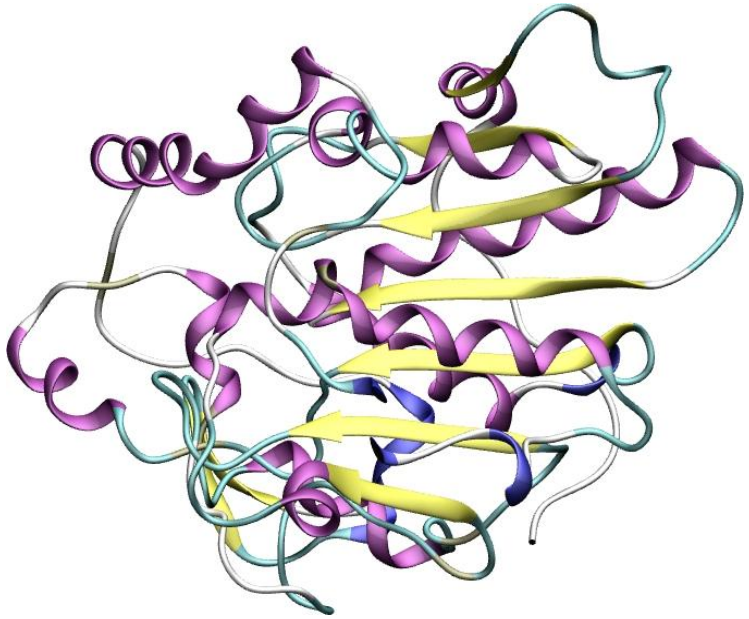
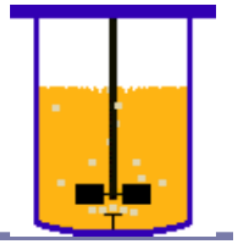
Fermentations for the production of amino acids &, antibiotics



Fermentations



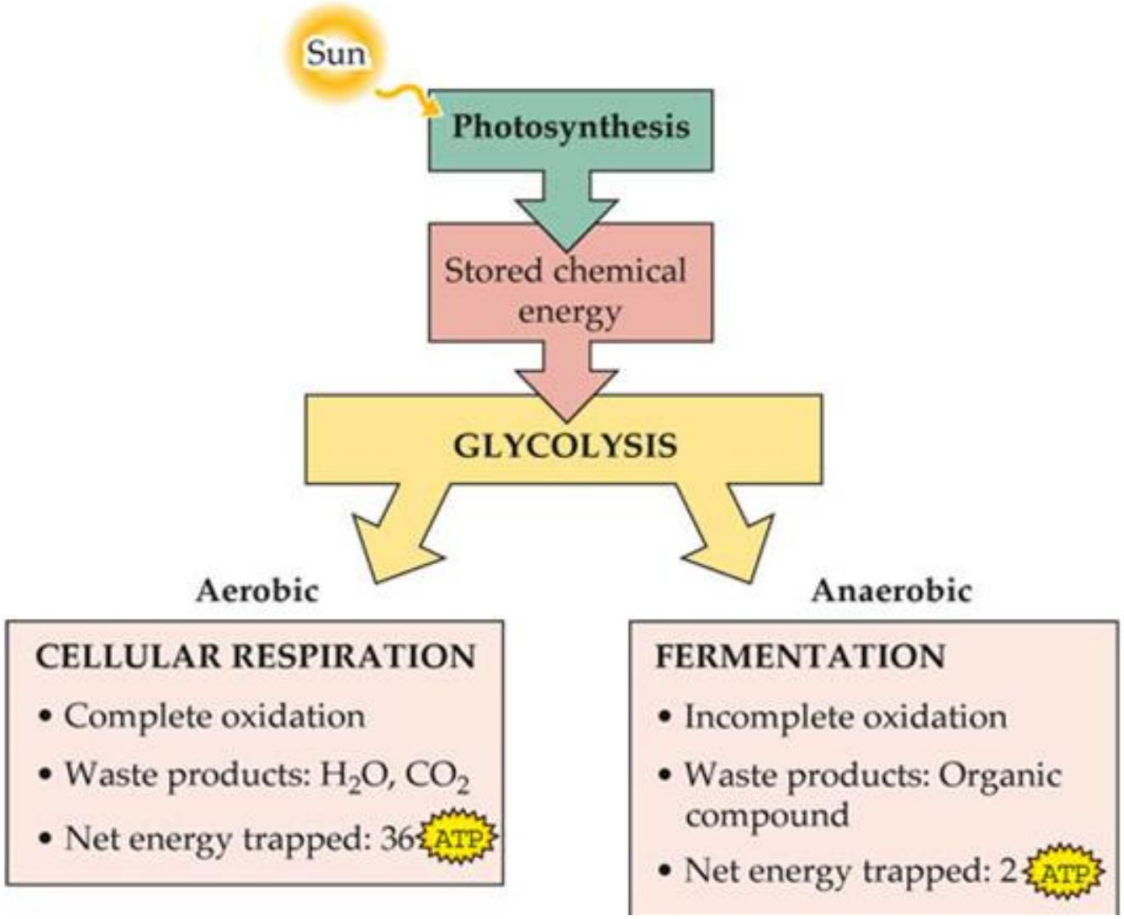
Definition(s)

1) *sensu stricto*: a metabolic process where O_2 is replaced by an organic molecule as final acceptor of electrons

KEY CONCEPT

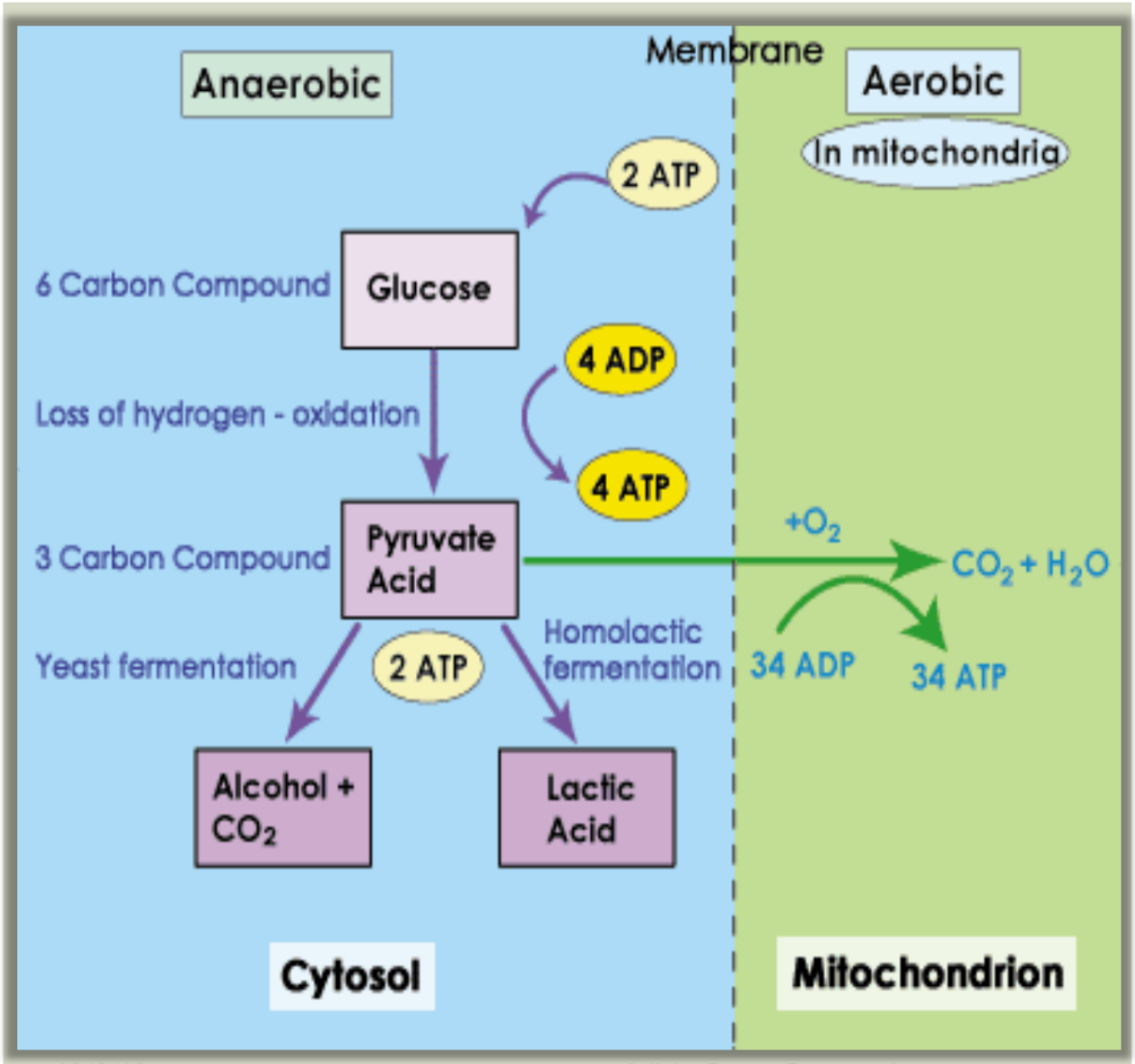
Fermentation allows the production of a small amount of ATP without O_2

- **Fermentation allows Glycolysis to continue producing ATP when oxygen is unavailable.**
  - **Anaerobic process**



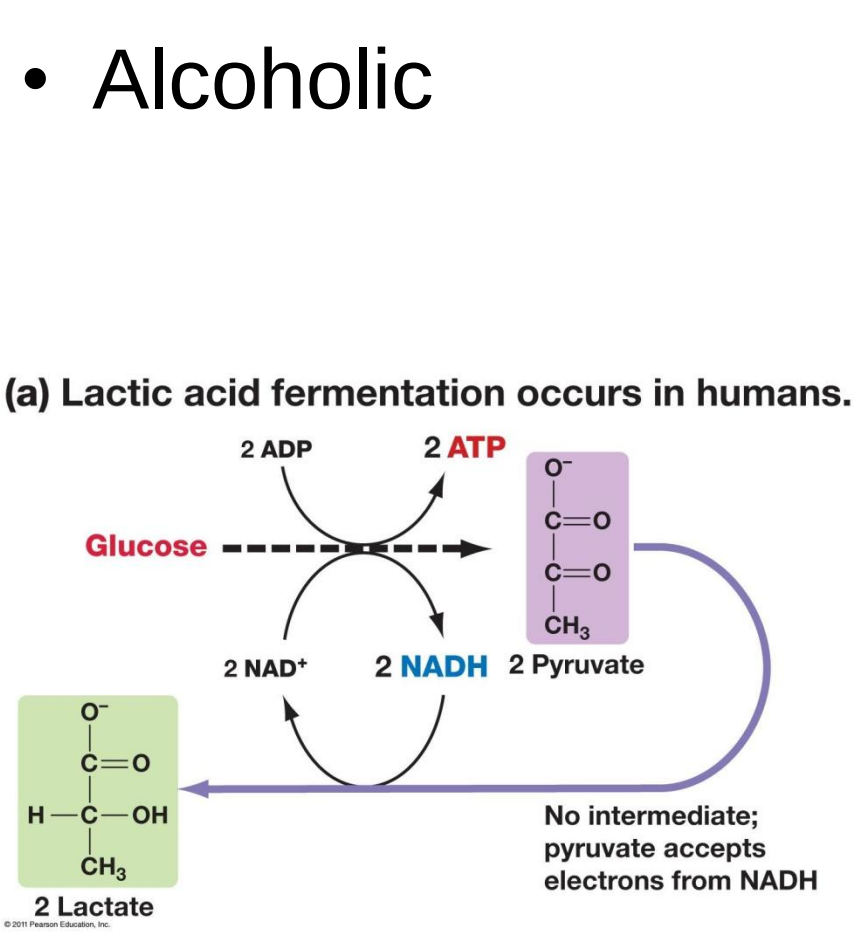
FERMENTATION

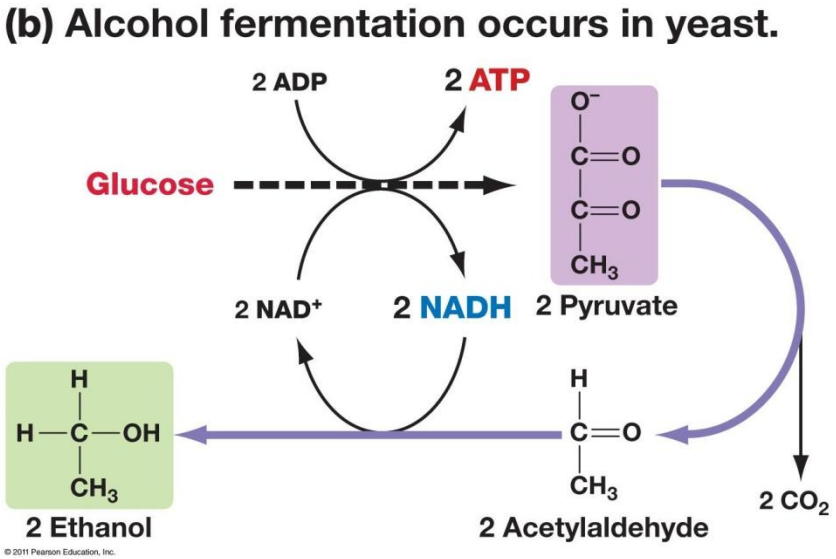
$$C_6H_{12}O_6 \xrightarrow{\text{energy}} \text{low } O_2 \rightarrow CO_2 + \text{waste} + \text{energy}$$

