African Journal of Biotechnology Vol. 10(20), pp. 4175-4182, 16 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2285
ISSN 1684–5315 © 2011 Academic Journals

*Full Length Research Paper*

# Improved taxol production in *Nodulisporium sylviforme* derived from inactivated protoplast fusion

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Accepted 6 April, 2011

**Inactivated protoplast fusion by UV irradiation and UV+LiCl mutation was conducted using *Nodulisporium sylviforme* strain $UV_{40-19}$ and $UL_{50-6}$ to breed a high taxol-producing fungus. Qualitative and quantitative analysis of taxol production was confirmed using thin-layer chromatography, high performance liquid chromatography and mass spectrometry. The protoplasts of $UV_{40-19}$ and $UL_{50-6}$ were fully inactivated by heating at 54°C for 5 min and by UV irradiation (30 w UV light and vertical distance 30 cm) for 85 s. The highest fusion rate (14.31 ± 1.13%) between $UV_{40-19}$ and $UL_{50-6}$ was obtained under the conditions of 35% PEG, 90 s fusion time and the addition of 0.01 mol/l $CaCl_2$. One high taxol production strain HDF-68 was obtained. The taxol production was up to 468.62 ± 37.49 μg/l, which was increased by 24.51 and 19.35% compared with the parental strain $UV_{40-19}$ and $UL_{50-6}$, respectively. This study provided a good basis for the application of this technique to the breeding of the strains with high taxol output.**

**Key words:** Taxol, endophytic fungi, protoplast preparation, protoplast fusion.

## INTRODUCTION

Taxol is a diterpenoid with anticancer activities. It was first isolated from the bark of *Taxus brevifolia* Nutt by Wani et al. (1971) and is still mainly extracted from the bark of yews. The action of taxol is to inhibit the depolymerization of microtubulin and disturb the function of microtubes thus, affect the formulation of spindle and inhibit tumor cell mitosis (Zhou et al., 2003). Recently, great effort has been made to develop alternative means of taxol production, including complete chemical synthesis (Holton et al., 1994a, b; Nicolaou et al., 1994), semi-synthesis (Comnercon et al., 1995; Holton et al., 1995) and the *Taxus* spp. plant cell culture (Christem et al., 1991; Arteca and Wickremesinhe, 1993; Ketchum et al., 1995; Furmanowa and Syklowska-Baranek, 2000; Wang at al., 2001).

Using microbe fermentation to produce taxol is a very prospective method for obtaining a large amount of taxol. Several endophytic fungi that produce taxol have been isolated (Stierle et al., 1993; Strobel et al., 1996; Zhou et al., 2009). Since 1993, the authors have isolated five endophytic fungal species that can produce taxol by screening samples from the inner bark (phloem-cambium) and xylem of *Taxus cuspidata* Sieb. et Zucc. These fungi are *Nodulisporium sylviforme* (Zhou et al., 2001), *Pleurocytospora taxi* (Sun et al., 2003), *Alternaria taxi* (Ge et al., 2004), *Botrytis* (Zhao et al., 2008a) and *Aspergillus niger* var. *taxi* (Zhao et al., 2009).

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 bottleneck lies in the breeding of high yield strain (Zhou et al., 2003). Two high taxol-production mutants $UV_{40-19}$ and $UL_{50-6}$, were screened from *N. sylviforme* $HQD_{33}$ by UV irradiation and UV+LiCl mutating in our lab to induce mutagenesis. The common mutagenesis may have little effect on taxol production. Alternatively, protoplast fusion can be used to produce high yield stable strain and is widely applied to

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fungal breeding (Gokhale, 1992; Muralidhar and Panda, 2000). Up to date, no report on the breeding of high taxol-production strain by inactivated protoplast fusion is available. In this study, we report the production of a high taxol-producing strain by inactivated protoplast fusion of two parental strains.

## MATERIALS AND METHODS

### Strains

The spores from *N. sylviforme* $HQD_{33}$ (a taxol-producing endophytic fungus isolated from *T. cuspidata* in China, CCTCC M 202049) with a taxol output of 51.06 to 125.70 µg/l were subjected to a series of mutagenesis screening (UV, EMS, $^{60}Co$ and NTG). A mutagenesis-derived strain NCEU-1 with a taxol output of 314.07 µg/l was used as the primary starting strain. $UV_{40-19}$ with taxol output of 376.38 µg/l and $UL_{50-6}$ with taxol output of 392.63 µg/l were the mutants from strain NCEU-1 protoplasts which underwent UV mutagenesis and the combined mutagenesis by UV and LiCl, respectively (Zhao and Zhou, 2004). $UV_{40-19}$ and $UL_{50-6}$ were used as parental strains in this study for inactivated protoplast fusion.

