Study on Methods of Protoplast Preparation of Aspergillus Niger

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Abstract: Filamentous fungi are important industrial strains. Aspergillus niger is a safe organism for the production of proteins, organic acids, and chemicals. In order to improve the efficiency of genetic manipulation of A. niger, we studied the method of preparing protoplasts and improving the regeneration rate, and established a method of efficient transformation of protoplasts by exogenous DNA. Studies have shown that the preparation and regeneration of A. niger protoplasts are affected by many factors, such as mycelial growth state, osmotic pressure stabilizer, cell wall lyase system, enzymatic hydrolysis time, regeneration medium composition and so on. The highest efficiency of protoplast release and regeneration was obtained by using the mycelium of A. niger fresh spores cultured for 13-14 hours, using a mixture of snailase and lyticase, 0.6 M sorbitol as an osmotic pressure stabilizer, and enzymatic hydrolysis at 32 °C for 2 hours.

1 INTRODUCTION

Filamentous fungi are important industrial strains. The typical filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*, *Trichoderma reesei* are widely used in the production of organic acids, antibiotics, enzymes and proteins. The filamentous fungi *A. niger*, *A. oryzae* and *T. reesei* are recognized as safe production strains with mature fermentation and post-processing technology (Frisvad JC, 2018). As the host of genetic engineering, filamentous fungi have unique advantages different from that of bacteria and yeast. They can correctly perform various post-translational processing similar to higher eukaryotes. They have high protein secretion capacity. They can degrade and utilize a variety of biopolymers, such as starch, cellulose, hemicellulose, pectin, xylan and protein, so that they can use renewable resources such as plant biomass for culture (Knuf C, 2012).

The commonly used transformation methods of filamentous fungi are protoplast olyethylene glycol (PEG) transformation, electrotransformation, and Agrobacterium-mediated transformation. To obtain higher transformation efficiency, we must obtain more and higher quality protoplasts, and more efficient transformation method.

In this study, we first established a method for the preparation and regeneration of a large number of niger protoplasts.

2 MATERIALS AND METHODS

2.1 Strains and Media

A. niger N593 (ATCC Number : 64973) is a uridine-deficient strain. *A. niger* were inoculated in yeast extract peptone dextrose (YPED) plus uridine agar (peptone 2%, glucose 2%, yeast extract 1%, uridine 0.25%, agar 1.5%, natural pH) and cultured at 30ºC for 5 days to obtain mature spores. Collect the spores from the agar with sterile water, fully disperse spores with oscillator to obtain spore concentration 10^6/mL. 2 ~ 3 mL of spore suspension was inoculated in a 500 mL flask containing 80 mL of modified Czapek 's medium (glucose 1 %, citric acid 0.3 %, potassium dihydrogen phosphate 0.5 %, ammonia nitrate 0.2 %, magnesium sulfate 0.02 %, yeast extract 0.05 %, Tween 80 0.1 %, uridine 0.25 %, pH6.5), and cultured for 13-15 hours on a shaker at 30 ºC and 180 rpm.

2.2 Preparation of Protoplasts

The mycelium liquid of *A. niger* cultured for 13 ~ 14 h was put into two 50 mL centrifuge tubes, centrifuged at 6000 rpm for 10 min. The supernatant was removed, and the mycelium precipitate was retained. Add 20 mL 0.6 M KCl solution to the centrifuge tube, fully suspend the precipitate, centrifuge at 6000 rpm for 10 minutes, and discard the supernatant. Wash repeatedly 2 times. Then 30

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mL 0.6 M KCl solution was used to re-suspend the precipitate, and different proportions of snailase (S10083, Yuanye biotech, Shanghai, China) and lyticase (from *Arthrobacter luteus*, L2524, Sigma-Aldrich) (totoal 0.01g) were added. The centrifuge tube was placed at 32ºC water bath for 1~3 hours, intermittently shaken slightly and sampled regularly to monitor protoplasts formed. After enzymatic hydrolysis, the protoplast fluid was filtered with four layers of sterile wipe mirror paper. The filtrate was centrifuged at 4000 rpm for 10 min, and the supernatant was discarded. The precipitate was gently washed twice with 0.6 M KCl solution and centrifuged. The protoplast precipitation was resuspended in 0.5~2 mL of 0.6 M KCl solution and placed in an ice bath for later use.