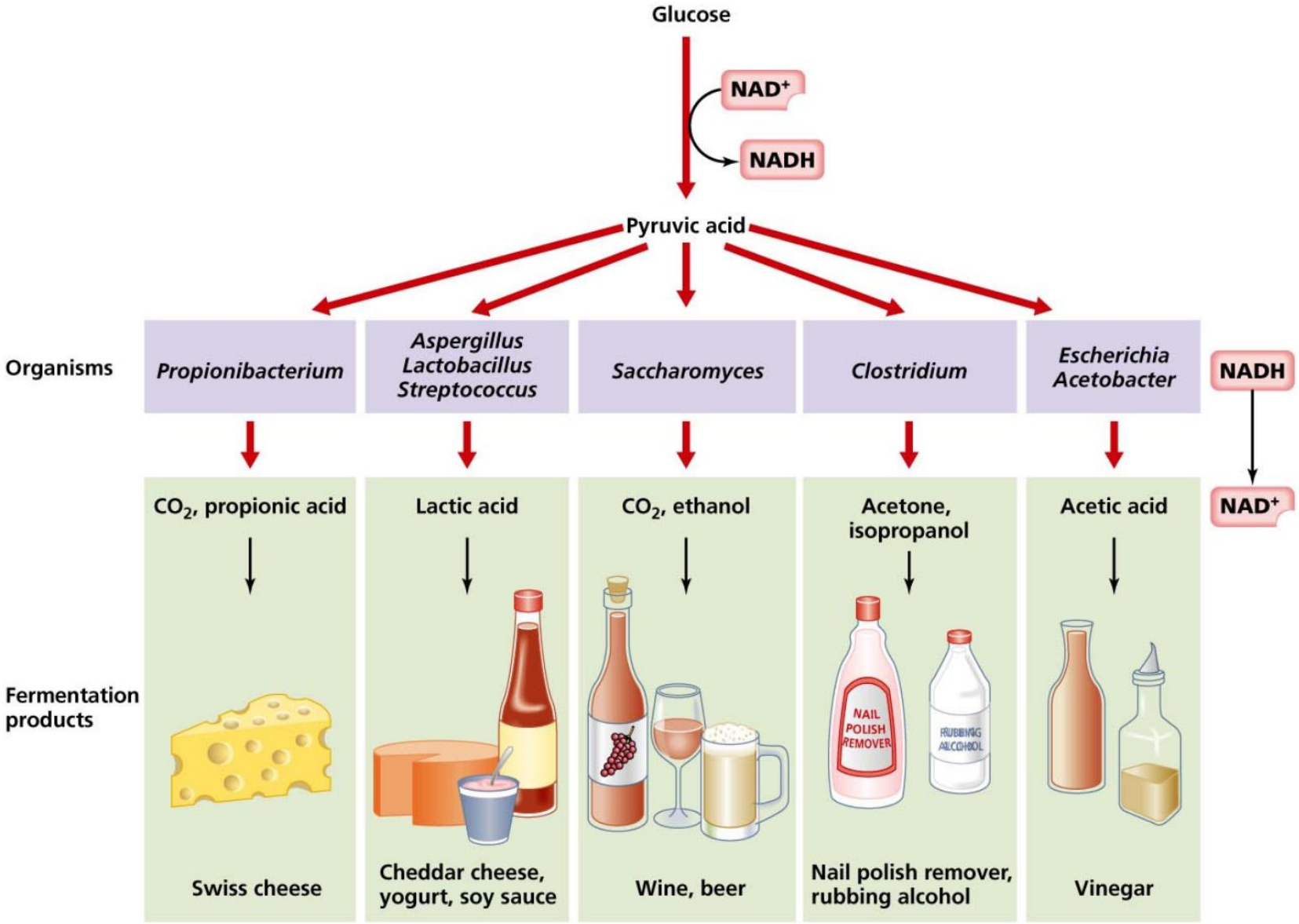

Anaerobic
Membrane
Aerobic
In mitochondria
2 ATP
6 Carbon Compound
Glucose
4 ADP
Loss of hydrogen - oxidation
4 ATP
3 Carbon Compound
Pyruvate Acid
+O2
CO2 + H2O
Yeast fermentation
2 ATP
Homolactic fermentation
34 ADP
34 ATP
Alcohol + CO2
Lactic Acid
Cytosol
Mitochondrion

Two examples of Fermentation

- Lactic Acid
- Alcoholic





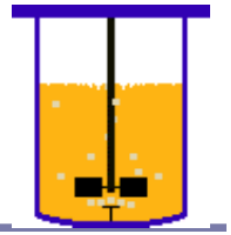


Uses of fermentation in industry

- Sewage treatment
- Biofuels
- Hydrogen gas

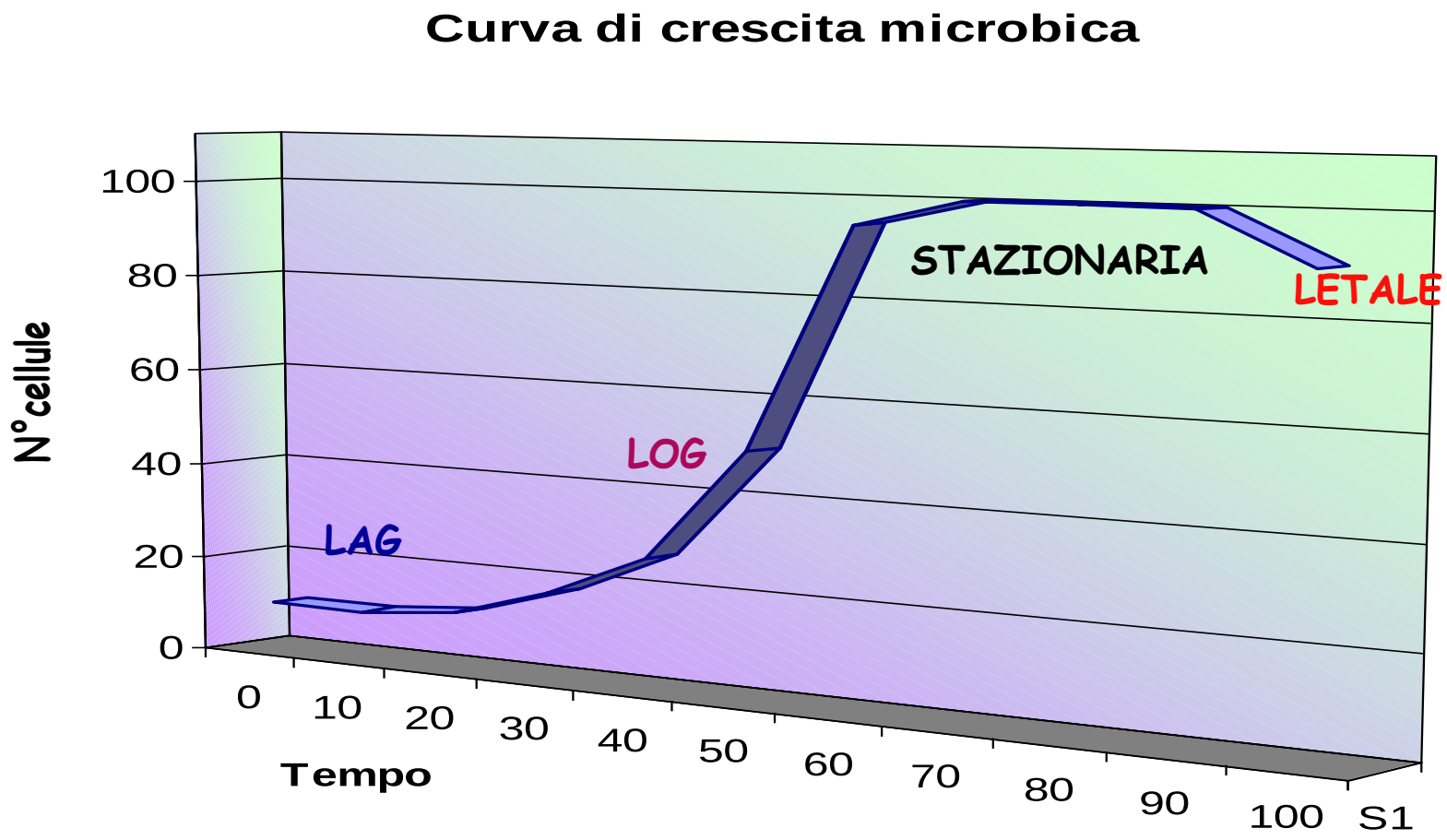
TABLE 5.4	Some Industrial Uses for Different Types of Fermentations		
Fermentation End-Product(s)	**Industrial or Commercial Use**	**Starting Material**	**Microorganism**
Ethanol	Beer	Malt extract	*Saccharomyces cerevisiae* (yeast, a fungus)
	Wine	Grape or other fruit juices	*Saccharomyces cerevisiae* var. *ellipsoideus*
	Fuel	Agricultural wastes	*Saccharomyces cerevisiae*
Acetic acid	Vinegar	Ethanol	*Acetobacter* (bacterium)
Lactic acid	Cheese, yogurt	Milk	*Lactobacillus, Streptococcus* (bacteria)
	Rye bread	Grain, sugar	*Lactobacillus bulgaricus* (bacterium)
	Sauerkraut	Cabbage	*Lactobacillus plantarum* (bacterium)
	Summer sausage	Meat	*Pediococcus* (bacterium)
Propionic acid and carbon dioxide	Swiss cheese	Lactic acid	*Propionibacterium freudenreichii* (bacterium)
Acetone and butanol	Pharmaceutical, industrial uses	Molasses	*Clostridium acetobutylicum* (bacterium)
Glycerol	Pharmaceutical, industrial uses	Molasses	*Saccharomyces cerevisiae*
Citric acid	Flavoring	Molasses	*Aspergillus* (fungus)
Methane	Fuel	Acetic acid	*Methanosarcina* (bacterium)
Sorbose	Vitamin C (ascorbic acid)	Sorbitol	*Acetobacter*

Fermentations in wide sense: the products



Microbial biomasses	starters for the food industry, probiotics etc.
Fermentation products (*sensu stricto*)	ethanol, butanol, lactic acid etc.
Primary metabolites	aminoacids, vitamins etc.
Products from incomplete oxidation	organic acids
Secondary metabolites	**antibiotics, anticancers, statins etc.**
Recombinant proteins	**insulin, growth hormone, industrial enzymes**
Polisaccharides	

