table 3). The coefficient R^2 of the first-order model was 0.8976, indicating that up to 89.76% of the data variability could be explained by the model (Equation 3):

$$y=0.83534-0.04491\times x_1-0.02916\times x_2-0.00634\times x_3-0.00209\times x_4+0.00441\times x_5-0.0734\times x_6 \quad (3)$$

Table 3. Identifying significant variables for biomass density using Plackett–Burman design.

Variable	Coefficient	*t*-Value	*p*-Value
Intercept	0.83534	226.30	<0.0001
x_1	-0.04491	-12.17	<0.0001
x_2	-0.02916	-7.90	<0.0001
x_3	-0.00634	-1.72	0.0981
x_4	-0.00209	-0.57	0.5756
x_5	0.00441	1.19	0.2433
x_6	-0.0734	-1.99	0.0577

Table 4. Experimental results of the path of steepest ascent (descent).

Run	Temperature	pH	Culture time	OD_{600}
1	20	6.0	72	0.883
2	18	5.8	66	0.918
3	16	5.6	60	0.964
4	14	5.4	54	0.949
5	12	5.2	48	0.104

However, the results of *t*-test for variance between average of observation of 2-level experiment and center point showed that the difference was not significant ($P>0.05$). This indicated that the optimum point was not in the domain of our experiment. Experimentation of steepest ascent path was thus suggested to reach the optimum domain.

Path of steepest ascent

Based on Table 3, the path of steepest ascent and the proper direction of changing variables were determined by increasing or decreasing the concentration of the main effects. The path of steepest ascent started from the center of the factorial design and moved along the path in which the temperature (x_1), pH (x_2) and culture time (x_6) decreased. The design and results of the path of steepest ascent experiments are shown in Table 4. It is shown that the highest biomass density is 0.964 where the temperature is 16℃, pH is 5.6, and culture time is 60 h. It suggested that this point was near the region of maximum production response.

Central composite design and response surface analysis

Based on the Plackett-Burman design and the method of steepest ascent, three variables including temperature, pH and culture time, which significantly influenced biomass density, were investigated. A central composite

Table 5. The matrix of the CCD experiment and the corresponding experimental data.

Run	x_1	x_2	x_6	OD_{600}
1	-1	-1	-1	0.924
2	-1	-1	1	0.916
3	-1	1	-1	0.899
4	-1	1	1	0.885
5	1	-1	-1	0.891
6	1	-1	1	0.873
7	1	1	-1	0.861
8	1	1	1	0.821
9	-1.681	0	0	0.931
10	1.681	0	0	0.878
11	0	-1.681	0	0.914
12	0	1.681	0	0.895
13	0	0	-1.681	0.942
14	0	0	1.681	0.912
15	0	0	0	0.918
16	0	0	0	0.932
17	0	0	0	0.931
18	0	0	0	0.925
19	0	0	0	0.926
20	0	0	0	0.930

$x_1 = (X_1-16)/2$; $x_2 = (X_2-5.6)/0.2$; $x_6 = (X_6-60)/6$.

design (CCD) with five coded levels was used to fully explore the sub-region of the response surface in the neighborhood of the optimum. The design matrix and the corresponding experimental data are shown in Table 5.

The application of response surface methodology (RSM) yielded the following regression equation (Equation 4) which is a second-order polynomial relationship between the response variable (biomass density) and the test variables in coded units:

$$y=0.927883-0.01956*x_1-0.012445*x_2-0.009552*x_6-0.013724*x_1*x_1-0.00325*x_1*x_2-0.0045*x_1*x_6-0.013724*x_2*x_2-0.0035*x_2*x_6-0.005769*x_6*x_6 \quad (4)$$

where, x_1, x_2 and x_6 are the coded values of the test variables temperature, pH and culture time, respectively. The significance of each coefficient was determined by Student's *t*-test and *p*-value, which are listed in Table 6. The larger the magnitude of *t*-test and smaller the *p*-value, the more significant is the corresponding coefficient (Shih et al., 2008). This implies that the first order main effect of the temperature is highly significant as is evident from its respective *p*-values ($px_1 = 0.0022$) and its second order main effect ($px_1^2 = 0.0148$), whereas pH has a significant effect at second order level ($px_2^2 = 0.0148$) than the first order main effect ($px_2 = 0.0268$). These suggest that the temperature has a direct relationship with the biomass density.

