

Article

## Mathematic Modeling for Optimum Conditions on Aflatoxin B<sub>1</sub> Degradation by the Aerobic Bacterium *Rhodococcus erythropolis*

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**Abstract:** Response surface methodology was employed to optimize the degradation conditions of AFB<sub>1</sub> by *Rhodococcus erythropolis* in liquid culture. The most important factors that influence the degradation, as identified by a two-level Plackett-Burman design with six variables, were temperature, pH, liquid volume, inoculum size, agitation speed and incubation time. Central composite design (CCD) and response surface analysis were used to further investigate the interactions between these variables and to optimize the degradation efficiency of *R. erythropolis* based on a second-order model. The results demonstrated that the optimal parameters were: temperature, 23.2 °C; pH, 7.17; liquid volume, 24.6 mL in 100-mL flask; inoculum size, 10%; agitation speed, 180 rpm; and incubation time, 81.9 h. Under these conditions, the degradation efficiency of *R. erythropolis* could reach 95.8% in liquid culture, which was increased by about three times as compared to non-optimized conditions. The result by mathematic modeling has great potential for aflatoxin removal in industrial fermentation such as in food processing and ethanol production.

**Keywords:** *Rhodococcus erythropolis*; degradation efficiency; optimization; Plackett–Burman design; central composite design; response surface methodology; aflatoxins

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## 1. Introduction

Mycotoxins are secondary metabolites produced by *Aspergillus*, *Fusarium* and other fungal species that may be present in agricultural commodities and other food [1]. Aflatoxins (AFs) are produced mainly by *Aspergillus flavus* and *A. parasiticus*. There are mainly four types of aflatoxins, namely AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>. They are detected in most food, such as corn, peanuts, tree nuts, milk and seafood [2,3]. Among the aflatoxins, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most widespread and so the most investigated mycotoxin because of its hepatotoxic, hepatocarcinogenic, mutagenic and teratogenic properties [4,5]. Due to the great health risk posed by aflatoxin contamination of food and feed, many countries have established Maximum Residue Limits (MRL) allowed in food and feed supplies for consumption and trading. The European Union has set more stringent regulations that limits the amount of aflatoxin B<sub>1</sub> to no more than 5 ng g<sup>-1</sup> in nuts and oils and zero tolerance of total aflatoxins in infant formula [6]. The U.S. Food and Drug Administration has set the amount of total aflatoxins for interstate trading and consumption to no more than 20 ng g<sup>-1</sup>. To minimize potential human exposure to aflatoxins, numerous strategies have been used to control the growth of fungi and inhibit aflatoxin formation [7]. Physical methods such as adsorption, heating, UV light, and ionizing radiation are effective only to some degree. Chemical degradation by the addition of chlorinating, oxidizing or hydrolytic agents, are not widely accepted, except ammoniation, which requires expensive equipments and may result in loss of nutritional quality and flavor [8]. Therefore, it is important to find a practical, cost effective and non-toxic method for aflatoxin removal. Use of natural plant extracts and biological methods are one kind of efficient, low-hazardous method for removing toxins [9]. Natural products of plants, such as the extracts of *Agave* species [10], *Garcinia indica* extract [11] and *Satureja hortensis* L. essential oil [12] can inhibit the growth of *Aspergillus flavus* and aflatoxin biosynthesis. Bacteria isolated from almonds also showed inhibition of *A. flavus* growth [13]. Previous studies have demonstrated that some bacterial species could degrade aflatoxin. *Flavobacterium* NRRL B-184 could detoxify aflatoxin in contaminated materials irreversibly [14]. A strain of *Mycobacterium fluoranthenorans* screened from soil of a former coal gas plant was able to degrade aflatoxin B<sub>1</sub> [15]. Several species of Actinomycetales could degrade aflatoxins, too. Recently, two families of F<sub>420</sub> H<sub>2</sub>-dependent reductases from Mycobacteria that catalyze aflatoxin degradation have been identified [16]. *Bacillus megaterium* is a rod-shaped, Gram-positive, endospore-forming, aerotolerant species of bacteria. Some researches showed that *B. megaterium* could inhibit the growth of *A. flavus* and the biosynthesis of aflatoxin B<sub>1</sub> [17]. The effectiveness of *Rhodococcus erythropolis* on AFB<sub>1</sub> degradation was first reported in 2006 [18].

Response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, described first by Box and Wilson, is a much more efficient technique for optimization [19]. RSM consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. RSM defines the effect of the independent variables, alone or in combination, on the process. This method has been successfully applied to the optimization of medium composition, conditions of enzymatic hydrolysis, and parameters of food preservation and fermentation processes.

The purpose of this study was to screen and to establish the optimum conditions for aflatoxin degradation involving the variable factors: temperature, pH, liquid volume, inoculum size, agitation

speed, incubation time and to investigate the application of response surface methodology using central composite design to model the degradation of AFB<sub>1</sub>.

## 2. Materials and Methods

### 2.1. 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). *R. erythropolis* was cultivated in flasks containing ATYP medium for 58.2 h at 15.3 °C with shaking at 180 rpm. The concentration of the viable organisms was estimated to be 10<sup>8</sup> colony forming units (CFU)/mL by plate count method.

