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7. Fungal Recombinant Technology

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7.1 Information:

As human we have a long heritage of using fungi and fungal products in fungal biotechnology such as baking, brewing and the numerous fermented food products originated hundreds or even thousands of years ago and largely by the chance of association between natural fungi and one or more of substances of food material.

Although the original discovery of first antibiotic was also a matter of chance by Sir Alexander Fleming, a Scottish researcher, is credited with the discovery of penicillin in 1928. At the time, Fleming was experimenting with the influenza virus in the Laboratory of the Inoculation Department at St. Mary's Hospital in London. Further all improvements of penicillin were at the molecular level. Techniques were found that enabled cultivation of particular organisms and strains were selected that had advantageous biological characteristics.

From ancient times, fungi have been used to produce products such as beer, wine, bread, and cheese. The twentieth century, a golden age of industrial microbiology, yielded thousands of products made by fermentation processes: solvents, antibiotics, enzymes, vitamins, amino acids, polymers, and many other useful compounds.

The development of molecular biology techniques provided new ways to use yeasts and molds as microbial cell factories for the production of homologous and heterologous (especially mammalian) proteins as well as other metabolites, such as antibiotics, pigments, and fatty acids. The choice of the strain is made on the basis of production yields and regulatory issues, especially for fungi used in the food industry. The strains are usually chosen from among those which have attained the so called GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration (FDA). Several species of fungi have that status and are currently being used for large-scale production of recombinant proteins and metabolites.

Citric acid was the first biotechnology product and the techniques developed for production of citric acid by surface cultures of a filamentous fungus in batch mode were applied in the 1940s to production of a much more revolutionary fungal product, the antibiotic penicillin. We have probably heard the story about penicillin being discovered by chance when Alexander Fleming noticed that his bacterial strain *Staphylococcus aureus* had killed by *Penicillium notatum*, a contaminating fungus on his culture plate. However, the details history of penicillin is as bellow:

- **Alexander Fleming (1928)** in St. Mary's Hospital London discovered his cultures of *Staphylococcus aureus* were killed by contaminating colonies of *Penicillium notatum* and have said some time later: "I must have had an idea that this was of some importance, for I preserved the original culture plate".
- **Howard Florey** and **Ernst Chain** (1940) in Oxford University, as part of a survey of antibacterial substances produced by micro-organisms showed the chemotherapeutic properties of penicillin.
- **Dorothy Hodgkin** and **Barbara Law** (1945) in Oxford used X-ray crystallography to establish the β-lactam structure of penicillin.
- **John Sheehan** (1956) in USA produced penicillin by chemical synthesis.

Penicillin contributed in a major way to a revolutionary change in medical treatment which in turn changed the human lifestyle to such an extent that diseases which were common causes of death and disability before penicillin are now rarely encountered. This dramatic change to the every-day experience of everyone on the planet is the most remarkable aspect of the discovery and introduction of penicillin.

Arachidonic acid is another important medicine which is a fermentative production of zygomycetous fungi. Arachidonic acid is one of the essential fatty acids required by most of the mammals and a major constituent of the membranes of many animal cells contributing to their fluidity. Arachidonic acid has a wide variety of physiological functions in animals and consequently used in many ways in medicine, pharmacology, cosmetics, the food industry and agriculture. It is a precursor of prostaglandins, thromboxane and leukotrienes.

- **Prostaglandins** which modulate immune function via the lymphocyte. They are mediators of the vascular phases of inflammation and are potent vasodilators. They increase vascular permeability. Prostacyclin is a vasodilator hormone that works with thromboxane in a homeostasic mechanism in relation to vascular damage.
- **Thromboxane** is a powerful vasoconstrictor that also increases platelet aggregation
- **Leukotrienes** which mediate inflammation causing vasoconstriction but increased microvascular permeability.

The physiological pathways to which arachidonic acid contributes in the whole animal are major mechanisms for production of pain and inflammation and control of homeostatic function.

