Study on the Preparation and Regeneration of Protoplast from Taxol-producing Fungus *Nodulisporium sylviforme*

Kai Zhao [1, 2], Dongpo Zhou [1], Wenxiang Ping [1], Jingping Ge [1]
(1. Life Science College of Heilongjiang University, Harbin, Heilongjiang 150080, China;
2. Food Science College of Northeast Agricultural University, Harbin, Heilongjiang 150030, China)

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 combination of various enzymes, digesting time and temperature, and pH value, osmotic stabilizers, pretreation, culture medium and culture method. Under the condition of the mixed enzymes in osmotic pressure stabilizer (0.7 mol/L NaCl) combination of 3% lywallzyme, 2% snailase and 1% lysozyme, pH 5.5～6.0, digesting time 9h and digesting temperature 30℃, the results showed that the highest preparation frequency of protoplasts is a concentration of 2.0×10^7 protoplasts per microlitre. After the obtained protoplasts were purified, they would be regenerated by bilayer plate culture method in PDA regenerative medium. This study laid the foundation to construct the engineering taxol-producing strains by protoplast mutagensis, transformation, fusion, and turn to a new page of research of producing anti-tumor drug taxol by microbiological fermentation. [Nature and Science, 2004,2(2):52-59]

Keyword: taxol; *Nodulisporium sylviforme*; protoplast; preparation; regeneration

1 Introduction

Taxol is a complicated diterpene alkaloid with anti-tumor activity, which was first isolated from *Taxus brevifolia* by Wani *et al* (1971) [1]. 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 [2]. The taxol has been used to cure many malignant tumors, such as breast cancer, ovarian cancer, choriocaicinoma, hysteromyoma *et al* [3,4,5]. *Nodulisporium sylviforme* is an endofungus isolated from phloem of *Taxus cuspidata* by Zhou *et al*(1993) [6], and it is a genus new to China [7]. The output of taxol measured first by HLPC was 51.06~125.70μg/L with higher fermentility than of other known fungi. Thus it becomes a prospective taxol-producing fungus.

Protoplasts are the cytosolic constituents of fungal cells. Their cytoplasm can be considered equivalent to cytoplasm in higher cells. Protoplasts contain all the intracellular organelles of cells and form a vital link in transfer of micromolecules between cyto-organelles. Currently, most of the laboratories engaging in fungal genetics are using gene manipulation procedures based on protoplasts. Therefore, to further improve the genetic properties of these strains using protoplast fusion, we attempted to develop methods for preparation and regeneration of their protoplasts. In addition, fungus breeding by protoplast mutagensis and fusion has become a useful method for the breeding of microbiology. In this paper, we describe conditions for the preparation and regeneration of the protoplasts from the taxol-producing fungus *Nodulisporium sylviforme*. The study laid the foundation to construct the engineering strains of taxol-producing fungus by protoplast mutagensis, transformation, and fusion.

2 Materials and Methods

2.1 Materials

2.1.1 Strain

The strain HQD_{33} that isolated from *Taxus cuspidata* is an endofungus--*Nodulisporium sylviforme* [6,7]. Its spore experienced a series of mutagensis screening (UV EMS ^{60}Co NTG), ⟶ and were finally obtained a mutagensis-derived strain NCEU-1 (the output of taxol:314.07 μg/L), which was used as parent strain.

2.1.2 Media

PDA liquid medium [8]; PDA solid medium [8]; Wort medium [8]; Czapek-Dox medium [8];Yeast extract medium [8];CM medium contains $MgSO_4\cdot7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, pepton 2 g, Yeast extract 2

g, glucose 20 g and agar 20 g. For the regeneration of protoplasts, NaCl, KCl, mannitol, glucose, sucrose were respectively added to above mentioned the medium except for PDA liquid medium, and their final concentration is 0.7 mol/L; Modified S-7 medium [9].

2.1.3 Reagents

(1) osmotic pressure stabilizer: NaCl 0.7 mol/L, pH5.5～6.0;

(2) enzymes: lywallzyme, snailase, lysozyme. (Shanghai Lizhu & Dongfeng Biotechnology CO. LTD, China);

(3) Extracting solvent: methanol, ethylacetate, hexane, acetonitrile (Tianjing Bo Di Chemical Reagent CO. LTD, China);

(4) Developer: chloroform and methanol (7:1 v/v);

(5) Color developing reagent: 1% vanillin in concentrated sulfuric acid.