The statistical significance of the second-order model equation was checked by an *F* test (ANOVA) with data shown in Table 7. The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.8169, indicating that 81.69% of the variability in the response could be explained by the model.

The 3-dimensional graphs obtained from the calculated RSM are shown in Figures 2 to 4. Figure 2 shows the effects of temperature and pH on the biomass density. An increase in OD_{600} could be achieved when the temperature was increased from 12 to 16°C, and beyond this, OD_{600} decreased with the temperature increase. OD_{600} increased as pH was raised from 5.2 to 5.6, and then decreased with further increase in pH. Figure 3 shows the effects of temperature and culture time on the biomass density. At 48 h, OD_{600} up to maximum temperature of 25°C then decreased with further increase in temperature. With the culture time increase, temperature showed an antagonistic effect on OD_{600}. At a low temperature, the effect of culture time on OD_{600} is slight. With the temperature increase, OD_{600} decreased with time increase. Figure 4 shows the effects of pH and culture time on the biomass density. It is evidence that OD_{600} slightly increased at a low pH when prolonging the culture time. However, further elevation of culture time showed a declining trend for OD_{600} at pH 5.9. With the increase of pH, OD_{600} increased at first and then at about pH 5.6, it began to decrease with the increase of pH. According to the results of the statistical design, the maximum OD_{600} was 0.938. The optimal temperature, pH and culture time were calculated by the SAS software to be 15.3°C, 5.56, 58.2 h, respectively.

The validation of the statistical model and regression equation were conducted by temperature of 15.3°C, pH 5.56, culture time 58.2 h. Under these optimized conditions, the observed experimental value (OD_{600}) was 0.934. Also, the number of viable organisms of *R. erythropolis* could reach 10^8 CFU/ml, which was 100 times higher than that incubated in the initial medium. These results confirm the validity of the model, and the experimental values were determined to be quite close to the predicted values. Furthermore, after 58.2 h incubation in this optimum cultivating conditions, 53.9 ± 2.1% of aflatoxin B_1 was degraded (initial concentration of aflatoxin B_1 in medium was 10 ppm), while only 20.6 ± 1.4% of aflatoxin B_1 was degraded in the initial conditions (initial concentration of aflatoxin B_1 in medium was 10 ppm).

Conclusion

With the main aim of testing as many factors as possible and selecting those that affected the biomass density of

Table 6. The significance of the regression coefficients of the model.

Parameter	DF	Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	0.927883	0.007233	128.29	<.0001
x_1	1	-0.019561	0.004799	-4.08	0.0022
x_2	1	-0.012445	0.004799	-2.59	0.0268
x_6	1	-0.009552	0.004799	-1.99	0.0745
x_1*x_1	1	-0.013724	0.004671	-2.94	0.0148
x_2*x_1	1	-0.003250	0.006270	-0.52	0.6155
x_2*x_2	1	-0.013724	0.004671	-2.94	0.0148
x_6*x_1	1	-0.004500	0.006270	-0.72	0.4894
x_6*x_2	1	-0.003500	0.006270	-0.56	0.5890
x_6*x_6	1	-0.005769	0.004671	-1.24	0.2450

Table 7. ANOVA results for central composite design (CCD).

