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Full Length Research Paper

Protoplast formation, regeneration and transformation from the taxol-producing fungus *Ozonium* sp.

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The effects of some factors on protoplast isolation and regeneration from taxol-producing fungus *Ozonium* sp. BT2 were investigated, including the enzymolysis time and temperature, the osmotic pressure stabilizer, mycelial incubation time, the culture medium, the culture methods and preprocessing. The mycelia were digested by enzyme combination of 1.5% lywallzyme, 0.5% snailase, 1.5% cellulase and 1.0% lysozyme for 3 h at 30°C, and a high yield of protoplasts (1.31×10^8/g moist mycelia) were obtained. The protoplasts were purified, and then regenerated by different culture methods. The results showed that regeneration frequency was not different. The protoplasts could regenerate on both PDA and Czapek medium with 0.6 M sucrose, 0.6 M sodium chloride and 0.6 M mannitol, respectively. The regeneration rate was about 2.56% under 25°C on Czapek medium using 0.6 M sodium chloride as osmotic pressure stabilizer. Furthermore, transformants were obtained by transforming the protoplasts with plasmid pAN7-1 carrying hygromycin B phosphotransferase gene (*hph*) conferring hygromycin resistance. This study provides the foundation to develop an engineered strain of taxol-producing fungus by protoplast mutagenesis, fusion and genetic transformation.

Key words: Endophytic fungus, enzyme combination, regeneration, protoplast, transformation, *Ozonium* sp.

INTRODUCTION

Protoplasts of fungi are suitable for transformation experiments with plasmid DNA or protoplasts fusion crosses. They have widened the interest in improving methods for obtaining protoplasts from a variety of fungi of biotechnological interest (Cocking, 1979). The higher frequency of regeneration from fungal protoplasts is not only the foundation of fungi genetic manipulation and improvement but also a good experimental system for the study of gene expression and *in vitro* cell differentiation. Protoplast fusion and transformation technologies are viewed as a technically potential tool for strain improvement. The efficient transfer of genetic material is based on the formation of stable and viable protoplasts. It is necessary to study the critical factors affecting the protoplast formation and regeneration to enable protoplasts to be used in various areas of biotechnology.

The diterpenoid taxol is widely recognized as an important anticancer agent. Taxol, though isolated more than 34 years ago, has been the subject of several investigations (Wani et al., 1971). Since 1993, observations of taxol producing endophytic fungi, *Taxomyces andreanae* and *Pestalotiopsis microspora*, have been reported, demonstrating that organisms other than *Taxus* sp. could produce taxol (Stierle et al., 1993; Strobel et al., 1996). Thus, fermentation processes using taxol-producing microorganisms may be an alternative way to produce taxol. Subsequently, much research had focused on fermentations condition of taxol-producing endophytic fungus (Zhang and Dong, 2002; Liu et al., 2002; Chen et al., 2004). However, due to the low content of taxol and

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taxol-related compounds present in endophytic fungi isolated from yew, industrial taxol production with endophytic fungi cannot be commercially carried out (Lin et al., 2003). Therefore, researchers attempted to improve endophytic fungi strains by means of genetic engineering to enhance taxol content of strains (Wang et al., 2007). Recently, the endophytic fungus has been successfully isolated from the inner bark of *Taxus chinensis* var. *mairei* by aseptic techniques in our laboratory and extract from the cultures fungus was analyzed by liquid chromatography coupled to mass spectrometry and found to produce taxol and taxol-related compounds (Guo et al., 2006). Consequently, the objectives of this study were to develop an efficient protocol for protoplast production and regeneration from endophytic fungus (*Ozonium* sp. BT2) with the ultimate purpose of making this fungal species amenable to genetic studies and transformation.

MATERIALS AND METHODS

Fungal strain and cultivation

The strain of endophytic fungi used in the work was previously isolated from *T. chinensis* var. *mairei* in our study group. The extracts of the fungal fermentation were proved containing taxol and its related compounds (Guo et al., 2006). The cultivation method of entophytic fungi was described as reference (Zhou et al., 2006).

Effects of combination of several commercial enzymes on production of protoplast

The lytic enzyme was studied by the production of protoplasts from *Ozonium* sp., and compared with the effect of combinations of several commercial enzymes. Then, selection of combinations of the various enzymes was tested by orthogonal experiment (Zhou et al., 2006).

