Fundamentals of Fermentation Technology

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Edited by

Milind H. Gajbhiye

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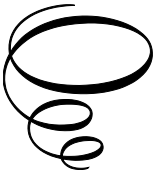

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CHAPTER ONE

STRAIN IMPROVEMENT OF INDUSTRIALLY IMPORTANT MICROORGANISMS

MILIND H. GAJBHIYE

Introduction

In general, industrially important microbes are isolated from soil and water during primary screening. These natural isolates are called 'wild type'. Once isolated, these natural organisms undergo rigorous testing to assess their potential to produce desirable metabolites, often quantitatively evaluated as a part of secondary screening. However, despite their potential, these natural isolates typically exhibit low yields of commercially significant products, which renders them less suitable for industrial applications. Consequently, considerable efforts are invested in enhancing the productivity of the selected microorganism. One approach to augment productivity involves optimizing cultural conditions, including factors such as temperature, pH, nutrient availability, and oxygenation. By manipulating these variables, it is possible to create an environment that is conducive to heightened metabolite production. The organism's maximum ability to synthesize the product, however, will limit this approach. Therefore, while cultural optimization is a valuable tool in enhancing microbial productivity, it must be supplemented with other strategies such as genetic engineering or strain selection to unlock the potential of industrially important microbes fully. The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the potential yield. Modification or manipulation of microbes for the overproduction of desirable metabolites is called strain improvement. Simply, inferior producers (wild type or natural isolate) are converted into superior producers (mutants). Thus, the process of strain improvement involves the continuous genetic modification of the culture, followed by re-evaluations of its cultural requirements and productivity. Thus, strain improvement is a program that consists of multiple procedures.

The major objective of strain improvement program is to obtain strains with superior productivity. The superior productivity is achieved when the maximum amount of precursors or substrates are diverted towards the formation of desirable products/metabolites, supported by modified control systems. Thus, knowledge of biochemical pathways and control systems is essential to plan the modifications/changes for the overproduction of desirable metabolites. This information is useful for the construction of a "blueprint". These blueprints are useful for predicting changes, selecting techniques for modification, or designing techniques to increase productivity. Detailed information about biosynthetic routes and control mechanisms is available for many primary metabolites such as amino acids, nucleotides, enzymes, vitamins etc. but little is known about secondary metabolites (eg. antibiotics, inhibitors). Thus, combinations of several procedures have been used for the improvement of microbial strains for the production of primary metabolites.

Primary metabolites are produced continuously during active growth phase of microbes (trophophase). These metabolites are considered essential to microorganisms for proper growth. Ex. sugars, organic acids, enzymes, amino acids, vitamins, nucleic acids, proteins etc.

Secondary metabolites are formed during the end or near the stationary phase of growth (idiophase). They are the products of secondary metabolism. They do not play a significant role in growth and development. But they may be essential for the survival of microbes sometimes. For example, antibiotics, enzyme inhibitors, compounds with pharmacological properties.

It is important to note that secondary metabolism may occur in continuous cultures at low growth rates, which is a property of slow-growing and non-growing microbes. Thus, in a natural environment when microbes grow at relatively low growth rates, it suggests that it is the idiophase state that exists in nature rather than the trophophase.

The objective of strain improvement

The major objective of strain improvement is to increase the yield/productivity of metabolites produced by industrial microorganisms. The microbes produce metabolites in limiting concentrations as per their need and if synthesized in excess, its biosynthesis is temporarily stopped by the control system present in the cell. Thus, metabolites/biochemicals are never produced in excess due to the presence of feedback control systems. In this view, logically, if these control systems are lifted/removed, the strains

shall produce the metabolites in an uncontrolled manner (overproduction). Thus, the strains are modified to increase productivity and efficiency during strain improvement program.

YouTube Video:
https://youtu.be/9QxuavYPxfM?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

Properties other than overproduction

There are several other properties of organisms that may affect the design and economics of a commercial fermentation process. Thus, properties other than productivity are also important. For example, a strain may produce a very high level of a metabolite, but if it is extremely unstable, then it is not suitable for commercial fermentation. If the strain's productivity is high but its oxygen demand is very high and beyond the fermenter's capacity, then the strain is unsuitable. Therefore, characteristics of the producing organism which affect the process are also important for the commercial success of fermentation. The following are a few examples of such important characteristics other than productivity that should be considered during the selection of strains for commercial fermentations.

1. Selection of a stable strain - A very important quality is the ability of the producing strain to maintain its high productivity during both culture maintenance and fermentation. Yield decay, a decrease in yield, may occur during culture storage. Controlling the loss of productivity during fermentation is very difficult. Normally, a decrease in the productivity of a commercial strain is due to the occurrence of lower-yielding, spontaneous revertant mutants, which frequently have a higher growth rate than the high-producing parents. Thus, yield decay is especially problematic in fed-batch or continuous fermentations, which are long-term processes. Here, the faster-growing, lower producer may predominate, or even replace, the high-yielding original strain.

2. Selection of non-foaming strains – Foaming during fermentation leads to several effects such as loss of culture medium, cells and product via the air outlet, risk of contamination etc. Foaming may be controlled by either the addition of antifoam agents or by mechanical foam breakers. However, these efforts may be decreased if a non-foaming strain of the commercial organism is selected. Foaming in the early stages of fermentation is usually due to components present in the medium whereas foaming during the late stages is generally due to growing microbes that produce foam-

enhancing substances. Therefore, the latter type of foam may be controlled by the selection of non-foaming strain.

3. Selection strains resistant to contamination – Phages are one of the major contaminants of bacterial fermentations that may affect the fermentation very seriously due to the lysis of producing strains. Thus, the selection of strains resistant to the phages that are isolated from the fermentation plant is very important. Additionally, it is important to ensure that the isolated resistant strains are not lysogenic since they carry the population of phages in fermentation, potentially releasing them as lytic phage mutants. Therefore, maintaining fermentation plant hygiene is essential to minimize the risk of contamination. There may be the addition of chemical agents in the fermentation which selectively inhibit phage replication.