2.2 Methods

2.2.1 Culture and collection of mycelium

Mutant NECU-1 derived from HQD $_{33}$ 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℃ 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.

2.2.2 Preparation of enzymolysis solution

Preparation of recombination of various enzymes saw Table 2.

2.2.3 Pretreatment

The collected mycelium should be respectively treated for 30 min using 0.2%, 0.5%, β-mercapoethanol before enzymolysis. At the same time, untreated mycelium as control.

2.2.4 Preparation of protoplasts

The mixed enzymes in osmotic pressure stabilizer with recombination of various enzymes were respectively added to the tube that contained moist mycelia at the ratio of 250 mg mosit mycelia for enzymolysis solution of 1 mL, then the mosit mycelia was enzymlized in warm-water bath at 30℃. 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 [10].

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

2.2.5 Regeneration of protoplasts

Firstly, the protoplast suspension was diluted by ten times at 0.7 mol/L NaCl or above mentioned the osmotic pressure stabilizer, then they would be respectively cultured as follow.

Monolayer plate culture method: the diluted a series of the protoplast suspension was directly put out regenerative media solid-plate 28℃, cultured for 3~5 days.

Bilayer plate culture method: 0.5 mL the suspension was added to tubes containing 4.5 mL regenerative soft agar medium in turn. After twisted equally with hands, the solution was put out accordingly regenerative solid-plate, 28℃, cultured for 3~5 days.

At the same time, another 0.5 mL protoplast suspension was added into 4.5 mL distillated water and schizolysed for 30 min, spreaded the solid plate, which was not added osmotic pressure stabilizer 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 preparation frequency of protoplast (Fp).

3 Results

3.1 Observation of the formation and release of protoplasts

The diameter of *Nodulisporium Sylviforme* hyphae ranges between 2.58 μm and 6.45 μm(Figure 1-A), the hyphal contents were distributing equably. The shape of mycelia began to change after enzymolysis for l hour or so and protoplasts began to form. With the increasing of enzymolysis time, mycelia broke gradually, the contents presented to be beaded (Figure l-B) and protoplasts began to be prepared in large amounts. During the formation of the protoplasts, the protoplast were released from the top of hyphae or at hyphal original location (Figure 1-C). The diameter of protoplast ranges between 3.4 μm and 10.3 μm (Figure 1-D).

3.2 Effects of some factors on the preparation of

protoplast

3.2.1 Effects of recombination of various enzymes on preparation of protoplast

The paper studied the effect of lywallzyme, snailase, lysozyme and cellulase on the preparation of the *Nodulisporium sylviforme* protoplast by orthogonal experiment (Table 1). The result indicated that the optimal condition of the preparation of protoplast is 3% lywallzyme+1% snailase+1% lysozyme+3% cellulase. However, the best condition was 3% lywallzyme+3% snailase+1% lysozyme+3% cellulase. The effect order is successive: celluase, lywallzyme, snailase, lysozyme.

3.2.2 Effects of pH value on preparation of protoplast

The relationship between pH 4.5~7.0 and the preparation frequency of protoplast was studied using recombination of 3% lywallzyme+3% snailase+1% lysozyme+3% cellulase (Table 2), the results showed that the maximum concentration of the preparation frequency of protoplast was $2.5\sim2.7\times10^7$ per milliliter when the pH value was 5.5~6.0.

3.2.3 Effects of enzymolysis temperature on the preparation of protoplast

The optimal enzymolysis temperature was studied using the enzyme combination of 3% lywallzyme+3% snailase+1% lysozyme+3% cellulase and pH 5.5~6.0. The preparation frequency of protoplast at different enzymolysis temperature were presented in Table 3. It was clear that 30℃ was the optimal enzymolysis temperature on the base of the optimal enzyme combination when the preparation frequency of protoplasts reached 1.47×10^7 per milliliter.

3.2.4 Effects of enzymolysis time on the preparation of protoplast

Effects of enzymolysis time on the preparation frequency of protoplasts were studied under the condition of 3% lywallzyme+3% snailase+1% lysozyme+3% cellulase, 30℃ and pH 5.5~6.0. The result showed that with prolonging of enzymolysis time, the preparation frequency of protoplasts increased, the preparation frequency of protoplasts 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.

