Protoplast preparation

Introduction

This procedure efficiently produces clean protoplasts that can be used for transformation or DNA extraction

Materials

- Modified Vogel's minimal medium: Vogel's salts in 1% glucose
- 0.6 M $MgSO_4$
- Novozyme buffer :
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    - 1.2 M $MgSO_4$
    - 10 mM potassium phosphate pH 5.8
- Novozyme lysing enzyme (Interspex). NB See note 4 below.
- Separation buffer A :
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    - 0.6 M sorbitol
    - 100 mM Tris-Cl pH 7.0
- Separation buffer B :
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    - 1.2 M sorbitol
    - 10 mM Tris-Cl pH 7.5

Equipment

- Water bath (shaking) at 33°C
- Microcentrifuge and tubes
- Whatmann 54 paper
- Buckner funnel
- Centrifuge
- Universal tubes

Procedure

1) Inoculate 50 ml of Vogel's minimal medium in a 250 ml conical flask with a final concentration of 10^7 spores / ml and incubate with shaking at 200 rpm until late exponential phase (18-24 h) at 37°C. Alternatively, use Saborauds dextrose broth / malt extract broth and grow for 16 h.

2) Dry down the mycelium onto Whatmann 54 paper using a Buckner funnel and a side-arm flask attached to a vacuum pump and wash with 0.6 M $MgSO_4$. Alternatively, the mycelium can be filtered through sterile muslin. Resuspend in Novozyme buffer to give 40 mg of mycelium per ml buffer.

3) Add Novozyme to a final concentration of 2 mg/ml and mix. Incubate the mycelia at 33°C with shaking for 1-3 h to generate protoplasts. Alternatively, the incubation can be done at 37°C at 100 rpm on a shaking incubator or with slow stirring on a magnetic stirrer.

4) Overlay the protoplast suspension with separation buffer A and centrifuge at 1500 x g at room temperature for 15 min. The protoplasts will form an interface layer.

5) Remove the interface protoplast layer to a universal tube and make up to 10 ml with separation buffer B.

6) Centrifuge at 1000 x g for 10 min and repeat the 10 ml wash twice. Resuspend the pellets in 3 ml of separation buffer B and divide into 3 microcentrifuge tubes, centrifuge at 1000 x g for 10 min and pour off the buffer. Add 0.5 ml of separation buffer B per tube. Protoplasts may be stored overnight at 4°C.

Timetable

Fungal culture (1) 24 h
Protoplast production (2 - 6) 3 - 5 h

Tips and general comments

1) Protoplast production is affected by several variables and has been investigated in detail by Birch *et al.*, (1998).

2) With *A.nidulans* we have observed that protoplast formation is most efficient when prepared from a filamentous culture. Filamentous growth of *A.nidulans* is easily obtained by growth in thin layer culture: 50 µl of a 10^7 *A. nidulans* stock spore suspension was inoculated into 10 ml of YEPD broth in a 90 mm petri dish and incubated O/N at 37°C.

3) The final concentration of Novozyme can be increased to 6 mg/ml in order to speed up the production of protoplasts. It is better to harvest protoplasts earlier rather than later.

4) Novozyme 234 is no longer commercially available and currently there are no enzyme preparations which are equivalent in activity to this preparation. Interspex recommend using a combination of driselase and beta D-glucanase. Glucanex can be used and is available from Sigma as lysing enzyme (L1412) - our suggested concentration to use is 12 mg/ml or from Novozymes Switzerland AG. There is a discussion of which enzymes might be used to replace Novozyme in the Fungal Genetics Newsletter Number 47 (2000) p 65-66.

Alternative protoplasting buffers

The following buffers can be tried for washing mycelium and generating protoplasts.

- 1 M NaCl, 10 mM $MgCl_2$, 10 mM potassium phosphate pH 5.8
- 0.6 M KCl

Alternative protoplast purification

1) Filter protoplasts through miracloth, household cleaning cloth (J-Cloth) or three layers of lens cleaning tissue.

2) Collect by centrifugation at 800 x g for 10 min and wash protoplasts twice in protoplasting buffer.

3) Resuspend in appropriate buffer at desired concentration.

References

Vogel, H.J. (1956) A convenient growth medium for *Neurospora* (medium N). Microbiol. Gen. Bull. 13, 42 - 44

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