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Screening and breeding of high taxol producing fungi by genome shuffling

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To apply the fundamental principles of genome shuffling in breeding of taxol-producing fungi, *Nodulisporium sylviform* was used as starting strain in this work. The procedures of protoplast fusion and genome shuffling were studied. Three hereditarily stable strains with high taxol production were obtained by four cycles of genome shuffling. The qualitative and quantitative analysis of taxol produced was confirmed using thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and LC-MS. A high taxol producing fungus, *Nodulisporium sylviform* F4-26, was obtained, which produced 516.37 μg/L taxol. This value is 64.41% higher than that of the starting strain NCEU-1 and 31.52%—44.72% higher than that of the parent strains.

taxol, taxol-producing fungi, protoplast fusion, genome shuffling

Taxol is a diterpenoid with anticancer activities which was first isolated from the bark of Pacific yew *Taxus brevifolia* by Wani et al.[1]. It is used widely in the treatment of a variety of cancer diseases, including carcinomas of the ovary, breast and cervix[2−4]. Intensive efforts have been made to develop alternative approaches of taxol production to the isolation of taxol from yew, in order to protect the wild yew. Using microbe fermentation in the production of taxol would be a very prospective method for obtaining a large amount of taxol. However, the method is still at the experimental stage due to the low yield of the isolated and bred strain, which makes industrial production difficult. Therefore, the primary problem is the breeding of high yield strain[5].

Genome shuffling is a new breeding method in microbiology in recent years. The advantage of this technique is that the genetic breeding can be performed on the tested microbes without knowing its genetic background, making it a highly effective method[6,7]. The concept of genome shuffling was first put forward by Zhang et al. [8]. This technique was used to improve the ability of *Streptomyces fradiae* to synthesize Tailexing. Patnaik et al. (2002)[9] and Wang et al. (2007)[10] reported the screening of acid-resistant lactic acid bacillus by genome shuffling, which increased the ability of lactic acid bacillus to synthesize large amounts of lactic acid. Dai et al. (2004)[11] increased up to 10 times of the tolerable concentrations of *Sphingobium chorophenoclicum* to pentachlorolphenol by triplicate genome shuffling, and as much as 3 mmol/L pentachlorolphenol contained in the medium could be degraded completely.

The starting strain HQD_{33} used in this study was isolated by Zhou et al.[12,13] in 1993 from the inner bark of *Taxus cuspidate*, a taxol-producing endophytic fungus. This fungus was identified as *Nodulisporium sylviforme* (strain HQD_{33}), a new genus and new species to china. In view of the little knowledge about its genetic background, the high output taxol-producing strains were

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common mutagenesis may have little effect on the in screened in our lab by long-term mutagenesis. The crease of taxol production. Furthermore, no report on the breeding of high taxol-production strain by genome shuffling is available. Therefore, this study aims to breed the taxol-producing strain by genome shuffling method and study the application of this technique in taxol-producing fungus to screen the variance strain with high taxol output.

1 Materials and methods

1.1 Materials

1.1.1 Strain.

HD_{100-23}, UV_{40-19} and UL_{50-6} were used as starting strain.

The spores from *Nodulisporium sylviforme* taxol-producing strain HQD_{33} with a taxol output of 51.06—125.70 μg/L were subjected to a series of mutagenesis screening (UV, EMS, ^{60}Co and NTG), then the mutagenesis-derived strain NCEU-1 screened with a taxol output of 314.07 μg/L, was used as the primary starting strain.

The strain HD_{100-23} with taxol output at 356.80 μg/L was a mutant derived from the strain NCEU-1 of which the spores were subjected to combined mutagenesis by UV and NTG. The strains UV_{40-19} with taxol output of 376.38 μg/L and UL_{50-6} with taxol output of 392.63 μg/L were the mutants from NCEU-1 protoplasts which underwent UV mutagenesis and the combined mutagenesis by UV and LiCl, respectively[14].

1.1.2 Medium.

(1) PDA liquid medium[15];

(2) PDA solid medium: sterilized PDA liquid medium containing 2% agar;

(3) PDA liquid medium with high osmotic pressure: PDA liquid medium containing 0.7 mol/L NaCl after sterilization;

(4) PDA semi-solid regeneration medium: PDA liquid medium with high osmotic pressure containing 0.7% agar after sterilization;

(5) Regeneration medium: PDA liquid medium with high osmotic pressure containing 2% agar after sterilization;

(6) Barley medium: 200 g barley was washed and boiled for 30 min, and filtered using Microcloth. 20 g of glucose was added to the filtered liquid and diluted to 1000 mL using distilled water at neutral pH. 2% agar was added and sterilized.

(7) Yeast extract medium: Yeast extract 5 g, peptone 10 g, and glucose 15 g were diluted to 1000 mL at pH6.0 on 2% agar, sterilized at 1.05 kg/cm^2 for 30 min and packaged for use.

