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ORIGINAL ARTICLE

Penicillium ochrochloron MTCC 517 chitinase: An effective tool in commercial enzyme cocktail for production and regeneration of protoplasts from various fungi

Nilambari S. Patil [a], **Jyoti P. Jadhav** [a,b,*]

[a] *Department of Biotechnology, Shivaji University, Kolhapur 416004, India*
[b] *Department of Biochemistry, Shivaji University, Kolhapur 416004, India*

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Abstract *Penicillium ochrochloron* MTCC 517 is a potent producer of chitinolytic enzymes. Novozyme 234, traditional enzyme cocktail for protoplast generation is not available in the market. So, new enzyme cocktail is prepared for protoplast formation from various filamentous fungi which consists of 5 mg ml^{-1} lysing enzymes from *Trichoderma harzianum*, 0.06 mg ml^{-1} β-glucuronidase from *Helix pomatia* and 1 mg ml^{-1} *P. ochrochloron* chitinase. The greatest number of protoplasts could be produced from most of the fungi in 0.8 M sorbitol and by incubation for about 2 h at 37 °C, but the number was decreased by incubation for more than 3 h. About twice as many protoplasts were produced from different species of fungi by involvement of *P. ochrochloron* chitinase than with combined commercial enzymes.

1. Introduction

Fungal protoplasts have recently become an effective experimental tool and an important technique with an increasing number of applications in pure and applied biological research (Peberdy and Ferenczy, 1985). Strain improvement programmes facilitate the production of potential strains with desirable properties by protoplast fusion of industrially important fungi. Fusion and transformation systems depend on the yield and speed of protoplast formation (Hamlyn et al., 1981).

In recent years, efforts are taken by researchers for improving the methods for protoplast formation from mycelial cells for fungal genetic study. For every fungus, concentration of enzymes and osmotic stabilizers must be estimated properly in order to obtain maximum yield of protoplasts without affecting their viability. The efficient transfer of genetic material is based on the formation of stable and viable protoplasts (Xuanwei et al., 2008). Various treatments for protoplast

* Corresponding author at: Department of Biotechnology, Shivaji University, Vidyanagar, Kolhapur 416004, India. Tel.: +91 231 2609365; fax: +91 231 1691533.
E-mail address: jpjbiochem@gmail.com (J.P. Jadhav).
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preparation have been developed by the use of combinations of commercial enzymes.

Furthermore, most of these studies used Novozyme 234; however, this enzymatic complex is not commercially available. Henceforth, the present study is undertaken to investigate new enzyme cocktail for production and regeneration of protoplasts from different filamentous fungi. Various conditions were optimized along with the effect of various enzyme concentrations and osmotic stabilizers to enhance protoplast yield.

2. Materials and methods

2.1. Microorganisms

Penicillium ochrochloron MTCC 517 was obtained from MTCC, Chandigarh, India. The deuteromycotina strains; *Aspergillus sojae* NCIM 1198, *Trichoderma harzianum* NCIM 1185, *Aspergillus oryzae* NCIM 1272, *Rhizopus oligosporus* NCIM 1215 and *Neurospora crassa* NCIM 870 belonging to ascomycotina were procured from NCIM, Pune.

2.2. Chemicals and enzymes

The cell-wall-lytic enzyme, chitinase from *P. ochrochloron* used for the study was purified in author's laboratory (Patil et al., 2013). Elaborately, acetone precipitation of a culture filtrate of *P. ochrochloron* MTCC 517 followed by dialysis and DEAE–cellulose ion exchange chromatography. Other enzymes used were lysing enzymes from *T. harzianum* and β-glucuronidase from *Helix pomatia* (Sigma, St. Louis, MO). Other chemicals used were of highest purity and analytical grade.