The ingredients of the ATYP medium for propagating *R. erythropolis* consist of (g/L): KH<sub>2</sub>PO<sub>4</sub>, 1; CaCl<sub>2</sub>, 0.1; NaHCO<sub>3</sub>, 3; CH<sub>3</sub>COONa, 1; MgCl<sub>2</sub>, 0.5; NH<sub>4</sub>Cl, 1; NaCl, 1; C<sub>4</sub>H<sub>4</sub>Na<sub>2</sub>O<sub>4</sub>, 0.5; yeast extract, 0.5; peptone, 0.5. One milliliter trace element solution and 1 mL vitamin solution were supplemented to each liter of the ATYP medium to maintain the necessary nutrient requirement of the cells. The trace element solution consists of (g/L): FeCl<sub>2</sub>·4H<sub>2</sub>O, 1.8; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.25; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.01; CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.01; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.7; ZnCl<sub>2</sub>, 0.1; H<sub>3</sub>BO<sub>3</sub>, 0.5; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.03; Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 0.01. The ingredients of the vitamin solution consist of (g/L): V<sub>H</sub>, 0.1; nicotinic acid, 0.35; V<sub>B1</sub>, 0.3; *p*-aminobenzoic acid, 0.2; C<sub>8</sub>H<sub>12</sub>N<sub>2</sub>O<sub>2</sub>·2HCl, 0.1; calcium pantothenate, 0.1; V<sub>B12</sub>, 0.05. The pH of the medium was adjusted to 5.56.

The aflatoxigenic strain, *A. flavus* 2810, was kindly provided by Prof. Weijian Zhuang, Fujian Agriculture and Forestry University, China and was maintained on potato dextrose agar (PDA) medium (containing the extract from 200 g boiled potato, 20 g glucose and 20 g agar in 1 liter distilled water) at 4 °C. Spore suspensions were prepared by harvesting seven-day-old sporulating *A. flavus* cultures with sterile distilled water. Spores were counted with a hemocytometer and then were diluted with sterile distilled water to the desired concentration.

### 2.2. Degradation of AFB<sub>1</sub> by *R. erythropolis* in Liquid Culture

*R. erythropolis* (10<sup>8</sup> CFU/mL) and 0.5 mL aflatoxin B<sub>1</sub> (10 mg/L, Sigma, Saint Louis, Missouri, USA) were added to autoclaved ATYP medium in 100 mL flasks. ATYP medium supplemented with AFB<sub>1</sub> without *R. erythropolis* was used as control. After incubation, the cells were removed by centrifugation at 12000 rpm for 10 min, and AFB<sub>1</sub> was extracted from the supernatants by methyl alcohol and quantified by aflatoxin B<sub>1</sub> ELISA test kit (Beacon Analytical Systems Inc., Portland, Maine, USA).

### 2.3. Experimental Design

#### 2.3.1. Plackett-Burman Design

The Plackett–Burman (PB) experimental design is widely used as a screening tool [20]. Compared with the conventional full factorial design, which is labor-intensive and time-consuming, the PB design significantly decreases the number of experiments needed to effectively achieve experimental objectives [21]. The technique is based on the first-order polynomial model:

$$[y = \beta_0 + \sum \beta_i x_i] \quad (1)$$

where  $y$  is the response (the degradation rate of AFB<sub>1</sub>),  $\beta_0$  is the model intercept and  $\beta_i$  is the linear coefficient and  $x_i$  is the level of the independent variable [22]. 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 calculation software SAS 8.0 (SAS Inst. Inc, Cary, N.C., USA) was used for the regression analysis of the data obtained.

**Table 1.** Range of values for Plackett-Burman (PB) <sup>a</sup>.

| Code           | Variables (unit)          | Levels <sup>a</sup> |     |     |
|----------------|---------------------------|---------------------|-----|-----|
|                |                           | −1                  | 0   | +1  |
| X <sub>1</sub> | Temperature (°C)          | 15                  | 25  | 35  |
| X <sub>2</sub> | pH                        | 6.0                 | 7.0 | 8.0 |
| X <sub>3</sub> | Liquid volume (mL/100-mL) | 10                  | 20  | 30  |
| X <sub>4</sub> | Inoculum size (%)         | 6                   | 10  | 14  |
| X <sub>5</sub> | Agitation speed (rpm)     | 160                 | 180 | 200 |
| X <sub>6</sub> | Incubation time (h)       | 48                  | 72  | 96  |

<sup>a</sup>  $x_1 = (X_1 - 25)/10$ ;  $x_2 = (X_2 - 7.0)/1$ ;  $x_3 = (X_3 - 20)/10$ ;  $x_4 = (X_4 - 10)/4$ ;  $x_5 = (X_5 - 180)/20$ ;  $x_6 = (X_6 - 72)/24$ .

### 2.3.2. Central Composite Design (CCD) and Response Surface Analysis (RSM)

A Central composite design (CCD) with five coded levels was used for exploring the sub-region of the response surface in the neighborhood of the optimum. The experimental results of the response surface analysis (RSM) were fitted via the response surface exploring the sub-region of the response surface in the neighborhood of the optimum. The experimental results of RSM were fitted via the response surface regression procedure, as expressed by the following second order polynomial equation:

$$[y = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j] \quad (2)$$

where  $y$  is the predicted response (the degradation rate of AFB<sub>1</sub>),  $x_i x_j$  are independent variables,  $\beta_0$  is the offset term,  $\beta_i$  is the linear coefficient,  $\beta_{ii}$  is the quadratic coefficient, and  $\beta_{ij}$  is the second-order interaction coefficient [23]. Data were analyzed using the response surface regression procedure (SAS 8.0) where  $x$  is the coded level of the independent variable.