(8) Nystatin-resistant culture: the sterilized PDA medium was cooled to 50℃—60℃, and the sterilized Nystatin was added at the concentration of 135 μg/mL for use.

(9) Modified S-7 medium: Modified S-7 medium was based on S-7 medium[16] with the addition of tyrosine, linoltic acid and phenylalanine at the final concentrations of 1.5, 1.5 and 5.0 mg/L, respectively.

1.1.3 Reagents. Lywallzyme (Institute of Microbiology of Guangdong, Guangzhou, China), snailase (Beijing BioTech Co., China), cellulase (Shanghai Lizhu Biotech, Co. Ltd) and lysozyme (Institute of Biochemistry, Chinese Academy of Sciences) were dissolved in 0.7 mol/L NaCl, respectively, and centrifuged at 4000 r/min for 15 min. The supernatants were collected by filtration and the pH was adjusted to 5.5—6.0. Taxol standard (from *Taxus brevifolia*, at least 95% in purity) was purchased from Sigma.

PEG6000 35%—40% (*w/v*), $CaCl_2$ 0.01 mol/L, glycine 0.5% (*w/v*) were dissolved in an osmotic stabilizer. All other reagents were of analytical grade.

1.2 Methods

1.2.1 Culture and collection of mycelium. See ref. [17].

1.2.2 Preparation and regeneration of protoplasts. See refs. [17, 18].

1.2.3 Inactivation of protoplasts. Heat inactivation: The purified HD_{100-23} protoplast suspension was transferred into sterilized test tubes, and then put into 50℃ water 1bath for 1, 2, 3, 4, 5 and 6 min, respectively for inactivation. The results were examined using the regenerative medium.

UV inactivation: The purified UV_{40-19} protoplast suspension was transferred into a sterilized Petri dish with a diameter of 6 cm, which was then put under a preheated 30 W UV lamp with a vertical distance of 30 cm and irradiated for 70, 75, 80, 85, 90, 95, 100 and 105 s, respectively for inactivation. The regenerative medium was used to examine the inactivation results.

UV+LiCl inactivation: 0.6% LiCl was added to the purified $UL_{50\text{-}6}$ protoplast suspension, and then irradiated for 10, 20, 30, 40, 50, 60, 70 and 80 s under a preheated 30 W UV lamp with a vertical distance of 30 cm. The results of combined inactivation by UV plus LiCl were examined using regenerative medium.

The inactivation rate of protoplasts was calculated as follows:

$$a=[1-(b-c)/(d-e)]\times 100\%,$$

where a refers to inactivation rate of protoplasts; b the number of colonies on regenerative medium after inactivation; c the number of colonies on PDA medium after inactivation; d the number of colonies on regenerative medium before inactivation; e the number of colonies on PDA medium before inactivation.

1.2.4 Protoplast fusion. Two inactivated protoplast suspensions were selected randomly at the concentration of 1.0×10^6 protoplasts/mL. 1 mL of each suspension was blended and centrifuged at 3000 r/min for 10 min. The protoplasts were collected and suspended in 0.2 mL osmotic stabilizer, then 1.8 mL of different concentrations of PEG preheated at 30℃ was added to the suspensions. The mixed liquid was treated in a 30℃ water bath for different time, then 5 mL osmotic stabilizer at 4℃ was added to terminate the fusion, followed by washing and centrifugation to discard the fusion agent. The washed protoplasts were resuspended into osmotic stabilizers, and regenerated by double-layer culture for 3—5 d at 28℃. The fusion rate was calculated based on the regeneration of syzygies and inactivated parents.

The fusion time was set at 60 s with PEG concentrations at 25%, 30%, 35%, 40% and 45%; then the fusion time was set at 30, 60, 90 and 120 s under the optimal PEG concentration. In addition, the effect of Ca^{2+} (0.01 mol/L$CaCl_2$) in fusion reagents on the fusion rate of the protoplasts was also studied. The fusion rate of the protoplasts was calculated as follows:

$$a=[(b-c)/d]\times 100\%,$$

where a refers to the fusion rate of the protoplasts; b the number of colonies on regeneration plate; c the number of colonies on inactivated parent regeneration plate; d the number of parent colonies on regeneration plate.

1.2.5 Genome rearrangement. Protoplasts of $HD_{100\text{-}23}$, $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$ parent strains were inactivated by heating, UV and UV+LiCl, respectively. The inactivated protoplasts were fused randomly under suitable conditions and regenerated by double-layer culture method at 28℃ for 4—5 d. All the regenerated colonies were collected and used as F1 fused colonies. Then the F1 heterozygosis was used to prepare the protoplasts which were then inactivated using the above-mentioned methods and F2 heterozygosis colonies were acquired. F3 and F4 heterozygosis colonies were also prepared as above. All the regenerated strains were screened primarily and then fermented. Metabolites were extracted and purified, and were analyzed by TLC, MS and HPLC methods to test the genome rearrangement strains for high taxol yield.

