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Fungal protoplast fusion – a revisit

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Abstract Protoplast fusion is a non-specific recombination technique used for transfer of cytosolic organelles including genetic material. The process involves cell wall breakdown, regeneration of protoplasts, chemofusion and electrofusion. This review article discusses all the stages involved in fusion of protoplasts and some of the applications of protoplast fusion technique in fungal systems.

1 Introduction

Protoplasts are the cytosolic constituents of fungal cells. Their cytoplasm can be considered equivalent to cytoplasm in higher cells. Protoplasts contain all the intracellular organelles of cells and form a vital link in transfer of micromolecules between cyto-organelles. Protoplast fusion is a cyto-transformation technique in genetic recombination technology. Fusion involves heterokaryon formation, nuclear fusion, diploidization and haploidization [1]. Fusion of two protoplasts of fungi may result in hybridization of the characteristics of the parental strains and the progeny will express characteristics of either of parental strains or a hybrid expression may occur. This property leads to extensive research activity in the field of protoplast fusion in fungal systems as they form a large part of micro-organisms used in bioengineering, biotechnology, and fermentation industry. Fusion can be done with either wild type strains or mutated strains, strains with incompatible heterokaryon, auxotrophic wild type strains or auxotrophic mutant strains and prototrophic strains [2].

Protoplast fusion can be achieved by two techniques namely chemofusion and electrofusion. This review article discusses various steps involved in fusion of protoplasts including cell wall lysis, regeneration, chemofusion, electrofusion and the various applications of protoplast fusion.

2 Cell wall lysis

Cell wall lysis is done by enzymatic digestion of the cell wall in the presence of an isotonic buffer having optimal pH and containing divalent cations and chelating agents. Fungal cell wall is made up of chitin and polymannan [3]. Peberdy et al. [4] have reviewed methods for producing protoplasts. Factors considered were composition and structure of cell walls and the enzymes which lyse the walls. The two important parameters necessary to be considered for proper generation of protoplasts are age of mycelia and contact time with lytic enzyme. The various sources of enzymes which were used for lysis of fungal cell wall are:

- A culture filtrate of *Trichoderma harzianum* [5].
- A culture filtrate from *Trichoderma viride* [6].
- Enzymes from snail gut juice [7].
- By using commercially available enzyme [8].
- From a culture filtrate of *Actinomyces kurssanovii*, *Actinomyces cellulosae* [9].
- Lysozyme [9] and zymolyase [10].

Enzymes from a culture filtrate of *Trichoderma harzianum* were found to dissolve cell walls of a wide variety of filamentous fungi belonging to Ascomycotina, Deuteromycotina, Basidomycotina and Zygomycotina [5]. Ogawa et al. [11] have obtained protoplasts of *Aspergillus awamori* by using enzymes obtained from *Trichoderma viride*. Protoplasts from auxotrophic mutant strains of *Candida albicans* and *Candida tropicalis* were obtained using enzymes from snail gut juice [7]. Novozyme 234 has been used to obtain protoplasts from *Trichoderma reesei* [12]. The isotonic solutions used in the protoplasting process mainly consisted of buffers. Some of the different types of buffers used were:

- 0.8 M NH_4Cl solution for obtaining protoplasts from *Candida utilis* [6].
- Citrate-phosphate buffer was used to obtain protoplasts from *Candida lipolytica* [10].
- Phosphate buffer (25 mM) has been used by Anjani and Panda to obtain protoplasts from *Trichoderma reesei* [13].

The buffers used for protoplast generation contained osmotic stabilizers consigns of monovalent salts such as sodium chloride [14], potassium chloride [13] or polyhydroxy alcohols such as mannitol [9]. N-tris(hydroxy methyl)-methyl-2-amino ethane sulfonic acid (TES) and N-2-hydroxy ethyl piperazine sulfonic acid (HEPES) buffer were used by Davis et al. [15]. The incubation temperature was found to be between 28 °C [14] to 40 °C [15]. The generation of protoplasts was done by agitation of the reaction mixture by vibration as shaking and stirring damaged the protoplasts [13].

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Regeneration of protoplasts

Regeneration of protoplasts is done to check their viability. Regeneration is carried out using complete glucose mineral medium [16]. The regeneration frequency is defined as the ratio of the number of colonies regenerating from the protoplasts to the total number of plated protoplasts. Regeneration has been done using a medium containing 3% agar and 30 mM calcium chloride [2]. Another method to check the regenerating ability is to study the ability of protoplast to uptake [^{14}C] isotope and to incorporate it in cellular proteins [12]. A direct method to study viability is by staining with Trypan blue. The protoplasts are stained with trypan blue. The viable count is performed and the number of viable cells are determined from the count [13].

