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RESEARCH ARTICLE

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Optimization of Protoplast Preparation and Regeneration of a Medicinal Fungus *Antrodia cinnamomea*

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ABSTRACT

Antrodia cinnamomea is a unique medicinal fungus in Taiwan. It has been found rich in some pharmacologically active compounds for anti-cancer, hangover, and immune regulation etc. With the in-depth study of these components, it would be interesting and important to establish a molecular system for basic studies of *A. cinnamomea*. Thus, we would like to set up a foundation for this purpose by studying the *A. cinnamomea* protoplast preparation and regeneration. Firstly, we studied the optimization method of protoplast preparation of *A. cinnamomea*, and found various factors that may affect the yield during protoplast preparation, such as mycelial ages, pH values, and osmotic stabilizers. Secondly, in the regeneration of protoplasts, we explored the effects of various conditions on the regeneration of protoplasts, including different media and osmotic pressure. In addition, we found that citrate buffer with pH value around 3 dramatically increased the regeneration of protoplasts of *A. cinnamomea*, and provided a set of regeneration methodology for *A. cinnamomea*.

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Antrodia cinnamomea; citrate buffer; medicinal fungus; protoplast preparation; protoplast regeneration

1. Introduction

Filamentous fungi are widely existing in the field, and their secondary metabolites are commonly used in all aspects of our daily life. The *Antrodia cinnamomea* is a unique slow growing filamentous and medicinal fungus in Taiwan. Its medicinal use was reported in many studies, such as functions in antioxidant [1,2], anti-cancer [3], anti-virus [4], and anti-biotics [3] activities. Because of its broad spectrum of medicinal application and extremely slow growth rate, *A. cinnamomea* is now considered as one of the most expensive herbal medicines in the market.

In the natural environment, *A. cinnamomea* only parasitizes on the hollow trunk of *Cinnamomum kanehirai*, a large evergreen broad-leaved tree that grows on broad-leaved forests at altitude between 200 and 2000 meters in Taiwan [5]. Because of decreasing population of *C. kanehirai* caused by deforestation, the wild *A. cinnamomea* is fewer and more expensive. Thus, researchers have been working on all aspects with this fungus, including medical effects [6], secondary metabolite identification and profiling [7], fruiting body formation and production [8], gene transformation [9], and molecular biology [10]. Based on the research perspective, protoplast preparation and regeneration may provide a useful tool for further basic and applied researches of *A. cinnamomea*.

The cell wall is a very important cellular structure of certain organisms such as plants, fungi, algae and bacteria. It carries various functions such as supporting cell configuration, providing permeability barriers, and protecting cells in harmful environments. The cells without cell walls are called protoplasts. As well as plant cells, the protoplasts produced from fungi have totipotency which can be induced to regenerate a new individual by external stimuli [11]. Cell walls can be removed through mechanical or enzymatic methods to produce protoplasts [12]. The fungal cell walls are composed of polymers such as dextran, chitin, mannose, and manno-proteins. The chemical compositions of the cell walls varied depending on the fungal species, growth environments, and cell growth patterns [13]. These variables make the composition and structure of the cell wall different, so the digestion of cell wall. The required digestive enzymes, such as chitinases, cellulases and proteases, vary depending on the cell wall structure. Therefore, the enzyme mixture selected from the appropriate components and ratios is a key factor for protoplast preparation.

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Supplemental data for this article can be accessed here.

In addition, protoplasts are very sensitive to osmotic pressure during the cell wall digestion of filamentous fungi. It requires isotonic solution of specific salts or sugars to preserve protoplast integrity during the entire preparation process. Such substances used to stabilize osmotic pressure are called osmotic stabilizers and used to maintain cell conformation and prevent cell membrane breakage [14]. Due to the lack of a cell wall barrier, basic studies on cell membranes and organelles can be performed easier, such as cell fusion, gene transformation, microinjection [15].

In many studies, the *A. cinnamomea* extract has been found to contain many therapeutically effective ingredients even with the potential to develop anticancer drugs [16]. With the in-depth study of *A. cinnamomea*, more functional genes and metabolic pathways will be explored in the future. Therefore, we would like to establish a procedure of high-efficiency system for protoplast preparation and regeneration which may be applied in subsequent *A. cinnamomea*-related researches.

2. Materials and methods

2.1. Fungal strains and chemicals

The *A. cinnamomea* strain 1337-3 is a monokaryotic haploid strain of a single basidiospore isolate derived from the field-collected dikaryotic haploid *A. cinnamomea* strain 1337 and is publicly available from Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan (http://www.bcrc.firdi.org.tw) with catalog number BCRC MU30687 *Antrodia cinnamomea* 1337-3. All the chemicals used were of analytical grade or higher.

2.2. Growth of A. cinnamomea

The *A. cinnamomea* was grown in a so-called 50% malt extract agar (MEA) composed of 1% glucose (Merck KGaA, Darmstadt, Germany), 1% malt extract, 0.05% peptone and 2% agar (Becton, Dickinson & Co, Sparks, MD, USA) by weight/volume [10]. The medium is sterilized at 121 °C for 30 min. A 90 × 10 mm petri dish containing 50% MEA was inoculated with a 2 × 2 mm agar blot with full grown fungus. The fungus was incubated at 25 °C in the dark. The whole processes were under aseptic condition.

2.3. Preparation of the conidia of A. cinnamomea 1337-3

The conidia of *A. cinnamomea* 1337-3 were collected from a four weeks old 50% MEA culture. The samples were gently rinsed and collected with 10 mL of sterilized water. Then, the samples were passed through a so-called cell strainer with 40 μm pore size (BD Falcon^{TM}, Sparks, MA, USA) three times to remove the broken hyphae, and the conidia were washed with sterilized water at 3000 *g* for 10 min to remove any debris by 3 times. The conidia collected were counted on a cell counter and the concentration of the conidia was adjusted. A total of 1×10^9 conidia were cultured in 50 mL malt extract broth (MEB; 2% glucose, 2% malt extract, 0.1% peptone) at 160 rpm and 25 °C.

2.4. Hyphal preparation for protoplast preparation

For preparation of protoplasts, the hyphal materials were collected from the MEB liquid media inoculated with conidia with the addition of 0.024% $MgSO_4$ (Merck KGaA). The MEB was adjusted to pH 4.5 with HCl. The cultured hyphae were collected by centrifugation at 3000 *g* for 10 min. The new mycelia, called germlings, germinated from the conidia is about 20–40 µm.