### Media

Potato dextrose agar (PDA) medium and potato dextrose liquid medium (Shen et al., 1999) were used as the growing medium. For the regeneration of protoplasts, solid regeneration medium (PDA medium containing 0.7 mol/l NaCl) and semi-solid regeneration medium (reducing the content of agar in solid regeneration medium to 6 to 8 g/l) were used as hypertonic media. S-7 medium (Stierle et al., 1993) with the addition of tyrosine, linoltic acid and phenylalanine at a final concentration of 1.5 to 5.0 mg/l was used as the fermentation medium.

### Lytic enzyme solution

Lytic enzyme solution was prepared by dissolving 30 mg/ml lywallzyme (Institute of Microbiology of Guangdong, Guangzhou, China), 20 mg/ml snailase (Beijing BioTech Co., China) and 10 mg/ml lysozyme (Institute of Biochemistry, Academy of China) in 0.7 mol/l NaCl, followed by clarifying the solution by centrifuged at 4000 r/min at 4°C for 15 min. The supernatants were collected by filtration and the pH was adjusted to 5.5 to 6.0.

### Osmotic stabilizer and fusogen

NaCl at 0.7 mol/l was used as the osmotic stabilizer. Polyethylene glycol (PEG, MW 6000) was dissolved in the osmotic stabilizer to yield final concentration of 25, 30, 35and 40% (w/v), respectively. The PEG solutions containing 0.01 mol/l $CaCl_2$ and 0.05 mol/l glycine were used as fusogen.

### Preparation and regeneration of protoplasts

Preparation and regeneration of protoplasts were carried out as previously described (Zhao and Zhou, 2004).

### Inactivation and fusion of protoplasts

Protoplasts of strain $UV_{40-19}$ were inactivated by heating at 54°C for 5 min. Protoplasts of strain $UL_{50-6}$ were inactivated by UV irradiation (30 w UV light and vertical distance 30 cm) for 85 s. Two inactivated protoplast suspensions were selected randomly with a concentration of $1.0\times10^6$ protoplasts/ml. 1 ml of each was mixed 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 different concentrations of PEG preheated at 30°C was added to the suspensions. The mixed liquid was treated at a 30°C water bath for different periods, then 5 ml osmotic stabilizer at 4°C were added to stop 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 to 5 days at 28°C (Zhao et al., 2008b). 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 and 40%. 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 the fusion reagents on the fusion rate of the protoplasts was estimated. The fusion rate of the protoplasts was calculated as follows (Zhao et al., 2008b):

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

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

### Screening of fusants with high taxol yield

The obtained fusants with good mycelia growth and spore-production abilities were successively transferred and cultured. They were transferred onto plates containing 135 µg/ml Nystatin and cultured at 28°C for 3 days (Zhao et al., 2005). Single colony growing fast on the plates with large diameter was selected as resistant strains through primary screening which were used for the screening of high-output strains. Other colonies were discarded. The fusants obtained by primary screening were activated on PDA slope culture at 28°C, then transferred into 50 ml PDA liquid medium in 250 ml flask and cultured at 28°C for 3 days. The products were inoculated into modified S-7 culture at the concentration of 3% (v/v) and fermented at 28°C and 150 r/min for 12 days. At the end of fermentation, the filtrate and mycelium were collected. The filtrate was extracted twice using acetic ether, one hour 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 and then, distilled to remove the organic solvent.

### Purification by column chromatography

The silica gel used for 60 to 100 um column chromatography was dipped in $CHCl_3$ overnight and packed (15 × 260 mm) by gravity settling. The impurities were washed out from silica gel thoroughly using $CHCl_3$. The sample was dissolved into the $CHCl_3$ and loaded. The unabsorbed impurities were washed out using $CHCl_3$. Thereafter, methanol: $CHCl_3$ (3:97, v/v) was used for elution and the peaks were collected. All the process was carried out at room temperature.

### Thin-layer chromatography (TLC) analysis

The chloroform-methanol (7:1, v/v) was used as developer

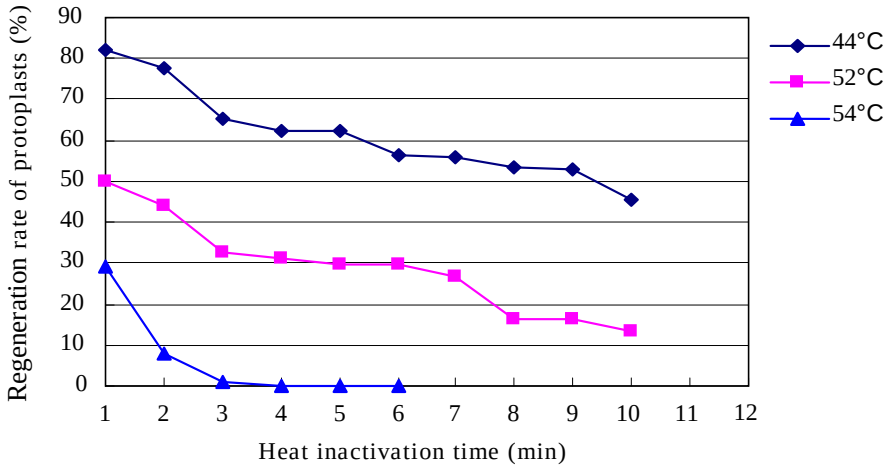


**Figure 1.** Effect of heat inactivation time and temperature on regeneration rate of strain $UV_{40-19}$ protoplasts.

and taxol standard (Sigma Ltd) was used as the control. 1% vanillin-concentrated sulfuric acid was used as chromatography agent. After TLC developing, chromategenic agent was sprayed and dried at 90 to 105°C for chromatogenic reaction.

