Chapter IV. Production improvment

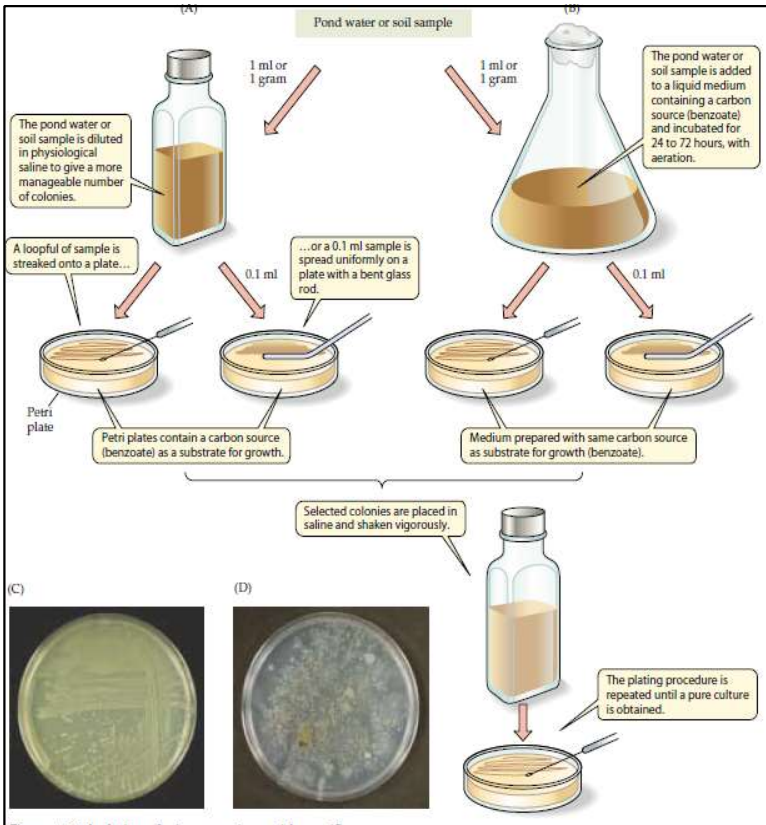


Figure 5.13 Isolation of microorganisms with specific nutritional requirements
Isolation of a bacterium that can utilize a specific compound as carbon and energy source. (A) Enrichment on a solid medium. (B) Enrichment in a liquid medium. (C) Plate streaked from a liquid enrichment. The liquid enrichment technique tends to favor rapidly growing organisms. Note that only one type of colony has developed. (D) Spreading samples directly onto the surface of an agar plate yields a variety of microorganisms. Photos courtesy of Jerome Perry.

Primary screening

- Primary screening involves the qualitative evaluation of all isolated microorganisms (MOs) based on their biotechnological suitability.
- This initial assessment aims to reduce the number of MOs subjected to subsequent secondary (quantitative) screening.

Secondary screening

- In this stage, a quantitative comparison of microorganisms (MOs) is conducted.
- The MOs are cultured in a liquid medium with a defined composition, under uniform conditions.
- The desired activity, typically represented by specific metabolites, is measured using appropriate methodologies, such as biochemical, microbiological, or immunological techniques.
- The objective is to identify the most efficient strains, characterized by their highest levels of production.

Production improvement: optimization

- Optimization aims to improve the biotechnological characteristics of the selected MO, this consists of modifying the cultivation conditions or genetically modifying the MO (lifting the inhibition for overproduction)

a. Optimization of culture conditions (medium design)

This refers to the combination of various essential elements required for the growth and production of microorganisms, including water, carbon sources, nitrogen sources, oxygen, growth factors, pH, temperature, and other factors.

The aim is to optimize these conditions to ensure that the microorganisms in culture achieve maximum productivity.

Classic methods

Statistical methods

b. Strain development

Natural evolution is based on three processes:

1. The generation of mutants,
2. The (natural) selection of mutants with desirable properties
3. The reassortment of traits among strains through genetic exchange.

