Isolation, Preservation and Improvement of Industrially Important Microorganisms
Criteria to select an industrially important microorganism

- **The nutritional characteristics of the organism:** It is frequently required that a process be carried out using a very cheap medium or a pre-determined one, e.g. the use of methanol as an energy source. These requirements may be met by the suitable design of the isolation medium.

- **The optimum temperature of the organism:** The use of an organism having an optimum temperature above 40° considerably reduces the cooling costs of a large-scale fermentation and, therefore, the use of such a temperature in the isolation procedure may be beneficial.

- The reaction of the organism with the equipment to be employed and the suitability of the organism to the type of process to be used.

- The stability of the organism and its amenability to genetic manipulation.

- The productivity of the organism, measured in its ability to convert substrate into product and to give a high yield of product per unit time.

- The ease of product recovery from the culture.
<table>
<thead>
<tr>
<th>Culture Collection</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>National Collection of Type Cultures (NCTC)</td>
<td>PHS Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK</td>
</tr>
<tr>
<td>National Collection of Industrial and Marine Bacteria Ltd. (NCIB, NCMB)</td>
<td>23 St Mochtar Drive, Aberdeen AB2 1RY, UK</td>
</tr>
<tr>
<td>National Collection of Yeast Cultures (NCYC)</td>
<td>AFRC Institute of Food Research, Norwich Laboratory, Celney Lane, Norwich NR4 7UA, UK</td>
</tr>
<tr>
<td>Collection of International Mycological Institute (IMI)</td>
<td>Culture Collection and Industrial Service Division, Perry Lane, Kew, Surrey TW9 3AF, UK</td>
</tr>
<tr>
<td>American Type Culture Collection (ATCC)</td>
<td>12811 Parklawn Drive, Rockville, MD 20852, USA</td>
</tr>
<tr>
<td>Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM)</td>
<td>Mascheroder Weg 1 b, D-3300 Braunschweig, Germany</td>
</tr>
<tr>
<td>Centraalbureau voor Schimmelcultures (CBS)</td>
<td>P.O. Box 273, Oosterbussingel 1, NL-3740 AV Baarn, Netherlands</td>
</tr>
<tr>
<td>Czechoslovak Collection of Microorganisms (CCM)</td>
<td>Masaryk University, Jozefova 10, 662 43 Brno, Czech Republic</td>
</tr>
<tr>
<td>Collection Nationale de Cultures de Microorganismes (CNM)</td>
<td>Institut Pasteur, 25, rue du Docteur Roux, F-75248 Paris Cedex 15, France</td>
</tr>
<tr>
<td>Japan Collection of Microorganisms (JCM)</td>
<td>Riketsuka, Wako-shi, Saitama, 351-01 Japan</td>
</tr>
<tr>
<td>Culture Collection of the Institute for Fermentation (IFO)</td>
<td>Institute for Fermentation, 17-85 Juge-Honchimaichi, 2-chome, Yodogawa-ku, Osaka, Japan</td>
</tr>
</tbody>
</table>
Isolation methods utilizing selection of the desired characteristic

Liquid culture enrichment

- A model of the competition between two organisms capable of growth in a continuous enrichment culture is represented in Fig. 3.2.

- Consider the behaviour of the two organisms, A and B, in Fig. 3.2.

- In continuous culture the specific growth rate is determined by the substrate concentration and is equal to the dilution rate, so that at dilution rates below point Y in Fig. 3.2 strain B would be able to maintain a higher growth rate than strain A, whereas at dilution rates above Y strain A would be able to maintain a higher growth rate.

- Thus, if A and B were present in a continuous enrichment culture, limited by the substrate depicted in Fig. 3.2, strain A would be selected at dilution rates above Y and strain B would be selected at dilution rates below Y. Thus, the organisms which are isolated by continuous enrichment culture will depend on the dilution rate employed which may result in the isolation of organisms not so readily recovered by batch techniques.
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Continuous enrichment techniques

- Continuous enrichment techniques are especially valuable in isolating organisms to be used in a continuous-flow commercial process.

- Organisms isolated by batch enrichment and purification on solid media frequently perform poorly in continuous culture, whereas continuous enrichment provides an organism, or mixture of organisms, adapted to continuous culture.

- The enrichment procedure should be designed such that the predicted isolate meets as many of the criteria of the proposed process as possible (using the carbon as the sole source of organic carbon in the enrichment medium, and that the medium should be carbon limited)

- The inclusion of other organic carbon sources, such as vitamins or yeast extract, may result in the isolation of strains adapted to using these, rather than the principal carbon source, as energy sources.
The isolation of an organism capable of growth on a simple medium should also form the basis of a cheaper commercial process and should be more resistant to contamination – a major consideration in the design of a commercial continuous process.

The use of as high as possible an isolation temperature should also result in the isolation of a strain presenting minimal cooling problems in the subsequent process.

