Korean J. Chem. Eng., **25**(6), 1416-1421 (2008)

Protoplast from β-carotene-producing fungus *Blakeslea trispora*: Preparation, regeneration and validation

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(*Received 6 December 2007 • accepted 22 April 2008*)

Abstract–The effects of key parameters on the preparation and regeneration of protoplast from the β-carotene-producing fungus *Blakeslea trispora* were discussed in this paper, including the combination of various enzymes, mycelial age, digesting time and temperature, pH value, osmotic stabilizers, pretreatment, culture medium and culture method. Under the condition of mixed enzymes in osmotic stabilizer (0.6 M NaCl) combined with 2% lysozyme, 3% cellulase and 3% snailase, the highest protoplast yield, as high as 7.48×10^6 protoplasts/mL, was obtained when mycelial age was 60 h at pH 5.0-6.0 with digesting for 14-16 h at 28 °C. After purification of the obtained protoplasts, they were regenerated in PDA regenerative medium using bilayer plate culture method. To validate the usability of the protoplasts, a novel plasmid with green fluorescent protein (GFP) was used in transformation for easy visual observation. The results showed that the protoplasts prepared by the optimized method were active and applicable in further gene manipulation experiments.

Key words: *Blakeslea trispora*, Protoplast, Preparation, Regeneration, Validation

INTRODUCTION

Lycopene is a member of the carotenoid family of chemical substances that are found in many fruits, vegetables and other green plants. Lycopene, which is mainly found in tomatoes and tomato-based products, is the most prevalent carotenoid in the Western diet. It is also the most predominant carotenoid in human plasma, present naturally in greater amounts than β-carotene and other dietary carotenoids. This indicates its greater biological significance in the human defense system. It is a powerful antioxidant that has been shown to neutralize free radicals, especially those derived from oxygen, thereby conferring protection against prostate cancer, breast cancer, atherosclerosis and associated coronary artery disease. It reduces LDL (low-density lipoprotein) oxidation and helps reduce cholesterol levels in the blood. In addition, preliminary research suggests lycopene may reduce the risk of macular degenerative disease, serum lipid oxidation, and cancers of the lung, bladder, cervix, and skin. The chemical properties of lycopene responsible for these protective actions are well-documented [1-6].

Due to the diverse biological functions of lycopene, the demand is increasing and the industry has tried to satisfy this demand by manufacturing lycopene. At present, three methods, chemical synthesis [7], extraction from plant [8] and fermentation [9], are known for the production of lycopene. The synthesis method has been used for the commercial production of lycopene. But this method has the drawback of not yielding a product that is perceived as natural, which has limited its market application. The extraction method obtains the lycopene through tomato, but it is a very expensive process because of being short of the rare sources containing high levels of this natural dye. The fermentation method has been widely used for the production of various valuable substances [10-23] and regarded as the efficient route to obtain lycopene. More than 100 study organizations focus on this area and more attention has been paid to this method.

Blakeslea trispora is the best strain used for the production of β-carotene. The output of β-carotene measured by HPLC was 1.5 g/L [24] with higher fermentability than of other known fungi. As described in previous work [25], lycopene is an intermediate in the biosynthesis pathway of other carotenoids. So there is an interest in constructing a lycopene-producing genetically engineered strain that can promote the accumulation of lycopene. Currently, most of the laboratories engaged in fungal genetics are using gene manipulation procedures which are based on protoplasts. Therefore, the methods for preparation and regeneration of protoplast are important to improve the genetic properties of these strains using protoplast transformation. Bélanger et al. [26] described a specific protocol for yielding and regenerating protoplasts from spores of *Pseudozyma flocculosa*. The authors argued that this protocol would be useful in further genetic studies. Deshpande and Chitnis [27] investigated the experimental parameters for isolation and regeneration of protoplasts from the mycelial and yeast form cells of the dimorphic zygomycete *Benjaminiella poitrasii*. However, the studies on preparation and regeneration of *B. trispora* protoplast have not yet been reported in the literature. Because the method for isolating and regenerating protoplast is specific for each strain, the aim of this paper was to investigate the effect of each experimental parameter on preparation and regeneration of the protoplast from the β-carotene-producing fungus *B. trispora* and finally determine the most suitable experimental conditions. Subsequently, to validate the usability of the protoplasts in constructing the engineered strains of lycopene-producing fungus by protoplast mutagenesis and transformation, the transformation has been done by using a novel plasmid with GFP which can be observed visually.

