Optimization of Protoplast Preparation Conditions for Pleurotus giganteus (Postprint)

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Abstract

To optimize the preparation conditions for Pleurotus giganteus protoplasts, this study utilized two strains of P. giganteus with different temperature types, PG46 and PG79, as materials. Using single-factor and orthogonal experimental methods, the effects of mycelial culture age, osmotic stabilizer type, lytic enzyme concentration, enzymatic hydrolysis temperature, and enzymatic hydrolysis time on protoplast preparation were investigated. The results showed: (1) In single-factor experiments, the suitable conditions for P. giganteus protoplast preparation were a culture age of 5 d, lytic enzyme concentration of 2.5%, 0.6 mol · L^{-1} mannitol as the osmotic stabilizer, and enzymatic hydrolysis at 32 ℃ (for PG46) or 27–35 ℃ (for PG79) for 4 h. (2) Orthogonal experiments verified and optimized the single-factor experimental results. Combination 2 (culture age of 5 d, lytic enzyme concentration of 2.5%, 0.6 mol · L^{-1} mannitol, enzymatic hydrolysis at 32 ℃ for 4 h) was the optimal condition for protoplast preparation in both PG46 and PG79, with protoplast yields of 1.12$×10^{7}$ CFU · mL^{-1} and 7.28$×10^{6}$ CFU · mL^{-1}, respectively. (3) In F-test analysis, the influence degree of each factor on protoplast preparation was, in descending order: culture age > lytic enzyme concentration > enzymatic hydrolysis temperature > enzymatic hydrolysis time (for PG46), and culture age > enzymatic hydrolysis time > enzymatic hydrolysis temperature > lytic enzyme concentration (for PG79). In summary, the protoplast preparation conditions for the two P. giganteus strains with different temperature types were essentially consistent, with culture age having the most significant effect on protoplast yield for both strains. These results may provide a foundation for subsequent work on hybrid breeding, genetic transformation, whole genome sequencing, and other research in P. giganteus, thereby further advancing the development of molecular genetics in this species.

Full Text

Optimization of Protoplast Preparation Conditions for *Pleurotus giganteus*

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Abstract: To optimize the protoplast preparation conditions for *Pleurotus giganteus*, two strains with different temperature requirements—PG46 (high-temperature type) and PG79 (medium-temperature type)—were selected as experimental materials. Using single-factor and orthogonal experimental methods, we investigated five key factors: mycelial age, osmotic stabilizer type, lywallzyme concentration, enzymatic hydrolysis temperature, and enzymatic hydrolysis duration. The results demonstrated: (1) In single-factor experiments, the optimal conditions for protoplast preparation were mycelial culture for 5 days, 2.5% lywallzyme, 0.6 mol · L^{-1} mannitol as osmotic stabilizer, and enzymatic hydrolysis at 32 °C (PG46) or 27–35 °C (PG79) for 4 h. (2) Orthogonal experiments verified and refined these results, identifying combination 2 (mycelial age 5 d, lywallzyme concentration 2.5%, 0.6 mol · L^{-1} mannitol, 32 °C for 4 h) as the optimal condition for both strains, yielding 1.12$×10^{7}$ CFU · mL^{-1} and 7.28$×10^{6}$ CFU · mL^{-1} for PG46 and PG79, respectively. (3) F-test analysis revealed that the factors influencing protoplast preparation followed the order: mycelial age > lywallzyme concentration > enzymatic hydrolysis temperature > enzymatic hydrolysis duration for PG46, and mycelial age > enzymatic hydrolysis duration > enzymatic hydrolysis temperature > lywallzyme concentration for PG79. In conclusion, the optimal protoplast preparation conditions were essentially identical for both temperature-type strains, with mycelial age exerting the most significant effect on protoplast yield. These findings establish a foundation for subsequent hybrid breeding, genetic transformation, whole-genome sequencing, and molecular genetics research in *P. giganteus*.

