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A Method for Improving Microbial Conversion of Diosgenin and Separation and Identification of the Product

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Abstract: Diosgenin, a hydrolysis product from *Dioscorea* plants, can be used as a precursor of steroid drugs (e.g., progesterone, testosterone, and glucocorticoid). However, traditional acid hydrolysis production wastes water and causes severe environmental pollution. The extraction of diosgenin through microbial transformation is the most green and environmentally friendly method at present. In order to improve the efficiency of the extraction of diosgenin through microbial transformation, we proposed a new method of strain mutagenesis. After mutagenesis, the response surface methodology was used to optimize the solid-state fermentation medium, thereby improving the diosgenin yield. We found that the optimal formulation was 5.5% sucrose, 0.6% $NH_4H_2PO_4$, and 26.6% wheat bran. The final extraction rate of diosgenin reached 0.439% (the value of diosgenin per g. of starting plant dry material). Compared with 0.338% before optimization, it had increased 1.29 times. Furthermore, two other compounds were isolated from the fermentation products. These were identified as diosgenone ($C_{27}H_{41}O_3$) and yuccagenone ($C_{27}H_{42}O_3$). Traditional diosgenone is obtained through the oxidation of diosgenin with oxalic acid, but the method in this study is directly obtained from *Dioscorea* rhizome powder. The price of *Dioscorea* rhizome powder is much lower than diosgenin, thus greatly reducing the cost of obtaining diosgenone. This method provides a basis for subsequent research on other pharmacological compounds.

Keywords: endophytic fungus; diosgenin; strain mutagenesis; fermentation; response surface methodology

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1. Introduction

Diosgenin, a C27 spirostan steroid obtained from Dioscorea plants, is a derivative of steroidal sapogenin. It was discovered in Dioscoreaceae plants by the Japanese scientists Matsukawa and Fujii in 1935.

Diosgenin has a variety of biological activities [1]. Sun et al. (2020) found that diosgenin can increase the concentration of Ca^{2+} through the release and inflow of Ca^{2+}, while it leads to the unrelated death of Ca^{2+} in PC3 cells, revealing the potential of using diosgenin for human prostate cancer treatment [2]. Sirotkin et al. (2019) tested the direct effects of plant steroidal saponins on basic ovarian cell function in livestock and explored its promotion of ovarian cell function, potentially improving female reproduction [3]. Yang et al. (2021) added diosgenin to yam and fed it to mice. This enhanced the memory of the mice and promoted neurite outgrowth in their neurons [4]. Li et al. (2018) injected lipopolysaccharide into mice to induce motor dysfunction, stimulate inflammation and an oxidation irritable reaction, and activate the TLR/NF-κB pathway in vitro and in vivo [5].

They found that diosgenin could effectively alleviate the LPS-induced response by inhibiting the TLR/NF-κB signal pathway, curing LPS-induced rat Parkinson's disease (PD) to some extent.

Diosgenin is the synthetic precursor of steroid drugs (e.g., progesterone, testosterone, and glucocorticoid) widely used in clinical practice and it has important industrial application value [6]. However, it mainly exists as glycosides in plants such as turmeric [7], and inversing glycoside is the main strategy used in the extraction of diosgenin [8]. In the traditional industry, direct acid hydrolysis, including hydrochloride acid ethanol extraction and sulfuric acid ethanol extraction, is often used. However, this method requires high volumes of water and results in severe environmental pollution [9]. In order to explore an environmentally friendly extraction method of diosgenin, Shen et al. (2021) proposed the use of phosphorus tailings to prepare solid acid and convert Dioscorea zingiberensis C.H. Wright (DZW) to diosgenin [10]. Zhao et al. (2022) developed and synthesized a new solid acid based on TiO_2 as an effective solid acid catalyst by sulfonating the prepared TiO_2, which can be used to extract diosgenin from saponin [11]. Although the methods mentioned above solve the problem of the severe environmental harm caused by the large-scale application of sulfuric acid, they have several disadvantages. For example, the concentration of diosgenin extracted from steroidal saponins is low and still involves the production of pollutants. Cheng et al. (2021) developed and optimized a method of homo-enzyme catalysis to produce diosgenin at yields of up to 96.5%. This method's diosgenin yield is high, with low by-product generation [12]. However, the cost is very high, bringing a new challenge to the industrial production of diosgenin.

Therefore, the purpose of this study is to improve the yield of diosgenin through the mutagenesis of endophytic fungus $SY_{fx2}13.2$ using UV-lithium chloride combined mutagenesis and the optimization of the fermentation culture conditions. In our study, the environmental pollution caused by traditional acid hydrolysis was eliminated, and the steroidal saponins were fully transformed using microorganisms, which reduced the production of by-products and reduced the production cost.

2. Experimental

2.1. Identification of Fungal Strains

In our research, we isolated the $SY_{fx2}13.2$ strain (Preservation number: CGMCC 11517), which can efficiently convert diosgenin from *Dioscorea* obtained from National Engineering Research Center for Phytochemistry. This strain is an endophytic fungus isolated from Dioscoreaceae. The strain was identified by sequencing the ribosomal ITS sequence. Genomic DNA of $SY_{fx2}13.2$ strain was isolated with DNA extraction kit (Solarbio Science & Technology Co., Ltd., Beijing, China) and used as PCR amplification template. The forward primer ITS1 and reverse primer ITS4 were utilized. The used forward (ITS1) primer was 5′-TCCGTAGGTGAACCTGCGG-3′, and the reverse (ITS4) primer was 5′-TCCTCCGCTTATTGATATGC-3′. Then, 10 μL mix enzymes (Tsingke Biotechnology Co., Ltd., Beijing, China) were mixed with 0.5 μL 10 μmol/L ITS1 primers, 0.5 μL 10 μmol/L ITS4 primer, 5 μL DNA templates, and 4 μL ddH_2O. ITS rDNA sequences were amplified using PCR. PCR was performed as follows: (1) the initial denaturation temperature was 94 °C for 5 min; (2) 30 cycles, each cycle 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; (3) the terminal extension step was kept at 72 °C for 5 min [13]. PCR products were sent to China Beijing Aoko Biotechnology Co., Ltd. and purified for sequencing. The ITS sequences obtained were compared in NCBI Blast database to determine the species. The morphology and structure of the colonies were analyzed using scanning electron microscopy (No. 2021252903, Hitachi High-tech Company, Tokyo, Japan) and optical microscopy (NIKON, Tokyo, Japan).

2.2. Preparation of Spore Solution

The stored $SY_{fx2}13.2$ was removed from the refrigerator at −80 °C and inoculated into PDA medium by spreading plate method, then put into an incubator. The activated

SY_{fx2}13.2 plate was taken from the incubator, and the spores in the plate were washed off with sterile water in the sterile operating table. The spores were placed in a conical flask with glass beads, fully shaken, and scattered. Then the single spore suspension was obtained using filtration with a syringe stuffed with cotton. The concentration of spores was adjusted to 1×10^6 cfu/mL, and it was added to the fermentation medium under the condition of 5% inoculation amount [14]. The germination state of spores was observed every two hours, and the spore solution was taken for the next experiment.

2.3. UV-LiCl Combined Mutagenesis Method

From the UV mutagenic lethal curves and LiCl mutagenic lethal curves, we obtained the respective lethality condition of 80% (materials), as shown in Table 1. We did crossover experiments with compound mutagenesis to obtain a lethality of 80% for the compound conditions for the next experiment. The spore solution was mutagenized using compound conditions of UV irradiation time under light-protected conditions. After the mutagenesis was completed, the indoor fluorescent light was turned on and the single spores were restored using light for 30 min. Then the fluorescent light was turned off again and UV irradiation was maintained, re-exposing them to an additional 2 min under dark conditions. The mutagenized spore solution was diluted to 1×10^2 cfu/mL with distilled water, and 0.1 mL was taken and coated on the activation medium with the suitable LiCl concentration. Single spores untreated with UV and lithium chloride were used as controls. Cultures were incubated at 33 °C in the dark for 3–5 days.