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‡This work was presented at 13th YABEC symposium held at Seoul, Korea, October 20-22, 2007.

MATERIALS AND METHODS

1. Materials

1-1. Strains

A strain of *B. trispora* ATCC 203, mating type (-) was used throughout this investigation. It was obtained from the American type culture collection (ATCC) (Rockville, MD) and maintained at 4 °C on potato dextrose agar (PDA, Merck 10130) slants. A strain grown on petri-dishes at 28 °C for 4 days and then at 20 °C for 2 days to induce the formation of spores [28] was used for the inoculation of the PDA liquid medium.

1-2. Media

PDA liquid medium; PDA solid medium; yeast extract medium; RM medium (per L: $NaNO_3$ 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, KH_2PO_4 1 g, KCl 0.5 g, dextrose 40 g, yeast extract paste 2 g, agar 20 g). For the regeneration of protoplast, NaCl was added to the above medium except for PDA liquid medium, and the final concentration was 0.7 M.

2. Methods

2-1. Culture and Collection of Mycelium

50 μL spores of *B. trispora* ATCC 203 (-) at concentration of 1×10^6 spores/mL from 5-6-day old PDA plates were inoculated into a 250 mL Erlenmeyer flask with 50 mL medium and shaken at 180 rpm at 28 °C for 60 h. Finally, the cultured mycelium was collected by centrifugation at 3,000 rpm for 10 min, then the collected mycelium was washed twice using phosphate buffer (0.2 M pH 6.8) and transferred to the sterilized beakers.

Table 1. Orthogonal experiment of the enzymolysis factors for preparation of the protoplasts

No.	Enzyme				Protoplast yield ($\times 10^6$)
	Lywallzyme (%)	Lysozyme (%)	Cellulase (%)	Snailase (%)	
1	0	0	1	1	1.25±0.11
2	0	1	2	2	3.75±0.32
3	0	2	3	3	7.25±0.63
4	1	2	1	2	2.92±0.27
5	1	0	2	3	3.50±0.41
6	1	1	3	1	4.58±0.51
7	2	1	1	3	2.00±0.17
8	2	2	2	1	4.00±0.45
9	2	0	3	2	3.83±0.36
K_1	12.25	8.58	6.17	9.83	
K_2	11.00	10.33	11.25	10.5	
K_3	9.83	14.17	15.66	12.75	
k_1	4.08	2.86	2.06	3.28	
k_2	3.67	3.44	3.75	3.50	
k_3	3.28	4.72	5.22	4.25	
R	0.80	1.86	3.16	0.97	

Where K_1 is the sum of three protoplast yields under the first content level of four different enzymes, K_2 is the sum of three protoplast yields under the second content level of four different enzymes, K_3 is the sum of three protoplast yields under the third content level of four different enzymes, k_1 is the average value of three protoplast yields under the first content level of four different enzymes ($k_1=K_1/3$), k_2 is the average value of three protoplast yields under the second content level of four different enzymes ($k_2=K_2/3$), k_3 is the average value of three protoplast yields under the third content level of four different enzymes ($k_3=K_3/3$), R is the maximum difference of three values of k (max (k_1, k_2, k_3) - min(k_1, k_2, k_3)), which indicate the effect degree of four different enzymes.

2-2. Preparation of Enzymolysis Solution

The enzymes used for cell wall removal were lywallzyme, lysozyme, snailase, cellulase Onozuka R-10 from Yakult (Japan). Preparation and combination of those enzymes is listed in Table 1.

2-3. Pretreatment

The collected mycelia were respectively pretreated directly and indirectly. In direct pretreatment, the mycelia were permeated in the mixture of pretreatment solution (0.6 M sucrose, 0.01 M $MgSO_4$, 0.05 M DTT) and enzymolysis solution. In indirect pretreatment, the mycelia were pretreated in the pretreatment solution before enzymolysis.