Regression	DF	Sum of square	R-Square	F Value	Pr > F
Linear	3	0.008586	0.5000	9.10	0.0033
Quadratic	3	0.005097	0.2968	5.40	0.0181
Crossproduct	3	0.000345	0.0201	0.37	0.7797
Total model	9	0.014028	0.8169	4.96	0.0099

$$OD_{600} = -10.4524+0.1409*x+3.7502*y-0.0033*x*x-0.0081*x*y-0.3288*y*y$$

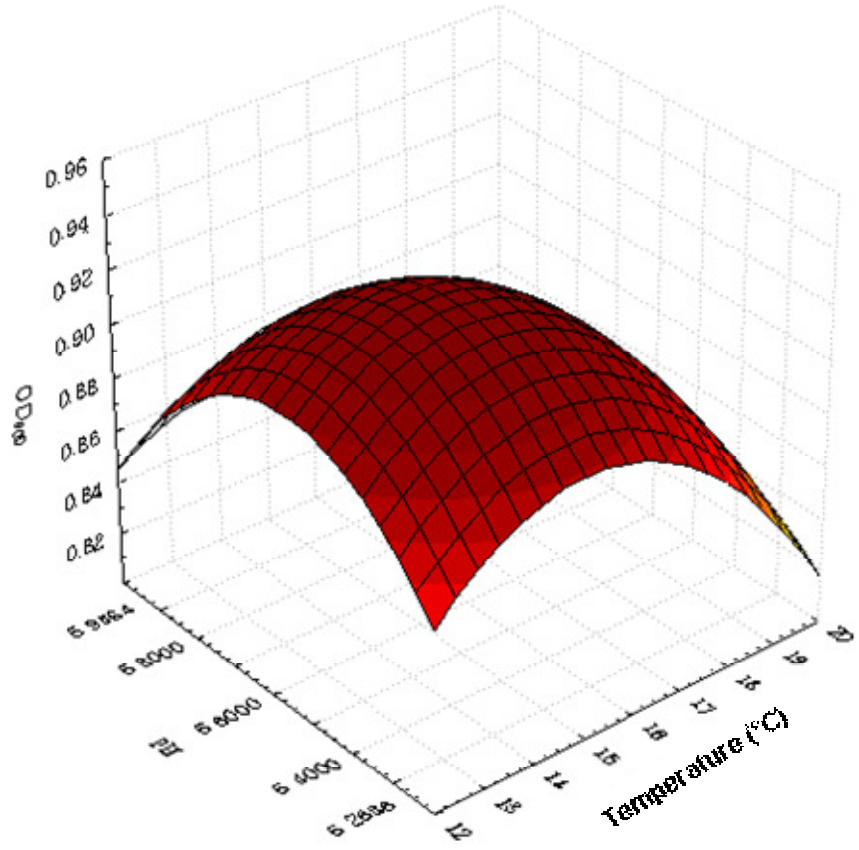

Figure 2. The response surface plot showing the effects of temperature (x_1) and pH (x_2) on OD_{600}.