The main difficulty in using a continuous-enrichment process is the washout of the inoculum before an adapted culture is established. To address this, the isolation process should be started in batch culture using a 20% inoculum and as soon as growth is observed, the culture should be transferred to fresh medium and than isolation.
<table>
<thead>
<tr>
<th>Selective agent</th>
<th>Target organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruneomycin</td>
<td>Micromonospora</td>
</tr>
<tr>
<td>Dihydroxymethylfuratrizone</td>
<td>Microtetraspora</td>
</tr>
<tr>
<td>Gentamycine</td>
<td>Micromonospora</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Actinomadura</td>
</tr>
<tr>
<td>Nitrofurazone</td>
<td>Streptomyces</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Micromonospora</td>
</tr>
<tr>
<td>Tellurite</td>
<td>Actinoplanes</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>Micromonospora</td>
</tr>
</tbody>
</table>

Table 3.3 Selective substrates for the isolation of actinomycetes and antibiotic-producing actinomycetes

<table>
<thead>
<tr>
<th>Substrates selective for Actinomycetes</th>
<th>Substrates selective for antibiotic-producing actinomycetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>Proline</td>
</tr>
<tr>
<td>Glucose (1.0%)</td>
<td>Glucose (1.0%)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Starch</td>
<td>Starch</td>
</tr>
<tr>
<td>Humic acid (0.1%)</td>
<td>Humic acid (0.1%)</td>
</tr>
<tr>
<td>Propionate (0.1%)</td>
<td>Zinc</td>
</tr>
<tr>
<td>Methanol</td>
<td>Alanine</td>
</tr>
<tr>
<td>Nitrate</td>
<td>Potassium</td>
</tr>
<tr>
<td>Calcium</td>
<td>Vitamins</td>
</tr>
<tr>
<td></td>
<td>Cobalt (0.05%)</td>
</tr>
<tr>
<td></td>
<td>Phenol (0.01%)</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
</tr>
</tbody>
</table>
The most desirable isolation medium would be one which selects for the desired types and also allows maximum genetic expression. Cultures grown on such media could then be used directly in a screen.

However, it is more common that, once isolated, the organisms are grown on a range of media designed to enhance productivity.

Some guidelines for the design of overproduction media

**Table 3.4 Guidelines for ‘overproduction media’ (Nisbet, 1982)**

1. Prepare a range of media in which different types of nutrients become growth-limiting e.g. C, N, P, O
2. For each type of nutrient depletion use different forms of the growth-sufficient nutrient
3. Use a polymeric or complexed form of the growth-limiting nutrient
4. Avoid the use of readily assimilated forms of carbon (glucose) or nitrogen (NH$_4^+$) that may cause catabolite repression
5. Ensure that known cofactors are present (Co$^{3+}$, Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$).
6. Buffer to minimize pH changes.
Role of molecular biology in screening

The progress in molecular biology, genetics and immunology has also contributed extensively to the development of innovative screens, by enabling the construction of specific detector strains, increasing the availability of enzymes and receptors and constructing extremely sensitive assays.

(i) The provision of test organisms that have increased sensitivities, or resistances, to known agents. For example, the use of super-sensitive strains for the detection of β-lactam antibiotics.

(ii) The cloning of genes coding for enzymes or receptors that may be used in inhibitor or binding screens makes such materials more accessible and available in much larger amounts.

(iii) The development of reporter gene assays. A reporter gene is one which codes for an easily assayable product so that it can be used to detect the activation of a control sequence to which it is fused. Such systems have been used in the search for metabolites that disrupt viral replication.

(iv) Molecular probes for particular gene sequences may enable the detection of organisms capable of producing certain product groups. This information may be used to focus the search on these organisms in an attempt to find novel representatives of an already known commercially attractive chemical family.

(v) The development of immunologically based assays such as ELISA.
THE PRESERVATION OF INDUSTRIALLY IMPORTANT MICRO-ORGANISMS

- The isolation of a suitable organism for a commercial process may be a long and very expensive procedure and it is therefore essential that it retains the desirable characteristics that led to its selection.

- Also, the culture used to initiate an industrial fermentation must be viable and free from contamination.

- Thus, industrial cultures must be stored in such way as to eliminate genetic change, protect against contamination and retain viability.

- An organism may be kept viable by repeated sub-culture into fresh medium, but, at each cell division, there is a small probability of mutations occurring and because repeated sub-culture involves very many such divisions, there is a high probability that strain degeneration would occur.

- Also, repeated sub-culture carries with it the risk of contamination.

- Thus, preservation techniques have been developed to maintain cultures in a state of 'suspended animation' by storing either at reduced temperature or in a dehydrated form.
Storage at reduced temperature

STORAGE ON AGAR SLOPES

Cultures grown on agar slopes may be stored in a refrigerator (5°) or a freezer (-20°) and sub-cultured at approximately 6-monthly intervals. The time of subculture may be extended to 1 year if the slopes are covered with sterile medicinal grade mineral oil.

STORAGE UNDER LIQUID NITROGEN

- The metabolic activities of micro-organisms may be reduced considerably by storage at the very low temperatures (-150° to -196°) which may be achieved using a liquid nitrogen refrigerator.

- Fungi, bacteriophage, viruses, algae, yeasts, animal and plant cells and tissue cultures have all been successfully preserved.

- The technique involves growing a culture to the maximum stationary phase, resuspending the cells in a cryoprotective agent (such as 10% glycerol) and freezing the suspension in sealed ampoules before storage under liquid nitrogen.

- Some loss of viability is suffered during the freezing and thawing stages but there is virtually no loss during the storage period. Thus, viability may be predictable even after a period of many years.

