Food Sci. Biotechnol. Vol. 14, No. 4, pp. 543 ~ 546 (2005)

RESEARCH NOTE

Food Science and Biotechnology

Protoplast Preparation and Regeneration from Young Hyphae of the Citrinin Producing Fungus *Monascus ruber*

Tenzin Norlha and Inhyung Lee*

Food & Life Sci. Major, Kookmin University, Seoul 136-702, Korea

Abstract Optimized conditions for protoplast preparation and regeneration from young hyphae of *Monascus ruber* were established. Heat shock treatment of spores gave rapid and synchronized germination. Spores collected from cultures grown for 7-8 days at 30°C were germinated until over 70% germ tubes reached to 3-5 spore length. Enzymatic digestion of young hyphae was optimal with 50 mg/mL Glucanex in 0.1 M sodium citrate buffer containing 0.8 M mannitol as an osmotic stabilizer. Regeneration rate was around 10% when 0.8 M sorbitol was used as an osmotic stabilizer in regeneration medium. These conditions will be applied in genetic study of *M. ruber* that produces citrinin at high level and thus is good model strain for molecular genetic dissection of citrinin biosynthesis.

Keywords: *Monascus ruber*, protoplast formation, Glucanex, regeneration

Introduction

Beni-koji or ang-kak prepared by growing *Monascus* on steamed rice has been traditionally used as natural pigment for red soybean gels, meats, and vegetables, preservative, and koji for producing red wine in East Asia (1-3). Recently, *Monascus* has been gaining more interests from food and fermentation industries as it has been proven to produce various bioactive secondary metabolites. For example, many *Monascus* species produce monacolin K (also known as lovastatin, mevinolin, and mevacor) that is known to lower the levels of plasma cholesterol (4) and γ-aminobutyric acid (GABA), which shows anti-hypertensive effects in humans (5). However, *Monascus* also produces citrinin that has nephrotoxic and hepatotoxic properties (6-8), thus limiting the application of *Monascus* in the production of valuable substances.

Understanding the biosynthesis of secondary metabolites in *Monascus* will lead to efficient industrial applications. Only a few studies have been reported on the biosynthesis of secondary metabolites, on the biochemical and molecular genetic levels (9-10). The production of secondary metabolites differs among various species of *Monascus*. Because *M. ruber* is known to produce a high level of citrinin (7), this fungus is the reasonable choice for the study of citrinin biosynthesis. Recently, genetic transformation was successfully performed in certain *Monascus* species (11-14). However, protoplast preparation and regeneration protocol have not yet been reported in *M. ruber*. Protoplasts are involved in many genetic tools of filamentous fungi such as transformation and preparation of intact chromosomal DNA for the genomic library construction and pulsed field gel electrophoresis. In addition, protoplasts fusion technique is one of the conventional methods for the strain development (15).

In this study, we established a protocol for optimized protoplast preparation and regeneration, which should be the basis of developing molecular genetic tools in *M. ruber*, which is poorly characterized at the molecular genetic level but is still a good model strain for the molecular genetic analysis of citrinin biosynthesis.

*Corresponding author: Tel: 82-2-910-4771; Fax: 82-2-911-4771
E-mail: leei@kookmin.ac.kr
Received May 24, 2005; accepted June 25, 2005

Materials and Methods

Fungal strain, media, and preparation of spores *Monascus ruber* KCTC6122 was obtained from Korean Collection for Type Cultures (Daejeon, Korea). Stock culture was maintained in slant tubes at 4°C on potato dextrose agar (PDA) (Acumedia Manufacturers, Inc., Baltimore, MA, USA). To prepare spores, *M. ruber* KCTC6122 was grown on medium C agar plates (10% sucrose, 0.3% yeast extract, 0.5% casamino acids, 0.1% KH_2PO_4, 0.2% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 1.5% agar) at 30°C for 7-8 days (16). Spores were harvested by scrapping them with a spreading rod after adding 5 mL of sterile distilled H_2O. Collected spores were washed twice with sterile distilled H_2O before use. YM media containing mannitol, sorbitol, or sucrose as an osmotic stabilizer were used for regeneration of protoplasts.