The implementation of programs designed to enhance microbial strains beneficial for industrial applications, particularly for the overproduction of metabolites, primarily relies on these initial two processes.

The generation of mutants

- Natural variation, or mutagenesis, has been observed to occur at a low frequency.
- For practical purposes, it is essential to increase this frequency through the application of mutagens.
- Early research employed potent mutagens such as X-rays or nitrogen mustard; however, these agents often induce additional adverse effects and pose safety risks.
- Consequently, milder and more controllable agents, such as ultraviolet irradiation, are generally preferred in contemporary mutagenesis studies.

- Given that mutations frequently result in detrimental effects on the affected gene, one might anticipate challenges in isolating mutants with enhanced productivity of a metabolite.

However, this assumption is only partially accurate.

- Increased metabolite production can be achieved by either removing regulatory mechanisms governing the synthetic pathway or by eliminating competing metabolic activities that hinder the accumulation of the desired product.
- Such mutations can be induced empirically, without prior knowledge of the pathways and their regulatory control, by screening for the desired phenotype.
- Alternatively, gene cloning may be employed, though this approach necessitates a more comprehensive understanding of the relevant genes involved.

Selection of desired mutants

- In the context of evolution, improved strains are selected based on their enhanced potency.
- Similarly, in microbial genetics, the term "selection" typically refers to the application of conditions that allow only the desired strain to thrive.
- A prime example of this is the selection of antibiotic-resistant mutants. However, in the implementation of industrial microbial strain improvement programs, such selective conditions are rarely feasible.

- Producing high levels of an antibiotic does not necessarily provide a selective advantage for the producing mutant strain. Consequently, it is advisable to isolate individual colonies and evaluate the production levels of each. This process is commonly referred to as "screening," though it can be somewhat confusingly termed "selection" as well.
- In addition to production levels, various other characteristics are crucial for industrial applications. These include a high growth rate, effective substrate utilization, adaptability to different fermentation conditions, and the absence of undesirable by-products. The presence of such by-products not only adversely affects product formation but also contaminates the final product, thereby increasing the costs associated with downstream processing.

Overproduction of primary metabolites

I. Simple metabolic pathway

- A primary metabolite can be considered the end product of a series of reactions that convert an initial substrate (S) into a final product (P).
- One of the main factors likely to limit P production is the availability of the initial substrate, S.
- Increasing intracellular levels of S should enhance the speed of the pathway. However, since S is also utilized by other metabolic pathways within the cell, it can be challenging to influence its availability

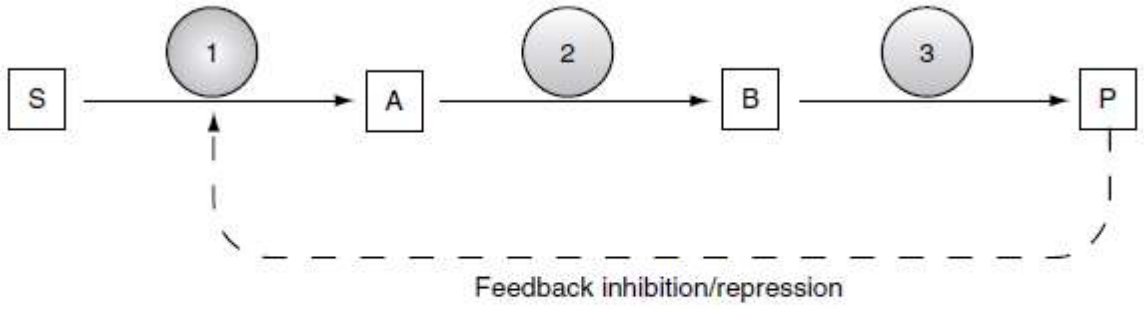


Figure 8.1 Regulation of primary metabolite production: a simple unbranched pathway

Additionally, the rate of enzymatic reactions affects P production. This rate depends on the number of enzyme molecules, the catalytic activity of the enzyme, and its affinity for the substrate.