Keywords: *Pleurotus giganteus*, different temperature types, tropical regions, protoplast yield, preparation conditions

Introduction

Pleurotus giganteus is a rare edible mushroom domestically developed in China, known commercially as "pork belly mushroom" due to its unique taste and slippery texture reminiscent of pork belly (Dong et al., 2010; Karunarathna et al., 2012). This species is rich in nutrients, including polysaccharides, proteins, and crude fiber, and exhibits various medicinal properties such as anti-inflammatory, anti-tumor, anti-fungal, and hepatoprotective effects, making it highly popular among consumers (Phan et al., 2014; Paravamsivam et al., 2016). Previous studies have demonstrated that *P. giganteus* possesses strong environmental adaptability, high biological conversion efficiency, and suitability for summer cultivation in high-temperature conditions, which is significant for regulating mushroom market supply, off-season cultivation, and particularly for edible fungi production in tropical regions (Yu et al., 2021). As cultivation areas continue to expand, genetic breeding of *P. giganteus* has become increasingly important (Wu, 2020).

Protoplasts are spherical, physiologically active structures formed by removing the cell wall from intact cells while retaining the complete genetic information, making them excellent materials for molecular genetics research in edible fungi (Muralidhar & Panda, 2000). In recent years, protoplast monokaryotization technology has been successfully applied to physiological and biochemical studies, strain rejuvenation, genetic transformation, breeding, and whole-genome sequencing of edible fungi (Dai et al., 2017; Sugano et al., 2017; Raman et al., 2021; Liu, 2021), greatly advancing research in this field. Therefore, systematic investigation of protoplast preparation for *P. giganteus* will facilitate further genetic breeding efforts.

Protoplast preparation has been extensively studied in various edible fungi species, including *Pleurotus ostreatus* (Peng et al., 1993), *Pleurotus eryngii* (Obatake et al., 2003), *Pleurotus cornucopiae* (Mizoguchi et al., 2006), *Ganoderma lucidum* (Li et al., 2016), *Auricularia* spp. (Cui et al., 2019), and *Agaricus bisporus* (Li et al., 2020). These studies have shown that protoplast preparation is a complex enzymatic process influenced by numerous factors, with conditions varying among different strains and even among different physiological states of the same strain. Su et al. (2020) found that mycelial age is a crucial factor, with significant differences in protoplast preparation conditions for *Auricularia cornea* at different ages. Peng et al. (2000) investigated protoplast isolation conditions for a wild *P. giganteus* strain using single-variable principles, demonstrating that protoplast yield was affected by enzymatic solution, osmotic stabilizer, mycelial age, and pH, though both preparation and regeneration rates remained low. Zou and Deng (2020) and Sun et al. (2022) further optimized single-factor results using orthogonal experiments and response surface methodology for *Agaricus blazei* and *Auricularia cornea*, respectively, substantially improving protoplast yield and quality. These findings indicate that protoplast preparation is influenced by multiple factors with important interactive effects. However, research on protoplast preparation in *P. giganteus*

remains limited, and the relative importance of various factors and their interactions are poorly understood, constraining rapid development of genetic breeding programs for this species.

Therefore, this study employed two *P. giganteus* strains with different temperature requirements as materials to investigate five key factors affecting protoplast preparation: mycelial age, lywallzyme concentration, osmotic stabilizer type, enzymatic hydrolysis temperature, and duration. Using single-factor and orthogonal experimental approaches, we aimed to clarify the optimal protoplast preparation conditions and differences between the two strains, thereby improving preparation efficiency. These results will provide a solid foundation for subsequent hybrid breeding, whole-genome sequencing, and variety improvement in *P. giganteus*, effectively promoting genetic breeding research for this important mushroom.

Materials and Methods

1.1 Materials Two *Pleurotus giganteus* strains with different temperature requirements were used: high-temperature strain PG46 and medium-temperature strain PG79 . Both strains are currently preserved at the Culture Collection Center of the Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences.

TABLE:1 Information on the two *Pleurotus giganteus* strains

Culture Media: MYG liquid medium contained 10 g maltose, 5 g glucose, and 5 g yeast extract per liter. Regeneration medium consisted of MYG liquid medium supplemented with 20 g agar and 0.6 mol $\cdot$ L^{-1} mannitol.

