THE KOREAN JOURNAL OF MYCOLOGY
Vol. 23, No 4, p305-309 December 1995
Printed in S. KOREA

Protoplast Formation and Regeneration of the Wood-Rot Basidiomycete *Phanerochaete chrysosporium*

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목재부후균인 *Phanerochaete chrysosporium*의 원형질체 생성 및 재생

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ABSTRACT: To investigate optimal conditions for the protoplast formation and regeneration of *Phanerochaete chrysosporium*, preparations of three enzymes were used to liberate protoplasts from its 20 hrs-old mycelium on cellophan membrane covered agar media. Novozym 234 alone with 0.6M sucrose was the most effective for isolation of protoplasts from the mycelium with 3 hrs incubation time at 39°C in shaking condition of 120 rpm. The poly-R medium stabilized with 0.6M mannitol was the best for regeneration of the protoplasts.

KEYWORDS: *Phanerochaete chrysosporium*, protoplast formation, protoplast regeneration, Basidiomycotina

The basidiomycete *Phanerochaete chrysosporium* has already been used in numerous studies on lignin degradation and its biotechnological applications (Eriksson *et al.*, 1991). Protoplasts are widely being used for the physiological, biochemical, and genetic research in microorganisms (Peberdy, 1979; Peberdy *et al.*, 1985). Recently, formation and regeneration of protoplasts have been extensively studied in basidiomycetes, especially mushrooms in Korea (Yoo *et al.*, 1987a; 1987b; Um *et al.*, 1988; Park *et al.*, 1988; Bok *et al.*, 1990; 1994; Yi *et al.*, 1993; Kim *et al.*, 1994). It is also reported that the formation and regeneration of protoplast is generally affected by culture age, osmotic stabilizers, lytic enzymes and reversion media (Peberdy, 1979; Peberdy *et al.*, 1985), although the precise and optimal conditions for formation and regeneration of protoplasts are different among species. This experiment was to find out optimal conditions for the production and regeneration of protoplasts from the mycelium of *Phanerochaete chrysosporium* ME 446.

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Materials and Methods

Organism and culture conditions

Phanerochaete chrysosporium ME446 was a gift from Dr. Reddy at Michigan State University and maintained on slants of malt extract media. After 7 to 10 days of growth at 39°C, conidia were harvested from the plates, filtered, counted and diluted when they were used. For liquid culture, cells were grown in Erlenmeyer flasks (100 m*l*) containing 10 m*l* of a medium on a stationary condition. Flasks were inoculated with 1% homogenized mycelium suspension, which was grown for 2~3 days at 39°C. For the reversion of protoplasts, an osmotically stabilized agar medium was prepared. It consisted of poly-R (Tien *et al.*, 1988), YGA (Yoo *et al.*, 1987) and SCM (Um *et al.*, 1988) medium and supplemented with 0.6M $MgSO_4$,

mannitol, sorbitol and sucrose, respectively. Bottom agar was 2.0% while overlaying soft agar was of 0.75% Bacto agar (Difco).

Protoplast formation

Three enzymes were used for the protoplast formation. They were cellulase, β-glucuronidase and Novozym 234 and were all purchased from Sigma Chemical Company, Ltd. Each enzyme (5 mg/ml for Novozym 234, 10 mg/ml for cellulase and 2.5 mg/ml for glucuronidase) was dissolved in 0.6M sucrose solution and sterilized by Millex filter unit (0.22 μm pore size). Disks of sterile cellophan membrane (commercial) were placed on the surface of malt extract media in petri dishes. The mycelial disks were ready for protoplast production after 18~20 hrs incubation at 39°C. Mycelial disks of *Phanerochaete chrysosporium* were removed to clean sterile petri dishes and the lytic enzyme in 0.6M sucrose solution was added immediately. The petri dishes were incubated on rotary shaker (120 rpm) at 39°C for 1~3.5 hours. The number of protoplasts in the suspension was determined with a hemacytometer.

