

# **Protoplast Fusion Technology and Its Biotechnological Applications.**

Nitin Verma\*, M.C. Bansal, Vivek Kumar

Department of Paper Technology, Indian Institute of Technology, Roorkee, Saharanpur  
Campus, Saharanpur, 247001, India.

\*Corresponding author: nitiniit2004@rediffmail.com

Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells. Protoplast can be obtained by specific lytic enzymes to remove cell wall. Protoplast fusion is a physical phenomenon, during fusion two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents. By protoplast fusion it is possible to transfer some useful genes such as disease resistance, nitrogen fixation, rapid growth rate, more product formation rate, protein quality, frost hardness, drought resistance, herbicide resistance, heat and cold resistance from one species to another. Protoplast fusion an important tools in strain improvement for bringing genetic recombinations and developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. These are the powerful techniques for engineering of microbial strains for desirable industrial properties. Protoplast fusion would continued to be an existing area of research in modern biotechnology. This technique in the future will be one of the most frequently used research tools for tissue culturists, molecular biologists, biochemical engineers and biotechnologists. This review describes the protoplast fusion technology and its biotechnological applications.

## **Introduction:**

The protoplast includes the plasmalemma and everything contained within i.e. the entire cell without its inherent cellulosic cell wall. In protoplast technology, two genetically different protoplasts isolated from the somatic cells and are experimentally fused to obtain parasexual hybrid protoplasts. The hybrid protoplast contained heteroplasmic cytoplasm and two fused parent nuclei. Fusion of protoplast is relatively a new versatile technique to induce or promote genetic recombination in a variety of prokaryotic and eukaryotic cells (Bhojwani S.S. et al 1977). Protoplast fusion may be used to produce interspecific or even intergeneric hybrids. Protoplast fusion becomes an important tool of gene manipulation because it breakdown the barriers to genetic exchange imposed by conventional mating systems. Protoplast fusion technique has a great potential for genetic analysis and for strain improvement. It is particularly useful for industrially useful microorganisms (Murlidhar R.V and Panda T. 2000).

### **Enzymes used for breaking of cell walls:**

For protoplast fusion it is important that the cell wall of plant and microorganisms is degraded. So various enzymes used for this process. cellulase and pectinase or macerozyme acting on plant cell wall. Bacterial cell wall are degraded by the action of lysozyme. Fungal wall degraded by Novozyme -234 which includes glucanase and chitinase. *Streptomyces* cell wall degraded by action of lysozyme and achromopeptidase (Narayanswamy S 1994)(Jogdand S.N 2001).

### **Methods of protoplast fusion:**

Protoplast fusion can be broadly classified into two categories:

**Spontaneous fusion:** Protoplast during isolation often fuse spontaneously and this phenomenon is called spontaneous fusion. During the enzyme treatment, protoplast from adjoining cells fuse through their plasmodesmata to form multinucleate protoplasts.

**Induced fusion:** Fusion of freely isolated protoplasts from different sources with the help of fusion inducing chemicals agent is known as induced fusion. Normally isolated protoplast do not fuse with each other because the surface of isolated protoplast carries negative charges (-10mV to -30mV) around the outside of the plasma membrane. And thus there is a strong tendency in the protoplast to repel each other due to their same charges. So this type of fusion needs a fusion inducing chemicals which actually reduce the electronegativity of the isolated protoplast and allow them to fuse with each others (Narayanswamy S 1994).

The isolated protoplast can be induced to fuse by three ways;

**Mechanical fusion:** In this process the isolated protoplast are brought into intimate physical contact mechanically under microscope using micromanipulator or perfusion micropipette.

**Chemofusion:** Several chemicals has been used to induce protoplast fusionsuch as sodium nitrate, polyethylene glycol, Calcium ions ( $\text{Ca}^{++}$ ). Chemical fusogens cause the isolated protoplast to adhere each other and leads to tight agglutination followed by fusion of protoplast (Pasha C.R et al 2007) (Jogdand S.N.2001). In order to convert cellulosic materials to ethanol by single step process, Srinivasan R and Panda T(1997) carried out chemofusion between protoplasts of *Trichoderma reesei* QM9414 and *Saccharomyces cerevesei* NCIM 3288. Observed successful fusion suggest that endoglucanase is the key enzyme in the success of fusion. Iwata M et al(1986) were obtained Tetracycline resistant ( $\text{Tet}^r$ ), erythromycin resistant ( $\text{Ery}^r$ ) fusants of *Lactobacillus fermentatum* 604 carrying a 10 megadalton  $\text{Tet}^r$  plasmid and *L.fermentatum* 605 carrying a 38 megadalton  $\text{Ery}^r$  plasmid by means of polyethylene glycol induced protoplast fusion.