1.2 Methods 1.2.1 Protoplast Preparation

Activated mycelia were inoculated into MYG liquid medium and cultured at 26 °C in darkness at 150 rpm for 7 days. Under sterile conditions, mycelia were filtered and washed twice with sterile water and twice with osmotic stabilizer (0.6 mol $\cdot$ L^{-1} mannitol). The mycelia were blotted dry on filter paper, and approximately 300 mg were transferred to a 1.5 mL centrifuge tube. Three volumes of 2.0% lywallzyme solution (0.02 g lywallzyme in 1 mL 0.6 mol $\cdot$ L^{-1} mannitol) were added, gently mixed, and incubated at 30 °C for 4 h to obtain crude protoplast suspension. After filtration through sterile cotton, the suspension was centrifuged at 3,000 rpm for 5 min, the supernatant was discarded, and the pellet was gently rinsed twice with 0.6 mol $\cdot$ L^{-1} mannitol to remove residual enzyme solution. Finally, the pellet was resuspended in 1 mL of 0.6 mol $\cdot$ L^{-1} mannitol to prepare the protoplast stock solution.

1.2.2 Protoplast Regeneration

The protoplast stock solution was diluted to different concentrations using 0.6

mol · L^{-1} osmotic stabilizer and counted with a hemocytometer. Aliquots (200 L) of each dilution were spread onto regeneration medium, with sterile water-diluted protoplasts serving as controls. Both experimental and control plates were incubated at 26 °C in darkness, and colony regeneration was observed daily.

1.2.3 Single-Factor Experiments

Five factors were investigated: mycelial age (3, 5, 7, 9, 11 days), lywallzyme concentration (1.0%, 1.5%, 2.0%, 2.5%, 3.0%), osmotic stabilizer type (mannitol, sorbitol, sucrose, $MgSO_4$, KCl), enzymatic hydrolysis temperature (25, 27, 30, 32, 35, 38 °C), and enzymatic hydrolysis duration (2, 3, 4, 5, 6 h) (Cui et al., 2019; Su et al., 2020). Each experiment was performed with five replicates to determine the effects of each factor on protoplast yield in the two temperature-type strains.

1.2.4 Orthogonal Experiments

Based on single-factor results, orthogonal experiments were designed with four factors (mycelial age, lywallzyme concentration, enzymatic hydrolysis temperature, and duration) at three levels each, using protoplast yield as the response variable .

TABLE:2 Factors and levels of orthogonal experimental design

Results

2.1 Protoplast Release Process Mycelia of both strains were observed under microscopy during enzymatic hydrolysis, with samples taken every 30 minutes. Protoplast release occurred in three distinct stages [Figure 1: see original paper]: initially, a small number of protoplasts were released from hyphal tips; subsequently, lateral hyphal walls ruptured, releasing protoplasts from the sides; finally, with prolonged enzymatic hydrolysis, most hyphal cell walls disintegrated, resulting in massive protoplast release.

FIGURE:1 Microscopic images of protoplast release stages. A. Early stage: tip release; B. Middle stage: side release; C. Late stage: massive release.

2.2 Single-Factor Experiment Results 2.2.1 Effect of Mycelial Age on Protoplast Preparation

Protoplast yield in both strains increased initially and then decreased with mycelial age [Figure 2A: see original paper]. Maximum yields were achieved at 5 days for both PG46 (4.82$×10^{6}$ CFU · mL^{-1}) and PG79 (2.74$×10^{6}$ CFU · mL^{-1}), which were significantly higher than other ages. Beyond 5 days, protoplast yield declined sharply, reaching minimum values at 11 days (0.62$×10^{6}$ and 0.86$×10^{6}$ CFU · mL^{-1} for PG46 and PG79, respectively), even lower than the 3-day yields. Thus, the optimal mycelial age for protoplast preparation was 5 days for both strains.

2.2.2 Effect of Osmotic Stabilizer Type on Protoplast Preparation

Different stabilizers produced varying protoplast yields [Figure 2B: see original paper]. For PG46, yields ranked: mannitol > sorbitol > sucrose > $MgSO_4$ > KCl, with a maximum of 2.74$\times$10^{6}$ CFU $\cdot$ mL^{-1}. No significant differences were observed among the three organic sugar alcohols (mannitol, sorbitol, sucrose), but all were significantly superior to $MgSO_4$ and KCl. For PG79, yields ranked: sucrose > mannitol > sorbitol > KCl > $MgSO_4$, with a maximum of 1.88$\times$10^{6}$ CFU $\cdot$ mL^{-1}. Again, the top three organic stabilizers showed no significant differences but outperformed the inorganic salts. These results indicate that organic sugar alcohols (mannitol, sorbitol, sucrose) are suitable osmotic stabilizers for *P. giganteus* protoplast preparation.