### 2.4. Inhibition of AFB<sub>1</sub> by *R. erythropolis* in Peanuts

Peanuts that reach commercial level of maturity were harvested and used immediately or stored at 4 °C for use within 48 h. For preparation, the peanut kernels were washed with tap water, then surface-disinfected with 0.1% sodium hypochlorite for 1 min, cleaned with tap water and air dried [17]. Twenty grams of peanuts were placed in each of the four autoclaved 100 mL flasks (three treatments plus one control). The three treatments were: (A) Two milliliters of *R. erythropolis* cell suspension (10<sup>8</sup> CFU/mL); (B) 2 mL *B. megaterium* cell suspension (10<sup>8</sup> CFU/mL); (C) 1 mL each of *R. erythropolis* and *B. megaterium* cell suspension (10<sup>8</sup> CFU/mL); and (D) sterile distilled water (control). After incubation at room temperature for three hours, 200 µL of *A. flavus* spores at a concentration of 10<sup>6</sup> spores/mL were inoculated into each flask. The flasks were placed in growth

chamber (QHX-300BS-III, Shanghai Xinmiao) with controlled humidity at 85% and temperature at 30 °C for incubation. The concentration of aflatoxin B<sub>1</sub> was examined after seven days' inoculation using high performance liquid chromatography (HPLC) [24].

### 3. Results and Discussion

#### 3.1. Plackett-Burman Design

The design matrix selected for the screening of significant variables for the degradation of AFB<sub>1</sub> and the corresponding responses are shown in Table 2. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's *t*-test for ANOVA (Table 3). It is indicated that temperature, pH, liquid volume and incubation time were the most important factors. The polynomial model describing the correlation between the formulation and the variables ( $x_1 - x_6$ ) and the degradation rate of AFB<sub>1</sub> (*y*) can be expressed by the following equation:

$$[y = 31.34688 - 13.38438 \times x_1 + 5.92813 \times x_2 + 6.17188 \times x_3 + 1.93438 \times x_4 - 1.97187 \times x_5 + 8.99063 \times x_6] \quad (3)$$

**Table 2.** Experimental designs and the results of the PB design.

| Run | $x_1$ | $x_2$ | $x_3$ | $x_4$ | $x_5$ | $x_6$ | <i>y</i> (%) |
|-----|-------|-------|-------|-------|-------|-------|--------------|
| 1   | −1    | −1    | −1    | −1    | −1    | −1    | 18.1         |
| 2   | 1     | −1    | −1    | −1    | −1    | 1     | 10.6         |
| 3   | −1    | 1     | −1    | −1    | −1    | 1     | 80.9         |
| 4   | 1     | 1     | −1    | −1    | −1    | −1    | 12.6         |
| 5   | −1    | −1    | 1     | −1    | −1    | 1     | 75.0         |
| 6   | 1     | −1    | 1     | −1    | −1    | −1    | 13.4         |
| 7   | −1    | 1     | 1     | −1    | −1    | −1    | 26.2         |
| 8   | 1     | 1     | 1     | −1    | −1    | 1     | 11.8         |
| 9   | −1    | −1    | −1    | 1     | −1    | 1     | 51.4         |
| 10  | 1     | −1    | −1    | 1     | −1    | −1    | 10.9         |
| 11  | −1    | 1     | −1    | 1     | −1    | −1    | 27.2         |
| 12  | 1     | 1     | −1    | 1     | −1    | 1     | 11.3         |
| 13  | −1    | −1    | 1     | 1     | −1    | −1    | 49.0         |
| 14  | 1     | −1    | 1     | 1     | −1    | 1     | 9.2          |
| 15  | −1    | 1     | 1     | 1     | −1    | 1     | 90.6         |
| 16  | 1     | 1     | 1     | 1     | −1    | −1    | 34.9         |
| 17  | −1    | −1    | −1    | −1    | 1     | 1     | 26.6         |
| 18  | 1     | −1    | −1    | −1    | 1     | −1    | 15.8         |
| 19  | −1    | 1     | −1    | −1    | 1     | −1    | 14.5         |
| 20  | 1     | 1     | −1    | −1    | 1     | 1     | 12.5         |
| 21  | −1    | −1    | 1     | −1    | 1     | −1    | 22.9         |
| 22  | 1     | −1    | 1     | −1    | 1     | 1     | 22.8         |
| 23  | −1    | 1     | 1     | −1    | 1     | 1     | 85.7         |
| 24  | 1     | 1     | 1     | −1    | 1     | −1    | 21.2         |
| 25  | −1    | −1    | −1    | 1     | 1     | −1    | 12.2         |