### High performance liquid chromatography (HPLC) analysis

Using taxol standard as control, the corresponding TLC area of 1 $cm^2$ of the sample was scratched and dissolved using 1 ml methanol and eluted using ultra sonication. The eluate was filtered. A Waters Millennium32 HPLC workstation equipped with a photodiode array detector was used for quantitative analysis. An aliquot of taxol extract (10 µl) was lyophilized and dissolved in 1% methanol and was injected onto a 250 × 4.6 mm Taxsil-3 $C_{18}$ reverse phase column. The mobile phase was a mixture of methanol-water (60:40) and the flow-rate was set at 1.0 ml/min. Taxol in the eluent was detected by measuring the absorbance at 227 nm where taxol has the maximum absorbance. Qualification was achieved using the standard curve generated from the taxol standard over a concentration range of 0.05 to 1.00 mg/ml at which the peak area showed linear relationships with the absorbance (r = 0.9988).

### Mass spectrometry analysis

The HPLC peak with the same retention time as the standard was collected and dried. The structure of the extracted taxol was confirmed with a waters triple quadrupole tandem LC-MS system (Waters, MA, USA). The HPLC portion was run isocratically with acetonitrile: water (49:51) as mobile the phase. The sample was loaded onto a 250 × 4.6 mm Taxsil-3 $C_{18}$ reverse phase column (Metachem, Co. Ltd) and separated at a flow rate of 0.8 ml/min with the column temperature at 35°C. The MS scanning ranged from 100 to 1000 m/z and the shell gas ($N_2$) and assistant gas ($N_2$) were 65 international units (IU) and 20 IU, respectively. The discharge current was 5 µA. The evaporator and capillary temperatures were 465 and 180°C, respectively.

### Measurement of hereditary stability of fusants

Fusants with high taxol output were cultured for ten generations. Strains with good hereditary stability and high yield of taxol were stored.

## RESULTS

### Preparation, inactivation and fusion of protoplasts

The yield of *N. sylviforme* $UV_{40-19}$ and $UL_{50-6}$ protoplasts was up to 1.26 × 10 and 1.82 × $10^7$/ml, respectively. Regeneration tests of inactivated protoplasts of both strains showed that no colony grew on the regeneration medium (Figures 1 and 2), demonstrating that the inactivation process was successful.

The protoplast fusion rate at different PEG concentration and different fusion time was shown in Tables 1 and 2, respectively. It can be seen that both PEG concentration and fusion time had great effects on the protoplast fusion. The optimal PEG concentration was 35% and the optimal fusion time was 90 s.

The addition of $Ca^{2+}$ into the fusion agent can improve the fusion rate of the protoplasts significantly. Figure 3 showed that under conditions of 90 s and 35% PEG, the addition of 0.01 mol/l $CaCl_2$ increased the fusion rate of the protoplasts, from 10.92 ± 0.76 to 14.31 ± 1.13%.

### Screening of fusants with high taxol output

Isolated fusants were transferred onto the PDA medium and incubated at 28°C for 2 to 3 days. There were great differences on the morphological characters and growth rate among fusants. The fusants growing faster were selected out and employed to fermentation and hereditary stability tests. From thirty five fusants, one high yield fusant HDF-68 was obtained. Morphological comparisons between parents and HDF-68 were shown in Table 3.

