

Study on the Mutagenesis of Protoplasts from Taxol-producing Fungus *Nodulisporium sylviforme*

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Abstract: The effects of some factors on the preparation and regeneration of protoplasts from the taxol-producing fungus *Nodulisporium sylviforme* were discussed in this paper, including recombination of various enzymes, digesting time and temperature, and pH value. The results showed that the highest preparation frequency of protoplasts is a concentration of 2.0×10^7 protoplasts per microlitre. The mutagenesis of the protoplasts from *Nodulisporium sylviforme* mycelia induced with UV or UV-0.6% LiCl were carried out. The condition of the best mutagenesis was achieved when the protoplasts of *Nodulisporium sylviforme* were irradiated by a 30 w UV lamp in a 30 cm distance for the 40 s. By primary and secondary screen, the mutant that the yield of taxol raised 25.01% (from 314.07 μ g/L to 392.63 μ g/L) was obtained. In addition, we made a study on analysis of peroxidase isozyme of taxol-producing fungus parent strain NCEU-1 and its mutant. [The Journal of American Science. 2005;3(1):55-62].

Key words: Taxol; *Nodulisporium sylviforme*; protoplast; preparation; regeneration; mutagenesis; peroxidase isozyme

Introduction

Taxol is a complicated diterpene alkaloid with anti-tumor activity, which was first isolated from *Taxus brevifolia* by Wani et al (1971). The effect mechanism of taxol is to inhibit the drepolymerization of microtubulin, disturb the function of microtube, thus affecting the formulation of spindle, prohibiting from mitosis of tumor cell (Zhou, et al,2003) . The taxol has been used to cure many malignant tumors, such as breast cancer, ovarian cancer, choriocarcinoma, hysterymyoma et al (Woo H.L, et al, 1996; Jones W.B, et al, 1996; Puldduinen J. O, et al, 1996). *Nodulisporium sylviforme* is an endofungus isolated from phloem of *Taxus cuspidata* by Zhou et al (1993), and it is a genus new to china (Zhou, et al, 2001). The output of taxol measured first by HPLC was 51.06~125.70 μ g/L with higher fermentility than of other known fungi. Thus it becomes a prospective taxol-producing fungus.

Remarkable results have been achieved in fungus breeding by genetic engineering in recent years, however, the traditionally physical or chemical mutagenesis is still an important method to raise the output of industrial fungi worldwide. Because protoplast is lack of the cell wall, it is more sensitive to the environment. Therefore, fungus breeding by

protoplast mutagenesis has become a useful method for the breeding of microbiology. The protoplast mutagenesis can be operated simply and conveniently, and to a large extent the protoplast can be capable of raise the output of fungi metabolisity in a short time, especially for filamentous fungi. In addition, the regenerative strain can be easily obtained by the mutagenesis of protoplast (Zhu, et al, 1994). However, by now little systematic research on *Nodulisporium Sylviforme* has been done, therefore, this study aims to screen taxol strains with higher output through the preparation and regeneration of protoplasts, mutagenesis of the protoplasts of *Nodulisporium Sylviforme* by ultraviolet and by ultraviolet or 0.6% LiCl, respectively. The complex of ultraviolet and LiCl for the protoplasts mutagenesis of taxol-producing fungi has not been reported by now.

1. Materials and Methods

1.1 Materials

1.1.1 Strain

The strain HQD₃₃ that isolated from *Taxus cuspidata* is an endofungus--*Nodulisporium sylviforme* (Zhou, et al, 2001). Its spore experienced a series of mutagenesis screening(UV→ EMS→ ⁶⁰Co→ NTG), and were finally obtained a mutagenesis-derived

strain NCEU-1 (the output of taxol: 314.07 μ g/L), which was used as parent strain.