**Table 2.** *Cont.*

|    |    |    |    |   |   |    |      |
|----|----|----|----|---|---|----|------|
| 26 | 1  | −1 | −1 | 1 | 1 | 1  | 8.7  |
| 27 | −1 | 1  | −1 | 1 | 1 | 1  | 62.8 |
| 28 | 1  | 1  | −1 | 1 | 1 | −1 | 26.7 |
| 29 | −1 | −1 | 1  | 1 | 1 | 1  | 40.3 |
| 30 | 1  | −1 | 1  | 1 | 1 | −1 | 19.8 |
| 31 | −1 | 1  | 1  | 1 | 1 | −1 | 32.3 |
| 32 | 1  | 1  | 1  | 1 | 1 | 1  | 45.2 |
| 33 | 0  | 0  | 0  | 0 | 0 | 0  | 79.4 |
| 34 | 0  | 0  | 0  | 0 | 0 | 0  | 81.4 |
| 35 | 0  | 0  | 0  | 0 | 0 | 0  | 79.8 |
| 36 | 0  | 0  | 0  | 0 | 0 | 0  | 80.9 |

**Table 3.** Identifying significant variables for the degradation of AFB<sub>1</sub> using Plackett–Burman design.

| Variable       | Coefficients | <i>t</i> Value | <i>p</i> -value |
|----------------|--------------|----------------|-----------------|
| Intercept      | 31.34688     | 10.52          | <0.0001         |
| x <sub>1</sub> | −13.38438    | −4.49          | 0.0001          |
| x <sub>2</sub> | 5.92813      | 1.99           | 0.0578          |
| x <sub>3</sub> | 6.17188      | 2.07           | 0.0489          |
| x <sub>4</sub> | 1.93438      | 0.65           | 0.5223          |
| x <sub>5</sub> | −1.97187     | −0.66          | 0.5143          |
| x <sub>6</sub> | 8.99063      | 3.02           | 0.0058          |

The results of the *t*-test for variance between the average of observation of the 2-level experiment and the center point showed that the difference was significant ( $P < 0.05$ ). This indicated that the optimum point was in the domain of our experiment. The next step in the optimization of the degradation efficiencies was to determine the optimum levels of significant variables. For this purpose, the RSM, using a central composite design, was adopted for optimization of the degradation efficiencies.

### 3.2. Central Composite Design and Response Surface Analysis

A central composite design (CCD) under RSM was used to analyze the interactive effect of temperature, pH, liquid volume and incubation time to reach an optimum level. The design matrix and the corresponding results of the RSM experiments to determine the effects of four independent variables are shown in Table 4.

**Table 4.** The matrix of the central composite design (CCD) experiment and the corresponding experimental data.

| Runs | x <sub>1</sub> | x <sub>2</sub> | x <sub>3</sub> | x <sub>6</sub> | y (%) |
|------|----------------|----------------|----------------|----------------|-------|
| 1    | −1             | −1             | −1             | −1             | 57.6  |
| 2    | −1             | −1             | −1             | 1              | 47.8  |
| 3    | −1             | −1             | 1              | −1             | 74.9  |
| 4    | −1             | −1             | 1              | 1              | 80.4  |
| 5    | −1             | 1              | −1             | −1             | 64.2  |

Table 4. Cont.

|    |    |    |    |    |      |
|----|----|----|----|----|------|
| 6  | −1 | 1  | −1 | 1  | 72.4 |
| 7  | −1 | 1  | 1  | −1 | 65.3 |
| 8  | −1 | 1  | 1  | 1  | 90.4 |
| 9  | 1  | −1 | −1 | −1 | 47.0 |
| 10 | 1  | −1 | −1 | 1  | 46.3 |
| 11 | 1  | −1 | 1  | −1 | 64.8 |
| 12 | 1  | −1 | 1  | 1  | 62.0 |
| 13 | 1  | 1  | −1 | −1 | 54.6 |
| 14 | 1  | 1  | −1 | 1  | 59.7 |
| 15 | 1  | 1  | 1  | −1 | 60.5 |
| 16 | 1  | 1  | 1  | 1  | 65.1 |
| 17 | −2 | 0  | 0  | 0  | 68.1 |
| 18 | 2  | 0  | 0  | 0  | 53.7 |
| 19 | 0  | −2 | 0  | 0  | 69.1 |
| 20 | 0  | 2  | 0  | 0  | 92.2 |
| 21 | 0  | 0  | −2 | 0  | 68.5 |
| 22 | 0  | 0  | 2  | 0  | 95.5 |
| 23 | 0  | 0  | 0  | −2 | 60.7 |
| 24 | 0  | 0  | 0  | 2  | 95.8 |
| 25 | 0  | 0  | 0  | 0  | 82.6 |
| 26 | 0  | 0  | 0  | 0  | 84.4 |
| 27 | 0  | 0  | 0  | 0  | 83.7 |
| 28 | 0  | 0  | 0  | 0  | 83.9 |
| 29 | 0  | 0  | 0  | 0  | 83.4 |
| 30 | 0  | 0  | 0  | 0  | 82.2 |
| 31 | 0  | 0  | 0  | 0  | 84.0 |

$$x_1 = (X_1 - 25)/5; x_2 = (X_2 - 7.0)/0.5; x_3 = (X_3 - 20)/5; x_6 = (X_6 - 72)/12.$$