1.2.6 Non-genome-rearrangement control. The protoplasts of starting colonies used for genome rearrangements were not fused and regenerated by double-layer culture. The obtained regeneration strains underwent the next cycle of the preparation and regeneration of the protoplasts. The filial generation P_n was obtained after n cycles of the protoplast preparation and regeneration, which was then fermented and compared with F_n.

1.2.7 Screening of recombinants with high yield. *Primary screening*: The obtained rearrangement strains with good mycelia growth and spore-production abilities were successively transferred and cultured. Then they were transferred onto the plate 135 μg/mL Nystatin[19] cultured at 28℃ for 3 d. Single colonies growing fast on the plates with a larger diameter were selected as resistant strains through primary screening, and used for the screening of high-output strains. Other colonies were discarded.

Secondary screening: The strains obtained by primary screening were activated in PDA slope culture at 28℃, then transferred into 50 mL PDA liquid medium in a 250 mL flask and cultured at 28℃ for 3 d. The products were inoculated into modified S-7 culture at the concentration of 3% (*v*/*v*)and fermented at 28℃ and 150 r/min for 12 d. At the end of fermentation, the filtrate and mycelium were collected. The filtrate was extracted twice using acetic ether, 1 h for each time, and supernatants were collected. The mycelium was whetted fully and extracted using 30 mL acetic ether for 1 h. The organic phase was combined with the above sample, and then distilled to remove the organic solvent. The extractant was dissolved into 2 mL acetonitrile for use.

1.2.8 Purification by column chromatography. The silica gel used for 60—100 μm column chromatography

was dipped in $CHCl_3$ overnight and packed (15 mm×260 mm) by gravity settling. The impurities were washed out from silica gel thoroughly using $CHCl_3$ until the column bed became transparent. The sample was dissolved into the $CHCl_3$ and then packed. The unabsorbed impurities were washed out using $CHCl_3$. Thereafter, methanol:$CHCl_3$ (3:97, *v/v*) was used for elution and the elution apex was collected. All the process was carried out at room temperature and atmospheric pressure.

1.2.9 Thin-layer chromatography analysis. The chloroform-methanol (7:1, *v/v*) was used as developer, and taxol standard (Sigma Ltd) was used as control. 1% vanillin-concentrated sulfuric acid was used as chromatography agent. The plate was dotted triplicate. After the complement of developing, the chromatogenic agent was sprayed and dried at 90℃—105℃ for chromatogenic reaction.

1.2.10 HPLC analysis. The blue site after the developing chromatogenic reaction using Taxol standard was used as control. The gel silica of 1 cm^2 was scratched and dissolved using 1 mL methanol and eluted by ultrasonic method. The eluate was filtered. Quantitative analysis of taxol was performed using a Waters Millennium32 HPLC work station, equipped with a photodiode array detector with a 200 mm×4.6 mm phenyl column at room temperature. An aliquot of taxol extract (20 μL) was injected. The eluate was a mixture of methanol-water (60:40, *v/v*) and the flow-rate was 1.0 mL/min. Taxol absorbance was measured at 254 nm. Taxol amounts were quantified using standard curves generated with the taxol standard.

1.2.11 Mass spectrometric analysis. The apex with the same residence time as that of the standard sample was collected, and dried. The structure of the taxol produced was confirmed using a Waters LC-MS system. The MS scan range was between 100 and 1000 *m*/*z*, and the shell gas (N_2) and assistant gas (N_2) were 65IU and 20IU respectively. The discharge current was 5 μA. The evaporator and capillary temperature was 465℃ and 180℃, respectively. The HPLC was run isocratically with acetonitrile: water (49:51) as the mobile phase using Taxsil-3column (250 mm×46 mm) (Metachem Ltd.). The samples were loaded onto a 250 mm×4.6 mm Taxsil-3 C_{18} reverse phase column (Metachem, Co. Ltd.) and separated at a flow rate of 0.8 mL/min at the column temperature of 35℃. The taxol standard sample was used as control.

1.2.12 Measurement of hereditary stability of the recombinant strains. The recombinant strains with high taxol output were cultured for six generations. Strains with good hereditary stability and high yield of taxol were stored.

2 Results and discussions

2.1 Preparation and regeneration of protoplasts

Preparation and regeneration of protoplasts are the prerequisite for such breeding work including protoplast mutagenesis, fusion and genome recombination. Therefore, the authors carried out the work based on the previous reports on the factors of the preparation and regeneration of *Nodulisporium sylviforme* taxol-producing fungus protoplasts such as the types and components of media, culture methods, enzymes and osmotic stabilizers[17]. This study provides new insight into the improvement of a key technique for the preparation and regeneration of protoplasts.