Host-restriction and modification system (HRM) is a mechanism that exists in certain bacterial cells whereby foreign nucleic acids which enter a cell are destroyed. This system is a set of genes that produce nucleases, and foreign nucleic acids are degraded. Thus, the introduction of HRM in the producer strains is also possible. The genes for many HRM systems can be cloned, introduced, and expressed into host strains. For the control of other contaminants antibiotics or inhibitors can be used. The antibiotics to which the commercial strain is resistant may be used to control the level of contaminants.

4. Selection of strains resistant to medium components – Some medium components that are required for product formation may be toxic for the producer organism itself. Therefore, it is desirable to select strains which are resistant to such toxic medium components. For example, phenylacetic acid is a precursor of penicillin G and is toxic to *P. chrysogenum* at high concentrations. Thus, mutants of *P. chrysogenum* strains that are resistant to high concentrations of precursors may be selected. This enables the use of precursors at high concentrations in the medium and ultimately increases the yield of penicillin G. In vitamin B12 fermentation, cobalt chloride is a precursor, however, it is also toxic to *Streptomyces griseus*, a producer strain. Thus, strains resistant to high concentrations of cobalt chloride may be isolated.

5. Selection of morphologically favorable strains – The morphology of a microorganism in submerged culture frequently affects the economics or ease of operation of a fermentation process. For example, in the case of filamentous microorganisms such as molds and actinomycetes, its

morphological form affects the aeration of the system, but it will make filtration of the fermentation broth easier. In the case of yeast cells, due to their tendency to flocculate in clumps, they can be easily separated from fermentation broth. Thus, this flocculating property of yeasts may be described as a morphologically favorable characteristic. This property of flocculation is beneficial in ethanol, wine and beer fermentation in which the yeast cells either rise to the top of the vessel (in case of 'top-fermenting' yeast) or settle to the bottom of the vessel (in case of 'bottom-fermenting' yeast), thus help in clarification of broth.

6. Selection of strains tolerant to low oxygen tension – The provision of oxygen is frequently one of the critical factors in many fermentations. Thus, it would be desirable to select an organism which can produce the product at a lower oxygen tension than normal. This may be achieved by screening for strains for increased production under low oxygen tension conditions. For example, a lysine-producing strain was selected that maintained its productivity at decreased aeration conditions.

7. Selection of strains that do not produce undesirable products – Although an industrial strain may produce desirable metabolite in large quantities, it may also produce a large amount of unwanted metabolites. Such metabolites may be toxic or may interfere with the extraction and purification process. Thus, the strains should be selected such that undesirable products are no longer produced. For example, the penicillin-producing molds produce a yellow pigment, chrysogenin, that affects the purification of penicillin. Thus, the modified strains that do not produce this pigment may be selected, which makes the extraction of the antibiotic much simpler.

Processes of feedback control for the biosynthesis of microbial metabolites

As mentioned above, it is necessary to study the mechanisms of control of biosynthesis of metabolites for the construction of 'blueprints'. These blueprints are needed to plan the changes for the possible overproduction of metabolites. The biosynthetic pathways of primary metabolites in microorganisms are regulated by feedback control systems. Some of the pathways are shared by primary and secondary metabolites. The major systems of control are feedback inhibition and feedback repression. The end products or the intermediates of the biochemical pathways are responsible for such control.

Feedback inhibition is the condition where the end product of a biochemical pathway inhibits the activity of an enzyme catalysing the reactions of the pathway. Inhibition occurs by the binding of the end product to the enzyme at an allosteric site which results in the interference with the attachment of the enzyme to its original substrate. Thus, feedback inhibition occurs at the enzyme level. *Feedback repression* is the condition where 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 by the binding of the end product to the operator site on a gene. This prevents the transcription of the gene (formation of mRNA), resulting in the prevention of enzyme synthesis. Thus, feedback repression occurs at the gene level.

The biosynthetic pathways may be branched or unbranched type. The control systems of the branched biosynthetic pathways that give rise to several end products are more complex than the control of unbranched pathways. These mechanisms of control may occur by inhibition, repression or a combination of both systems. These mechanisms are shown below.

1. Concerted or multivalent feedback control – In this control system, the biochemical pathways are controlled by more than one end product. Thus, as shown in Fig. 1.1 (i), the first enzyme of the pathway is inhibited or repressed only when all end products are synthesized in excess.

2. Co-operative feedback control - The system is similar to concerted control; however, end products of different branches may have an independent weak control (partial control). In addition, if all the end products are synthesized in excess, a synergistic repression or inhibition occurs. As shown in Fig. 1.1 (ii), if the synthesis of D is in excess, it will control its own synthesis by inhibiting the conversion of B to C, thus, diverting the flow of B towards the synthesis of F. If both are synthesized in excess then a combined effect will takes place and conversion of A to B will be inhibited.

3. Cumulative feedback control – In this system, every end product of the pathway inhibits the first enzyme by a certain percentage independently. As shown in Fig. 1.1 (iii), both D and F independently cause 50 % reduction in the activity of the first enzyme. This may result in 100 % inhibition when both end products are synthesized in excess. Also, each end product has control immediately after the branch point, for example, D and F

control the conversion of B to C and, B to E, respectively. Thus, if D is synthesized in excess, B will be available for the synthesis of F.

4. Sequential feedback control – This system occurs in sequence, as each end product of the pathway controls the enzyme that catalyzes the previous reaction after the branch point. As a result of this, the intermediates accumulate and control the enzyme of the previous reaction of the pathway. As shown in Fig. 1.1 (iv), D and F control the conversion of B to C and B to E, respectively. The inhibitory action of D, F, or both would increase the concentration of B, which, in turn, would inhibit the conversion of A to B.