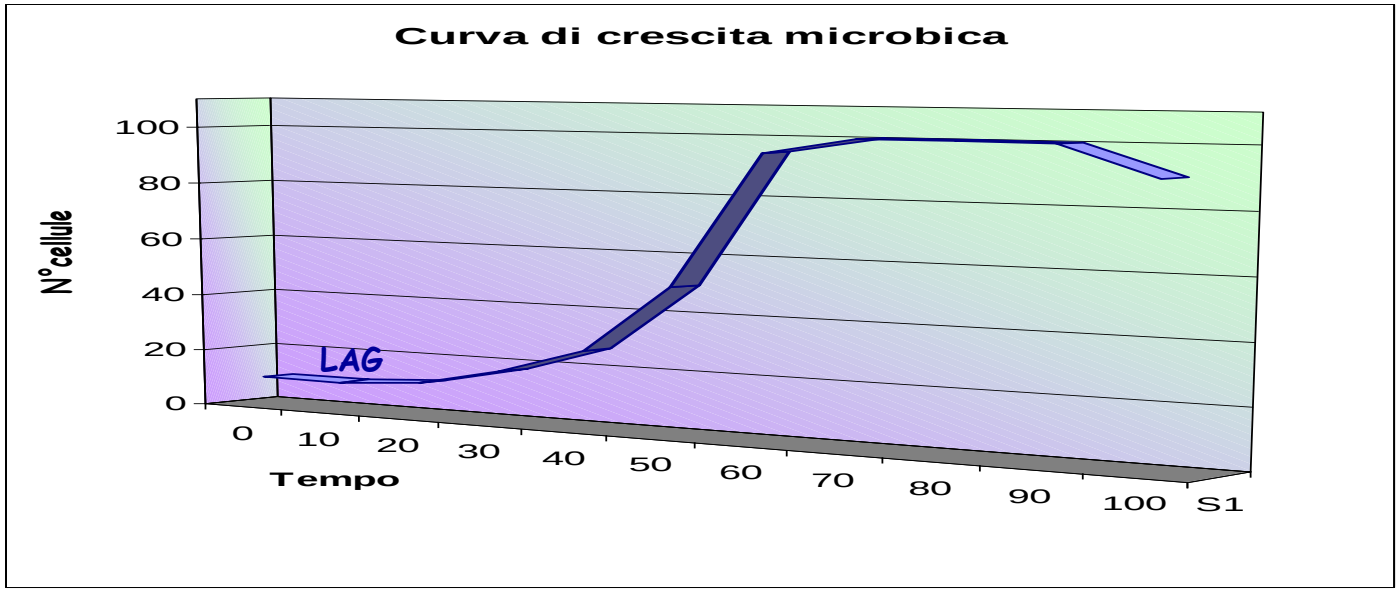
Produzione di biomassa (per es. produzione di enzimi)



Fase Lag

Stasi prima di una rapida crescita, può dipendere da:

- Le cellule potrebbero essere danneggiate
- Le cellule si debbono adattare al terreno
- Le cellule possono essere vecchie o fredde
- Le cellule producono nuovi ribosomi
- Le cellule sintetizzano nuovi enzimi
- Le cellule iniziano a fare cellule

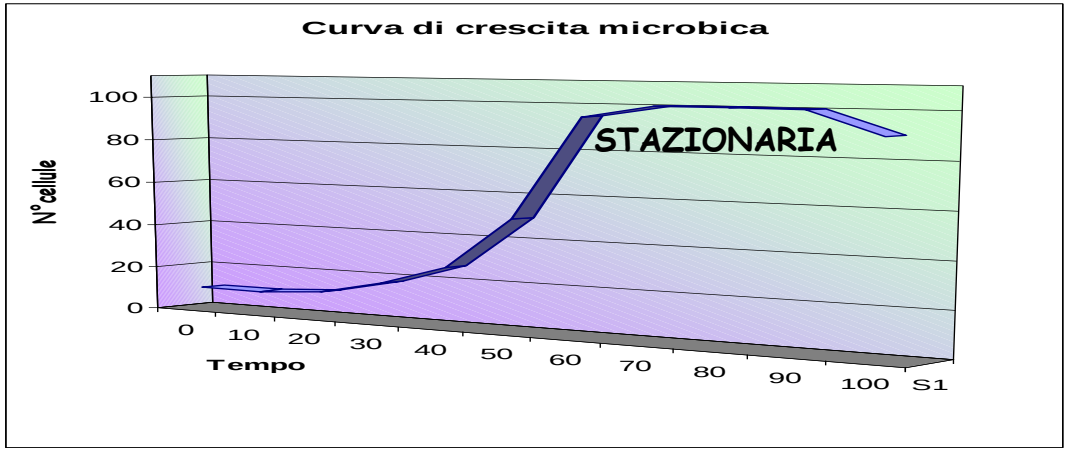


Fase Log

- Le cellule si riproducono velocemente
- La biomassa aumenta con rapidità
- Le sostanze nutrienti sono consumate in fretta
- L'Ossigeno (se usato) è consumato rapidamente
- Alcune colture producono calore
- Variazioni di pH dovute ai microrganismi
- Le proteine nel brodo possono formare schiuma
- La coltura può mutare reologia (mixing)

➢Fase Stazionaria

- ➢Esaurimento dei Nutrienti
- ➢L'Ossigeno può essere limitato
- ➢Rilascio di sostanze cellulari: per es. tossine
- ➢Cellule che crescono ~= cellule che muoiono
- ➢La divisione cellulare non è più logaritmica
- ➢Possono essere prodotti metaboliti secondari



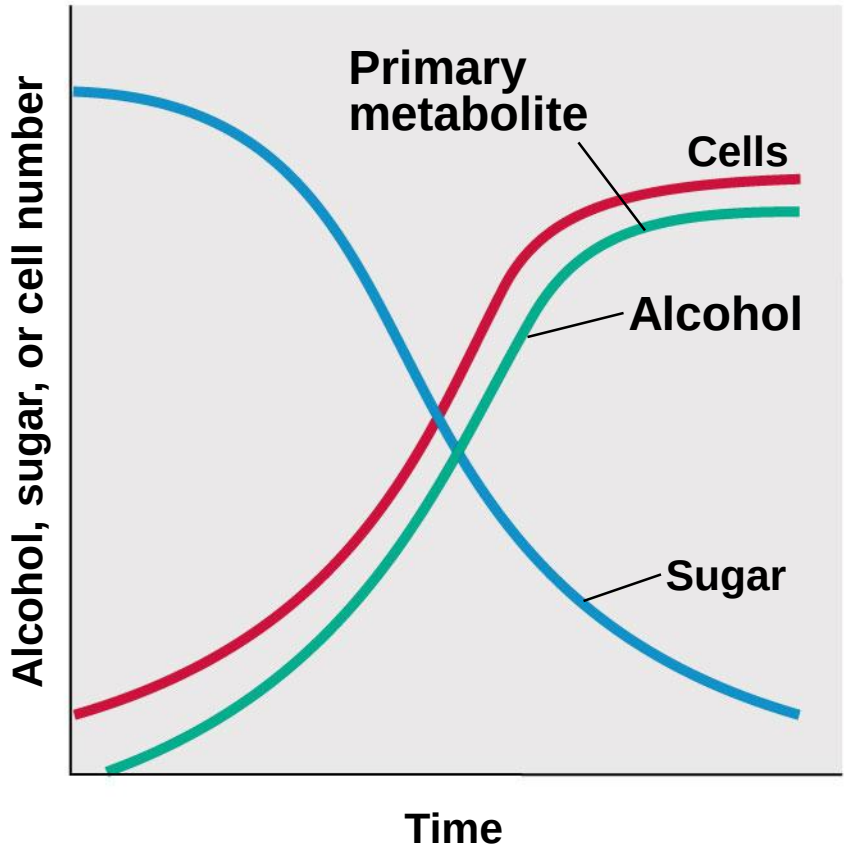
Fase letale

- Le cellule diminuiscono esponenzialmente
- Può verificarsi autolisi cellulare
- Le cellule sopravvissute non si duplicano

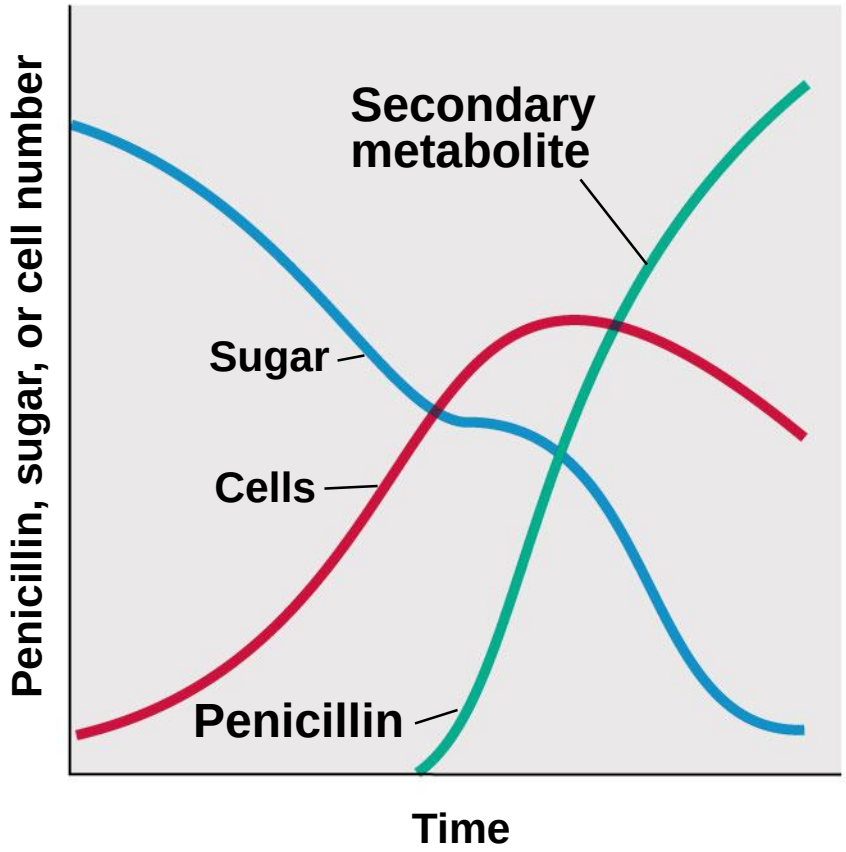
Fermentazione Industriale

- *Primary metabolite*
  - Produced during exponential growth
  - Example: alcohol
- *Secondary metabolite*
  - Produced during stationary phase

- *Secondary metabolites*
  - Not essential for growth
  - Formation depends on growth conditions
  - Produced as a group of related compounds
  - Often significantly overproduced
  - Often produced by spore-forming microbes during sporulation



(a)

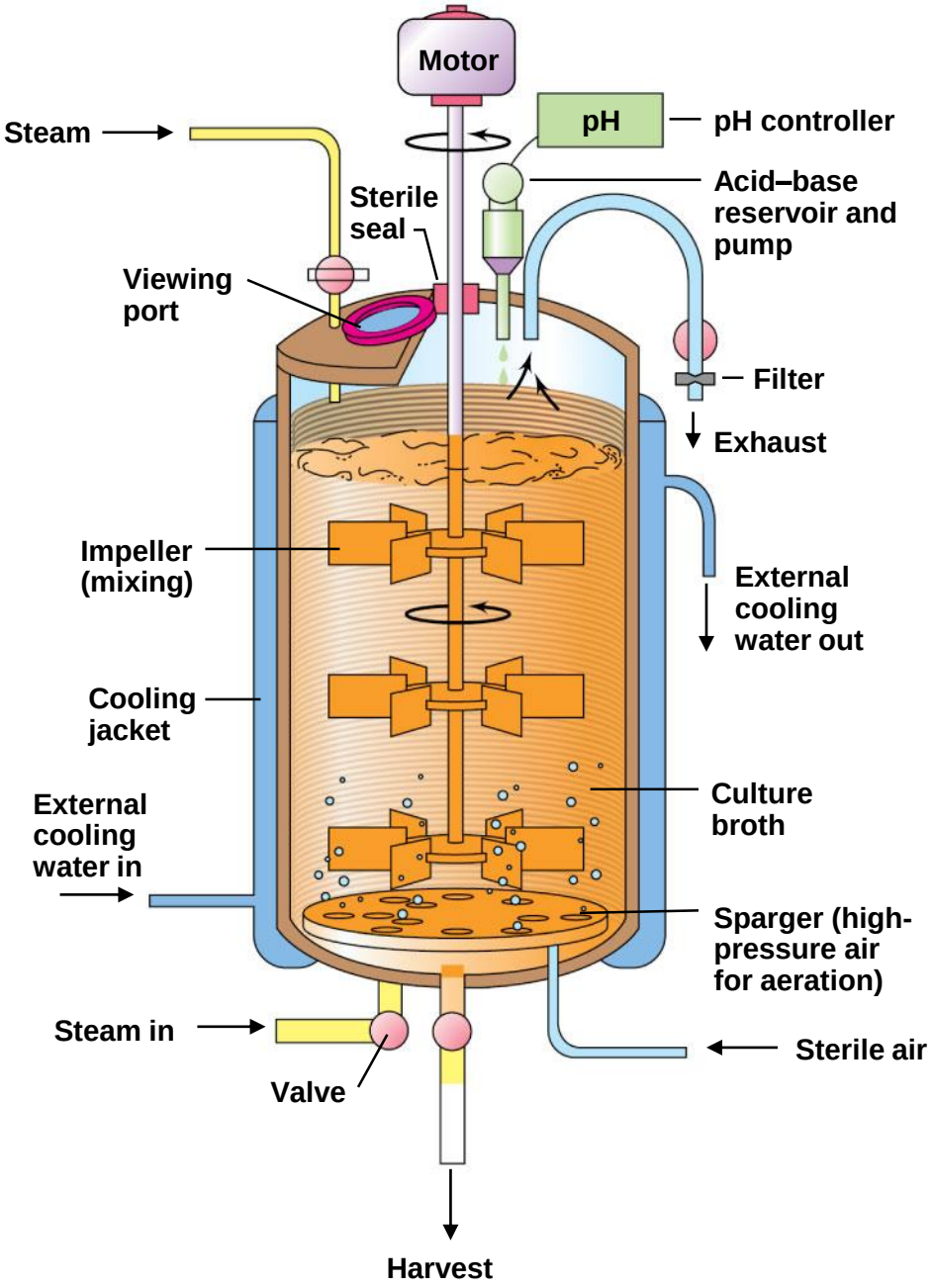


(b)