Experimental data were used in the response surface regression (RSREG) procedure of SAS to find the coefficients of the response function. The coefficients of the response function for the degradation efficiencies are listed in Table 5. In this case, temperature, pH, liquid volume and incubation time had a significant effect on degradation of AFB<sub>1</sub> ( $P < 0.05$ ), as well as the quadratic terms of temperature. The fitness of the model was examined by the coefficient of determination  $R^2$ , which was found to be 0.8096, indicating that the sample variation of 80.96% was attributed to the variables. The model can be shown as follows:

$$\begin{aligned}
 y = & 83.457143 - 5.075 \times x_1 + 4.066667 \times x_2 + 6.991667 \times x_3 + 4.391667 \times x_6 \\
 & - 7.662202 \times x_1 \times x_1 - 0.7375 \times x_1 \times x_2 - 1.5125 \times x_1 \times x_3 - 1.425 \times x_1 \times x_6 \\
 & - 2.724702 \times x_2 \times x_2 - 3.3125 \times x_2 \times x_3 + 3.175 \times x_2 \times x_6 - 2.387202 \times x_3 \times x_3 \\
 & + 1.85 \times x_3 \times x_6 - 3.324702 \times x_6 \times x_6
 \end{aligned} \quad (4)$$

Table 6 shows the analysis of variance of the regression parameters of the predicted response surface quadratic models for the degradation of AFB<sub>1</sub>. As can be seen, both linear and quadratic parameters were very significant. However, the statistical analysis showed that the interaction among parameters was insignificant. This means that all factors considered in this study had a specific effect

on the degradation. However, there were no effects of multi-variable interaction present. The effect of individual variable factors is more significant than that of the multi-factor interaction [25].

**Table 5.** Regression coefficients of the response function for the degradation of AFB<sub>1</sub>.

| Parameter                       | DF | Estimate  | Standard Error | <i>t</i> Value | Pr >   <i>t</i> |
|---------------------------------|----|-----------|----------------|----------------|-----------------|
| Intercept                       | 1  | 83.457143 | 3.273286       | 25.50          | <0.0001         |
| x <sub>1</sub>                  | 1  | −5.075000 | 1.767777       | −2.87          | 0.0111          |
| x <sub>2</sub>                  | 1  | 4.066667  | 1.767777       | 2.30           | 0.0352          |
| x <sub>3</sub>                  | 1  | 6.991667  | 1.767777       | 3.96           | 0.0011          |
| x <sub>6</sub>                  | 1  | 4.391667  | 1.767777       | 2.48           | 0.0244          |
| x <sub>1</sub> * x <sub>1</sub> | 1  | −7.662202 | 1.619505       | −4.73          | 0.0002          |
| x <sub>2</sub> * x <sub>1</sub> | 1  | −0.737500 | 2.165075       | −0.34          | 0.7378          |
| x <sub>2</sub> * x <sub>2</sub> | 1  | −2.724702 | 1.619505       | −1.68          | 0.1119          |
| x <sub>3</sub> * x <sub>1</sub> | 1  | −1.512500 | 2.165075       | −0.70          | 0.4948          |
| x <sub>3</sub> * x <sub>2</sub> | 1  | −3.312500 | 2.165075       | −1.53          | 0.1456          |
| x <sub>3</sub> * x <sub>3</sub> | 1  | −2.387202 | 1.619505       | −1.47          | 0.1599          |
| x <sub>6</sub> * x <sub>1</sub> | 1  | −1.425000 | 2.165075       | −0.66          | 0.5198          |
| x <sub>6</sub> * x <sub>2</sub> | 1  | 3.175000  | 2.165075       | 1.47           | 0.1619          |
| x <sub>6</sub> * x <sub>3</sub> | 1  | 1.850000  | 2.165075       | 0.85           | 0.4055          |
| x <sub>6</sub> * x <sub>6</sub> | 1  | −3.324702 | 1.619505       | −2.05          | 0.0568          |

**Table 6.** ANOVA results for central composite design (CCD).

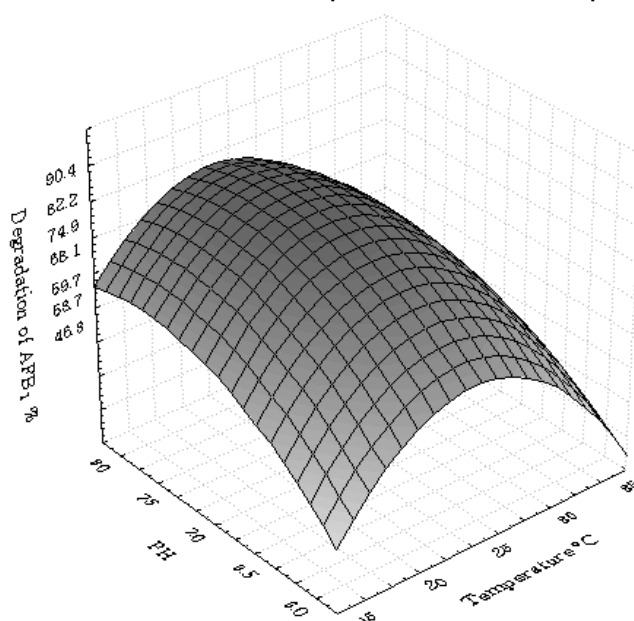
| Regression   | DF | Type I Sum of Squares | R-Square | <i>F</i> Value | Pr > <i>F</i> |
|--------------|----|-----------------------|----------|----------------|---------------|
| Linear       | 4  | 2651.125000           | 0.4205   | 8.84           | 0.0006        |
| Quadratic    | 4  | 1983.513233           | 0.3146   | 6.61           | 0.0024        |
| Crossproduct | 6  | 469.407500            | 0.0745   | 1.04           | 0.4345        |
| Total Model  | 14 | 5104.045733           | 0.8096   | 4.86           | 0.0017        |