Some mammals lack the ability to convert linoleic acid into arachidonic acid or have a very limited capacity to produce arachidonic acid which makes arachidonic acid an essential part of their diet. As plants have little or no arachidonic acid so these mammals are obligate carnivores.

Humans get their arachidonic acid from both dietary animal sources (meat, eggs, and dairy products) or by synthesis from linoleic acid. Animal liver, fish oil and egg yolk are well known sources of arachidonic acid but zygomycete fungi of the genus *Mortierella* are also prominent sources of arachidonic acid and production levels can be modified by mutations in genes contributing to fatty acid synthesis in *M. alpina* (Sakuradani *et al*., 2004). Conventional fermentation with *Mortierella* is a promising production method for easily-purified arachidonic acid particularly in a two-step fed-batch fermentation process. Arachidonic acid has useful clinical effects in lowering cholesterol and triacylglycerols in plasma with good effects on arteriosclerosis and other cardiovascular diseases. It is present in breast milk and is important in brain and retina development in newborn infants and for these reasons it is added to infant feeding 'formula milk'. Studies have demonstrated that feeding formulas supplemented with arachidonic acid resulted in enhanced growth of infants and provided better developmental outcomes than without supplemented formulas (Clandinin *et al*., 2005).

7.2 Improvement of Fungal Strain:

Production of new fungal metabolites by application of recombinant DNA technologies is of great interest. Continued progress in the area of metabolic engineering has led to overproduction of limiting enzymes of important biosynthetic pathways, thus increasing production of the final products. For production of good quality and quantity product through fermentation, new microbial strains are developed. Improvement of industrial strains means improvement of the productivity of the microorganisms which are used in industrial production of fermented products.

7.3 Techniques Applicable to Yeast Strain improvement:

Strains of baker's yeast have improved in recent years to accommodate extensive marketing strategies. Traditional strains have now become inadequate to satisfy consumer's demand. Strain selection and improvement are essential ongoing research programs for baker's yeast producers. Two principal objectives are:-

7.3.1 Improving baking activities through developing new strains with:

a. Rapid maltose fermenting ability
b. Enhanced osmotolerance
c. Increased fermentation rate
d. Improved brew dough activity
e. Better freeze tolerance
f. Increased stability

7.3.2 Manufacturing Low-Cost, High Yield Baker's Yeast:

Wild yeast strains generally show limited use in commercial operations. However, improvement of wild type can be achieved by selecting cultures that exhibit the desired characteristics. Techniques used to obtain novel yeast strains include strain selection, mutagensis, hybridisation and protoplast fusion and/or contemporary transformation and recombinant DNA techniques.

A. Strain Selection:

One of the earliest methods of strain selection is the use of single-cell clones from naturally occurring yeast population that performs certain activities. This technique involves the recognition of desirable characteristics and direct selection from a given yeast population. One of the major disadvantages of this method is the lack of desired genetic homogenicity in the strain overtime.

B. Mutagenesis:

Mutagenesis which stand for the use of chemical mutagens, ultraviolet light or radiation to generate in one step strains with improved expression and secretion of the product of interest. UV light or ethyl methanesulfonate can be used to induce mutation in yeast. These reactions alter the DNA sequence by changing the hydrogen bonds between the DNA bases within the helix. Mutations carried out with industrial strains of yeast are mostly recessive and therefore are not expressed due to heterozygocity.

The mutation is irreversible. It is also more difficult to induce a phenotypic change in diploid or polyploid commercial strains than the haploid laboratory counterpart. Most baker's yeast strains are aneuploidy, with an average number of ploidy higher than two. This special feature is the main reason why strain improvement by mutation techniques is rarely successful. One of the disadvantages of this method is that variation is always present within a yeast population. Successful isolation of mutants depends on the frequency within which they occur and to devise appropriate selection procedures. This technique has been used successfully to improve several products such as α-amylase by *Aspergillus oryzae*, cellulase activity by *Trichoderma reesei* and penicillin by *Penicillium chrysogenum*.