$$OD_{600} = -0.4232+0.1116*x+0.0191*y-0.0031*x*x-0.0004*x*y-0.0001*y*y$$

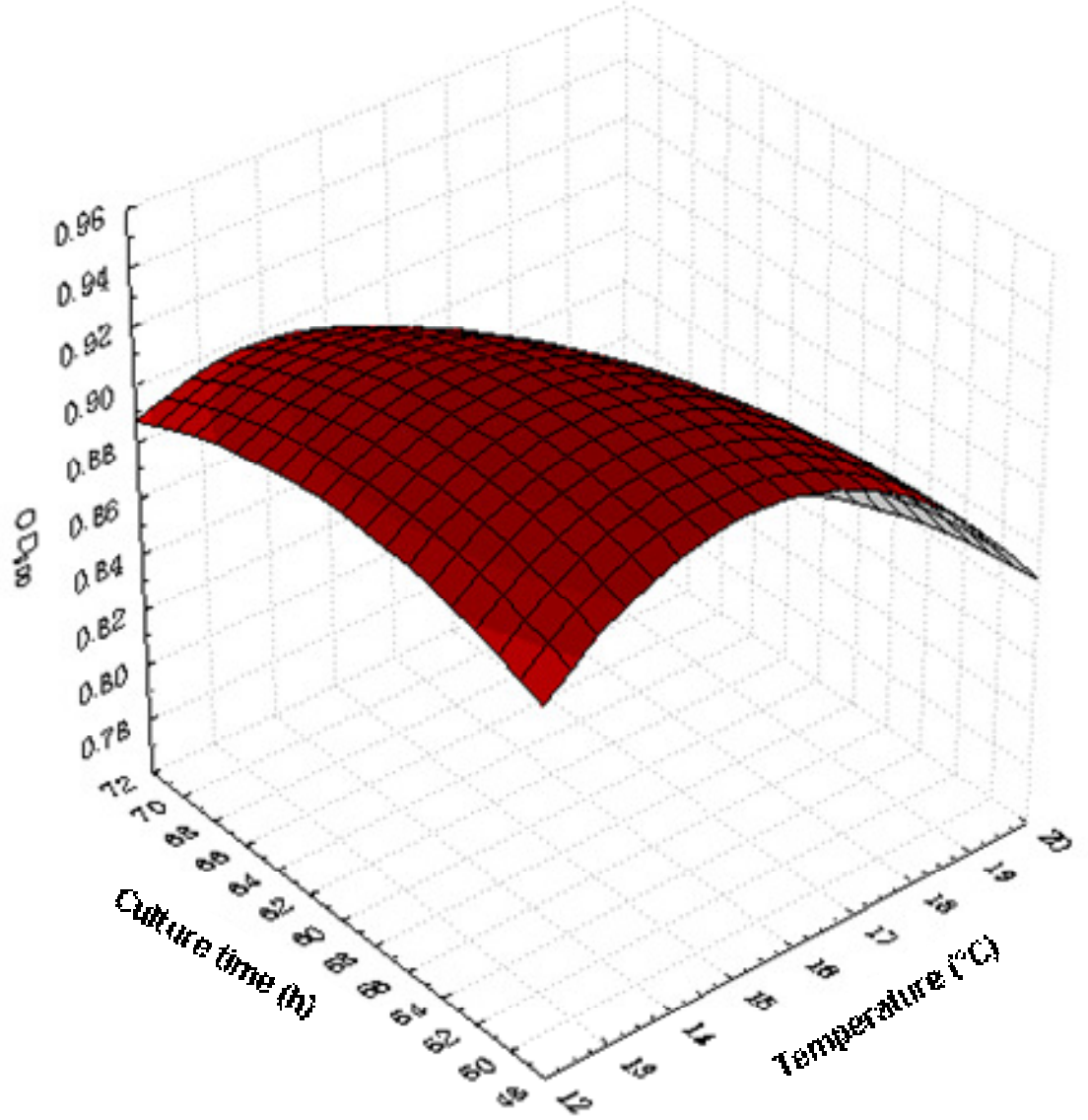

Figure 3. The response surface plot showing the effects of temperature (x_1) and culture time (x_6) on OD_{600}.

R. erythropolis most significantly, a Plackett–Burman design was used. In order to estimate effects of temperature, pH and culture time on the response and factor interactions, the central composite design was employed. The results suggest that statistical design methodology offers an efficient and feasible approach for the optimization of culture conditions. The number of viable organisms of *R. erythropolis* could reach 10^8 CFU/ml under the optimal culture condition: temperature, 15.3℃; pH, 5.56; inoculum size, 4%; liquid volume, 70 ml in 250 ml flask; agitation speed, 180 rpm; culture time, 58.2 h. Through the statistically designed optimization, the population of viable organisms of *R. erythropolis* is considerably higher than that obtained using pre-optimization conditions which was 10^6 CFU/ml. Consequently, the degrading rate of aflatoxin B_1 was dramatically enhanced by *R. erythropolis*.