Theoretically, it is possible to modify the enzyme's structure to increase its maximum activity or substrate affinity, but in practice, such mutations are extremely rare. A more practical approach would be to increase the enzyme's production rate, often by modifying the promoter site to enhance transcription of the gene of interest.

Example: Over-production of ornithine by a mutant strain of *Corynebacterium glutamicum*

Ornithine is an intermediate in the urea cycle; it is a non-proteinogenic amino acid, meaning it is not encoded by the genetic code and thus does not contribute to protein composition.

Ornithine is used in pharmaceuticals and food products.

The producing strain is auxotrophic for citrulline and, consequently, for arginine.

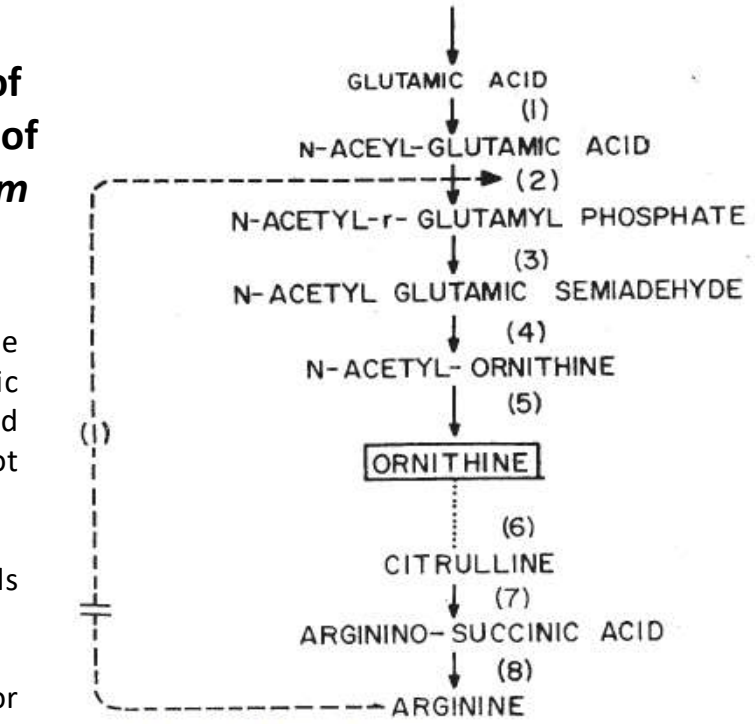


Fig. 6.6 Scheme for the Overproduction of Ornithine by a Citrulineless Mutant of *Corynebacterium glutamicum*

II. Branched metabolic pathway

Many amino acids are synthesized through branched metabolic pathways.

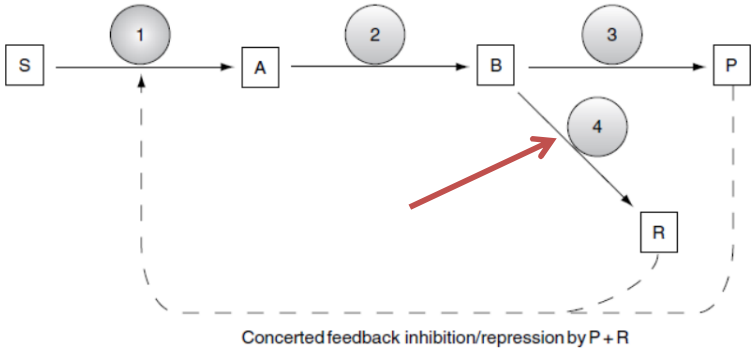


Figure 8.2 Regulation of primary metabolite production: a branched pathway

- The production of R diverts resources away from the production of P.
- A mutant deficient in enzyme 4, and therefore unable to produce R, would be able to generate higher levels of P.
- An additional advantage of the absence of R is that branched pathways often exhibit concerted (or multivalent) repression.
- In this example, enzyme 1 is repressed only when both P and R are present in sufficient quantities.