4
Chemofusion technique

Chemofusion technique is carried out between two viable protoplasts using a fusogen in the presence of divalent cations at optimal pH and under suitable related conditions. The most commonly used fusogen was polyethylene glycol [17]. The divalent ions used were calcium ions. All the fusogen solutions contained an osmotic stabilizer. Potassium ions were used as the osmotic stabilizer [18]. Different combinations of protoplasts can be fused to study the fusion characteristics of a particular strain. Protoplasts with double amino acid requirement was used for fusion by Toyama et al. [17]. Nutritionally complimentary strains were considered for fusion by Sung et al. [2]. The molecular weight of polyethylene glycol used for fusion varied from 4000 [2] to 6000 [17]. Mutant strains were obtained by exposure to UV-rays [16]. They were then screened for the stable strain. Differentiation between the wild type strains and the mutant strains was done by using markers. Amino acid markers were used to study auxotrophic mutants of *Aspergillus niger* and *Aspergillus kawachi* [19]. After fusion, the fusant strains were studied by phenotypic or genotypic characteristics. Special "knobs" were studied in *Trichoderma reesei* cells by Toyama et al. [17]. Colour of the fusant strain was used to differentiate between wild fusants and mutant strains [16]. Fusants can also be characterized by their genotypic expressions. Identification of newer genotypes indicate that genetic recombination occurs during fusion [1]. Production characteristics of the fusion strains in terms of enzyme activity or production of a metabolite can also be compared with the parental strain to asses recombination. Usami et al. [20] reported higher citric acid yield in fusants obtained from *A. niger* than from the parental strain. Electrophoretic property of the alkaline protease was studied to characterize the fusant strain. A study of the genotypes of some of the fusant strains have also indicated an increase in the DNA content of the fusant strain over the parent strain. Fusion resulted in increase in the cellulase activity of the fusant over the parent strain [21]. Higher ethanol production has been reported by Anjani and Panda [16]. They reported two fusants exhibiting higher alcohol production which were obtained from *Sachharomyces cerevisae* and *Trichoderma reesei*. Diploidy in fusants can be obtained by selective treatment with d-camphor [20] or by treatment with benomyl [16].

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Electrofusion technique

This is done by applying high DC pulse voltage to protoplasts for a specified time interval. Protoplasts of mutant strains of *A. oryzae* and *A. sojae* were fused by this technique. Phenotypically expressible mutants having pant and met markers were used for this purpose. The optimal conditions for fusion were 1.1 MHz AC frequency, 400 V/cm AC voltage, 4 KV/cm DC pulse voltage, 60 μs pulse duration [22]. Protoplasts from *A. niger* were electrofused by Zhou et al. [23]. Electrofusion was performed at pulse amplitude of 4.5 KV/cm, and pulse width 62.5 μm. The fusants obtained were stable and an increase in the enzymatic activity was observed from the fusants obtained by electrofusion.

6
Applications of protoplast fusion

Protoplast fusion has got extensive application in fermentation industry as well as in genetic engineering and molecular biology. Protoplast fusion has the following applications.

Transfer of cyto-organelles between different species of fungi:

- An oligomycin resistant mitochondria has been transferred between two species of *Aspergillus* [24].
- Transfer of cytoplastic genetic elements [25].

Strain improvement in filamentous fungi

- New fusants strains for use in dairy industry [1].
- Enhancement in stability of fusant strains [26].

Development of hyper producing strains:

- A new strain producing citric acid from xylose [10].
- New strains producing ethanol from cellulose [16].
- Increased antibiotic production in variants of *Penicillium* sp. [27].
- Increased enzyme production than the parental strain [28].
- Generation of interspecific hybrids with high cellulolytic activity [29].
- Increase citric acid production following protoplast fusion [30].

7
Conclusion

Fusion results in enhancement of strains over the parental strains. Gene manipulation during fusion can be used as a tool in cell biology. Out of many fusants generated from a particular strain, only a few have been found to be stable. Intergeneric fusion of protoplasts is a means of acquiring desirable strain characteristic from other genera. The methods presently available for mutation studies and screening of the mutant strains were found to be very cumbersome and time consuming.

8
Future study

Protoplast fusion can be used to produce high yielding stable strains of fungi which can thrive on cheaply avail-

able substrate such as agarowaste to produce products including antibiotics so that the cost of the product is greatly reduced for the benefit of mankind. Fusion technique can be used to produce newer classes of therapeutically important molecules. More research is to be carried out for development of newer mutation and screening techniques for accelerating fusion studies.

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