2.5. Effects of fungal ages on protoplast preparation

In order to test the effect of enzyme digestion on the protoplast yield of different fungal ages, the new mycelia were cultured in MEB for 16, 24, 30, 36 and 48 h. After completion of the culture, the hyphae were collected by centrifugation at 2000 *g* for 10 min, and adjusted to 2×10^8 germlings per digestive tube through cell counting plate. Digestive solution with 0.9 M sucrose, 50 mM citrate buffer at pH 6.0, and 20 mg mL^{-1} lysing enzyme (Sigma-Aldrich, St. Louis, MO, USA) were filter sterilized with a 0.45 µm filter tube (Acrodisc® Syringe Filters with Supor® Membrane; Pall Biotech, Port Washington, New York, USA). Digestive solution and the hyphae were mixed on a rotary oscillator (RT-01B; TKS, Keelung, Taiwan) with 120 rpm at 30 °C. After 6 h shaking, the digest solutions were checked under microscope for the protoplast morphology and counting.

2.6. Effects of pH on protoplast preparation

For determination of pH effects on protoplast preparation, 2×10^8 30 h old germlings were added to a final volume of 1 mL digestive mix with 20 mg mL^{-1} lysing enzyme and 1.1 M $MgSO_4$ at different pH buffers. The pH buffers were prepared with 50 mM potassium phosphate buffer at pH 7.2, pH 6.8 and pH 6.4, and with 50 mM citrate buffer at pH 6.0, pH 5.6, pH 5.2 and pH 4.8. The reaction mixes were gently shaken with 120 rpm at 30 °C. The reaction conditions were checked at 3, 4, 5, and 6 h for the protoplast production.

2.7. Effects of osmotic stabilizers on protoplast preparation

Four osmotic stabilizers were tested for the protoplast preparation, including mannitol, KCl, $MgSO_4$ and sucrose. In addition, different concentrations of these osmotic stabilizers were also tested for the effects on protoplast production at 0.7 M, 0.8 M, 0.9 M, 1.0 M, 1.1 M, 1.2 M and 1.3 M. Each reaction mix contained a fixed concentration of 20 mg mL^{-1} lysing enzyme, 50 mM citrate buffer at pH 6.0, and 2×10^8 30 h old germlings as described above. The reaction mixes were gently mixed with 120 rpm at 30 °C for digestion. The protoplast production was checked for quality and quantification at digestion time of 3, 4, 5, 6, 7, 8 and 9 h, respectively.

2.8. Effects of digestive enzyme concentrations on protoplast preparation

Different concentrations of digestive enzymes were tested for efficiency of the protoplast preparation. The reaction mixes contained 2×10^8 germlings mL^{-1} (30 h old) with 1.1 M $MgSO_4$, 50 mM citrate buffer at pH 6.0, the optimal pH for protoplast preparation based on above results, with 5 different concentrations of 6.25, 12.5, 25, 37.5 and 50 mg mL^{-1} lysing enzyme at 30 °C, 120 rpm. The protoplast preparation was checked for quality and quantification after 4 h of digestion.

2.9. Protoplast regeneration in solid media

The protoplasts produced through the optimal preparation procedure described above were washed with 0.8 M sucrose and span at 2000 *g* for 10 min. The protoplasts were then counted in a cell counting plate. The protoplast concentration was diluted to about 2×10^4 cells mL^{-1}, and 100 µL of protoplast preparation was plated onto a 90×20 mm petri dish plate. Different nutrient sources were tested, including potato dextrose broth (PDA with 2% agar; Becton, Dickinson & Co.), MEA with 2% agar, and non-nutrient medium (2% agar only) as the control. In addition, different concentrations of sucrose were tested at 0 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M, respectively. The protoplasts were cultured in the dark at 25 °C for 7 days. After 7 days of growth, the number of colonies on the culture plate was calculated for regeneration rate.

2.10. Protoplast regeneration in liquid media

The protoplast regeneration in liquid culture was carried out in five independent culture experiments. A 10 µL protoplast suspension with a concentration of 2×10^8 cells mL^{-1} was added to the liquid medium with different conditions, and cultured at 27 °C, 25 rpm for 2 days. The number of regenerated protoplasts and un-regenerated protoplasts was calculated on cell counter and the regeneration rate was calculated.

2.11. Effects of osmotic pressure

The protoplasts prepared according to the optimal condition obtained in the above experiment were washed three times with 0.8 M sucrose and 2000 *g* for 10 min, and then counted and diluted to a concentration of about 2×10^8 cells mL^{-1}. A 10 µL of protoplast suspension was mixed with 990 µL of MEB with concentration of 0.6 M, 0.8 M and 1.0 M sucrose, respectively, and 50 mM citrate buffer at pH 3, 27 °C and 25 rpm for 2 days. The samples were counted and observed for the effects of different osmotic pressure on the growth morphology and regeneration rate of protoplasts.

2.12. Effects of nutrient sources

A 10 µL of protoplast suspension was mixed with 990 µL of PDB, MEB and nutrient-free medium, respectively, with 0.8 M sucrose and incubated at 27 °C, 25 rpm for 2 days. The samples were checked for regeneration and growth morphology.

2.13. Effects of citric acid concentrations in three different nutrient sources

The effect of different citric acid concentrations on the regeneration of protoplasts in liquid culture was carried out in combination with different nutrient sources. A 1 mL protoplast suspension with a concentration of 2×10^8 cells mL^{-1} was precipitated at 2000 *g*, and re-suspended with PDB, MEB and non-nutrient medium with 0.8 M sucrose. The final concentration of citrate was 0, 1, 10, 20, 40, 80, 160 and 320 mM, respectively. The samples were then cultured at 25 °C, 25 rpm for 2 days. The samples were checked for regeneration and growth morphology.

2.14. Effects of pH values

A 1 mL protoplast suspension with a concentration of 2×10^8 cells mL^{-1} was precipitated at 2000 *g*, and re-suspended with PDB, MEB and non-nutrient medium with 0.8 M sucrose at pH 3, pH 4, pH 5 and pH 6 buffers with a final concentration of 50 mM citrate buffer, and pH 7 and pH 8 phosphate buffers. A culture medium without pH buffer is used as a control. The samples were grown at 27 °C and 25 rpm for 2 days. The culture samples were checked for the effect of different pH values on the

growth morphology of protoplasts and the protoplast regeneration rate.