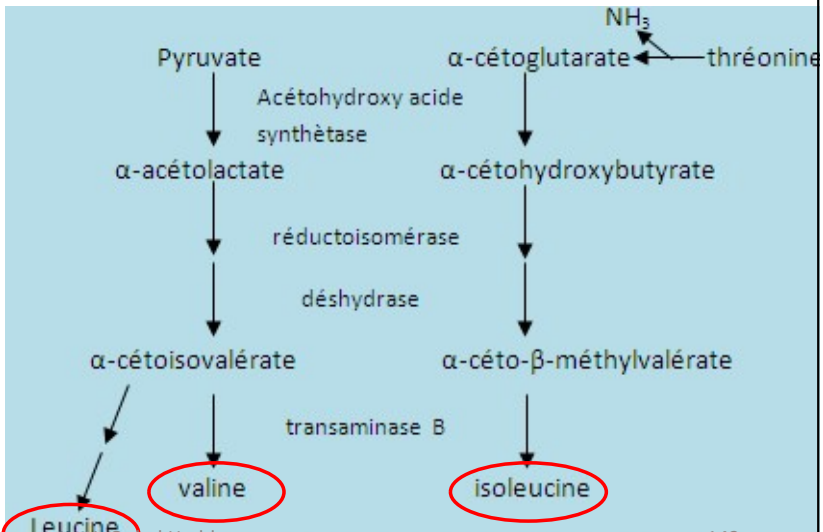
If R, the undesirable byproduct, is essential for cell growth, a mutant lacking enzyme 4 will be auxotrophic for R and can be isolated through replica plating.

Growth of these mutants will then require the addition of R to the growth medium. Feedback inhibition or repression of enzyme 1 may occur.

However, it is typically possible to add R in very small amounts that are sufficient to support growth without causing inhibitory effects.

- For example, attenuation of the ilv operon requires the simultaneous presence of leucine and valine, which are the end products of a branched pathway, as well as isoleucine, which shares enzymes with valine in its synthesis.

Another example is the production of lysine,…



Voies de biosynthèse de la valine, de la leucine et de l'isoleucine

Production of lysine

- A specific example is the commercial production of lysine, which is widely used as an additive in cereal-based animal feed. A simplified representation of the lysine production pathway in *Corynebacterium glutamicum* (Figure 8.3) shows that, as in other bacteria, the initial steps of lysine synthesis are shared with those of the threonine, isoleucine, and methionine synthesis pathways.

- Mutants of *C. glutamicum* that are deficient in the enzyme homoserine dehydrogenase are auxotrophic but can grow if homoserine (or a combination of threonine and methionine) is added.

- These mutants produce high levels of lysine (more than 50 g/L), partly due to the diversion of metabolites away from other amino acids and partly due to reduced feedback inhibition.