1.1.2 Media

- (1) PDA liquid medium (Shen, et al, 1999);
- (2) PDA solid medium: 2% agar was added to above mentioned the PDA liquid medium;
- (3) Regenerative medium: NaCl was added to above mentioned PDA solid medium, and its final concentration is 0.7 mol/L, then autoclaved;
- (4) Modified S-7 medium (Sterle A, et al, 1993): On the basic of the primary S-7 culture, phenylalanine with the final concentrations at 5.0 mg/L, tyrosine 1.5 mg/L, linoleic acid, 1.5 mg/L were added, respectively.

1.1.3 Reagents

- (1) Osmotic pressure stabilizer: NaCl 0.7 mol/L, KCl 0.7 mol/L, mannitol 0.7 mol/L, glucose 0.7 mol/L, sucrose 0.7 mol/L pH 5.5~6.0;
- (2) LiCl solution: 1 g/ml stored liquid;
- (3) Enzymes: lywallzyme, snailase, lysozyme;
- (4) Extracting solvent: methanol, ethylacetate, hexane, acetonitrile;
- (5) Developer: choloform and methanol (7:1 v/v);
- (6) Color developing reagent: 1% vanillin in concentrated sulfuric acid;
- (7) Clotrimazole: 10 mg/ml stored liquid, stored at 4°C;
- (8) Nystatin: 10 mg/ml stored liquid, stored at 4°C;
- (9) Streptomycin: 10 mg/ml stored liquid, stored at 4°C for a short time.

1.2 Methods

1.2.1 Culture and collection of mycelium

Mutant NECU-1 derived from HQD₃₃ was activated in PDA slant, and then in PDA liquid (50 ml/250 ml flask). After activated it was cultured in PDA liquid (200 ml/500 ml flask) with 3% inoculating ratio at 28°C for 3 days. Finally the cultured mycelium was collected by centrifugation at 3000 r/min for 10 min, then the collected mycelium was washed twice using osmotic pressure stabilizer and transferred to the tubes with level bottom, 1 g moist mycelia for each.

1.2.2 Preparation of protoplasts

The mixed enzymes in osmotic pressure stabilizer composed of 3% lywallzyme, 2% snailase and 1% lysozyme were added to the tube that contained moist mycelia at the ratio of 250 mg moist mycelia for enzymolysis solution of 1 ml, then the moist mycelia was enzymolized in warm-water bath at 30°C. A little quantity of the enzymolysis solution was taken out from the tube every regular time, and was filtered using three layers of lens paper to get rid of the remained mycelia and its fragments, the filtrate was centrifuged at 3000 r/min for 10 min. The protoplasts were collected and washed in 0.7 mol/L NaCl solution for twice, then resuspended in 0.7 mol/L NaCl solution. Finally, the protoplast was observed by microscope and counted using hemacytometer (Moriguchi M, et al, 1994).

The frequency of protoplast preparation (Fp, the number of protoplasts per 1 ml) = the number of protoplast (Np)/the volume of enzymolysis solution (V).

1.2.3 Regeneration of protoplasts (Zhou, et al, 1990)

Using bilayer plate culturing method. The protoplast suspension was diluted by ten times at 0.7 mol/L NaCl or above mentioned the osmotic pressure stabilizer, and then 0.5 ml the suspension was added to tubes containing 4.5 ml PDA soft agar medium in turn. After twisted equally with hands, the solution was put out PDA solid-plate, 28°C, cultured for 3~5 days. At the same time, another 0.5 ml protoplast suspension was added into 4.5 ml distilled water and schizolysed for 20 min, spread the PDA plate after it was diluted. The regeneration frequency of protoplasts was calculated by the number of colonies according to the following formula:

Regeneration frequency of protoplast (Rpf) (%) = [the number of colonies on regenerative culture (Cr) - the number of colonies on PDA culture (Cp)] × dilution frequency / the frequency of protoplast preparation (Fp)