- Secondary metabolites are often large organic molecules that require a large number of specific enzymatic steps for production
  - Synthesis of tetracycline requires at least 72 separate enzymatic steps
  - Starting materials arise from major biosynthetic pathways

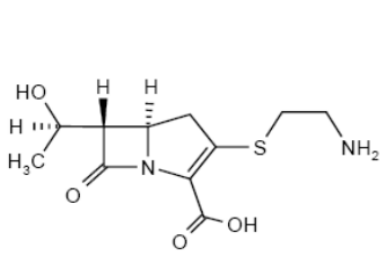
Fermentors

- *Fermentor* is where the microbiology process takes place
- Fermentors vary in size from 5 to 500,000 liters
  - Aerobic and anaerobic fermentors
- Large-scale fermentors are almost always stainless steel
  - Impellers and spargers supply oxygen

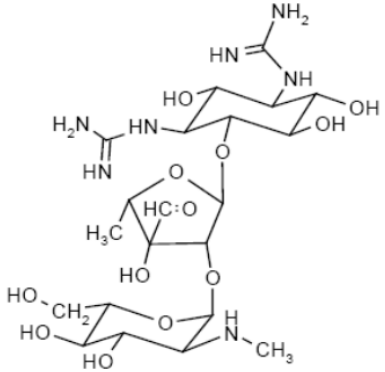

Motor
pH
pH controller
Steam
Sterile seal
Acid–base reservoir and pump
Viewing port
Filter
Exhaust
Impeller (mixing)
External cooling water out
Cooling jacket
Culture broth
External cooling water in
Sparger (high-pressure air for aeration)
Steam in
Sterile air
Valve
Harvest

(b)

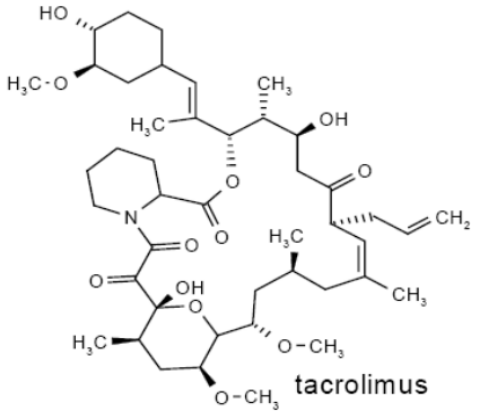
Secondary metabolites: a wide array of chemical structures



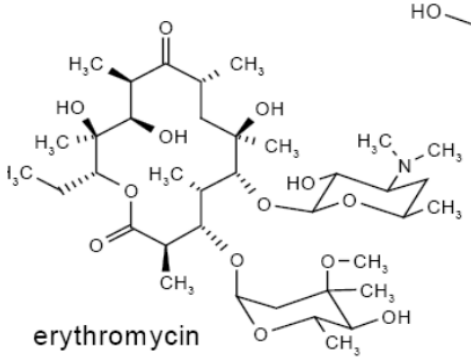
thienamycin



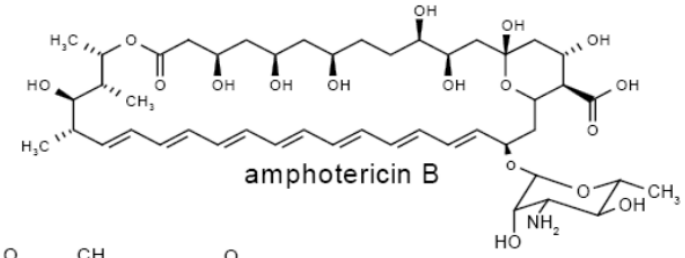
streptomycin



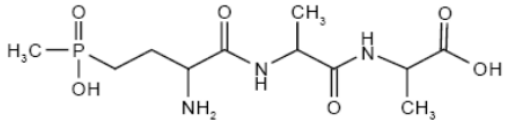
tacrolimus



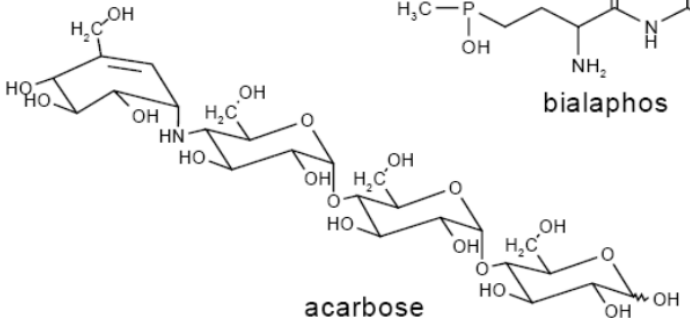
erythromycin



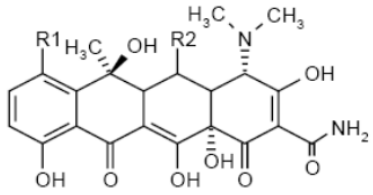
amphotericin B



bialaphos

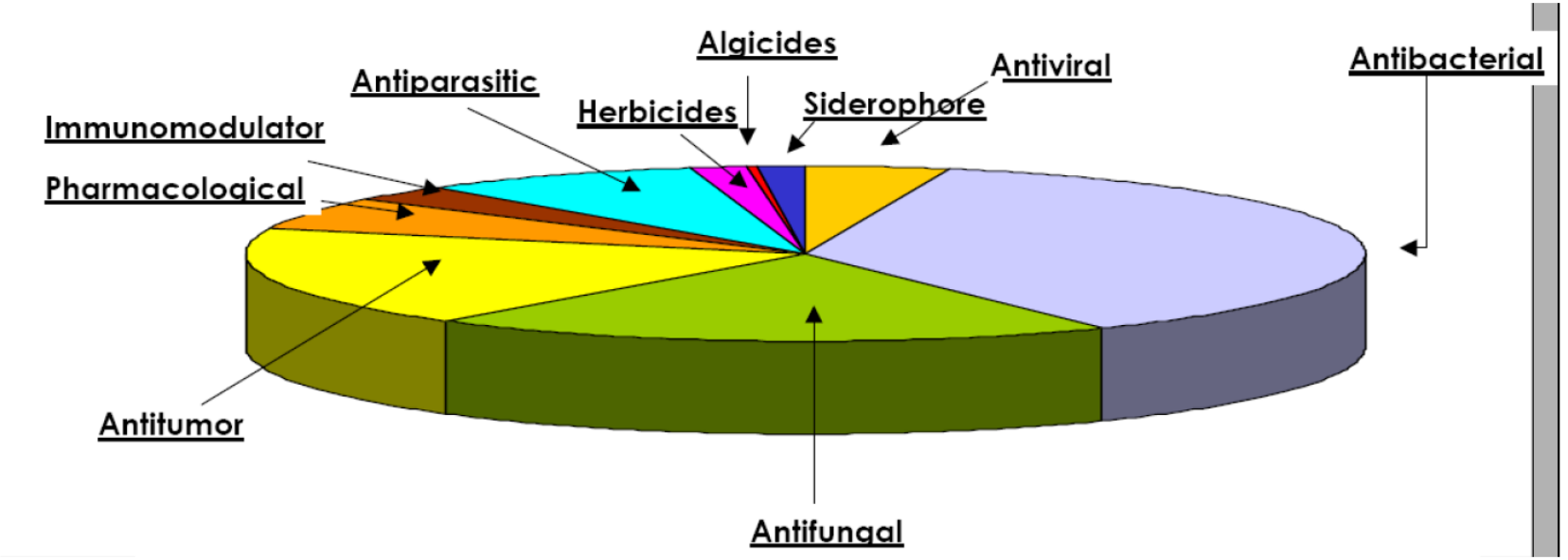


acarbose



tetracycline

Secondary metabolites: pharmaceutical properties



Data kindly furnished by prof. Flavia Marinelli

(VICURON ANTIBIOTIC LITERATURE DATABASE)

Secondary metabolites : pharmaceutical relevance

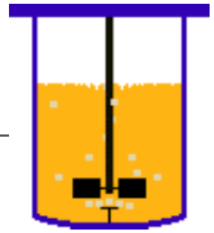
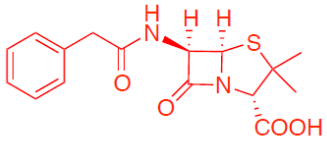


Table 1 The 50 most important microbial secondary metabolites or derivatives

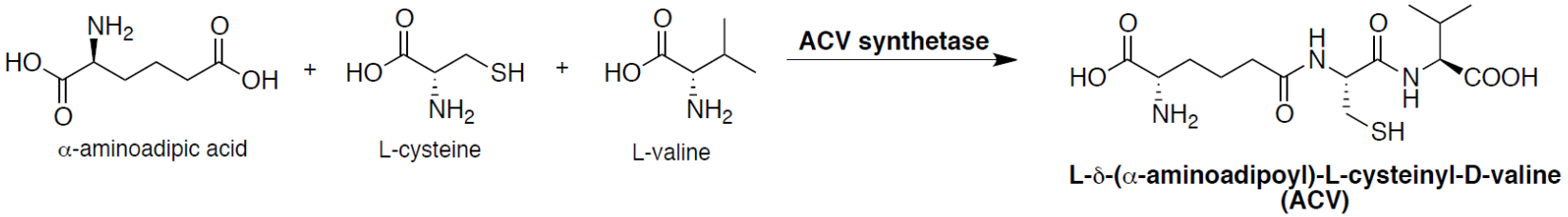
Amikacin sulfate	Antibiotic, semisynthetic aminoglycoside
Amoxacillin	Antibiotic, semisynthetic penicillin
Amoxacillin + clavulanate (Augmentin)	Antibiotic, semisynthetic penicillin + β-lactamase inhibitor
Amphotericin B	Antifungal, natural polyene
Ampicillin	Antibiotic, semisynthetic penicillin
Ampicillin + sulbactam (Sultamicillin, Unasyn)	Antibiotic, semisynthetic penicillin + β-lactamase inhibitor
Azithromycin	Antibiotic, semisynthetic erythromycin
Benzylpenicillin	Antibiotic, natural penicillin
Bialaphos	Herbicide, natural peptide
Cefaclor	Antibiotic, semisynthetic cephalosporin
Cefixime	Antibiotic, semisynthetic cephalosporin
Cefotaxime	Antibiotic, semisynthetic cephalosporin
Cefoxitin	Antibiotic, semisynthetic cephalosporin
Cefpodoxime Proxetil	Antibiotic, semisynthetic cephalosporin
Ceftazidime	Antibiotic, semisynthetic cephalosporin
Ceftrioxone	Antibiotic, semisynthetic cephalosporin
Cefuroxime	Antibiotic, semisynthetic cephalosporin
Cephalexin	Antibiotic, semisynthetic cephalosporin
Chlortetracycline	Antibiotic, natural polyketide, growth promotant
Clarithromycin	Antibiotic, semisynthetic erythromycin
Clindamycin	Antibiotic, semisynthetic lincomycin
Cyclosporin A	Natural immunosuppressant, cyclic peptide
Daunorubicin HCI	Antitumor, natural anthracycline
Doxorubicin HCI	Antitumor, natural anthracycline
Erythromycin A	Antibiotic, natural macrolide
Flomoxef	Antibiotic, semisynthetic cephalosporin
Gentamicin sulfate	Antibiotic, natural aminoglycoside
Imipenem	Antibiotic, semisynthetic thienamycin, carbapenem
Ivermectin	Antihelmintic, semisynthetic avermectin, macrolide
Kanamycin sulfate	Antibiotic, natural aminoglycoside
Lasalocid sodium	Natural polyether ionophore, coccidiostat, growth promotant
Lincomycin HCI	Antibiotic, natural lincosaminide
Lovastatin	Hypocholesterolemic polyketide, natural statin
Methicillin	Antibiotic, semisynthetic penicillin
Mitomycin C	Antitumor, natural mitosane
Monensin sodium	Natural polyether ionophore, coccidiostat, growth promotant
Oxytetracycline	Antibiotic, natural polyketide, feed additive
Paclitaxel (taxol)[a]	Antitumor, natural dipertene
Phenoxymethylpenicillin (penicillin V)	Antibiotic, biosynthetic penicillin
Pravastatin	Hypocholesterolemic polyketide, statin made by bioconversion
Rifampin	Antibiotic, semisynthetic rifamycin, ansamycin
Salinomycin	Natural polyether ionophore, coccidiostat, growth promotant
Spiramycin	Antibiotic, natural macrolide
Streptomycin sulfate	Antibiotic, natural aminoglycoside
Tacrolimus (FK506)	Natural immunosuppressant, macrolide
Teichoplanin	Antibiotic, natural glycopeptide
Tetracycline HCl	Antibiotic, natural polyketide
Tylosin phosphate	Natural macrolide, growth promotant
Vancomycin HCl	Antibiotic, natural glycopeptide