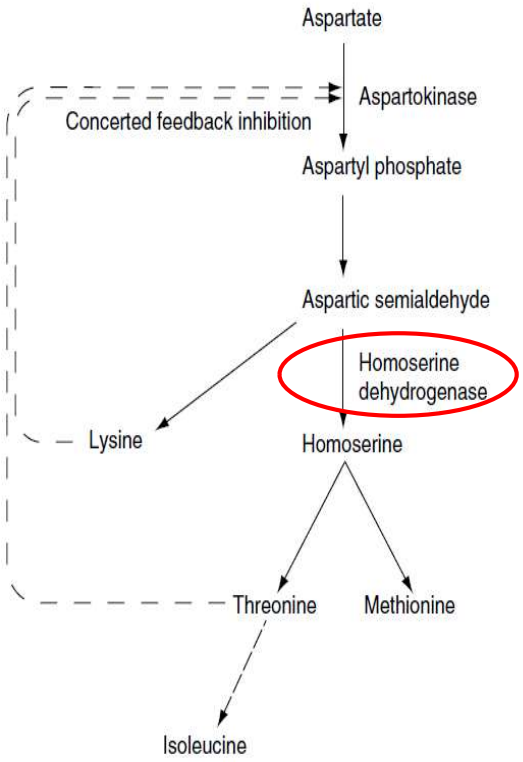


Figure 8.3 Lysine synthesis in *Corynebacterium glutamicum*

- The first enzyme in the pathway, aspartokinase, is subject to concerted (multivalent) regulation by lysine and threonine. However, in the auxotroph fed limited amounts of homoserine, the concentration of threonine remains too low to inhibit aspartokinase.

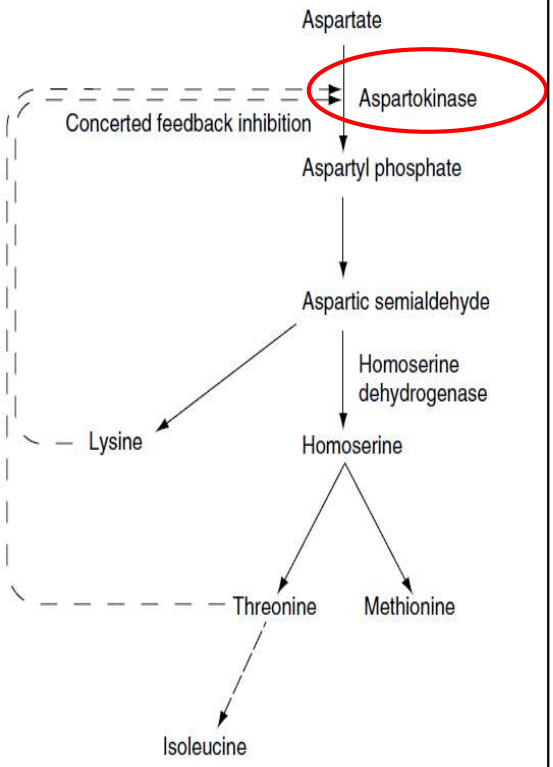
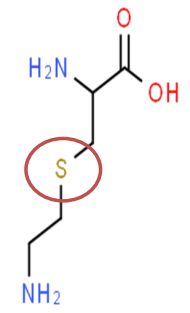
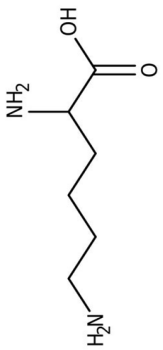


Figure 8.3 Lysine synthesis in *Corynebacterium glutamicum*

- **Amino acid analogs**
- can be used to obtain mutants resistant to feedback inhibition.
- For example, the lysine analog S-(2-aminoethyl)-L-cysteine mimics lysine's feedback inhibition of aspartokinase.
- This binding can signal the enzyme to reduce its activity, similar to how lysine would normally inhibit the enzyme when present in high concentrations.



S-(2-aminoéthyl)-L-cystéine



L-Lysine

- Mutants that have been exposed to this analog can develop resistance to its effects. These mutants have modified forms of aspartokinase that do not respond as strongly to lysine, meaning that even when lysine levels rise, the enzyme continues to function and synthesize lysine.
- As a result, these mutants can accumulate significantly higher levels of lysine because the normal feedback inhibition mechanism is less effective.

- **Reverse mutation of an auxotrophic mutant**
- When an auxotrophic mutant undergoes reverse mutation, the result may be an altered version of the enzyme that was affected by the original mutation. This enzyme may have new properties, such as:

- Increased Activity: The altered enzyme may function more effectively than the wild-type enzyme.

- Insensitivity to Feedback Inhibition: The new enzyme may not be inhibited by the end product of the metabolic pathway, allowing for continuous production of the desired metabolite even when its concentration is high.