- Snell (1991) suggested that liquid nitrogen is the method of choice for the preservation of valuable stock cultures and may be the only suitable method for the long term preservation of cells that do not survive freeze-drying.

- The method has the major disadvantage that liquid nitrogen evaporates and must be replenished regularly. It is also expensive.
Storage in a dehydrated form

DRIED CULTURES

- Dried soil cultures have been used widely for culture preservation, particularly for sporulating mycelial organisms.

- Moist, sterile soil may be inoculated with a culture and incubated for several days for some growth to occur and then allowed to dry at room temperature for approximately 2 weeks.

- The dry soil may be stored in a dry atmosphere or, preferably, in a refrigerator.

- The technique has been used extensively for the storage of fungi and actinomycetes.

- 1800 actinomycetes dried on soil about 50% were viable after 20-years storage.

- Silica gel and porcelain beads are suggested alternatives and inexpensive techniques.
LYOPHILIZATION

- Lyophilization, or freeze-drying, involves the freezing of a culture followed by its drying under vacuum, which results in the sublimation of the cell water.

- The technique involves growing the culture to the maximum stationary phase and resuspending the cells in a protective medium such as milk, serum or sodium glutamate.

- A few drops of the suspension are transferred to an ampoule, which is then frozen and subjected to a high vacuum until sublimation is complete, after which the ampoule is sealed.

- The ampoules may be stored in a refrigerator and the cells may remain viable for 10 years or more.

- Lyophilization is very convenient for service culture collections because, once dried, the cultures need no further attention and the storage equipment (a refrigerator) is cheap and reliable.

- The freeze-dried cultures are tedious to open and revitalize and several sub-cultures may be needed before the cells regain their typical characteristics.
Approaches of Strain Preservation

- Low Temperature Storage: -2-6°C (2-6 months)
- Storage as Frozen Culture: -20 to -100°C.
- Storage as Lyophilized cells: Under high Vacuum at low temperature (5/ even -20 to -70°C)
- Storage of Vegetative cells/spores in Liquid Nitrogen: -196°C / -167°C
- Air dried at room temperature on sterile loam sand or on other natural substrate: Like maize seed, rice, bran, etc., (bacterial culture may remain viable up to 70-80 years)
- Storage in Glycerin Stabs: 0.85ml of cell suspension mixed with 0.15ml of sterile glycerol and stored at -70 or -75°C.
THE IMPROVEMENT OF INDUSTRIAL MICRO-ORGANISMS

- Natural isolates usually produce commercially important products in very low concentrations and therefore every attempt is made to increase the productivity of the chosen organism.

- Increased yields may be achieved by optimizing the culture medium and growth conditions, but this approach will be limited by the organism's maximum ability to synthesize the product.

- The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the potential yield.

- The cultural requirements of the modified organism would then be examined to provide conditions that would fully exploit the increased potential of the culture, while further attempts are made to beneficially change the genome of the already improved strain.

- Thus, the process of strain improvement involves the continual genetic modification of the culture, followed by reappraisals of its cultural requirements.

- Genetic modification may be achieved by selecting natural variants, by selecting induced mutants and by selecting recombinants.
There is a small probability of a genetic change occurring each time a cell divides and when it is considered that a microbial culture will undergo a vast number of such divisions it is not surprising that the culture will become more heterogeneous.

The heterogeneity of some cultures can present serious problems of yield degeneration because the variants are usually inferior producers compared with the original culture.

However, variants have been isolated which are superior producers and this has been observed frequently in the early stages in the development of a natural product from a newly isolated organism.

An explanation of this phenomenon for mycelial organisms may be that most new isolates are probably heterokaryons (contain more than one type of nucleus) and the selection of the progeny of uninucleate spores results in the production of homokaryons (contain only one type of nucleus) which may be superior producers.

However, the phenomenon is also observed with unicellular isolates which are certainly not heterokaryons. Therefore, it is worthwhile to periodically plate out the producing culture and screen a proportion of the progeny for productivity.
Therefore, selection of natural variants may result in increased yields but it is not possible to rely on such improvements, and techniques must be employed to increase the chances of improving the culture.

These techniques are the isolation of induced mutants and recombination.

The most dramatic examples of strain improvement come from the applications of recombinant DNA technology which has resulted in organisms producing compounds which they were not able to produce previously.

Furthermore, the advances in these techniques have resulted in very significant improvements in the production of conventional fermentation products.

However, it should be remembered that these methods have not replaced mutant isolation but have made an invaluable addition to an impressive repertoire.
The techniques of mutant isolation have contributed enormously to the development of present-day industrial strains and these techniques will be considered first.

There are more inferior producers amongst the survivors of the ultraviolet treatment. There are also strains producing more than twice the parental level, far greater than the best of the natural variants. The use of ultraviolet light is only one of a large number of physical or chemical agents which increase the mutation-rate - such agents are termed mutagens.

The vast majority of induced mutations are deleterious to the yield of the desired product but, a minority are more productive than the parent.

The problem of obtaining the high-yielding mutants may be approached from two theoretical standpoints; the number of desirable mutants may be increased by 'directed mutation', i.e. the use of a technique which will preferentially produce particular mutants at a high rate; or techniques may be developed to improve the separation of the few desirable types from the large number of mediocre producers.
Fig. 3.3. The spread in productivity of chlortetracycline of natural variants of *Streptomyces viridifaciens* (Dulaney and Dulaney, 1967).