Osmotic pressure stabilizers and other factors

Based on the published literature, we selected three osmotic pressure stabilizers of them, potassium chloride, mannitol and sucrose, which were all tested at concentrations of 0.6, 0.8 and 1.0 M in order to determine the most suitable osmoticum. The osmotic pressure stabilizers and their various concentrations were tested using the optimal concentration of enzymes determined in section 0.6 M potassium chloride. Additional parameters such as lytic buffer and pH value, mycelia incubation time, enzymatic digested time and temperature, and cultured with medium were tested throughout the experiment.

According to the testing results of the other factors (data not shown), it was established that under the following conditions, osmotic pressure stabilizers which dissolved the enzyme was 0.6 M potassium chloride, the temperature was 30°C.

Viability assessment of protoplasts

The obtained protoplasts were treated with fluorescein diacetate and observed by phase contrast and fluorescence microscopy. Fluorescein diacetate was stored in an acetone stock solution (5 mg per milliliter) at -20°C, and added to the protoplast suspension to give a final concentration of 0.01~0.1%. After 5 min at room temperature, the protoplasts were examined for fluorescence using an Olympus IX71 microscope (Olympus, Japan).

Protoplast regeneration

The crude protoplast suspension was filtered through four layers of abrasive mirror paper. The prepared protoplasts were diluted serially to 10^3, 10^4 and 10^5 per milliliter by adding osmotic pressure stabilizers. The regeneration medium was both PDA medium and Czapek medium that composed of (w/v) $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4$ 0.001%, sucrose 3% and agar 2%. The osmotic pressure stabilizer of additional supplements was sodium chloride, mannitol and sucrose, which were all tested at concentrations of 0.6 M. The culture methods of protoplasts regeneration were used both the single layer and double layers culture. Czapek medium without adding osmoticum was used as control. In single layer culture, 50 µl of protoplasts suspension in STC buffer was diluted and plated onto the regeneration medium in Petri dishes and incubated. In double layers culture, the same protoplasts solution was plated on regeneration medium and covered with 5 ml of the regeneration medium containing 0.7% agar and incubated. The cultures were incubated for 48 h at 28°C until colonies became visible. The additional supplements included sodium deoxycholate 0.04% and Triton X-100 0.1% as colony restrictor.

Mycelium incubation time on the protoplasts formation and regeneration

According to the program of preparation of fungal material, various incubation times of mycelia was experimented and subsequently picked up respectively for digestion by the enzyme combination solution for 3 h at the condition of 28°C, and then the protoplast yield was obtained. With the same condition, the protoplast regeneration was carried out and the regeneration frequency of protoplast was obtained after 48 h.

Transformation and PCR analysis

Protoplast transformation of *Ozonium* sp. was done as described before (Chen et al., 2001). For transformation, 1×10^8 protoplasts suspended in 100 µl transformation buffer (STC) were incubated for 30 min at room temperature with 10 µg pAN7-1, a common transformation vector for filamentous fungi containing the *Escherichia coli hph* gene as a dorminant selectable marker under transcriptional control of the glyceraldehyde-3- phosphate dehydrogenase (*gpd*) promoter and the tryptophan synthetase (*trpC*) terminator signals from *Aspergillus nidulans* (Punt et al., 1987), and for an additional 30 min at room temperature after the addition of 1.25 ml PEG 4000 (w/v in transformation buffer) in small increments with no mixing. Transformed protoplasts were suspended in 8 ml STC and harvested at 4°C by centrifugation at 4000×*g* for 15 min. The harvested protoplasts were then incubated on a rotary shaker at 150 rpm at 28°C for 2 h in 3 ml PDB containing 1.2 M sorbitol and plated onto potato dextrose agar (PDA) containing 0.6 M sucrose and hygromycin (150 mg l^{-1}). After 2 days, transformants were transferred to PDA containing hygromycin (150 mg l^{-1}).

DNA was extracted from the control untransformed and the transformed endophytic fungi by standard method. Based on the conserved sequence of the hygromycin B phosphotransferase gene (*hph*) (Long et al., 1998), the specific primers fhph (5'-GTC GAGAAGTTTCTGATCG-3') and rhph (5'-GTTTCCACTATCGGCGAGTACT-3') were designed and synthesized. The PCR amplification for the *hph* gene was performed in a PTC-100™ programmable (MJ Research, INC) by 32 cycles (94°C for 50 s, 58°C for 1 min, 72°C for 80 s) of amplification followed by

Table 1. Factors and levels of the orthogonal experiment (%, w/v).