[a] Plant product, also made by microorganisms

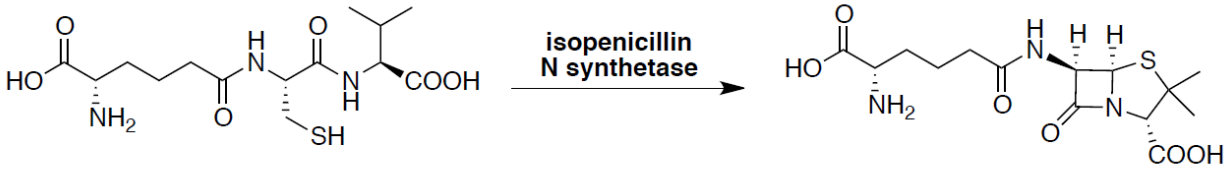
Penicillin G biosynthesis



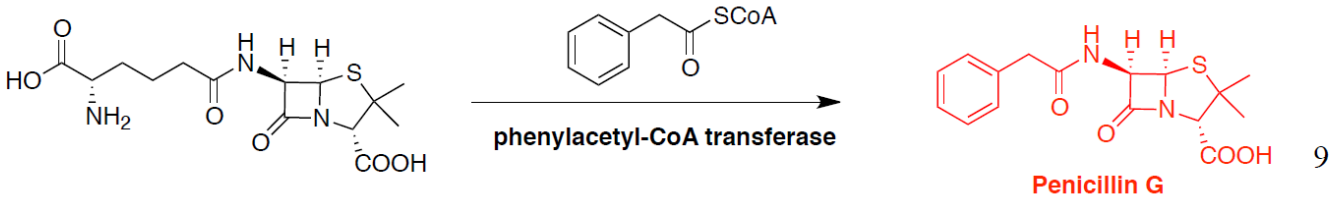
1) Polymerization: A tripeptide is formed by sequential addition of 3 aminoacids



2) Cyclization: the peculiar structure of penicillins (2 fused rings) is formed

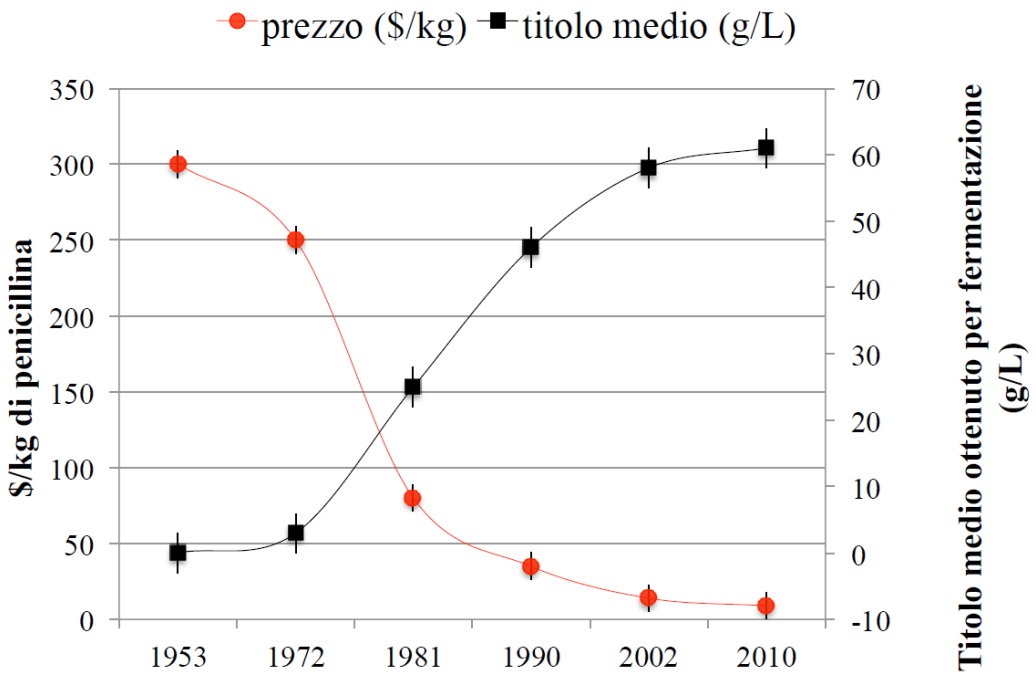


3) Decoration: transacylation with introduction of the phenylacetic moiety



Production of penicillins

Year	Production (kg)	Cost ($/kg)
1945	2,300	11,000
1963	3,000,000	150
1978	15,000,000	18.50
1992	22,000,000	–
1995	31,000,000	4.5



Amino acids: industrial impact

Of the 20 standard protein amino acids, the **9 essential amino acids** L-valine, L-leucine, L-isoleucine, L-lysine, L-threonine, L-methionine, L-histidine, L-phenylalanine, and L-tryptophan occupy a key position in that they are not synthesized in animals and humans but must be **ingested with feed or food**.

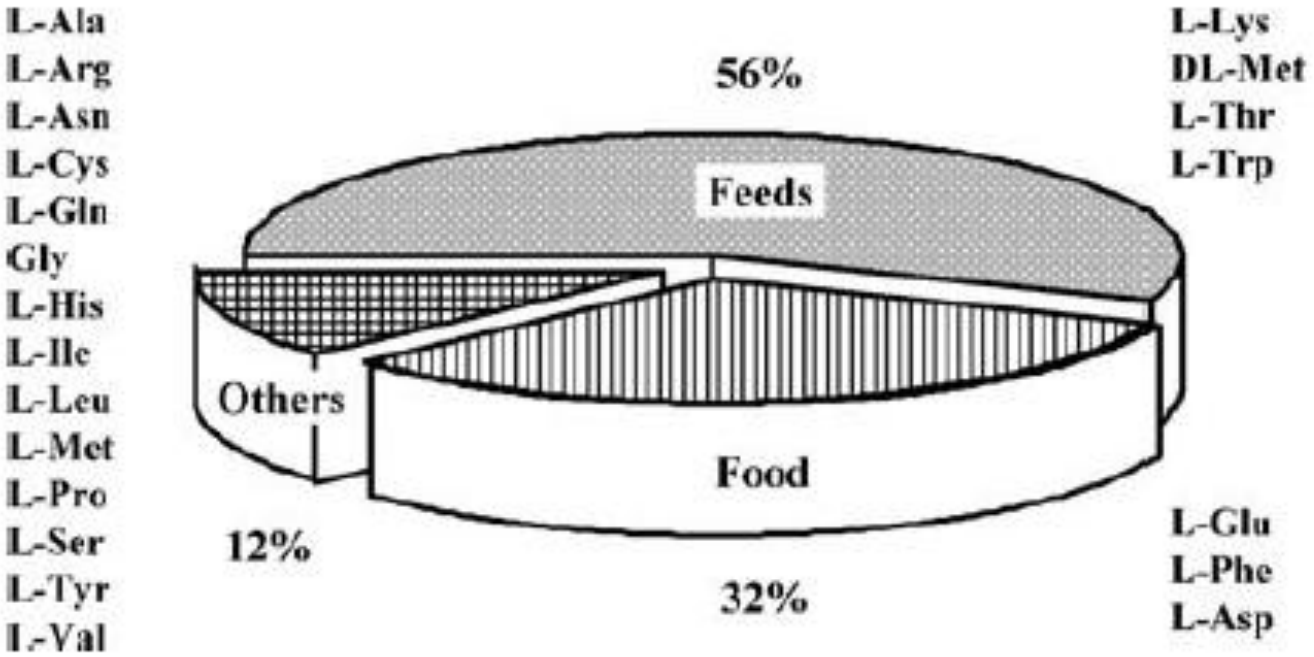
In terms of market volume, development over the last 20 years has been tremendously bullish in the so-called **feed amino acids** L-lysine, DL-methionine, L-threonine, and Ltryptophan, which constitute the largest share (56%) of the total amino acid market, estimated in 2004 at approximately US $4.5 billion.

Also substantial is the share of the **food sector**, which is determined essentially by three amino acids: **L-glutamic acid** in the form of the flavorenhancer monosodium glutamate (MSG) and the amino acids **L-aspartic acid and L-phenylalanine**, both of which are starting materials for the peptide sweetener L-aspartyl Lphenylalanyl methyl ester (Aspartame), used, for example, in “lite” colas.

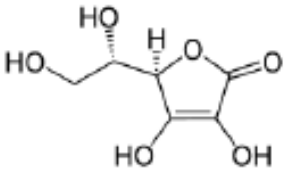
The amino acid market for synthesis applications is growing at an annual rate of 7% (US $1 billion in the year 2009), of which the share of amino acids for **peptide sweeteners** alone is expected to be more than US $400 million.

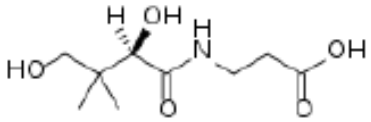
Amino acids: industrial impact

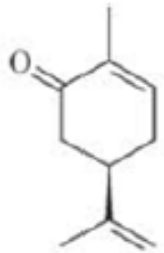
The use of enzymes and whole cell biocatalysts has proven particularly valuable in production of both **proteinogenic** and **nonproteinogenic** L-amino acids D-amino acids, and enantiomerically pure amino acid derivatives, which are of great interest as **building blocks** for active ingredients that are applied as **pharmaceuticals, cosmetics, and agricultural products**.

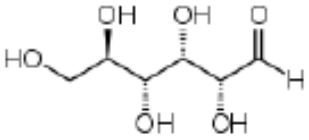


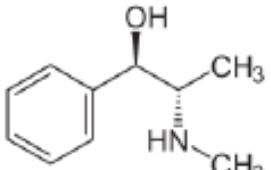
Chiral pool











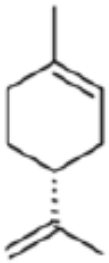
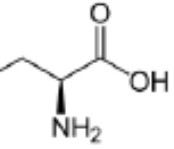
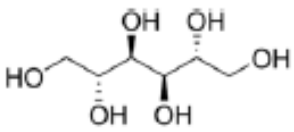


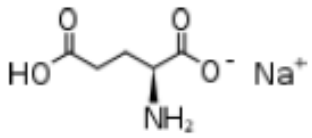
Table 2. Representative substances from the chiral pool[a]

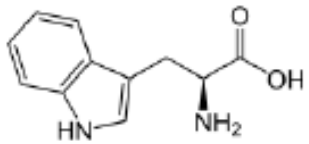
Compound	Approx. price (US dollars kg^{-1})
Ascorbic acid	13
(+)-Calcium pantothenate	16
(−)-Carvone	23
Anhydrous dextrose	1.2
Ephedrine hydrochloride	62
(+)-Limonene	3
→ L-Lysine	3.2
Mannitol	7.5
Monosodium glutamate	2
Norephedrine hydrochloride	24
Quinidine sulphate	130
Quinine sulphate	75
Sorbitol	1.7
→ L-Threonine	12–50, depending on grade
→ L-Tryptophan	68

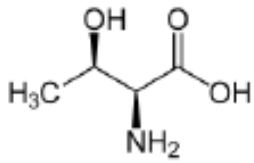
[a] Data from *Chemical Marketing Reporter*, Schnell Publishing, New York, 13 April (1990); reproduced by permission of the Editor.