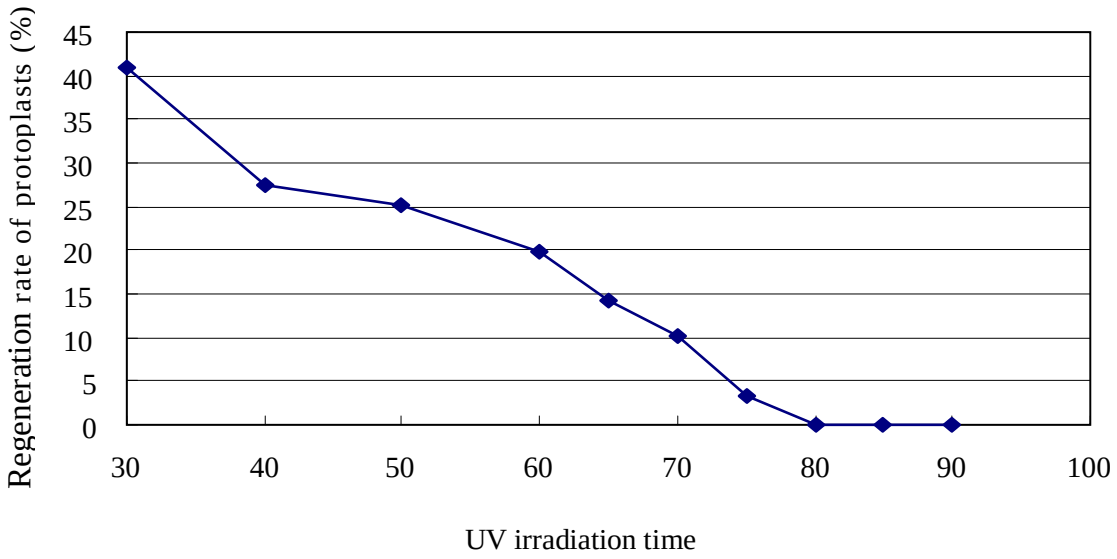


**Figure 2.** Effect of UV irradiation time on regeneration rate of strain $UL_{50-6}$ protoplasts.

**Table 1.** Effect of PEG concentration on fusion rate.

| PFG concentration (%, w/v) | Fusion rate ($\times10^{-2}$)* |
|---|---|
| 25 | 1.53 ± 0.11 |
| 30 | 4.92 ± 0.21 |
| 35 | 9.61 ± 0.52 |
| 40 | 6.44 ± 0.48 |

*Data were shown as Means ± SD and mean values were based on five replicates.

**Table 2.** Effect of fusion time on fusion rate.

| Fusion time (s) | Fusion rate ($\times10^{-2}$)* |
|---|---|
| 30 | 4.08 ± 0.26 |
| 60 | 6.18 ± 0.28 |
| 90 | 10.92 ± 0.76 |
| 120 | 8.27 ± 0.41 |

*Data were shown as Means ± SD and mean values were based on five replicates.

Clearly, the morphological characters of fusant HDF-68 were not identical to either of the parents. It displayed mixed characters of both parents' strains.

### Taxol production in the fusant HDF-68

TLC analysis showed that the fermentation extractions from the fusant HDF-68 appeared one blue dot located at the same place as the standard taxol Rf. This test indicated that the fusant HDF-68 can produce taxol or taxol-like compounds. Extracted from fermented fungal culture, the taxol sample showed the characteristic peaks as the standard taxol in the HPLC chromatogram (Figure 4a and b). After the addition of the taxol standard into the samples, the peaks from the samples increased, supporting that the purified fermentation product from the fusant HDF-68 is taxol. Based on taxol standard curve, the taxol content produced in the sample was 468.62 ± 37.49 μg/l. The taxol output of the fusant HDF-68 was higher than their parent strains *N. sylviforme* $UV_{40-19}$ and $UL_{50-6}$ by 24.51 and 19.35%, respectively ($p < 0.01$). Waters LC-MS analysis confirmed that purified product is taxol with the expected molecular ion mass $(M+H)^+$ (m/z 854.92) (Figure 5).

### Stability of strain HDF-68

After ten generations of successive subculturing, the colony morphological characteristics of strain HDF-68 were identical to those of the original fusant strain and the taxol output of successive strain of the fusant HDF-68 was almost the same as the original fusant strain (Table 4). The results showed that strain HDF-68 remained stable after being cultured for ten generations.

## DISCUSSION

### Effects of PEG concentration and fusion time on fusion rate

PEG is the most commonly used fusogen and its concentration has a significant effect on the rate of protoplast fusion (Gokhale, 1992). Previous studies showed that, in