Level	Factor			
	Lywallzyme (A)	Snailase (B)	Cellulase (C)	Lysozyme (D)
1	0.5	0.5	0.5	0.5
2	1.0	1.0	1.0	1.0
3	1.5	1.5	1.5	1.5

Table 2. Design of $L_9(3^4)$ table head and experimental results.

Item	Factors				Protoplasts production × 10^6·ml^{-1*}
	(A)	(B)	(C)	(D)	
1	**1**	**1**	**1**	**1**	**1.24**
2	1	2	2	2	0.88
3	1	3	3	3	0.93
4	2	1	2	3	1.22
5	2	2	3	1	2.21
6	2	3	1	2	1.41
7	3	1	3	2	2.41
8	3	2	1	3	1.40
9	3	3	2	1	1.91
K_1	3.05	4.87	4.05	5.36	
K_2	4.84	4.49	4.01	4.7	
K_3	5.72	4.25	5.55	3.55	
$\overline{K}_1$	1.02	1.62	1.35	1.79	
$\overline{K}_2$	1.61	1.50	1.34	1.57	
$\overline{K}_3$	1.91	1.42	1.85	1.12	
R	0.89	0.21	0.51	0.67	

Note: 500 mg moist mycelia was resuspended in 1 ml enzymolysis solution.

extension for 7 min at 72°C. The PCR products were purified using Gel Extraction Mini Kit (Watson, China), ligated to pMD18-T vectors (TaKaRa, China), transformed into *E. coli* strain DH5α and then sequenced with DYEnamic Direct dGTP Sequencing Kit (Amersham Pharmacia, England) and a 373A DNA sequencer.

RESULTS AND DISCUSSION

Optimization of enzyme combinations on protoplast formation

Lywallzyme, snailase, cellulase, and lysozyme were experimented by various combinations to produce protoplasts from *Ozonium* sp. The results are shown in Table 2.

We applied conventional analytic methods of orthogonal experiment and obtained the effect weight of each factor for experimental results and gave the principal-secondary sequence of each factor and optimal project (Zhao et al., 2004). The best combination of various enzymes is expected to get to after carrying out repeated experiment by specific analysis. According to the factors of orthogonal experiment contributed as shown in Table 1, (i) the range of each factor for orthogonal experiment was calculated and contributed as seen in Table 2. Because the range of fact A, B, C and D were 0.89, 0.21, 0.51 and 0.67 respectively, the effect order was successive: lywallzyme, lysozyme, cellulose, snailase. The conclusion was that the lywallzyme was the most influential factor, the lysozyme took the second place, the cellulase took the third place and the snailase was the subordination factor. (ii) Figure 1 showed that, along with levels of the factors were increased, the average of the factor A was revealed gradually to an upward trend, the factor D and B was revealed gradually to a decline trend and the factor C were revealed gradually to decline trend early then to an upward trend. The result indicated that the optimal condition of the preparation of protoplast is 1.5% lywallzyme + 0.5% snailase + 1.5% cellulose + 0.5%lysozyme. (iii) In the course of releasing protoplasts, various kinds of fungi needed different enzyme combination because there was

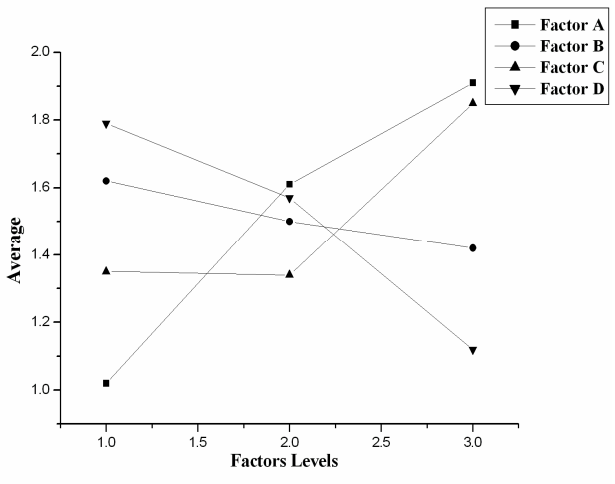

Figure 1. The factors levels average index of L_9 (3^4). Factor A, Factor B, Factor C, and Factor D indicate the average value of protoplast yield under the condition of lywallzyme, snailase, cellulase and lysozyme action, respectively.