Table 1. Cross experiment table of compound mutagenesis.

Impact Factor	A: Uv Irradiation Time (s)	B: LiCl Concentration (%)
Level 1	180 s	1.5%
Level 2	210 s	2.0%
Level 3	240 s	2.5%

2.4. Preparation of Fermented Seed Liquid

The seed medium liquid (5 g sucrose, 1 g beef extract-peptone, 7 g *Dioscorea* root powder, 0.75 g K_2HPO_4, 0.25 g $MgSO_4$, 0.25 g KCl) was prepared in 1 L distilled water and distributed into ten 250 mL conical flasks. The flasks were closed with eight layers of gauze and then sterilized through autoclaving for 20 min. Sterilized sterile water was drawn into the activated plate (Potato Dextrose Agar Medium), sterilized glass beads were placed on the plate, and the plate was shaken for 1–2 min to elute the spores. The mycelium was filtered out, and the suspension was diluted to a final spore concentration of 10^7 unit/mL. After the seed media cooled to room temperature, 5 mL of the suspension was inoculated into 100 mL media. The flasks were covered with gauze and incubated at 33 °C and 140 rpm agitation for 24 h.

2.5. Preparation of Solid-State Medium

The raw solid-state fermentation medium (80 g *Dioscorea* root powder, 3 g yeast extract powder, and 20 mL distilled water) was mixed with the seed liquid culture for 24 h and then inoculated with 50% of the inoculum, stirred evenly, and transferred to a 100-mesh screen. After the transfer, the medium was spread evenly on the screen, incubated in a constant temperature incubator for fermentation at 33 °C, and stirred once a day. The fermentation product was obtained after 10 d of fermentation [15].

2.6. Selection of Significant Factors by the Plackett–Burman Design

The Plackett-Burman design (PBD) was used to screen the variables that significantly affected the transformation of diosgenin by the fungus [16]. A single factor test was performed to determine the low and high levels of each variable (Supplementary Materials), and then PBD (Design Expert v8.0.6) was used to evaluate the seven factors [17]. The

contents include (Table 2) sucrose (%), $NH_4H_2PO_4$ (%), $MgSO_4$ (%), Tween (g/L), Rice Husk (%), KH_2PO_4 (%), and Wheat Bran (%), which are represented as A, B, C, D, E, F, and G. Diosgenin yield (%) was determined by calculating the average of three replicates of independent measurements. The significant variables obtained by this method are used for further optimization of biological processes.

Table 2. The Plackett-Burman experimental design.

Code	Factor	−1	1
A	Content of sucrose	6%	4%
B	Content of $NH_4H_2PO_4$	0.6%	0.8%
C	Content of $MgSO_4$	0.4%	0.8%
D	Content of Tween-80	1 g/L	3 g/L
E	Content of rice husk	20%	30%
F	Content of KH_2PO_4	0.5%	1.5%
G	Content of wheat bran	20%	30%

2.7. Levels of Each Factor Tested in the Box-Behnken

After determining the main influencing factors using PBD, Box-Behnken (BBD) analysis was carried out to obtain the significant influence on the biotransformation of DZW and the interaction between the selected factors, and the optimal value of each variable significantly affecting the yield of diosgenin was determined, so as to obtain the highest yield of diosgenin [18].

2.8. Chromatographic Purification

Chromatographic silica gel was loaded into the column using the wet loading method. The crude diosgenin obtained through extraction was ground and pulverized with liquid nitrogen, and 1.6 g was dissolved in 10–15 mL ethylacetate and added to 5 g of silica gel powder. The sample was stirred and incubated in a 50 °C water bath until the ethyl acetate evaporated. The sample was loaded onto the normal phase column, the eluent (light petroleum) was added, and the effluent liquid was collected. When the first substance was detected by using thin-layer chromatography, it was collected in a test tube. The same compounds were combined, and the liquid was dried with a rotary evaporator.

2.9. Thin Layer Chromatography

The samples collected in Section 2.4 were applied to a TLC plate at 1 cm intervals using a 0.3 mm glass capillary spotter. The TLC plate was placed vertically in the developing solvent tank containing cyclohexane and ethyl acetate at a 4:1 ratio. When the liquid level was 1 cm from the top edge, it was removed and dried. Subsequently, it was immersed in 10% sulfuric acid ethanol developer for 2 s and dried.

2.10. High Performance Liquid Chromatography (HPLC)

The solid fermentation product was dried in an oven at 60 °C. The dried products (4 g) were added to 20 mL petroleum ether in 50 mL centrifuge tubes and extracted with ultrasonic waves for 30 min [19]. The products were shaken for three hours at 180 rpm. The supernatant was collected into a flask and dried using a rotary evaporator, and the above extraction steps were repeated three times. The flask contents were washed with $CHCl_3$ and diluted to 10 mL in a centrifuge tube. Next, 2 mL of liquid were transferred into a 2 mL centrifuge tube, evaporated, then dissolved with 2 mL of chromatographic grade methanol. The liquid passed through an organic filter with a pore diameter of 0.45 microns. Then, 1 mL was aspirated into the HPLC vial using a 1 mL syringe as the completed preparation sample. The concentrations of diosgenin standards were 0.125, 0.250, 0.500, 1.000, and 2.000 g/L. The HPLC column (XTERRA MS C18, 4.6 × 250 mm, 5 um; Waters; MA, USA) was used. The column temperature was 25 °C. The wave length of UV detector was

210 nm. Loading quantity of sample was 10 uL. Acetonitrile: water = 8:2 was the eluent (1.0 mL/min). The yield of diosgenin was calculated with the following Equation:

$$\text{diosgenin yield (\%)} = \text{diosgenin production (mg/mL)/DZW (mg/mL)} \times 100\%$$

2.11. Nuclear Magnetic Resonance Method

Diosgenin standard and sample (30 mg each) were added to nuclear magnetic resonance tubes with a diameter of 5 mm and dissolved in 0.6 mL of chloroform-d using sonication [20]. ^{1}H-NMR, ^{13}C-NMR, and distortionless enhancement by polarization transfer (DEPT) were carried out on a nuclear magnetic resonance spectrometer with tetramethylsilane (TMS) as the standard material [21].

2.12. High Resolution Mass Spectrometry (HR-MS)

The samples were analyzed using mass spectrometry [22]. Next, 2 mg of purified sample was dried in a 5 mL centrifuge tube, and 1 mL of chromatographic grade methanol was added, sonicated until the sample was completely dissolved, and filtered through a 0.22 μm organic filter. The mass spectrometry conditions were as follows: EMS ion source was used with a mass-to-charge ratio (m/z) of 300–500 Da, positive ion mode, spray gas and auxiliary gas of 50 psi, curtain gas of 35 psi, ion source temperature of 550 °C, and spray voltage of 5500 V.

3. Results and Discussion

3.1. The Species Identification of the Target Strain

The optical microscopy and scanning electron microscopy of $SY_{fx2}13.2$ are shown in Figure 1. The mycelium was staggered, the diameter of the conidial pedunculum was about 8 μm, and the diameter of the thin-walled rough conidial head was about 35 um. After microscopy, we performed biological identification. Like Baquiao et al., we used ITS1 and ITS4 as primers for the amplification of specific gene fragments [23]. The obtained gene sequences (Supplementary Materials) were compared in the NCBI blast database, and gene sequence alignment analysis revealed that $SY_{fx2}13.2$ (MZ618696.1) is *Aspergillus flavus* [24]. It had been preserved in China General Microbiological Culture Collection Center (CGMCC), preservation number: 11517. Its evolutionary tree is detailed in Figure 2.

