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# Development of a New Bioprocess for Clean Diosgenin Production through Submerged Fermentation of an Endophytic Fungus

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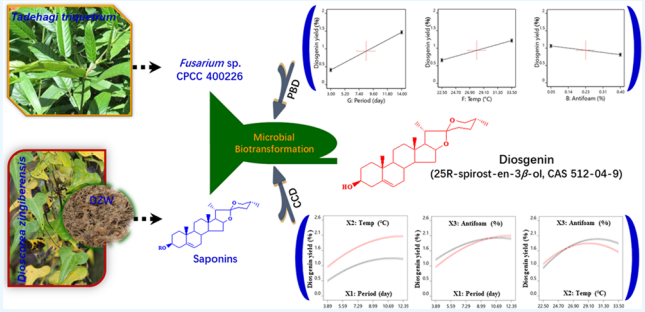
**ABSTRACT:** Diosgenin is used widely to synthesize steroidal hormone drugs in the pharmaceutical industry. The conventional diosgenin production process, direct acid hydrolysis of the root of *Dioscorea zingiberensis* C. H. Wright (DZW), causes large amounts of wastewater and severe environmental pollution. To develop a clean and effective method, the endophytic fungus *Fusarium* sp. CPCC 400226 was screened for the first time for the microbial biotransformation of DZW in submerged fermentation (SmF). Statistical design and response surface methodology (RSM) were implemented to develop the diosgenin production process using the *Fusarium* strains. The environmental variables that significantly affected diosgenin yield were determined by the two-level Plackett−Burman design (PBD) with nine factors. PBD indicates that the fermentation period, culture temperature, and antifoam reagent addition are the most influential variables. These three variables were further optimized using the response surface design (RSD). A quadratic model was then built by the central composite design (CCD) to study the impact of interaction and quadratic effect on diosgenin yield. The values of the coefficient of determination for the PBD and CCD models were all over 0.95. *P*-values for both models were 0.0024 and <0.001, with *F*-values of ∼414 and ∼2215, respectively. The predicted results showed that a maximum diosgenin yield of 2.22% could be obtained with a fermentation period of 11.89 days, a culture temperature of 30.17 °C, and an antifoam reagent addition of 0.20%. The experimental value was 2.24%, which was in great agreement with predicted value. As a result, over 80% of the steroidal saponins in DZW were converted into diosgenin, presenting a ∼3-fold increase in diosgenin yield. For the first time, we report the SmF of a *Fusarium* strain used to produce diosgenin through the microbial biotransformation of DZW. A practical diosgenin production process was established for the first time for *Fusarium* strains. This bioprocess is acid-free and wastewater-free, providing a promising environmentally friendly alternative to diosgenin production in industrial applications. The information provided in the current study may be applicable to produce diosgenin in SmF by other endophytic fungi and lays a solid foundation for endophytic fungi to produce natural products.




## 1. INTRODUCTION

Steroidal saponins are used extensively in the pharmaceutical industry as starting materials for the chemical synthesis of various steroid hormone drugs.[1] Diosgenin (25[R]-spirost-en-3$\beta$-ol), a naturally occurring steroidal saponin broadly present in a wide range of *Dioscorea* plants (e.g., *D. zingiberensis* C. H. Wright and *D. nipponica* Makino), *Trigonella* species, and *Costus* species, is a critical pharmaceutical precursor for the synthesis of hundreds of steroidal drugs including cortisone, progesterone, androstane, and androstene compounds.[2,3] In recent years, many biological activities such as antitumor effect, antimalarial action, antagonistic effect, and cardiovascular action have been ascribed to diosgenin.[4−6]

Diosgenin mostly exists in plants in the form of glycosidic saponins (chemical structures are presented in Figure S1), such as $\alpha$-L-(1 → 2)-rhamnoside, $\alpha$-L-(1 → 4)-rhamnoside, $\beta$-D-(1→3)-glucoside, and $\beta$-D-(1 → 4)-glucoside.[7,8] The conversion of saponins is the primary method for diosgenin preparation. Due to severe steric effects, the glycosyl at the *C*-3 position of steroidal saponins was more challenging to be hydrolyzed than other substituents in compounds 1−6.[9,10] In the industry, diosgenin is prepared annually using the "acid hydrolysis-chemical extraction" strategy, where steroidal saponins in the root of *Dioscorea zingiberensis* C. H. Wright (DZW) are hydrolyzed by sulfuric acid and then are extracted by gasoline.[11] However, this traditional acid hydrolysis process, generating massive amounts of acid wastewater (3 $m^3$ per produced 1 kg diosgenin) with a high chemical oxygen demand (∼80 g/L), has led to severe water waste and environmental problems,[12,13] which is the main bottleneck restricting the

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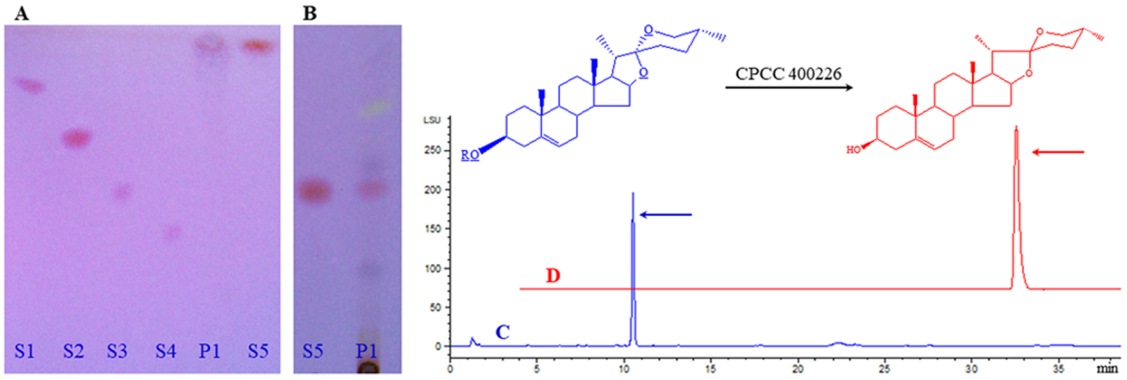


**Figure 1.** Analysis of biotransformation products converted by *Fusarium* sp. CPCC 400226. Products converted from TS were analyzed by thin layer chromatography (TLC). The developing solvents were (A) chloroform/methanol/water (70:26:6, v/v) and (B) petroleum ether/ethyl acetate (2:1, v/v). S1−S5, standard contrast of trillin, prosapogenin A of dioscin, deltonin, zingiberensis newsaponin, and diosgenin, respectively. P1 is the product of TS converted by CPCC 400226 in the YPG medium. Products converted from zingiberensis newsaponin were analyzed by high-performance liquid chromatography-evaporative light-scattering detector (HPLC-ELSD). The blue arrow and the red arrow indicate the substrate of zingiberensis newsaponin (C) and the resulting product of diosgenin (D), respectively.

development of the diosgenin industry. Unlike chemical reactions, biological hydrolysis offers many unique advantages.[14−16] Biological methods have been implemented to develop clean and efficient processes for the preparation of diosgenin. Among these, microbial biotransformation of DZW using native microorganisms is becoming increasingly attractive,[17−19] but the diosgenin yield cannot fully satisfy the industrial purposes, including the previous process developed in our laboratory.[20]

Microbial biotransformation is often affected directly and indirectly by many environmental factors. Optimization of these factors is primarily crucial for efficient biotransformation by the chosen microorganisms. Moreover, process optimization can reduce the processing time and decrease the production cost. This can be manipulated by the conventional one-factor-at-a-time (OFAT) or statistical design. Only one variable is adjusted at each experiment when the OFAT method is employed, and all other variables are maintained at constant levels. The experiments can be done easily; however, OFAT is time-consuming, laborious, and expensive.[21] Additionally, the mutual factor interactions that affect processing efficiency are not considered in process optimization procedures. The conventional OFAT method may be unable to determine the significant variables and frequently fails to generate the optimum response.[22] In contrast to this, the statistical design can effectively identify the effect of significant factors and mutual factor interactions.[23] The statistical method has many advantages, such as reduced experiments, increased efficiency, less time-consuming, and effortless.[24] This method is being used commonly to investigate a phenomenon for better understanding and improvement.[25−27]