2.2 Fusion of the protoplasts

2.2.1 Heat-inactivated $HD_{100\text{-}23}$ protoplasts. The purified $HD_{100\text{-}23}$ protoplasts were suspended into 50℃ water bath for treatment. The protoplasts at different time points were dispersed onto the double-layer regenerative plates, and cultured at 28℃ for 4—5 d, the regenerative colonies were counted, and the lethal rate was calculated. Figure 1 shows that when the $HD_{100\text{-}23}$ protoplasts were treated in the 50℃ water-bath for 5 min, the lethal rate

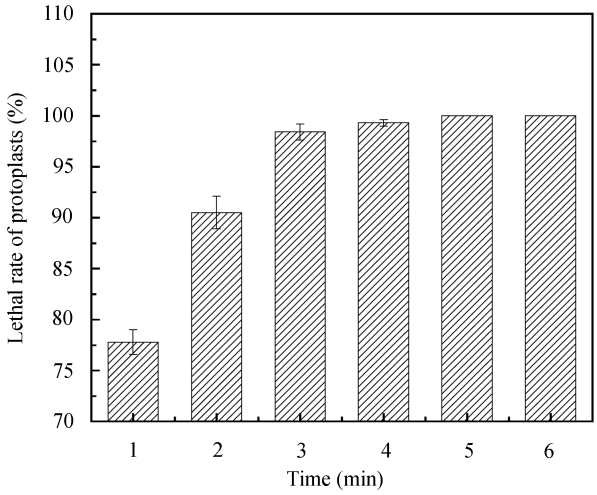

Figure 1 Effects of heat inactivation time on the lethal rate of $HD_{100\text{-}23}$ protoplasts.

reached 100%, which was used as the inactivation con-

dition for the $HD_{100\text{-}23}$ protoplast inactivation.

2.2.2 UV-inactivated $UV_{40\text{-}19}$ protoplasts. The purified $UV_{40\text{-}19}$ protoplast suspension was put under a preheated 30W UV-lamp with a vertical distance of 30 cm and irradiated for different time points, and then spread onto the regenerative plates using double-layer plate culture at 28℃ for 4—5 d. The lethal rate was calculated. Figure 2 showed that, when the irradiation time was 100 s, the lethal rate of the protoplasts was 100%. Therefore, the UV-irradiation was set at 100 s, which was used as the inactivation condition for the $UV_{40\text{-}19}$ protoplast inactivation.

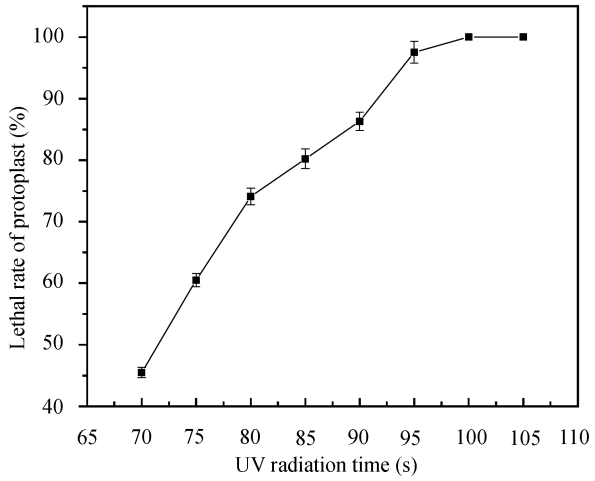

Figure 2 Effects of UV inactivation time on the lethal rate of $UV_{40\text{-}19}$ protoplasts.

2.2.3 UV+LiCl inactivation of $UL_{50\text{-}6}$ protoplasts. 0.6% LiCl was added to the purified $UL_{50\text{-}6}$ protoplast suspension, and then irradiated for different time under a preheated 30W UV lamp at a vertical distance of 30 cm, which was then diluted serially and spread onto the regenerative plates using double-layer plate culture at 28℃ for 4—5 d. The lethal rate was calculated. Figure 3 showed that, when the UV irradiation time was 70 s, the lethal rate of the $UL_{50\text{-}6}$ protoplasts was 100%. Therefore, the 0.6% LiCl combined with 70 s UV-irradiation was used as the optimized inactivation condition for the $UL_{50\text{-}6}$ protoplasts.