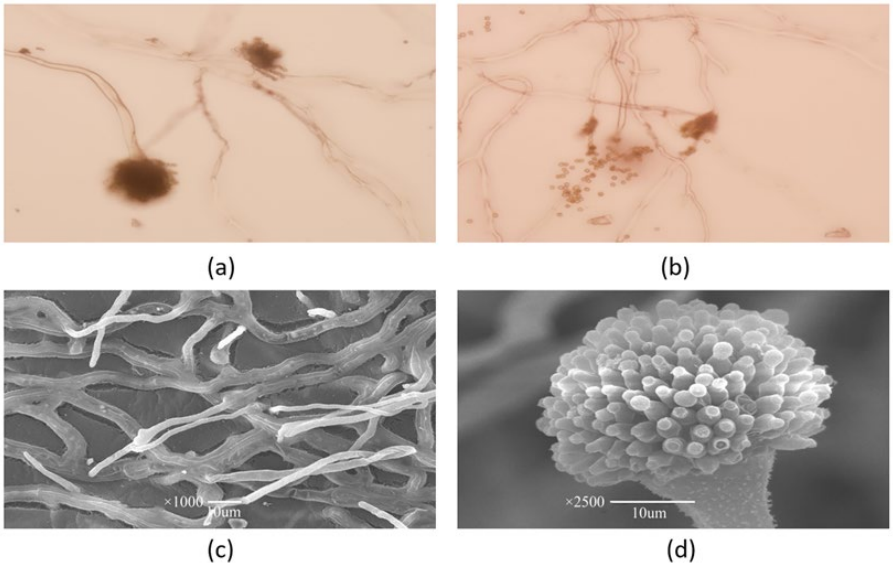

Figure 1. (**a**) The sporangia of $SY_{fx2}13.2$ fungus under the optical microscope at 800-times magnification; (**b**) spores of $SY_{fx2}13.2$ fungus under the optical microscope at 800-times magnification; (**c**) 1000-times magnification of mycelia under the scanning electron microscope; (**d**) 2500-times magnification of Conidial head under the scanning electron microscope.

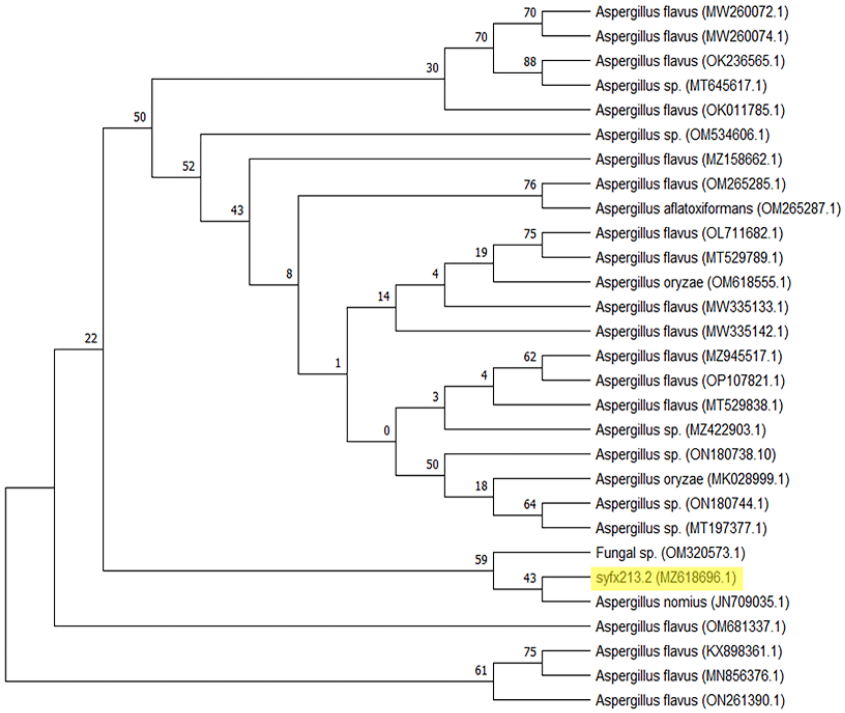

Figure 2. Fungal strain $SY_{fx2}13.2$ and its internal transcribed spacer ITS DNA phylogenetic tree. Numbers in brackets indicate GenBank accession number.

3.2. Screening of High Yield Diosgenin Strains by Compound Mutagenesis

Feng et al. screened for strains with high protease production using the UV mutagenesis of Bacillus subtilis [25]. Wang et al. combined Co-60 γ irradiation with LiCl mutagenesis to obtain strains with high Nosiheptide production [26], and our study combined UV mutagenesis and LiCl mutagenesis to screen for strains with high diosgenin production. It can be seen in Table 3 that the fatality rate is 80% when the UV irradiation time is 180 s and the dosage of lithium chloride is 2.0%. Therefore, this condition will be selected in subsequent experiments to carry out compound mutagenesis on $SY_{fx2}13.2$. After the mutagenesis, the strains were fermented and cultured, and the saponin yield was detected using HPLC. The test results are shown in Figure 3a.

According to the figure, all the samples in this batch produced saponin, among which the saponin spots of sample No. 11 were the largest and brightest, and it could be preliminarily determined that strain No. 11 had the highest saponin yield [27]. From Figure 3b, using the bar chart to compare the saponin yield after the fermentation of the screened strains, it can be concluded that, among them, the saponin yield of No. 11 was the highest, at 58.15 mg/L, while the saponin yields of No. 15, 8, 6, 23, 38, and 42 decreased successively, which were 54.02 mg/L, 53.34 mg/L, 51.43 mg/L, 50.6 mg/L, 47.47 mg/L, and 43.68 mg/L, respectively. The saponin production of these seven strains was higher than that of the control group (39.56 mg/L). The yield of the other strains was lower than that of the control group. After UV-LiCl compound mutagenesis, we evaluated the No. 11. strain, which we named as $^{11\#}SY_{fx2}13.2$, to have the highest yield of diosgenin. Ten consecutive passages were made and fermentation was performed, and it was found to be genetically stable, with a stable yield of diosgenin. This mutant was used for later experiments.

Table 3. The results of crossover experiments with compound mutagenesis conditions.

Number of Trials	A: Uv Irradiation Time (s)	B: LiCl Concentration (%)	Fatality Rate (%)
1	180 s	1.5%	70%
2	180 s	2.0%	80%
3	180 s	2.5%	90%
4	210 s	1.5%	70%
5	210 s	2.0%	90%
6	210 s	2.5%	100%
7	240 s	1.5%	90%
8	240 s	2.0%	90%
9	240 s	2.5%	100%