Production of glutamic acid

Glutamic acid is used as a flavor enhancer in the form of its monosodium salt, sodium glutamate. It is also produced by *Corynebacterium glutamicum* from α-ketoglutarate, an intermediate in the Krebs cycle, through the action of glutamate dehydrogenase (Figure 8.4).

This reaction competes with the next enzyme in the Krebs cycle, α-ketoglutarate dehydrogenase, for its substrate.

Mutants deficient in α-ketoglutarate dehydrogenase activity tend to accumulate glutamate.

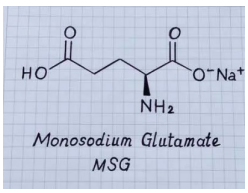


Figure 8.4 Synthesis of glutamic acid

Overproduction of secondary metabolites

- Antibiotics are the most important secondary metabolites produced by microorganisms.
- The first "true" antibiotic, penicillin, is produced by the mold *Penicillium*, but the primary sources of naturally produced antibiotics are filamentous bacteria known as actinomycetes, particularly *Streptomyces*.
- Generally, the level of antibiotic production by natural strains is far too low for industrial applications, so a strain improvement program is necessary.
- However, the metabolic pathways for the synthesis of these secondary metabolites are more complex and diverse than those for the production of primary metabolites. Therefore, the strain improvement program must be carried out empirically due to the lack of clarity regarding metabolic pathways and regulatory mechanisms.

- This challenge turned out to be easy to overcome. Screening large numbers of colonies is generally effective in identifying those with significant production.
- These selected colonies are then subjected to a new cycle of mutagenesis followed by selection.
- Repeated cycles of this process can lead to a strain that produces higher levels of the antibiotic compared to the original (isolated or wild) strain.
- It should be emphasized that the production of most antibiotics relies on strains that have been largely or entirely developed through these empirical processes.

- Today, although many pathways have been elucidated and genome sequencing has enhanced our understanding of the processes involved, random mutations and selection still remain the most effective methods for achieving higher production levels.

- On the other hand, in-depth knowledge of these pathways and their regulatory mechanisms opens new perspectives for manipulating the pathways to produce different derivatives of the desired antibiotic.

Tableau 3 – Amélioration d'une souche productrice de pénicilline

Identification du micro-organisme	Mutation spontanée ou agent mutagène	Multiplication du rendement par rapport à la souche de Fleming (*Penicillium notatum*)		
Penicillium chrysogenum NRRL 1951		1		
↓ NRRL 1951 B 25	spontanée	× 0,5	perte	
↓ X 1612	UV	× 4		
↓ WIS Q 176	UV	× 4,9		Amélioration obtenue par les chercheurs de l'université du Wisconsin
↓ WIS BL 3 D10	UV	× 9		
↓ 47.1564	spontanée	× 8,5	perte	
↓ 48.701	spontanée	× 8,1	perte	
↓ 49.133	Nitrosoguanidine (3 intermédiaires)	× 12,7		
↓ 51.20	Nitrosoguanidine (10 intermédiaires)	× 22,6		Amélioration réalisée dans les laboratoires pharmaceutiques Eli Lilly
↓ E.15	Nitrosoguanidine	× 50,6		
↓ E.15.1	spontanée	× 55		