Fig. 3.4. The spread in chlortetracycline productivity of the survivors of a UV-treated population of *Streptomyces viridifaciens* (Dulaney and Dulaney, 1967).
Inherent in the concept of directed mutation is the assumption that a mutation programme can be optimized to produce mutants of a particular kind.

Ultraviolet radiation was the most effective mutagen for increasing the yield of tetracycline by strains of *Streptomyces aureofaciens*.

DeWitt *et al.* (1989) emphasized that as well as certain mutagens being more beneficial, the dose will affect the generation of the desired types. Despite these observations it is frequently the case that it is difficult to predict what type of mutation is required at the molecular level to improve a strain.
<table>
<thead>
<tr>
<th>Mutagen class</th>
<th>Mutagen type</th>
<th>Type of mutation</th>
<th>Impact of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td>X-rays; γ-rays</td>
<td>Deletions; structural changes</td>
<td>Single-stranded or double-stranded breakage of DNA</td>
</tr>
<tr>
<td></td>
<td>UV rays</td>
<td>Transversion, deletion, frameshift, transitions GC→AT</td>
<td>Pyrimidine dimerisation and cross links in the DNA</td>
</tr>
<tr>
<td>Chemical</td>
<td>5- Bromouracil</td>
<td>Transitions, AT→GC, GC→AT</td>
<td>Faulty pairing</td>
</tr>
<tr>
<td></td>
<td>5- Chlorouracil</td>
<td>Transitions, AT→GC, GC→AT</td>
<td>Faulty pairing</td>
</tr>
<tr>
<td></td>
<td>Hydroxylamine (NH₂OH)</td>
<td>GC→AT transition</td>
<td>Deamination of cytosine</td>
</tr>
<tr>
<td></td>
<td>Nitrous acid (HNO₂)</td>
<td>Bidirectional translation, deletion, AT→GC, GC→AT</td>
<td>Deamination of A, C and G</td>
</tr>
<tr>
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<td>N-methyl-N’-nitro-N-nitrosoguanidine</td>
<td>GC→AT transition</td>
<td>Methylation, high pH, alkylation of C and A</td>
</tr>
<tr>
<td></td>
<td>Ethyl methane sulphonate</td>
<td>GC→AT transition</td>
<td>Alkylation of bases C and A</td>
</tr>
</tbody>
</table>
The selection of induced mutants synthesizing improved levels of primary metabolites

- The levels of primary metabolites in micro-organisms are regulated by feedback control systems. The major systems involved are feedback inhibition and feedback repression.

- Feedback inhibition is the situation where the end product of a biochemical pathway inhibits the activity of an enzyme catalysing one of the reactions (normally the first reaction) of the pathway.

- Inhibition acts by the end product binding to the enzyme at an allosteric site which results in interference with the attachment of the enzyme to its substrate.

- Feedback repression is the situation where the end product (or a derivative of the end product) of a biochemical pathway prevents the synthesis of an enzyme (or enzymes) catalysing a reaction (or reactions) of the pathway.

- Repression occurs at the gene level by a derivative of the end product combining with the genome in such a way as to prevent the transcription of the gene into messenger RNA, thus resulting in the prevention of enzyme synthesis.
Feedback inhibition and repression frequently act in concert in the control of biosynthetic pathways, where inhibition may be visualized as a rapid control which switches off the biosynthesis of an end product and repression as a mechanism to then switch off the synthesis of temporarily redundant enzymes.

The control of pathways giving rise to only one product (i.e. unbranched pathways) is normally achieved by the first enzyme in the sequence being susceptible to inhibition by the end product and the synthesis of all the enzymes being susceptible to repression by the end product, as shown in Fig. 3.5.

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**Fig. 3.5.** The control of a biosynthetic pathway converting precursor A to end product E via the intermediates B, C and D.
➢ The control of biosynthetic pathways giving rise to a number of end products (branched pathways) is more complex than the control of simple, unbranched sequences.

➢ The end products of the same, branched biosynthetic pathway are rarely required by the microorganism to the same extent, so that if an end product exerts control over a part of the pathway common to two, or more, end products then the organism may suffer deprivation of the products not participating in the control.

➢ Thus, mechanisms have evolved which enable the level of end products of branched pathways to be controlled without depriving the cell of essential intermediates. The following descriptions of these mechanisms are based on the effect of the control, which may be arrived at by inhibition, repression or a combination of both systems.
Concerted or multivalent feedback control

This control system involves the control of the pathway by more than one end product the first enzyme of the pathway is inhibited or repressed only when all end products are in excess, as shown in Fig. 3.6.

Fig. 3.6. The control of a biosynthetic pathway by the concerted effects of products D and F on the first enzyme of the pathway.
Co-operative feedback control.

The system is similar to concerted control except that weak control may be effected by each end product independently. Thus, the presence of all end products in excess results in a synergistic repression or inhibition. The system is illustrated in Fig. 3.7 and it may be seen that for efficient control to occur when one product is in excess there should be a further control operational immediately after the branch point to the excess product. Thus, the reduced flow of intermediates will be diverted to the product which is still required.