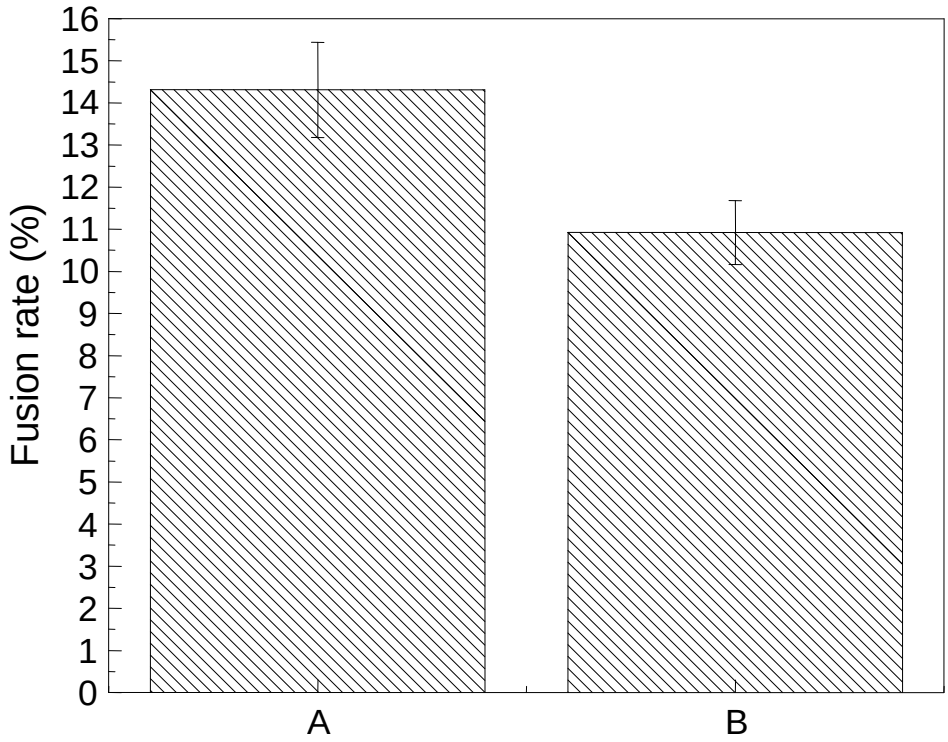


**Figure 3.** Effect of $Ca^{2+}$ on fusion rate. (A) With 0.01 mol/l $Ca^{2+}$; (B) without $Ca^{2+}$.

**Table 3.** Morphological characters of parents and fusant HDF-68.

| Strain | $UV_{40-19}$ | $UL_{50-6}$ | HDF-68 |
|---|---|---|---|
| Colony morph | Colonies loose, hoary, reverse light yellow, margin irregular, mycelium effuse | Colonies dense, hoary, reverse light brown, margin regular, mycelium effuse | Colonies dense, hoary, reverse light yellow, margin irregular, mycelium effuse |
| Colony diameter (PDA, 5 days) | 5.8-6.7 cm | 5.4-6.2 cm | 6.1-6.2 cm |
| Spore volume* | 33.68 ± 7.58 $\mu m^3$ | 29.45 ± 12.77 $\mu m^3$ | 36.95 ± 6.36 $\mu m^3$ |

*Spore volume was calculated by the equation: $V = 4/3 \cdot \pi \cdot a/2 \cdot (b/2)^2$. a: length of long axis; b: length of minor axis. Data were shown as Means ± SD and mean values were based on ten replicates.

case of fungi, 25 to 40% PEG had the optimal effect on improving fusion rate (Zhou and Ping, 1990). In this study, 35% PEG was the optimal concentration for protoplast fusion. Compared with the concentration of fusogen, fusion time has less influence on the fusion rate (Wesseling, 1982; Zhou and Ping, 1990). Only short period (about 1 min) of exposure to PEG was enough to induce protoplast fusion and with the prolonged fusion time, the fusion frequency did not increase accordingly (Hopwood and Wright, 1979; Baltz and Matsushima, 1981). The results of this study showed that, over the range of 30 to 90 s, with the increasing of fusion time, the fusion rate increased. The optimal fusion time for *N. sylviforme* was 90 s. Further prolonging the fusion time will reduce the fusion rate. The optimal fusion time varies with the diffe- rent species.

## Production of HDF-68 using inactivated protoplast fusion

In this study, the inactivation rates of both parents were 100%, so all the colonies growing on the regeneration medium should be fusants, which made the selection procedure of fusants easier. The regeneration rate in this study was up to 14.31 ± 1.13%. This is because heat