New strategies for searching for microbial metabolites

Genome mining

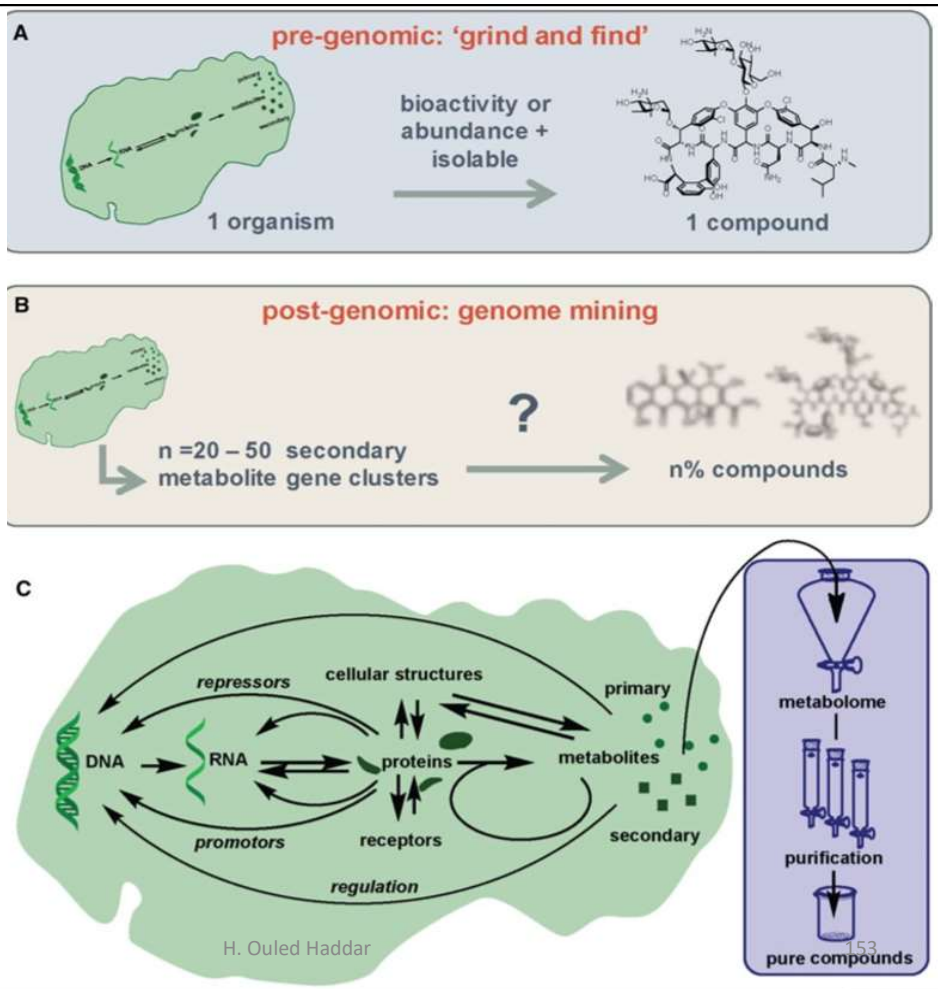
Genomic mining can be technically defined as “the process of translating sequence data from genes encoding secondary metabolites into purified molecules in test tubes.”

Fig 1. Strategies for natural product discovery.

a. Conventional methods involve "grinding and searching" for new secondary metabolites.

b. Post-genomic approaches now seek to leverage predictive data from gene sequences to identify new metabolites or improve yields.

c. The central dogma has been extended to exploit the genome for secondary metabolism.



Which microbes should we exploit?

Debate continues over which groups of microorganisms to target for a better source of novel natural products. Some scientists suggest that unculturable microorganisms could also serve as "untapped" sources of new secondary metabolites.

With the advent of inexpensive microbial DNA sequencing, it is now possible to explore the genetic capacity of different taxa of microorganisms and postulate:

- Which microbial taxa have the greatest potential to produce large numbers of complex secondary metabolites with drug-like properties (e.g., actinomycetes)?
- Which taxa have moderate potential?
- Which taxa have the lowest potential?