The Plackett−Burman design (PBD), a factorial experimental design with a small size and two levels, is commonly implemented for screening large factors. Using this method, the statistically significant variables are determined from the $k$ number of variables in $k + 1$ runs of experiment. One of the critical points for PBD is that the method does not take recourse to the mutual factor interactions between and among the various variables.[28] The resulting significant factors (usually three or four) are brought to the response surface method (RSM) for further optimization. By studying the mutual factor interactions among the variables over various values in a statistically effective manner, RSM simplifies the process optimization based on general statistics principles.[29] Due to the accuracy of the experiment, the central composite design (CCD) is applied frequently in RSM. By finding out the mutual influence and comprehensive effects of the main variables, informative results can be obtained. Followed by a simulation of the residual plots in CCD, the model adequacy and the uniformity of the error distribution are checked. Then, a regression model is further built based on the least-squares technique.[23] Recently, a combination of PBD and CCD has been used successfully to optimize many bioprocesses.[30−32]

Considering the particular existing form of diosgenin in plants and environmental issues of the acid hydrolysis method, progress has been made to develop clean and efficient methods for diosgenin production.[33−37] In our previous study, we found that the endophytic fungi *Fusarium* sp. CPCC 400709, isolated from *Dioscorea zingiberensis* C. H. Wright on Czapek's medium, was able to effectively biotransform DZW and produce diosgenin in solid-state fermentation (SSF).[20] However, there is still a long way ahead in industrial applications. In contrast to SSF, SmF has many advantages, such as low input, short cultivation time, high profits, and easy scale-up.[38] In the current study, a new bioprocess for clean and efficient diosgenin production through SmF of an endophytic fungus that belongs to the *Fusarium* genus was developed for the first time and successfully used to produce diosgenin. First, the fungal strain capable of transforming DZW was investigated for the first time. Statistical techniques were then employed to optimize the microbial biotransformation process for achieving enhanced diosgenin yield by *Fusarium* sp. CPCC 400226 in SmF. At last, the predicted response was validated with actual experimentation. This study presents a wastewater-free, acid-free, environmentally friendly, simple-operation, and low-cost bioprocess to produce diosgenin through the SmF of an endophytic fungus.

## 2. RESULTS AND DISCUSSION

**2.1. Screening of the Active Fungal Strain for DZW Biotransformation.** More than 16% of the fungal strains showed activities of hydrolyzing both substrates TS and zingiberensis newsaponin, and the desired product of diosgenin was obtained in the YPG medium. It was found that much less intermediates and more diosgenin were observed when the endophytic fungus CPCC 400226 was


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**Table 1. PBD Matrixes for the Evaluation of Diosgenin Yield through Biotransformation of DZW by CPCC 400226**

| | | | | | | | | | | | | diosgenin yield (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| run order | *A*: beads | *B*: antifoam | *C*: surfactant | *D*: volume | *E*: agitation | *F*: temp | *G*: period | *H*: pH | *J*: inoculum | *K*: DV1 | *L*: DV2 | predicted value | experimental value |
| 1 | +1 | −1 | +1 | +1 | −1 | +1 | +1 | +1 | −1 | −1 | −1 | 1.87 | 1.86 ± 0.124 |
| 2 | −1 | +1 | −1 | +1 | +1 | −1 | +1 | +1 | +1 | −1 | −1 | 1.08 | 1.07 ± 0.119 |
| 3 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | +1 | +1 | −1 | +1 | 0.49 | 0.50 ± 0.091 |
| 4 | −1 | −1 | −1 | +1 | −1 | +1 | +1 | −1 | +1 | +1 | +1 | 1.93 | 1.94 ± 0.102 |
| 5 | −1 | −1 | +1 | −1 | +1 | +1 | −1 | +1 | +1 | +1 | −1 | 0.84 | 0.82 ± 0.081 |
| 6 | +1 | −1 | −1 | −1 | +1 | −1 | +1 | +1 | −1 | +1 | +1 | 1.22 | 1.23 ± 0.133 |
| 7 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 0.26 | 0.25 ± 0.046 |
| 8 | −1 | +1 | +1 | −1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | 1.66 | 1.67 ± 0.112 |
| 9 | +1 | +1 | -1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | 0.53 | 0.51 ± 0.095 |
| 10 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | +1 | +1 | −1 | 1.05 | 1.03 ± 0.137 |
| 11 | +1 | −1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | +1 | 0.27 | 0.28 ± 0.057 |
| 12 | −1 | +1 | +1 | +1 | −1 | −1 | −1 | +1 | −1 | +1 | +1 | 0.08 | 0.09 ± 0.042 |

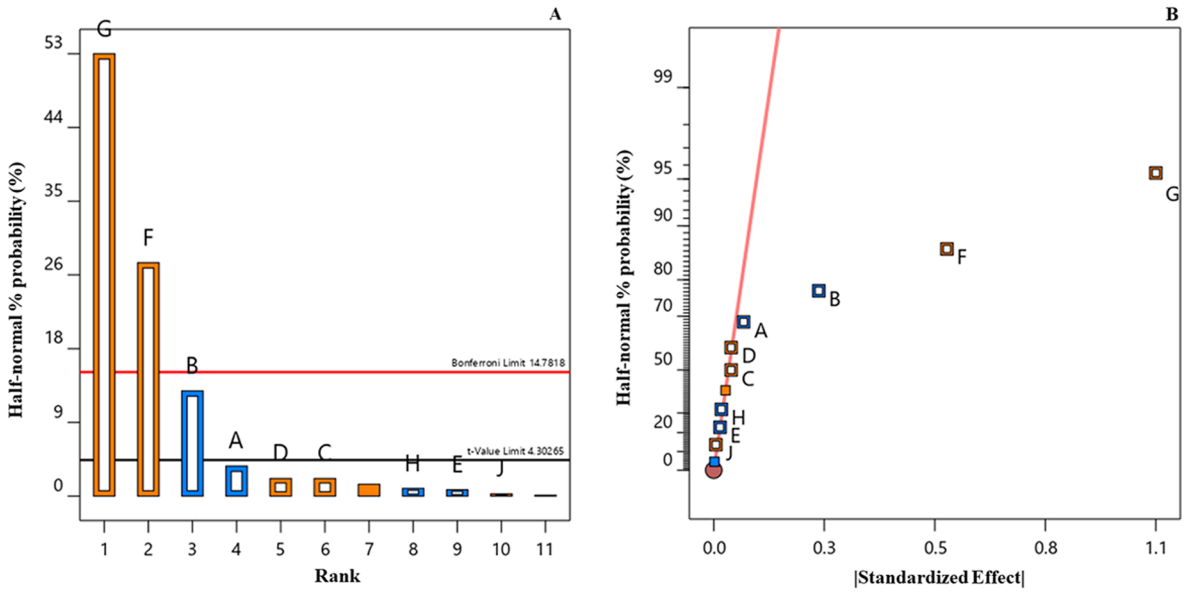


**Figure 2.** Plots of effect for screening the statistically significant factors in PBD. (A) Pareto chart. (B) Half-normal probability plot. Yellow points indicate positive effects, and blue points indicate negative effects. Glass bead addition (%), antifoam reagent addition (%), surfactant addition (g/L), working volume (mL), agitation (rpm), culture temperature (°C), fermentation period (days), fermentation pH, and inoculum size (%) are denoted *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, and *J*, respectively.

used. As shown in Figure 1A, the most active strain CPCC 400226 was therefore selected among all of the fungi because of the best biotransformation activity against steroidal saponins. After five days of microbial biotransformation by CPCC 400226, more than 90% of the zingiberensis newsaponin were converted to diosgenin, and the maximal diosgenin production was ∼40 μg/mL (Figure 1B).