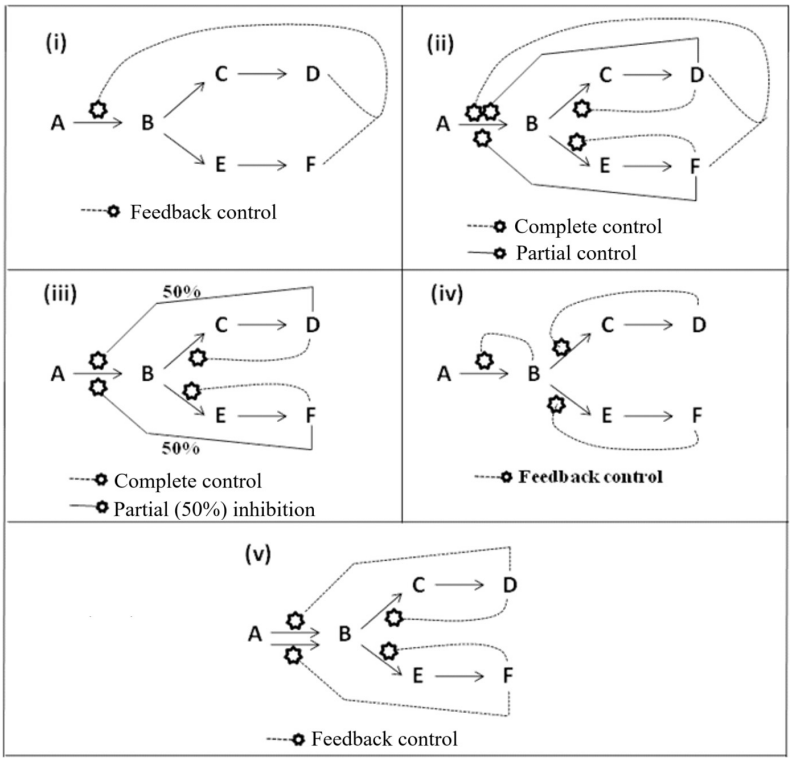

Fig. 1.1 Feedback control systems; (i) Concerted or multivalent (ii) Co-operative (iii) Cumulative (iv) Sequential and (v) Isoenzyme feedback control

5. Isoenzyme control - The enzymes which catalyze the same reaction but differ in their control properties are called isoenzymes. This property is

related to controlling metabolites. Thus, they catalyze the same reaction, but each of the isoenzymes is controlled by different end products of the pathway. This control system is very efficient if additional control exists immediately after the branch point. As shown in Fig. 1.1 (v), two isoenzymes converting A to B are controlled by two different end products, D and F. Thus, if one of the end products is synthesized in excess, the flow of intermediate is diverted towards another branch.

YouTube Video:
https://youtu.be/kVWbDmddhy0?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

Methods for Strain Improvement

As stated above, the levels of microbial metabolites may be controlled by a variety of mechanisms as shown above. Thus, the metabolites are synthesized in quantities sufficient for growth and thereafter, it is temporarily stopped. However, the ideal industrial microbe must produce metabolites in excess concentration than required for growth. Therefore, knowing the mechanisms of control systems and pathways may enable the construction of a 'blueprint'. This blueprint may be useful for the construction of an industrial mutant that overproduces microbial metabolites due to the modification of control systems. The control systems in such mutants are not inhibited by metabolites. In such mutants, the enzymes that are under control are termed 'key enzymes'.

These modifications are divided into two categories: (i) mutation and selection techniques and (ii) recombination techniques. The use of the modern approach of recombinant DNA technology leads to the most spectacular examples of strain improvement. This resulted in the construction of microbes that not only produce the natural metabolites in excess but also produce non-natural products (which they were not able to produce previously).

Mutation and Selection Techniques

Isolation of the mutants for the improved production of primary metabolites

In mutation and selection procedures, the appropriate microbial population is exposed to mutagenic agents (for example, UV rays, chemical mutagens etc.) for a definite period under standard conditions. The survivors of this

treatment may contain desirable mutants. The desirable mutants are then selected based on productivity and selective improved characters (eg. resistance to antibiotics, resistance to analogue, auxotrophic mutants etc.). Again, the superior producers are exposed to mutagenic agents to increase productivity further. Thus, this process may be repeated several times till the best sturdiest strains are obtained.

These types of proposed microbes with modified characteristics may be obtained through the below-mentioned ways of modifications. Such modified microbes may be the overproducers of desirable metabolites. (Remember: the enzymes that are under control are termed as 'key enzymes'.)

1. Alteration in cellular permeability character of microbe: The cultural conditions of the microbe may be modified such that the end products (feedback inhibitors/repressors) that control the key enzymes of the pathway are leaked out of the cell due to permeability defects in the cell membrane/cell wall of microbe.

2. Isolation of auxotrophic mutants: The microbe may be modified/mutated such that it does not produce the end products (feedback inhibitors/repressors) that control the key enzymes of the pathway.

3. Isolation of analogue resistant mutants and revertants: The microbe may be modified/mutated such that it does not identify the presence of inhibiting or repressing levels of the normal control metabolites (inhibitors/repressors).

1. Modification of the cellular permeability of producer microbial strain

One of the well-known examples of this kind of modifications is the production of glutamic acid by Kinoshita's isolate. Kinoshita and his coworkers from Japan, in l957 isolated a biotin-requiring, glutamate-producing bacterium. Subsequently, it was identified as *Corynebacterium glutamicum.* The unique characteristic of this bacterium was sensitivity to the levels of biotin, due to which the cellular permeability of this organism could be modified. This level of biotin in the production medium was suggested to be approximately 5 µg/ml. At this level of biotin, glutamic acid would be excreted in the medium. However, in a condition of higher concentrations of biotin would not result in the accumulation of glutamic acid in medium. It implies that the permeability of *C. glutamicum* may be controlled by the composition of the culture medium.