1.2.4 Mutagenesis of protoplasts

The obtained protoplast was diluted by 0.7 mol/L NaCl, and the final concentrations of the protoplast were 10⁶ per millilitre. Ultraviolet lamp was opened 30 min for preheating before irradiation (Li, et al, 2001). Then 5 ml of the suspension of diluted protoplast was added into plate of diameter 6 cm with containing aseptic rotor. The condition of ultraviolet

irradiation is: 30 watt lamp and 30 cm away, agitating by magnetic agitator during irradiation. After irradiating for a fixed time, the suspension of protoplast was diluted under red lamp. The regenerative mutagenesis strains were respectively cultured on bilayer plate containing regenerative culture and compound mutagenesis regenerative culture containing 0.6% LiCl, the regenerative strains were counted after culturing for 6 days at 28°C. At the same time, using non-mutagenesis plate as control, the lethal and survival rate was measured according to the following formula: $R_{pl} = (1 - R_{ipr}) / R_{pr} \times 100\%$.

R_{pl} represents lethal rate caused by mutagenesis, R_{ipr} represents regeneration frequency of protoplast after treated by mutagenesis, R_{pr} represents regeneration frequency of protoplast before treated by mutagenesis.

1.2.5 Screening of mutagenesis strain

Screening of mutagenesis strains with resistance: The obtained regenerative strains were successively transferred cultured, then they were transferred onto the plate of containing Clotrimazole and Nystatin with different concentration, respectively. The resistance mutagenesis frequency was computed after cultured for 3 days: $R_{rm} = C_r / C_t \times 100\%$.

R_{rm} represents resistance mutagenesis frequency, C_r represents the number of colonies with resistance activity, C_t represents the number of colonies transferred.

Primary screening: The single colonies with rapid growth rate and larger diameter were selected, and colonies of the small and slower growth rate were discarded.

Secondary screening: After all of the strains that obtained during primary screening and parent strain NECU-1 were activated, they were respectively inoculated onto the resistant plate with the different concentrations of clotrimazole and nystatin, at the same time, using the diameter of parent strain NECU-1 colonies as control. The positive mutagenesis strains were finally screened again.

Final screening: The strains obtained by secondary screening were cultured using modified S-7 culture (200 ml/500 ml flask) at 25°C in 120 r/min shaker. After cultured for 14 days, the fermentation liquid was filtered and taxol was extracted. Quality and quantity of the taxol was determined by thin layer chromatography and high performance liquid phase

chromatography, respectively. At last, taxol-producing strains with higher output of taxol were obtained.

1.2.6 Method of taxol extraction (Zhou, et al, 2001)

The fermentation liquid was filtered, what obtained the solid was grinded using a little quartz sand, and the volume of liquid was measured. The solid was extracted using ethyl acetate 30 ml, and then the mycelium was discarded and ethyl acetate was remained after a while. The filtrate was extracted using ethyl acetate (1/2, v/v), the water phase below was extracted using ethyl acetate again (1:1 v/v). Then obtained ethyl acetate in extraction of the solid and liquid was mixed and distilled by decompressure. The obtained solid was washed by methanol, and then extracted by hexane, the methanol phase was collected and extracted by adding ethyl acetate and water (1:2, v/v). Finally, the collected ethyl acetate was distilled by decompressure and the solid obtained was washed by acetonitrile, stored at 0°C.

1.2.7 Method of qualitative assay of taxol and semi-quantitative analysis

Preparation of thin-layer chromatography plate: 5 g silica gel (10-40 μ) with 20 ml water was mixed and grinded and spreaded the chromatography plate. Drying was done at dark environment at least 12 hours. Then the plate was activated at 105°C for 2 hours.

Dot sampling: the extracted liquid was absorbed using capillary tube (20 μ l) and dotted at 2 cm below silica plate with 2 cm interval between two points. Then the silica plate was placed into the saturated chromatography jar containing developer. The process was stopped when the front line was 2~3 cm away from the top of the chromatography plate. The silica plate was taken out and dry, then was sprayed using color-developing reagent. The taxol (Sigma Ltd) was used as control.