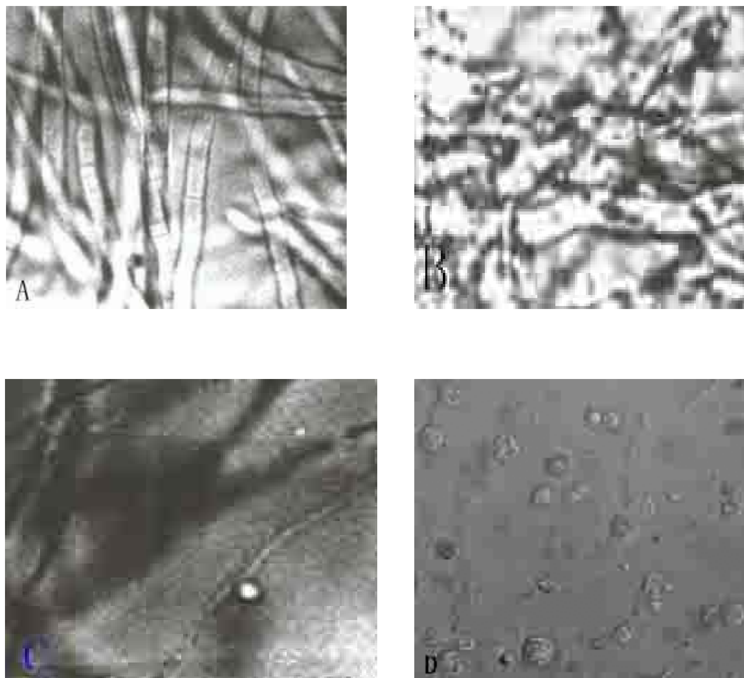

Figure 1 A. The shape of hyphae before enzymolysis; B. The shape of hyphae after enzymolysis; C. The release of protoplasts; D. The shape of protoplasts

Table 1 Orthogonal experiment of the enzymolysis factors for preparation of the protoplast

No	Enzymes				preparation frequency ($\times 10^6$/mL)
	lywallzyme（%）	snailase（%）	lysozyme（%）	cellulase（%）	
1	2	1	0	1	0.85
2	2	2	1	2	1.27
3	2	3	2	3	4.07
4	3	2	2	1	1.10
5	3	3	0	2	4.30
6	3	1	1	3	5.58
7	4	3	1	1	1.00
8	4	1	2	2	1.58
9	4	2	0	3	1.20
T_1	6.19	8.01	6.35	2.95	
T_2	10.98	3.57	7.85	7.15	
T_3	3.78	9.37	6.75	10.85	
X_1	2.06	2.67	2.12	0.98	
X_2	3.66	1.19	2.62	2.38	
X_3	1.26	3.12	2.25	3.62	
R	2.4	1.93	0.50	2.64	

Table 2 Effects of pH on preparation of protoplast

pH	Preparation frequency（$\times 10^7$/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

Table 3 The effect of digesting temperature on the preparation frequency of protoplast

Enzymolysis temperature	The preparation frequency of protoplast ($\times 10^7$/mL)
27℃	0.83
30℃	1.47
33℃	0.67

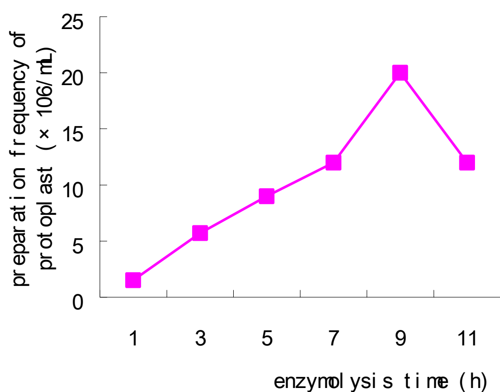

Figure 2 Effect of enzymolysis time on the preparation frequency of protoplast

3.2.5 Effects of pretreatment on the preparation of protoplast

The collected mycelia were treated for 30min using 0.2%, 0.5%, β-mercapoethanol before they would be enzymolysized, respectively. At the same time, untreated mycelia as control. The results showed that pretreatment couldn't raise the preparation frequency of protoplasts, on the contrary, the preparation frequency of protoplasts decreased.