Another feature of Kinoshita's isolate was the absence of α-ketoglutarate dehydrogenase. This enzyme is needed for the conversion of α-ketoglutarate to succinate in the tricarboxylic acid (TCA) cycle. This block in the metabolic pathway results in the accumulation of a large concentration of α-ketoglutarate which the bacterium converts to glutamic acid. As shown in Fig. 1.2, oxaloacetate is regenerated in glutamate producers through the glyoxylate pathway.

When *C. glutamicum* was grown in a medium containing a high concentration of biotin, the organism synthesized glutamate at a low level, due to feedback control by glutamate of its own synthesis. Under conditions of biotin limitation, glutamate was released from the cells and accumulated in the medium to a high level. Biotin limitation conditions cause a change in the phospholipid content of the cell membrane and permeability increases. Due to this, glutamate leaks out of the cells and accumulates in the medium. This is because biotin is required in the synthesis of oleic acid which is necessary for the synthesis of phospholipids of cell membrane. Thus, the permeability change occurs due to the synthesis of defective membranes because of insufficient amounts of phospholipids. As a result, these phospholipid-poor cells excrete L-glutamic acid.

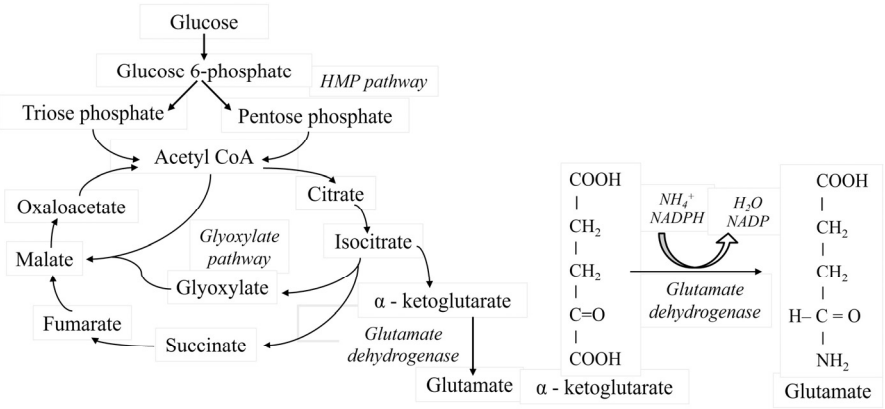

Fig. 1.2 Glutamate synthesis in *C. glutamicum*. Heavy lines indicate the pathway of glutamate synthesis and dashed lines show the regeneration of oxaloacetate via glyoxylate pathway

The role of biotin in glutamate fermentation is very important and thus it should be added in limiting concentration in the medium. Thus, the use of crude carbon sources such as cane molasses, which are rich in biotin,

presents difficulties. However, the addition of antibiotics such as penicillin or cephalosporin C during active growth phase allows the cells to accumulate and excrete L-glutamic acid even in the presence of excess biotin. Penicillin inhibits cell wall synthesis, and the cells become elongated and swollen. Thus, the cell membrane remains unprotected and gets damaged, breaking the permeability barrier. Saturated fatty acids (C_{16}-C_{18}) also permit cells to excrete L-glutamic acid in the medium. Thus, penicillin and saturated fatty acids function as anti-biotin agents when biotin-rich raw materials such as beet or cane molasses are used in the production of glutamic acid. Also, addition of polyoxyethylene sorbitan mono-oleate (Tween 80) during logarithmic growth in biotin-rich medium causes an aberration of the cell envelope permeability, resulting in the release of glutamate in the medium. In these examples, the cultural conditions for the producer strain have been modified, thus, it is not an example of strain improvement by the mutation and selection approach.

YouTube Video:
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2. Isolation of auxotrophic mutants i.e., Isolation of mutants which do not produce feedback inhibitors or repressors

Mutants that do not produce certain feedback inhibitors or repressors may be useful for the production of intermediates of unbranched pathways and intermediates & end products of branched pathways. Several hypothetical examples of possible mutations are shown in Fig. 1.3. These are the 'blue prints' of hypothetical mutants producing intermediates and end products of biosynthetic pathways. The mutants shown in the figure do not produce some of the inhibitors or repressors of the pathways. Thus, the control of the pathway is removed. At the same time if these inhibitors or repressors are essential for growth, then they must be added to the medium in feed limiting concentration (concentration sufficient for growth but not for feedback control).

In the case of an unbranched pathway, as shown in Fig. 1.3 (i), the first key enzyme of the pathway is normally controlled by feedback inhibition or repression by the end product, E. However, this organism may be mutated to an auxotrophic mutant. The mutant shown in the figure is auxotrophic for E due to the inability to convert C to D. Thus, the control of the pathway is lifted and C will accumulate or be overproduced. However, E should be added to the medium at a concentration sufficient to support growth but insufficient to cause inhibition or repression. Thus, E

should be added in the feed limiting concentration in the production medium.

In the case of branched pathways as shown in Fig. 1.3 (ii), first enzyme is controlled by the concerted inhibition by the combined effects of E and G. The mutant demonstrated in the figure is auxotrophic for E due to the absence of enzyme required for conversion of C to D. This will result in the removal of the concerted control of the first key enzyme and C will C will accumulate as the end product G controls the conversion of C to F. However, as stated earlier, E should be added to the medium in feed limiting concentration to allow sufficient growth but not inhibition. A similar example is shown in Fig. 1.3 (iii), however, it is a double auxotroph that requires the addition of both E and G to the medium in feed limiting concentration. Fig. 1.3 (iv) is the same pathway and shows another double auxotrophic mutant not synthesizing E & G. This is due to the lack of enzyme required for the conversion of C to D and F to G. This will result in the overproduction of F.