2.3. Protoplast formation

Protoplast formation was carried out according to the method of Yutaka et al. (1988) with slight modifications. The spore suspensions from different species of fungi have been inoculated into 100 ml medium containing 20% potato infusion and 2% dextrose having pH 6.0. The flasks were incubated on a rotary shaker at 120 rpm for 24–72 h at 30 °C. After incubation, mycelia were harvested by filtration through cheese cloth and collected by centrifugation at $1000 \times g$ for 15 min and washed twice with distilled water. Protoplasting was performed using 50 mg wet mycelia in 1.0 ml of 25 mM sodium phosphate buffer, pH 7.0. Enzyme preparation of *P. ochrochloron* was added at different concentrations and with different osmotic stabilizers (0.8 M) such as mannitol, sorbitol, KCl and sucrose; after that mycelium suspensions were incubated at 37 °C on a rotary shaker (120 rpm). Commercial lytic enzymes from *T. harzianum* and β-glucuronidase from *H. pomatia* were also tested alone and in combination with *P. ochrochloron* chitinase for their capability of releasing protoplasts from different fungi. Protoplast yield (protoplasts ml^{-1}) was determined by using a Neubauer haemocytometer (Marienfeld).

2.4. Protoplast regeneration

The crude protoplast suspension was filtered through a glass funnel and centrifuged at $1000 \times g$ for 5 min. 100 µl of protoplast suspension was spread on the regeneration medium containing 0.1% peptone, 0.1% KH_2PO_4, 0.03% $MgSO_4$. 7 H_2O, 0.05% yeast extract and 0.8 M sucrose and incubated at 25 °C. The incubated plate was observed for mycelia development and spore formation. Regeneration frequency of protoplasts has been calculated as the ratio of number of protoplasts regenerated to the number of protoplasts plated in the regeneration medium. Regeneration in liquid phase was checked by suspending aliquots of protoplasts in the regeneration medium and observed under microscope at different intervals.

3. Results and discussion

3.1. Protoplast formation from various fungi

P. ochrochloron enzyme possesses immense potential to digest the cell wall of various fungi and production of large number of protoplasts. Among the fungi tested, the highest production of protoplasts has been obtained from *N. crassa* species of ascomycotina. The yield of fungi belonging to zygomycotina and deuteromycotina varied depending on species which is probably due to variability in the degree of digestibility in the cell walls of mycelia. In addition digestibility was varied among fungal species belonging to same subdivision (Yutaka et al., 1988). Cell wall of *N. crassa*, *A. sojae* and *A. oryzae* was easily digested followed by *R. oligosporus* and *T. harzianum*.

3.2. Effect of osmotic stabilizers

Protoplasts were released from various fungi using lytic enzyme preparation from *P. ochrochloron*. Protoplasting has been performed as mentioned earlier in 0.8 M sorbitol and enzyme preparation was added to a final concentration of 1.0 mg ml^{-1}. Osmotic stabilizers are one of the important parameters for release and high yield of protoplasts. They support the protoplasts from being lysed. Besides sorbitol, various other osmotic stabilizers were tested, all at 0.8 M concentration. In sorbitol, good results of protoplast formation were observed as compared to mannitol, sucrose and KCl (Fig. 1). Several other osmotic stabilizers like sucrose (Kim et al., 2000), magnesium sulphate (Gupta et al., 1997) and mannitol (Wakabayashi et al., 1985) had been successfully used for maximum protoplast yield in fungi.

3.3. Effect of incubation time

The maximum yields of protoplasts were reached after 2 h incubation, but the number decreased above 3 h due to bursting and prolonged incubation caused lysis of protoplasts. Protoplasts released after 1 h of incubation from partially digested mycelia and large size protoplasts have been obtained after 2 h of incubation (Fig. 1). These results seem to be in accord with preparations of cell-wall-lytic enzymes from *T. harzianum* (Yutaka et al., 1988) and *Trichoderma koningii* UC174 (Liu and Zhu, 2000).

3.4. Effect of enzyme concentration

During incubation with *P. ochrochloron* chitinase at concentrations of 0.25–2.0 mg ml^{-1}, discrepancies in the yield of protoplast formation were observed (data not shown). The initial