Protoplast fusion and regeneration

Protoplasts from the lytic mixture were separated from the mycelial debris by filtration through sintered glass filters (porosity No.1), and sedimented by centrifugation at 3,000 rpm for 15 min, and washed twice with 10 ml of 0.6M sucrose. The suspensions (5×10^6 cells/ml) were then mixed and sedimented together by centrifugation at 3,000 rpm for 20 min. The protoplasts were resuspended in 1 ml of 30% polyethylene glycol (PEG) 8,000 (prewarmed to 38°) - 10 mM $CaCl_2$ - 0.05M glycine (pH 7.5) and incubated for 15 min at 38°C. Sucrose (0.6M, 5 ml) was then added and the protoplasts were resedimented by centrifugation at 1,000 rpm for 10 min and resuspended in 0.6M sucrose. Protoplasts in 0.6M sucrose were plated in regeneration poly-R media with soft top agar of 0.6M mannitol in 0.02M Na-phosphate buffer (pH 5.8). Plates were incubated at 28°C for one week.

Results and Discussion

Factors affecting protoplast formation

Several different enzyme preparations were tested to induce protoplast formation (Table 1). Novozym 234 alone was the most effective for *Phanerochaete chrysosporium* as *Pleurotus ostreatus* (Byun *et al.*, 1984; Yoo *et al.*,

Table 1. Comparison of different enzyme preparations for the release of protoplasts.

Enzyme[a] \ RT[b]	2.5	3.0	3.5
Novozym-234	22.5[c]	21	20.7
Cellulase	1.5	2	2.3
Glucuronidase	4.5	2.8	1.3
Nov+Cell	10.5	10.8	9
Nov+Cell	10.5	9.5	8.5

a: Novozym-234 (5 mg/ml), Cellulase (10 mg/ml), Glucuronidase (2.5 mg/ml)
b: reaction time (hour)
c: 22.5→22.5×10^5 /ml

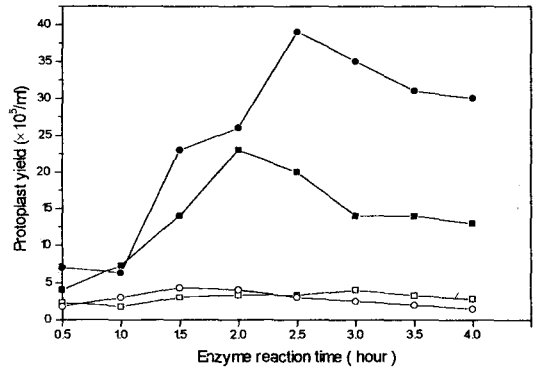

Fig. 1. Effect of different osmotic stabilizers on the formation of protoplasts.
Symbols; $MgSO_4$ (■), Mannitol (□), Sucorse (●) and Sorbitol (○)

1985). Unlike the result of Gold *et al.* (1983) from *Phanerochaete chrysosporium*, several kinds of combination among three enzymes, that is, Novozym 234, β-D-glucuronidase and cellulase, showed less effective than Novozym 234 alone. When Novozym 234 was used alone, the yield of protoplasts was approximately 200% of the yield when the enzymes were used together. The osmotic stabilizers varied in their ability to support the formation and maintain the stability of protoplasts. As shown in Fig. 1, sucrose gave the maximum yield of protoplasts among the various stabilizers. The use of sucrose led to two times of that of $MgSO_4$ and ten or fifteen times of that of mannitol or sorbitol in the production of protoplasts. The growth phase of the mycelium used for the preparation of protoplasts also influenced the yield of protoplast formation. Experiments with different phases of the mycelium showed that maximum yields of the protoplasts were obtained from the cultures in the exponential phase of growth as in other reports (Yoo *et al.*, 1987a; 1987b; Bok *et al.*, 1990). As showen in Fig. 2, maximum yields were obtained from 42 hrs culture of the mycelium grown on malt extract medium.

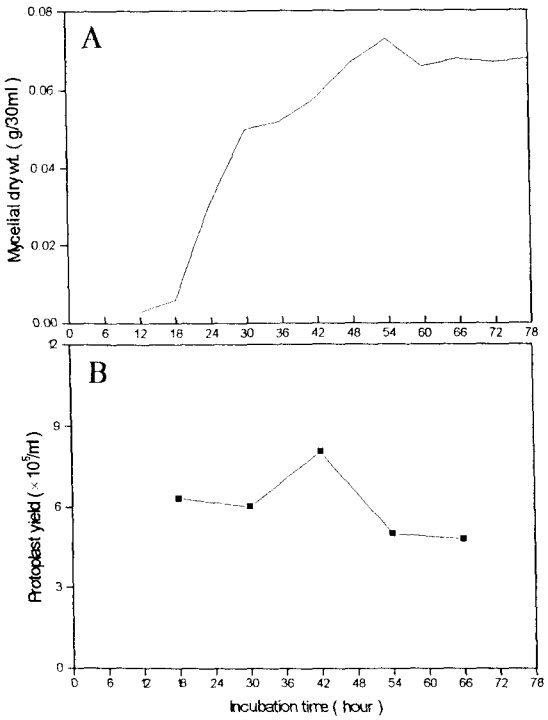

Fig. 2. Production of protoplasts (B) according to the growth phase of mycelium (A).