C. Hybridisation:

This was the pioneering effort of Winge and Lausten in 1938. Hybridisation led to the production of inters specific yeast hybrids by mating haploids of opposite mating type to give a heterozygous diploid. Recombinant meiotic progeny were recovered by sporulating the diploid and recovering the individual haploid spores. Researchers tried to apply hybridisation directly to manipulate industrial strains and encountered numerous difficulties, including homothallic nature of these yeast strains, lack of mating ability, poor sporulation and poor spore viability. These problems are due to industrial yeast strains having polyploid or aneuploid.

D. Protoplast Fusion:

This technique establishes recombination through fusion in yeast strains that do not have the ability to mate naturally. Complementary biochemical mutants are used and protoplasts are generated following the treatment of whole cells with lytic enzymes. It was first described by van Solingen and van der Plaat in 1977.

The fusion has been used in the past to genetically improve both brewers and baker's yeasts. An important application of this method is the recombination through fusion of strains that do not have the ability to mate naturally.

This technique overcomes the problem of reduced mating response and has also been put forward as a mean to produce interspecific and intergenic hybrids. The major problem with spheroplast fusion is that wide ranges of cell types are produced which are often mitotically unstable.

This being especially true for inter- species and inter-genetic fusion. Spheroplast (or sphaeroplast in British usage) is a microbial cell from which the cell wall has been almost completely removed, as by the action of penicillin or lysozyme. According to some definitions, the term is used to describe Gram-negative bacteria. Various antibiotics convert Gram-negative bacteria into spheroplasts.

These include peptidoglycan synthesis inhibitors such as fosfomycin, vancomycin, moenomycin, lactivicin and the β-lactam antibiotics. Antibiotics that inhibit biochemical pathways directly upstream of peptidoglycan synthesis induce spheroplasts too (e.g. fosmidomycin, phosphoenolpyruvate).

In addition to the above antibiotics, inhibitors of protein synthesis (e.g. chloramphenicol, oxytetracycline, several aminoglycosides) and inhibitors of folic acid synthesis (e.g. trimethoprim, sulfamethoxazole) also cause Gram-negative bacteria to form spheroplasts.

E. Transformation:

Transformation is an important technique in which exogenous DNA is introduced into a cell, resulting in genetic modification. In the case of fungi, the spheroplasts of the budding yeast *Saccharomyces cerevisiae* were first successfully transformed in 1978. Several methods to transform intact cells, including the lithium, electroporation, biolistic and glass bead methods, have been developed, and the efficiency of each method has been improved. These methods can be used for transforming other fungi such as yeasts (e.g., *Schizosaccharomyces pombe, Candida albicans* and *Pichia pastoris*) and filamentous fungi (e.g., *Aspergillus species*), although the efficiency of transformation of these fungi is generally lower than that of *S. cerevisiae*.

Transformation of *S. cerevisiae* by the spheroplast method was first performed by Hinnen et al., 1933. They transformed spheroplasts, prepared by enzymatic digestion of *S. cerevisiae leu2 3–112* mutant cells, with chimeric plasmid DNA containing *LEU2* but not a yeast replicon.

The transformation efficiency was only 30–50 transformants/μg of plasmid DNA, which must integrate into chromosomal DNA for establishing stable transformants. Transformation of *S. cerevisiae* involves the isolation of genetic material, *in vitro* manipulations and introduction of new genetic materials into yeast in such a way that it can be expressed, replicated and transmitted to daughter cells at division.