2-4. Preparation of Protoplast

The mycelium was collected by centrifugation at 3,000 rpm for 10 min and washed with 0.2 M phosphate buffer, pH 6.8. The mixed enzymes in osmotic stabilizer (0.6 M NaCl pH 5.8-6.2) were added to the beaker that contained 300 mg wet mycelia per mL enzymolysis solution. The mycelia were digested in an incubator at 70 rpm at 28 °C. A sample of the enzymolysis solution was taken out from the beaker in the interval of 2 h, and was filtered by using four layers of lens paper to get rid of the remaining mycelia and fragments. The filtrate was centrifuged at 3,000 rpm for 10 min. The collected protoplasts were washed twice by osmotic stabilizer and resuspended in it. Finally, the protoplasts were observed by microscope and counted with a hemacytometer. The protoplast yield was calculated by the number of protoplast according to the following formula:

$$Fp = \frac{Np}{V} \tag{1}$$

where Fp is the protoplast yield (the number of protoplasts/mL), Np is the number of protoplasts, and V is the volume of enzymolysis solution.

2-5. Regeneration of Protoplast

First, the protoplast suspension was diluted by 0.7 M NaCl in ten times and was cultured by monlayer or bilayer method. In the monolayer plate culture method, 0.5 mL diluted protoplast suspension was directly plated on regenerative solid-plate and cultured at 28 °C for 3-4 days. In the bilayer plate culture method, 0.5 mL suspension was added to tubes containing 4.5 mL regenerative soft agar medium. After being shaken equably by hand, the solution was plated on a regenerative solid-plate and cultured at 28 °C for 3-4 days. At the same time, another 0.5 mL protoplast suspension was added as a control, into 4.5 mL distillated water for 30 min and then plated on PDA solid-plate without osmotic stabilizer. The regeneration frequency of protoplast was calculated by the number of colonies according to the following formula:

$$Rpf = \frac{Cr - Cp}{Np} \tag{2}$$

where Rpf is regeneration frequency of protoplast (%), Cr is the number of colonies on regenerative culture, Cp is the number of colonies on PDA culture, and Np is the number of protoplasts.

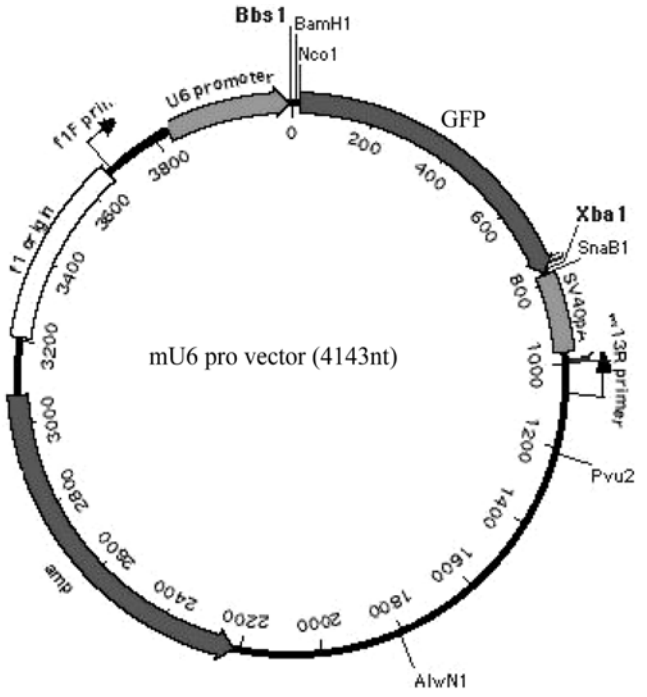

Fig. 1. Map of plasmid vector used for transformation.

2-6. Validation of Protoplast

The mU6pro vector (Fig. 1) constructed from an RNA pol II expression vector [29] was used in transformation. In this plasmid, the cDNA for eGFP is present at downstream of the U6 promoter, with an SV40 late polyadenylation site. Transformation of protoplasts was carried out as previously described [30,31], with minor modification. Protoplasts were separated from the mycelium and cell debris by filtering through four layers of sterile lens paper. The filtrate was centrifuged for 5 min at 2,500 rpm and washed twice with ST (0.55 mM sucrose, 10 mM Tris HCl, pH 7.5), after which 10^7 protoplasts were resuspended in 2 mL STC (0.55 mM sucrose, 10 mM Tris HCl, 25 mM $CaCl_2$, pH 7.5). 200 μL of protoplast suspension was mixed with 100 μL of mU6pro vector (0.5 μg/mL), and incubated on ice for 20 min. 10 volume of 60% PEG solution was added to the tube, with gentle mixing, and was incubated for 20 min at 25 °C. At the end of the incubation, 2 volume of STC was added to the tube, and the contents were gently mixed again. Samples were then centrifuged at 2,500 rpm for 5 min and protoplasts were collected. The protoplasts were washed with ST, and then resuspended in 1 mL of ST. 100-400 aliquots were spread on PDA for regeneration. All experiments were repeated at least three times, and the results represented the mean values.