2.15. Effects of citrate buffer concentration at pH 3

The citric acid at pH 3 was found to have dramatic effects on protoplast regeneration. Therefore, we made a detail test for its concentration effect on protoplast regeneration. A 1 mL protoplast suspension with a concentration of 2×10^8 cells mL^{-1} was precipitated at 2000 *g*, and re-suspended with PDB with 0.8 M sucrose at pH 3 citrate buffer of 20, 40, 80, 160 and 320 mM, and cultured at 27 °C, 25 rpm for 2 days. The culture samples were checked for the effect of different pH values on the growth morphology of protoplasts and the protoplast regeneration rate.

2.16. Protoplast counting and statistical analysis

For protoplast preparation, all experiments were carried three repeats. The number of protoplasts was calculated on cell counter. The protoplasts have an average diameter around 12 µm. The protoplasts with a diameter less than 8 µm were excluded. The final values were represented as mean ± standard error. For protoplast regeneration experiments, the solid state culture was carried out in three independent culture experiments. The number of colonies on each plate was measured and the regeneration rate was calculated. The values of the protoplast regeneration rate were represented as mean ± standard error. The calculation method of the regeneration rate is referred to (17]. The protoplast regeneration data analysis was based on two sample *t*-test to compare the mean values between the two groups of samples, when $p < .05$ is a significant difference.

3. Results

3.1. Optimization of protoplast preparation of A. cinnamomea

3.1.1. Fungal ages

The effect of conidia cultural time was tested for protoplast preparation. We found the sample from 30-h MEB suspension culture producing the most protoplasts as shown in Figure 1. The concentration of protoplast based on this study reached to 5.2×10^7 cells mL^{-1}.

3.1.2. pH values

The reaction mix with various pH was examined at digestive time of 3, 4, 5 and 6 h, respectively. As shown in Figure 2, the reaction produced most

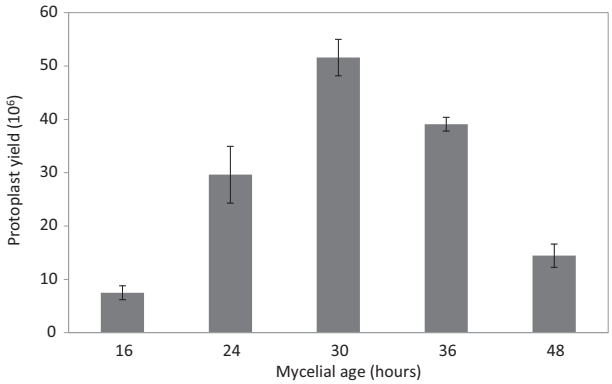

Figure 1. The effects of fungal ages on protoplast yields of *A. cinnamomea*. The fungal samples were from different cultural time of conidia at 16, 24, 30, 36 and 48 h, respectively. Digestive solution contained 0.9 M sucrose, 50 mM citrate buffer at pH 6.0, and 20 mg mL^{-1} lysing enzyme.

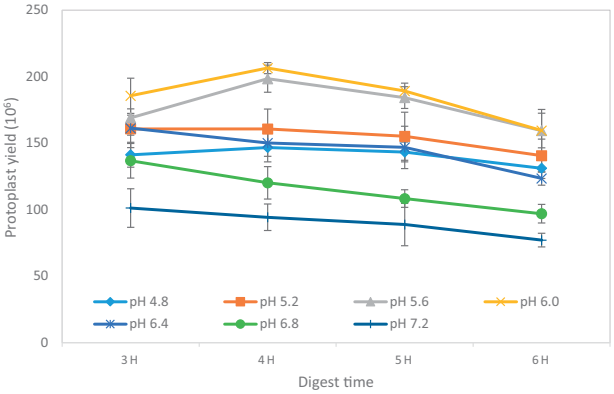

Figure 2. The pH effects of digestive solution on *A. cinnamomea* protoplast yields. A total of 2×10^8 30 h old germlings were digested in digestive solution with 20 mg mL^{-1} lysing enzyme and 1.1 M $MgSO_4$ at different pH buffers. The pH buffers were prepared with 50 mM potassium phosphate buffer at pH 7.2, pH 6.8 and pH 6.4, and with 50 mM citrate buffer at pH 6.0, pH 5.6, pH 5.2 and pH 4.8.

protoplasts at pH 6. This condition at pH 6 yielded highest concentration of protoplasts at 2.1×10^8 cells mL^{-1} with 4-h digestion.

3.1.3. Osmotic stabilizers

We tested 4 different osmotic stabilizers for the effects of protoplast preparation. As shown in Figure 3, the reaction mix with 1.1 M $MgSO_4$ as osmotic stabilizer carried the best result with protoplast production at 2.1×10^8 cells mL^{-1} after 4-h digestion. In addition, we would like to examine the effect of osmotic stabilizer on the stability of protoplasts. Thus, we examine the protoplast yield every h from 3 to 9 h of reaction time. As shown in Figure 3, the protoplast yields in the reaction mix with $MgSO_4$ and KCl as osmotic stabilizers gradually decreased after 4-h digestion. On the other hand, sucrose as the osmotic stabilizer maintained a stable protoplast yield throughout the 9-h digestion. Thus, in order to obtain the maximum yield and