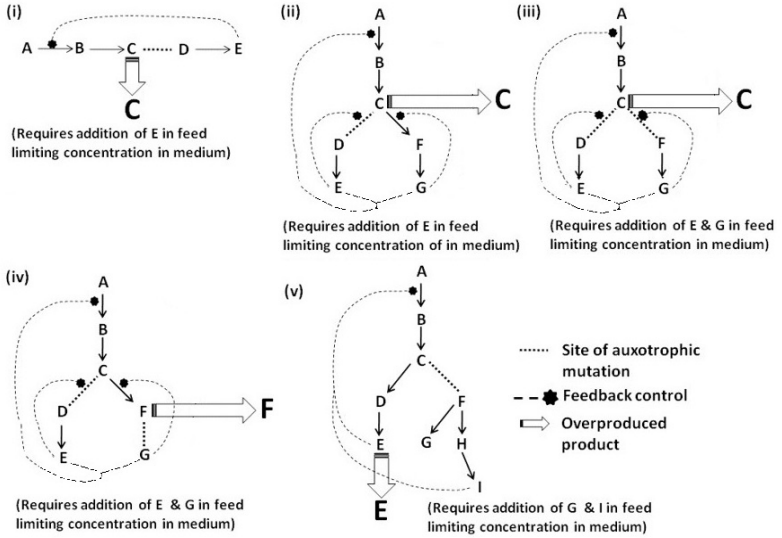

Fig. 1.3 Hypothetic examples of auxotrophic mutants for the possible overproduction of primary metabolites. The possible sites of mutation are shown for desired overproduction

A different branched pathway is shown in Fig. 1.3 (v). End products E & I have combined control over the first key enzyme of the pathway. The mutant illustrated is auxotrophic for I and G due to the lack of enzyme required for the conversion of C to F. Thus, G and I should be added to the medium in feed feed-limiting concentration. Due to the absence of I in excess, the inhibition of the first key enzyme never happens, as a result, end product E will be overproduced.

Therefore, all the above examples suggest that such auxotrophic mutants may overproduce the desirable metabolite, provided that the mutation for auxotrophy occurs at the correct site in the genome. The isolation of auxotrophic mutants is a simpler process; however, the recovery of higher producer strains is difficult. Thus, after mutation, higher producing auxotrophs from the survivors of the mutation treatment are screened with respect to productivity.

Methods of isolation of auxotrophic bacteria

i) Enrichment culture technique - The population (survivors of mutation treatment) is grown first in a minimal medium with an antimicrobial agent. This antimicrobial agent affects only growing or dividing cells, and this would result in the killing of prototrophs but not non-growing auxotroph. This population is then transferred into a complete medium to obtain the growth of auxotrophic mutants. Different antimicrobial agents are used with various types of microbes. For example, the enrichment culture technique utilizes penicillin as the inhibitory agent. The survivors of a mutation treatment are first cultured in a complete medium and harvested by centrifugation, followed by washings. The cells are then resuspended in a minimal medium plus penicillin. Only the growing prototrophic cells are susceptible to penicillin, and the non-growing auxotrophs survive. The cells are then separated by centrifugation, washed (to remove the penicillin and products released from lysed cells) and resuspended in a complete medium to allow the growth of the auxotrophs (Fig. 1.4).

ii) Replica plate technique – This is another method for the separation of auxotrophs from prototrophs. First, the survivors of the mutation treatment are transferred to a complete medium, on which both auxotrophs and prototrophs form colonies. A replica of this plate is then taken on a plate containing minimal medium with the help of a velvet pad. Only prototrophs grow on this medium. Thus, after incubation, the colonies missing on the minimal medium and present on the complete medium are auxotrophic mutants.

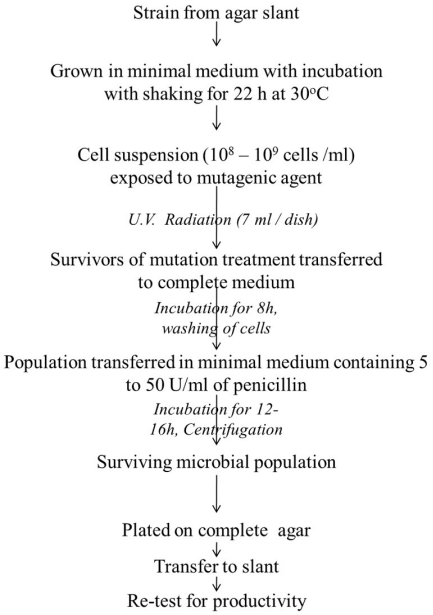

Fig. 1.4 Penicillin selection technique for the isolation of auxotrophic mutants (Abe, 1972)

Example of the use of auxotrophs for the production of primary metabolites – Many auxotrophic mutants have been used for the production of amino acids and nucleotide-related compounds. Here, we are describing one example of an auxotrophic mutant of *C. glutamicum* that has been used for the production of lysine. The control of the production of amino acids of the aspartate family in *C. glutamicum* is shown in Fig. 1.5, where aspartokinase, the first key enzyme in the pathway, is controlled by the concerted feedback inhibition by lysine and threonine, if synthesized in excess. Homoserine dehydrogenase is another key enzyme, controlled by feedback inhibition by threonine and repression by methionine. The first enzyme in the route from aspartate semialdehyde to lysine is not under feedback control. Thus, the control system found in *C. glutamicum* is relatively simple one. Nakayama et al. (1961) used the mutation and selection technique and selected a homoserine auxotroph of *C. glutamicum,* by the penicillin selection and replica plating technique. This improved strain produced lysine in a medium containing a low level (feed limiting concentration) of homoserine, or threonine plus methionine. The mutant lacked homoserine dehydrogenase, which is required for the

synthesis of homoserine from aspartyl semi-aldehyde, thus, no synthesis of threonine. In this way, the concerted feedback inhibition of aspartokinase is removed. Therefore, aspartyl semialdehyde is converted solely to lysine.