1.2.8 Quantitative analysis of taxol

Taxol was determined by HPLC (Waters model). Using a C_{18} column (200 \times 4.6 mm; particle size 5 μ m). The mobile phase consisted of methanol-water (60 : 40, v/v) and the flow rate was 1.0 ml per minute, 10 μ l of each samples was injected into the HPLC column. Detection operating was performed with photodiode array (PDA) detector at 227 nm. Taxol was quantified by comparing the average peak response of the sample to that of the standard. There is

a linear relationship ($r = 0.9988$) for both peak area and peak height over the range of 0.05~1.00 mg per milliliter.

1.2.9 Analysis of peroxidase isozyme

The strains U₅₀₋₂, U₁₋₆ and U₅₋₈, which were obtained by ultraviolet mutagensis, were derived from NECU-1. The strains L₃₀₋₂, L₅₀₋₄ which were obtained by the complex of mutagensis ultraviolet and LiCl were also derived from NECU-1. Firstly, parent strains NECU-1 and all of the above mentioned mutagensis strains were cultured and collected, what collected mycelium was grinded in ice-bath using Tris-lemon and Tris-HCl, respectively. And then centrifuged, what collected the enzyme solution was electrophorized and dyed by peroxidase isozyme. The band of peroxidase isozyme appeared blue after 1~6 min. Finally, gotten rid of the color using distilled water, and stored in 3% CH₃COOH for fixed.

2. Results

2.1 Preparation of protoplast

2.1.1 Effects of pH value on preparation of protoplast

The relationship between pH 4.5~7.0 and the preparation frequency of protoplast was studied (Table 1), the result showed that the maximum concentration of the preparation frequency of protoplast was 2.5~2.7 × 10⁷ per millilitre when the pH value was 5.5~6.0.

Table 1. Effects of pH on preparation of protoplast

pH	Preparation frequency (×10 ⁷ /ml)
4.5	0.82
5.0	1.50
5.5	2.50
6.0	2.70
6.5	1.30
7.0	0.23

2.1.2 Orthogonal experiment of the enzymolysis factors of the preparation of protoplast

The experiment studied the effect of lywallzyme, snailase, lysozyme and enzymolysis temperature on the preparation of the *Nodulisporium sylviforme* protoplast (Table 2). The result indicated that under the conditions of pH 5.5~6.0 the optimal conditions of the preparation of protoplast is 3% lywallzyme+2% snailase+1% lysozyme, 30°C. The effect order is successive: lywallzyme, enzymolysis temperature, snailase, lysozyme.

Table 2. Orthogonal experiment of the enzymolysis factors for preparation of the protoplast

No	Factors				preparation frequency (×10 ⁷ /ml)
	lywallzyme (%)	snailase (%)	lysozyme (%)	temperature (°C)	
1	2	1	1	27	0.14
2	2	2	2	30	0.85
3	2	3	3	33	0.36
4	3	1	2	33	0.97
5	3	2	3	27	1.42
6	3	3	1	30	2.04
7	4	1	3	30	1.52
8	4	2	1	33	1.08
9	4	3	2	27	0.94
T ₁	1.25	2.73	3.26	2.50	
T ₂	4.53	3.35	2.76	4.41	
T ₃	3.54	3.34	3.30	2.41	
X ₁	0.42	0.91	1.07	0.83	
X ₂	1.51	1.12	0.92	1.47	
X ₃	1.18	1.11	1.10	0.80	
R	1.09	0.21	0.18	0.67	