RESULTS AND DISCUSSION

1. Effects of Key Factors on the Preparation of Protoplast

1-1. Effect of Enzymolysis on Preparation of Protoplast

To improve the production of protoplasts, we attempted first to choose the best enzymatic system. Table 1 shows that the effects of lywallzyme, lysozyme, cellulase and snailase on the preparation of the *B. trispora* protoplast by orthogonal experiments. The results indicated that the optimal condition for the preparation of protoplast is a combination of 2% lysozyme, 3% cellulase and 3% snailase. Enzymolysis effect of each enzyme is in decreasing order of cellulase>lysozyme>snailase>lywallzyme.

Subsequently, the effect of enzymolysis methods was investigated. Herein, two enzymolysis methods were used: at rest; and with shaking at 75 rpm. When *B. trispora* was digested at rest, the mycelia usually massed tightly and suspended as granules in the enzymolysis solution and the protoplast yield was low. On the contrary, the mycelia were digested loosely with shaking and the protoplast yield was higher. A similar conclusion on enzymolysis method was reported in previous work [32], which proved that the enzymolysis method plays an important role in protoplast preparation.

The effect of pretreatment on preparation of protoplast was also studied. In this paper, the collected mycelia were pretreated directly and indirectly. In direct pretreatment, the mycelia were permeated in the mixture of pretreatment solution and enzymolysis solution. In indirect pretreatment, the mycelia were pretreated in the pretreatment solution at 28 °C for 20 min before enzymolysis. When compared with untreated mycelia as control, the protoplast yield was lower in the case of direct pretreatment and almost the same for indirect pretreatment. That is, pretreatment could not increase the protoplast yield [32]. However, the protoplast yield decreased in the mixture of pretreatment solution and enzyme solution. A similar phenomenon was also found by Sergi [33].

1-2. Effect of Mycelial Age on Preparation of Protoplast

Mycelial age plays an important role in protoplast formation. In this paper, effects of mycelial age on the protoplast yield were studied under the condition obtained in section 1-1. As shown in Fig. 2, the optimal mycelial age for preparation of protoplast was 60 h. When culturing the mycelia for a short time, the cells in mycelia did not mature completely and released protoplasts easily. However, the released protoplasts were not stable and broken easily [34,35]. On the contrary, if the mycelia age was too old, the cell wall would become thicker and the mycelia would be digested more difficultly, which led to the decrease of protoplast yield.

1-3. Effect of Treatment Condition on Preparation of Protoplast

The treatment conditions studied here included pH, enzymolysis temperature and enzymolysis time. The relationship between pH

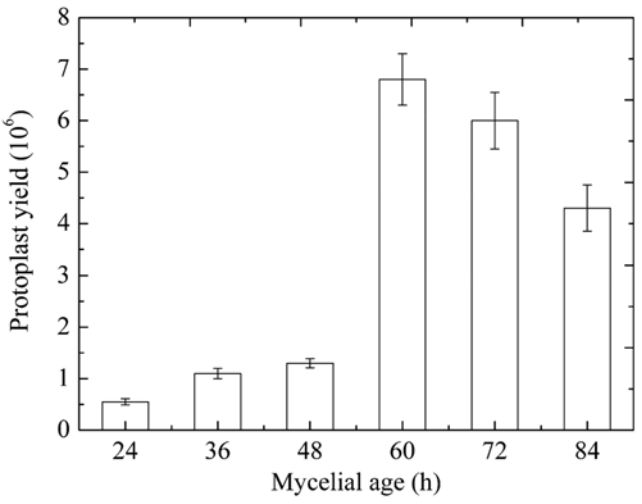

Fig. 2. The effect of mycelial age on protoplast yield.