In this work, a total of 184 endophytic fungi isolated from Chinese medicinal plants and preserved in the CPCC were brought to the diosgenin-producing activity screening using both substrates TS and zingiberensis newsaponin. According to the ITS rRNA gene sequence analysis, the most dominant genera were determined as *Preussia* (20.1%), *Paraphoma* (14.7%), and *Fusarium* (13.6%). Among these, *Fusarium* sp. was the most active genus, and ∼41% of the *Fusarium* strains could produce diosgenin. It was found that DZW could be converted by the fungi belonging to *Trichoderma* and *Aspergillus* genera.[12,39] We previously found that *Fusarium* strains isolated from *Dioscorea zingiberensis* C. H. Wright could convert steroidal saponins and produce diosgenin.[20] The fungus CPCC 400226, initially isolated from *Tadehagi triquetrum* (L.) Ohashi using Czapek yeast extract agar medium (http://www.cpcc.ac.cn/fungus/?id=5095), also belonged to *Fusarium*. It is once more demonstrated that *Fusarium* strains are key bioresources for the production of diosgenin through microbial biotransformation. Moreover, the *Fusarium* strains may have great potential to produce other natural products.

**2.2. Screening of Bioprocess Factors Affecting DZW Biotransformation Using PBD.** Prediction of the significant influence of each independent variable is critical for diosgenin yield. The PBD method has been used extensively for identifying the most significant variables from various conditions. In this study, PBD was implemented for investigating the effect degrees of each independent variable on diosgenin yield and for screening dominant environmental factors. It is well-recognized that the performance of SmF can be influenced by the fermentation period, culture temperature, fermentation pH, agitation, inoculum size, and working volume. Thus, these six factors were included in the PBD


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experiments. We found that large amounts of foam were generated in the bioreactor when the SmF of CPCC 400226 was conducted. The strain formed densely packed mycelia in the shake flask, and glass bead were added to the fermentation culture. It was found that surfactants could improve the performance of the SmF process.[40,41] In this case, the addition of glass bead, antifoam reagent, and surfactant was also included. Nine factors were investigated on diosgenin yield by running 12 experiments between low (−1) and high (+1) levels. The experimental design matrix with the results is shown in Table 1. Selected variables affected the diosgenin yield, which varied from 0.09 to 1.94%. The highest diosgenin yield was obtained in run 4 followed by runs 1, 8, and 6. On the other hand, the lowest diosgenin yield was detected in run 12 followed by runs 7, 11, and 3.

It is well-acknowledged that the Pareto chart can check the statistical significance and present the effect of factors on response.[42] To identify the significant factors affecting microbial biotransformation efficiency by CPCC 400226 in SmF, the Pareto chart was plotted. As shown in Figure 2A, the Pareto chart was plotted by the *t*-values of effect versus various variables. The length of each variable is proportional to the absolute values of the estimated effects. Two straight lines presenting the *t*-value limit and the Bonferroni limit are included as horizontal reference lines. The variable can be considered significant when the *t*-value of this variable is higher than the *t*-value limit line. If a variable has a *t*-value higher than the Bonferroni limit line, it can be considered that this variable has a very significant effect.[43] In the Pareto chart, the Bonferroni limit line with a value of 14.7818 and the *t*-value limit line with a value of 4.30265 were obtained. The two *t*-value limit lines were then implemented for identifying the significant variables that affect the microbial biotransformation of DZW through the SmF of CPCC 400226. It was found that three factors, i.e., fermentation period, culture temperature, and antifoam reagent addition, had a significant influence on the desired response of diosgenin yield.

The half-normal probability plot, plotted by the half-normal probability (%) versus the absolute value of standardized effect of each variable, is also often used to identify significant factors. The variable having an effect near the straight line through zero indicates that this variable is more likely not significant. In contrast, the variable deviating from the straight line is considered to be significant.[44] The half-normal plot for diosgenin yield is shown in Figure 2B. It was seen that five of the nine tested variables (i.e., fermentation period, culture temperature, working volume, surfactant addition, and inoculum size) positively affected diosgenin yield, while the other variables had a negative effect, which were antifoam reagent addition, glass bead addition, fermentation pH, and agitation. The factor *G* (fermentation period) was the most significant variable with the highest positive impact on diosgenin yield. The factor *F* (culture temperature) also demonstrated a significant enhancement effect. On the other hand, the highest significant negative effect on the yield of diosgenin was observed in factor *B* (antifoam reagent addition). Other variables, including glass bead addition, working volume, surfactant addition, fermentation pH, agitation, and inoculum size, reveal no significant effects, which is in agreement with the results obtained in the Pareto chart. The two plots indicate that the fermentation period (*G*), culture temperature (*F*), and antifoam reagent addition (*B*) are statistically significant.

Using the analysis of variance (ANOVA), experimental data were further analyzed. As seen in Table 2, the fermentation

**Table 2. Statistical Analysis of the Model from the PBD[a]**

| source | SS | Df | MS | *F*-value | *P*-value |
|---|---|---|---|---|---|
| model | 4.51 | 9 | 0.5014 | 414.99 | 0.0024* |
| *A*, glass bead addition | 0.0154 | 1 | 0.0154 | 12.75 | 0.0703 |
| *B*, antifoam reagent addition | 0.1900 | 1 | 0.1900 | 157.25 | 0.0063* |
| *C*, surfactant addition | 0.0052 | 1 | 0.0052 | 4.31 | 0.1735 |
| *D*, working volume | 0.0052 | 1 | 0.0052 | 4.31 | 0.1735 |
| *E*, agitation | 0.0007 | 1 | 0.0007 | 0.56 | 0.5327 |
| *F*, culture temperature | 0.9352 | 1 | 0.9352 | 773.97 | 0.0013* |
| *G*, fermentation period | 3.3600 | 1 | 3.3600 | 2780.86 | 0.0004* |
| *H*, fermentation pH | 0.0010 | 1 | 0.0010 | 0.83 | 0.4574 |
| *J*, inoculum size | 0.0001 | 1 | 0.0001 | 0.06 | 0.8265 |
| residual | 0.0024 | 2 | 0.0012 | | |
| cor total | 4.52 | 11 | | | |

[a]Model summary: $R^2$, 0.9995; adjusted $R^2$, 0.9971; predicted $R^2$, 0.9807. *indicates $P < 0.05$, 5% significant level. SS, sum of squares; Df, degree of freedom; MS, mean sum of squares.

period represents the most significant effect on diosgenin yield approved by the largest *F*-value and the lowest *t*-value. It further reveals that this variable has the largest positive coefficient, which agrees with the results demonstrated in the Pareto chart and the half-normal probability plot. This variable had the strongest enhancement effect on DZW biotransformation. The antifoam reagent addition had the strongest negative effect on the microbial biotransformation of DZW by CPCC 400226. Meanwhile, the $R^2$ (coefficient of correlation), predicted $R^2$, and adjusted $R^2$ were 0.9995, 0.9807, and 0.9971, respectively. Generally, the acceptance of any model is emphasized with $R^2 > 0.75$.[45] In this case, the values of $R^2$, predicted $R^2$, and adjusted $R^2$ were all acceptable, showing good fitness of the model. The "model *F*-value" to occur due to noise was 0.24%, and the model *F*-value was 414.99, which implies that the model was significant. PBD experiments on diosgenin yield by CPCC 400226 indicate that the dominant variables are culture temperature, fermentation period, and antifoam reagent addition. These three independent variables were chosen for RSD.