Chemofusion is a non specific, inexpensive, can cause massive fusion product, can be cytotoxic and non selective and having less fusion frequency.

**Electrofusion:** Recently, mild electric stimulation is being used to fuse protoplast. In this two glass capillary microelectrode are placed in contact with the protoplast. An electric field of low strength ( $10\text{Kvm}^{-1}$ ) gives rise to dielectrophoretic dipole generation within the protoplast suspension. This leads to pearl chain arrangement of protoplasts. Subsequent application of high strength of electric fields ( $100\text{kvm}^{-1}$ ) for some microseconds results in

electric breakdown of membrane and subsequent fusion(Ushishima S.T et al 1991) (Jogdand S.N.2001).Groth DI(1987) et al carried out electrofusion of *Penicillium* protoplasts,after diaelectrophoresis and found viable fusion products.Dimitrov AP and Christov AM(1992) reported electrically induced protoplast fusion using pulse electric field for diaelectrophoresis and suggest the possibility of electrically induced protoplast fusion at cation concentration that prevents fusion when sine – wave fields are applied.Gaint protoplast of *Pleurotus cornucopiae* were fused using the glass microelectrode fusion technique.To induce fusion  $Ca^{++}$  was necessary .Polyethylene glycol 400 (PEG) promoted fusion but also increased the adhesion of protoplasts(Magae Y et al 1986).In order to regulate electrofusion Urano N et al(1998) studied electrofusion procedures for yeast breeding and reported that cell membrane fusion behaviour of respiration deficient yeasts( $P^-$ ) was remarkably different from the normal yeasts( $P^+$ ).Induction of cell membrane fusion in  $P^+$  protoplast appeared under the pulse conditions (height 2.5-5.5  $kVcm^{-1}$  and duration 25-430 $\mu s$ ) and the time interval of morphological change from the long to short state was 3–11 s. On the other hand, induction of cell membrane fusion in  $P^-$  protoplasts appeared under the higher pulse condition (height: 4.0–7.0  $kV cm^{-1}$  and duration: 20–500  $\mu s$ ). The time interval of the morphological change from the long to short state was 110–170 s in cell membrane fusants of  $P^-$  protoplasts. The  $\xi$ -potential of  $P^+$  protoplasts was –10 to–30 mV and that of the  $P^-$  protoplasts was –25––60 mV. The surface charge of the  $P^-$  protoplasts was more negative than that of  $P^+$  protoplasts; therefore, regulation of electrofusion among various kinds of yeast strains was possible by changing the surface charge of the protoplasts using mitochondrial mutations.

Electrofusion is easy to control having fusion frequency upto 100%.gives reproducibility.less cytotoxic.But equipment is sophisticated and expensive.

#### **Mechanism of protoplast fusion:**

The mechanism of protoplast fusion is not fully known .Several explanations have been put forward to understand the mechanism of protoplast fusion.Some are explained here:When the protoplasts are brought into close proximity ,this is followed an induction phase thereby changes induced in electrostatic potential of the membrane results in fusion .After the fusion,The membranes stabilizes and the surface potential returns to their former state.Other literature showed when the protoplasts are closely adhered ,the external fusogens cause disturbance in the intramembranous proteins and glycoproteines.This increases membrane fluidity and creates a region where lipid molecule intermix,allowing coalescence of adjacent membranes.The negative charge carried by protoplast is mainly due to intramembranous phosphate groups .The addition of  $Ca^{++}$  ions causes reduction in the zeta potential of plasma membrane and under this situation protoplasts are fused(Peberdy J.F 1980).The high molecular weight polymer (1000-6000) of PEG acts as a molecular bridges connecting the protoplasts. calcium ions linked the negatively charged PEG and membrane surface .On elution of the PEG ,the surface potential are disturbed ,leading to intramembrane contact and subsequent fusion, Besides this ,the strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity,thus inducing fusion.Protoplast fusion

takes place when the molecular distance between the protoplasts is 10A or less .This indicates that protoplast fusion is highly a traumatic events.(Jogdand S.N,2001)(Narayanswamy S 1994)