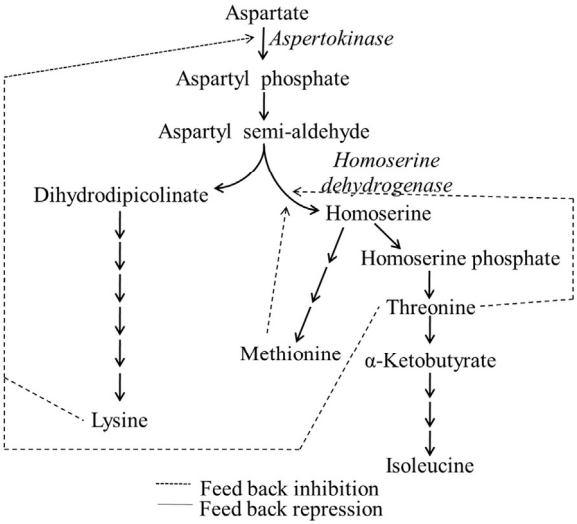

Fig. 1.5 Biosynthetic pathway and feedback control system of aspartate family of amino acids in *Corynebacterium glutamicum*

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https://youtu.be/ub1pCKSPcis?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

3. Isolation of mutants that do not recognize the presence of inhibitors and repressors

The auxotrophic mutants have been used in the production of many microbial products in large concentrations as described earlier. But obviously, such mutants are not suitable for the synthesis of products which control their own synthesis independently. A hypothetical example as shown in Fig. 1.6 where the end product E controls its own biosynthesis by feedback inhibition of the first enzyme in the pathway. If it is required to produce the intermediate D in large concentrations, then this may be achieved by the isolation of a mutant auxotrophic for E (by a block between D and E). However, if E is required to be synthesized in large concentrations it is not possible by auxotrophy.

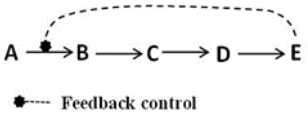

Fig. 1.6 The control of production of an end product E in an unbranched pathway

The answer to this problem is to modify the organism such that the first enzyme in the pathway no longer recognizes the presence of inhibiting levels of end product E. The isolation of mutants with changes in the recognition of control factors can be achieved mainly using two techniques: (i) The isolation of analogue-resistant mutants. (ii) The isolation of revertants.

(i) The isolation of analogue-resistant mutants - Analogue is a compound which is very similar in structure to another compound. Analogues of amino acids and nucleotides are frequently growth inhibitory, and their inhibitory properties may be due to several possible mechanisms. For example, the analogue may be used in the biosynthesis of macromolecules, resulting in the production of defective cellular components. Sometimes, the analogue is not incorporated in place of the natural product, but it may interfere with its biosynthesis by mimicking (imitating) its control properties (FBI or FBR). For example, consider the pathway illustrated in Fig. 1.6 where the end product, E, inhibits the first enzyme in the pathway. If E* is an analogue of E, then it will inhibit the first enzyme in a similar way to E. But this would cause permanent damage to the enzyme resulting in the inhibition of the growth of the organism.

Mutants may be isolated which are resistant to the inhibitory effects of the analogue and, if the site of toxicity of the analogue is the mimicking of the control properties of the natural product, such mutants may overproduce the compound to which the analogue is analogous. To return to the example of the biosynthesis of E where E* is inhibitory, due to its mimicking control properties with E; a mutant may be isolated which may be capable of growing in the presence of E* because the first enzyme in the pathway is no longer sensitive to inhibition by the analogue. This modified enzyme of the resistant mutant may not only be resistant to inhibition by the analogue but may also be resistant to the control effects of the natural end product, E. This will result in the uninhibited (continuous) production (overproduction) of E. If the control system were the repression of enzyme synthesis, then the resistant mutant may be modified such that the enzyme synthesis machinery (operon) does not recognize the presence of the analogue.

However, the site of resistance of the mutant may be different the control system; for example, the mutant may be capable of degrading the analogue, in which case the mutant would not be expected to overproduce the end product.

Thus, analogue resistant mutants may be expected to overproduce the end product to which the analogue is analogous provided that:

(i) The toxicity of the analogue is due to its similar effect on the control system of the natural product.

(ii) The site of resistance of the resistant mutant is the site of control by the end product.

Method of isolation of analogue-resistant mutants - Analogue-resistant mutants are isolated from the survivors of the mutation treatment on media containing analogue in variable concentrations. A method of isolation of analogue-resistant mutants has been described by Szybalski (1952), called the gradient plate technique. In this method, the survivors of a mutation treatment are exposed to different concentrations of analogue on a single plate. This plate is prepared by pouring 20 ml of analogue containing molten agar medium while placing the petri dish into a slightly slanted position. Agar is allowed to set and a layer of medium without the analogue is poured and allowed to set with the plate level. The analogue will diffuse into the upper layer, giving a concentration gradient across the plate and the survivors of a mutation treatment may be spread over the surface of the plate and incubated. Resistant mutants should be detected as isolated colonies appearing in a zone with high analogue concentrations, as shown in Fig. 1.7. The resistant isolates should then be screened to produce the desired compound.