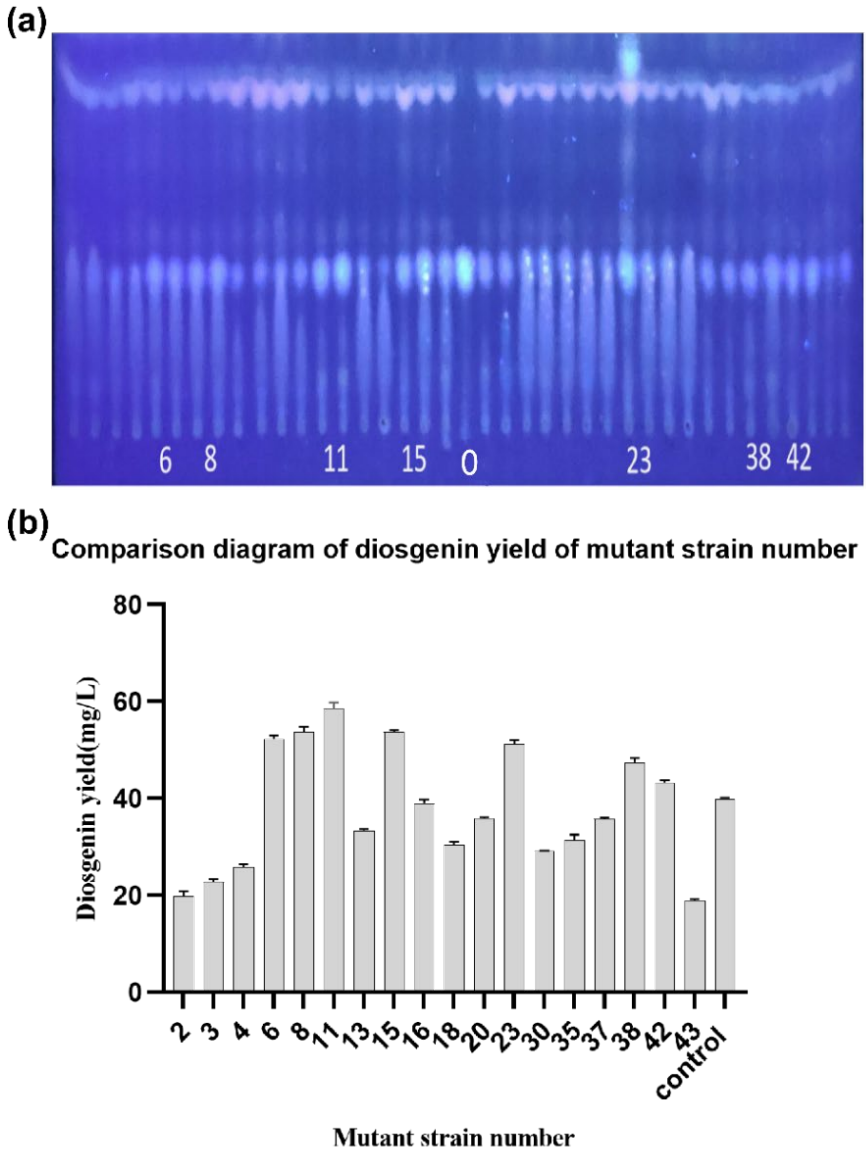

Figure 3. (**a**) Thin layer chromatogram of diosgenin from screened mutant strains. Strain 0 was the unmutagenized strain $SY_{fx2}13.2$ as the control. Strain 6, 8, 11, 15, 23, 38, 42 were strains with higher yield of diosgenin than the control strain. (**b**) Comparison diagram of diosgenin yield of mutant strains. Unmutagenized strain $SY_{fx2}13.2$ used as the control group.

3.2.1. Plackett-Burman Experiment Results

The mutagenic high-yielding fungi $^{11\#}SY_{fx2}13.2$ were used for subsequent solid fermentation. In this experiment, taking the transformation rate of saponin as the response value (R1), the above seven factors were selected at two levels, i.e., high and low.

The specific Plackett-Burman experimental table and corresponding experimental results are shown in Table 4. (A: Content of sucrose; B: Content of $NH_4H_2PO_4$; C: Content of $MgSO_4$; D: Content of Tween-80; E: Content of rice husk; F: Content of KH_2PO_4; G: Content of wheat bran; R1: Extraction rate of diosgenin).

Table 4. Solid-state fermentation to produce diosgenin Plackett-Burman experimental table and results.

Number of Tests	A	B	C	D	E	F	G	R1/%
1	−1	1	1	−1	1	1	1	0.419
2	1	1	1	−1	−1	−1	1	0.427
3	−1	1	1	1	−1	−1	−1	0.203
4	−1	−1	1	−1	1	1	−1	0.291
5	−1	−1	−1	−1	−1	−1	−1	0.275
6	1	−1	−1	−1	1	−1	1	0.392
7	1	1	−1	−1	−1	1	−1	0.376
8	−1	1	−1	1	1	−1	1	0.244
9	1	−1	1	1	1	−1	−1	0.258
10	−1	−1	−1	1	−1	1	1	0.241
11	1	1	−1	1	1	1	−1	0.303
12	1	−1	1	1	−1	1	1	0.285

The Pareto charts (Figure 4) showed a Bonferroni limit of 5.74651 and a t-value limit of 2.77645, reflecting significant factors in a group. It was concluded that the influence of Tween-80 is the most significant. However, it had a negative effect and greatly inhibited improving the extraction rate of diosgenin. De Oliveira et al. produced beta-fructofuranosidase (FFase) using solid-state fermentation and found that the sucrose level had a significant effect on FFase production [28]. In Li et al.'s study, wheat bran had a greater positive effect on the bacterial community [29], so we ventured to add ammonium dihydrogen phosphate to the solid-state fermentation medium and found that it had a significant positive effect on the production of diosgenin. In our research, the effects of sucrose, wheat bran, and $NH_4H_2PO_4$ were also significant; and their effects were positive. Adding $MgSO_4$, rice husk, and KH_2PO_4 had no significant effect on the extraction rate of diosgenin.

The significance of this experiment was analyzed using the F test, and the results are shown in Table 5.

$$\begin{aligned} \text{R1 (\%, diosgenin yield)} = {} & 0.31 + 0.031 \times A + 0.019 \times B + 4.333 \times 10^{-3} \times C - 0.054 \times D \\ & + 8.333 \times 10^{-3} \times E + 9.667 \times 10^{-3} \times F + 0.025 \end{aligned} \quad (1)$$

The fitting formula is Formula 1.

The whole model's p-value ≤ 0.01, which is significant. The effect of Tween-80 was the most significant (p-value = 0.0011). The p-value of sucrose was 0.0084, the p-value of $NH_4H_2PO_4$ was 0.039, and wheat bran's p-value was 0.0165. These three factors were significant, and consistent with the results shown in the Pareto chart.

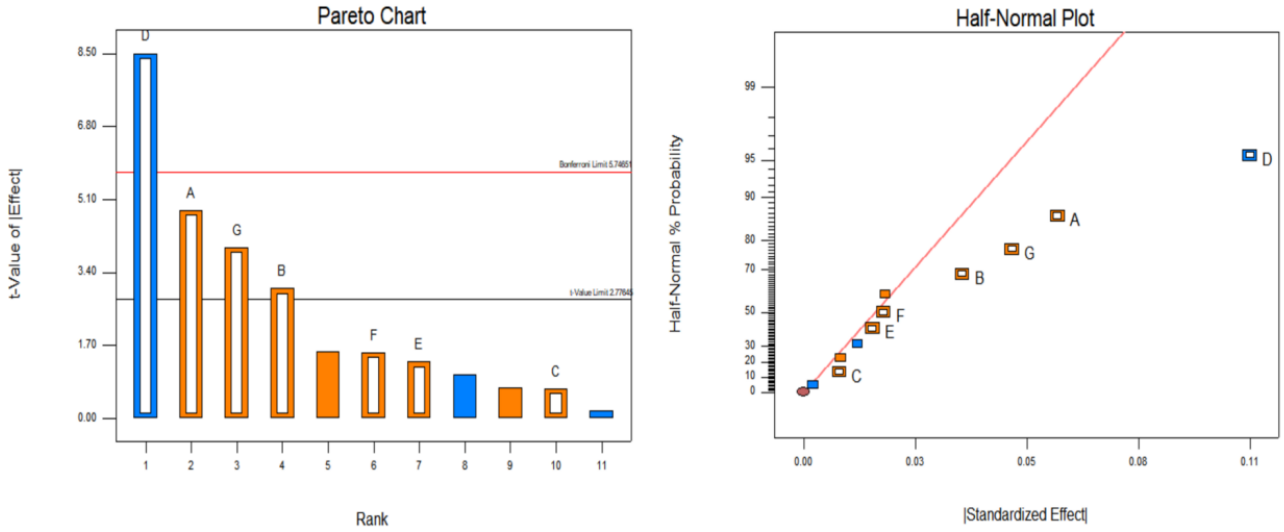

Figure 4. (**a**) Pareto chart of the Plackett-Burman experiment; (**b**) distribution chart of the Plackett-Burman experiment results. (A: Content of sucrose; B: Content of $NH_4H_2PO_4$; C: Content of $MgSO_4$; D: Content of Tween-80; E: Content of rice husk; F: Content of KH_2PO_4; G: Content of wheat bran; R1: Extraction rate of diosgenin).