In general, the growth of a fungal strain can be directly or indirectly influenced by the basic environmental conditions, such as culture temperature, period, pH value, etc. Meanwhile, microbial hydrolysis is also affected by these variables because these conditions can dramatically affect the enzymatic activity of the enzymes produced by the strain. As we assumed, the fermentation period and culture temperature had a significant effect on the microbial biotransformation of DZW through SmF of the *Fusarium* strain. However, no significant effect was observed in the fermentation pH although it is well-acknowledged that the pH values can dramatically affect the enzymatic reaction. We assumed that the fungal strain CPCC 400226 might produce a variety of glycosidases with abundant diversity and broad reaction pH, which supported the high efficiency of microbial biotransformation of DZW by this strain, at least in part. Interestingly, the $\beta$-glucosidase FBG1 purified from *Fusarium* sp. CPCC 400709 was still capable of catalyzing trillin and producing diosgenin even when the reaction pH values were lower than 3 or higher than 7. On the


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other hand, it made it easier to perform large-scale SmF without pH control for future industrial applications.

Agitation and aeration may often cause excessive foam formation and thus influence cell growth and biotransformation efficiency. In our previous study, a certain quantity of antifoam reagent was added into the fermentation medium and reaction broth for the SmF of yeast and recombinant enzyme catalysis, respectively.[46−48] The antifoam reagent has been used commonly in SmF, and the mechanisms of action were also summarized, such as bridging-stretching, spreading fluid entrainment, bridging-dewetting, etc.[49] However, there is very little knowledge on the effect of antifoam reagent addition on the growth of *Fusarium* strains. Investigation on the influence of antifoam reagents on the production of diosgenin through SmF of a *Fusarium* strain is more limited. In this study, we found that the diosgenin yield was negatively affected by antifoam reagent addition, thereby significantly influencing the microbial biotransformation of DZW by CPCC 400226 in SmF. It is suggested that the use of an antifoam reagent and its amounts should be considered carefully for the *Fusarium* strains to produce diosgenin. On the other hand, adding a certain amount of antifoam reagent could apparently enhance the production of diosgenin in SmF. We assumed that antifoam reagent addition might refresh the growth conditions for the fungal strain and make it more effective to continue the communication between enzymes (in or secreted from CPCC 400226) and substrates (of steroidal saponins in DZW). The information obtained from the PBD experiment provides a critical basis for the bioreactor-scale SmF of CPCC 400226.

**2.3. Optimization of Significant Variables Affecting Diosgenin Yield by CCD.** *2.3.1. Model Building for Bioprocess Optimization.* The CCD with experimental and predicted values is presented in Table 3. Selected primary variables significantly affected the diosgenin yield, which varied from 0.11 to 1.98%. The highest diosgenin yield was obtained in run 7 followed by runs 16, 14, and 4. Conversely, the lowest diosgenin yield was detected in run 19 followed by runs 1, 9, and 6.

**Table 3. CCD Matrixes for the Optimization of Diosgenin Yield**

| | | | | diosgenin yield (%) | |
|---|---|---|---|---|---|
| run order | *X*1: period | *X*2: temp | *X*3: antifoam | predicted value | experimental value |
| 1 | −1 | −1 | +1 | 0.29 | 0.28 ± 0.079 |
| 2 | +1 | −1 | −1 | 0.98 | 0.97 ± 0.123 |
| 3 | 0 | 0 | 0 | 1.95 | 1.94 ± 0.151 |
| 4 | 0 | 0 | 0 | 1.95 | 1.96 ± 0.157 |
| 5 | 0 | 0 | 0 | 1.95 | 1.92 ± 0.152 |
| 6 | −$\alpha$ | 0 | 0 | 0.25 | 0.27 ± 0.063 |
| 7 | +1 | +1 | −1 | 1.99 | 1.98 ± 0.146 |
| 8 | +1 | −1 | +1 | 1.31 | 1.30 ± 0.054 |
| 9 | −1 | −1 | −1 | 0.34 | 0.33 ± 0.083 |
| 10 | −1 | +1 | −1 | 1.03 | 1.02 ± 0.094 |
| 11 | 0 | +$\alpha$ | 0 | 1.10 | 1.11 ± 0.100 |
| 12 | 0 | 0 | +$\alpha$ | 1.56 | 1.58 ± 0.119 |
| 13 | 0 | 0 | 0 | 1.95 | 1.92 ± 0.112 |
| 14 | 0 | 0 | 0 | 1.95 | 1.97 ± 0.120 |
| 15 | 0 | 0 | −$\alpha$ | 1.70 | 1.71 ± 0.111 |
| 16 | 0 | 0 | 0 | 1.95 | 1.96 ± 0.166 |
| 17 | −1 | +1 | +1 | 0.54 | 0.53 ± 0.083 |
| 18 | +$\alpha$ | 0 | 0 | 1.92 | 1.93 ± 0.131 |
| 19 | 0 | −$\alpha$ | 0 | 0.05 | 0.07 ± 0.029 |
| 20 | +1 | +1 | +1 | 1.88 | 1.87 ± 0.117 |

The second-order polynomial equation was as follows

$$Y(\%,\ \text{diosgenin yield}) = 1.95 + 0.49X_1 + 0.31X_2 - 0.04X_3 + 0.08X_1X_2 + 0.10X_1X_3 - 0.11X_2X_3 - 0.30X_1^2 - 0.48X_2^2 - 0.11X_3^2 \quad (1)$$

where $Y$ represents the diosgenin yield (%); 1.95 is the intercept; 0.49, 0.31, and −0.04 are the linear coefficients; 0.08, 0.10, and −0.11 are the interactive coefficients, −0.30, −0.48, and −0.11 are the quadratic coefficients; and *X*1, *X*2, and *X*3 are the fermentation period, culture temperature, and antifoam reagent addition, respectively. Among the three variables, antifoam reagent addition demonstrated the lowest regression coefficient. The highest value was observed in the fermentation period followed by culture temperature.

*2.3.2. Mathematical Validation of the Model.* Based on the above regression equation, the interactions of the primary variables are indicated by the statistical significance of each coefficient. As shown in Table 4, each variable, model terms, and the mutual factor interactions were significant for the microbial biotransformation of DZW through SmF of CPCC 400226. A *P*-value of 0.51 implies that the lack of fit is not significant relative to the pure error. It was seen that the model could fit the experimental values and predict the yield of diosgenin excellently. Usually, the high adequacy, precision, and reliability of the model can be indicated by a low coefficient of variation (CV). A high $R^2$ can indicate that the model is workable.[42] In this study, a CV value of 1.62% was observed, along with an $R^2$ of 0.9995 for diosgenin yield. The predicted $R^2$ and the adjusted $R^2$ were 0.9978 and 0.9990, respectively. In addition, the "adeq precision" value (∼127) was greater than 4. Thus, the model was adequate, precise, and reliable.

**Table 4. Statistical Analysis of the Model from the CCD[a]**

| source | SS | Df | MS | *F*-value | significance by *P*-value |
|---|---|---|---|---|---|
| model | 9.28 | 9 | 1.03 | 2215.06 | * |
| *X*1, fermentation period | 3.34 | 1 | 3.34 | 7173.29 | * |
| *X*2, culture temperature | 1.33 | 1 | 1.33 | 2867.79 | * |
| *X*3, antifoam reagent addition | 0.0212 | 1 | 0.0212 | 45.65 | * |
| *X*1*X*2 | 0.0512 | 1 | 0.0512 | 110.03 | * |
| *X*1*X*3 | 0.0722 | 1 | 0.0722 | 155.16 | * |
| *X*2*X*3 | 0.0968 | 1 | 0.0968 | 208.02 | * |
| $X1^2$ | 1.33 | 1 | 1.33 | 2867.06 | * |
| $X2^2$ | 3.38 | 1 | 3.38 | 7272.1 | * |
| $X3^2$ | 0.1794 | 1 | 0.1794 | 385.56 | * |
| residual | 0.0047 | 10 | 0.0005 | | |
| lack of fit | 0.0023 | 5 | 0.0005 | 0.9802 | 0.5085 |
| pure error | 0.0024 | 5 | 0.0005 | | |
| cor total | 9.28 | 19 | | | |

[a]Model summary: $R^2$, 0.9995; adjusted $R^2$, 0.9990; predicted $R^2$, 0.9978. *$P < 0.05$, 5% significant level. SS, sum of squares; Df, degree of freedom; MS, mean sum of squares.