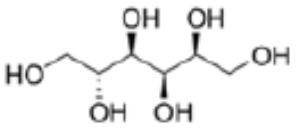


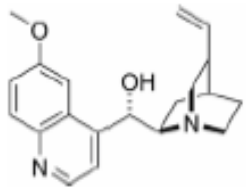


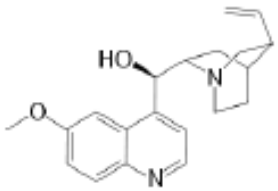


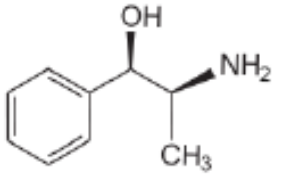












PRODUCTION OF AMINOACIDS

Extraction of amino acids from protein hydrolysate as a method of obtaining L-amino acids is now of only limited importance; although still relevant for production of **L-serine, L-proline, L-hydroxy-proline, and L-tyrosine**, for example, it is not suitable for large-scale production of amino
acids.

The extraction method for obtaining **L-glutamate** was superseded nearly 50 years ago by fermentation, following a sharp increase in demand.

MICROBIAL PRODUCTION OF AMINOACIDS

The discovery of the soil bacterium, ***Corynebacterium glutamicum***, which is capable of producing L-glutamic acid with high productivity from sugar, paved the way for the success of the fermentation technique in amino acid production
(Kinoshita et al. 1957). It was advantageous that the wild strain could be used on an industrial scale under optimized fermentation conditions for **mass production of glutamate.**

Biotechnological production of lysine: *Corynebacterium glutamicum*

Lysine is a preferred additive to animal feeds for pig breeding (as the first limiting amino acid) and poultry (second limiting amino acid, after methionine).

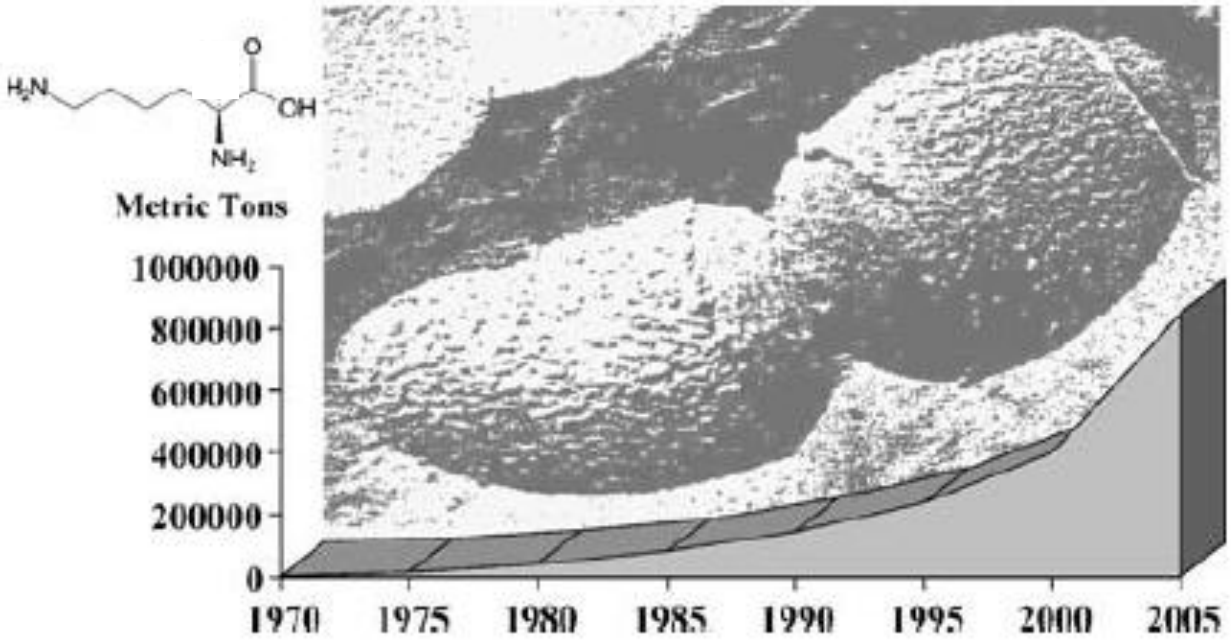


Fig. 3 Global market for L-lysine (1970–2005). The picture shows the lysine-producing mutant of *C. glutamicum*—after cell division

Selected amino acid producing strains

The amino acids **L-phenylalanine and L-cysteine**, both of which were previously produced mainly with the help of enzymes, can now be obtained more cost effectively by fermentation with ***E. coli*** strains and are thus available to a larger and growing market. Almost all proteinogenic amino acids, with a few exceptions, can be produced industrially by specially developed mutants of ***C. glutamicum*** or *E. coli*.

Amino acid	Strain/mutant	Titer (g/l)	Estimated yield (g/100 g sucrose)
L-Lysine HCl	*C. glutamicum* B-6	100	40–50
L-Threonine	*E. coli* KY 10935	100	40–50
L-Tryptophan	*C. glutamicum* KY9218/pIK9960	58	20–25
L-Tryptophan	*E. coli*	45	20–25
L-Phenylalanine	*E. coli* MWPWJ304/pMW16	51	20–25
L-Arginine	*Brevibacterium flavum* AJ12429	36	30–40
L-Histidine	*C. glutamicum* F81/pCH99	23	15–20
L-Isoleucine	*E. coli* H-8461	30	20–30
L-Serine	*Methylobacterium* sp. MN43	65	30–35
L-Valine	*C. glutamicum* VR 3	99	30–40

Enzymatic production of enantiomerically pure aminoacids

- For other amino acids, there is no comparable enzyme system for conversion of the D-form, and there is no fermentation process with adequate yield.
- For these amino acids, it is necessary to produce the enantiomerically pure form using enzymatic procedures.
- The racemates are generally produced by chemical synthesis.

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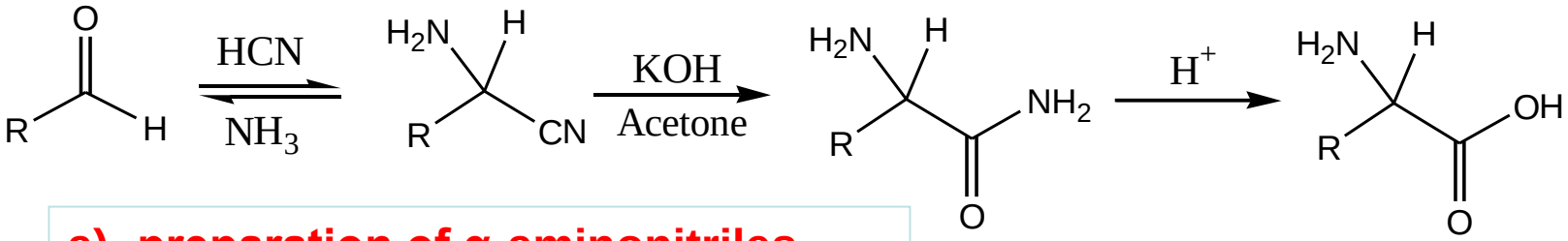
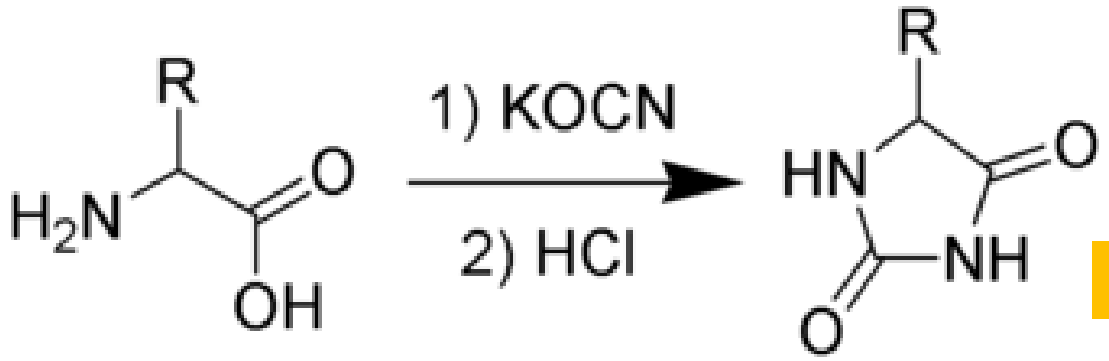
Synthesis of intermediates of D,L-amino acids:



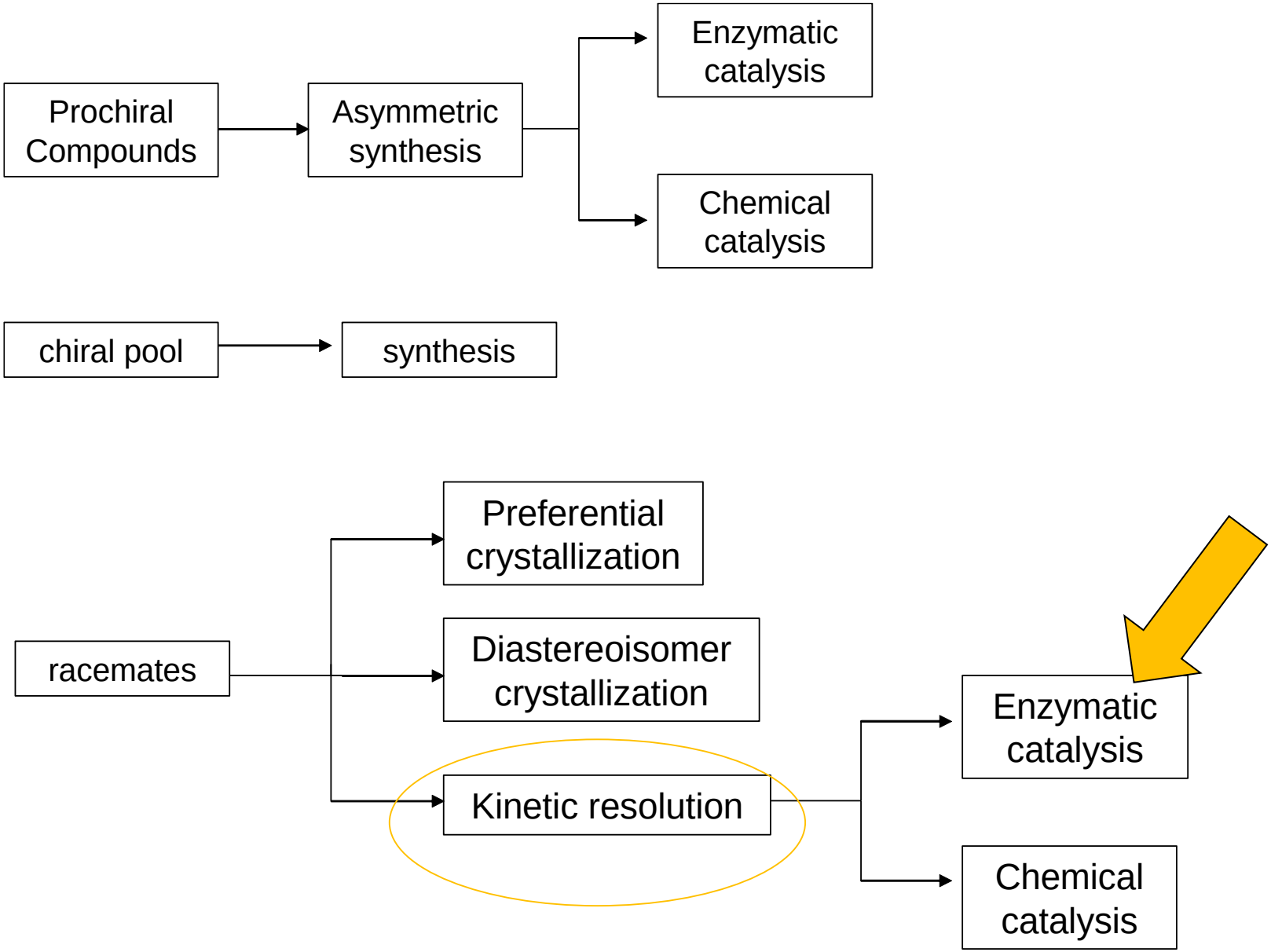
a) preparation of α-aminonitriles,
b) hydrolysis of the nitrile.