2.2.4 Conditions for protoplast fusion. (1) Effects of PEG concentrations on fusion.

The concentrations of PEG have great effects on the protoplast fusion. Low PEG concentrations can stabilize protoplasts, and accelerate karyokinesis favoring the formation and regeneration of the cell wall. This paper studies the fusion effects of fusion time of 60 s and the

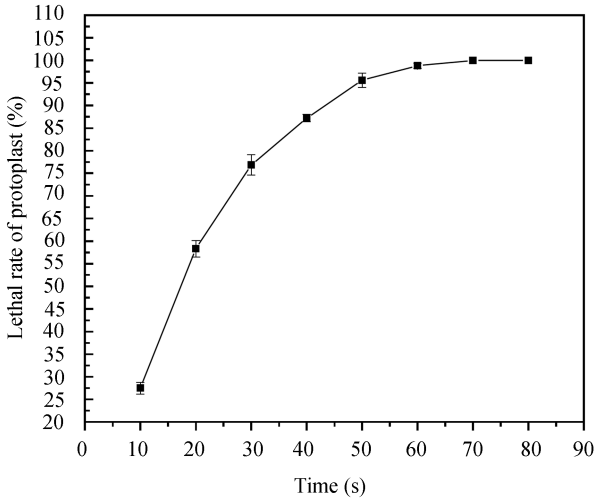

Figure 3 Effect of UV + LiCl inactivation time on the lethal rate of $UL_{50\text{-}6}$ protoplasts.

PEG6000 concentrations at 25%, 30%, 35%, 40% and 45%. Figure 4 showed that, with the increase of PEG concentration, the fusion rate increased as well and reached the maximal fusion rate (10.5±0.6%) when the PEG concentration was 35%. When the concentration of PEG increased further, the fusion rate decreased, therefore, 35% PEG was used as the fusing agent for taxol-producing protoplasts.

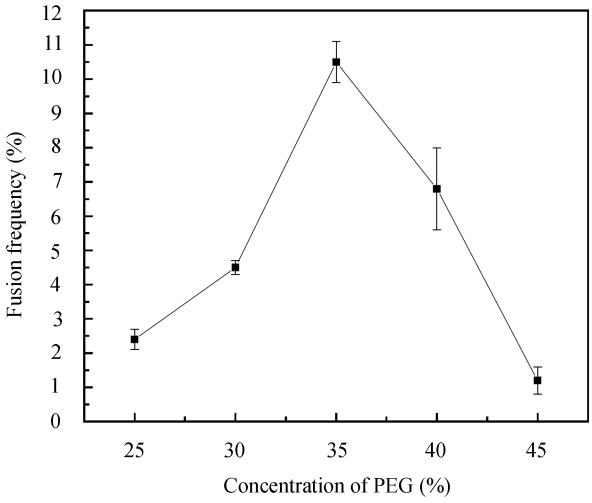

Figure 4 Effect of PEG concentrations on fusion.

(2) Effects of fusion time and concentration of Ca^{2+} on fusion efficiency

The protoplasts of taxol-producing fungus $HD_{100\text{-}23}$, $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$ were inactivated using the above methods, then were fused using 35% PEG6000 in the

30℃ water-bath for a while. The PEG was then discarded by centrifugation. Then the protoplasts were spread onto the regenerative plates using double-layer plate culture at 28℃ for 4—5 d. The fusion rate was calculated. Figure 5 shows that, with the increase of fusion time, the fusion rate increased as well and reached the maximal fusion rate (12.8±0.6)% at 90 s, then decreased thereafter. Baltz et al. (1981)[20] fused several *Streptomyces* using PEG for 30—60 s. Wang (1996)[21] studied the fusion of *Candida* sp. protoplasts and found that the fusion of the protoplasts can be completed in a very short time. When the fusion time is longer than 30 min, the fusion body cannot be obtained. These studies showed that the fusion of the protoplasts occurred quickly when the PEG was added, therefore, when the fusion time was longer, the fusion rate could not increase. On the contrary, because of the toxicity of PEG, the activities of the protoplasts and the regeneration rate of the fusion bodies decreased, resulting in the decrease of the fusion rate.

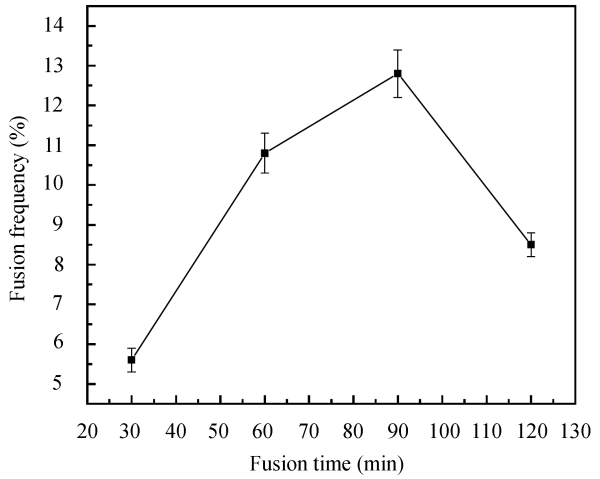

Figure 5 Effect of fusion time and concentration of Ca^{2+} on fusion efficiency.

