3 INDUSTRIAL MICROORGANISMS

Microorganisms are used extensively to provide a vast range of products and services (Table 1.3). They have proved to be particularly useful because of the ease of their mass cultivation, speed of growth, use of cheap substrates (which in many cases are wastes) and the diversity of potential products. Their ability to readily undergo genetic manipulation has also opened up almost limitless further possibilities for new products and services from the fermentation industries.

Traditional fermentations were originally performed (and still are in many cases) by a mixture of wild microorganisms emanating from the raw materials or the local environment, e.g., some food and alcoholic beverage fermentations. Initial attempts to improve the microorganisms involved occurred little more than 120 years ago, when they were first isolated from these processes as pure cultures from which the most useful strains were then selected. Those fermentation processes developed in the first 80 years of the 20th century have mostly used monocultures. The specific microorganisms employed were often isolated from the natural environment which involved the random screening of a large number of isolates. Alternatively, suitable microorganisms were acquired from culture collections. Most of these microorganisms, irrespective of their origins, were subsequently modified by conventional strain improvement strategies, using mutagenesis or breeding programmes, to improve their properties for industrial use. Several processes developed in the past 20 years have involved recombinant microorganisms and genetic engineering technologies have increasingly been used to improve established industrial strains.

In most cases, regulatory considerations are of major importance when choosing microorganisms for industrial use. Fermentation industries often prefer to use established GRAS (Generally Regarded As Safe) microorganisms, particularly for the manufacture of food products and ingredients. This is because requirements for process and product approval using a "new" microorganism are more stringent and associated costs are much higher. Where pathogens and some GMMs are used as the producer organism, additional safety measures must be taken. Special containment facilities are employed and it may be possible to use modified ("crippled") strains that cannot exist outside the fermenter environment.

3.1 ISOLATION OF SUITABLE MICROORGANISMS FROM THE ENVIRONMENT

Strategies that are adopted for the isolation of a suitable industrial microorganism from the environment can be divided into two types, "**shotgun"** and **objective** approaches. In the shotgun approach, samples of free living microorganisms, biofilms or other microbial communities are collected from animal and plant material, soil, sewage, water and waste streams and particularly from unusual man-made and natural habitats. These isolates are then screened for desirable traits. The alternative is to take a more objective approach by sampling from specific sites where organisms with the desired characteristics are considered to be likely components of the natural microflora. For example, when attempting to isolate an organism that can degrade or detoxify a specific target compound, sites may be sampled that are known to be contaminated by this material. These environmental conditions may select for microorganisms able to metabolize this compound.

Once the samples have been collected, a major problem is deciding on the growth media and cultivation conditions that should be used to isolate the target microorganism(s). An initial step is often to kill or repress the proliferation of common organisms and encourage the growth of rare ones. **Enrichment cultures** may then be performed in batch culture, or often more suitably in continuous systems. This encourages the growth of those organisms with the desired traits and increases the quantity of these target organisms, prior to isolation and screening. However, this mode of selection is suitable only for cases where the desired trait provides a competitive advantage for the organisms.

Subsequent isolation as pure cultures on solid growth media involves choosing or developing the appropriate selective media and growth conditions. Once isolated as pure cultures, each must be screened for the desired property, production of a specific enzyme, inhibitory compound, etc. However, at this stage the level of activity or concentration of the target product *per se* is not of major concern, as strain development can normally be employed to vastly improve performance. Selected isolates must also be screened for other important features, such as stability and where necessary, non-toxicity.

These isolation and screening procedures are more easily applied to the search for a single microorganism. However, it is much more difficult to isolate consortia which together have the ability/characteristic that is sought and whose composition may vary with time. Such groups can be more efficient, particularly where the ability to degrade a complex recalcitrant compound is involved.