1. Strecker



2. Hydantoine

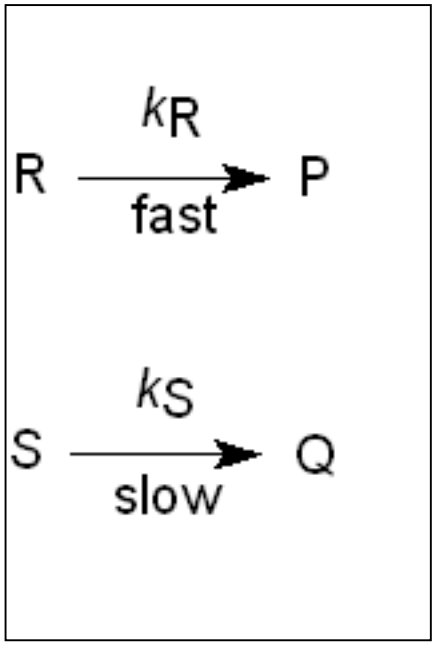
Synthetic methods for the production of pure enantiomers



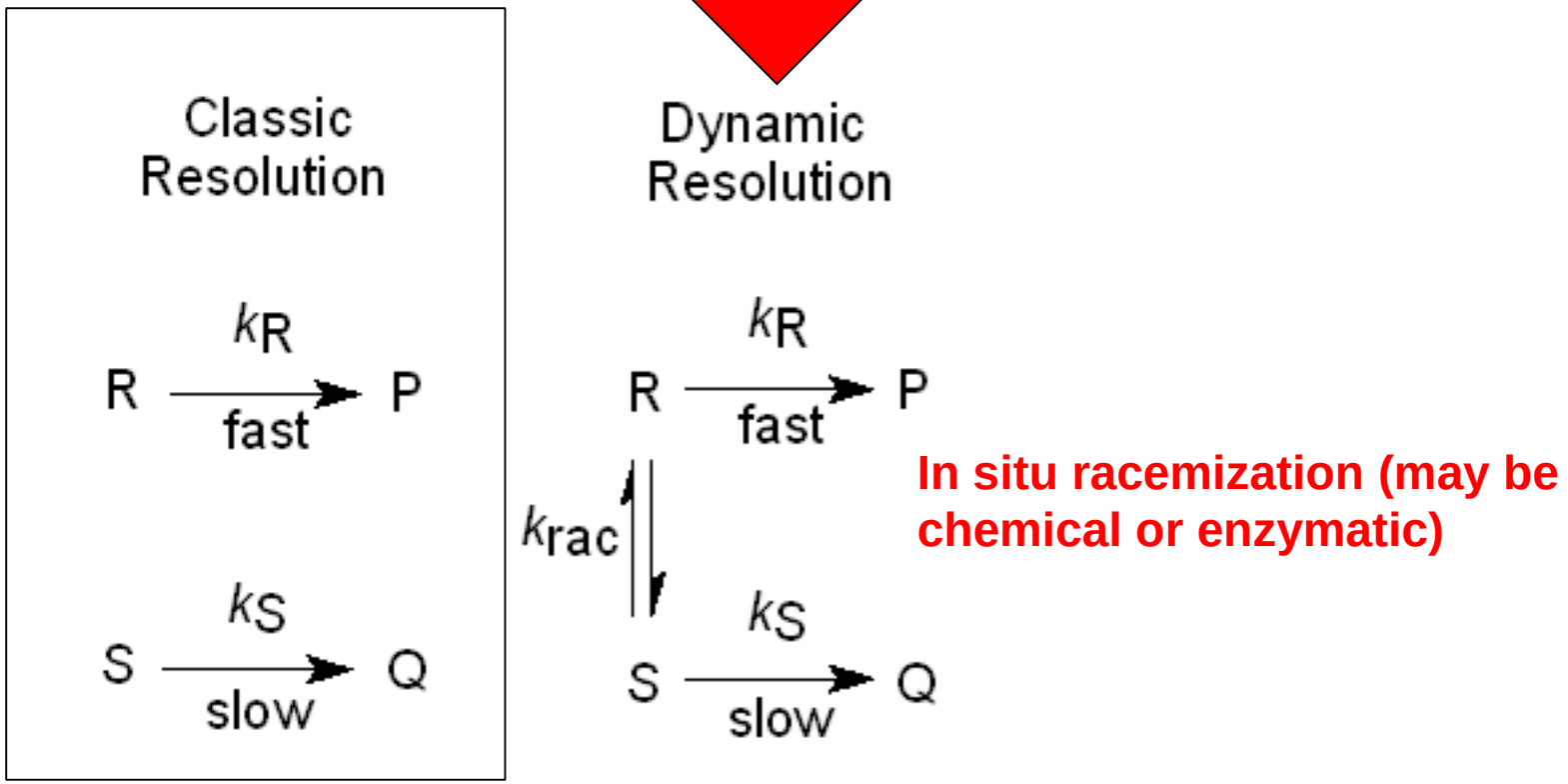
Kinetic resolution of racemates: typical application of hydrolases

When an enzymatic catalytic reaction is followed in time, *ideally* only one enantiomer reacts and the reaction stops at 50% conversion.

- **Resolutions have a maximum theoretical yield of 50%**
- **Unwanted enantiomer is wasted or at best recycled**



How to overcome the maximum 50% yield?
Dynamic Kinetic Resolution

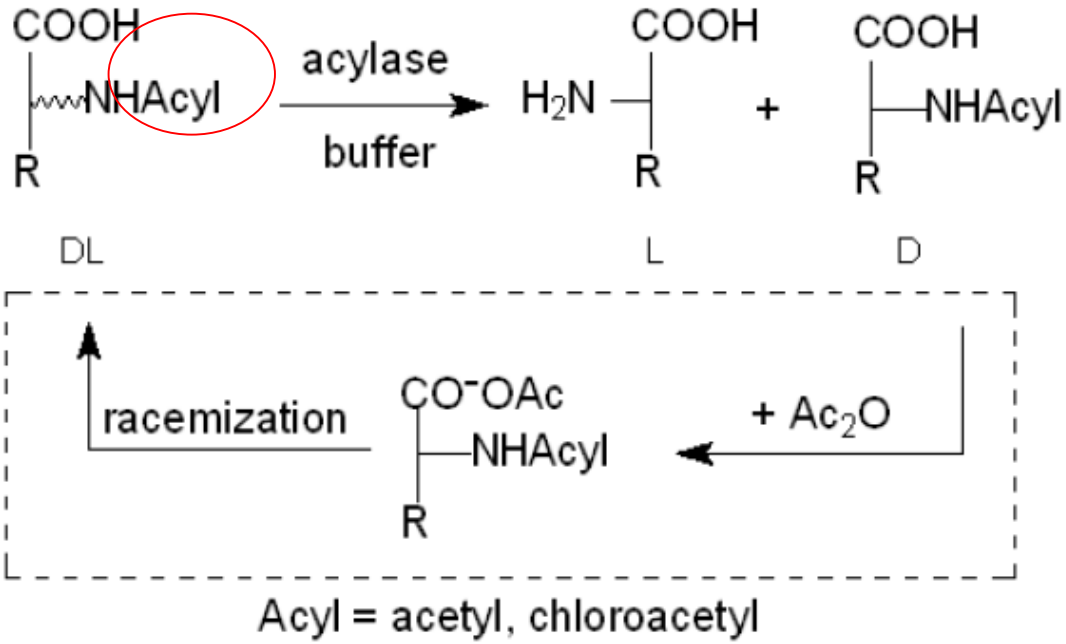


In situ racemization (may be chemical or enzymatic)

R, S = substrate enantiomers

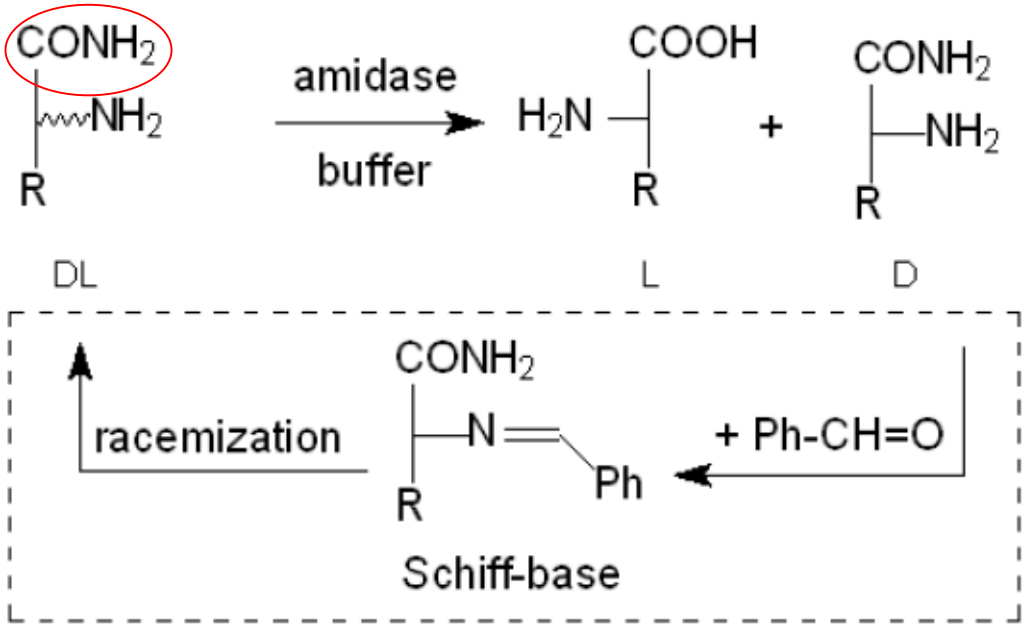
P, Q = product enantiomers

DKR: Hydrolysis of N-acylated amminoacids



- *rac* - *N* -acyl amino acids as substrates.
- use of acylases from porcine kidney or from *Aspergillus* or *Penicillium* sp.
- resolution of *N* -acetyl tryptophan and -phenylalanine on an industrial scale using immobilized enzymes in column reactors.
- the non-reacting D-enantiomer may be recycled *via* **racemization** of the corresponding **mixed anhydride** intermediate in a separate step.

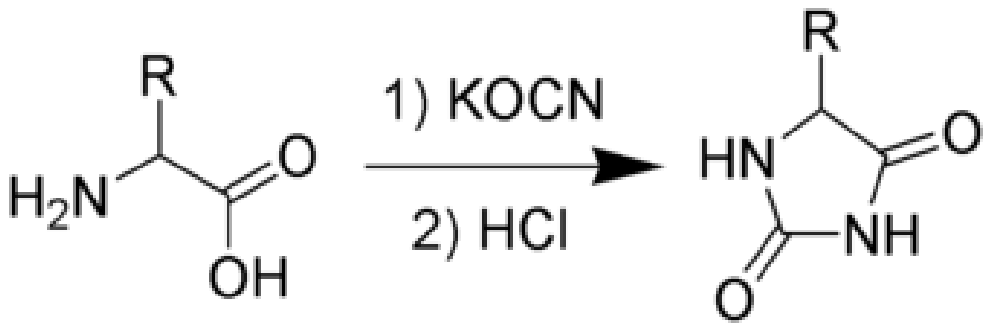
DKR: Hydrolysis of amides of aminoacids



- use of L-selective amidases from *Pseudomonas* , *Aspergillus* or *Rhodococcus* sp.,
- hydrolyze **L-amino acid amides** from a racemate.

The possibility to **recycle** the unreacted D-configured amide *via* its corresponding Schiff-base with benzaldehyde in a separate step makes this procedure economical.