a great distinction in structure of cell wall between one fungus and another (Hamlyn et al., 1981). Although the literature describes several recipes for obtaining protoplasts from various fungal species, and the availability of several commercial enzymes has facilitated the release of protoplasts, each fungus or fungal group would usually require its own specific conditions (Lalithakumari, 1996). Hydrolytic enzymes play a key role in protoplast isolation. Nowadays, we have the opportunity to select from a diverse variety of commercially available enzymes. These enzymes, such as lywallzyme, snailase, cellulase and lysozyme, have been reported to be active against a wide range of fungal species. The results of many researchers had proved that using the enzyme combination was better than using single enzyme on preparation protoplasts of fungus (Gallmetzer et al., 1999; Sun et al., 2001; Xu et al., 2006).

However, how did several commercial enzymes combine an efficient enzyme system? We do not have a standard rule to follow. Based on comparing influence of each enzyme on releasing protoplasts of *Ozonium* sp., we selected a rational combination of the four enzymes by orthogonal test, which means each enzyme has its unique proportion in the complex.

Effect of enzymolysis time and temperature on the protoplast production

The optimal enzymolysis time and enzymolysis temperature were studied using the enzyme system. The results showed that the optimal enzymolysis time was 3 h. The experimental result was identical as recent literature (Zhao et al., 2004). Along with increasing enzymolysis time of, protoplast production of mycelia was increased gradually among 1, 2 and 3 h. A longer incubation time resulted in the presence of lysed protoplasts.

According to the principle of enzyme reaction kinetics, the enzymatic reaction speeds were affected directly by enzymolysis temperature. The enzymolysis temperature for most of filamentous fungus was between 24 and 35℃ (Sun et al., 2001). We have routinely repeated this process. At the conditions of 25, 28, 30 and 33℃, protoplasts formation was compared. The results showed that the highest preparation frequency of protoplasts is a concentration of 6.55×10^7 protoplasts per milliliter under the condition of mixed enzyme in digesting time 3 h and digesting temperature 30℃.

Viability assessment of protoplasts

Protoplast viability was assessed both by microscopic observation with fluorescein diacetate and by direct regeneration in an osmotically stabilized medium. The results of observation were contributed as seen in Figure 2A and Figure 2B. By superimposing these images, it appeared that all protoplasts were viable based on their absorption of fluorescein diacetate (Cheng et al., 2000).

Protoplast regeneration was examined on PDA and Czapek medium containing various osmotic pressure stabilizers. Although the protoplast regeneration was fast (about 36 h) on PDA medium, mycelia colony was mistiness. So the regeneration of protoplast was carried out on Czapek medium. Two additional supplements, 0.1% Triton X-100 and 0.04‰ sodium deoxycholate, were used as colony restrictors in regeneration medium. When 0.1% Triton X-100 as colony restrictor was added in regeneration medium, regeneration of protoplasts did not present. When 0.04‰ sodium deoxycholate was used as colony restrictor, mycelia colony of regeneration of protoplasts was nakedness.

Various osmotic pressure stabilizers adding in the regeneration medium were tested, in which, results showed that 0.6 M sodium chloride was the most efficient amendment on Czapek medium. The protoplasts regenerated as mycelia colonies after 60 h (Figure 3B); the regeneration frequency was 2.56%. When 0.6 M sucrose was used as the osmoticum, the regeneration frequency was high and colony formation was also faster, but colonies were mistiness (Figure 3A). When 0.6 M mannitol was used as the osmoticum, the regeneration frequency was low (about 1.5%) and colony formation was slower (Figure 3C).

It has been observed that protoplasts obtained in shorter exposure times to lytic enzymes have greater capacity to regenerate than those which have been in contact with these enzymes for longer periods, since the membrane is liable to be damaged. For this reason, the digestion time was kept to a minimum with *Ozonium* sp. The regeneration of *Ozonium* sp. with spread plates was completed in 60 h (Liu and Zhu, 2000).