2.3 Regeneration of Protoplasts and Determination of Regeneration Rate

Preparation of protoplast regeneration medium (PRM) (glucose 1%, citric acid 0.3%, potassium dihydrogen phosphate 0.5%, ammonia nitrate 0.2%, magnesium sulfate 0.02%, yeast extract 0.025%, uridine 0.25%, potassium chloride 0.6 mol / L, agar (upper 0.5 %, lower 2%), pH 6.5). Double-layer plate coating regeneration: After preparing the regenerated solid medium (lower layer), the freshly prepared protoplasts were gently mixed with the cooled regenerated solid medium (upper layer) and poured onto the lower regenerated solid medium. cultured at 30 ºC for 3~5 days, the formed colonies were counted (A); in order to eliminate the error caused by the mycelial fragments remaining in the prepared protoplast solution and the colonies formed by the regrowth of hyphal fragment, the prepared protoplast solution was spread on the regeneration medium without osmotic stabilizer, and the number of regenerated colonies was used as control (B). The number of protoplasts observed under microscope was C. Regeneration rate was calculated as follows: Protoplast regeneration rate = [(A-B) / C] × 100 %

3 RESULTS AND DISCUSSION

3.1 Effect of Lyase on Protoplast Formation

In order to obtain a higher protoplast yield, it is necessary to select the appropriate lyase. Fungal cell wall composition is complex. It is mainly composed of four layers, from outside to inside is the amorphous glucan layer, glycoprotein layer, protein layer, chitin layer (Free SJ, 2013). Therefore, using mixture of different lyase is better than using one kind of enzyme alone. Snailase contains chitinase, cellulase, pectinase, amylase, protease, etc., often used for yeast cell wall disruption (Cheng, 2018). lyticase, also known as N-acetylmural glycan hydrolase, can destroy the β-1,4 glycosidic bond between N-acetylmural acid and N-acetylglucosamine in the cell wall (Tang, 2015).

The mycelia cultured in modified Czapek 's medium for 14 h were collected. 0.6 M KCl was used as osmotic pressure stabilizer. Firstly, the effects of snailase and lyticase on protoplast formation were determined. The results showed that the yield of protoplasts was 5.41 × 106 / mL and 2.32 × 106 / mL respectively when snailase (0.01 g / 30 mL hyphal suspend) or lyticase (0.01 g / 30 mL hyphal suspend) was used alone after 2 hours of enzymolysis (Fig. 1A). When snailase: lyticase was 2:1, 3:1, 1:1, 1:2, 1:3 (total 0.01 g / 30 mL hyphal suspend), the production of protoplasts was 9.09×10^6 / mL, 8.17×10^6 / mL, 1.19×10^7 / mL, 1.21×10^7 / mL, 1.35×10^7 / mL, respectively. When snailase: lyticase = 3:1, protoplasts reached the highest (Fig. 1B).

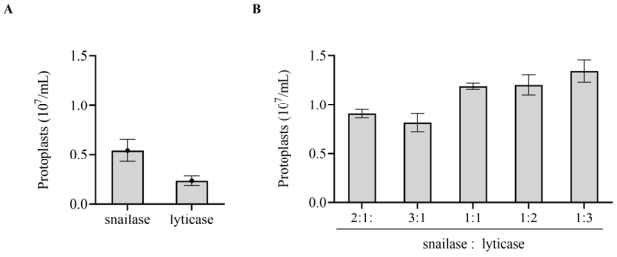

Figure 1: Effect of cell wall lyase on protoplast formation. (A) The number of protoplast formation when snailase or lyticase was used alone. (B) Effects of different proportions of snailase and lyticase on protoplast formation. For ease of comparison, the vertical coordinates of (A) and (B) are consistent.

3.2 Effect of Osmotic Pressure Stabilizer on Protoplast Formation and Regeneration

The type and concentration of osmotic stabilizer is the key factor to maintain and control the number of protoplasts. It can not only maintain the osmotic balance, but also promote the active reaction of lyase. On the other side, osmotic stabilizer may aggravate the damage of protoplasts or inhibit the synthesis of cell wall during regeneration process. The mycelium was cultured for 14 h in modified Czapek's medium, 0.6 mol / L KCl was used as osmotic stabilizer, and the ratio of snailase: lyticase = 1:3 was used as mixed enzyme to prepare protoplasts. The effects of two sugars (glucose, sorbitol) and two salts (KCl, NaCl) as osmotic pressure stabilizer on the production and regeneration of protoplasts were studied.

The results showed that the formation of protoplasts was the highest when sorbitol was used as a stabilizer. When 0.6 mol / L sorbitol was used, the production of protoplasts was the highest, reaching 1.93 × 10^7 / mL. When the concentration of each osmotic pressure stabilizer was between 0.4~0.8 mol / L, it had little effect on the formation of protoplasts. However, when the concentrations of KCl and NaCl reached 1.0 mol/L, the protoplast formation was inhibited (Fig. 2A). The osmotic pressure stabilizers also had a certain effect on the regeneration of protoplasts. When 0.6 mol / L KCl was used as the osmotic pressure stabilizer, the regeneration rate of protoplasts was the highest, reaching 25.40 % (Fig. 2B). Combining the production and regeneration rate of protoplasts, the number of regenerated protoplasts was the highest when 0.6 mol / L sorbitol was used.