Figures 1–6 portray the 3D response surface plots constructed using the quadratic regression model generated by regression analysis. Figure 1 shows the effects of temperature and pH on the AFB<sub>1</sub> degradation. The degradation of AFB<sub>1</sub> increased with temperatures up to 25 °C. In temperature ranges over 25 °C, a negative effect of the temperature on AFB<sub>1</sub> degradation was observed. AFB<sub>1</sub> degradation increased with an increase in pH. The effects of temperature and liquid volume are shown in Figure 2. An increase in temperature above 25 °C resulted in less degradation of AFB<sub>1</sub>. As the liquid volume increased, the degradation of AFB<sub>1</sub> increased at all temperatures probably due to the fact that a high liquid volume is conducive to the growth of bacteria. Figure 3 shows the effects of the interaction of temperature and incubation time on the degradation of AFB<sub>1</sub>. As the length of the incubation time increased to 84 h, the degradation of AFB<sub>1</sub> increased at all temperatures. However, further increase in incubation time resulted in little increase in the degradation of AFB<sub>1</sub>. Figure 4 is a response surface plot indicating the effects of pH and liquid volume on the degradation of AFB<sub>1</sub>. pH and liquid volume had a positive linear effect on the degradation of AFB<sub>1</sub>. The plot also reveals that properly increasing pH is conducive to increasing degradation. Figure 5 shows the effects of the interaction of pH and

incubation time on the degradation of AFB<sub>1</sub>. At 96 h, a linear increase in degradation was observed when the pH was increased. In alkaline conditions, increase in incubation time resulted in increased degradation. A similar profile is observed in Figure 6 with liquid volume and incubation time.

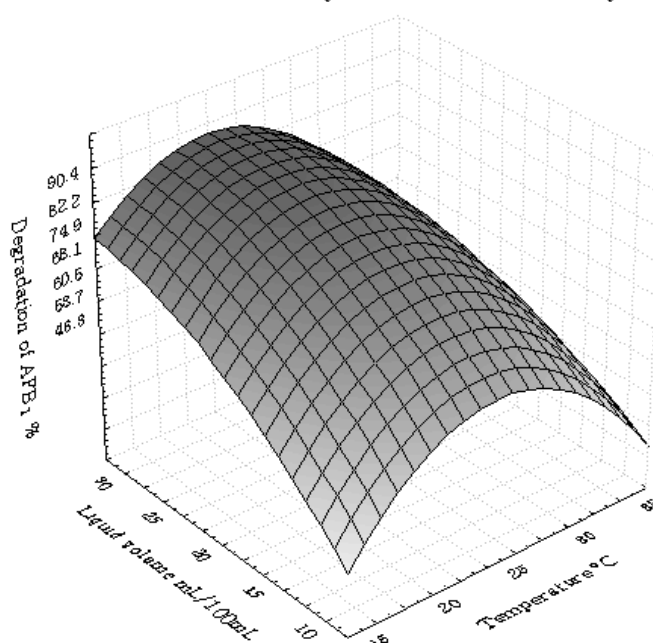
**Figure 1.** The response surface plot showing the effects of temperature ( $x_1$ ) and pH ( $x_2$ ) on AFB<sub>1</sub> degradation.

$$Z = -609.2175 + 15.276 * x + 137.3353 * y - 0.2845 * x * x - 0.295 * x * y - 8.7019 * y * y$$



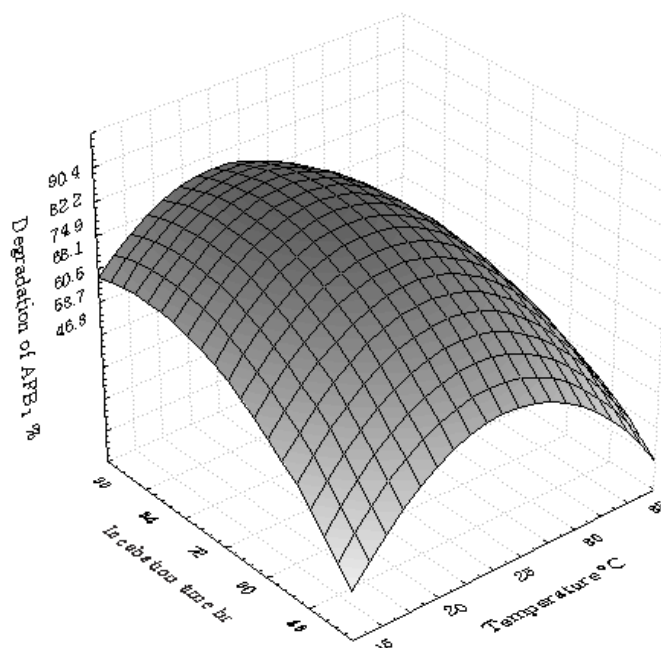
**Figure 2.** The response surface plot showing the effects of temperature ( $x_1$ ) and liquid volume ( $x_3$ ) on AFB<sub>1</sub> degradation.