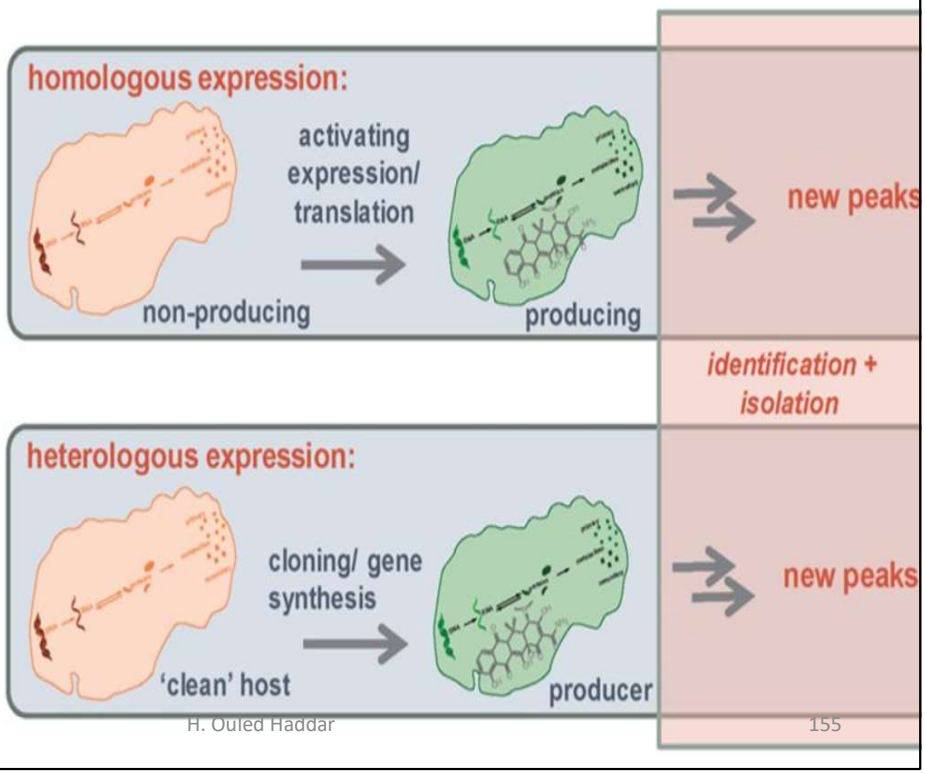
It is clear that efforts should be focused on the most promising microorganisms, while those with the lowest potential should not be heavily emphasized.

How to exploit microbial genomes?

Fig. 2 Two main categories of approaches are studied in the context of genomic exploitation.

In heterologous expression, entire gene cassettes are introduced into expression strains and the products are then differentially analyzed to identify new compounds.

In homologous expression approaches, endogenous transcriptional, translational or metabolic elements are manipulated, either by mutation or by (bio)chemical stimulants to activate the production of secondary metabolites. In both cases, these approaches involve an intense process of identifying new peaks in a metabolome and isolating the corresponding compounds for comprehensive structural characterization,



Examples of genetic strain improvement techniques

Among the promising approaches for activating cryptic biosynthesis gene cassettes in *Streptomyces* species are:

Ribosomal engineering

ribosomal engineering for strain improvement involves the strategic modification of the ribosomal machinery to enhance protein synthesis capabilities in microorganisms. This can lead to increased yields, improved product specificity, and greater resilience, making it a promising approach in biotechnology and industrial microbiology.

Table 1 Improvement of antibiotic/enzyme production and cell's physiology by subjecting to ribosome engineering

Strain	Antibiotic/enzyme	Mutation	Comment	Reference
Actinomycetes				
S. coelicolor	Actinorhodin	*rpsL*(K88E or K88R)	Using *relC* mutant	Ochi et al. (1997)
S. coelicolor	Actinorhodin	*rpsL*(K88E)	Streptomycin resistance	Hesketh and Ochi (1997)
S. coelicolor	Actinorhodin	*rpsL*(P91S)	Paromomycin resistance	Okamoto-Hosoya et al. (2000)

Appl Microbiol Biotechnol (2013) 97:87–98
DOI 10.1007/s00253-012-4551-9

MINI-REVIEW

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Kozo Ochi · Takeshi Hosaka

J Ind Microbiol Biotechnol (2014) 41:175–184
DOI 10.1007/s10295-013-1389-9

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REVIEW

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157