2.2.3 Effect of Lywallzyme Concentration on Protoplast Preparation

Protoplast yield increased initially and then decreased with rising lywallzyme concentration [Figure 2C: see original paper]. Both strains achieved maximum yields at 2.5% concentration (7.64$\times$10^{6}$ and 7.38$\times$10^{6}$ CFU $\cdot$ mL^{-1} for PG46 and PG79, respectively). At 3.0% concentration, yields decreased significantly compared to the 2.5% level. Therefore, 2.5% lywallzyme was determined as the optimal concentration for protoplast preparation.

2.2.4 Effect of Enzymatic Hydrolysis Temperature on Protoplast Preparation

Protoplast yield increased initially and then decreased with rising temperature [Figure 2D: see original paper]. PG46 reached maximum yield at 32 °C (6.22$\times$10^{6}$ CFU $\cdot$ mL^{-1}), significantly higher than other temperatures. PG79 also achieved maximum yield at 32 °C (3.54$\times$10^{6}$ CFU $\cdot$ mL^{-1}), though this was not significantly different from yields at 27, 30, or 35 °C. Thus, the optimal temperature for PG46 was 32 °C, while PG79 tolerated a broader range of 27–35 °C.

2.2.5 Effect of Enzymatic Hydrolysis Duration on Protoplast Preparation

Protoplast yield increased initially and then decreased with prolonged hydrolysis time [Figure 2E: see original paper]. Both strains achieved maximum yields at 4 h (9.12$\times$10^{6}$ and 6.48$\times$10^{6}$ CFU $\cdot$ mL^{-1} for PG46 and PG79, respectively), with no significant difference from the 5 h treatment. Yields decreased significantly beyond 5 h. Therefore, 4–5 h was determined as the suitable enzymatic hydrolysis duration.

FIGURE:2 Effects of various factors on protoplast yield of two *Pleurotus giganteus* strains. A. Mycelial age; B. Osmotic stabilizer type; C. Lywallzyme concentration; D. Enzymatic hydrolysis temperature; E. Enzymatic hydrolysis duration. Different lowercase and uppercase letters indicate significant differences for PG46 and PG79, respectively ($P<0.05$).

2.3 Orthogonal Experiment Analysis To investigate interactive effects among factors, orthogonal experiments were conducted to determine optimal protoplast preparation conditions. For PG46, all nine orthogonal combinations supported protoplast preparation and regeneration . Combination 2 (mycelial age 5 d, lywallzyme concentration 2.5%, 0.6 mol · L^{-1} mannitol, 32 °C for 4 h) yielded the highest protoplast production (11.22$×10^{6}$ CFU · mL^{-1}), significantly surpassing other combinations. However, maximum regeneration rate (1.20%) was observed in combination 1 (mycelial age 5 d, lywallzyme concentration 2.0%, 0.6 mol · L^{-1} mannitol, 30 °C for 3 h), indicating that optimal preparation and regeneration conditions differed for PG46. Variance analysis showed that factors affecting PG46 protoplast preparation ranked as: mycelial age > lywallzyme concentration > enzymatic hydrolysis temperature > enzymatic hydrolysis duration, with all factors being highly significant ($P<0.001$).

For PG79, all nine combinations supported protoplast preparation, though some were unsuitable for regeneration . Maximum protoplast yield (7.28$×10^{6}$ CFU · mL^{-1}) was achieved in combination 3 (mycelial age 5 d, lywallzyme concentration 3.0%, 0.6 mol · L^{-1} mannitol, 35 °C for 5 h), though this was not significantly different from combinations 1 and 2. Maximum regeneration rate (0.45%) occurred in combination 1, but no regeneration was observed under optimal preparation conditions. Variance analysis revealed that factors influencing PG79 protoplast preparation ranked as: mycelial age > enzymatic hydrolysis duration > enzymatic hydrolysis temperature > lywallzyme concentration, with all factors being significant ($P<0.05$).

Discussion and Conclusion

This study optimized key factors affecting protoplast preparation (mycelial age, osmotic stabilizer type, lywallzyme concentration, enzymatic hydrolysis temperature, and duration) in two temperature-type strains of *P. giganteus* using single-factor and orthogonal experiments. The results demonstrated that optimal preparation conditions were essentially identical for both strains, with combination 2 (mycelial age 5 d, lywallzyme concentration 2.5%, 0.6 mol · L^{-1} mannitol, 32 °C for 4 h) producing the highest yields, significantly exceeding single-factor experiment results. This confirms that interactive effects among factors are important and cannot be ignored during optimization. F-test analysis revealed that mycelial age had the most significant influence on protoplast yield in both strains, while the relative importance of lywallzyme concentration, temperature, and duration differed between strains, likely due to genetic diversity and differentiation between PG46 and PG79 (Dai et al., 2019).