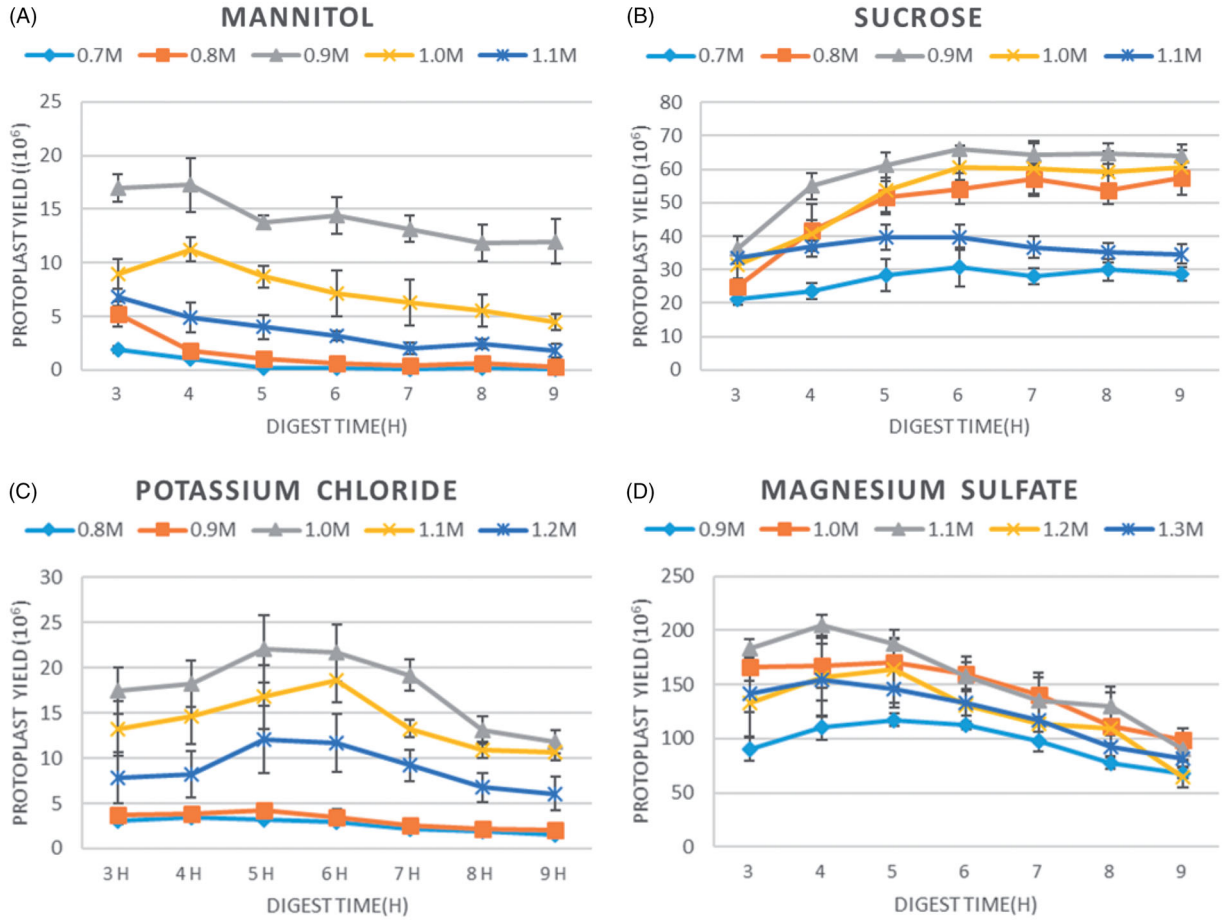

Figure 3. Effects of osmotic stabilizers on *A. cinnamomea* protoplast yields. Four osmotic stabilizers were tested for the protoplast preparation, including mannitol (A), sucrose (B), KCl (C), and $MgSO_4$ (D). Each reaction mix contained 20 mg mL^{-1} lysing enzyme, 50 mM citrate buffer at pH 6.0, and 2×10^8 30 h old germlings.

optimal stability of protoplasts, the protoplast preparation experiments used $MgSO_4$ as the osmotic stabilizer, and the protoplast regeneration experiments used sucrose as the osmotic stabilizer.

3.1.4. Enzyme concentrations

To obtain the optimal enzyme concentration for protoplast preparation, various concentrations of lysing enzyme were tested. As shown in Figure 4, the protoplast preparation reached the highest production when the lysing enzyme concentrations were 25 mg mL^{-1}or higher. The yield of protoplasts is about 2.0×10^8 cells mL^{-1}.

Taken together, based on the above experiments, we concluded the optimal condition for protoplast preparation of *A. cinnamomea* was as follows: fungal sample from conidial MEB culture for 30 h and at the concentration of 2×10^8 germlings mL^{-1}, the reaction mix was prepared with 1.1 M $MgSO_4$, 25 mg mL^{-1} lysing enzyme, a final concentration of 50 mM citrate buffer at pH 6.0, and incubated at 30 °C, 120 rpm for 4 h. The concentration of protoplasts yielded was about 2.1×10^8 cells mL^{-1}. The morphology of the protoplasts prepared was shown in Figure S1.

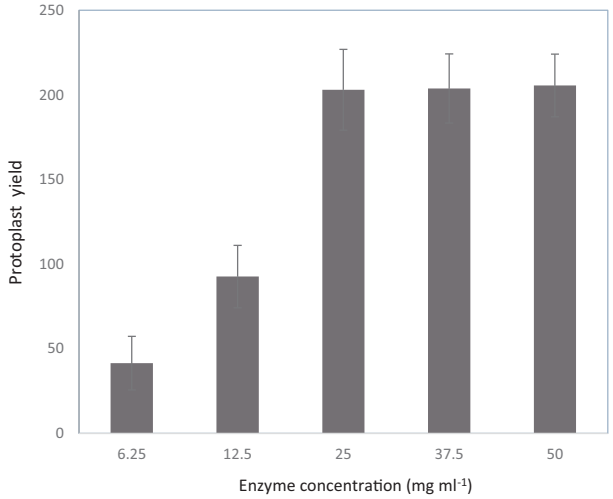

Figure 4. Effects of digestive enzyme concentrations on *A. cinnamomea* protoplast yields. The reaction mixes contained 2×10^8 30 h old germlings mL^{-1} with 1.1 M $MgSO_4$, 50 mM citrate buffer at pH 6.0 and 5 different concentrations of lysing enzyme at 6.25, 12.5, 25, 37.5 and 50 mg mL^{-1}.

3.2. Protoplast regeneration of A. cinnamomea in solid media

Three different nutrient media, PDA, MEA and nutrient-free media, combined with different osmotic pressures were tested for protoplast

regeneration in solid media. The colonies at different osmotic pressures and different nutrient sources were examined as shown in Figure S2. The protoplasts under different nutrient sources were compared with each other. It showed that the mycelial growth on PDA and MEA media was stronger than that of the nutrient-free medium. The colony diameter is relatively larger on PDA and MEA media rather than that on nutrient-free medium under the same osmotic pressure. The protoplast regeneration rates as shown in Figure 5 indicate that they are not statistically significant under the same osmotic pressure. However, the regeneration rate gradually increased with the increase of osmotic pressure indicating the osmotic pressure may be an important factor for the protoplast regeneration of *A. cinnamomea*.