3.2 CULTURE COLLECTIONS

Microbial culture collections provide a rich source of microorganisms that are of past, present and potential future interest. There are almost 500 culture collections around the world; most of these are small, specialized collections that supply cultures or other related services only by special agreement. Others, notably national collections, publish catalogues listing the organisms held and provide extensive services for industrial and academic organisations. In the UK for example, the National Culture Collection (UKNCC) is made up of several collections. They are housed in separate institutions and tend to specialize in bacteria, yeasts, filamentous fungi or algae of either industrial or medical importance; whereas in the USA there is a main centralized collection, the American Type Culture Collection (ATCC), which holds all types of microorganisms.

The prime functions of a culture collection are to maintain the existing collection, to continue to collect new strains and to provide pure, authenticated culture samples of each organism. Problems of culture maintenance have been aided by the development and use of cryopreservation and freeze-drying (lyophilization) techniques, along with miniaturized storage methods. One convenient method involves adsorption of cells to glass beads (2 mm diameter) that may be placed in frozen storage, from which individual beads may be removed without thawing the whole sample.

Use of microorganisms selected from a culture collection obviously provides significant cost savings compared with environmental isolation and has the advantage that some characterization of the microorganism will have already been performed. However the disadvantage is that competitors have access to the same microorganisms.

3.3 INDUSTRIAL STRAINS AND STRAIN IMPROVEMENT

Irrespective of the origins of an individual microorganism, it should ideally exhibit:

1. Genetic stability.
2. Efficient production of the target product, whose route of biosynthesis should preferably be well characterized.
3. Limited or no need for vitamins and additional growth factors.
4. Utilization of a wide range of low-cost and readily available carbon sources.
5. Amenability to genetic manipulation.
6. Safety, non-pathogenicity and should not produce toxic agents, unless this is the target product.
7. Ready harvesting from the fermentation.
8. Ready breakage, if the target product is intracellular.

In biotechnological processes the aim is primarily to improve/optimise the particular characteristics sought in an organism. Advances have been achieved in this area by using screening and selection techniques to obtain better organisms. In a selection system, all rare or novel strains grow while the rest do not. In a screening system, all strains grow but certain strains or cultures are chosen because they show the desired qualities required by the industry in question.

3.3.1 GENETIC MANIPULATION OF MICROORGANISMS

Genetic manipulations are used to produce microorganisms with new and desirable characteristics. The classical methods of microbial genetics play a vital role in the development of cultures for industrial microbiology.

3.3.1.1 Mutation

Once a promising culture is found, a variety of techniques can be used for culture improvement, including chemical mutagenesis and ultraviolet light. However, such methods normally lead only to the loss of undesirable traits or increased production due to loss of control functions. It has rarely led to the appearance of a new function or property. Thus, an organism with a desired feature will be selected from the natural environment, propagated and subjected to a mutational programme, then screened to select the best progeny. As an example, the first cultures of *Penicillium notatum*, which could be grown only under static conditions, yielded low concentrations of penicillin. In 1943 a strain of *P. chrysogenum* was isolated – strain NRRL 1951 – which was further improved through mutation (using X-ray treatment, UV and mustard gas the yield was increased from 120 IU to 2 580 IU). Today most penicillin is produced with *P. chrysogenum* grown in aerobic stirred fermenters, which gives 55-fold higher penicillin yields than the original static cultures.

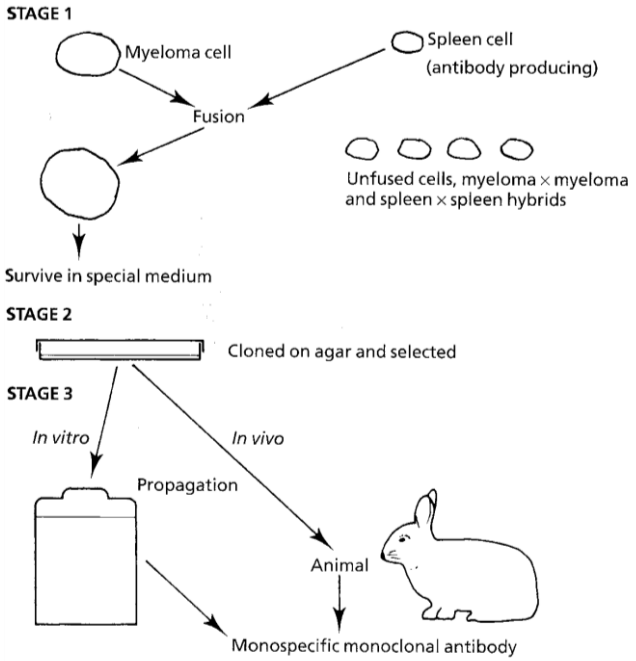
In recent years industrial genetics has come to depend increasingly on two new ways of manipulating DNA – protoplast and cell fusion and recombinant DNA technology (genetic engineering).