3.3 Effect of Lyase Action Time on Protoplast Formation and Regeneration

The mycelium was cultured in modified Czapek 's medium for 14 h. The ratio of snailase: lyticase = 1:3 was used as mixed enzyme to prepare protoplasts. 0.6 mol / L sorbitol was used as osmotic pressure stabilizer, and protoplasts were prepared at 32 °C. The process of protoplast release was observed by regular sampling. The number of protoplast formation was monitored (Fig. 3). The mycelium began to release protoplasts after 30 min of enzymatic hydrolysis.With the extension of enzymolysis time, the number of protoplasts gradually increased, and reached the highest after 3 h, reaching 2.55× 10^7/mL.

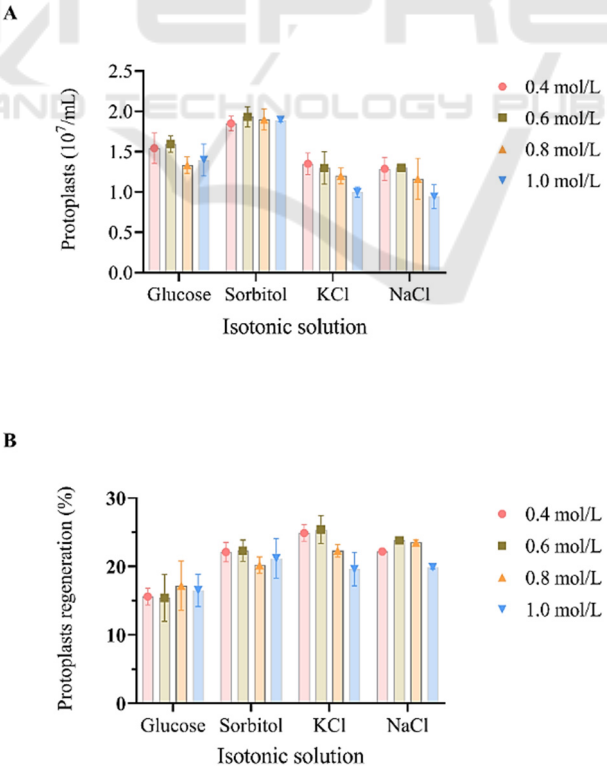

Figure 2: Effects of osmotic stabilizers on protoplast formation and regeneration. (A) Protoplast formation. (B) Protoplast regeneration.

Continue to extend the enzymolysis time, the number of protoplasts did not continue to increase, but decreased slightly (Fig. 3). Protoplast is not stable due to the lack of cell wall. In addition, lysing enzyme will damage the plasma membrane system of protoplast to some extent, so the length of enzymolysis time will seriously affect the regeneration of protoplast. The enzymolysis time is short, and the protoplast has not been fully released; with the extension of enzymolysis time, the number of protoplasts increased greatly, and the regeneration rate increased continuously. Continue to extend the enzymolysis time, the regeneration rate will decline although the total number of protoplasts will increase. When the enzymolysis time was 3 h, although the protoplast production reached the highest, the regeneration rate decreased significantly, only 27.1 % of the regeneration rate when the enzymolysis time was 2 h (Fig. 3). When the enzymolysis time was 4 h, the regeneration rate decreased to nearly 0. This may be due to the lack of cell wall in the formed protoplasts. Therefore, considering the results of protoplast production and regeneration, the enzymolysis time should not exceed 2 h. After enzymolysis, the lysate should be removed as soon as possible.

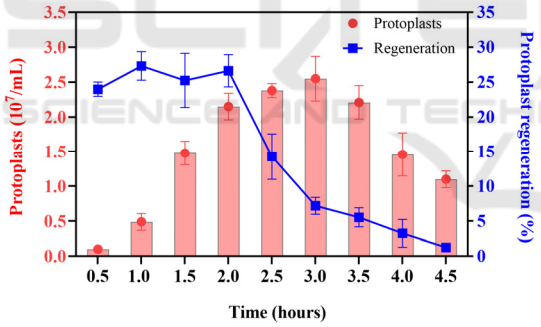

Figure 3: Effect of lysing time on protoplast formation and regeneration.

4 CONCLUSIONS

In this study, the method of preparation and regeneration of protoplasts and the method of efficient transformation of *A. niger* protoplasts by exogenous DNA were established. The study provides technical support for improving the efficiency of gene targeting in *A. niger* and helps realizing the genetic engineering with *A. niger* as cell factory in future.

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