Influence of culture methods on protoplast regeneration

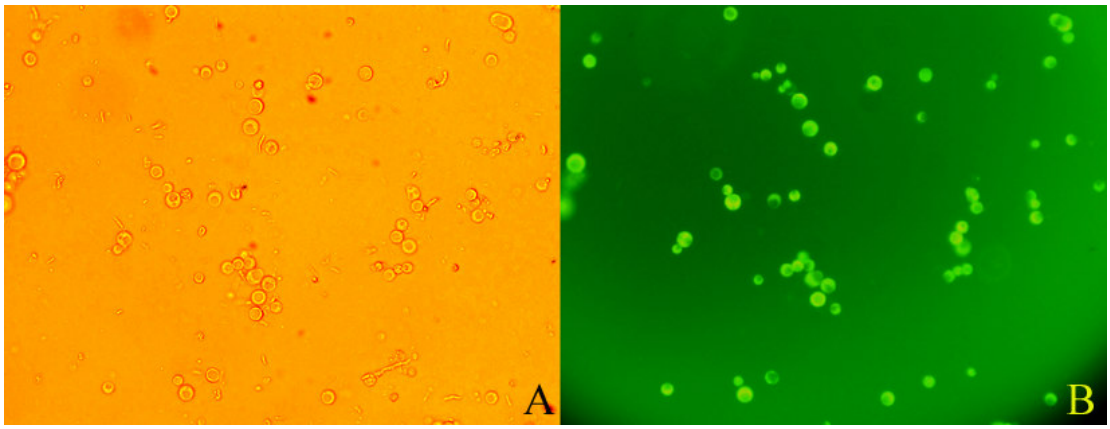

Figure 2. The protoplast viability assessment of endophytic fungus. **A:** Phase contrast micrograph of protoplasts in the presence of the fluorescein diacetate. **B:** micrograph of the same field like in A but illuminated with UV light (×400).

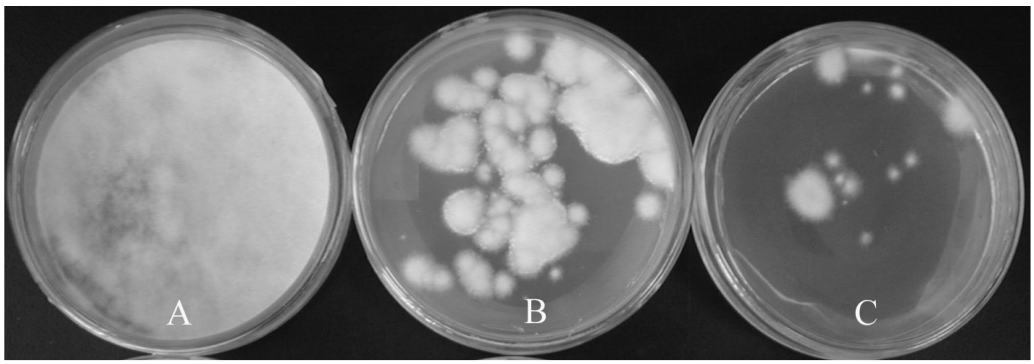

Figure 3. The regenerative morphology of the colonies of protoplasts of *Ozonium* sp. The protoplast solution (2.7×10^3) was spread out on Czapek Petri dishes containing 0.6 M sucrose (A), 0.6 M sodium chloride (B) and 0.6 M mannitol (C), respectively, and incubated for 60 h at 25°C.

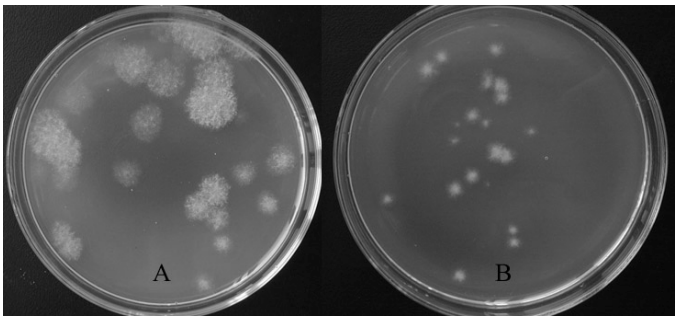

Figure 4. The regenerative morphology of the colonies with double layer culture method (A) and single layer culture method (B). The protoplasts regenerations were in Czapek medium containing 0.6 M sodium chloride for 48 h at 25°C.

was investigated both by single layer culture and double layers culture method, the results showed that using of soft-agar overlays (double layers culture) did not significantly increase the regeneration frequencies of *Ozonium* sp. However, the regeneration was faster with the overlays (Figure 4A) than with the single layer culture (Figure 4B), while the regeneration was complete after 48 h.