Table 5. Regression Equation F test table of this experiment.

	Quadratic Sum	Degree of Freedom	Variance	F	Prob > F
Model	0.060	7	8.607×10^{-3}	17.87	0.0072
A-sucrose	0.011	1	0.011	23.43	0.0084
B-$NH_4H_2PO_4$	4.408×10^{-3}	1	4.408×10^{-3}	9.15	0.0390
C-$MgSO_4$	2.253×10^{-4}	1	2.253×10^{-4}	0.47	0.5316
D-Tween-80	0.035	1	0.035	72.20	0.0011
E-rice husk	8.333×10^{-4}	1	8.333×10^{-4}	1.73	0.2587
F-KH_2PO_4	1.121×10^{-3}	1	1.121×10^{-3}	2.33	0.2018
G-wheat bran	7.600×10^{-3}	1	7.600×10^{-3}	15.78	0.0165
Residual	1.927×10^{-3}	4	4.817×10^{-4}		
Cor Total	0.062	11			

3.2.2. Response Surface Analysis of the Interaction of Various Factors

The Plackett-Burman experiment indicated that there are four significant factors. However, adding Tween-80 has a clear inhibitory effect on the yield and transformation rate of diosgenin, which is contrary to the purpose of this experiment. Therefore, as shown in the Table 6. Tween-80 was not added in the response surface experiments. Only sucrose, $NH_4H_2PO_4$, and wheat bran were used in the additive optimization experiment.

Design expert v8.0.6 software was used to conduct quadratic regression analysis on the results of the above experimental design, and the final Equation (Formula 2) was obtained.

$$\begin{aligned} \text{R1(\%, diosgenin yield)} = 0.45 - 6.750 \times 10^{-3} \times \text{A} - 9.375 \times 10^{-3} \times \text{B} + 8.125 \times 10^{-3} \times \text{C} + 5.250 \times 10^{-3} \times \text{A} \times \text{B} \\ - 9.750 \times 10^{-3} \times \text{A} \times \text{C} - 5.000 \times 10^{-3} \times \text{B} \times \text{C} - 0.022 \times \text{A2} - 1.500 \times 10^{-3} \times \text{B2} - 0.02 \times \text{C2} \end{aligned} \quad (2)$$

Each symbol in the formula represents the following factors: R1 is the diosgenin yield (mg/L); A is the concentration of sucrose (g/L); B is the concentration of $NH_4H_2PO_4$ (g/L); C is the concentration of wheat bran (g/L). The analysis of the model from the BBD shown in Table 7.

Table 6. Solid-state fermentation to produce diosgenin response surface experimental table and results.

Number of Tests	A: Content of Sucrose	B: Content of $NH_4H_2PO_4$	C: Content of Wheat Bran	R1/%
1	5	0.6	20	0.426
2	5	0.7	25	0.452
3	5	0.7	25	0.437
4	5	0.8	20	0.419
5	4	0.8	25	0.418
6	6	0.7	30	0.401
7	4	0.7	30	0.436
8	6	0.7	20	0.402
9	4	0.7	20	0.398
10	5	0.7	25	0.459
11	4	0.6	25	0.449
12	5	0.7	25	0.455
13	5	0.7	25	0.452
14	6	0.8	25	0.417
15	5	0.6	30	0.45
16	6	0.6	25	0.427
17	5	0.8	30	0.423

Table 7. Statistical analysis of the model from the BBD.

	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	0.0061	9	0.0007	15.77	0.0007
A-sucrose	0.0004	1	0.0004	8.44	0.0228
B-$NH_4H_2PO_4$	0.0007	1	0.0007	16.28	0.0050
C-wheat bran	0.0005	1	0.0005	12.23	0.0100
AB	0.0001	1	0.0001	2.55	0.1541
AC	0.0004	1	0.0004	8.81	0.0209
BC	0.0001	1	0.0001	2.32	0.1719
A^2	0.0020	1	0.0020	46.13	0.0003
B^2	9.474×10^{-6}	1	9.474×10^{-6}	0.2194	0.6537
C^2	0.0017	1	0.0017	39.01	0.0004
The rest of item	0.0003	7	0.0000		
Lack of fit	0.0000	3	8.083×10^{-6}	0.1163	0.9459
Pure error	0.0003	4	0.0001		
Total	0.0064	16			

As shown in Table 7, the additive effects of sucrose, $NH_4H_2PO_4$, and wheat bran on the yield of diosgenin are significant. The interactions between sucrose and $NH_4H_2PO_4$ and between $NH_4H_2PO_4$ and wheat bran were not significant. However, the interaction between sucrose and wheat bran had a notable effect on the diosgenin yield, with a *p*-value = 0.0209, indicating that the effects of sucrose and wheat bran on the yield are not simple linear relationships [30]. The strength of various additives' influence on the diosgenin yield, judged by the F value, are $NH_4H_2PO_4$ > wheat bran > sucrose. Tanyildizi et al. used response surface methodology (RSM) to optimize the medium composition for α-amylase production by solid substrate fermentation (SSF) and found that $(NH_4)_{(2)}SO_4$ had the greatest effect on α-amylase production [31]. Based on the results of this experiment, we conjecture that it is the effect of NH_4^+ that makes the endophytic *Aspergillus flavus* strain $^{11\#}SY_{fx2}13.2$ produce more α-amylase and thus decompose the polysaccharide material wrapped around diosgenin and contribute to the conversion of diosgenin. There is not much difference in the lack of fit of the model as prob > F is 0.9459, indicating that the model can simulate the experimental results well [32].

The response surface analysis (Figure 5) confirmed that the interactions between sucrose and $NH_4H_2PO_4$, $NH_4H_2PO_4$ and wheat bran, and sucrose and wheat bran affect diosgenin yield greatly [18].