#### **Protoplast fusion in fungi:**

Production and regeneration of protoplasts is a useful technique for fungal transformations.Commercial preparation of enzymes which contain mixture of products to digest fungal cellwall used .Novozyme 234 includes (glucanase and chitinase) enzyme mixture is added to rapidly growing fungal tissue suspended in an osmotic buffer (e.g.0.6 mol<sup>-1</sup>,KCl,1.2 mol<sup>-1</sup>,Sorbitol or 1.2 mol<sup>-1</sup>MgSO<sub>4</sub>).The protoplasts and DNA are mixed in presence of 15%(w/V)PEG 6000 and pH buffer (TRIS HCl).10 mml<sup>-1</sup>.PEG causes clump formation in protoplasts. At 37<sup>0</sup>C ,grow mycelium on cellophane placed on agar overnight.Incubte with enzyme at 30<sup>0</sup>C for 1.5 hours in empty petridish having KCl,than filter protoplasts,wash protoplast in KCl (Centrifuge and resuspended the pellets).Protoplast fusion frequency in fungi is 0.2 -2%(Srinivas R.T and Panda T 1997)(Jogdand S.N 2001)

#### **Protoplast technology for *Streptomyces* species:**

*Streptomyces* spp also do not have natural means of mating .For obtaining protoplasts from *Streptomyces* lysozyme is used which breakes glycan portion of peptidoglycan wall.Cultures from spore suspension (2 days in shaker at 30<sup>0</sup>C) harvest by centrifugation ,Resuspended in 0.03 mol<sup>-1</sup> sucrose, washed and reharvest,Than resuspended in lysozyme solution in protoplasting medium(30 min – 2 hr at 30<sup>0</sup>C) (Kohlar J and Darland G et al 1988) (Tehrani J.L et al 1992).

#### **Protoplast fusion in bacteria:**

In bacteria protoplast can be obtained and fusion can be carried out with low frequency in some gram positive organisms.For gram negative bacteria it is possible to obtain protoplast but regeneration is difficult. The procedure is highly efficient and yields upto 80% transformants (Iwata M. et al 1986) (Jogdand S.N.2001).

#### **Biotechnological applications of protoplast fusion:**

Protoplasts contained all the intracellular organelles of cells and form a vital link in transfer of micromolecules between cyto organelles,currently most of the laboratories engaging in fungal genetics are using gene manipulation procedures based on protoplasts.Therefore to further improve the genetic properties of these strains using protoplast fusion are attempt to develop methods for preparation and regeneration of protoplasts.The process involves protoplast mutagenesis,transformation and protoplast fusion ( Evans D.A.1983). The direct bioconversion of cellulosic materials to ethanol by the intergeneric fusants between *T.reesei* and *Saccharomyces cerevesie* appears to be are of the best technique for an alternative approaches for ethanol production Also this process is helpful in the production of a complete set of cellulases by the protoplast fusion of *T.reesei* and *A.niger* (one produced more amount of endo and exoglucanase and other produced more β- glucosidase(Ahmed M