Based on the microscopic observations, the results of protoplast viability assessment were astonishingly high with nearly 100% viability rate (Figures 2A, 2B). Through further regeneration experiments, only 2.56% of all protoplasts regenerate to form mycelia colonies in Czapek medium containing 0.6 M sodium chloride. As a matter of fact, Peberdy reported that frequency of regeneration of fungal protoplasts ranged from less than 0.1 up to 50% in various fungi (Peberdy, 1991). More recent reports have not exceeded this range which classifies these results as the highest protoplast regeneration rate ever reported for a fungus. For different results between microscopic observations and direct regeneration, we can only speculate that whether this general regeneration rate is attributable to the conditions described for this protocol. However, additional studies implementing this protocol with closely and remotely related fungi would answer this question.

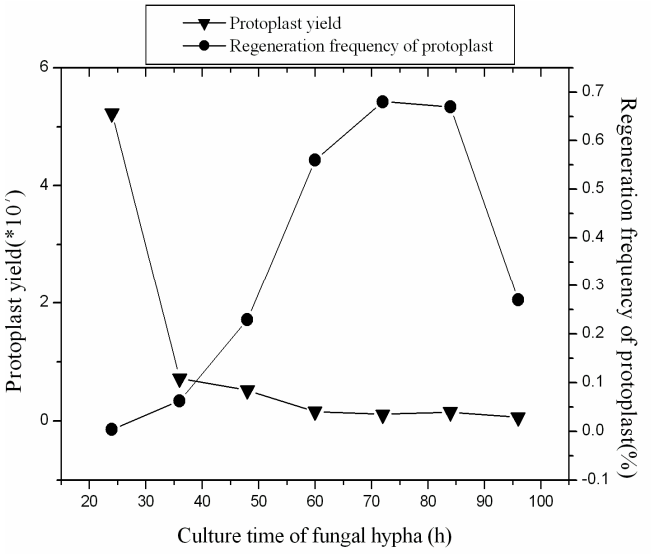

Figure 5. Effect of mycelia incubation time on protoplast formation and regeneration. Mycelia were digested by the enzyme combination of 1.5% lywallzyme, 0.5% snailase, 1.5% cellulase and 1.0% lysozyme for various times at 28℃. Protoplasts of *Ozonium* sp. BT2 were cultured and regenerated on Czapek medium containing 0.6 M sodium chloride for 48 h at 25℃. Colony formation from protoplasts adopted double layer culture method.

Effect of mycelium incubation time on the protoplasts formation and regeneration

The growth curve of fungal hypha was carried out by mycelium weight method. With the same treating time and enzymatic combinations, the mycelia of various incubation times were used to experiment for isolation and regeneration of protoplasts. The result showed that there is a contrary relationship between protoplast release and regeneration in logarithmic growth phase (from 24 to 84 h) (Figure 5). The prior period of logarithmic growth phase, protoplast yields was higher but regeneration frequency was lower, such as growth 24 h mycelia protoplast yield was 1.05×10^8/g moist mycelia, but regeneration frequency of protoplast was only 0.00382%. In later stage of logarithmic growth phase, protoplast yields were lower but regeneration frequency of protoplast was higher comparatively, such as growth 72 h mycelia, protoplast yield was only 2.2×10^6/g moist mycelia, but regeneration frequency increased to 0.68%. Fungal cells possess a form of exoskeleton called cell wall that protects the organism from osmotic pressure and environmental insults. Synthesis of the various building blocks that make up the cell wall occurs in the cytoplasm, and thus fungal cells face specific biochemical and biophysical problems related to the polymerization, transport, and assembly of building blocks into the final wall structure at an extra-cellular site (Goldman and Branstrom, 1999). Then, we know the fact when the enzyme dissolves cell wall of fungi, the protoplasts will be released. But in different stage of the fungi growing period, the lyses' efficiency of the enzyme varies from the component of the cell wall. In addition, the regeneration situation was the various with different species and different assimilation times. Based on the results of growth curve of endophytic fungus under shaking condition (data not shown), mycelia incubation time was established under the following conditions, cultures were in YPS liquid medium on a rotary shaker at 150 rpm for 36 h at 28℃.