Pretreatment of spores For heat shock pretreatment of spores, the washed spores were suspended in 5 mL YM medium. Spore suspensions were incubated at 50°C in a water bath for 90 sec (11). For Tween 80 pretreatment of spores, spore suspensions were treated with a solution of 0.1 M phosphate buffer, pH 7.5, and 0.01% Tween 80 for 3 hr at 30°C, followed by incubation in 0.1 M phosphate buffer, pH 7.5, for additional 3 hr at 30°C (17). Germina-tion was monitored every hour using a microscope.

Enzymatic digestion of young hyphae A variety of lysing enzymes were tested at different concentrations and combinations. The selected enzymes were 50 mg/mL Glucanex (Novozymes Switzerland AG, Dittingen, Switzerland), 20 mg/mL driselase (Sigma-Aldrich, Inc., St

Louis, MO, USA), and 20 μg/mL β-glucuronidase (Sigma-Aldrich). Initial evalua-tions of the enzymes were carried out in sodium citrate buffer (0.1 M sodium citrate, 0.01 M EDTA, pH 5.8) containing 0.8 M $(NH_4)_2SO_4$ as an osmotic stabilizer.

Osmotic stabilizers A wide range of inorganic salts (KCl, $CaCl_2$, NH_4Cl, $(NH_4)_2SO_4$, $MgSO_4$, and NaCl) and organic compounds (sugars and sugar alcohols) have been used as osmotic stabilizers for fungal protoplast preparation and regeneration (18-19). We therefore selected KCl, $(NH_4)_2SO_4$, mannitol, glycerol, sorbitol, and sucrose, which were tested at concentrations of 0.4, 0.6, 0.8, 1.0, and 1.2 M to determine the most suitable osmoticum. The osmotic stabilizers at various concentrations were tested using the optimal concentration of enzymes determined in the above section.

Protoplast preparation Spores (1-2.5 × 10^9/mL) pretreated by heat shock as described above were inoculated into 100 mL YM broth and grown for 7-8 hr at 30°C with shaking at 170 rpm. Young hyphae were collected by centrifugation at 7,000 × g at 4°C and washed twice with 15 mL sterile H_2O. The washed young hyphae were resuspended in different enzyme solutions prepared in sodium citrate buffer containing a suitable osmotic stabilizer. The mixture was incubated at 30°C for 210 min in an incubator under gentle shaking of 50 rpm. One milliliter each of the reaction solutions was taken every 30 min, and the enzyme reaction was stopped by washing the solution twice with respective osmoticum through centrifugation at 1,200 × g for 10 min. The number of protoplasts was counted using a haemacytometer.

Protoplasts regeneration YM media containing different concentrations of osmotic stabilizers (mannitol, sorbitol, and sucrose) were used as the regeneration media. The protoplast suspension was tenfold diluted with the respective osmotic stabilizer. Diluted protoplast solution (0.5 mL) was added to 10 mL YM soft agar pre-cooled to 45°C and overlaid onto the regeneration media. As a control, protoplast suspension diluted with sterile H_2O and YM medium without an osmotic stabilizer was used. Protoplasts were incubated at 30°C for 2-3 days, and regenerated protoplasts were counted as individual colonies on the Petri dishes.

Results and Discussion

Pretreatment of spores Because the conidia cell wall is more rigid than that of the young hyphae, protoplasts are usually prepared from young hyphae in filamentous fungi. The conidia of *M. ruber* germinated slowly, some remaining ungerminated even after 10 hr, at which some of the early-germinated hyphae extended too long (Fig. 1). To speed up and synchronize the germination process, spores were treated by heat shock (11) and Tween 80 (17). Both treatments of spores were effective, resulting in about 70% of spore germination after 8 hr (Fig. 1). The heat shock method was applied in protoplast preparation, because it was easier and simpler to perform.