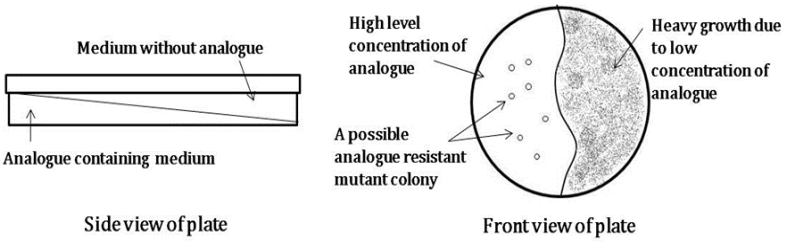

Fig. 1.7 Isolation of analogue resistant mutants by gradient plate technique

Example of the use of analogue resistant mutants for the production of primary metabolites –

Lysine analogue-resistant mutants of *Brevibacterium flavum* were investigated for the production of lysine by Sano and Shiio (1970). The control of the biosynthesis of the aspartate family of amino acids in *B. flavum* is as shown in Fig. 1.5, which is like *C. glutamicum.* The major key enzyme in lysine synthesis is aspartokinase, controlled by the concerted feedback inhibition by lysine and threonine. Sano and Shiio demonstrated that S-(2 aminoethyl) cysteine (AEC) completely inhibited the growth of B. flavum in the presence of threonine, but only partially in its absence. Also, the inhibition by AEC and threonine could be reversed by the addition of lysine. This evidence suggested that the inhibitory effect of AEC was due to its lysine-like effect of the concerted inhibition of aspartokinase. AEC-resistant mutants were isolated by plating the survivors of a mutation treatment on minimal agar containing 1 mg/ml of both AEC and threonine. Investigation of the lysine producers indicated that their aspartokinase is not sensitive to the concerted inhibition by lysine and threonine.

YouTube Video:
https://youtu.be/9ZsUJdpSCTs?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

(ii) The isolation of revertants - The second technique used for the isolation of mutants altered in the recognition of control factors is the isolation of revertant mutants. The wild types are mutated to auxotrophic mutants. These auxotrophs are again mutated to the parent type. These reverted mutants are termed revertants.

As shown in Fig. 1.6, where E controls its own production by feedback inhibiting the first enzyme of the pathway. This organism is mutated to a mutant that does not synthesize the key enzyme (enzyme under feedback control), i.e., an auxotrophic mutant which is, therefore, auxotrophic for E. This auxotroph is again mutated to the parent type i.e., revertant. However, a revertant mutant produces large concentrations of E. The explanation of the behavior of the revertant is that, with two mutations having occurred at loci concerned with the production of key enzyme, the enzyme of the revertant is different from the enzyme of the original prototrophic strain and is not susceptible to the control by E. The revertants are isolated by plating cells on medium which would allow the growth of only the revertants, i.e. in the above example, on medium lacking E.

Example of the use of revertants for the production of primary metabolites – The use of prototrophic revertants of *B. flavum* for the production of lysine has been described by Shiio and Sano (1969). The pathway for the synthesis of amino acids is shown in Fig. 1.5. They isolated prototrophic revertants from a homoserine dehydrogenase-deficient mutant. The overproduction of lysine was due to the very low level of homoserine dehydrogenase in the revertants, which, presumably, resulted in the synthesis of threonine and methionine in quantities sufficient for some growth only, but insufficient to cause inhibition or repression.

YouTube Video:
https://youtu.be/9ZsUJdpSCTs?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

Isolation of mutants for the improved production of secondary metabolites

Production of secondary metabolites has been tried for a long time without the knowledge of biosynthetic pathways by random selection technique. A technique proposed by Davis is shown in Fig. 1.8.

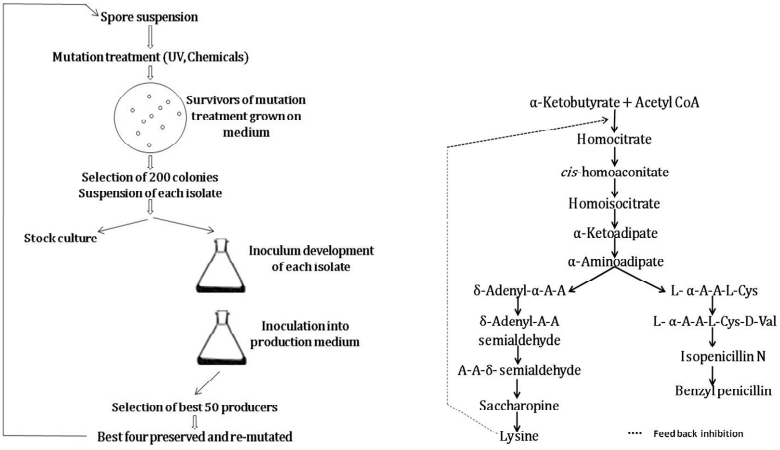

Fig. 1.8 A general strain-improvement program for a strain producing secondary metabolite (Davies, 1964) (Left side). Biosynthetic pathway for benzyl penicillin synthesis and lysine in *Penicillium chrysogenum*. (right side)

Secondary metabolites are also produced by the modifications that are described earlier for primary metabolites.

The isolation of auxotrophic mutants – In many cases, primary metabolites and secondary metabolites share biochemical pathways. Many secondary metabolites are the end products of branched pathways, which also give rise to primary metabolites. Thus, a mutation to auxotrophy for the primary end product may also influence the production of the secondary metabolites. In *P. chrysogenum,* lysine and penicillin share the same common biosynthetic route to α-aminoadipic acid, as shown in Fig. 1.8. Thus, lysine auxotrophs would produce higher levels of penicillin due to the diversion of the intermediate towards penicillin synthesis and the removal of any control of homocitrate synthase by endogenous lysine.