$$Z = -160.8703 + 14.3561 * x + 5.7997 * y - 0.2832 * x * x - 0.0605 * x * y - 0.0722 * y * y$$



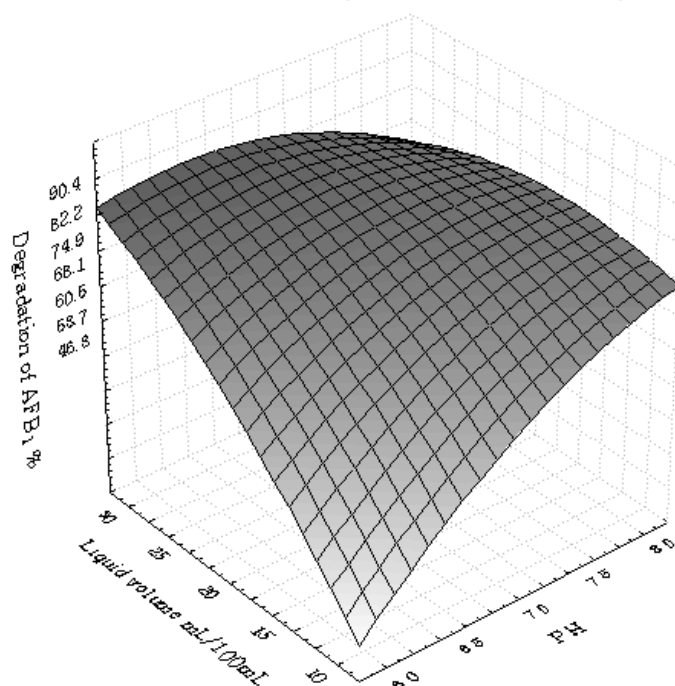
**Figure 3.** The response surface plot showing the effects of temperature ( $x_1$ ) and incubation time ( $x_6$ ) on AFB<sub>1</sub> degradation.

$$Z = -246.2476 + 15.0363 * x + 3.7929 * y - 0.2868 * x * x - 0.0238 * x * y - 0.0197 * y * y$$



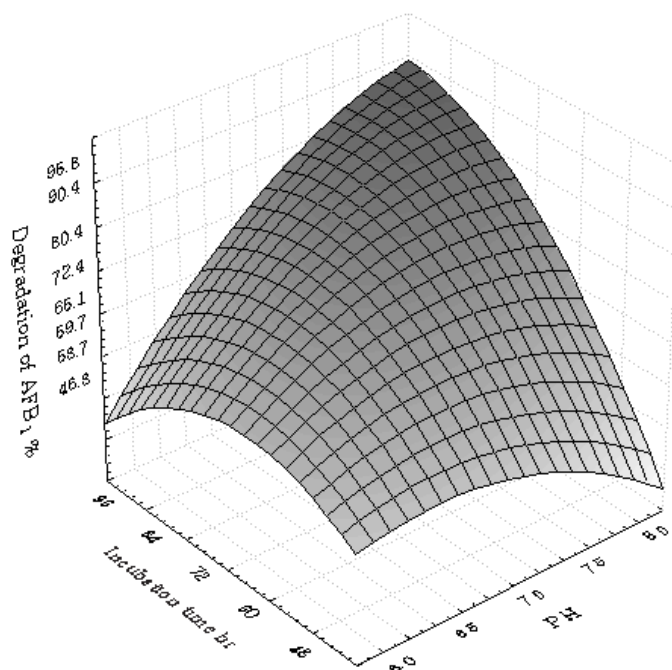
**Figure 4.** The response surface plot showing the effects of pH ( $x_2$ ) and liquid volume ( $x_3$ ) on AFB<sub>1</sub> degradation.

$$Z = -545.3577 + 128.0564 * x + 12.8026 * y - 6.6731 * x * x - 1.325 * x * y - 0.0532 * y * y$$



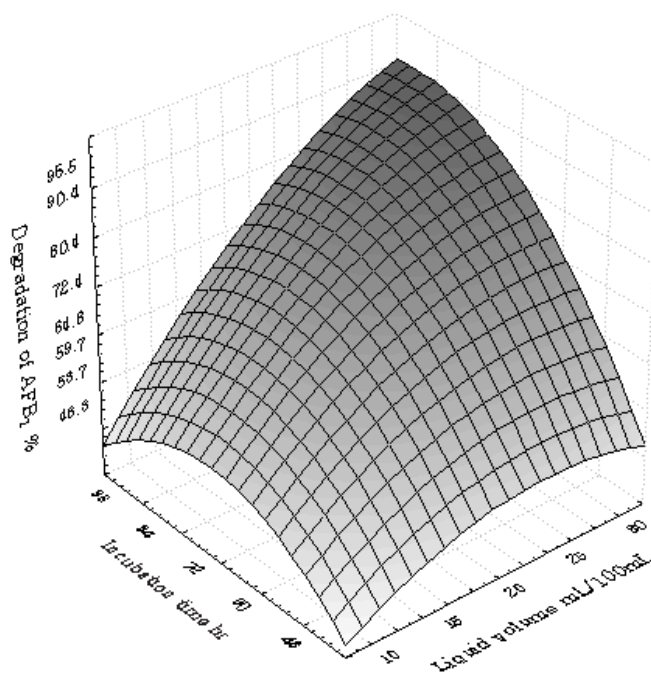
**Figure 5.** The response surface plot showing the effects of pH ( $x_2$ ) and incubation time ( $x_6$ ) on AFB<sub>1</sub> degradation.