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**Fig. 3.7.** The control of a biosynthetic pathway by the co-operative control by end products D and F.
**Cumulative feedback control**

- Each of the end products of the pathway inhibits the first enzyme by a certain percentage independently of the other end products.

- In Fig. 3.8 both D and F independently reduce the activity of the first enzyme by 50%, resulting in total inhibition when both products are in excess.

- As in the case of co-operative control, each end product must exert control immediately after the branch point so that the common intermediate, B, is diverted away from the pathway of the product in excess.

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**Fig. 3.8.** The control of a biosynthetic pathway by the cumulative control of products D and F.
Sequential feedback control

- Each end product of the pathway controls the enzyme immediately after the branch point to the product. The intermediates which then build up as a result of this control earlier enzymes in the pathway.

- Thus, in Fig. 3.9, D inhibits the conversion of B to C, and F inhibits the conversion of B to E. The inhibitory action of D, F, or both, would result in an accumulation of B which, in turn, would inhibit the conversion of A to B.

--- Feedback control

**Fig. 3.9.** The control of a biosynthetic pathway by sequential feedback control.
Isoenzyme control

- Isoenzymes are enzymes which catalyse the same reaction but differ in their control characteristics.

- Thus, if a critical control reaction of a pathway is catalysed by more than one isoenzyme, then the different isoenzymes may be controlled by the different end products.

- Such a control system should be very efficient, provided that control exists immediately after the branch point so that the reduced flow of intermediates is diverted away from the product in excess.

- An example of the system is shown in Fig. 3.10. Thus, the levels of microbial metabolites may be controlled by a variety of mechanisms, such that end products are synthesized in amounts not greater than those required for growth.

![Diagram](image_url)

**Fig. 3.10.** The control of two isoenzymes (catalysing the conversion of A to B) by end products D and F.
However, the ideal industrial micro-organism should produce amounts far greater than those required for growth and, as suggested earlier, an understanding of the control of production of a metabolite may enable the construction of a 'blueprint' of the most useful industrial mutant, i.e. one where the production of the metabolite is not restricted by the organism's control system.

**Such postulated mutants may be modified in three ways:**

1. The organism may be modified such that the end products which control the key enzymes of the pathway are lost from the cell due to some abnormality in the permeability of the cell membrane.

2. The organism may be modified such that it does not produce the end products which control the key enzymes of the pathway.

3. The organism may be modified such that it does not recognize the presence of inhibiting or repressing levels of the normal control metabolites.
Selection for Auxotrophic Mutants

1. Mutate a prototrophic strain (UV, chemicals)
2. Enrich by growing in Minimal media plus penicillin (only kills actively growing prototrophs)
3. Centrifuge, wash penicillin away, and plate and grow on Complete medium
4. Replica plate

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Complete media
All kinds of auxotrophs grow

Replica plating

Minimal media
Plus nutrient (e.g. adenine)

Minimal media
No Growth (Negative Control)
Modification of the permeability

- The best example of the modification of the permeability of a micro-organism is provided by the glutamic acid fermentation.

- Kinoshita et al. (1957a) isolated a biotin-requiring, glutamate-producing organism, subsequently named *Corynebacterium glutamicum*, the permeability of which could be modified by the level of biotin.

- Provided that the level of biotin in the production medium was below 5 μg dm⁻³ then the organism would excrete glutamate, but at concentrations of biotin optimum for growth the organism produced lactate (Kinoshita and Nakayama, 1978).

- Thus, the permeability of *C glutamicum* may be controlled by the composition of the culture medium.

- However, the isolation of *C glutamicum* was a major advance in the microbial production of amino acids and the demonstration of the role of permeability in the production of glutamate suggested the possibility of the genetic modification of permeability to achieve high levels of productivity.

- Thus, it is relevant at this stage to consider the physiology of glutamate production by biotin-limited culture.
Kinoshita's isolate was not only deficient in its ability to produce biotin but also in the level of α-ketoglutarate dehydrogenase, which normally converts α-ketoglutarate to succinate in the tricarboxylic acid cycle.

Thus, a metabolic block results in the accumulation of a large concentration of α-ketoglutarate which the organism converts to glutamic acid.

Oxaloacetate is regenerated in glutamate producers by the activity of the glyoxylate pathway as shown in Fig. 3.11.

When *C. glutamicum* was grown in a medium containing a high concentration of biotin, the organism synthesized glutamate at a level of 25-36 μg mg⁻¹ dry weight of cells, further production being assumed to be prevented by some form of feedback control by glutamate of its own synthesis.

Under conditions of biotin limitation, glutamate was released from the cells and accumulated to a level of up to 50 g dm⁻³. The increased permeability of the biotin-limited cells to glutamate corresponds with a change in the fatty acid and phospholipid content of the cell envelopes.

The crucial factor in the control of permeability appears to be the synthesis of membranes deficient in phospholipids.
Fig. 3.11. Biosynthesis of glutamate by *C. glutamicum*. Heavy lines indicate the route to glutamate and the light lines indicate the route in the regeneration of oxaloacetate via the glyoxylate cycle.
The role of biotin in the glutamate fermentation obviously necessitates the inclusion of limiting levels of biotin in the medium.