2.1.3 Effects of enzymolysis time on the preparation of protoplast

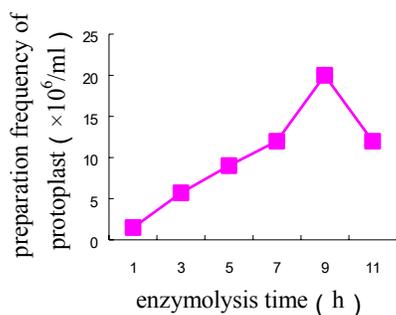


Figure 1. Effect of enzymolysis time on the preparation frequency of protoplast

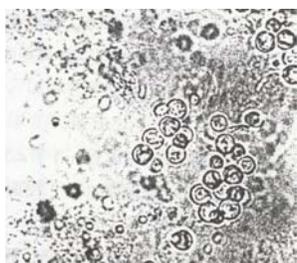


Figure 2. The morphology of the protoplast after purification

Effects of enzymolysis time on the preparation of protoplast were studied under 3% lywallzyme+2% snailase +1% lysozyme, 30 0°C and pH 5.5~6.0. The result showed that with prolonging of enzymolysis time, the preparation frequency of protoplast increased, the preparation frequency of protoplast came to the maximum of 2.0×10^7 when enzymolysis time was 9 hours (Figure 2). If the enzymolysis time is longer than 9 hours, the preparation frequency of protoplast decreased. Figure 1 gives the morphology of the protoplast after purification.

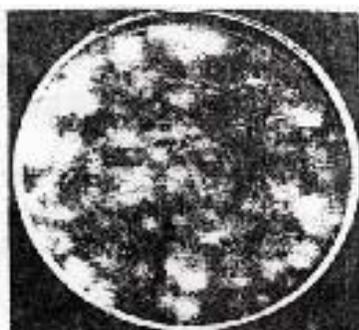


Figure 3. The morphology of protoplast in PDA high osmosis plate

2.2 Regeneration of protoplast

After the concentrations of prepared protoplasts from different enzymolysis time were modulated, by using bilayer plate culture the prepared protoplasts were regenerated in the PDA high osmosis regenerative culture which contained 0.7 mol/L NaCl, whose regeneration frequency is highest than any of osmosis pressure stabilizer (Zhou, et al,1990) The regenerative morphology of the colonies is shown in Figure 3. The Figure 4 indicated that the regeneration frequency of protoplast changed little in short time, while with the enzymolysis time prolonged, the regeneration frequency decreased. Therefore, taking account of the preparation frequency of protoplast, enzymolysis time 7~9 hours were selected in this study in order to obtain higher regeneration frequency.

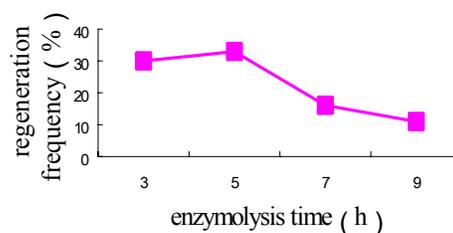


Figure 4. Effect of enzymolysis time on the regeneration frequency of protoplast.

2.3 Mutagenesis of protoplast

2.3.1 Mutagen and the mutagen dose

After *Nodulisporium sylviforme* protoplasts were directly irradiated for 10, 20, 30, 40, 50, 60 second, the lethal rate was shown in Table 3 and Figure 5 by direct regeneration and complex mutagenesis regeneration of containing 0.6% LiCl solution, respectively.

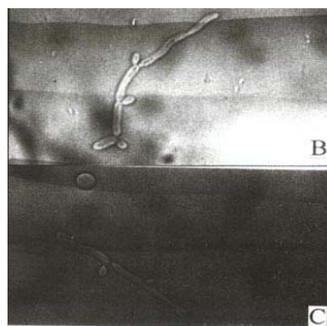


Table 3. The relationship of lethal rate and mutagenesis dose

UV irradiation time (s)	UV irradiation lethal rate (%)	UV-LiCl lethal rate (%)
30	60.0	74.2
40	72.5	90.2
50	85.3	97.6