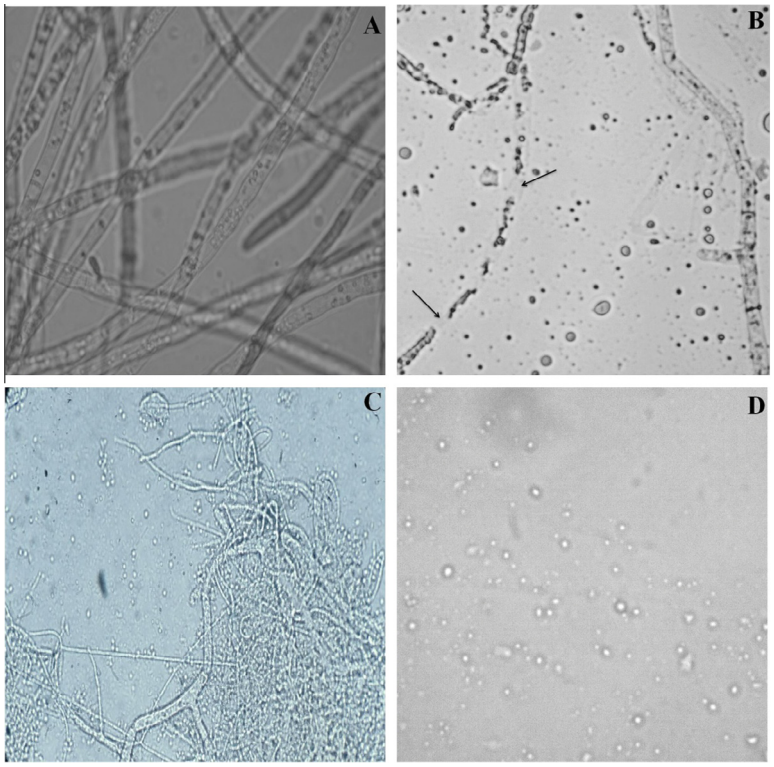

Figure 1 (A) Parent hyphae. (B) Arrow indicated digested mycelia. (C) Release of protoplasts from mycelia. (D) Protoplasts (400× magnification).

Table 1 Comparison of production of protoplasts using commercial enzyme and in combination with *P. ochrochloron* chitinase.

Fungal strain	Lysing enzymes from *Trichoderma harzianum* + β-glucuronidase from *Helix pomatia*	Lysing enzymes from *Trichoderma harzianum* + β-glucuronidase from *Helix pomatia* + 1 mg ml^{-1} *P. ochrochloron* chitinase
Neurospora crassa NCIM 870	0.7×10^8	2×10^8
Aspergillus sojae NCIM 1198	4.3×10^6	7.8×10^7
Aspergillus oryzae NCIM 1212	3.6×10^6	5.2×10^7
Rhizopus oligosporus NCIM 1215	0.2×10^5	2×10^6
Trichoderma harzianum NCIM 1185	8.0×10^5	1×10^6

rate of protoplast formation and the yields of protoplasts from various fungi were increased by the addition of an increasing concentration of the enzyme, but were not improved over 1.0 mg ml^{-1} which is probably due to high concentrations resulting in the lysis of protoplasts soon after their appearance.

Effectiveness of commercial preparations of lytic enzymes in releasing protoplasts from fungi was investigated. Lytic enzyme mixture from *T. harzianum* and β-glucuronidase from *H. pomatia* gave good yields of protoplasts from various fungi. Nevertheless, the yield of protoplasts obtained with the *P. ochrochloron* enzyme when used in combination with the above mentioned commercial enzymes was about 2 times than alone commercial enzymes (Table 1). Enzyme cocktail containing 5 mg ml^{-1} lysing enzymes from *T. harzianum*, 0.06 mg ml^{-1} β-glucuronidase from *H. pomatia* and 1 mg ml^{-1} *P. ochrochloron* chitinase has been used in the present study for generation of protoplasts from various fungi.

Chitin and β-glucan are the major components of fungal cell wall and chitinases and β-1,3glucanase have been known to lyse the fungal cell wall. *P. ochrochloron* MTCC 517 has been reported earlier for β-1,3glucanase (Jadhav et al., 2006) and chitinase activity (Patil et al., 2013). The addition of chitinase to the lytic enzyme systems is not uncommon and has been shown to be necessary for hydrolysis of the cell walls of many organisms (Skujins et al., 1965; Liu and Zhu, 2000). Bekker et al. (2009) reported that the cocktail consists of lysing enzymes from *T. harzianum*, chitinase from *Streptomyces griseus* and β-glucuronidase from *H. pomatia* for production of protoplasts from *Aspergillus niger*. Considering the chitinase activity of *S. griseus* (0.15 U ml^{-1}) used in above enzyme cocktail is significantly lower than *P. ochrochloron* (61 U ml^{-1}). In this aspect, use of *P. ochrochloron* chitinase in combination with commercial enzymes would be superior in generation of protoplasts from various fungi.