3.3. Protoplast regeneration of A. cinnamomea in liquid media

3.3.1. Osmotic pressure

As shown in Figure 6(a), the sucrose concentration at 0.8 M carried the highest regeneration rate around 69.5% compared with 55.5% of 0.6 M sucrose and 30.6% of 1 M sucrose. The morphology of the protoplasts after regeneration in the liquid culture is shown in Figure 6(b). From the Figure 6(b), hyphae grown in MEB with 0.8 M sucrose are more uniform in length than that with 0.6 M sucrose. The growth and regeneration of the protoplasts in MEB with 1.0 M sucrose was poor compared with those with 0.6 M sucrose or 0.8 M sucrose.

3.3.2. Nutrient sources

The regeneration rate for MEB culture was 24.0%, followed by 23.3% in PDB culture and 21.3% with the nutrient-free medium. However, the regeneration rates of the three media were not statistically different indicating the nutrient sources were not an important factor in the protoplast regeneration in liquid culture (data not shown).

3.3.3. Citric acid concentrations

From our preliminary experiments, we found citric acid may carry novel effects on protoplast regeneration. Thus we used above condition with addition of various concentrations of citric acid for

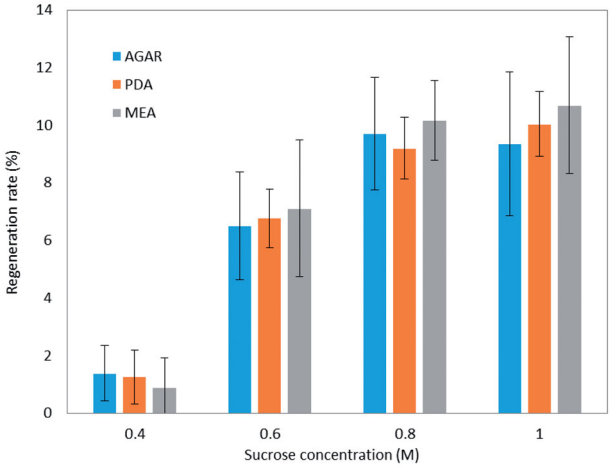

Figure 5. Protoplast regeneration in solid media with different nutrient sources (MEA, PDA, and nutrient-free 2% agar) and osmotic pressures (0.4 M, 0.6 M, 0.8 M and 1.0 M sucrose). The protoplasts were cultured in the dark at 25 °C for 7 days.

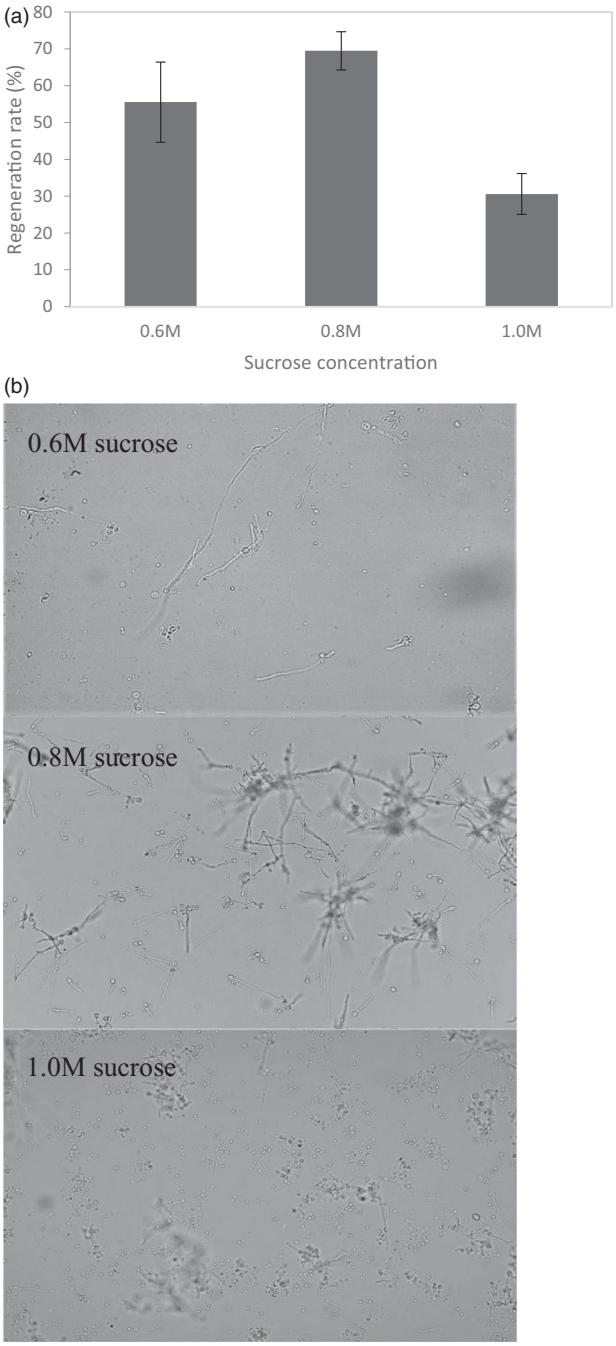

Figure 6. The osmotic pressure effects for protoplast regeneration in liquid culture. The *A. cinnamomea* was cultured in MEB medium containing 0.6 M, 0.8 M and 1.0 M sucrose, respectively, in addition to a final concentration of 50 mM citrate buffer at pH 3, 25 rpm shaking for 2 days at 27 °C.

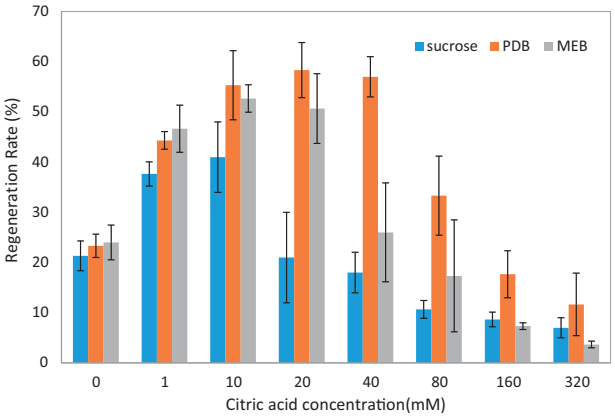

Figure 7. Effects of citric acid concentrations in three different nutrient sources, PDB, MEB and nutrient-free medium, on *A. cinnamomea* protoplast regeneration in liquid culture.