The addition of Ca^{2+} into the fusion agent can improve the fusion rate of the protoplasts significantly. Figure 6 shows that under conditions of 90 s and 35% PEG, the addition of 0.01 mol/L $CaCl_2$ can increase the fusion rate of the protoplasts, from (12.8±0.6)% to (15.4±0.7)%.

2.3 Breeding of high output of taxol-producing strain by genome rearrangement

2.3.1 Acquisition of starting colonies. The taxol-producing fungus $HD_{100\text{-}23}$, $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$ were obtained from the same wild strains with different breeding methods, that is, they have the same genetic background and different mutagenesis, and therefore, this study selected $HD_{100\text{-}23}$, $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$ as the starting strains of the first round genomic rearrangement. And the first round multiple-parent protoplast fusion was carried out according to the above optimal conditions and cultured on the regenerative plates. All the regenerated colonies were marked as F1 generation. This study did not calculate the recombination rate, but collected the filial generation of regeneration, therefore, no dilution was required. After double-layer culture, the regeneration filial generation only needs to grow into dense colonies. The regeneration colonies were collected and used as the parents for the next generation. This process was repeated four times; the well-growth filial generation was selected and seeded onto the PDA slant culture to screen recombinants with high taxol output.

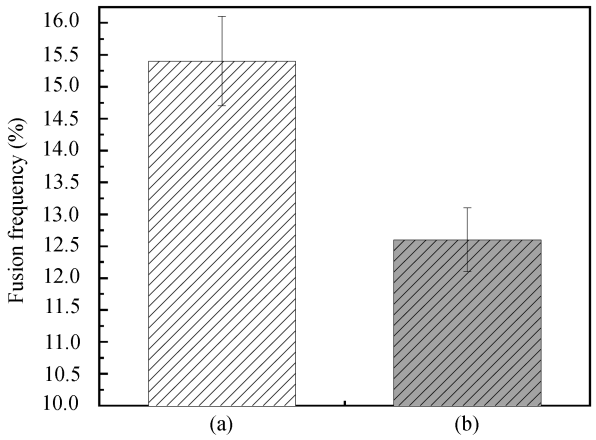

Figure 6 Effect of Ca^{2+} on fusion rate. (a) With Ca^{2+}; (b) without Ca^{2+}.

2.3.2 Screening of recombinants with high taxol output. The colonies with good growth state from the third and forth regeneration were selected and inoculated onto the PDA slant medium and cultured for three generations, then were screened on PDA plate containing 135 μg/mL nystatin. 326 colonies with fast growth rates and larger diameters were observed on the resistance plate, which were used as the resistance strain from the primary screening. The study screened 50 strains from the F3 generation and 88 strains from the F4 generation. Thereafter, the strains from the primary screening were fermented for 12 d, then extracted and purified for quantitative and qualitative analysis.

TLC analysis showed that the fermentation extractions from the 12 strains showed one blue point located

near or at the same place as the standard taxol Rf (0.6—0.7). This test indicated that these strains can excrete taxol or taxol-like compounds. HPLC analysis showed that, among the 12 strains, only 3 of them (F4-17, F4-26 and F4-70) had the purified products with the same retention time as that of the standard taxol (Figure 7(a)—(d)). After the addition of the taxol standard into the samples, the apexes became overlapped, which can further prove that the purified fermentation products of F4-17, F4-26 and F4-70 are taxol, with taxol contents at 487.93, 516.37 and 496.74 μg/L, respectively based on the apex area. The taxol output of F4-26 is 64.41% higher than that of the NCEU-1 and 31.52%—44.72% higher than its parent strain.

Waters LC-MS analysis indicated that the purified products contained taxol-specific $[M+H]^+$ (*m/z* 854.92), proving that the purified gradients of F4-17, F4-26 and F4-70 have the same specific mass spectra (Figure 8).

2.3.3 Control experiment. In order to prove that the improvement of the target traits of the strains was not the result of mutagenesis caused by the repeated preparation and regeneration of the protoplasts, but due to genome rearrangement, this study set the control group to assess the effects of preparation and regeneration of the protoplasts on the taxol output. The results showed that significant improvement of the F4 generation phenotype is related to the genome rearrangement. Meanwhile the repeated preparation and regeneration of the protoplasts had little effect on these traits. The recombinants with specific phenotype were produced from the genome recombination.

2.4 The genetic stability of the rearrangement strain

The three strains with high taxol output were generated six times, and cultured. TLC and HPLC analysis showed that the strains after passages showed no change in the size and color of the specific blue points, with the same Rf value as that of the taxol standard. The capability of the strains to produce taxol did not decrease. These findings showed that these strains had good genetic stability.