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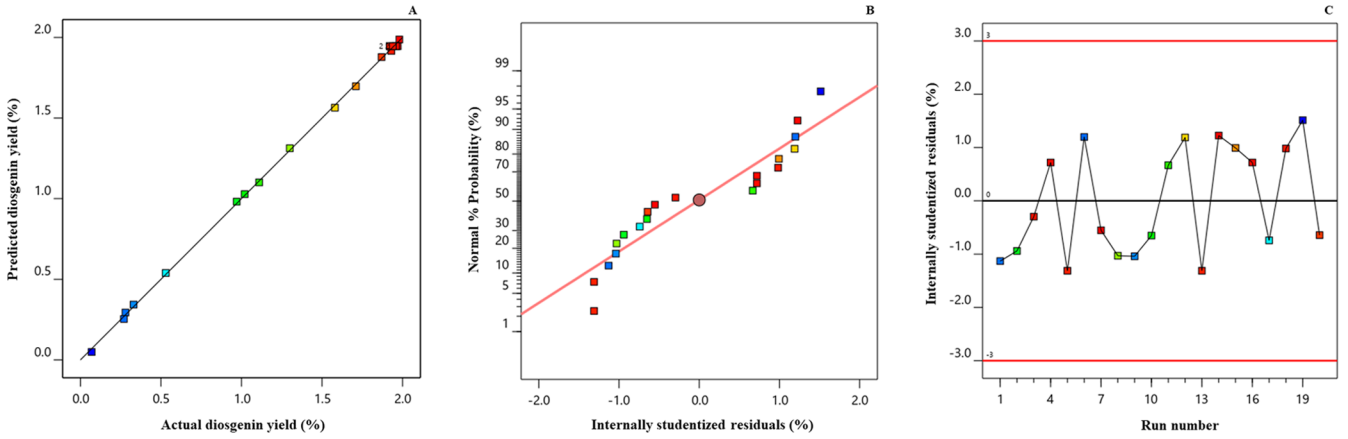


**Figure 3.** Diagnostic plots of the CCD model adequacy for diosgenin yield. (A) Plot of predicted values versus experimental values. (B) Plot of normal % probability. (C) Plot of internally studentized residuals. Each value in the plots was presented by different color points.

*2.3.3. Diagnostics Plots of Model Adequacy.* Various diagnostic plots generated using the experimental values, probability values, and residuals were applied for checking the adequacy of the model. As shown in Figure 3A, data points in the plot of predicted values versus experimental values were reasonably aligned, suggesting that the model predicted values were in good agreement with the experimental values. The normal % probability plot is represented in Figure 3B. Most of the data points were close to a straight line, implying that the model was robust, accurate, and conforming to normal distribution. Figure 3C demonstrates the plot of internally studentized residuals. The absolute values of each data point were less than three, suggesting that the model is adequate. Therefore, the model developed in the current study possessed satisfactory fits for the yield of diosgenin. It was again validated that the model was reliable to fit the interactions between various variables.

*2.3.4. Mutual Factor Interactions Analysis.* The perturbation plot is often employed to estimate the effect of various variables. By moving each variable from the chosen reference point while keeping the other variables at constant reference values, the response changes are presented in the perturbation plot.[50] As shown in Figure S2, the curve with the most notable change was the fermentation period (A) followed by culture temperature (B). The least notable variable was defined as antifoam reagent addition (C). Moreover, the two-dimensional (2-D) contour maps and three-dimensional (3-D) response surfaces were plotted for further visualizing the influences of each variable and mutual factor interactions on diosgenin yield. The plots were generated by the response ($Z$-axis) according to two factors ($X$ and $Y$ coordinates) while holding the other factor at the zero level, and the optimum value of each variable was determined to reach a maximum response. Generally, the optimum point is inside the design boundary level unless there is no clear peak on each 3-D response surface. Perfect mutual factor interactions often show elliptical contours on the 2-D contour map, while a circular shape indicates less significant mutual factor interactions. In the optimal region of the contour map, the surface confined in the smallest ellipse often indicates the maximal predicted response.[51]

The mutual effect of fermentation period ($X1$) and culture temperature ($X2$) on diosgenin yield is depicted in Figure 4A, where the antifoam reagent addition ($X3$) was maintained at a constant zero level. The response surface is steep, indicating the apparent influence of fermentation period and culture temperature on the yield of diosgenin. It was found that the interaction between the fermentation period and culture temperature was significant for diosgenin yield since a uniformly elongated diagonal running pattern was seen in the 2-D contour plot. When the antifoam reagent addition was 0.2% (level zero), the yield of diosgenin first gradually increased and then maintained at a constant level on increasing the fermentation period. However, diosgenin yield first gradually increased and then decreased as the culture temperature increased.

The mutual interactions between the fermentation period ($X1$) and antifoam reagent addition ($X3$) are presented in Figure 4C,D, maintaining the culture temperature ($X2$) at level zero. The 2-D contour line is oval, and the 3-D response surface is steep. The linear and quadratic terms of the fermentation period and antifoam reagent addition led to a significant effect on diosgenin yield. Moreover, the mutual interaction between fermentation period and antifoam reagent addition also demonstrated a significant effect. It could be noticed from the plots that the lowest diosgenin yield was observed when the fermentation period was low. When the culture temperature was 28 °C, the decrease in diosgenin yield was followed by an increase as sharply increasing antifoam reagent addition. On the other hand, a decrease in antifoam reagent addition led to a slight reduction in diosgenin yield.

Figure 4E,F shows the plots of the culture temperature ($X2$) and antifoam reagent addition ($X3$), with a fixed fermentation period (level zero). The response surface in the 3-D response surface plot is steep, suggesting the notable effect of mutual interactions between culture temperature and antifoam reagent addition on the yield of diosgenin converted from DZW by SmF of CPCC 400226. A significant interaction between the culture temperature and antifoam reagent addition was also observed because the contour line is oval in the 2-D contour plot, which was in agreement with the shape of the response surface in the 3-D plot. Under a constant fermentation period of eight days (zero level), the diosgenin yield first gradually increased and then decreased as the antifoam reagent addition increased.