The physiological state of strains and enzymatic hydrolysis conditions differentially affect protoplast yield and quality in edible fungi (Sun et al., 2022). In this study, mycelial age was the most critical factor affecting protoplast preparation. Cell wall thickness and growth vigor vary during different growth

phases, influencing sensitivity to lywallzyme (Zhang et al., 2003). During early growth, thinner cell walls are more easily degraded, while increased age leads to thickened walls and accumulation of secondary metabolites, reducing enzymatic efficiency and protoplast yield (Kim et al., 2000). Our preliminary observations indicated that day 5 represented the exponential growth phase for *P. giganteus* mycelia, with optimal cell wall thickness and vigor, making it ideal for protoplast preparation.

Since protoplasts lack cell walls and are highly sensitive to environmental conditions, osmotic stabilizers are essential for maintaining pressure balance and preventing rupture or shrinkage (Pasternak et al., 2002). Stabilizers also mediate the reaction between cells and lywallzyme, with their properties affecting enzymatic activity (Liu et al., 2009). Our results showed that organic sugar alcohols (mannitol, sorbitol, sucrose) produced significantly higher yields than inorganic salts ($MgSO_4$, KCl). Previous studies have reported similar differences between organic and inorganic stabilizers in edible fungi (Zou & Deng, 2020; Sun et al., 2022), possibly because organic sugar alcohols not only maintain osmotic balance but also promote enzyme-substrate binding, thereby enhancing enzymatic reaction yields (Tan et al., 2006).

Enzymatic solution is critical for cell wall lysis and protoplast release, with enzyme type and concentration affecting protoplast viability and quantity (Kanchanapoom & Jantaro, 2001). Previous studies have shown that single lywallzyme is suitable for protoplast preparation in many edible fungi, offering high efficiency and low cost (Li, 2018). However, due to variations in fungal cell wall composition and structure, different lywallzyme concentrations produce varying effects. Our results indicated that protoplast yield increased with enzyme concentration up to 2.5%, beyond which yields declined, possibly because excessive enzyme concentrations damage protoplast membranes, causing overly complete cell wall removal and affecting subsequent regeneration (Sun & Zhou, 2002).

At constant lywallzyme concentration, temperature and duration are key determinants of cell wall degradation efficiency. According to enzyme kinetics principles, temperature directly affects reaction rate and cell wall physiological state, with optimal temperatures maximizing lywallzyme activity. Our study identified 32 °C as optimal for both strains; lower temperatures required extended hydrolysis time for adequate yields, while higher temperatures reduced both yield and regeneration rate. This may be because low temperatures insufficiently activate enzymes and slow mycelial metabolism, whereas high temperatures destabilize enzymes, reduce lywallzyme activity, and damage released protoplasts, impairing regeneration (Zhang & Guo, 2008; He & Guo, 2012). Enzymatic hydrolysis duration also affects reaction extent, with appropriate extension increasing protoplast release. Our results showed 4 h as optimal for both strains, with yields declining beyond this time point. This likely reflects decreased stability of cell wall-less protoplasts, which may rupture due to enzymatic and osmotic effects during prolonged treatment. Additionally, protoplast regeneration requires some cell wall debris, which may become depleted during

extended hydrolysis (Guo & Zhao, 2009). Wang et al. (2013) similarly reported that excessive hydrolysis time causes early-released protoplasts to rupture without cell wall protection, reducing viability and regeneration capacity. Therefore, appropriate hydrolysis duration is crucial.

In summary, the optimal protoplast preparation conditions were identical for both temperature-type strains, though the relative importance of factors differed. When preparing protoplasts from different *P. giganteus* strains, conditions should be adjusted according to specific strain characteristics and experimental requirements. These results provide a foundation for future hybrid breeding, genetic transformation, and whole-genome sequencing studies, promoting the development of the *P. giganteus* industry.

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Note: Figure translations are in progress. See original paper for figures.