3 Discussions

In recent years, the studies on the cell-mediated biosynthesis of taxol from yew have made breakthrough advances. However, there are altogether 19 enzymatic reactions, which is very complex[22,23]. The metabolizing way of taxol synthesis by microbe fermentation is still unclear, and its process is very complex. Therefore, in order to increase the yield of taxol, multiple genes must be coordinate. The traditional mutagenesis breeding is usually related to the functions of one or a few genes (possibly the mutation effects of the neighbor genes). Therefore, it is difficult to increase the taxol yield significantly. Genome shuffling is a technique in which several genomes are recombined simultaneously at different sites, therefore, multiple exchanges and multiple-gene recombination can happen easily. Finally different genes causing the positive mutations are recombined into one cell, and it is relatively easy to increase the taxol output to a great extent[24]. In this way, genome shuffling technique can improve the strains more quickly than the traditional mutagenesis, accelerating the evolution process of the nature[25−27]. In addition, the traditional breeding methods have such disadvantages as low mutagen efficiency, huge work of screening, long period, accumulation of the unrelated or negative mutagenesis caused by repeated mutagenesis. These factors can cause the insensitivity of the strains to mutagenesis conditions. The result is that it is very difficult to increase the taxol output by ordinary breeding. Sometimes such phenomenon as low growth rate, decrease in the capability of the assimilation to substrate, and low tolerance to the environment happens.

Genome shuffling needs a specific genetically positive mutant library for rearrangement, however, previous studies generally acquired the starting colonies of the genome rearrangement by using single mutagenesis condition[28]. Although the starting colonies in this study came from the same wild strain, the groups with large genetic differences could be obtained using multiple mutagenesis methods. Therefore, although the starting colonies in this study had similar genetic background, the positive mutagenesis groups with great differences were a great improvement.

Theoretically, a certain method of inactivation may cause injury at the same site of the chromosome. If several parent protoplasts were inactivated using the same method of inactivation, it would be difficult for the fusion body to regenerate. This is the first time that we try to use different inactivation methods to inactivate different parent protoplasts before fusion. The lethal injury at the different sites of the chromosome can be complemented, and thus the fusion body with physiological activities can be screened. Furthermore, inactivation of the multiple-parent protoplasts can avoid the numerous