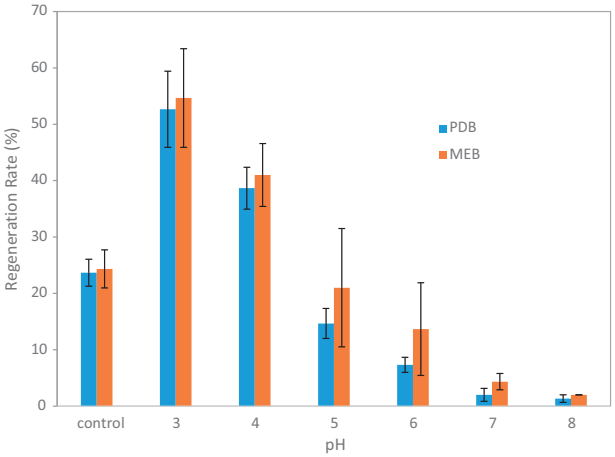

Figure 8. Effects of pH values on *A. cinnamomea* protoplast regeneration in PDB and MEB cultures with 0.8 M sucrose. The pH 3, pH 4, pH 5 and pH 6 buffers were prepared with a final concentration of 50 mM citrate buffer, and phosphate buffer for pH 7 and pH 8. A culture medium without pH buffer is used as a control (MEB with initial pH 5.1; PDB with initial pH 4.9).

protoplast regeneration in liquid culture. As shown in Figure 7, addition of 1 and 10 mM citric acid significantly increased the protoplast regeneration rate ($p < .05$) for all three culture media. With the citric acid concentration higher than 20 mM, the regeneration rate was gradually decreased. The effect in protoplast regeneration turned into negative when the citric acid concentration higher than 80 mM.

3.3.4. pH values

Based on the study of citric acid effect on protoplast regeneration, it is interesting to us to see whether pH value may play a role in this effect. As shown in Figure 8, the regeneration rate was highest both for MEB (54.7%) and PDB (52.7%) culture at pH 3. It also shown that as pH value increased higher than pH 3, the regeneration rate of protoplasts gradually decreased. The regeneration rates between pH 5 to pH 8 were even lower than that of the control

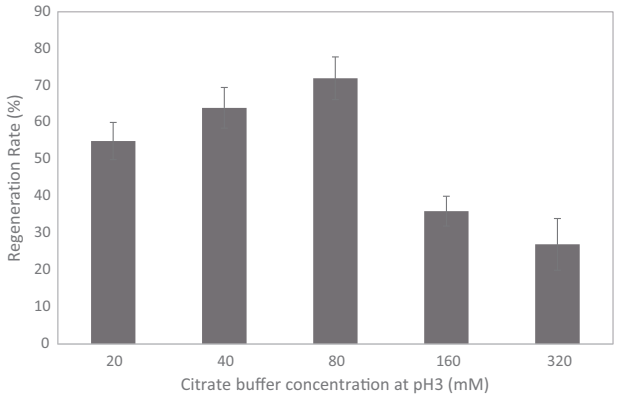

Figure 9. Effects of citric acid concentration at pH3 on *A. cinnamomea* protoplast regeneration in PDB with 0.8 M sucrose.

indicating a negative effect of higher pH value for protoplast regeneration.

3.3.5. Citrate buffer concentrations at pH 3

From the above studies, citric acid and pH value at 3 may carry a significant effect in the protoplast regeneration rate. Therefore, we further examine the effect of different citrate buffer concentrations at pH 3. As shown in Figure 9, the citrate buffer at a final concentration of 80 mM had the highest regeneration rate about 71.3%.

Taken together, the optimal regeneration condition for the protoplasts of *A. cinnamomea* was a suspension culture in PDB with 0.8 M sucrose, a final concentration of 80 mM citrate buffer at pH 3, and cultured at 27 °C, 25 rpm for 2 days. Under this condition, the morphogenesis of protoplast regeneration can be easily spotted as shown in Figure 10 that protoplast polarization, tip formation, cell wall synthesis and hyphal formation can be observed.

4. Discussion

Based on earlier study of fungal protoplast preparation [17], we examined various factors for protoplast preparation of *A. cinnamomea*, including strain varieties, mycelial ages, medium sources, osmotic stabilizers, digestive enzymes, digestive reaction time, temperature, and pH value. Several factors showed great impact on the yield of protoplasts.

The growth of filamentous fungi varies greatly with the external culture environment. The type of medium, pH value, temperature and culture time affect the structural composition of the fungal cell walls. As time increases, the cell wall structure reacts with various enzymes on the membrane or in the external environment, making it more complex and thickening. In order to make the structure