*2.3.5. Optimum Conditions Selection.* After optimization of the microbial biotransformation process evaluated from the model, the optimal environmental conditions for clean and efficient diosgenin production through SmF of CPCC 400 226


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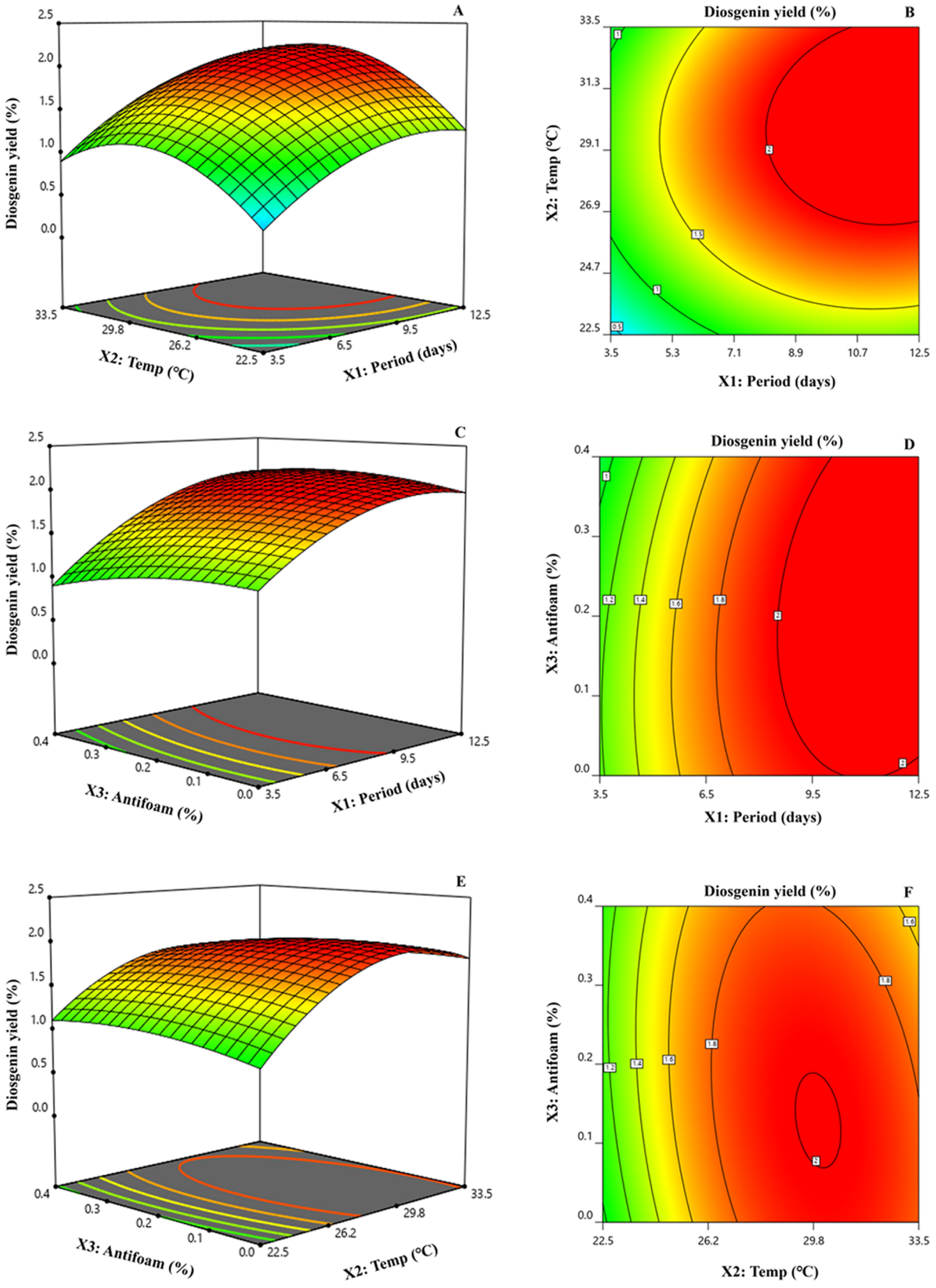


**Figure 4.** Mutual factor interactions among fermentation period, culture temperature, and antifoam reagent addition on diosgenin yield. (A) Three-dimensional response surface plot of mutual interaction between fermentation period and culture temperature. (B) Two-dimensional contour map of mutual interaction between fermentation period and culture temperature. (C) Three-dimensional response surface plot of interaction between fermentation period and antifoam reagent addition. (D) Two-dimensional contour map of mutual interaction between fermentation period and antifoam reagent addition. (E) Three-dimensional response surface plot of mutual interaction between culture temperature and antifoam reagent addition. (F) Two-dimensional contour map of mutual interaction between culture temperature and antifoam reagent addition.



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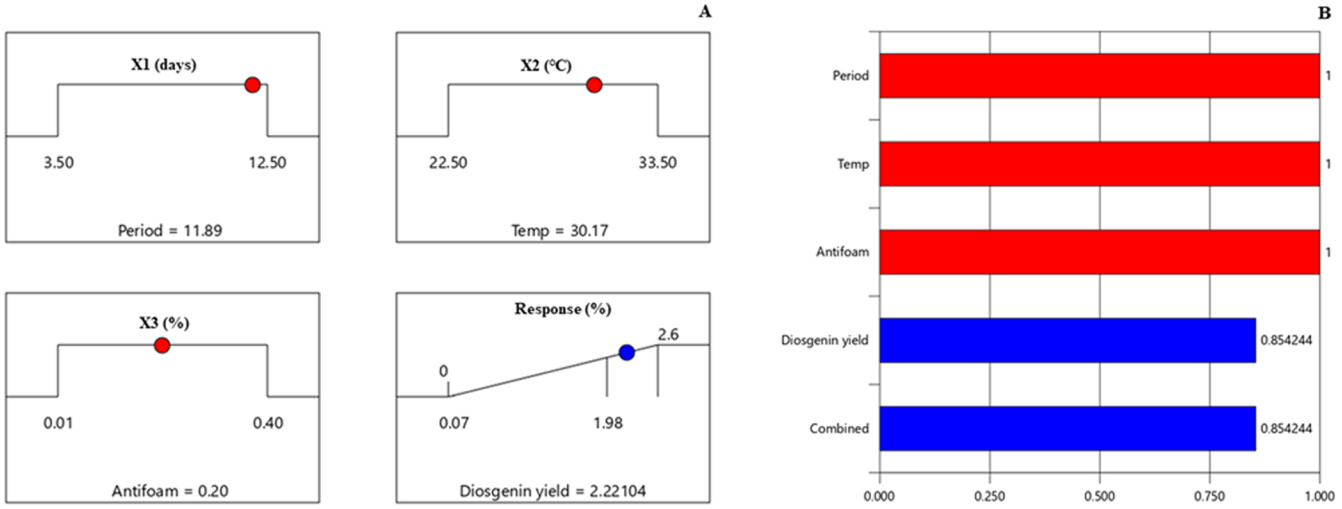


**Figure 5.** Numerical optimization in the CCD for maximal diosgenin yield. (A) Plot of solution ramp. (B) Bar graph for desirability. The optimum conditions of fermentation period, culture temperature, and antifoam reagent addition were determined for maximal diosgenin yield.

against DZW were determined as follows (Figure 5A): a fermentation period of 11.89 days, a culture temperature of 30.17 °C, and an antifoam reagent addition of 0.20%. Under these conditions, the predicted diosgenin yield was 2.22%. Theoretically, ∼85% of steroidal saponins in DZW could be transformed into diosgenin through SmF of CPCC 400226 (Figure 5B).

**2.4. Experimental Validation.** Under optimum conditions obtained from the PBD−CCD, the validity of the statistical model was evaluated by microbial biotransformation of DZW by CPCC 400226 in SmF. Three replicate verification experiments were carried out with the same conditions. The steroidal saponins in DZW were transformed into diosgenin, and the experimental yield of diosgenin reached 2.24 ± 0.17%.