3.3.1.2 Protoplast Fusion

Protoplast fusion is now widely used with yeasts and moulds. Most of these microorganisms are asexual or of a single mating type, which decreases the chance of random mutations that could lead to strain degeneration. To carry out genetic studies with these microorganisms, protoplasts are prepared by growing the cells in an isotonic solution while treating them with enzymes, including cellulose and ß-galacturonidase. The protoplasts are then regenerated using osmotic stabilizers such as sucrose. If fusion occurs to form hybrids, desired recombinants are identified by means of selective plating techniques. After regeneration of the cell wall, the new protoplasm fusion product can be used in further studies.

A major advantage of the protoplast fusion technique is that protoplasts of different microbial species can be fused, even if they are not closely linked taxonomically. For example, protoplasts of *Penicillium roquefortii* have been fused with *P. chrysogenum*. Even yeast protoplasts and erythrocytes can be fused.

One of the most exciting and commercially rewarding areas of biotechnology involves a form of mammalian cell fusion to the formation of monoclonal antibodies. In 1975, pure monoclonal antibodies were produced from the fusion product of (hybridoma) of ß-lymphocytes and myeloma tumour cells. The monoclonal antibody technique changes antibody secreting cells (with a limited life span) to cells that are capable of continuous growth (immortalisation) while maintaining their specific antibody secreting potential (Fig. 3.1). Monoclonal antibodies are now widely applied in many diagnostic techniques which require a high degree of specificity. Specific monoclonal antibodies have been combined into diagnostic kits in health care, in plant and animal agriculture and food manufacture.



Stage 1: myeloma cells and antibody-producing cells (derived from immunized animal or man) are incubated in a special medium containing polyethylylene glycol, which enhances fusion.
Stage 2: the myeloma spleen hybridoma cells are selected out and cultured in closed agar dishes.
Stage 3: the specific antibody-producing hybridoma is selected and propagated in culture vessels (*in vitro*) or in an animal (*in vivo*) and monoclonal antibodies are harvested.

Fig. 3.1: The formation of antibody-producing hybridomas by fusion techniques.

3.3.1.3 Genetic Engineering

Genetic recombination occurs during normal sexual reproduction and as a result of the breakage and rejoining of DNA molecules of the chromosomes, there is reassortment of genetic material – but this is restricted to close taxonomic relatives. Recombinant DNA technology or genetic engineering offers unlimited opportunities for creating new combinations of genes. These techniques allow the splicing of DNA molecules of quite diverse origin and when combined with the techniques of genetic transformation, facilitate the introduction of foreign DNA into other organisms (Fig. 3.2). DNA can be isolated from plants, animals or microorganisms (the donors), and fragmented into groups of one or more genes. Such fragments can then be couples to another piece of DNA (the vector) and then passed into the host or recipient cell, becoming part of the genetic complement of the new host. The host cell can then be propagated in mass to form novel genetic properties and chemical abilities that were unattainable by conventional ways of breeding or mutation.

Short lengths of chemically synthesized DNA sequences can be inserted into recipient microorganisms by the process of **site-directed mutagenesis**. This can create small genetic alterations leading to a change of one or several amino acids in a target protein. Such minor amino acid changes have been found to lead, in many cases, to unexpected changes in protein characteristics and have resulted in new products such as more environmentally resistant enzymes and enzymes that can catalyze desired reactions. These approaches are part of the field of **protein engineering**. Enzymes and bioactive peptides with markedly different characteristics (stability, kinetics, activities) can be created. The molecular basis for the functioning of these modified products also can be better understood. One of the most interesting areas **is the design of enzyme-active sites to promote the modification of "unnatural substrates".** This approach may lead to improved transformation of recalcitrant materials, or even the degradation of materials that have previously not been amenable to biological processing.