Synthetic intermediates of D,L-amino acids: hydantoines

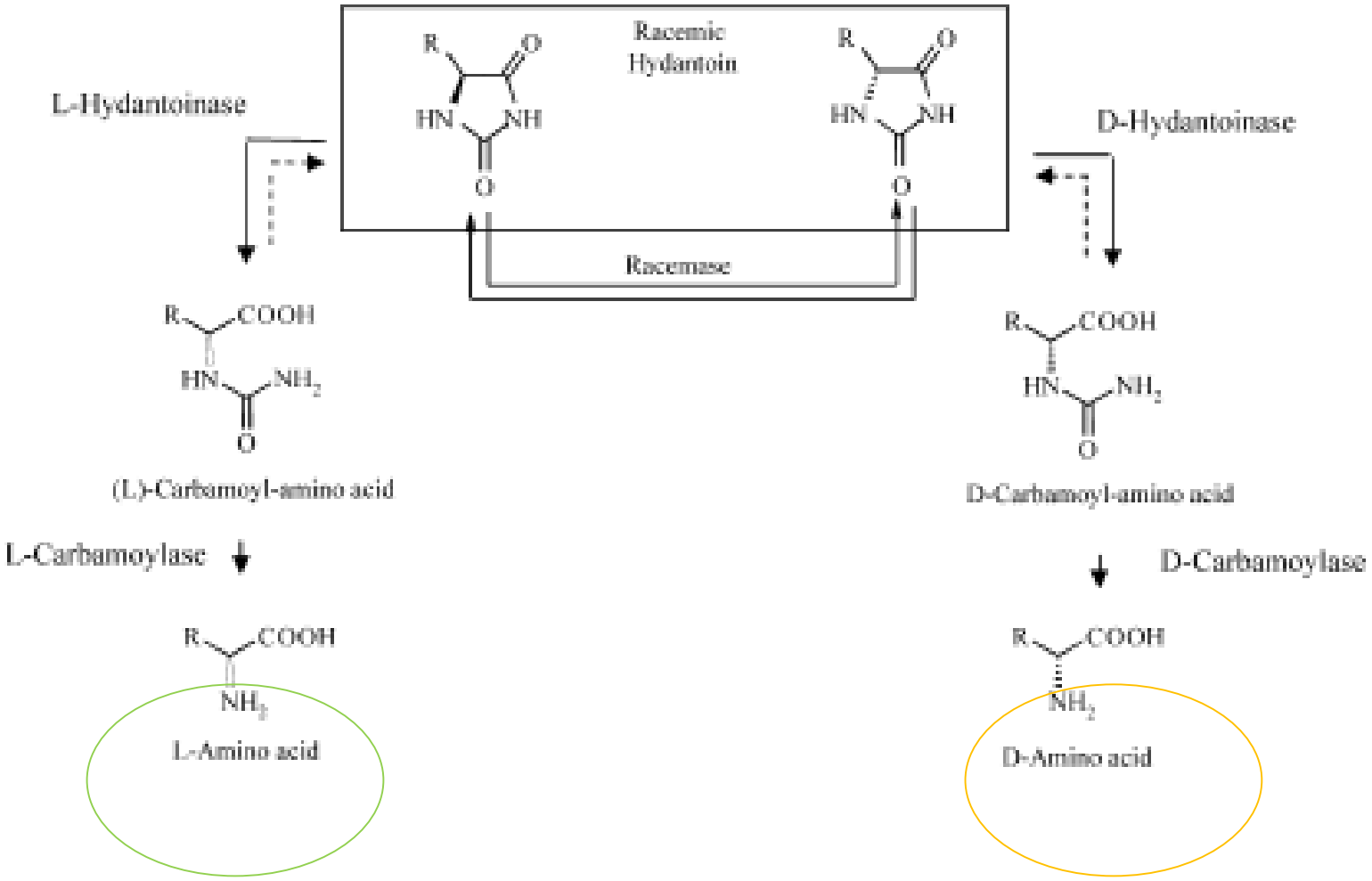


hydantoines

A promising route to enantiomerically pure amino acids, both L- and D-enantiomers, is based on conversion of hydantoins via hydantoinases and, additionally, carbamoylase.

5-substitued hydantoins

1. hydantoinase
2. carbamoylase,
3. hydantoin racemases



Dynamic Kinetic Resolution (DKR)

Racemization using enzymes

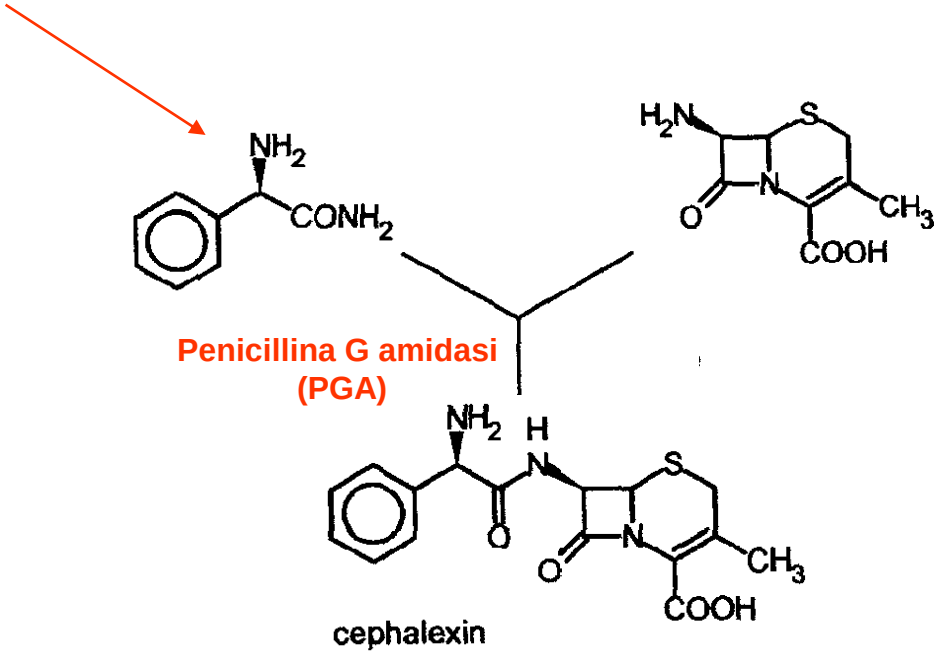
The use of an **enzyme,** rather than a transition metal catalyst, represents an attractive option for combined DKR reactions in view of the likely mild conditions associated with enzyme-catalyzed racemization processes.

Racemases belong to the group of enzymes EC 5.1.X.X and contain notable members such as mandelate racemase and various **amino acid racemases**.

Method of the 5-substitued hydantoins for side chain antibiotics production

D-Phenylglycin
D-p-hydroxyphenyglycine

Side chains of beta lactams antibiotics (ampicillin, cephalexin amoxycillin)



Recordati (IT)

Method of the 5-substitued hydantoins

D-serine
L-methionine

Degussa (D):
whole **cells coexpressing** **L-carbamoylase + hydantoin racemase + hydantoinase**

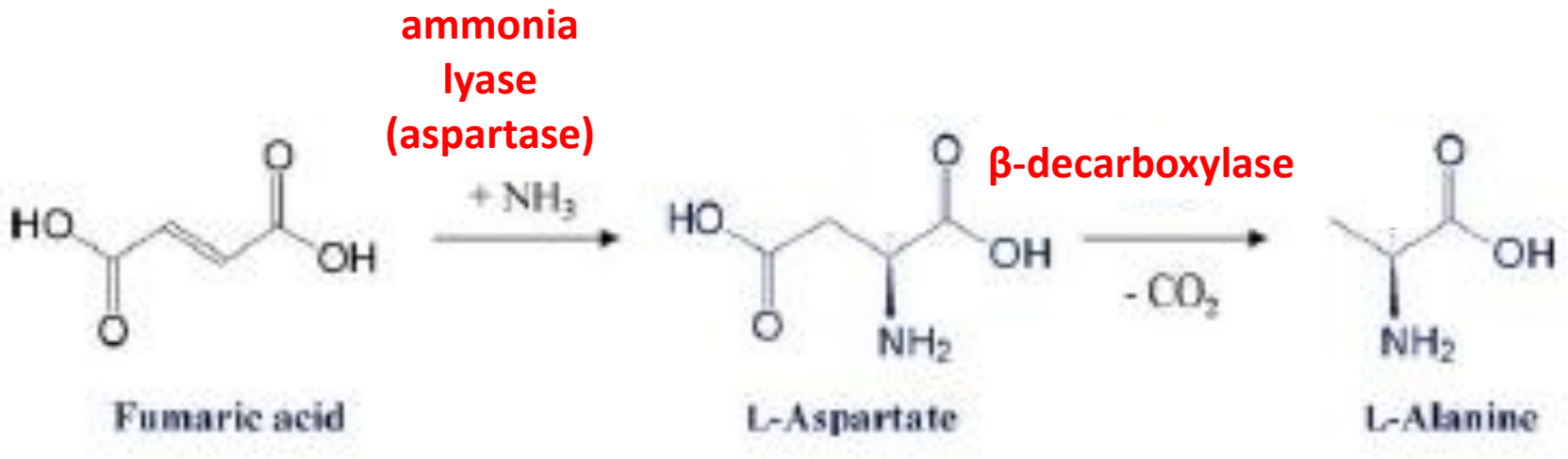
L-Aspartic acid: Asymmetric enzymatic synthesis

Achiral substrate + chiral (enantioselective) biocatalyst

1.addition of ammonia to fumaric acid catalyzed by **ammonia lyase** from *E.coli* , also called aspartase

2.L-aspartate (which is required in large quantities for the sweetener Aspartame).

3.aspartate **β-decarboxylase** from *Pseudomona dacunhae* trasforms aspartic acid into **L-alanine**



- **Lyases catalyze the addition or removal of a chemical group without passing through hydrolysis, oxidation, transfer**

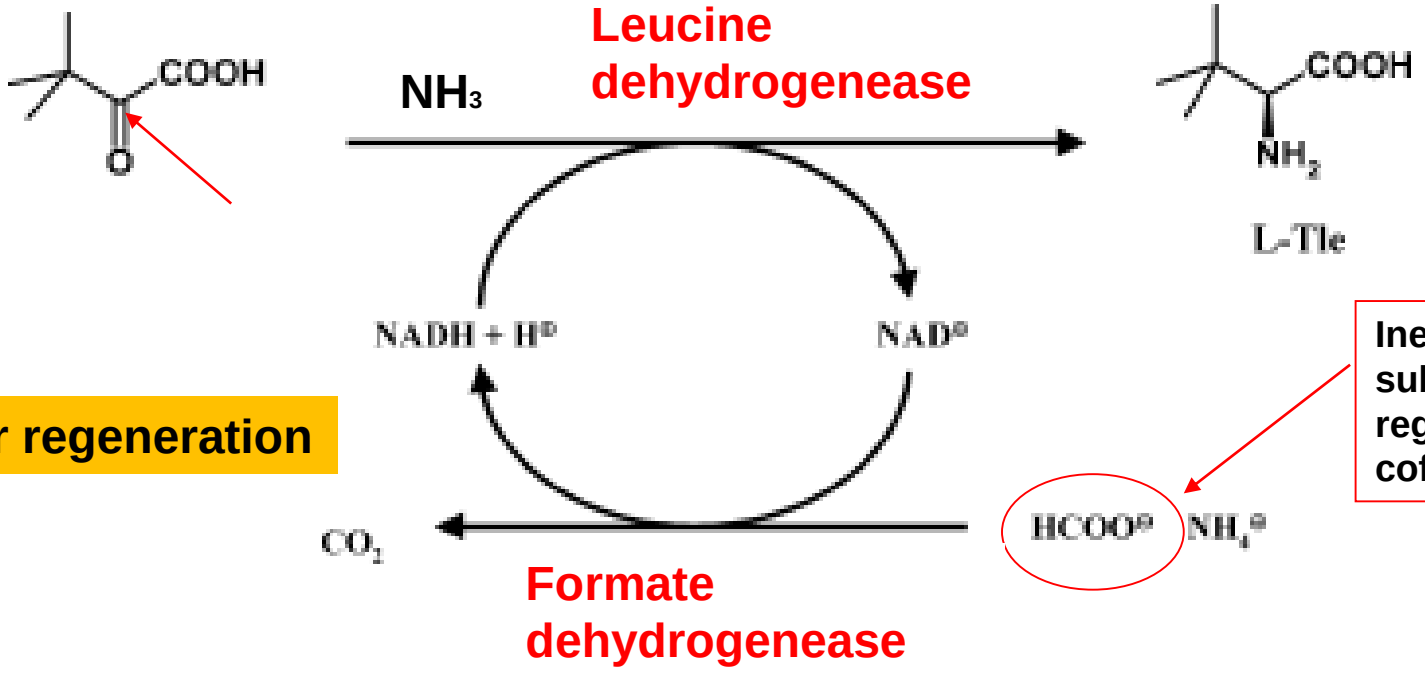
Lyases can act on

- **C - C bonds (decarboxylases; aldolases)**
- **C – O (hydratases or dehydratases)**
- **C – N**
- **C – S**
- **C – X**

Asymmetric enzymatic synthesis

Synthesis of L-*tert*-leucine via reductive amination of trimethyl pyruvate

***Prochiral compound*:**



Cofactor regeneration

Use of recombinant *E. coli* coexpressing **Leucine dehydrogenase** and NAD+-dependent **Formate dehydrogenase**.