Through microbial biotransformation, the steroidal saponins in DZW were converted into diosgenin. It was found that the fungal strains could convert DZW and produce diosgenin. These strains primarily belonged to the genera of *Trichoderma* and *Aspergillus*. Using a DZW concentration of 30 g/L, over 80% of the steroidal saponins were catalyzed by *Trichoderma harzianum* CGMCC 2979.[12] By comparison, ∼48% of the steroidal saponins in DZW were converted into diosgenin when DZW was fermented by *Trichoderma reesei* ACCC 30597.[52] A mixed culture of three filamentous fungi (*A. oryzae*, *Phanerochaete chrysosporium*, and *A. niger*) resulted in significantly enhanced diosgenin yield, although lower production was obtained when 50 g/L DZW was processed by either of them.[39] Therefore, the chosen strain plays a fundamental role in efficient diosgenin production through microbial biotransformation. Moreover, the diosgenin yield and biotransformation efficiency varied because of the different fermentation conditions and specific strains. Herein, the source of DZW and its concentration also directly affected the biotransformation efficiency and therefore determined the final diosgenin yield. A balance between the substrate concentration and product yield is required. Taking CPCC 400226 as an example, over 95% steroidal saponins in DZW were converted, and the diosgenin yield was ∼2.5% when ∼10 g/L DZW was applied, which offers benefits for the following product purification, but the production capacity cannot meet industrial demand. Conversely, a large decrease in diosgenin production was observed when the DZW concentration was over 60 g/L. The substrate conversion and diosgenin yield were less than 40% and 1%, respectively. We previously found that ∼75% of the DZW could be transformed and produce diosgenin when ∼25 g/L DZW was fermented for 21 days.[20] Generally, it is emphasized the acceptance of the microbial biotransformation with a DZW concentration not less than 30 g/L, which could meet the industrial purposes for diosgenin production factories unless the substrate conversion rate was lower than 60%. In the current study, the DZW concentration was maintained at 40 g/L. Verification experiments were performed according to the optimum values obtained by PBD−CCD. The agreement between experimental diosgenin yields and predicted values confirmed the validity of the statistical design. With this new bioprocess, over 80% of the steroidal saponins in DZW were efficiently converted into diosgenin in a clean and sustainable way, which further confirmed the strong catalytic activity of CPCC 400226 and the promising application prospect of this SmF process.

## 3. CONCLUSIONS

For clean and efficient diosgenin production, the fungal strain *Fusarium*. sp. CPCC 400226 was screened for the first time and selected. By taking the SmF of this fungal strain as a typical example, a new diosgenin production process based on the microbial biotransformation of DZW was suggested for the first time for *Fusarium* strains. Statistical design and RSM were used successfully as efficient techniques to optimize the yield of diosgenin. The impact of various variables was explored by PBD to decipher the main variables. CCD was then implemented to determine the mutual interactions and optimum conditions of the fermentation period, culture temperature, and antifoam reagent addition on diosgenin yield. Under optimum SmF conditions, the experimental diosgenin yield reached 2.24%, which was in great agreement with the yield predicted by the model. The final diosgenin production was significantly increased as compared with the initial fermentation conditions. The model generated by PBD−CCD was adequate, precise, and reliable. This model satisfied the necessary arguments for the development and optimization of the microbial biotransformation process. The current study provides a detailed investigation using statistical analysis to identify the optimal level of each variable and mutual factor


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interactions among the three independent variables in diosgenin yield through SmF of a *Fusarium* strain. Moreover, the study also provides a basis for further developing an acid-free and clean bioprocess in the industrial production of diosgenin. It therefore lays down a solid foundation for SmF of endophytic fungi to produce natural products.

## 4. EXPERIMENTAL SECTION

**4.1. Chemicals, Substrates, and Media.** The dried DZW was obtained from Ankang (Shanxi Province, China). Diosgenin standard was purchased from National Institutes for Food and Drug Control (Beijing, China). Other standards and total saponins (TS) were kindly gifted by Dr. Baiping Ma. TS was mainly composed of zingiberensis newsaponin, deltonin, prosapogenin A, and trillin. HPLC-grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (China) Co., Ltd. (Beijing). Other chemicals were of analytical grade unless otherwise stated. Potato dextrose agar (PDA) and potato dextrose broth (PDB) media were purchased from Becton Dickinson Co. (Franklin Lakes, NJ). Antifoam reagent OED60K, surfactant Tween-80, and glass bead (5−6 mm) were purchased from Shanghai Yuanye Biological Technology Co., Ltd (Shanghai). YPG medium (0.5 g of $MgSO_4{\cdot}7H_2O$, 1 g of $K_2HPO_4{\cdot}3H_2O$, 4 g of yeast extract, and 15 g of glucose per 1 L, pH 6.0) and the basic SmF medium (40 g/L DZW, 10 g/L phosphate, pH 6.0) were prepared in this laboratory.

**4.2. Microorganisms.** Endophytic fungi used in microbial biotransformation were selected for their potential ability to convert DZW and produce diosgenin. The isolate was earlier purified in this laboratory and maintained on the strain medium containing 15% glycerol. All of the fungal strains are now preserved in the China Pharmaceutical Culture Collection (CPCC, Beijing. http://www.cpcc.ac.cn).

**4.3. Preparation of Seed Suspension.** The strain was plated on a PDA slant and incubated at 28 °C for seven days. Culture from the PDA slant was picked and inoculated in a 125 mL flask containing 25 mL of sterilized PDB medium. The flask was placed in a thermostatic rotary shaker Innova 43 (New Brunswick Scientific Co., Brisbane, CA) at 30 °C, 200 rpm for 48 h. The resulting liquid culture was used for seed suspension.

**4.4. Screening of the Active Strain.** The heat-sterilized YPG medium (∼20 mL) containing 0.2 mg/mL TS was placed in the 125 mL flasks and inoculated by 0.5 mL of PDB seed suspension. These flasks were cultivated at 30 °C, 200 rpm for five days, followed by incubation at 50 °C, 200 rpm for 24 h. After 6 days of biotransformation, 20 mL of water-saturated *n*-butanol was added into the fermentation broth and treated by supersonic extraction at 40 kHz, 200 W, 28 °C for 30 min (SB-5200DT, Ningbo Scientz Biotechnology Co., Ltd., Zhejiang province, China). The extraction was repeated three times; the resulting *n*-butanol layer was collected by a centrifuge at 4000*g*, 25 °C for 30 min (Multifuge X3 FR, Thermo Fisher Scientific (China) Co., Ltd.) and concentrated under reduced pressure. The residue was dissolved in 0.5 mL of methanol and immediately subjected to TLC analysis.[20] Using zingiberensis newsaponin (0.1 mg/mL) as a substrate, the biotransformation activities of potential active fungi were confirmed by following the procedure mentioned above and analyzed by HPLC.

**4.5. Biotransformation of DZW in SmF.** Dried DZW were ground into powder by a grinder (FW100, Changzhou Jintan Youlian Instrument Research Institute, Jiangsu province, China). The powder was passed through an 80-mesh sieve and stored at 4 °C. Microbial biotransformation experiments were carried out in the 125 mL flasks containing 20 mL of basic SmF medium. Unless otherwise indicated, flasks were inoculated with 0.5 mL of PDB seed suspension and incubated at 30 °C, 200 rpm for seven days. The experiment using only basic SmF medium without a substrate was used as the blank control and processed as the same method above.

**4.6. Determination of Diosgenin by HPLC.** At the end of SmF, the fermentation broth was centrifuged at 25 °C, 4000*g* for 30 min. The precipitation containing the products was collected and placed in an oven (UFB400, Memmert GmbH$^+$ Co. KG, Schwabach, Bavaria, Germany) and dried at 80 °C to a constant weight. The resulting solid pellet was smashed and stored at 4 °C. Using the reflux extraction method, the smashed powder was transferred to a 500 mL distilling flask and extracted at 93 °C under reflux three times (100, 80, and 50 mL of ethyl acetate, 1 h each time). The extracts were combined, and the ethyl acetate from the 1 mL extract was recovered by a solvent recovery station at 45 °C (Genevac EZ-2.3 Elite, SP Scientific, Ipswich, Suffolk, U.K.). The residue was dissolved in 1 mL of methanol. The resulting samples were filtered and analyzed by HPLC equipped with an ELSD.