F. Recombinant DNA Technology:

Advances in molecular technology have provided an infinite scope for genetic engineering. Researchers can now understand and manipulate genes with precision. Recombinant DNA technology, when used in yeast strain selection, can overcome most of the problems associated

with the conventional strain selection methods. With this technique, it is possible to rearrange the yeast DNA in such a way that the resulting yeast transformant contains merely yeast DNA and no genetic material originating from other organisms. In 21st century, the recombinant DNA technology was applied in several model filamentous fungi. A few noteworthy examples are as bellow:

- **1973:** The first DNA-mediated transformation of a fungal species using genomic DNA without the use of vectors was carried out by Mishra & Tatum (1973) who transformed an inositol-requiring mutant strain of Neurospora crassa to inositol independence strain using DNA extracted from an inositol-independent strain.
- **1979:** Case *et al*. (1979) developed an efficient transformation system for *Neurospora crassa* that used sphaeroplasts and a recombinant *Escherichia coli* plasmid carrying *the N. crassa qa*-2^+ gene (which encodes the enzyme dehydroquinase).
- **1983:** Ballance, Buxton & Turner (1983) performed the first auxotrophic marker transformation in *Aspergillus nidulans* when they relieved an auxotrophic requirement for uridine in a mutant strain of *A. nidulans* by transformation with a cloned segment of *Neurospora crassa* DNA containing the corresponding (= homologous) gene coding for orotidine-5′-phosphate decarboxylase.
- **1985:** Successful transformation of a filamentous industrial fungus when Buxton, Gwynne & Davies (1985) transformed sphaeroplasts of a mutant of Aspergillus niger defective in ornithine transcarbamylase function with plasmids carrying a functional copy of the *argB* gene of *A. nidulans*, and Kelly & Hynes (1985) transformed *A. niger*, which cannot use acetamide as a nitrogen or carbon source, with the *amdS* (acetamidase) gene of *A nidulans*.

Recombinant proteins result from expression of genes which are introduced by recombinant DNA techniques. The product is described as being homologous protein where it results from expression of additional copies of a gene native to the species (for example, the transformation of the inositol-requiring mutant strain of *Neurospora crassa*.

The initial isolation of the gene of interest may be done by direct complementation. For example, an invertase-deficient mutant of *Saccharomyces cerevisiae* has been used to identify by complementation the corresponding invertase genes cloned from *Neurospora crassa* and *Aspergillus Niger*.

The most commonly employed method now, though, is by reverse genetics in which sequence information about the target protein and/or gene is used to design synthetic oligonucleotide probes that are then used to isolate the gene sequence from cDNA or genomic DNA libraries.

Natural genetic recombination which stand for classical ‘applied genetics’ involving cross-breeding to generate segregation and recombination of ‘desirable’ genes using parasexual cycle, heterokaryosis or protoplast fusion in combination with artificial selection of the required useful traits. This approach has been used successfully to improve productivity of glucoamylase by *Aspergillus Niger* and exoglucanase by *Trichoderma reesei*.

Gene manipulation involves extraction of the desired DNA fragment and ligation to the appropriate vector or DNA source. The recombinant DNA molecule is then propagated in a suitable host (Figure 7.1).

