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# Optimization of bilirubin oxidase production conditions by *Myrothecium* sp.IMER1under solid fermentation using response surface methodology

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## ABSTRACT

To improve bilirubin oxidase production by *Myrothecium* sp.IMER1, the fermentation conditions of bilirubin oxidase (BOX) production by Myrothecium sp.IMER1 under solid fermentation were optimized by response surface methodology. Based on pre-test results, Plackett-Burman design was used to evaluate the effects of five factors related to BOX production and three statistically significant factors moisture content, pH and temperature were selected. The path of steepest ascent was used to approach the optimal region of BOX production subsequently. Then, the optimal combined concentration for maximum enzyme activity were further optimized by response surface methodology and determined as follows: 4.6, 23°C and 83.5%. The optimization of culture conditions of IMER1 led to about 2 fold increases in BOX production compared to the initial, which indicate that single factor in combination with response surface methodology is an effective method for optimization of BOX production conditions by *Myrothecium*sp.IMER1. The maximum amount of enzyme under solid fermentation is found on the fifth day, but high BOX yield can be sustained; Its extracellular enzyme activity were analyzed by native electrophoresis, the results show that BOX can be detected and no BOX isozymes appear.

## KEYWORDS

Bilirubin oxidase; *Myrothecium* sp.; Response surface methodology; Solid fermentation.

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## INTRODUCTION

*Myrothecium sp.* is an asexual filamentous fungus in *Tuberculariaceae, Hyphomycetales, hyphomycetes, Deuteromycotina*. It is a major fungus to produce the bilirubin oxidase (BOX). Its strains are frequently used to produce the BOX. The BOX can be used to diagnose the jaundice levels clinically, and treat the neonatal jaundice and hyperbilirubinemia[1-4]; The BOX is a multi-copper ion oxidase which contains a Type I copper ion, a Type II copper ion, two Type III copper ions. Type I copper ion mediates the transfer of electrons from the substrate to other copper bits; Type II copper icon and 2 Type III copper ions form 3-cored center where the oxygen molecules are trapped and reduced to 2 water molecules. The catalytic mechanism is similar to the laccase[5-7]. In recent years, the BOX has been broadly applied and used in researches on biocells manufacture, sewage treatment, etc[8, 9]. Therefore, as BOX demand increases, how to improve the BOX production is a major issue challenging its substantial application. The Response Surface Methodology (RSM) is a statistic method to resolve the multivariate issues, with which some data is derived by sound experimental design and test, the functional relationship is fitted between factors and response values using the multivariate quadratic equation, and the optimal process is found through the regression equation analysis[10]. RSM is an ideal method frequently used in multivariate optimization thanks to fewer tests, shorter cycle, higher regression equation accuracy, capability of investigating the interaction among several factors in recent years[11]. Concurrently, *Myrothecium sp.* produces the BOX by liquid fermentation whilst rare studies have reported its solid fermentation. In this paper, the relevant parameters in solid fermentation of *Myrothecium sp*. IMER1 were investigated and the solid fermentation condition was optimized through RSM, thus laying a foundation for improving the BOX production and its further application.

## Materials and methods

### Materials and main apparatus

Test strains IMER1 (*Myrothecium sp*.IMER1); 2,2 - azino - bis (3-ethyl-benzothiazole-6-sulfonic acid ammonium) (ABTS), SIGMA,US; potato solid medium (PDA): 20% potato (chipped and boiled), 2% glucose, 2% agar; solid medium: 5g bran, 15ml water or water added according to the experimental conditions.

Model UNICO-UV-2000 UV-visible spectrophotometer (Unico(Shanghai) Instrument Co., Ltd.,); Model TGL-16/TGL16 desktop high-speed refrigerated centrifuge (Hunan Xiangyi Group); Model ZD-88 full-temperature air-bath shaker(JintuanChenghui Instrument Factory)

### Experimental methods

#### Solid culture

IMER1's PDA slant-surface strains usually produce large amounts of spores in 5-7 days. The spores are flushed with a proper amount of distilled water in the tube. The number of spores is adjusted to $1\times10^8$/m using a microscopic counting mean. Prior to use, the spore suspension is shaken evenly. 1ml pores solution is pipette and injected to the 5g sterile solid medium (100ml conic flasks), and then the flask is cultured in a constant-temperature incubator.