3.2.6 Effects of medium on the preparation of protoplast

Under the condition of 3% lywallzyme+3% snailase+1% lysozyme+3% cellulase, 30℃ and pH 5.5~6.0, the experiment studied the effect of PDA medium, Wort medium, Czapek-Dox medium, Yeast extract medium on the preparation of the *Nodulisporium sylviforme* protoplast (Figure 3). The results showed that the preparation frequency of protoplasts varied greatly with medium, among which PDA medium showed the best result and Czapek-Dox medium showed the most dissatisfactory result.

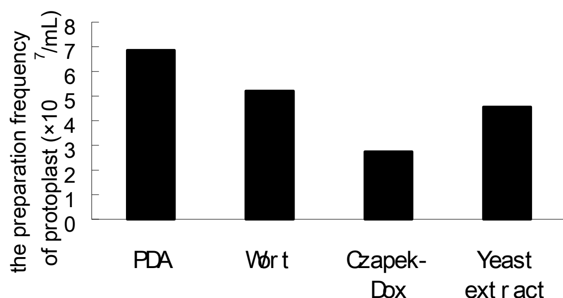

Figure 3 Effects of medium on preparation frequency of protoplast

3.2.7 Effects of culture method on the preparation of protoplast

When *Nodulisporium sylviforme* was cultured in liquid medium at shock, the mycelium usually twisted tightly and suspended as granule in liquid medium, and some mycelia had empty inside them. The preparation frequency of protoplasts was low at shock. While the mycelia were cultured in liquid at rest, they grew into loosely and the preparation frequency of protoplasts was higher than at shock.

3.3 Effects of some factors on the regeneration of protoplast

3.3.1 Effects of regenerative medium on the regeneration of protoplast

Three kinds of high osmotic agar media including PDA medium, CM medium and Yeast extract medium were applied to study on the effects of medium on regeneration of *Nodulisporium Sylviforme* protoplasts. We could saw from Table 4, the regeneration frequency of protoplasts was highest using PDA, moderate using CM, and lowest using Yeast extract.

Table 4 The effect of regeneration medium on the regeneration frequency of protoplast

Regeneration medium*	The number of regenerative colonies	Regeneration frequency (%)
PDA	108	72
CM	66	44
Yeast extract	17	11

Note: *All contain 0.7 mol/L NaC1

3.3.2 Effects of culture method on the regeneration of protoplast

After the concentrations of prepared protoplasts were modulated, respectively using bilayer plate culture and monolayer 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 [11]. The results showed that the regeneration frequency of protoplasts was more higher using bilayer plate culture than monolayer plate culture (Figure 4).

3.3.3 Effects of enzymolysis time on the regeneration of protoplast

After the concentrations of prepared protoplasts from different enzymolysis time were modulated, using

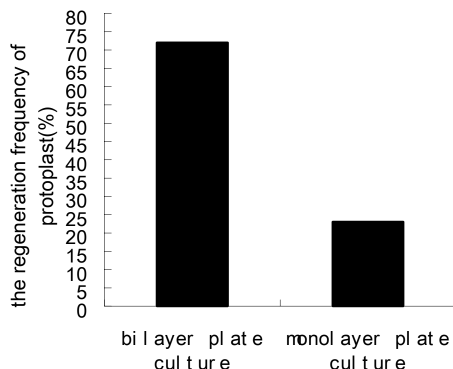

Figure 4 Effect of regeneration culture method on the regeneration frequenjcy of protoplast

dilayer plate culture the prepared protoplasts were regenerated in the PDA high osmosis regenerative culture which contained 0.7 mol/L NaCl. The regenerative morphology of the colonies is shown in Figure 5. The Figure 6 indicated that the regeneration frequency of protoplasts changed little in short time, while with the enzymolysis time prolonged, the regeneration frequency decreased. Therefore, taking account of the preparation frequency of protoplasts, enzymolysis time 7~9 hours were selected in this study in order to obtain higher the regeneration frequency of protoplasts.

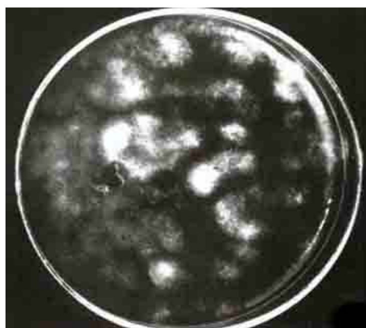

Figure5 The regenerative morphology of the colonies of protoplast in PDA high osmosis plate

4 Discussion

Taxol-producing fungus *Nodulisporium sylviforme* is isolated from phloem of *Taxus cuspidata* by Zhou *et al* (1993) [6], and it is a genus new to China [7]. The preparation of protoplasts and the regeneration of protoplasts for *Nodulisporium sylviforme* has not been reported.