Isolation of resistant mutants – This strategy can be employed in several ways for strain improvement in the production of secondary metabolites: (i) Mutants can be isolated that are resistant to analogs of primary metabolite precursors involved in secondary metabolite production, thereby increasing the precursor's availability. (ii) Mutants can be isolated that are resistant to the feedback inhibition exerted by the secondary metabolite. (iii) Mutants can be selected that are resistant to the toxic effects of secondary metabolite precursors when these are introduced during the organism's growth phase. (iv) Mutants can be isolated that are resistant to the toxic effects caused by the production of the secondary metabolite.

(i) Mutants resistant to the analogues of primary metabolic precursors of the secondary metabolite – This approach was employed by Elander et al. in isolating mutants of *Pseudomonas aureofaciens* that overproduced the antibiotic pyrrolnitrin. Tryptophan, a precursor of pyrrolnitrin, stimulates production but is economically impractical to add during industrial processes. To address this, they used the gradient plate technique to isolate mutants resistant to tryptophan analogs. They eventually identified a strain that produced two to three times more antibiotic than the parent strain and was resistant to feedback inhibition by tryptophan.

(ii) Mutants resistant to the feedback effects of the secondary metabolite - Many secondary metabolites, such as chloramphenicol, aurodox, cycloheximide, staphylomycin, ristomycin, puromycin, fungicidin, candihexin, mycophenolic acid, and penicillin, inhibit their own synthesis. However, the exact mechanisms behind this feedback control are often unclear. Selecting mutants resistant to feedback inhibition by a secondary metabolite is more challenging than isolating strains resistant to primary metabolic control. It is improbable that a toxic analog mimicking the feedback control of the secondary metabolite could be found, especially

since the compound is not essential for growth. Nonetheless, mutants resistant to feedback inhibition by antibiotics can be detected using solidified media screening techniques. Survivors of mutation treatments are cultured on solid media containing repressing levels of the antibiotic, and resistant mutants are identified and overlaid with indicator organisms for further analysis.

(iii) The isolation of mutants resistant to the toxic effects of the secondary metabolite in the trophophase - Demain (1974) demonstrated that many secondary metabolites are toxic to the producing cell during the trophophase (growth phase). A metabolic switch in the idiophase allows the organism to produce an otherwise autotoxic compound. In some cases, the higher the resistance to the secondary metabolite in the growth phase, the higher the productivity during the production phase. For example, Dolezilova et al. (1965) showed that the level of nystatin production by various strains of *Streptomyces noursei* correlated with resistance to the antibiotic during the growth phase. A non-producing mutant was inhibited by 20 units/ml, the parent strain produced 6,000 units/ml and was inhibited by 2,000 units/ml, while a mutant producing 15,000 units/ml was resistant to 20,000 units/ml.

Use of combined approach of mutation and selection for strain improvement to increase the productivity of primary metabolites–

The production of threonine by *C. glutamicum* has been achieved using a combined auxotrophic and analogue-resistant mutant. An example of this strategy is reported by Kase and Nakayama (1972), in which stepwise improvements were made in productivity of strain. First, they mutated strains of *C. glutamicum* to a methionine auxotroph and these auxotrophs were made resistant to α-amino-β-hydroxy valeric acid (a threonine analogue) and *S*-(β-aminoethyl)-L-cysteine (a lysine analogue). The protocol for the generation of such mutants, along with the yield obtained, is shown in Fig. 1.9.

In this example, the analogue-resistant strains were found to be altered in the susceptibility of aspartokinase and homoserine dehydrogenase to feed back control, and the lack of methionine removed the repression control of homoserine dehydrogenase. The use of recombinant DNA technology has resulted in the construction of far more effective threonine producers and these strains are discussed under recombinant DNA technology.

YouTube Video:
https://youtu.be/AnZgplugF6Y?list=PL2NriTGQb1Gw4KoAbw0xREuJ629bexv-7

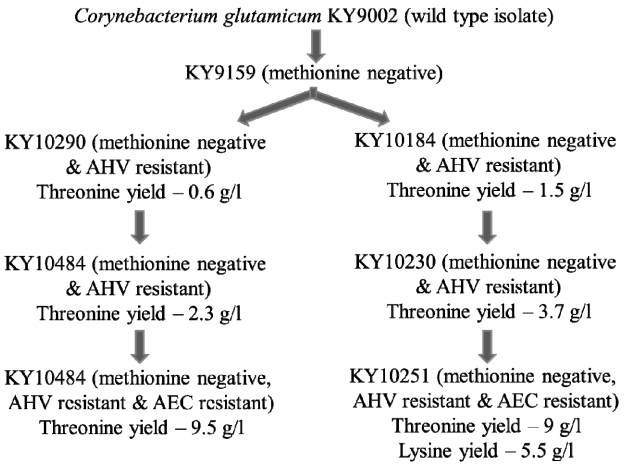

Fig. 1.9 A protocol designed by Kase and Nakayama (1972) for the improvement of *Corynebacterium glutamicum* for the overproduction of L-threonine or L-threonine & L-Lysine. AVH (α-amino-β-hydroxy valeric acid - a threonine analogue) & AEC (*S*-(β-aminoethyl)-L-cysteine - a lysine analogue) were used in this study

Recombination technique (The application of recombinant DNA technology)

The transfer of DNA between different bacterial species has been successfully achieved using both *in-vivo* and *in-vitro* techniques. This allows genetic material from one species to be incorporated into another, where it can be expressed. *In-vivo* techniques utilize phage particles that capture genetic information from the chromosome of one bacterial species and transfer it to another species through infection. As a result, the genetic information from the first species is introduced and may be expressed in the second host. In this process, vectors play a crucial role by collecting genetic material from one cell and incorporating it into another. On the other hand, *in-vitro* techniques involve inserting genetic information into a vector through laboratory manipulation. The vector, along with its inserted DNA, is then introduced into the recipient cell. This method allows for the