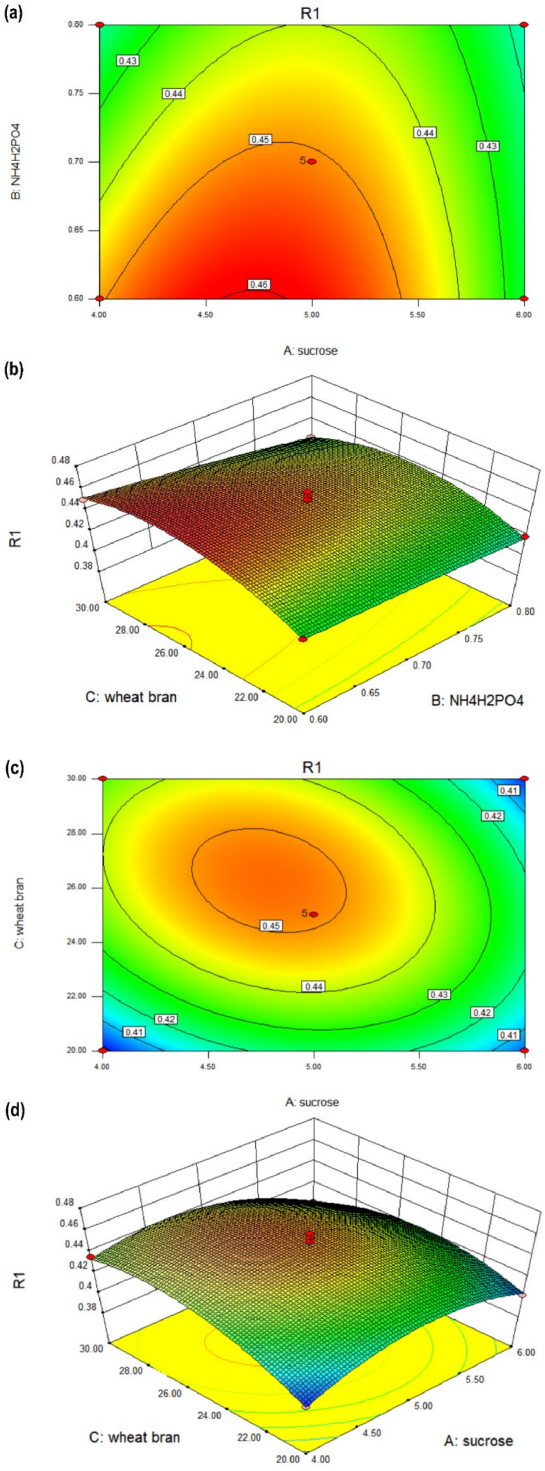

Figure 5. *Cont.*

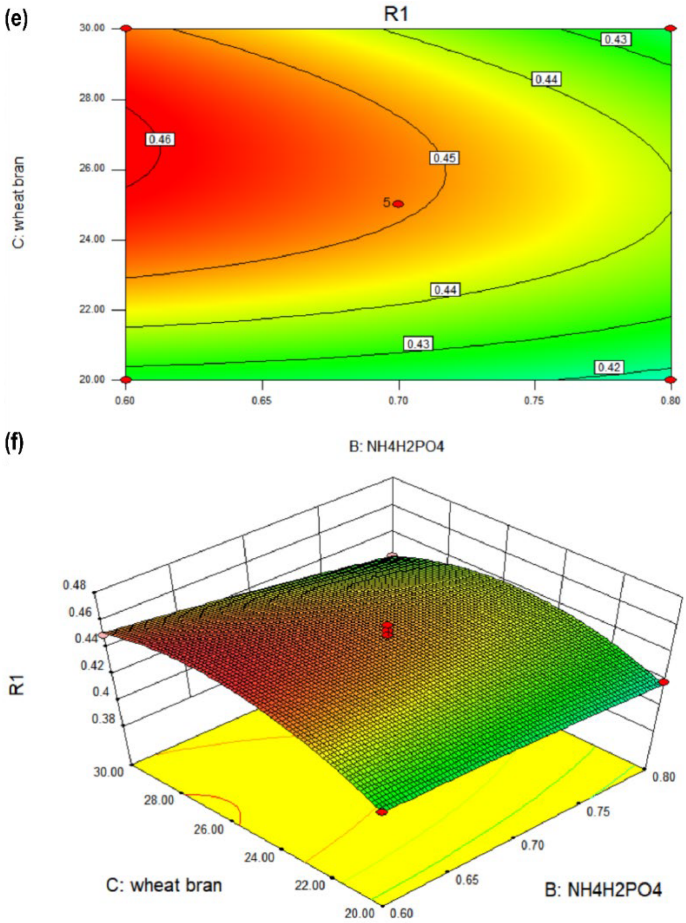

Figure 5. (**a**,**b**): sucrose × $NH_4H_2PO_4$ response surface plot and contour map; (**c**,**d**): sucrose × wheat bran response surface plot and contour map; (**e**,**f**): $NH_4H_2PO_4$ × wheat bran response surface plot and contour map.

3.3. Model Validation Experiment

The optimal fermentation medium composition was determined to be 5.47% of sucrose, 0.6% of $NH_4H_2PO_4$, and 26.6% of wheat bran. The extraction rate of diosgenin reached 0.4491%. Considering the operability, the addition amount was adjusted to 5.5% of sucrose, 0.6% of $NH_4H_2PO_4$, and 26.6% of wheat bran. Under the adjusted conditions, three repeated verification experiments were carried out and an average extraction rate of 0.439% was achieved.

3.4. Separation and Identification of Solid-State Fermentation Products

By separating the crude products using column chromatography, as shown in Figure 6. three substances were obtained, i.e., diosgenin, yuccagenone, and diosgenone.