Regeneration and stability of protoplasts

The frequency of protoplast regeneration was affected by various osmotic substances

Table 2. Effect of different osmotic stabilizers on the reversion of protoplasts from *Phanerochaete chrysosporium* ME446

Osmotic stabilizer (0.6M)	Percentage of protoplast reverted on poly-R
Mannitol	1.08
Sorbitol	0.85
Sucrose	0.52
$MgSO_4$	0.03

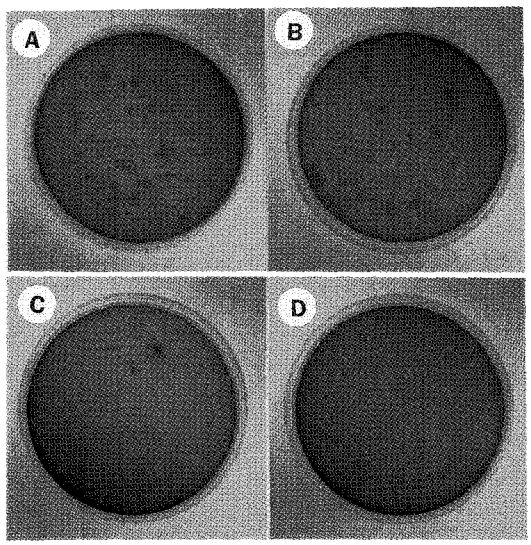

Fig. 3. Reversion of protoplasts from *Phanerochaete chrysosporium* ME446. Protoplasts were incubated at 28°C for 10 days on 0.6M mannitol (A), Sorbitol (B), Sucrose (C) and $MgSO_4$ (D) on poly-R media.

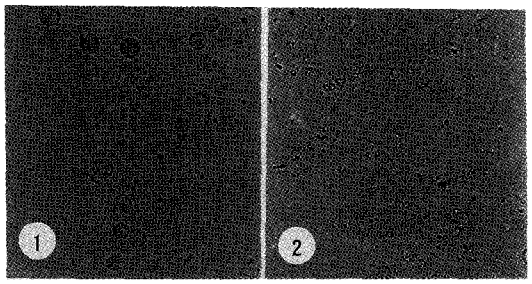

Fig. 4. Stability of protoplasts in 0.6M sucrose (1) and mannitol (2) after treatment of PEG.

which were added into the regeneration media. Among the osmotic stabilizers tested, the regeneration frequency was 1.08% on 0.6M mannitol as in Table 2. Protoplasts of *Phanerochaete chrysosporium* were able to form colonies at the highest frequency on poly-R media supplemented with 0.6M mannitol as an osmotic stabilizer (Fig. 3). The use of YGA and SCM instead of poly-R media led to similar results of protoplast regeneration, but these media resulted in colony spreading and dispersed when the protoplasts were subsequently plated in media. During the process of protoplast fusion, it involved the agregation of protoplasts in the presence of PEG and subsequently PEG was processed to dilute or wash away with a suitable osmotic stabilizer. In this process, the stability of protoplasts was maintained on 0.6M sucrose but not on 0.6M mannitol, which showed the best osmotic stabilizer for regeneration of protoplast as above (Fig. 4).

적 요

Phanerochaete chrysosporium ME446의 원형질체 생성 및 재생을 위한 원형질체 분리에 알맞은 세포벽 분해효소로는 Novozym 234이였으며 삼투압 조절제로서는 0.6M sucrose, 처리시간은 2.5~3시간이었다. 균사체 배양일수는 대수기에 해당하는 42시간이었으며 원형질체 재생에 알맞은 배지로는 0.6M mannitol을 첨가한 poly-R 배지였다.

Acknowledgement

We are grateful to Dr. Young-Bok Yoo and Professor Hee-Moon Park for their valuable discussions. This work was supported in part by the grant (931-0500-005-2) from KOSEF.

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