and Berkley EI 2006).Prabhavarthy V.R. et al (2006) reported that the isolated protoplast from *Trichoderma reesei* strain PTr2 showed high CMCase activity with 80% of fusants and more than two fold increment in enzyme activities with two fusants SFTr2 and SFTr3 as compared to the parental strain PTr2.Nazari R et al(2005) carried out intraspecific protoplast fusion with polyethylene glycol 1000 in *Streptomyces griseoflavus* to increase the production of desferrioxamine B chelator that absorbs additional iron from the blood of thalasemia patients.A haploid glucoamylase producing strains of *Saccharomyces diastiacus* lacking the pof gene and thus unable to produce phenolic off flavour in beer was constructed by classical genetic techniques.Protoplast fusion was performed between this strain and a brewing polyploidy *Saccharomyces uvarum* strain .Some clones derived from fusion products can produce low carbohydrate beer of acceptable flavor(Janderova B et al 1990).Rygielska J.K (2004) obtained interspecific fusants of starch fermenting yeasts *Schwanniomyces occidentalis* ATCC 48086 and yeasts *Saccharomyces diastiacus* ATCC 13007 and *Saccharomyces cerevisiae*.The fusants showed the tolerance to cyclohexamide(0.001%) in case of *Schwanniomyces occidentalis* ATCC 48086 and the ability of *Saccharomyces yeasts* to grow at 40°C. and also improved the ability to synthesize amylolytic enzymes to improve the biochemical ability of yeasts .Amylase hyper producing ,catabolite repression resistant recombinant strains were produced by intraspecific protoplast fusion of thermophilic fungus *Thermomyces lanuginosus* strains (Rubinder K et al 2000) (Zhao K et al(2004) studied on the preparation and regeneration of protoplast from Taxol producing fungus *Nodulisporium sylviforme* ,found that the combination of 4 enzymes (lywallzyme,snailase,lysozyme and cellulase) obtained higher the preparation frequency of protoplast than that of any of them used alone. Zhang ZB et al(2007) carried out protoplast fusion between the *Helminthosporium gramineum* sub sp,*Echinchloae* (HGE)strain ,HMI and *Curvularia lunata* (CL) to breed new strains with improved spore productivity and form fusant strains had increased production of the Phytotoxin ophiobolin A,compared with HMI.Liu WH etal(1996) carried out protoplast fusion between the protoplast of an inducible cholesterol oxidase producing bacterium *Arthrobacter simplex* USA 18 and a constitutive cholesterol oxidase producer strain US 3011 in the presence of 20-40% polyethylene glycol 6000 and the resultant fusants showed the cholesterol oxidase activity in a cholesterol containing medium with 20-60% higher than that of parental strains.Brume MD et al (1992) carried out protoplast fusion of morphologically and biochemically different *Claviceps purpurea* strains producing ergotoxins without introducing selective auxotrophic markers .Fused strains showed about ten fold higher alkaloid production than parental strains.Javedekar VS et al (1995)construct a highly flocculent yeast with a killer character by protoplast fusion .Resultant yeasts also being resistant to the fungicide at a concentration of 100 µg/ml.Pasha et al (2007) reported that protoplast fusion followed by sequential mutations gave a stable and good performing fusant with maximum utilization of reducing sugars in the media.They fused the protoplasts of thermotolerant *Saccharomyces cerevisiae* and mesophilic,xylose-utilizing *Candida shehatae* by electrofusion,and the selected best fusant was mutated sequentially.The mutant fusant CP11 was found to be stable and used for lignocellulosic fermentation and gave an ethanol

yield of 0.459 g g<sup>-1</sup> productivity of 0.67 g/l/h and fermentation efficiency of 90%. Electrically induced protoplast fusion employed for hybrid construction in ergosterol producing yeast strains. Some fusion products proved to be hybrid with respect to ergosterol content and to remain stable over several generations (Avram D et al 1992). Yari S et al (2002) studied the effects of protoplast fusion on  $\delta$ -endotoxins production in *Bacillus thuringiensis* spp (H14) found that *Bacillus thuringiensis* fusants have 1.48 time more  $\delta$ -endotoxins than wild type. According to US Patent 7241588 Fusants of *Penicillium chrysogenum* and *Cephalosporium acremonium* produced a novel lactam antibiotic. Kohlar J and Darland G (1988) investigated the protoplast fusion in *Streptomyces avermitilis* which involved in avermectin biosynthesis. resulting fusants showed improved properties in respect to rifampicin resistance and maintaining the ability to carry out the methylation of C-5 hydroxyl of the avermectin molecules. Arti Das and Anuradha Ghosh (1989) carried out protoplast fusion between two strains of *Aspergillus niger* 8-2 a fast growing strain and poor producer of glucoamylase and *Aspergillus niger* 8-7, a slow growing strain and good producer of enzyme and the resulting fusant produced 68% more glucoamylase than parental strains. Bakthiari M.R. et al (2007) carried out protoplast fusion between different strains of *Tolypocladium inflatum*. One of the recombinants produced cyclosporine 2.8 times more than parental strain.

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