Effect of other factors on protoplast production

The osmotic pressure stabilizers are another important component to consider with the choice of the lytic enzymes. 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 are benefit to improve enzyme activities. So to select the optimal osmotic pressure stabilizer is very important. Since the cell wall composition varies among species, it is safe to assume that various osmotica at different concentrations will be optimal for each species. The range of inorganic salts, sugars and sugar alcohols can be used as osmotic pressure stabilizers for fungal protoplast isolation and culture have been described by Davis (1985). Up to now, for a certain fungus, there is no reasonable explanation about that a kind of chemical reagent is more suitable to be an osmotic pressure stabilizer than another. However, there is a general assumption that inorganic salts are more effective with filamentous fungi, and sugar and sugar alcohols with yeasts and higher plants (Lalithakumari, 1996). In this study, the inorganic salts were found to be the optimal osmotic pressure stabilizer for *Ozonium* sp., which confirmed the viewpoint previously.

Pretreatment is by artificial controlling condition to influence structure of cell wall, which would become flexible or more sensitive with enzyme. In our experiment, mycelium was treated with 2-mercaptoethanol containing 0.1 M Tris-0.1 M EDTA, and its preparation frequency of protoplasts was enhanced in evidence. Peberdy (1979) found that when treated with 2-mercaptoethanol, mycelium could break off outside of mycelium and accordingly be propitious to preparation protoplast. This was confirmed in the present study, but in contradiction with the results by other studies (Zhou et al., 1984; Sun et al., 2001).

PCR detection for the transgene (hph) in the genome of transformed fungi

PCR amplification was a simple and quick method for determining the status of fungal transformants (Wang et al., 1999). After hygromycin selection, hygromycin-resistant fungi were regenerated from protoplasts transformed with pAN7-1 containing the *hph* gene.

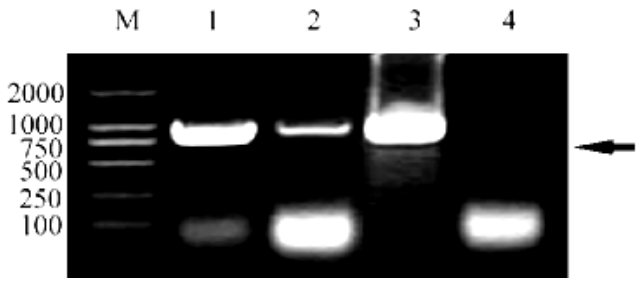

Figure 6. PCR analysis for the presence of the transgene (*hph*) in the genomes of randomly selected hygromycin-resistant protoplast-derived fungi. M: DL2000 (TaKaRa), 1: transformant 1; 2: transformant 2; 3: pAN7-1 (plasmid); 4: untransformed control fungi. The arrow indicated the amplified *hph* band (about 850 bp).

Hygromycin-resistant transformants were randomly picked up and examined by PCR for the presence of the trangene (*hph*) in the genomes of hygromycin-resistant transformants. The result showed that specific *hph* gene bands were amplified in hygromycin-resistant transformants, indicating the *hph* gene has been integrated into the genomes of transformed fungi (Figure 6).

Because the transformation frequency is not very high (about 1-2 transformants per microgramme vector DNA) in the present study, we should establish a better protoplast transformation system to support our further research, which includes transforming fungi with taxol biosynthetic pathway genes to improve the contents of taxol or taxol-related compounds in fungi in the future.

Conclusion

There are very few reports earlier on the isolation and regeneration of protoplasts for endophytic fungus from yew. In the present study, endophytic fungus of *Ozonium* sp. which can produce taxol and taxol-related compounds is successfully isolated from the inner bark of *T. chinensis* var. *mairei*. We also studied the conditions of protoplast isolation and regeneration of the isolated endophytic fungus by taking a step-by-step approach in which most of the parameters described earlier to be important in protoplast production and regeneration were considered. Primarily transformation also has been achieved. This study has laid the foundation to develop engineered strains of fungus producing high taxol and taxol-related compounds by protoplast mutagenesis, transformation and fusion.

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