Table 3.1: Strategies involved in genetic engineering

STRATEGY	METHOD
Formation of DNA fragment	Extracted DNA can be cut into smaller fragments by specific enzymes - restriction endonucleases found in many species of bacteria.
Splicing of DNA into vectors	The small sequences of DNA can be joined or spliced into the vector DNA molecules by an enzyme DNA ligase, creating an artificial DNA molecule.
Introduction of vectors into host cells	The vectors are either viruses or plasmids and are replicons and can exist in an extra-chromosomal state; they can be transferred normally by transduction or transformation.
Selection of newly acquired DNA	Selection and ultimate characterisation of the recombinant clone.

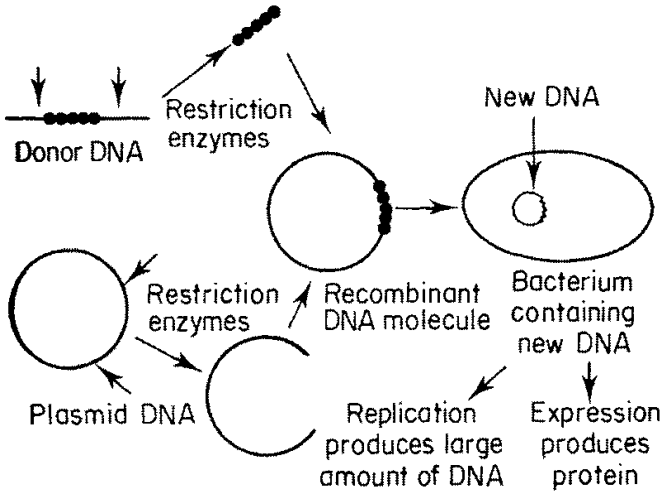


Fig 3.2: Recombinant DNA: the technique of recombining genes from one species with those of another.

3.4 PRESERVATION OF MICROORGANISMS

Once a microorganism or virus has been selected or created to serve a specific purpose, it must be preserved in its original form for further use and study. Periodic transfers of cultures have been used in the past although this can lead to mutations and phenotypic changes in microorganisms. To avoid these problems a variety of culture preservation techniques may be used to maintain desired culture characteristics (Table 3.2). **Lyophilization,** or freeze-drying and storage in liquid nitrogen are frequently employed with microorganisms.

3.4.1 CULTURE MAINTENANCE MEDIA

These media are used for the storage and sub culturing of key industrial strains. They are designed to retain good cell viability and minimize the possible development of genetic variation. In particular, they must reduce the production of toxic metabolites that can have strain-destabilizing effects. If strains are naturally unstable, they should be maintained on media selected for the specific characteristic that must be retained.

Table 3.2: Methods Used to Preserve Cultures for Industrial Microbiology and Biotechnology

METHOD	COMMENTS
Periodic transfer	Variables of periodic transfer to new media include frequency, medium used and holding temperature. This can lead to increased mutation rates and production of variants.
Mineral oil slant	A stock culture is grown on a slant and covered with sterilized mineral oil; the slant can be stored at refrigerator temperature.

Minimal medium, distilled water, or water agar	Washed cultures are stored under refrigeration; these cultures can be viable for 3-5 months or longer.
Freezing in growth medium	Not reliable, can result in damage to microbial structures, with some microorganisms, however this can be a useful means of culture maintenance.
Drying	Cultures are dried on sterile soil (solid stocks), on sterile filter paper disks, or in gelatine drops; these can be stored in a dessicator at refrigeration temperature or frozen to improve viability.
Freeze-drying	Water is removed by sublimation, in the presence of a cryoprotective agent; sealing in an ampoule can lead to long term viability, with 30 years having been reported.
Ultrafreezing	Liquid nitrogen at -196˚C is used, and cultures of fastidious microorganisms have been preserved for more than 15 years.