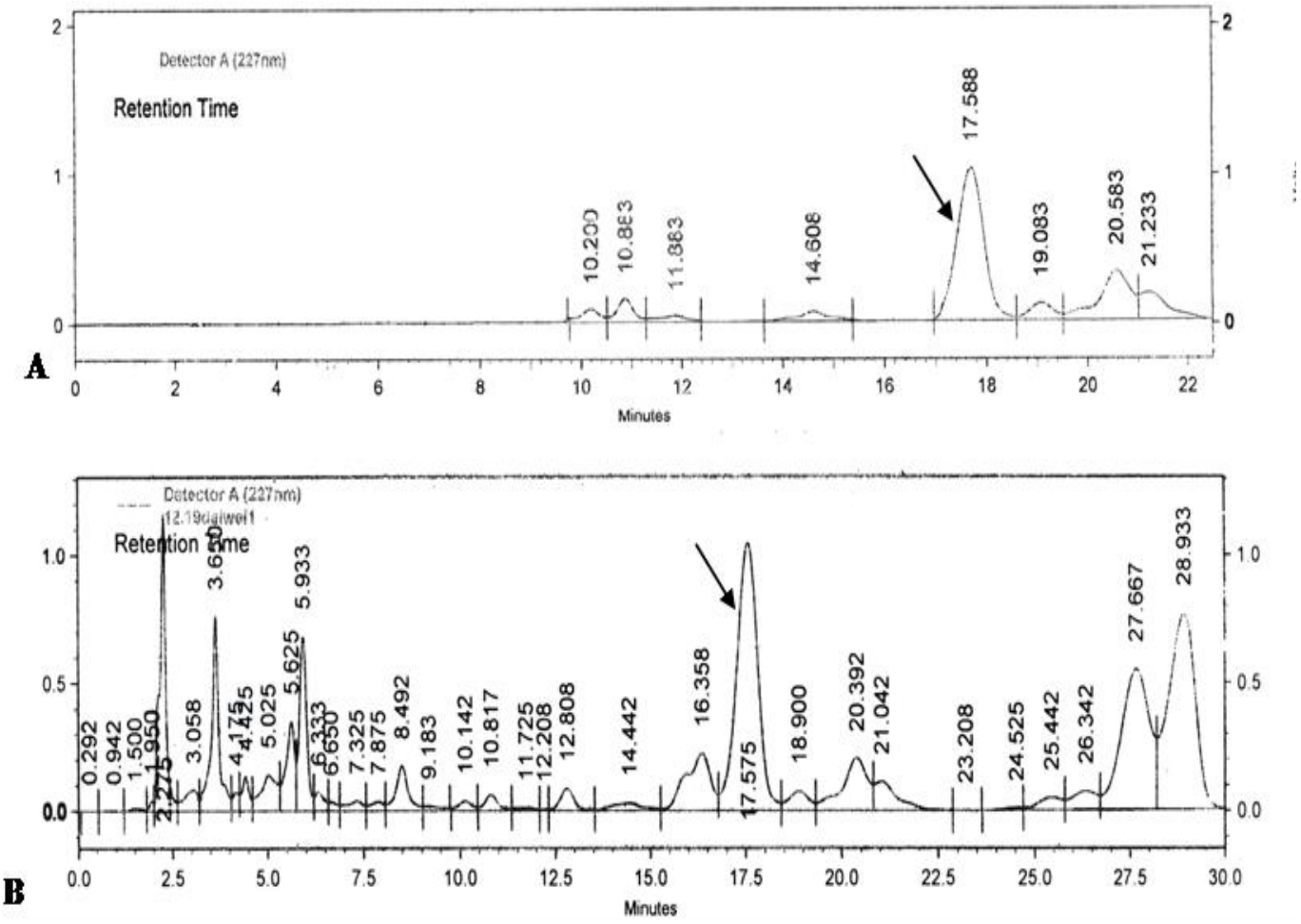


**Figure 4.** HPLC chromatograms of taxol extracted from strain HDF-68. Arrows indicate the taxol-specific peaks. A, Taxol sample extracted from strain HDF-68; B, taxol molecule standard.

treatment and UV-irradiation affected the different processes of the protoplasts. For example, UV-irradiation could enhance crossing over between the two genomes, leading to high complementation ability (Gokhale, 1992). Although, a lot of fusants were obtained, many were unstable. This may be because the protoplasts derived from mycelium of *N. sylviforme* contain several nuclei, which usually leads to multinucleate protoplasts (Gokhale, 1992).

Through protoplast fusion with inactivated parents, we have succeeded in obtaining a high yield fusant HDF-68 that could produce 468.62 μg/l taxol and the fusant HDF-68 exhibited characteristics of both parents and genetic stability after subculturing. These results suggest that intraspecific chromosomal recombination between *N. sylviforme* $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$ might have occurred since the parents were inactivated.

Up to date, there is no report on using inactivated protoplast fusion to produce a strain with high taxol output. This is the first successful application of the methods in improving taxol output of endophytic fungi. Although, the yield of taxol obtained in this study is insufficient for industrial production, we proved that the inactivated protoplast fusion is an effective method in fungus breeding and provided a good basis for the application of this technique to the breeding of the strains with high taxol output. Further improvement of strain HDF-68 and the optimization of fermentation conditions and culture medium might lead to higher production of taxol.

## ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (30970090), China Postdoctoral Science Foundation (20090450136), Innovation Foundation of Harbin (2010RFQXS043), Program for New Century Excellent Talents in Heilongjiang Provincial University, Research Program Heilongjiang Education Bureau (11551377), High-level Talents (innovation team) Project of