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 the preparation frequency of protoplasts. Taking into account the effect of lywallzyme, snailase, lysozyme and cellulase on the preparation frequency of protoplasts, it is found that the combination of the four enzymes will obtain higher the preparation frequency of protoplast than that of any of them used alone, and the results are the same as other authors' results [11,12,13,14]. This may be due to the fact that the strains have complicated structure and the four 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 protoplasts; while the enzymolysis time is too long, the preparation frequency protoplasts and the regeneration frequency of protoplasts 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.

pH value is also an effective factor that indirectly affect the preparation of protoplasts because it has effect on enzyme activity. The experimental results showed that when the pH value closed to the optimum of the compound enzyme systems (pH 6.0), it was the most beneficial for the enzymes to digest. In addition, it is obviously that the enzymolysis temperature can also affect the enzyme activity. The temperature that fungi

lose their cell wall by enzymolysis is between 24℃ and 35℃ [11].

The osmotic pressure stabilizers can keep the balance of interior and exterior osmotic pressure of the protoplasts, which have lost the protection of cell wall, and can prevent the protoplasts from being broken and is benefit to improve enzyme activities. So to select the optimal osmotic pressure stabilizer is very important [15]. Up to now, for a certain fungal, there is no reasonable explanation about that a kind of chemical reagent is more suitable to be an osmotic pressure stabilizer than another.

The function of pretreatment changes the structure of cell wall by artificial control, which can increase cell wall more sensitive to enzyme and further raise the preparation frequency of protoplasts [11]. Peberdy reported that β-mercaptoethanol could destroy the protective layer of protein outside the cell wall by breaking the disulfide bond of excretion protein of the mycelium, therefore, it was easy to prepare protoplast by enzymolysis method [16]. However, Qi-Wen Zhou considered that the pretreatment could lower the preparation frequency of protoplasts [17]. The result was consistent with what our experiment made conclusion. It seems that cell wall is sensitive to enzyme, not the method itself that determines whether pretreatment will promote the release of protoplasts or not.

The regeneration frequency of protoplasts is often a restricted factor in the application of protoplast technique. If the regeneration frequency of protoplasts 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. In conclusion, this study illustrates that it is fundamentally difficult to establish a universal method of the regeneration of protoplast for taxol-producing fungi *Nodulisporium sylviforme*, mainly due to differences in their requirement for medium in the regeneration of protoplast. In this study, several kinds of regeneration media containing different substances were developed. These media should enable efficient protoplast regeneration for *Nodulisporium sylviforme* described in this study, especially the regeneration frequency of protoplasts obtained on PDA medium was highest. Therefore, this study should provide a basis for the genetic improvement of *Nodulisporium sylviforme* with protoplast fusion.

It has been reported that bilayer plate culture couldn't obviously improve the regeneration frequency of *T. cutaneum* protoplasts, but it could grow faster [14]. In our experiment, we found that the regeneration frequency of protoplasts was higher when using bilayer plate culture method than that of monolayer. Although the single layer plate culture method was easily manipulated, it was apt to impair the protoplast. The bilayer plate culture method can easily keep the integrality of the protoplasts and assure the normal growth of protoplasts, furthermore, the bilayer plate culture method is apt to observe the regenerative colonies, which grew on the plates. In addition, effects of enzymolysis time on the regeneration of protoplast were studied in the paper. This study showed that with the prolonging of enzymolysis time, the regeneration frequency protoplast decreased, and much too high concentrations of enzyme can also affect the regeneration frequency of protoplasts. This may be because when the cell wall was removed too much, the primers for synthesizing cell walls will lost during regenerating[11], and when the concentration of the enzyme is too high, the heteroalbumose enzymes will affect the activities and the regeneration frequency of the protoplasts.

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Correspondence to:

Jingping Ge
74 Xuefu Road
Nangang District
Heilongjiang University
Harbin, Heilongjiang 150080, China
Office Telephone: 01186-451-86608586
Home telephone: 01186-451-86609178
Mobile phone: 01186-13836002907
E-mail: gejingping9178@hotmail.com

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