Table 2. The effect of pH on protoplast yield

pH	Protoplast yield ($\times 10^6$)
4.0	4.63±0.53
5.0	6.74±0.72
6.0	7.48±0.86
7.0	5.12±0.45
8.0	1.25±0.13

Table 3. The effect of enzymolysis temperature on protoplast yield

Enzymolysis temperature	Protoplast yield ($\times 10^6$)
25 °C	6.84±0.57
28 °C	7.38±0.71
31 °C	5.12±0.44

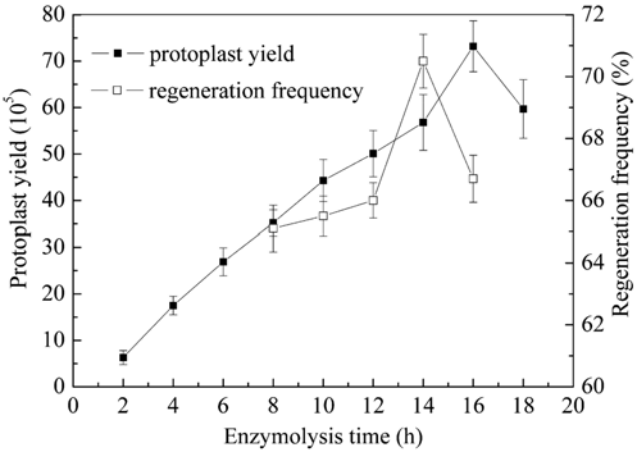

Fig. 3. The effect of enzymolysis time on the protoplast yield and regeneration frequency.

and the protoplast yield was investigated and the results are presented in Table 2. The maximum protoplast yield was achieved as high as 7.48×10^6 protoplasts/mL when the pH value was 6.0. Higher or lower pH value will decrease protoplast yield, which is due to the effect of pH on enzyme activities.

The protoplast yields at different enzymolysis temperature are presented in Table 3. It was clear that 28 °C was the most suitable enzymolysis temperature on the basis of the optimized enzymolysis conditions. Higher or lower temperature decreased the protoplast yield because of the decrease of enzyme activity.

The effect of enzymolysis time on the protoplast yield is shown in Fig. 3. According to the results, the protoplast yield first increased with prolonged enzymolysis time. The maximum was 7.32×10^6 protoplasts/mL when enzymolysis was for 16 h. However, if the enzymolysis time was longer than 16 h, the protoplast yield decreased, which can be explained as a result of rupture of protoplasts, loss of regenerative primers and reduction of protoplast activity.

2. Effects of Key Factors on Regeneration of Protoplast

2-1. Effects of the Regeneration Medium and Method on Regeneration of Protoplast

Three kinds of high osmotic agar media including PDA medium, RM medium and yeast extract medium were applied to investigate

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

Regeneration medium*	The number of regenerative colonies	Regeneration frequency (%)
PDA	98±3.39	77.5±2.70
RM	44±2.94	32.5±2.17
Yeast extract	9±0.82	3.3±0.30

Note: *All contain 0.7 M NaCl

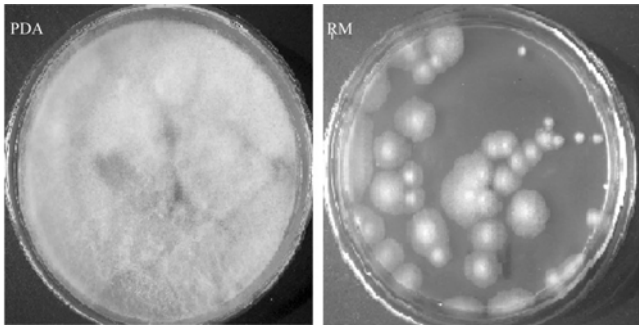

Fig. 4. The regenerated morphology of the colonies of protoplast in PDA and RM high osmosis plate.

the effect on regeneration of *B. trispora* protoplasts. As shown in Table 4, the regeneration frequency of protoplast was highest in PDA, moderate in RM, and lowest in yeast extract. The regenerative morphology of the colonies of protoplast in PDA and RM high osmosis plate is shown in Fig. 4. It is clear that more colonies were observed in PDA high osmosis plate after regeneration of about 70 h.

After determining the regeneration medium, two regeneration methods, bilayer plate culture and monolayer plate culture, were studied to choose the more appropriate one. The results showed that regeneration frequency was higher using the bilayer plate (71%) than the monolayer plate (49%) culture.