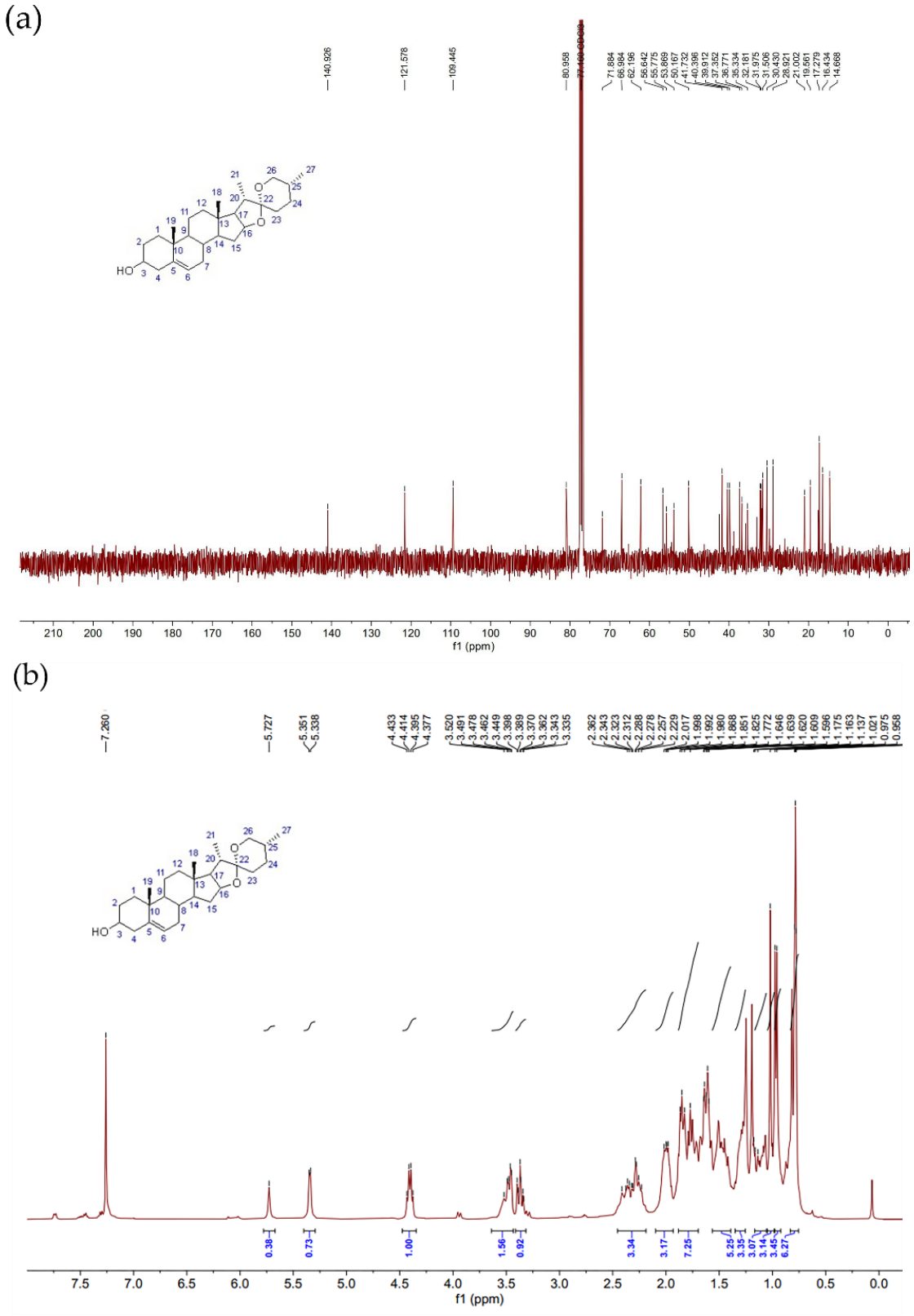

Figure 6. *Cont.*

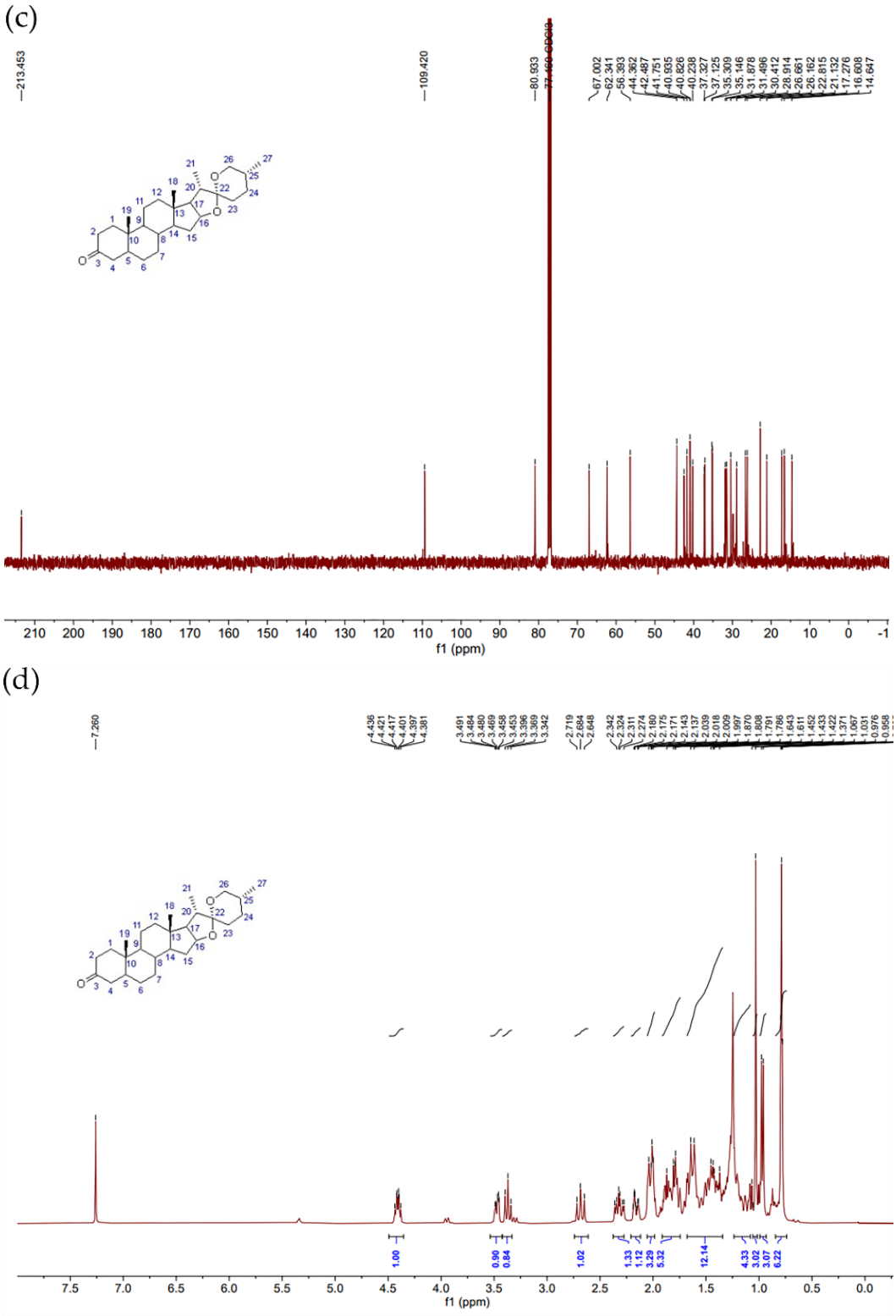

Figure 6. *Cont.*

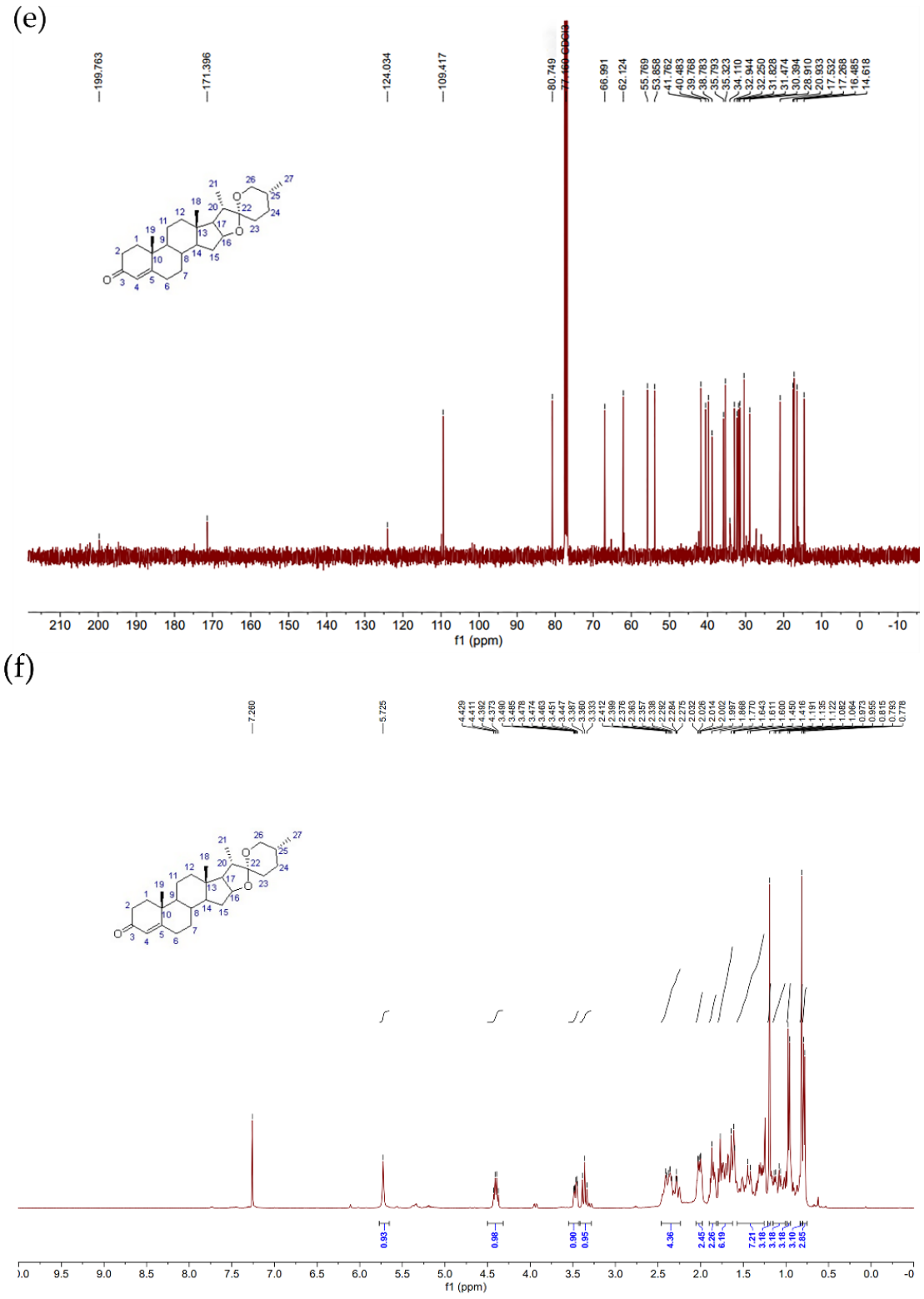

Figure 6. NMR spectra of the three compounds (**a**) diosgenin C-NMR, (**b**) diosgenin ^{1}H-NMR, (**c**) yuccagenone C-NMR, (**d**) yuccagenone ^{1}H-NMR, (**e**) diosgenone C-NMR, (**f**) diosgenone ^{1}H-NMR.