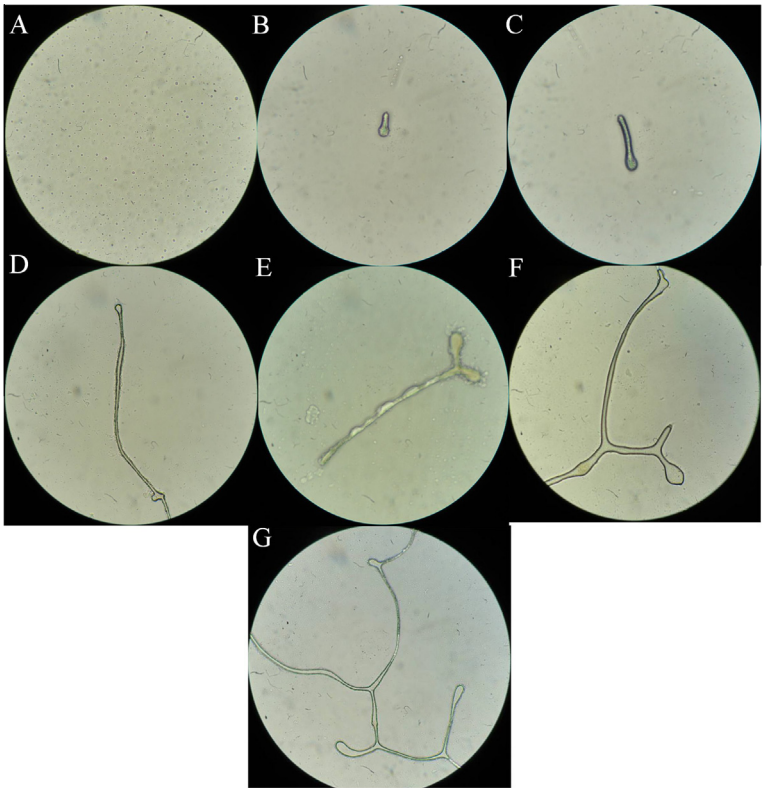

Figure 2 Microscopic observations (400× magnification) of regeneration of protoplasts of *Aspergillus oryzae*. (A) Formed protoplasts after the action of enzyme. (B) Formation of bud like structure and germ tube. (C) Emergence of hypha from germ tube. (D) Elongation of hypha (E, F, G) Branching of hyphae.

3.5. Regeneration of protoplasts

The higher frequency of regeneration from fungal protoplasts is not only the foundation of fungi genetic manipulation and improvement but also a good experimental system for the study of gene expression and molecular studies (Xuanwei et al., 2008). Successful reversion and regeneration of protoplasts to normal mycelia are important. The regeneration of protoplasts was observed in terms of morphological changes detected after 2 h. The protoplasts put forth bud like structures, which developed into germ tubes. These germ tubes later formed hyphal cells (Fig. 2) Lalithakumari (2000) reported similar patterns of hyphal regeneration (including a third type of a germ tube like hypha germinating from the protoplasts) in several filamentous fungi.

Among the different osmotic stabilizers used in the regeneration medium, regeneration frequency has been observed better with sucrose and it was found to be maximum for *A. oryzae* (Table 2). Protoplast regeneration provides a relative measure of the effects of enzyme treatment on cell viability. Protoplasts that lack the ability to regenerate presumably either lack nuclei or were damaged at some point during or after the enzyme treatment (Rui, 1993).

Table 2 Protoplast regeneration frequency of fungi.

Fungal strain	Regeneration frequency (%)
Aspergillus oryzae NCIM 1212	66
Aspergillus sojae NCIM 1198	58
Trichoderma harzianum NCIM 1185	55
Rhizopus oligosporus NCIM 1215	47
Neurospora crassa NCIM 870	38.8

4. Conclusion

The present study provides major finding purified chitinase from *P. ochrochloron* MTCC 517 having tremendous activity suitable for digestion of cell wall and protoplast formation from various fungi. *P. ochrochloron* chitinase can be used in enzyme cocktail in combination with commercial enzyme to obtain obstacle yield of protoplasts. It also provides intuition into conditions that control the formation and regeneration of protoplasts. Considering yield and regeneration capacity of protoplasts different fungal strains would definitely provide basis for protoplast fusion and strain improvement studies.

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