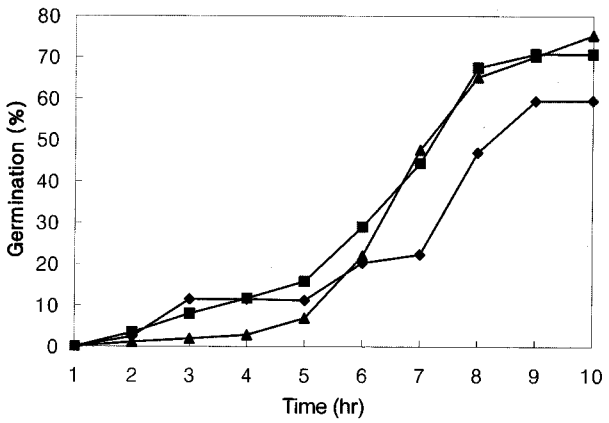

Fig. 1. Effect of pretreatment of spore on germination. Spore solutions were treated by heat shock (▲) or Tween 80 (■) and then subjected to germinate by incubation at 30°C. A control spore solution (◆) was germinated without pretreatment.

Enzymatic digestion of young hyphae Effectiveness of the cell wall lytic enzymes on the release of protoplasts differs among fungi, generally requiring conditions specific to each fungus. Of the three commercial enzymes, Glucanex (50 mg/mL), Driselase (20 mg/mL), and β-glucuronidase (20 μg/mL), Glucanex was the most efficient for the protoplast formation; Driselase and β-glucuronidase yielded very few protoplasts (data not shown). The addition of Driselase (20 mg/mL), and β-glucuronidase (20 μg/mL) to Glucanex (50 mg/mL) did not exert any significant influence on the protoplast formation (Table 1). Therefore, Glucanex (50 mg/mL) was chosen for the subsequent experiments.

Osmotic stabilizers Because the cell wall compositions of filamentous fungi vary among species, each species is assumed to have different optimal osmotic stabilizers at different concentrations. Of the five selected osmotic

Table 1. Protoplast release from young hyphae of *M. ruber* under different enzymatic treatments

Enzyme[a]	Number of protoplast (× 10^6) formed from 10^7 spores				
	30 min	60 min	90 min	120 min	150 min
50 mg/mL Glucanex	0.8±0.1	1.8±0.7	2.4±0.3	2.8±1.0	3.2±1.4
50 mg/mL Glucanex+20 mg/mL Driselase	0.3±0.0	0.9±0.5	1.2±0.8	1.9±0.4	2.3±0.3
50 mg/mL Glucanex+20 μg/mL β-glucuronidase	1.0±0.5	1.6±0.26	2.8±1.0	2.7±0.3	3.2±1.2
50 mg/mL Glucanex+20 mg/mL Driselase +20 μg/mL β-glucuronidase	0.3±0.0	0.9±1.0	1.3±0.90	1.3±1.0	2.5±0.3

[a]Enzyme solutions were prepared in 0.1 M sodium citrate buffer, pH 5.8. 0.8 M $(NH_4)_2SO_4$ was used as the osmotic stabilizer. Results shown represent the means of three to five replicates and values are given as mean±SEM.

Table 2. Protoplast release from young hyphae of *M. ruber* following treatment with different osmotic stabilizers

Osmoticum[a]	Number of protoplast (× 10^6) formed from 10^7 spores						
	30 min	60 min	90 min	120 min	150 min	180 min	210 min
0.6 M mannitol	1.0±0.1	1.8±0.1	4.5±2.3	4.9±1.8	5.7±1.9	6.2±1.9	6.6±0.8
0.6 M glycerol	0.8±0.4	2.9±1.9	3.6±1.2	3.5±1.2	4.2±1.3	5.1±1.2	4.8±1.1
0.6 M KCl	0.6±0.4	1.2±0.4	1.7±0.7	2.2±0.5	3.2±1.2	3.7±1.8	4.9±1.6
0.6 M sorbitol	0.4±0.2	1.6±0.9	2.4±1.5	3.2±2.7	3.8±1.9	4.4±2.2	4.6±1.9
0.8 M $(NH_4)_2SO_4$	0.8±0.1	1.8±0.7	2.4±0.3	2.8±1.0	3.2±1.4	2.4±1.2	2.6±1.3

[a]Osmotica were prepared in 0.1 M sodium citrate buffer, pH 5.8, 50 mg/mL Glucanex was used as enzyme. Results shown represent the means of two to five replicates and values are given as mean±SEM.