The results of our analysis of the above chart are shown in the Table 8.

Table 8. ^{1}H NMR-(400MHz) ^{13}C NMR-(400MHZ).

	1		**2**		**3**	
The Number of Carbons	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**
1		37.3		37.2		35.7
2		31.4		37.1		34.0
3	3.46 (br s)	71.8		213.3		199.8
4		42.3		42.4	5.66 (s)	124.1
5		141.0		44.3		171.4
6	5.28 (br s)	121.5		26.1		32.9
7		32.1		26.6		32.2
8		31.6		35.2		35.3
9		50.1		40.8		53.8
10		36.6		35.0		38.7
11		20.9		21.1		20.8
12		39.8		40.1		39.7
13		40.3		40.7		40.4
14		56.5		56.3		55.7
15		32.1		31.8		31.7
16	4.34 (q, 7.3)	80.9	4.34 (q, 7.2)	80.8	4.34 (q, 7.2)	80.7
17		62.1		62.3		62.0
18	0.72 (s, 3H)	16.3	0.72 (s, 3H)	16.6	0.75 (s, 3H)	16.4
19	0.96 (s, 3H)	19.4	0.97 (s, 3H)	22.7	1.13 (s, 3H)	17.5
20		41.6		41.6		41.7
21	0.90 (d, 6.8, 3H)	14.5	0.91 (d, 6.8, 3H)	14.6	0.90 (d, 6.8, 3H)	14.6
22		109.3		109.3		109.4
23		31.4		31.4		31.4
24		28.8		28.8		28.8
25		30.3		30.3		30.3
26	3.30 (t, 11.0) 3.41 (dd, 11.0, 3.3)	66.9	3.31 (t, 11.0) 3.41 (dd, 11.0, 3.3)	66.9	3.30 (t, 11.0) 3.41 (dd, 11.0, 3.3)	66.9
27	0.74 (d, 7.0, 3H)	17.2	0.72 (overlap, 3H)	17.2	0.73 (d, 6.5, 3H)	17.2

The chemical formula of compound 1 was deduced to be $C_{27}H_{42}O_3$ from the quasi-molecular ion peak m/z 415.3215, $[M+H]^+$, (cald. 415.3207 for $C_{27}H_{43}O_3$) in the HR-MS (Supplementary Materials). In the ^{1}H-NMR spectrum (Table 7), the presence of four methyl peaks in the high field, including two d peaks (δ_H 0.90, d, 6.8, 3H; 0.70, d, 7.0, 3H) and two s peaks (δ_H 0.96, s, 3H; 0.72, s, 3H), indicating that one could be a steroid. Olefinic protons (δ_H 5.28, brs, 1H) in the low field indicated that one had an olefinic fragment. In total, 27 carbon signals appeared in the ^{13}C-NMR spectrum. The DEPT experiment differentiated them into four methyl groups (δ_C 19.4, 17.2, 16.3, and 14.5), nine methines (including an olefinic methine (δ_C 121.5)), two oxygenated methines (δ_C 80.9 and 71.8), and four quaternary carbons (including an olefinic quaternary carbon (δ_C 141.0), a bi-oxygenated quaternary carbon (δ_C 109.3) and two aliphatic quaternary carbons (δ_C 40.3 and 36.6)). It shows that compound 1 is a typical spirostan compound. Compared with the NMR data of diosgenin in the literature Chen reported [33], we can confirm that this compound is diosgenin, the target product of diosgenin chain degradation by microorganisms.

The quasi-molecular ion peak of m/z 415.3215, $[M+H]^+$, (cald. 415.3207 for $C_{27}H_{43}O_3$) appears in the HR-MS spectrum of compound 2. The chemical formula of this compound is $C_{27}H_{42}O_3$, an isomer of diosgenin. However, compared with compound 1, it lacks olefinic protons at position 6 and even tertiary carbon-oxygen protons at position 3 in the ^{1}H-NMR spectrum. The ^{13}C-NMR spectrum lacks C-5, C-6 olefinic carbon signals, and even C-3 tertiary carbon-oxygen signals, but has an extra carbonyl group signal (δ_C 213.3). In addition, ring A and ring B's signals in the ^{13}C-NMR spectrum changed greatly, while the other signals were nearly unchanged. Therefore, it is speculated that this compound may be a diosgenin derivative with the 5,6-position double bond reduced and the 3-position

hydroxyl group oxidized to a ketone group. The NMR data in previous literature confirmed this compound to be yuccagenone [34].

The quasi-molecular ion peak of m/z 413.3051, $[M+H]^+$, (cald. 413.3050 for $C_{27}H_{41}O_3$), appears in the HR-MS spectrum of compound 3. The chemical formula of this compound is $C_{27}H_{40}O_3$. The NMR data of this compound are similar to those of compound 2, but with an added unsaturation appearing as two olefinic carbon signals (δ_C 171.4 and 124.1), indicating that this compound has an extra double bond compared with compound 2. Meanwhile, the signal of the carbonyl group significantly shifted to the high field (δ_C 199.8), and the carbon signals of the double bond are the typical signals of an α, β-unsaturated ketone fragment. Therefore, it is assumed that the carbonyl group of this compound is still at position 3, and the double bond is at positions 4 and 5. Combined with previous literature results, this compound is diosgenone [35].

4. Conclusions

We isolated *Aspergillus flavus* strains from an endophyte fungus. After mutagenesis, response surface methodology was used to optimize the solid-state fermentation medium, thereby improving the diosgenin yield [36]. The extraction of diosgenin using microbial conversion provides a new diosgenin production method to solve the problems of environmental pollution, resource waste, and high production cost caused by the traditional production process. In this study, the efficiency of the extraction of diosgenin using microbial transformation was greatly improved by means of strain mutagenesis and medium optimization. The most productive diosgenin strain was selected through strain mutation, and the solid-state fermentation was carried out. The optimization of the solid-state fermentation medium using the Plackett-Burman test indicated four substances that have a significant effect on diosgenin production, i.e., sucrose, $NH_4H_2PO_4$, Tween-80, and wheat bran. The BBD test was carried out to test the three significant positively influencing factors [25]. The optimal addition amounts were 5.5% sucrose, 0.6% $NH_4H_2PO_4$, and 26.6% wheat bran. The final extraction rate of diosgenin reached 0.439%. Compared with 0.338% before optimization, it had increased 1.29 times. Obviously, this method can effectively improve the extraction rate of diosgenin transformed by microorganisms.

This study not only produced diosgenin from the fermentation products, but also obtained and identified diosgenone after separation. This means that diosgenone was directly obtained using microbial transformation, avoiding the step of producing it from diosgenin through oxidation. This method greatly reduces the cost of producing diosgenone, and provides a basis for subsequent research on other pharmacological compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9010070/s1, Figure S1. Fatality rate curve of ultraviolet mutagenesis. Figure S2. Fatality rate curve of lithium chloride mutagenesis. Figure S3. Activation of strains and solid state fermentation. Table S1. The single factor experimental design. Figure S4. The effects of various additives on the extraction rate of diosgenin. Figure S5. HR-MS three isolated compounds. Figure S6. High performance liquid chromatography.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds (diosgenin, yuccagenone, and diosgenone) and plant endophytic fungi $SY_{fx2}13.2$ are available from the authors.

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