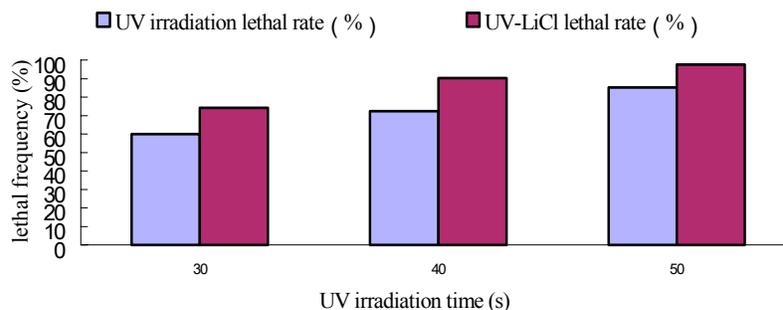


Figure 5. The relationship of lethal rate and mutagen dose

We can see from Table 3 and Figure 5, with the increasing of mutagen dose, the lethal rate increased also, however, no apparent direct ratio be appeared. In this study, in order to decrease working and increase to screen the possibility of taxol-producing fungi with high output of taxol, the mutagenesis strain with lethal rate at 60%~80% was selected during primary screening. The result showed that this selecting is feasible. Among the 165 mutants regenerated strains, 12 strains were screened with higher output of taxol, in which UV₄₀₋₁₉ and UL₅₀₋₆ have significantly higher the output of taxol than parent strain, which come to 392.63 μg/L using taxol sample (Sigma Ltd) as control, and can be further studied as material of the genetic differences.

2.3.2 Resistance mutagenesis

We can see from Figure 6 and Figure 7 that it was feasible to screen mutagenesis strains by using

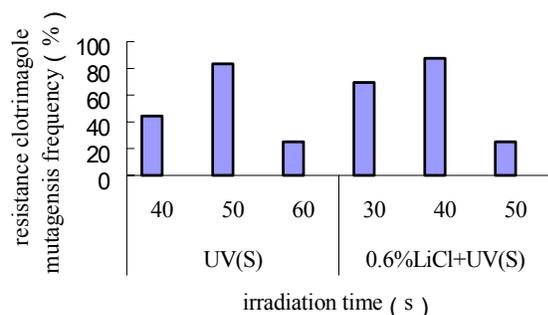


Figure 6. Resistance clotrimazole mutagenesis frequency and mutagen dose

resistance mutagenesis, and optimal sensitivity of mutagenesis strains were 65 μg/L for clotrimazole and 85 μg/L for nystatin.

2.4 Analysis of peroxidase isozyme

The Figure 8 showed that the strains NECU-1, L₃₀₋₂, U₅₀₋₂ and U₅₋₈ have five bands when the Rf was between 0.15 and 0.85, and every band almost has the same in mobility ratio and activity of enzyme. While the strain L₅₀₋₄ has six bands when the Rf was between 0.15 and 0.85, it has another band with Rf at 0.27. The strain U₁₋₆ has seven bands when the Rf is between 0.09 and 0.85. This indicates that treatment with ultraviolet and complex of ultraviolet and LiCl has no effect on peroxidase isozyme of the strains, except that L₅₀₋₄ increase a band whose Rf is 0.27 and U₁₋₆ increased 2 bands whose Rf is 0.27 and 0.09, respectively.