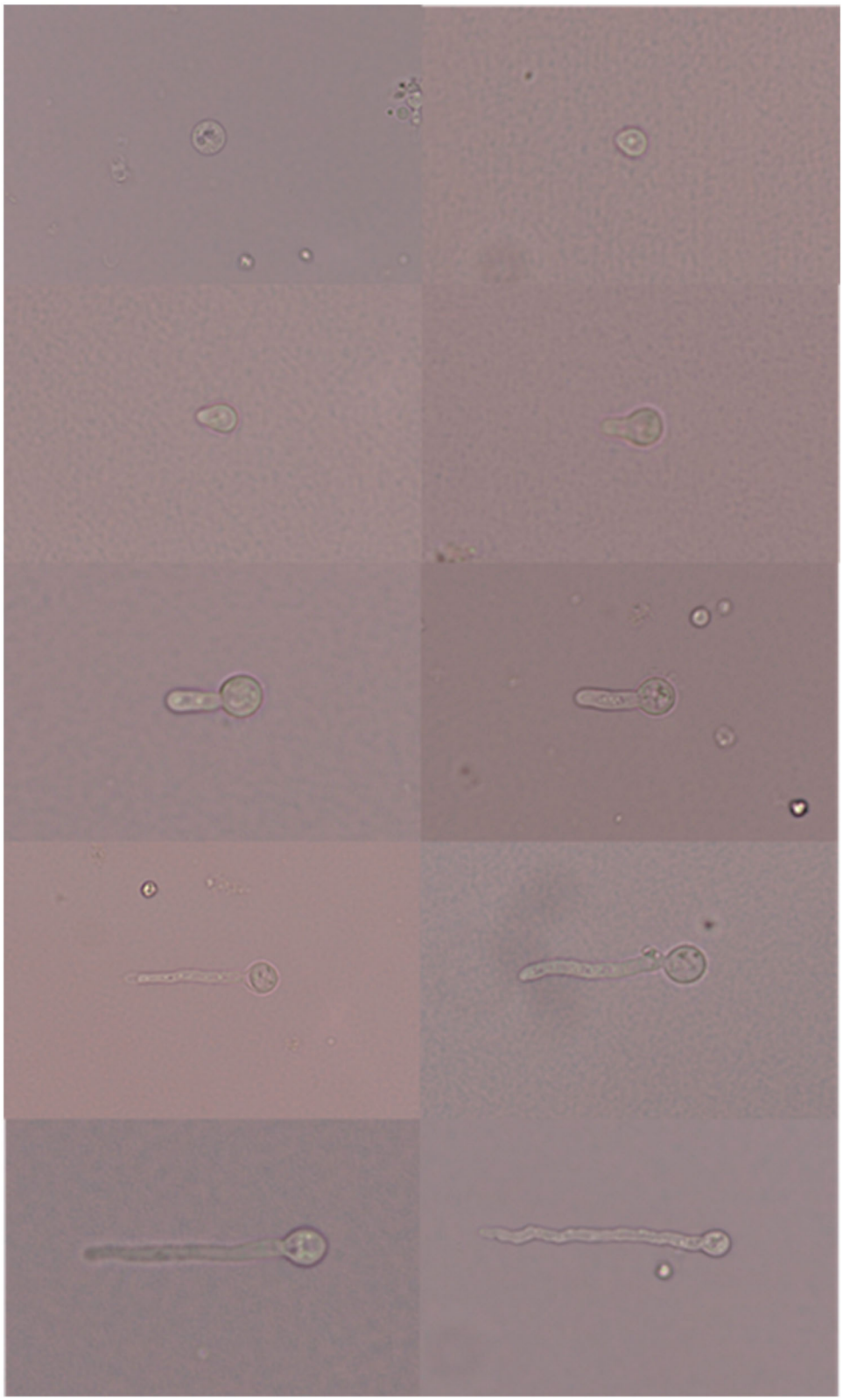

Figure 10. The morphogenesis of *A. cinnamomea* protoplast regeneration under optimal protoplast regeneration condition.

of the fungal cell wall consistent, the major medium used was the MEB formula as described earlier [10]. For the selection of mycelial materials, fresh hyphae with relatively simple structure and chemical composition of cell wall were used. We found the best results of protoplast preparation come from digestion of conidia with MEB cultured for 30 h. The hyphae under this condition are of about 20–50 μm in length and more evenly distributing in suspension media making it possible to quantify the number of hyphae and calculate the relationship between the mycelial concentration and the digestive enzyme. By controlling the concentration of the substrate and the digestive enzymes we may obtain a relatively clean protoplast solution.

Different osmotic stabilizers have a considerable effect on the protoplast yield. When the protoplasts are released from the hyphae, the osmotic pressure balance stabilized both sides of the cell membrane. The osmotic stabilizer concentration is required for protection of protoplasts. If the concentration is too high, the volume of the protoplasts will be reduced and the regeneration may be inhibited. If the concentration is too low, the protoplasts will be bulky and may be broken. The osmotic stabilizer will also effect of enzyme activity, which indirectly affects the yield of protoplasts. Based on the result (Figure 3), sucrose was chosen as main osmotic stabilizer in later experiments for protoplast regeneration. Under the conditions obtained, it showed that the mycelial cell wall was almost completely digested, and no further filtration step was required to remove the cell wall debris (Figure S1). The number of protoplasts obtained was 2.1×10^8 cells mL^{-1} which was much higher than earlier studies when compared with the literature on the preparation of protoplasts of *A. cinnamomea* [18].

After the protoplast preparation was optimized as described, subsequent experiments for protoplast regeneration were carried out. According to previous reports on fungal protoplast regeneration [19], the factors that may affect protoplast regeneration are hyphae material, osmotic stabilizer, protoplast digestion time, and medium type. Thus, we firstly selected the fungal sample at a culture stage of exponential growth phase for better protoplast regeneration. Since sucrose was the best osmotic stabilizer for the protoplast stability (Figure 3), the protoplast regeneration experiment used sucrose as the osmotic pressure stabilizer. Reaction time is also a factor that may affect the rate of protoplast regeneration. The longer the cell in protoplast form, the lower the survival rate and regeneration rate [17]. The $MgSO_4$ was found in digestion reaction as the best osmotic stabilizer tested for protoplast production (Figure 3). Therefore, $MgSO_4$ can shorten the digestion time and increase regeneration rate.

The reaction mix can be divided into two portions after centrifugation. The upper supernatant contained low-density and highly vacuolated protoplasts, and the bottom precipitate contained highly dense and healthy protoplasts. According to earlier studies [20], the protoplasts in bottom precipitate have higher translation ability.

In addition to cell quality, protoplast regeneration is also affected by the external environment. We studied the regeneration of protoplasts under solid and liquid culture environments. As shown in Figure 5, different nutrient sources on the solid cultures have no significant effects for protoplast regeneration. But the osmotic pressure significantly affected the regeneration rate. In the colony morphology, it showed that PDA, MEA and control groups could produce colonies after protoplast regeneration (Figure S2), but the hyphal growth in PDA or MEA was better than that in the control. In addition, the mycelial growth was slower with the increase of osmotic stabilizer. Together, this result indicates that no nutrient in media were needed at the beginning of protoplast regeneration, but the subsequent nutrient supply after regeneration of the protoplasts allows the hyphae to continue to grow and the colonies to expand.