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$$OD_{600} = -9.7516+3.574*x+0.0294*y-0.309*x*x-0.0029*x*y-0.0001*y*y$$

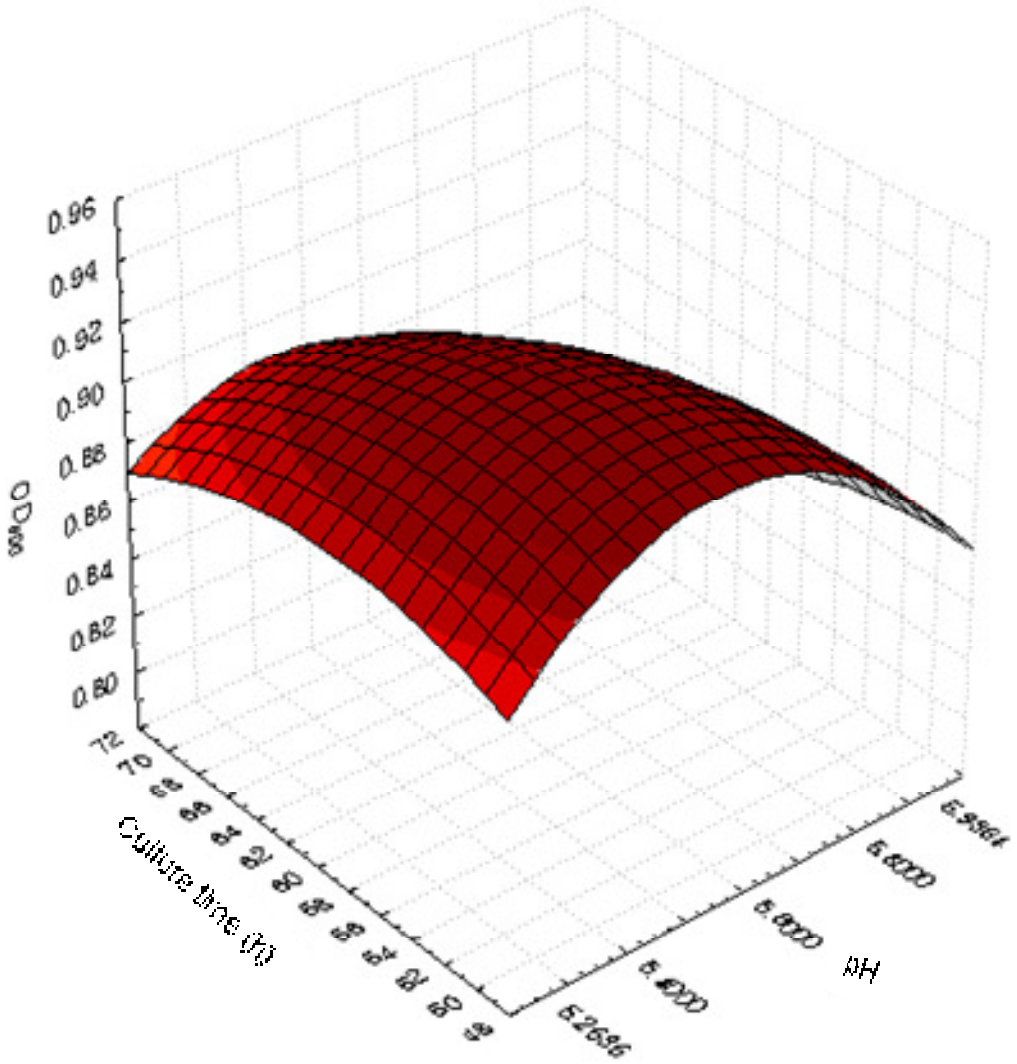

Figure 4. The response surface plot showing the effects of pH (x_2) and culture time (x_6) on OD_{600}.

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