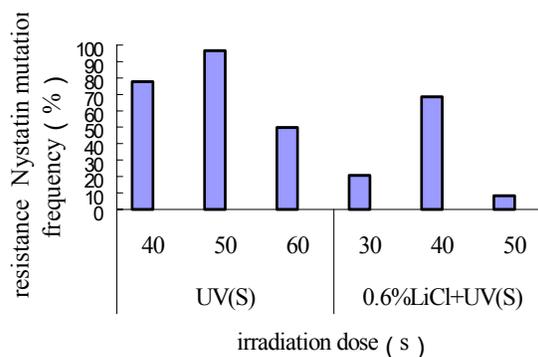


Figure 7. Resistance Nystatin mutagenesis frequency and mutagen dose

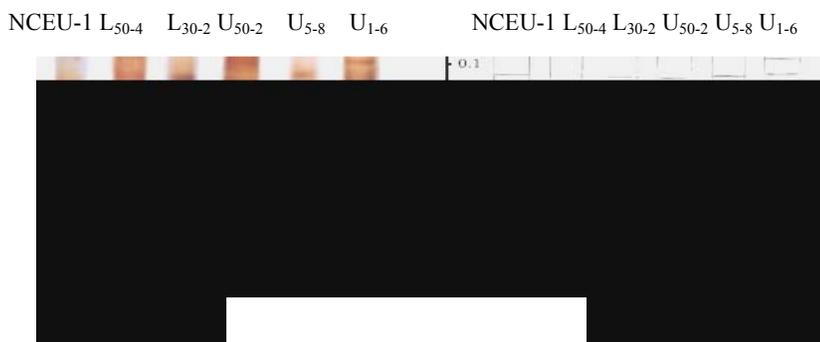


Figure 8. The analysis atlas of peroxidase isozyme

3. Discussion

Mycelium that obtained to disperse well is essential in the preparation of protoplast by enzymolysis. Culturing of mycelium at rest than at shock is more difficult to form twinning hypha mass, and dispersed hyphamass is more beneficial to touch and enzymolysis of mycelium, thus, it will obtain higher preparation frequency of protoplasts. Taking into account the effect of lywallzyme, snailase, lysozyme and enzymolysis temperature on the preparation frequency of derived strains from *Nodulisporium sylviforme* HQD₃₃, it is found that the combination of the three enzymes will obtain higher preparation frequency of protoplast than that of any of them used alone. This may be due to the fact that the strains have complicated structure and the three enzymes respectively have different active sites and different functional principle, and the combination of them may has synergistic effect. In addition, in a certain degree, prolonging of the enzymolysis time will be helpful to raise the preparation frequency of the protoplast; while the enzymolysis time is too long, the preparation frequency and regeneration frequency of protoplast will decrease, this may be because of breaking of the protoplasts or enzymolysis too much, causing the loss of regenerative primers, and the reduction of protoplast activity.

The regeneration frequency of protoplast is often a restricted factor in the application of protoplast technique. If the regeneration frequency of protoplast is too lower or the protoplast can't regenerate, it will lead to be lack of materials for experiment to select, and it is very difficult to count. Therefore, improvement of the regeneration frequency of protoplast is very important. This study showed that with the prolonging of enzymolysis time, the regeneration frequency decreased, and much too high

concentrations of enzyme can also affect the regeneration frequency. This may be because when the cell wall was removed too much, the primers for synthesizing cell walls will lost during regenerating [12], and when the concentration of the enzyme is too high, the heteroalbumose enzymes will affect the activities and the regeneration frequency of the protoplasts.

Using the complex of ultraviolet-LiCl to treat protoplast is a new method for the mutagenesis of taxol-producing fungi, which has not been reported by now. This study indicated that the complex of ultraviolet and LiCl will obtain higher mutagenesis frequency than ultraviolet alone, which may be because LiCl has to assist function for ultraviolet mutagenesis. In addition, the analysis atlas of peroxidase isozyme of the mutagenesis strains U₁₋₆, U₅₋₈, U₅₀₋₂, L₃₀₋₂, L₅₀₋₄ and the parent strain NECU-1 showed that these were significant differences between the parent strain and the mutagenesis strains, which were the result of DNA structural change, this inferred that mutagenesis of ultraviolet or the complex of ultraviolet-LiCl is a effective mutagenesis method. However, more work should be done to find whether the differences exist regularity, and whether it is related to the output of taxol. In addition, to select the mutagen dose, when taxol-producing fungus *Nodulisporium* were mutagened, we selected lethal frequency at 60%~80% mutagen dose and achieved a better effectiveness by using the complex of ultraviolet and LiCl treatment. The study has importantly practical significance to screen and breed taxol-producing fungi and industrially produce anti-cancer drug taxol.

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