Thus, the use of crude carbon sources such as cane molasses, which are rich in biotin, presented difficulties which have been overcome by the modification of the permeability by means other than biotin limitation.

The inclusion of penicillin or fatty acid derivatives such as polyoxyethylene sorbitan mono-oleate (Tween 80) during logarithmic growth in biotin-rich medium caused an aberration of the cell envelope permeability resulting in the release of glutamate.

The permeability of the producing organism may be controlled by the addition of penicillin but a genetic solution to this problem has also been found.

Nakao et al. (1970) isolated a glycerol requiring auxotrophic mutant of Corynebacterium alkanolyticum in which phospholipid synthesis was controlled by the supply of glycerol.

The mutant produced about 40 g dm$^{-3}$ glutamate when grown on n-paraffins in the presence of 0.01% glycerol.

Thus, an understanding of the mode of action of biotin limitation in the glutamate fermentation has led to the use of genetic modification of permeability as a method to overcome the normal control mechanisms of the producing organism.
THE ISOLATION OF MUTANTS WHICH DO NOT PRODUCE FEEDBACK INHIBITORS OR REPRESSORS

Mutants which do not produce certain feedback inhibitors or repressors may be useful for the production of intermediates of unbranched pathways and intermediates and end products of branched pathways.

Demain (1972) presented several 'blue-prints' of hypothetical mutants producing intermediates and end products of biosynthetic pathways and these are illustrated in Fig. 3.12.

The mutants illustrated in Fig. 3.12 do not produce some of the inhibitors or repressors of the pathways considered and, thus, the control of the pathway is lifted, but, because the control factors are also essential for growth, they must be incorporated into the medium at concentrations which will allow growth to proceed but will not evoke the normal control reactions.
In the case of Fig. 3.12(1) the unbranched pathway is normally controlled by feedback inhibition or repression of the first enzyme of the pathway by the end product, E.

However, the organism represented in Fig. 3.12(1) is auxotrophic for E due to the inability to convert C to D so that control of the pathway is lifted and C will be accumulated provided that E is included in the medium at a level sufficient to maintain growth but insufficient to cause inhibition or repression.

Fig. 3.12. Overproduction of primary metabolites by decreasing the concentration of a repressing or inhibiting end product. ——— Site of auxotrophic mutations; —— feedback regulation; → overproduced product (Demain, 1972).
Figure 3.12(2) is a branched pathway controlled by the concerted inhibition of the first enzyme in the pathway by the combined effects of E and G.

The mutant illustrated is auxotrophic for E due to an inability to convert C to D, resulting in the removal of the concerted control of the first enzyme. Provided that E is included in the medium at a level sufficient to allow growth but insufficient to cause inhibition then C will be accumulated due to the control of the end product G on the conversion of C to F.

The example shown in Fig. 3.12(3) is similar to that in Fig. 3.12(2) except that it is a double auxotroph and requires the feeding of both E and G.

Figure 3.12(4) is, again, the same pathway and illustrates another double mutant with the deletion for the production of G occurring between F and G, resulting in the accumulation of F.

---

**Fig. 3.12.** Overproduction of primary metabolites by decreasing the concentration of a repressing or inhibiting end product. ····· Site of auxotrophic mutations; ------ feedback regulation; → overproduced product (Demain, 1972).
Figure 3.12(5) illustrates the accumulation of an end product of a branched pathway which is normally controlled by the feedback inhibition of the first enzyme in the pathway by the concerted effects of E and I.

The mutant illustrated is auxotrophic for I and G due to an inability to convert C to F and, thus, provided G and I are supplied in quantities which will satisfy growth requirements without causing inhibition, the end product, E, will be accumulated.
All the hypothetical examples discussed above are auxotrophic mutants and, under certain circumstances, may accumulate relatively high concentrations of intermediates or end products.

Therefore, the isolation of auxotrophic mutants may result in the isolation of high-producing strains, provided that the mutation for auxotrophy occurs at the correct site, e.g. between C and D in Figs 3.12(1) and (2).

The recovery of auxotrophs is a simpler process than is the recovery of high producers, as such, so that the best approach is to design a procedure to select relevant auxotrophs from the survivors of a mutation and subsequently screen the selected auxotrophs for productivity.
Fig. 3.13. The use of the penicillin selection method for the isolation of auxotrophic mutants of *C. glutamicum* (Abe, 1972).
EXAMPLES OF THE USE OF AUXOTROPHS FOR THE PRODUCTION OF PRIMARY METABOLITES

- Many auxotrophic mutants have been produced from *C. glutamicum* for the synthesis of both amino acids and nucleotide related compounds.

- *C. glutamicum* is a biotin-requiring organism which will produce glutamic acid under biotin-limited conditions but it is important to remember that mutants of this organism, employed for the production of other amino acids, must be supplied with levels of biotin optimum for growth.

- Biotin limited conditions will result in these mutants producing glutamate and not the desired amino acid.

- Auxotrophic mutants of *C. glutamicum* have been used for the production of lysine. The control of the production of the aspartate family of amino acids in *C. glutamicum* is shown in Fig. 3.14.

- Aspartokinase, the first enzyme in the pathway, is controlled by the concerted feedback inhibition of lysine and threonine. Homoserine dehydrogenase is subject to feedback inhibition by threonine and repression by methionine.