The content of diosgenin in the products was determined according to the diosgenin standard curve. In brief, diosgenin standard was dissolved in methanol with a final concentration of 2.28 mg/mL and used as the stock solution. This stock solution was then gradually diluted by HPLC-grade methanol with various final working concentrations.[20] The working solutions were immediately analyzed by an Agilent 1290 series analytical HPLC (Agilent Technologies, Inc., Santa Clara, CA). The gradient HPLC program was as follows: 30−60% B in 14 min, 60−91% B in 6 min, 91% B in 12 min, 91−30% B in 2 min, and 30% B in 6 min (A = water and B = acetonitrile). The HPLC system was equipped with an Agilent XDB-C18 column (5 $\mu$m, 4.6 × 150 mm) and an Agilent 1290 Infinity II ELSD. Injection volume, flow rate, and column temperature for HPLC were 10 $\mu$L, 1 mL/min, and 25 °C, respectively. Drift tube temperature and gas flow rate for ELSD were 110 °C and 2.5 L/min, respectively. The yield of diosgenin was calculated with the following equation

$$\begin{aligned}&\text{diosgenin yield (\%)}\\&= \text{diosgenin production (mg/mL)/DZW (mg/mL)}\\&\quad \times 100\%\end{aligned} \tag{2}$$

Using traditional acid hydrolysis, the natural diosgenin yield in DZW was determined according to a previously reported method.[53]

**4.7. Selection of Significant Factors by the Plackett−Burman Design.** The Plackett−Burman design (PBD) was used to screen and select the primary variables that significantly influence the microbial biotransformation of DZW by CPCC 400226. A first-order polynomial model was used to fit PBD as follows

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \tag{3}$$

where $Y$ is the predicted response and $\beta_0$, $\beta_i$, $X_i$, and $k$ are the model intercept, linear coefficient, level of the independent variable, and the number of involved variables, respectively.



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To determine the low and high levels for each variable, the preliminary investigation of variables on diosgenin yield was previously explored through the SmF of CPCC 400226 (data not shown). Then, a total of 12 runs of PBD were used to evaluate the nine factors, including glass bead addition (%), antifoam reagent addition (%), surfactant addition (g/L), working volume (mL), agitation (rpm), culture temperature (°C), fermentation period (days), fermentation pH, and inoculum size (%), which were denoted *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, and *J*, respectively. These factors were tested at the two-level PBD (Table 5). The experimental errors in data analysis were estimated by introducing two unassigned variables (referred to as dummy variables) including DV1 and DV2, which were denoted *K* and *L*, respectively. The response (*Y*) of diosgenin yield (%) was determined by calculating the average value of three replicates measured independently. The statistically significant variables were thus used for further bioprocess optimization.

**Table 5. Levels of Each Factor Tested in the PBD**

| | | actual experimentation value | |
|---|---|---|---|
| variables | symbol | low (−1) | high (+1) |
| glass bead addition (%) | *A* (beads) | 2.4 | 3.9 |
| antifoam reagent addition (%) | *B* (foam) | 0.05 | 0.4 |
| surfactant addition (g/L) | *C* (surfactant) | 1.5 | 2.5 |
| working volume (mL) | *D* (volume) | 10 | 30 |
| agitation (rpm) | *E* (agitation) | 150 | 240 |
| culture temperature (°C) | *F* (temp) | 22.5 | 33.5 |
| fermentation period (days) | *G* (period) | 3 | 14 |
| fermentation pH | *H* (pH) | 4.5 | 6.5 |
| inoculum size (%) | *J* (inoculum) | 2 | 10 |

**4.8. Bioprocess Optimization by CCD.** After dominant factors were identified by PBD, CCD was performed to obtain the significant effects on biotransformation of DZW and the mutual factor interactions between the selected factors. To maximize the yield of diosgenin, the optimal value of each variable that significantly influenced diosgenin production was further identified. Three factors selected from PBD for further optimization were fermentation period (day), culture temperature (°C), and antifoam reagent addition (%), which were denoted *X*1, *X*2, and *X*3, respectively. Five different levels of design were implemented to assess each factor, which included the combining factorial points (−1, +1), axial points ($-\alpha$, $+\alpha$), and central point (0). A total of 20 runs of CCD were conducted for the three chosen factors. Table 6 shows the levels of each factor used in the CCD.

A second-order polynomial equation was applied for analyzing diosgenin yield. Using the multiple regression procedure, the model data was fitted in the equation. The following quadratic polynomial equation was applied for fitting CCD

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \quad (4)$$

where *Y* is the response and $\beta_0$ is a constant term. *X*1, *X*2, and *X*3 are significant independent variables; $\beta_1$, $\beta_2$, and $\beta_3$ are linear regression coefficients; $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ are quadratic regression coefficients; and $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are interactive regression coefficients.

**Table 6. Levels of Each Factor Tested in the CCD**

| | | levels | | | | |
|---|---|---|---|---|---|---|
| variables | symbol | $-\alpha$ | −1 | 0 | +1 | $+\alpha$ |
| fermentation period (days) | *X*1 (period) | 0.5 | 3.5 | 8 | 12.5 | 15.5 |
| culture temperature (°C) | *X*2 (temp) | 19 | 22.5 | 28 | 33.5 | 37 |
| antifoam reagent addition (%) | *X*3 (foam) | 0 | 0.01 | 0.2 | 0.4 | 0.5 |

**4.9. Verification Experiments.** According to the optimum values obtained by PBD−CCD, verification experiments were performed to verify the reliability of the experimental model. The microbial biotransformation by CPCC 400226 in SmF was carried out in three replicates, and the resulting values were averaged to obtain the final diosgenin yield.

**4.10. Statistical Analysis.** All experiments were performed in three replicates, and the data consisted of means of independent measurements. Results were presented as mean ± S.D. for three replicates. Design-Expert software (trial version, Minneapolis, MN) was utilized for statistical analysis and graph plotting. $P < 0.05$ was considered to be significant.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00010.

Chemical structures of trillin (1), prosapogenin A of dioscin (2), deltonin (3), gracillin (4), dioscin (5), and zingiberensis newsaponin (6) (Figure S1); overlay plot of perturbation for diosgenin yield (Figure S2) (PDF)

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### Author Contributions

W.L. and L.Y. designed the experiments. W.L. performed the experiments, and H.X., T.Z., X.P., J.S., and H.L. helped perform the work. W.L. and L.Y. drafted the manuscript. B.M. helped design the experiments. L.Y. supervised the project.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

DZW, root of *Dioscorea zingiberensis* C. H. Wright; PBD, Plackett−Burman design; CCD, central composite design; SmF, submerged fermentation; OFAT, one-factor-at-a-time; DOE, design of experiment; RSM, response surface method; SSF, solid-state fermentation; TS, total saponins; CPCC, China Pharmaceutical Culture Collection; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; ELSD, evaporative light-scattering detector; PDA, potato dextrose agar; PDB, potato dextrose broth; ANOVA, analysis of variance; CV, coefficient of variation; SS, sum of squares; Df, degree of freedom; MS, mean sum of squares

## ■ NOMENCLATURE

| | |
|---|---|
| $Y$ | predicted response of diosgenin yield (%) |
| $\beta_0$ | linear coefficient (in PBD) and constant term (in CCD) |
| $\beta_i$ | model intercept (in PBD) |
| $X_i$ | level of the independent variable (in PBD) |
| $k$ | number of involved variables (in PBD) |
| $A$ | glass bead addition (%) |
| $B$ ($X3$) | antifoam reagent addition (%) |
| $C$ | surfactant addition (g/L) |
| $D$ | working volume (mL) |
| $E$ | agitation (rpm) |
| $F$ ($X2$) | culture temperature (°C) |
| $G$ ($X1$) | fermentation period (days) |
| $H$ | fermentation pH |
| $J$ | inoculum size (%) |
| $K$ | dummy value 1 |
| $L$ | dummy value 2 |
| $\beta_1$, $\beta_2$, $\beta_3$ | linear regression coefficients |
| $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ | quadratic regression coefficients |
| $\beta_{12}$, $\beta_{13}$, $\beta_{23}$ | interactive regression coefficients |

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