In term of protoplast regeneration in liquid culture, it showed that the regeneration rate was better at 0.8 M sucrose osmotic pressure rather than 0.6 M and 1.0 M sucrose (Figure 6(a)). At 0.6 M sucrose, the protoplasts had worse regeneration rate and the hyphal length was less uniform compared with the 0.8 M sucrose culture media. Fuchino et al. [21] reported that mycelial polarization occurred more often when the hyphae grew in a lower osmotic pressure. It indicated the mycelial grew faster at lower osmotic pressure than that at higher osmotic pressure. This might also be the reason for the slower expansion of fungal colonies at solid culture with high concentration sucrose for regeneration experiment (Figure S2). The regeneration of protoplasts in liquid culture with 0.6 M and 0.8 M sucrose, showed germinated protoplasts grew faster in 0.6 M sucrose but the mycelia in 0.6 M sucrose was less uniform in the filament morphology than in 0.8 M sucrose (Figure 6(b)). Based on this result, we used 0.8 M sucrose as osmotic stabilizer for the regeneration experiments.

The regeneration rate was about 23% in MEB, PDB, and nutrient-free culture media and was about doubled systematically when compared with regeneration rate in solid culture. Similar to those studies in the solid culture regeneration experiment, it showed that there was no significant difference in the regeneration rate under different nutrient sources in liquid culture. Together, we concluded that the effects of the three culture environments for protoplast regeneration of *A. cinnamomea* were not obvious. Earlier study [22] showed that the regeneration rates of protoplasts in different medium cultures were found different by about 5 folds and concluded that nutrient sources may be important factors for regeneration of protoplasts. That study contradicted the results of current experiment may be caused by different fungal characteristics. The *A. cinnamomea* is a very slow growing fungus. The time course of observation on protoplast regeneration may be more delicate and differentiate the effects of nutrient sources. Later observation on the growth rate of germinated hyphae did show the

difference among the three medium cultures. Therefore this result provides a more dynamic view of the regeneration of fungal protoplasts.

When studied the pH effect on the protoplast regeneration, we found citric acid might increase the rate of protoplast regeneration. Thus, we further investigated this issue by adding citric acid with final concentrations of 1, 10, 20, 40, 80, 160 and 320 mM in the liquid culture of PDB, MEB and nutrient-free media. As shown in Figure 7, citric acid with a final concentration of 20 mM to PDB, measured by pH meter to be pH 2.81, with the highest regeneration rate of 58.3%. The MEB with 10 mM citric acid at pH value to be 2.74 carried the highest regeneration rate of 52.7%. These results were more than 100% increase of the regeneration rate than those of the same nutrient media without citric acid. However, as a tradeoff, the higher concentration of citric acid reduced regeneration rate overtime and caused the mycelial cell wall abnormal.

The effects of citric acid in PDB and MEB media on protoplast regeneration of *A. cinnamomea* showed that the optimal pH should be around pH 3. This led us to further investigation of whether the pH value is an important factor for protoplast regeneration. As shown in Figure 8, various pH value of the media significantly affected the protoplast regeneration of *A. cinnamomea*. On the protoplast morphology, we found that as the pH of the medium increased more protoplast vesicles were produced. When compared the media at pH 8 with pH 6, the protoplasts with vesicles increased and the intracellular vesicles were significantly larger (data not shown). Based on the previous study [20], it suggested that these vesicles may be vacuoles and as an acidic organelle involved in various cellular physiological processes, such as stabilizing and regulating activities of proteins, ions, pH, osmotic pressure … etc. The functions of vacuoles are highly related with vesicular transport and provide enzymes for cell organelles and cell membranes to maintain their physiological activities. Earlier studies [23] indicated cell polarization has a positive correlation with vacuole involvement. This is inconsistent with our results since the regeneration rate of protoplast with larger vacuoles is very low at pH 8 (Figure 8). It might be protoplasts initiated certain mechanisms to maintain their survival and adapt to adversity due to external stresses. It may change the gene expressions and cause some functional proteins and signal peptides to lose their activities, such as some cellular activities could not be effectively carried out their exocytosis [24]. Cell polarization was reduced and intracellular vacuoles may further forward autophagy pathway, when the environment continues to adversity. Induction of expanding vacuoles eventually leads to cell cycle arrest and even cell death.

In addition, we found the control group of PDB and MEB media without pH buffer had higher rates of protoplast regeneration when compared with those media with pH buffer at pH 5 or higher (Figure 8). This indirectly confirmed that *A. cinnamomea* better grew in a more acidic environment than pH 5. Based on earlier studies [25], the pH value of the growth environment of *A. cinnamomea* decreases over time. Therefore, it is believed that the protoplast may decrease the pH value of the culture environment through its own metabolic reaction to maintain an environment suitable for its growth and regeneration. According to the regeneration experiment, the optimal pH for protoplast regeneration of *A. cinnamomea* should be around pH 3. Therefore, we used the PDB with citrate buffer at pH 3 throughout the later experiments.

To test the effect of different concentrations of citrate buffer on the protoplast regeneration, protoplasts in PDB containing 0.8 M sucrose and citrate buffers at a final concentration of 20, 40, 80, 160 and 320 mM, respectively, pH 3, were tested. The regeneration rate was calculated after shaking for 2 days at 27 °C, 25 rpm. As shown in Figure 9, it had the highest regeneration rate of 71.3% at 80 mM citrate buffer in this experiment. The protoplast regeneration rate is dramatically increased from earlier report of 6.8% [18] to 71.3%. Based on the above optimal condition for protoplast regeneration of *A. cinnamomea*, it is easier for observation of subsequent results of protoplast regeneration, such as shown in Figure 10.

In the earlier experiments, Lu et al. [25] studied the liquid culture and fermentation efficiency of *A. cinnamomea* ATCC 200183 showed that the optimal germination rate after conidia culture for 24 h was between pH 2.5 and pH 3.5, which was similar to the result of this experiment. Therefore, we believe that fungal protoplast regeneration and hyphal tip polarization may have close relationship. Many studies related to the mycelial tip have been reported, including polarization, secretory pathways and various mycelial cell wall growth mechanisms [26]. Genetic studies related to fungal protoplast regeneration, however, still remain in simple culture experiments, such as testing different media types and osmotic pressures [19]. Therefore, it is needed for further studies on the relationship between tip polarization, cell wall synthesis and protoplast regeneration, and to develop a simple method for the regeneration system of fungal protoplasts.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Authors' contributions

JDW worked on the experiments of protoplast preparation and regeneration of *A. cinnamomea*; JCC provided funding, designed the experiments, and prepared the manuscript. Both authors read and approved the final manuscript.

Disclosure statement

The authors declare that they have no conflict of interest.

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