2-2. Effect of Enzymolysis Time on regeneration of Protoplast

After modulating the concentration of protoplasts prepared with different enzymolysis time, the protoplasts were regenerated in the PDA high osmosis regenerative medium. The results indicated that the regeneration frequency of protoplast changed little in short time. However, the regeneration frequency decreased with prolonged enzymolysis time. This may be due to the loss of primers for synthesizing cell walls at long enzymolysis time. Therefore, taking into account the protoplast yield and regeneration frequency, 14-16 h was selected as the optimal enzymolysis time in this study. Fig. 3 shows that the effects of enzymolysis time on regeneration frequency of protoplast.

3. Validation of Protoplast

To investigate the activity of protoplasts and usability for genetic manipulation, a transformation experiment was carried out. When we assayed genetic transformation of these protoplasts with the mU6pro vector, we obtained a frequency of approximately 2.7×10^{-3} transformants per viable protoplast. It is clear that the transformants with mU6pro-GFP showed strong GFP expression (Fig. 5), which can prove that the protoplasts were in good physiological condition and applicable in further gene manipulation experiments.

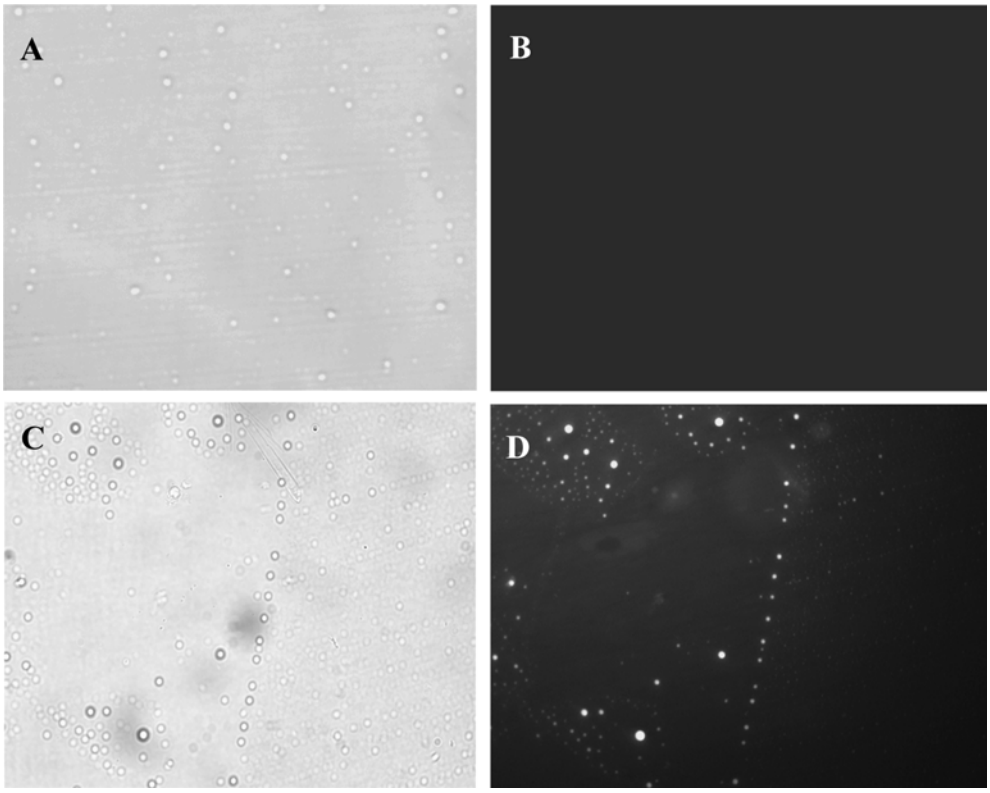

Fig. 5. GFP expression by *B. trispora* transformants, observed at a magnification of 1,000× under visible light (A, C,) and in the dark with GFP excitation at 488 nm (B, D). A, B: untransformed *B. trispora* (control); C, D: transformant.

CONCLUSION

The protoplast system developed in the present study provides a large number of viable protoplasts: 7.48×10^6 protoplasts/mL; 77.5% regeneration rate. Protoplasts produced in this way, can serve as a useful tool for studies about further gene manipulation experiments.

ACKNOWLEDGMENTS

The authors would like to acknowledge financial support of National Natural Science Foundation of China (No. 20376007).

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