- The first enzyme in the route from aspartate semi-aldehyde to lysine is not subject to feedback control. Thus, the control system found in *C. glutamicum* is a relatively simple one.

- Nakayama *et al.* (1961) selected a homoserine auxotroph of *C. glutamicum*, by the penicillin selection and replica plating method, which produced lysine in a medium containing a low level of homoserine, or threonine plus methionine. The mutant lacked homoserine dehydrogenase which allowed aspartic semi-aldehyde to be converted solely to lysine and the resulting lack of threonine removed the concerted feedback inhibition of aspartokinase.
Fig. 3.14. The control of the aspartate family of amino acids in *C. glutamicum*. 
The first enzyme in the pathway, is controlled by the concerted feedback inhibition of lysine and threonine. Homoserine dehydrogenase is subject to feedback inhibition by threonine and repression by methionine. The first enzyme in the route from aspartate semialdehyde to lysine is not subject to feedback control. Thus, the control system found in C. glutamicum is a relatively simple one. Nakayama et al. (1961) selected a homoserine auxotroph of C. glutamicum, by the penicillin selection and replica plating method, which produced lysine in a medium containing a low level of homoserine, or threonine plus methionine. The mutant lacked homoserine dehydrogenase which allowed aspartic semi-aldehyde to be converted solely to lysine and the resulting lack of threonine removed the concerted feedback inhibition of aspartokinase. Kinoshita and Nakayama (1978) quoted the homoserine auxotroph, C. glutamicum 901, as producing 44 g dm-3 lysine.

The control of the production of arginine in C. glutamicum is shown in Fig. 3.15. The major control of the pathway is the feedback inhibition of the second enzyme in the sequence, acetylglutamic acid phosphorylating enzyme, although the first enzyme may also be subject to regulation.

Kinoshita et al. (1957b) isolated a citrulline requiring auxotroph of C. glutamicum which would accumulate ornithine at a molar yield of 36% from glucose, in the presence of limiting arginine and excess biotin. The mutant lacked the enzyme converting ornithine to citrulline which resulted in the cessation of arginine synthesis and, therefore, the removal of the control of the pathway.

Inosine monophosphate has been shown to demonstrate flavour-enhancing qualities and is produced commercially by the chemical phosphorylation of inosine (Shibai et al. 1978) which is produced from auxotrophic strains of Bacillus subtilis The control of the production of purine nucleotides is shown in Fig. 3.16. The main sites of control shown in Fig. 3.16 are:
Fig. 3.15. The control of the biosynthesis of arginine in C. glutamicum.
1. Phosphoribosyl pyrophosphate (PRPP) amidinotransferase (the first enzyme in the sequence) is feedback inhibited by AMP but only very slightly by GMP.

2. The synthesis of PRPP amidinotransferase is repressed by the co-operative action of AMP and GMP, as are the syntheses of the other enzymes indicated in Fig. 3.16.

3. IMP dehydrogenase is feedback inhibited and repressed by GMP.

4. Adenylosuccinate synthase is repressed by AMP but is not significantly inhibited.
Mutants which are auxotrophic for AMP or doubly auxotrophic for AMP and GMP have been isolated which will excrete inosine at levels of up to 15 g dm\(^{-3}\) (Demain, 1978). AMP auxotrophs, lacking adenylosuccinate synthase activity, require the addition of small quantities of adenosine but will accumulate inosine due to the removal of the inhibition and co-operative repression of PRPP amidinotransferase, as shown in Fig. 3.17. AMP and GMP double auxotrophs will produce inosine due to the removal of the controls normally imposed by the two end products, as illustrated in Fig. 3.18. Such double auxotrophs require the addition of both adenosine and guanosine, in small concentrations.

Fig. 3.17. The control of the synthesis of purine nucleotides in a mutant with a defective adenylosuccinate synthase.

Fig. 3.18. The control of the synthesis of purine nucleotides in a mutant defective in adenylosuccinate synthase and IMP dehydrogenase activities.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Initial yield at time of discovery (units cm$^{-3}$)</th>
<th>Improved yield in France, 1972 (units cm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>20 (1943)</td>
<td>12,000–15,000</td>
</tr>
<tr>
<td>Steptomycin</td>
<td>50 (1945)</td>
<td>12,000–15,000</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>200 (1948)</td>
<td>12,000–15,000</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>100 (1955)</td>
<td>3000</td>
</tr>
</tbody>
</table>
Fig. 3.25. Biosynthesis of benzyl penicillin and lysine in *Penicillium chrysogenum*.
The isolation of resistant mutants

The isolation of analogue-resistant mutants has already been discussed in the field of primary metabolism, where the rationale was that a mutant resistant to the inhibitory effects of an analogue, which mimics the control characteristics of the natural metabolite, may overproduce the natural metabolite.

This approach has been adopted, or may be adopted, in a number of guises in the field of secondary metabolism.

1. Mutants may be isolated which are resistant to the analogues of primary metabolic precursors of the secondary metabolite, thus increasing the availability of the precursor.

2. Mutants may be isolated which are resistant to the feedback effects of the secondary metabolite.

3. Mutants may be selected which are resistant to the toxic effects of the secondary metabolite when added to the trophophase of the producing organism.