#### Collection of crude enzyme solution in solid fermentation products

After IMER1 is cultured in solid medium for a period of time, the flask is taken out, added with 100ml distilled water under the aseptic conditions, and oscillated for 3h. The crude enzyme solution is filtered and collected.

#### Determination of enzymatic activity

See reference[12] for ABTS method: a 3mL reaction system contains 2.7mlpH4.5 0.2mol/L acetic acid - sodium acetate buffer, 0.1ml enzyme solution, 0.2ml1mmol/L ABTS, which are sequentially mixed and shaken evenly. After 30 seconds, $A_{420}$ is noted once every 15s. An enzyme activity unit (U) is the amount of enzyme required for catalyzing the oxidation of 1μmol ABTS per minute under the above conditions.

#### Active PAGE electrophoresis

The glass plate, pads and combs are washed cleanly with double-distilled water, and wiped dryly withalcohol-dipped swab. The electrophoresis tank is installed, and the separating gel (12%) and stacking gel (5%) and condensation gel(5%) are prepared. During the preparation, no SDS is added and no heated denaturation is done in samples. After the electrode buffer is added, the samples (fermentation liquid) are loaded to the well bottom with a micro-injector for 60-volt electrophoresis. When the bromophenol blue reaches the separating gel, the voltage is tuned to 150 volts, the electrophoresis continues until the bromophenol blue reaches the bottom of the gel. The gel is peeled off, and cut into different strips along the runs. The strips are immersed in 100ml solution with different substrates (ABTS, or bilirubin), stained for 1-3 hours, and photographed immediately after the gel strips are developed.

#### Response surface design and statistical analysis

The experimental design procedures and variance analysis in SAS®8.0 are used.

## RESULTS AND ANALYSIS

### Determination of major factors affecting IMER1 to produce BOX in solid medium

Plackett- Burman (PB) method is a nearly-saturated level-2 test design method. Based on the principle of non-fully balanced block, it is capable of estimating the major effect of factors with the least test time, and screening the several most

important factors out of many investigated factors quickly and efficiently for further study. According to the result of previous pre-experiments that the single factor affects IMER1 to produce the enzyme, we elected five factors: water content, temperature, pH, inoculum and copper ions; and we fully investigated and optimized 5 factors using PB design. See TABLE 1 for the experimentally-derived activity. See TABLE 2 for the parameters and factor effects representative of every factor. The factors showing significant impact on the BOX production in solid medium fermentation include water content, pH and temperature; pH and temperature show a negative effect on the enzyme production whilst the water content shows a large positive effect. Therefore, to improve the BOX production, the water content must be increases and pH and temperature must be decreased.

**TABLE 1 : Experimental design of Plackett-Burman and corresponding results at N=8**

| RUN | Water(%) | $Cu^{2+}$(mM) | pH | IN ($1\times10^8$/mL) | T(°C) | Y1 Activity |
|---|---|---|---|---|---|---|
| 1 | 50% | 0.1 | 5 | 1.0 | 31 | 0.033 |
| 2 | 80% | 0.1 | 5 | 0.2 | 26 | 4.033 |
| 3 | 50% | 1.0 | 5 | 0.2 | 31 | 0.000 |
| 4 | 80% | 1.0 | 5 | 1.0 | 26 | 5.522 |
| 5 | 50% | 0.1 | 8 | 1.0 | 26 | 0.033 |
| 6 | 80% | 0.1 | 8 | 0.2 | 31 | 0.122 |
| 7 | 50% | 1.0 | 8 | 0.2 | 26 | 0.033 |
| 8 | 80% | 1.0 | 8 | 1.0 | 31 | 2.922 |

**Note: Water (%), water content; IN, inoculums**

**TABLE 2 : Factors and effect estimates of Plackett-Burman design**

| Factor | t value | Pr>t | Significance |
|---|---|---|---|
| W | 9.531687 | 0.010828 | 1 |
| $Cu^{2+}$ | 3.245349 | 0.08326 | 5 |
| pH | -4.9397 | 0.038624 | 3 |
| IN | 3.295676 | 0.081035 | 4 |
| T | -4.99003 | 0.037892 | 2 |

**Steepest ascent experiment determines the center point of RSM factor level**

The step length is set as per the magnitude of significant factor effect screened using PB method for steepest ascent experimental design to seek the area of maximum BOX production. Their change direction and step length are set according to the proportion of magnitude of 3 factor effects: water content, pH and temperature. The steepest ascent experimental design and experimentally-determined results are shown in TABLE 3 and 4. The area of maximum BOX production is in the vicinity of the fourth experiment. The conditions of this experiment are made the center point of RSM factor level.

**TABLE 3 : Experimental design of steepest ascent and corresponding results**

| Experiment No. | Water(%) | pH | T(°C) | Activity(u/g) |
|---|---|---|---|---|
| 1 | 78 | 5.2 | 26 | 8.4 |
| 2 | 80 | 5.0 | 25 | 6.2 |
| 3 | 82 | 4.8 | 24 | 10.5 |
| 4 | 83 | 4.6 | 23 | 13.5 |
| 5 | 85 | 4.7 | 22 | 12.3 |

**TABLE 4 : Factors and levels of response surface central composite design**

| Factor | Level | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| pH | 4.4 | 4.6 | 4.8 |
| W(%) | 82 | 83.5 | 85 |
| T | 22 | 23 | 24 |

**Response surface design determines the optimal level of significant factor**

After approaching the area of maximum BOX production, the response surface central composite design is done. The polynomial regression model is established with experimental results by fitting to create the relationship between response quantity (BOX production) and variables (factors affecting the BOX production). The experimental data is regressively fitted using SAS software and the significant test and analysis of variance are performed for the fitting equation. 3-factor 5-level response surface central composite experiment is designed for 15 experimental points. Such experiment design and experimentally-determined BOX activity results are shown in TABLE 5.

**TABLE 5 : Response surface central composite design and corresponding results**

| RUN | pH | W | T | Activity |
|---|---|---|---|---|
| 1 | 4.4 | 82.0 | 23 | 4.156 |
| 2 | 4.4 | 85.0 | 23 | 10.911 |
| 3 | 4.8 | 82.0 | 23 | 9.289 |
| 4 | 4.8 | 85.0 | 23 | 10.578 |
| 5 | 4.6 | 82.0 | 22 | 3.644 |
| 6 | 4.6 | 82.0 | 24 | 6.133 |
| 7 | 4.6 | 85.0 | 22 | 5.422 |
| 8 | 4.6 | 85.0 | 24 | 6.889 |
| 9 | 4.4 | 83.5 | 22 | 5.467 |
| 10 | 4.8 | 83.5 | 22 | 5.511 |
| 11 | 4.4 | 83.5 | 24 | 5.867 |
| 12 | 4.8 | 83.5 | 24 | 13.289 |
| 13 | 4.6 | 83.5 | 23 | 13.467 |
| 14 | 4.6 | 83.5 | 23 | 13.700 |
| 15 | 4.6 | 83.5 | 23 | 13.300 |

**Creation of regression model and confidence analysis**

The multivariable regression analysis is performed using SAS software and gives the results as shown in TABLE 6 and TABLE 7. As can be seen from the variance analysis table, some monomial and quadratic terms are significant and some are insignificant in the equation. The interactive terms with insignificant impacts can be ignored. The polynomial regression model is fitted according to the impact of experimental factors on the response value as below:

$$Y_1 = -12621.7 + 244.1654\times W+207.4944\times T-1.45679\times W\times W-4.477778\times T\times T$$

As can be seen from TABLE 6, the linear relationship is significant between the dependent and independent variables when the relationship between factors and response values are described using above regression equation. Its determinant coefficient is 96.39% indicating that the proposed regression equation has a better fitting degree.

**TABLE 6 : Partial regression coefficient estimates**

| Parameter | Estimate value | Standard deviation | t | Pr>\|t\| |
|---|---|---|---|---|
| pH | 1.5333333 | 0.418168 | 3.666791 | 0.014492 |
| W | 1.3222222 | 0.418168 | 3.161943 | 0.02504 |
| T | 1.5166666 | 0.418168 | 3.626934 | 0.015109 |
| pH×pH | -1.372222 | 0.615526 | -2.22935 | 0.076227 |
| pH×W | -1.366667 | 0.591378 | -2.31099 | 0.068823 |
| pH×T | 1.8444444 | 0.591378 | 3.118891 | 0.026285 |
| W×W | -3.383333 | 0.615526 | -5.49665 | 0.002722 |
| W×T | -0.255556 | 0.591378 | -0.43214 | 0.683645 |
| T×T | -4.583333 | 0.615526 | -7.4462 | 0.000689 |
| RMSE(response value) | 1.182757 | | | |
| R-square(determinant coefficient) | 96.39% | | | |

**Optimization and verification of significant factor level**

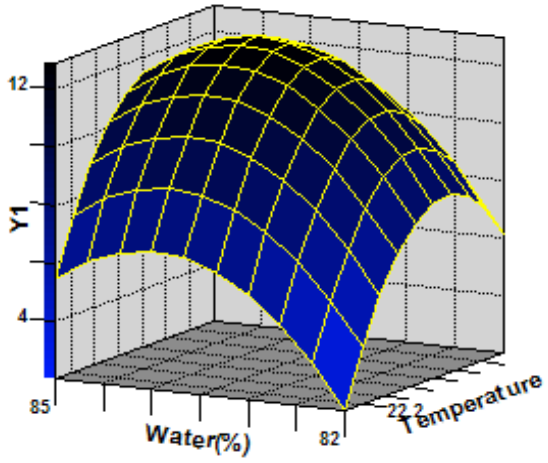


**Figure 1 : Surface and contour plots for the effects of temperature and water content on BOX production**

It is known from the regression equation's 3D surface and counter plots given in Figure 1 that theregression equation presents a stable point, the actual values of appropriate pH, T and water content are 4.6, 23 and 83.5 respectively. The maximal estimated BOX activity is 13.45U/g. The experiment is done under the optimal conditions by totally 3 batches of solid culture tests. The results are 12.1 U/g, 11.9 U/g and 12.9 U/g, and the average experimental value is 12.3 U/g. This proves that the predictive value 13.45 is more approximate to the average experimental value 12.3 whilst the BOX production is increased by 1.63 times from 1.63 prior to optimization.

**Time course curve of IMER1's BOX production in a solid-state formation system**

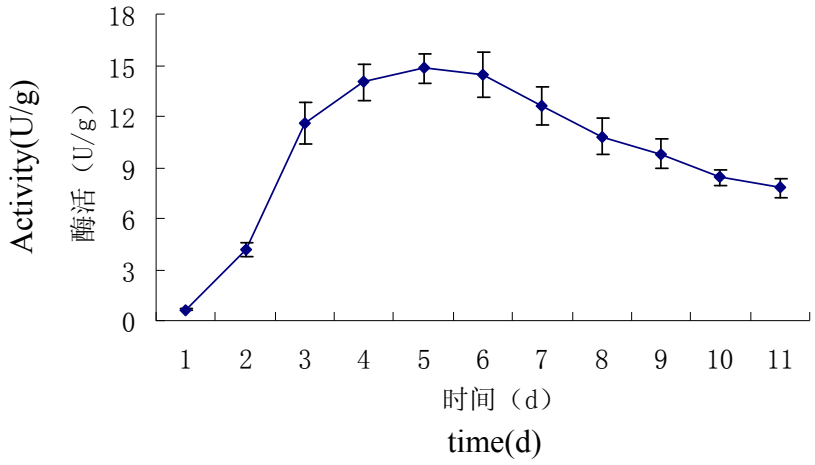


**Figure 2 : Time course of production of IMER1's BOX in solid-state fermentation**

As illustrated in Figure 2, BOX activity can be determined in 1d after IMER1's solid-state fermentation. The BOX activity reached the highest in the 5$^{th}$ day, and kept at a higher level from the 3$^{rd}$ to 10$^{th}$ day, suggesting that IMER1 in bran solid-state matrix can continue a higher BOX production. With increasing incubation time, BOX activity decreased slowly, but in a lower declining magnitude. The results showed that the BOX was produced early in solid-state formation and kept a higher production for a longer time. Though in more advantages of solid-state fermentation than the liquid-state once, some technical issues remain in the large-scale solid-state fermentation, for example, the equipment covers a large area, the work strength is intensive, the mass and heat transfer is difficult, and the parameters, such as pH, temperature, cell proliferation amount and production, etc, are determined difficultly[13,14]. If such issues can be well-solved, the solid-state fermentation will get a better development.

**Determination of extracellular BOX activity in IMER1's solid-state fermentation products**


**Figure 3 : Native-PAGE of extracellular enzyme from solid-state fermentation (1,ABTS; 2, Bilirubin).**

BOX is an extracellular enzyme of *Myrothecium sp*. There are two issues to be explored in solid-state fermentation to produce the BOX: 1. the extracellular enzymes in some fungi can produce isoenzymes after induction. Owing to the complexity of solid-state matrix, whether *Myrothecium sp.* can be induced to produce BOX isozyme? 2. Whether the extracellular enzymes of *Myrothecium sp.* have other oxidase that has a crossed activity as BOX substrate? To further solve these problems, the activity of extracellular enzyme solution produced in *Myrothecium sp.* solid-state fermentation was analyzed by electrophoresis in this paper. Theoretically, BOX can catalyze the yellow bilirubin to form a green substance, and additionally catalyze the colorless ABTS to form a green substance. The present experiment determined the activity after IMER1 fermentation liquid electrophoresis using BOX substrate (bilirubin and ABTS) (Figure 3). When the gel strip that the bilirubin solution (pH8.0) substrate is developed is used, it is found that the yellow background showed apparent single green strip; when the gel strip that the colorless ABTS solution substrate (pH4.0) is developed is used, it is found that the grayish background showed apparent single green strip. The outcome suggests that IMER1 under the described solid-state fermentation condition can only produce a BOX free from the isozyme whilst other extracellular enzymes produced by the strain do not generate the crossed activities with BOX.

## CONCLUSION

The factors significantly affecting the IMER1's BOX production include the water content, pH and temperature under the solid-state matrix fermentation conditions. The PB-designed experimental results showed that the pH and temperature presented a negative effect on BOX production whilst the water content presented a large positive effect. The outcomes from steepest ascent experiment and response surface central composite design showed that the BOX production reaches the largest when pH, T and water content are 4.6, 23 and 83.5% respectively. The polynomial regression model through fitting by SAS software showed that the linear relationship between the dependent and independent variables is significant and the determinant coefficient is 96.39%, suggesting that the fitting degree of the regression equation is very well. The estimated value 13.45 by simulative experiment to produce the BOX is more approximate to the actual experimental average 12.3, and increased by about 2 times comparing to the BOX production 6.21 prior to optimization. With the electrophoresis of crude BOX solution generated in solid-state fermentation, the single strip of BOX activity can be detected, suggesting that no BOX isoenzyme of the strain is induced and no other extracellular enzyme with crossed activity is produced. These experimental results provide a theoretic ground for the BOX production through solid-state fermentation, also provide a reference for the large-scale industrial BOX production and demonstrate some theoretical and practical significance.

## ACKNOWLEDGEMENT

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