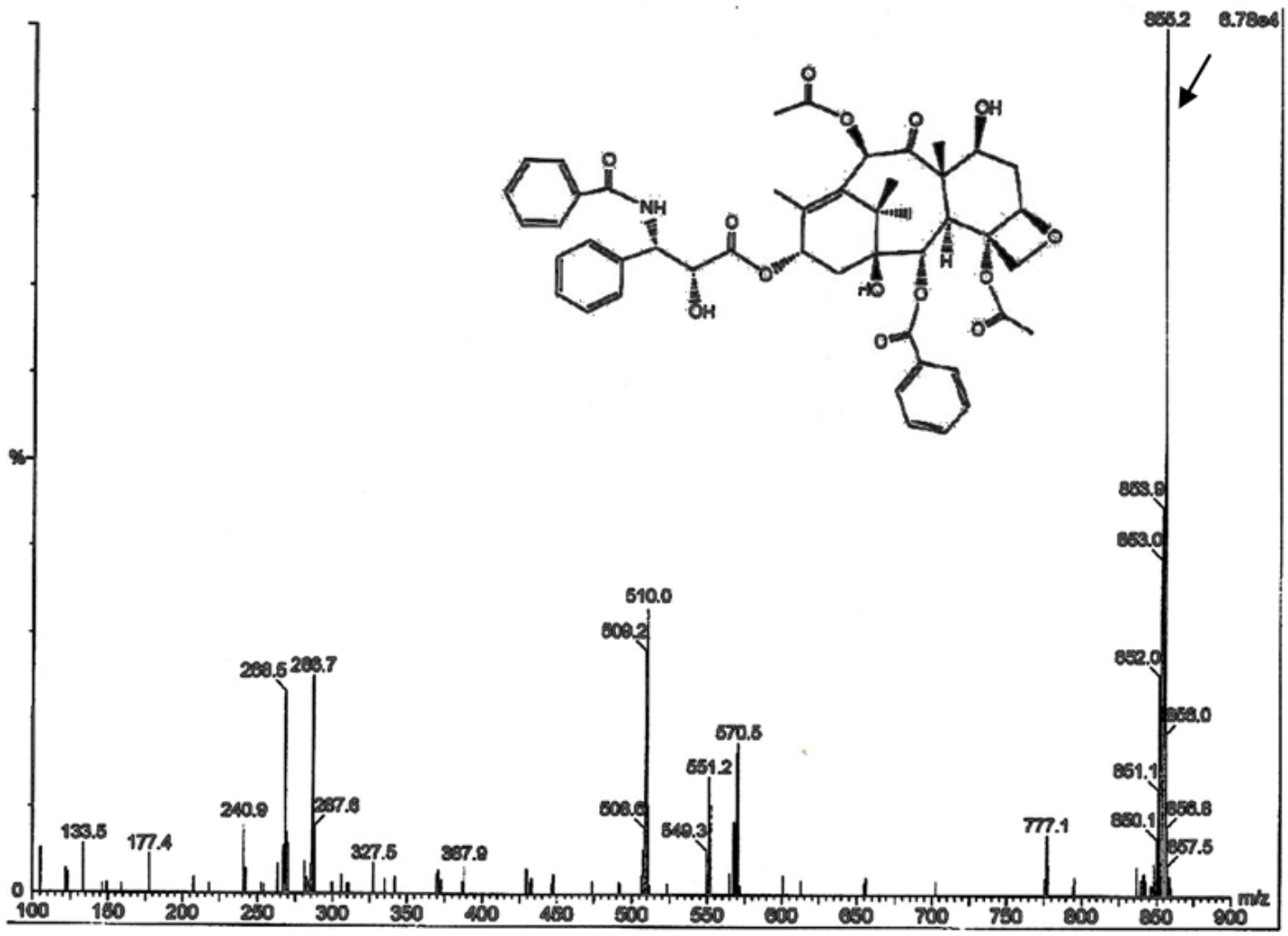


**Figure 5.** Mass spectrum of taxol extracted from strain HDF-68. Arrow indicates the molecular ion of taxol at m/z 855$(M+H)^+$

**Table 4.** Properties of HDF-68 after 10 generations.

| Colony morph # | Growth rate # | Taxol production* |
|---|---|---|
| + | + | 462.76 ± 25.01 μg/l |

**#** "+"means there were no changes between the original strain and the successive strain of HDF-68. *Data were shown as Means ± SD and mean values were based on four replicates.

Heilongjiang University (Hdtd2010-17) and Outstanding Youth Science Fund of Heilongjiang University.

## REFERENCES

Arteca RN, Wickremesinhe ERM (1993). Cultured *Taxus* tissues as a source of taxol, related taxanes and other novel anti-tumor/anti-viral compounds. WO Patent 93/23555.

Baltz RH, Matsushima P (1981). Protoplast fusion in *Streptomyces*: Conditions for efficient genetic recombination and cell regeneration. J. Gen. Microbiol. 127: 137-146.

Christem AA, Gibson DM, Bland J (1991). Production of taxol or taxol-like compounds in cell culture. US Patent 5019504.

Comnercon A, Bourzat JD, Didier E, Lavelle F (1995). Practical semisynthesis and antimitotic activity of docetaxel and side-chain analogs. ACS. Symp. Ser. 583: 233-246.

Furmanowa M, Syklowska-Baranek K (2000). Hairy root culturee of *Taxus×media* var. Hicksii Rehd. as a new source of palitaxel and 10-deactylbaccatin III. Biotechnol. Lett. 22: 683-686.