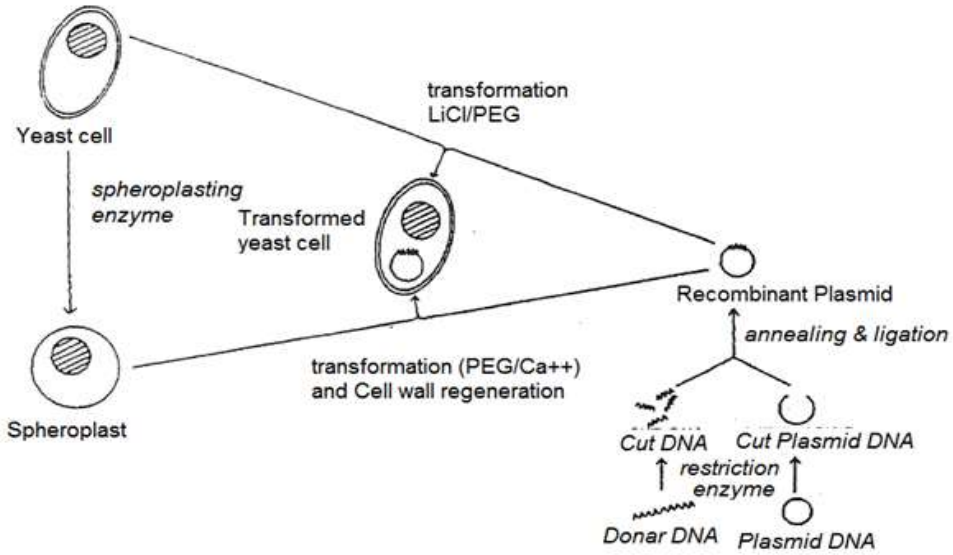

Figure 7.1: Two different gene transfer technique- spheroplast and rDNA

7.4 Recombinant Protein Production by Filamentous Fungi

Some recombinant proteins produced successfully by filamentous fungi are:

Xylanase is added to dough to improve bread production. 1, 4-β-endoxylanase from *Aspergillus awamori* is produced in *A. Niger* transformants containing multiple copies of the gene. The overproduced enzyme has the same biochemical properties as the original enzyme.

Phytase is a major phosphate reserve in plant seeds, cereals and oilseeds, up to 90% of total phosphorus can be stored as phytate which is almost indigestible to animals. *Aspergillus Niger* produces a 3-phytase enzyme that initiates dephosphorylation at the 3-phosphate (IP_3) position of inositolhexakisphosphate and efficiently breaks down phytate. In regions of high livestock density farmers use *Natuphos* as a tool to manage manure disposal and avoid overloading soil with high-phosphorus wastes.

The enzyme is also useful as a feed supplement to release digestible phosphorus and other phytate-bound nutrients to monogastric animals such as pigs and poultry. Phytase produced by *A. Niger* has high activity and is more thermostable than other microbial phytases. The enzyme is produced by an *A. Niger* strain transformed with multiple copies of the phytase gene (*phyA*).

7.4.1 Lipase: triglyceride lipases are added to biological detergents to remove grease stains during the wash cycle. The lipase gene from the thermophilic *Humicola* (=*Thermomyces*) *languinosus* has been used to transform *A. oryzae*. The recombinant lipase differs from the native lipase in having greater glycosylation and better thermos ability. Lipolase was first industrial enzyme made by a genetically engineered microorganism to be sold to the general public (*The world's first detergent lipase*).

Chymosin is an aspartyl protein which cleaves casein in milk to promote clotting. Rennilase® (rennin) from *Rhizomucor miehei* has been used since 1969 as an alternative to bovine chymosin. To produce the recombinant enzyme a chymosin cDNA sequence was fused to the last codon of the *Aspergillus awamori* glucoamylase gene and the construct was used to transform *A. awamori*. Yield of the proteinase by the transformed strain was improved further by classical mutagenesis and selection, and by site-directed mutagenesis to introduce a glycosylation site.

7.5 Production of Recombinant Polypeptides:

7.5.1 Mammalian Proteins:

Saccharomyces cerevisiae is considered to be a safe host for the production of pharmaceutical proteins. This yeast can be grown rapidly and to a high cell density, can secrete heterologous proteins into the extracellular broth, and knowledge of its genetics is more advanced than that of any other eukaryote. Mammalian genes have been cloned and expressed in *S. cerevisiae*, including human interferon, human epidermal growth factor, and human hemoglobin. The most commercially important yeast recombinant mammalian proteins have been the production of genes encoding surface antigens of the hepatitis B virus, resulting in the first safe hepatitis B vaccine. Despite these successful examples, *S. cerevisiae* is sometimes regarded as a less than optimal host for large scale production of mammalian proteins because of certain drawbacks, such as hyperglycosylation, the presence of alfa-1,3-linked mannose residues that may cause antigenic responses in patients, and the absence of strong and tightly regulated promoters. For these reasons, *Pichia pastoris* has become one of the most extensively used expression systems. Among the advantages of this methylotrophic yeast over *S. cerevisiae* are:

a. An efficient and tightly regulated methanol promoter (AOX1) which yields alcohol oxidase at 30% of soluble protein,
b. Less extensive glycosylation, due to shorter chain lengths of N-linked high-mannose oligosaccharides, usually up to 20 residues lacking the terminal alfa-1, 3-mannose linkages.
c. Integration of multiple copies of foreign DNA into chromosomal DNA yielding stable transformants.
d. The ability to secrete high levels of foreign proteins,
e. High-density growth and straightforward scale-up.

There are many examples of intracellular or extracellular recombinant products that have been made in *P. pastoris*. Nonetheless, one of the main drawbacks to this excellent expression system is its non-GRAS status, although some products made by this yeast are being evaluated in phase III clinical trials. For example, the production of recombinant hirudin, a thrombin inhibitor from the medicinal leeches Hirudo medicinalis.

7.6 Commercial Recombinant Enzymes:

Recombinant fungi are one of the main sources of enzymes for industrial applications. The industrial enzyme market reached 1.6 billion in 1998 for the following application areas: food, 45%; detergents, 34%; textiles, 11%; leather, 3%; pulp and paper 1.2%.

Over 60% of the enzymes used in the detergent, 192 food, and starch processing industries are recombinant products.

Although the number of heterologous fungal enzymes approved for food applications is not very large, the list is continuously increasing. Due to the low yields achieved with non-fungal proteins (see above), many recombinant food-grade proteins are of fungal origin. There is one exception in which the donor strain is not another fungus, i.e., calf rennin (chymosin), which is used for cheese making. Production of this bovine protein in recombinant *Aspergillus Niger* var *awamori* amounted to about 1g/l after nitrosoguanidine mutagenesis and selection for 2-deoxyglucose resistance. Further improvement was done by parasexual recombination, resulting in a strain producing 1.5 g/l from parents producing 1.2g/l. A recombinant strain of *Aspergillus oryzae* producing an aspartic proteinase from *Rhizomucor miehei* has been approved by FDA for cheese production. Microbial lipases have a huge potential in areas such as food technology, biomedical sciences, and chemical industries since they are:

- Stable in organic solvents,
- Possess broad substrate specificity,
- Do not require cofactors and
- Exhibit high enanti selectivity.

In the food industry, lipases are commonly used in the production of fruit juices, baked foods, desirable flavors in cheeses, and interesterification of fats and oils to produce modified acylglycerols. There are three fungal recombinant lipases currently used in the food industry, Rhizomucor miehi, *Thermomyces lanuginosus* and *Fusarium oxysporum*, all of which are produced in *A. oryzae*.

7.7 Thaumatin:

Thaumatin, a protein from the plant *Thaumatococcus danielli* with an intense sweetness (about 3,000 times more than sucrose), has been recently approved as a food-grade ingredient. Successful expression of thaumatin was achieved in *Penicillium roqueforti* and *A. Niger* var *awamori*. Recently, an impressive improvement in yield (up to 14 mg/l) has been obtained in *A. Niger* var *awamori* by use of stronger promoters and higher gene dosage. Production of the sweetener xylitol has also been improved by transforming the XYL1 gene of *Pichia stipitis* encoding a xylose reductase into *S. cerevisiae*.

Lactic Acid:

Lactic acid production in *S. cerevisiae* has been achieved by cloning and expression of a muscle bovine lactate dehydrogenase gene, reaching productivities of 11 g/l h.

Steroidal Drug Synthesis:

Combining heterologous gene expression of a single plant enzyme and eight mammalian proteins, as well as four targeted gene deletions, led to a recombinant *S. cerevisiae* strain able to produce hydrocortisone, the major adrenal glucocorticoid of mammals and an important intermediate of steroidal drug synthesis.

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