Table 3. Effect of concentrations of an osmotic stabilizer, mannitol on protoplast release

Concentration	0.4 M	0.6 M	0.8 M	1.0 M	1.2 M
Number of protoplast (× 10^6) formed from 10^7 spores	3.88	4.31	4.98	4.78	4.16

Osmotica were prepared in 0.1 M sodium citrate buffer, pH 5.8. 50 mg/ml Glucanex was used as enzyme. The yield of protoplasts was after 150 min incubation at 30°C.

stabilizers, mannitol, glycerol, KCl, sorbitol, and $(NH_4)_2SO_4$, 0.6 M mannitol and 0. 8 M $(NH_4)_2SO_4$ were the most and the least effective, respectively (Table 2). Under microscopic examination some protoplasts began to burst as enzyme treatment time increased. In addition, the long enzyme treatment negatively affected the protoplast regeneration. The optimal concentration of mannitol was then determined. The results showed that 0.8 M was the most suitable for maintaining the protoplasts (Table 3). Based on these results, protoplasts were prepared by treating young hyphae with 50 mg/mL Glucanex for 150 min in the presence of 0.8 M mannitol as an osmotic stabilizer.

Protoplast regeneration Protoplast regeneration was examined on YM agar medium containing different osmotic stabilizers. The regeneration frequency was the highest with 0.8 M sorbitol (Table 4). About 10% of protoplasts formed colonies after 2-3 days. Studies showed that regeneration of fungal protoplasts ranges from less than 0.1% up to 50% in various fungi.

We established the optimal condition for protoplast preparation and regeneration in *M. ruber* KCTC6122. The treatment of young hyphae of *M. ruber* with 50 mg/mL Glucanex yielded protoplasts with over 60% convertion ratio. In some *Monascus* species, propoplasts were prepared to use them mainly for transformation (11-13). Various cell wall lytic enzymes and osmotic stabilizers were adopted, and protoplast yields differed among research groups, with the maximum yield of over 90% conversion (12) or range from 1.3 × 10^7/ml to 2 × 10^9/mL

Table 4. Effect of different osmotic stabilizers on protoplast regeneration

Osmotic stabilizers	Regeneration (%)
0.8 M mannitol	1.85±0.2
0.8 M sorbitol	8.61±2.3
0.8 M sucrose	4.92±1.7

Protoplasts were prepared by treatment of 50 mg/ml Glucanex in 0.1 M sodium citrate buffer, pH 5.8 containing 0.8 M mannitol as an osmotic stabilizer for 150 min at 30°C. Results shown represent the means of three replicates and values are given as mean±SEM.

(11, 13, 15). In this study, over 80% of the young hyphae of *M. ruber* were converted into protoplasts by prolonged treatment of cell wall lytic enzymes; however, protoplast regeneration efficiency was optimum at 50-60% protoplast formation yield. Therefore, the condition of protoplast formation yield was set at 50-60%. About 10% of protoplast regeneration ratio was equivalent to 5 to 20% in *M. purpureus* DSM1379 (12). The established protocol for optimized protoplast preparation and regeneration in *M. ruber*, which produces citrinin at high level but is poorly characterized at the molecular genetic level, should be useful in developing molecular genetic tools, which is required for molecular genetic dissection of *Monascus*.

Acknowledgments

This work was supported by a grant of the Korea Health 21 R&D Project (03-PJ1-PG3-22000-0052), Ministry of Health & Welfare, Republic of Korea.

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