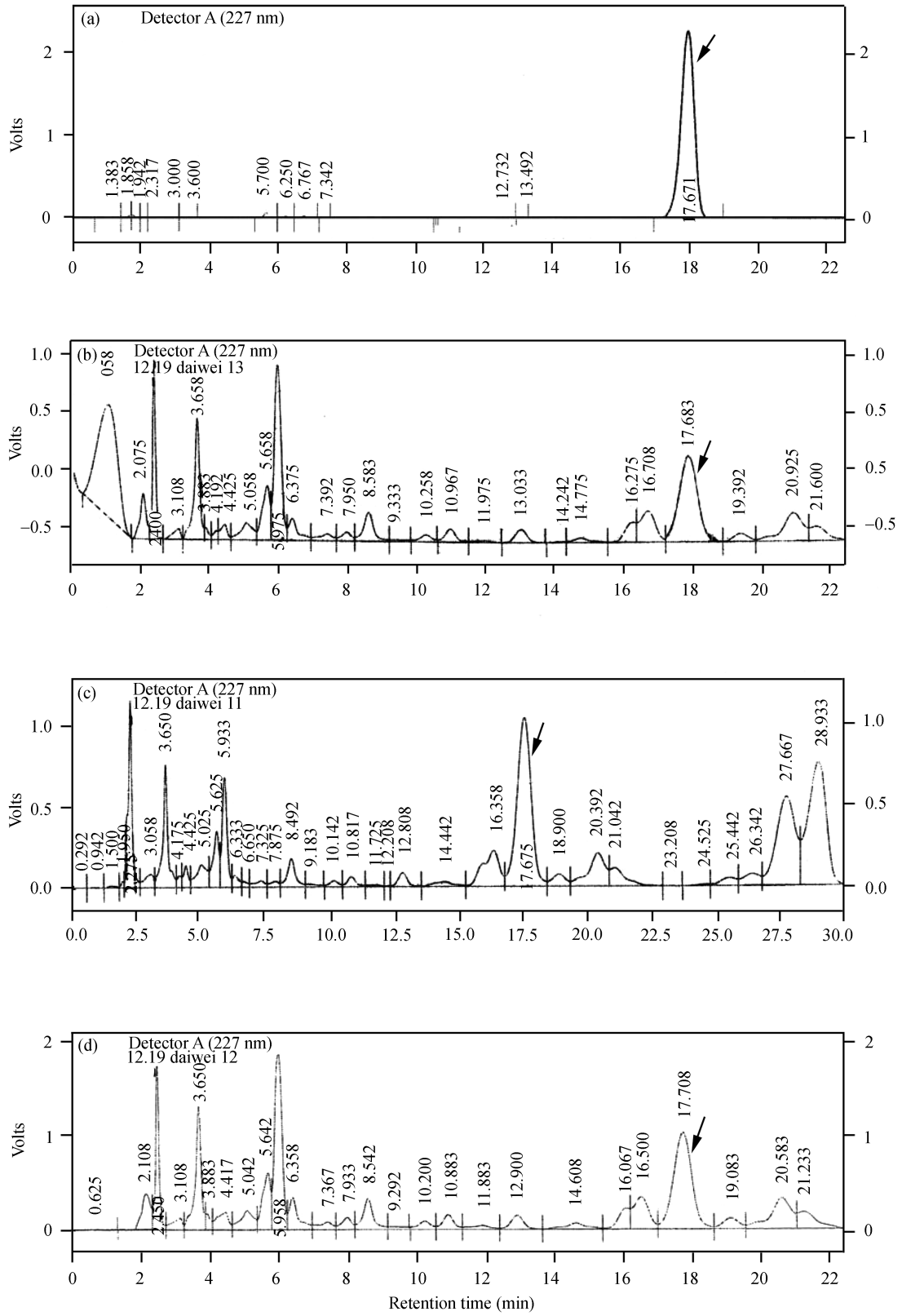

Figure 7 Chromatograms of HPLC analysis, arrows indicated the taxol-specific apex. (a) Taxol standard; (b) extracts from strain F4-17 fermentation; (c) extracts from strain F4-26 fermentation; (d) extracts from strain F4-70 fermentation.

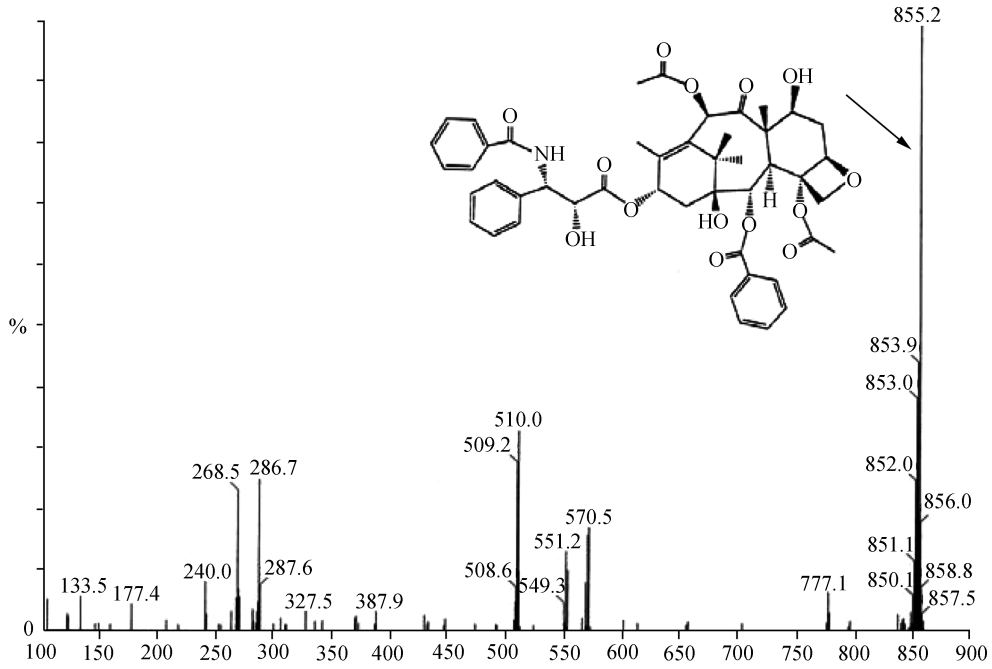

Figure 8 A representative MS spectrum of the purified product from strains F4-19, F4-26 and F4-70. The molecular mass shown as $[M+H]^+$ is indicated by an arrow.

processes of genetic marking, and increased the screening efficiency of the recombinants.

In addition, our previous work[19] found that the mutagenesis strain can not only acquire resistance to nystatin, but also increase the ability of the taxol-producing fungus to produce taxol. This is, with the increase of nystatin resistance, the output of the taxol increased as well. Therefore, during the screening of the recombinants with high taxol output, nystatin was added to the PDA plate as pressure selecting factor to reduce workload and increase screening efficiency. This result confirmed that this method is effective.

Up to now, there has been no report on the application of genome shuffling into breeding of the strain with high taxol output. This is the first report on the use of genome shuffling in improving taxol-producing fungus. We have successfully screened three strains with steady taxol output. In addition, we have proved that genome shuffling is effective in fungus breeding, and provide a good basis for the application of this technique to the breeding of the strains with high taxol output and studying the gene modulation of taxol-producing fungus.

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