Ge JP, Ping WX, Ma X, Zhou DP, Zhang YS (2004). Identification of taxol-producing strain HU1353. J. Microbiol. 3: 19-21.

Gokhale DV (1992). Fungal protoplast fusion-A tool for breeding industrial strains. J. Sci. Ind. Res. 51: 497-506.

Holton RA, Biediger RJ, Boatman PD (1995). Semisynthesis of taxol and taxotere. In: Suffness M (ed), taxol science and applications. CRS Press, Boca Raton, pp. 97-121.

Holton RA, Kim HB, Somoza C, Liang F, Biediger RJ, Boatman PD, Shindo M, Smith CC, Kim S, Nadizadeh H, Suzuki Y, Tao C, Vu P, Tang S, Zhang P, Murthi KK, Gentile LN, Liu JH (1994b). First total synthesis of taxol. 2. Completion of C and D rings. J. Am. Chem. Soc. 116: 1599-1600.

Holton RA, Somoza C, Kim HB, Liang F, Biediger RJ, Boatman PD, Shindo M, Smith CC, Kim S, Nadizadeh H, Suzuki Y, Tao C, Vu P, Tang S, Zhang P, Murthi KK, Gentile LN, Liu JH (1994a). First total synthesis of taxol. 1. Functionalization of Bring. J. Am. Chem. Soc. 116: 1597-1599.

Hopwood DA, Wright HM (1979). Factors affecting recombisnt frequency in protoplast fusion of *Streptomyces coelicolor*. J. Gen. Microbiol. 111: 137-143.

Ketchum REB, Gibson DM, Gallo LG (1995). Media optimization for maxium biomass production in cell cultures of pacific yew. Plant. Cell. Tissue. Organ. Cult. 42: 185-193.

Muralidhar RV, Panda T (2000). Fungal protoplast fusion-a revisit. Bioprocess. Engineering. 22: 429-431.

Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermet PG, Guy KK, Claiborne CF, Renaud J, Couladouros EA, Paulvannin K, Sorensen EJ (1994). Total synthesis of taxol. Nature, 367: 630-634.

Shen P, Fan XR, Li GW (1999). Guide to Microbial Experiments. Higher Education Press, Beijing, China, 214-222.

Stierle A, Strobel G, Stierle D (1993). Taxol and taxane production by Taxomyces andreannae, an endophytic fungus of Pacific yew. Science, 260: 214-216.

Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X (1996). Taxol from fungal endophytes and the issue of biodiversity. J. Ind. Microbiol. 17: 417-423.

Sun JQ, Zhou DP, Ping WX (2003). A new species of the genus *Pleurocytospora*. Mycosystema, 1: 12-13.

Wang CG, Wu JY, Mei XG (2001). Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl. Microbiol. Biotechnol. 55: 404-410.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brefolia*. J. Am. Chem. Soc. 93: 2325-2327.

Wesseling AC (1982). Protoplast among the actinomycetes and its industrial applications. Dev. Ind. Microbiol. 23: 31-40.

Zhao K, Ping WX, Li Q, Hao SS, Zhao LF, Gao TT, Zhou DP (2009). *Aspergillus niger* var. *taxi*, a new species variant of taxol-producing fungus isolated from *Taxus cuspidata* in China. J. Appl. Microbiol. 107: 1202-1207.

Zhao K, Ping WX, Zhang LN, Liu J, Lin Y, Jin T, Zhou DP (2008b). Screening and breeding of high taxol producing fungi by genome shuffling. Sci. China. Ser. C. 51(3): 222-231.

Zhao K, Zhao LF, Jin YY, Wei HX, Ping WX, Zhou DP (2008a). Isolation of a taxol-producing endophytic fungus and inhibiting effect of the fungus metabolites on HeLa cell. Mycosystema, 5: 210-217.

Zhao K, Zhou DP (2004). Study on preparation and regeneration of protoplast from taxol-producing fungus *Nodulisporum sylviforme*. Nat. Sci. 2(2): 52-59.

Zhao K, Zhou DP, Ping WX, Liu J, Ma X, Jin T (2005). Study on breeding up high yield strain of taxol protoplast mutagensis. Chin. J. Biotechnol. 21(5): 848-851.

Zhou DP, Ping WX (1990). Microbial protoplast fusion. Heilongjiang Sci Technol Press, Harbin, China.

Zhou DP, Ping WX (2003). Anti-cancer taxol produced by microbiology fermentation, Chinese science and technology publishing house, Beijing, China.

Zhou DP, Sun JQ, Yu HY, Ping WX, Zheng XQ (2001). *Nodulisporium*, a genus new to China. Mycosystema, 2: 277-278.

Zhou X, Zheng W, Zhu H, Tang K (2009). Identification of a taxol-producing endophytic fungus EFY-36. Afr. J. Biotechnol. 8(11): 2623-2625.