$$Z = -171.9545 + 68.5045 \cdot x - 0.9798 \cdot y - 7.0337 \cdot x \cdot x + 0.5292 \cdot x \cdot y - 0.0164 \cdot y \cdot y$$



**Figure 6.** The response surface plot showing the effects of liquid volume ( $x_3$ ) and incubation time ( $x_6$ ) on AFB<sub>1</sub> degradation.

$$Z = -41.9974 + 1.3999 \cdot x + 2.0753 \cdot y - 0.0555 \cdot x \cdot x + 0.0308 \cdot x \cdot y - 0.0162 \cdot y \cdot y$$



According to the canonical analysis, the results predicted by the model showed that the maximum degradation could be achieved when the temperature, initial pH, liquid volume and incubation time were set at 23.2 °C, 7.17, 24.6 mL/100 mL and 81.9 h, respectively. The predicted optimum rate of

AFB<sub>1</sub> degradation was 96.7%. In order to confirm the optimization results, a further degradation test was carried out under the optimal conditions based on the results from the model; the experimentally observed optimum rate of AFB<sub>1</sub> degradation was 95.8% which was quite in agreement with the predicted value.

### 3.3. Inhibition of AFB<sub>1</sub> by *R. erythropolis* in Peanuts

The strain of marine *B. megaterium* was isolated from the Yellow Sea of East China, and showed reduced postharvest infection of peanut kernels caused by *A. flavus* [17]. The results showed both *R. erythropolis* and *B. megaterium* could significantly reduce the biosynthesis of aflatoxins in peanuts ( $p < 0.01$ ) (Table 7). The effect of *R. erythropolis* plus *B. megaterium* was better than that of a single bacterial strain.

**Table 7.** Inhibition of aflatoxin B<sub>1</sub> by *R. erythropolis* in peanuts.

|                                                 | Control       | <i>R. erythropolis</i> | <i>B. megaterium</i> | <i>R. erythropolis</i> and<br><i>B. megaterium</i> |
|-------------------------------------------------|---------------|------------------------|----------------------|----------------------------------------------------|
| Aflatoxin B <sub>1</sub> (µg/kg)<br>(mean ± SD) | 195.69 ± 1.92 | 178.38 ± 2.47          | 148.27 ± 3.87        | 140.80 ± 3.59                                      |

Tejada-Castañeda *et al.*, reported that a strain of *Nocardia corynebacteroides* was safe to detoxify aflatoxin-contaminated feed for chickens, because it could transform AFB<sub>1</sub> to other less toxic compounds [26]. Can *R. erythropolis* degrade AFB<sub>1</sub> completely or can it only transform AFB<sub>1</sub> to other less toxic compounds? To answer this question, the metabolites of AFB<sub>1</sub> incubated with *R. erythropolis* should be studied further. Furthermore, the work of Taylor *et al.*, describes the enzymes in *Mycobacterium smegmatis* that degrade aflatoxin, which is subsequently corroborated by the work of Lapalikar *et al.*, who show that enzymes from *R. erythropolis* also degrade aflatoxin [17,27]. These enzymes are dependent upon the cofactor F<sub>420</sub>, whose production has been shown to be maximal at around 96 hours [28,29]. In a next step, the enzyme activity of F<sub>420</sub>H<sub>2</sub>-dependent reductases in the strain of *R. erythropolis* used in this study and the expression of aflatoxin pathway genes in *A. flavus* corresponding to the effect of *R. erythropolis* should be studied in greater detail, because the results could help us elucidate the mechanism of degradation of aflatoxin by *R. erythropolis*.

## 4. Conclusions

Plackett–Burman design and central composite design were adopted to screen the key factors and identify the optimal conditions for degradation of AFB<sub>1</sub>. Using RSM analysis, the four significant variables (temperature, pH, liquid volume and incubation time) selected by the Plackett–Burman design experiment were found to have linear effects on AFB<sub>1</sub> degradation at significant level. The optimum conditions of each variable were as follows: temperature, 23.2 °C; pH, 7.17; liquid volume, 24.6 mL/100mL; inoculum size, 10%; agitation speed, 180 rpm; and incubation time, 81.9 h. Under these conditions, the AFB<sub>1</sub> degradation efficiency of *R. erythropolis* was increased from 28.7% to 95.8%.

Our result by mathematic modeling has great potential for practical applications. It can be used in aflatoxin removal during industrial fermentation for food processing and fermentation for bioenergy

generation such as ethanol production. *R. erythropolis* can be used to reduce the biosynthesis of aflatoxins in peanuts and other agricultural food commodities. It can potentially be used in biocontrol to reduce aflatoxin contamination of food and feed utilizing its activity against the aflatoxins biosynthesis in *A. flavus*. The potential for commercial use in the market to prolong shelf life can also be explored.

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## Conflict of Interest

The authors declare no conflict of interest.

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