4. Mutants may be isolated which are resistant to the toxic effects of a compound due to the production of the secondary metabolite.
Fig. 3.32. Control of the aromatic amino acid family in Corynebacterium glutamicum.

--- ○ Feedback inhibition control
CM Chorismate mutase
DS DAHP synthase
P. Dehyd Prephenate dehydratase
P. Dehydrog Prephenate dehydrogenase
SK Shikimate kinase
The improvement of industrial strains by modifying properties other than the yield of product

- Selection of stable strains
- Selection of strains resistant to infection
- Selection of non-foaming strains
- Selection of strains which are resistant to components in the medium
- Selection of morphologically favourable strains
- Selection of strains which are tolerant of low oxygen tension
- Elimination of undesirable products from a production strain.
- Development of strains producing new fermentation products
Fig. 10.2 Strain improvement of *Acremonium chrysogenum* by UV mutagenesis programme for cephalosporin production
Fig. 10.3 Strain improvement of *Penicillium chrysogenum* by classical mutagenesis for penicillin production.
<table>
<thead>
<tr>
<th>Category of improvement</th>
<th>Microorganisms</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate uptake</td>
<td><em>Lactobacillus delbrueckii</em> and <em>Bacillus amyloliquefaciens</em></td>
<td>Non-fastidious strain obtained after three rounds of genome shuffling for direct conversion of starch to lactic acid</td>
<td>John et al. (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Sphingobium chlorophenolicum</em></td>
<td>Higher tolerance level of pentachlorophenol for degradation at a faster rate</td>
<td>Dai and Copley (2004)</td>
</tr>
<tr>
<td>Strain tolerance to end product</td>
<td><em>L. rhamnosus</em></td>
<td>More accumulation of lactic acid at 3.6 pH compared to wild type</td>
<td>Wang et al. (2007)</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces pristinaespiralis</em></td>
<td>Four rounds of protoplast fusion generated 100-μg/ml pristinamycin resistant recombinant</td>
<td>Xu et al. (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Improved strain maintaining cell viability till 55 °C and 25 % ethanol stress after three rounds of genome shuffling</td>
<td>Shi et al. (2009)</td>
</tr>
<tr>
<td>Product yield enhancement</td>
<td><em>Streptomyces fraiae</em></td>
<td>Two rounds of genome shuffling led to six times higher titre of tylosin</td>
<td>Zhang et al. (2002)</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces gilvosporeus</em></td>
<td>High natamycin-producing strain – approximately 153 % higher than the parent strains and 1.17 times more than present strain</td>
<td>Zhu et al. (2006)</td>
</tr>
<tr>
<td></td>
<td><em>Phaffia rhodozyma</em></td>
<td>Two cycles of recursive protoplast fusion, a shuffled strain was selected and 1.43 times higher yield of astaxanthin was obtained</td>
<td>Zheng and Zhao (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Sorangium cellulosum</em></td>
<td>The epothilone production of the fusant was increased about 130 times compared to the starting strain by 3 rounds of genome shuffling</td>
<td>Gong et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium diolis DSM 15410</em></td>
<td>Improvement of 1,3-propanediol production by 4 rounds of genome shuffling by 80 %</td>
<td>Otte et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>Propionibacterium shermanii</em></td>
<td>Enhanced vitamin B₁₂ production after genome shuffling</td>
<td>Zhang et al. (2010)</td>
</tr>
<tr>
<td>Drug/drug precursor</td>
<td>Host</td>
<td>Application</td>
<td>Engineering technology</td>
</tr>
<tr>
<td>---------------------</td>
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<td>-------------</td>
<td>----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Shikimic acid</td>
<td><em>E. coli</em></td>
<td>Antiviral</td>
<td>Heterologous expression of glucose facilitator protein, glucokinase both from <em>Zymomonas mobilis</em></td>
</tr>
<tr>
<td>Taxadien-5 α- acetoxy-10β-ol (taxol precursor)</td>
<td><em>S. cerevisiae</em></td>
<td>Antitumor</td>
<td>Heterologous expression of taxol biosynthetic genes</td>
</tr>
<tr>
<td>Lycopene</td>
<td><em>E. coli</em></td>
<td>Antioxidant</td>
<td>Heterologous expression of carotenoid genes from <em>Pantoea agglomerans</em></td>
</tr>
<tr>
<td>Tylactone</td>
<td><em>S. venezuelae</em></td>
<td>Antibiotic</td>
<td>Heterologous expression of tylosin polyketide synthase in <em>S. venezuelae</em></td>
</tr>
<tr>
<td>Macrolide 6-deoxyerythromycin-D</td>
<td><em>E. coli</em></td>
<td>Antibiotic</td>
<td>Heterologous expression</td>
</tr>
</tbody>
</table>
Fig. 10.5 Components of precision engineering technology for microbial strain improvement

**Metabolic Diversity**
- Mutant repository
- Transformant Collections
- Different set of growth conditions

**Profiling Technologies**
- Metabolite
- Genome
- Gene Expression

**Association Analysis**
- Novel genes
- Linking strain development & Process optimization

Precision Engineering Technology
FIGURE 3.26  Protoplast Fusion Between Two Strains, A and B
Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation