BIOLOGY, BIOTECHNOLOGY

2ND PART

BIOCHEMICAL ENGINEERING

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2014

1. INTRODUCTION. BIOENGINEERING AND BIOTECHNOLOGY

There are many definitions regarding biotechnology accordig to what wants the definition creator emphasize. Some says, biotechnology is only the modern biotechnology, including genetic engineering while others show that biotechnology is just an „old wine in a new barrell" .Let us accept the definition of the IUPAC that is the same as of EFB (European Federation of Biotechnology) defined it in 1981:

> BIOTECHNOLOGY is integrated application of
> BIOCHEMISTRY,
> MICROBIOLOGY AND
> ENGINEERING SCIENCES
> principles in order to the technological use of
> microorganisms
> animal and plant tissues
> or parts of these (e.g.. enzymes)
> to produce something

USA Congress gave an other definition in 1984:

> Biotechnologies are commercial techniques, that use living organisms or substances from those organisms, to make or modify a product, including techniques used for the improvement of the characteristics of economically important plants and animals and for the development of microorganisms to act on environment (Congress of the USA, 1984)

The expression *biotechnology* itself was introduced and first used in 1919 by a Hungarian engineer Ereky Károly . He defined it as: **„Biotechnology is every work with which products are produced from raw materials by the aid of living organisms."** Moreover he said that like once the stone age and ironage, a bio age will come some time.

Naturally neither this old nor the above more up to date definitions are everlasting, for biotechnology has been continuously developing with higher and higher speed, its teritories are widening, so definitions are to be permanently modified and refined.

In 2005 OECD gave a so called statistical definition that interpret biotechnology in a very wide meaning: **„the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services".** It was extended by a list of the actual territories, processes, approaches, methodologies etc.of the biotechnology.Some of these are as follow:

DNS/RNS: Genomics, pharmacogenomics, genetic engineering, DNS/RNS sequencing/syntheses/ amplification, genexpression, antisens technology.

Proteins and other molecules: sequencing/synthesis and engineering of proteins and peptides (hormones), proteomics, protein izolation and purification, signaling, identification of cell receptors .

Cell and tissue engineering (including biomedical engineering)

Cell fusion, vaccine-/immunostimulant production

Biotechnological processes, techniques. Fermentation, bioreactors, bioleaching, bioremediation.

Gentherapy, therapeutical use of viral vectors.

Bioinformatics: genoms, data bases of protein sequences, -structures, modelling complex bioprocesses, systems biology.

Nano-biotechnology.

Interdisciplinarity of the biotechnology can be seen on Fig.1.1

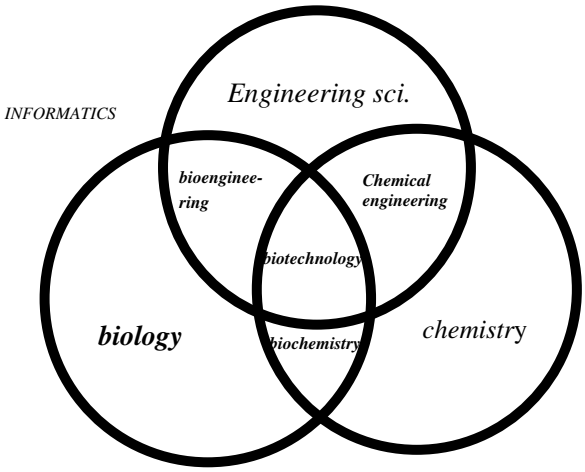

Fig 1.1.: Interdisciplinarity of biotechnology

1.1. Short hystory of the biotechnology

Here we give a very brief historical summary of the development of biotechnology, pointing on the most important discoveries and innovations contributing to its development.

B.C. 6000–3000 Egypt, Babilon, China: bread leavening. Alcoholic beverages (fruits, milk). Beer production. Cheese making. Vinegar.

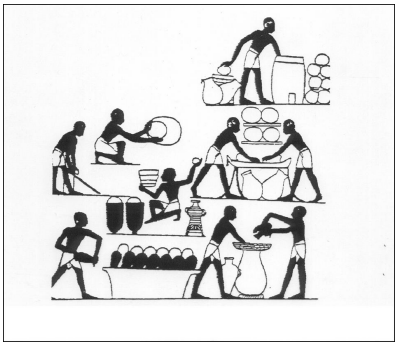

Fig.1.3.: Breadmaking Tomb painting in Egypt, Théba, BC. 1500

Fig 1.4.: Beermaking and beer sacrifice to godess Nin-Harra. Monument Blau, clay tablet, Sumerian Empire, BC 2500. (Louvre, Paris)

B.C. 2000 Vine making in Assíria.

500 First “antibiotic”: moldy soy curd is used for treatment of inflammation in China.

420	Sokrates (470?–399) raises first question of genetics:why do not resemble newborn boys to their father in every respect?
BC 1 about	Beer fermentation at celts and germans
AD 100 about	First insecticide: powdered chrisanthem (Chína).
3. century	Marcus Aurelius Probus: grape plantations in Germania
1150	Alcohol production from wine.
1300	Mexico: aztecs harvest algae from lake for food purposes.
1320	An arabic chief first apply artificial insemination in order to create a „superhorse”.
14. century	Vinager manufacture near to Orléans.
1590	Janssen: discovery of microscope
1630	William Harvey ascertains that either plant or animals reproduce by sexual way.
After 1650	Artificial mashroom breeding in French.
1663	Hooke: discovery of the existance of cells.
About 1680	Antoni van Leeuwenhoek(1632–1723): microscope, spermium, yeast, bacterium.

Fig 1.5.: Antoni van Leeuwenhoek and his microscope

1700	Camerarius, Rudolf Jakob (Camerer, 1665–1721) german botanist proves that plant flowers also have sexual organs.
1761	Kölreuter, Joseph Gottlieb (1733–1806) german botanist decribes the first crossbreed between plants belonging to different species.
1796	Edward Jenner (1749–1823) british physician developes the first vaccine against smallpox (vaccinus = from cow).
1838	Schleider–Schwann-cell theory: „Every cell arises from a cell.”
1857	Pasteur: yeast are responsible for fermentation, description of a lactic fermentation

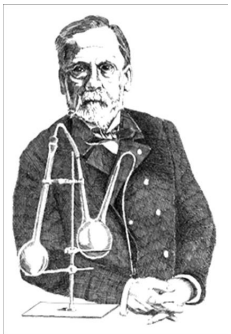

Fig.1.6.: Louis Pasteur (1822–1895)

(www.accessexcellence.org/RC/AB/BC/Louis_Pasteur.php)

1858	Traube supposes that fermentations are done by enzymes.

1859 Darwin publish his work: „On the origin of species”.

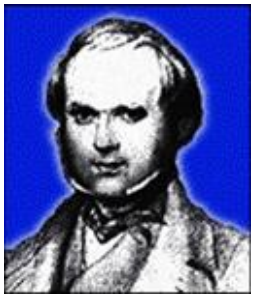

Fig 1.7.: Charles Darwin (1809–1882)

1863–64 Pasteur discovers the „pasteurization”.

1865 Mendel gives his views about his *laws of heredity*

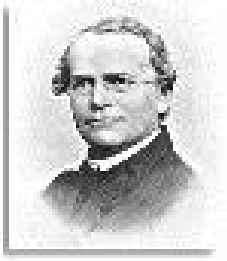

Fig 1.8.: Gregor Mendel (1822–1884)
(http://en.wikipedia.org/wiki/Gregor_Mendel)

1879 Hansen discovers Acetobacters.

1881 Industrial production of lactic acid

1882 Robert Koch identifies the tuberculosis bacterium.

~1885 Artificial mushroom production in the USA.

1893 Koch and Pasteur patented fermentation process.

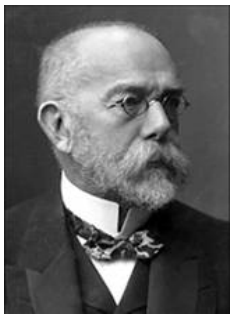

Fig 1.9.: Robert Koch (1843–1910)
(http://hu.wikipedia.org/wiki/Robert_Koch)

1897 Buchner established that in the yeasts there are fermenting enzymes.

Fig 1.10.: Eduard Buchner (1860–1917)
(http://nobelprize.org/nobel_prizes/chemistry/laureates/1907/buchner.html)

19. század vége The first municipal waste water treatment plants are built in Berlin, Hamburg, München, Paris etc.

1900 körül	Chromosome theory becomes generally accepted.
1902	The notion of IMMUNOLÓGY appears.
1906	Paul Ehrlich: Salvarsan, the first chemotherapeutic.
	Introduction of the notion GENETICS.
1908	Calmette and Guerin: BCG-vakccine against tbc. (introducing in 1921).
1910	Thomas H. Morgan proves that genes are localised on the chromosomes.

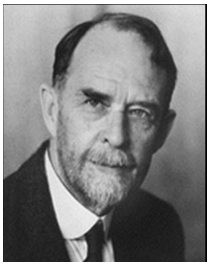

Fig 1.11.: Thomas H. Morgan (1866–1945)

(http://nobelprize.org/nobel_prizes/medicine/laureates/1933/index.html)

1915	Bakers yeast production with „german method”
1914–16.	Bakers yeast and fodder yeast large volume production by the leadership of Delbrück, Hayduck and Hanneberg.
1916	Weizmann’ process for aceton-butanol fermentation.

Fig 1.12. ábra: Chaim Weizmann (1874–1952)

(http://www.jewishvirtuallibrary.org/jsource/biography/weizmann.html)

1915	First finding of bacteriophage and bacteriovirus
1915–16	„Sulfite method” for glycerol fermentation
1919	BIOTECHNOLÓGY word fisrt time appearance in printed manner: Ereky Károly

Fig 1.13.: Ereky Károly (1878–1952)

1920-tól	Surface method of citric acid fermentation
1928–29	Fleming discovers penicillin.

Fig 1.14.: Alexander Fleming (1881–1955) accepts Nobel-prize in 1945

(www.bl.uk/onlinegallery/features/beautifulminds/flemingnobellge.html)

1937	Mamoli and Vercellone discover the possibility of microbial transformations.
1938	In France *B. thuringiensis* toxin production starts as insekticide.
1938	The expression „molecular biology" has launched.
1941	Beadle–Tatum: „one gene one enzyme" theory.
1941–44	Industrial production of penicillin started.
1944	Schatz and Waksman discover streptomycin.
	Sanger introduces chromatography for sequencing of insuline.
	Avery proves, that DNA carries the genetic informations.
1946	Tatum and Lederberg discover conjugation.

Fig 1.15.: Edward Lawrie Tatum (1909–1975)

(http://nobelprize.org/nobel_prizes/medicine/laureates/1958/tatum.html)

Fig 1.16.: Joshua Lederberg (1925–)

(http://nobelprize.org/nobel_prizes/medicine/laureates/1958/lederberg.html)

1948	Duggar discovers chlortetracyclin.
1949	Submerged acetic acid fermentation is launched. Vitamin B_{12} fermentation starts. Industrial scale biotransformations start.
1953	Watson, Crick and Wilkins discover double helix of DNA

Fig 1.17.:
Watson, Crick és Wilkins

(www.nobelprize.org/nobel_prizes/medicine/laureates/1962/)

1955	Discovery that animal cells can be grown in chemically defined culure media.
1956	Kornberg discovers DNA polymerase.
1957	Kinoshita and coworkers: glutamic acid fermentation.
1959	JACOB and MONOD discover gene level regulation.

Fig 1.18.: François Jacob (1920–)

(www.nndb.com/people/157/000129767/)

Fig 1.19.: Jacques Monod (1910–1976)

(http://nobelprize.org/nobel_prizes/medicine/laureates/1965/monod.html)

1955–60	Submerged citric acid fermentation
1960	Vegetative microbreeding of plants.
1961	Nierenberg: synthesis of poly-U, UUU codes the Phe.
1962	Watson, Crick and Wilkins get Nobel prize.
1965	Fusion of mice and human cells.
1966	Decoding of the genetic code.
1969	First *in vitro* enzyme synthesis
1970	First isolation of reverse transcriptase.
	Discovery of the restriction enzymes
1971	The whole plant can be regenerated from a protoplast
1972	First succesful DNA-clonong.
1973	Recombinant DNA-methods: „genetic engineering”
1975	Moratorium in Asilomar for the rDNA-experiments.

	First monoclonal AB (antibody) production.
1976	Launching of the first gentech company:GENENTECH.
1977	Genentech announces the bacterial production of the first human protein: somatostatin.
1978	Somatic hibridization of potatoes and tomatos (POMATO).
1980	The Chakrabarty-case: USA allows patenting genetically modified life forms: „superbug": HC-eating microbe. „Anything under the sun that is made by the hand of man is patentable" (USA Suprem Court, 1980).
1981	First stransgenic mammal: mice
1982	Human inzulin – first commercial rDNA-product.
1983	Kary Mullis (CETUS) developes PCR technique (1993: Nobel-prize).
1990	Human genom project starts (HUGO).
1992	Sheep cloning: animal cells are totipotent,too.
1996	The whole yeast gemon is known.
2000	The total human genom sequences are known.

1.2. Features of biotechnological processes

It is a general habit nowadays to give colour and group biotechnological processes accordig to their use into *red, white and green* groups. Red biotech means the health related, white biotech means the chemical industry related (raw and transitional materials as well as end products) while green biotechnology means the agriculture, environment protection and -menagement, bioremediation and biofuels related territories of the biotechnological processes and services.

This three level categorization, not beeing absolute and satisfactory, often a wider palettes of colours are applied for the various biotech fields as shows the list below[1]:

Red: health, medical diagnostics
Yellow: food and nutrition
Blue: aquaculture, sea-biotechnology
White: bioindustry
Gold: bioinformatics, nano-biotech
Green: agriculture, biofuel, biomanure, bioremediation, biological waste water treatment, geomicrobiology
Brown: biotech of dry deserted lands
Black: bioterrorism, bioweapons…
Purple: bio patents, publication, know how…
Grey: classical fermentation and bioprocess technology

[1] E. J. Da Silva (2005): The Colours of Biotechnology: Science, Development and Humankind Electronic Journal of Biotechnology.

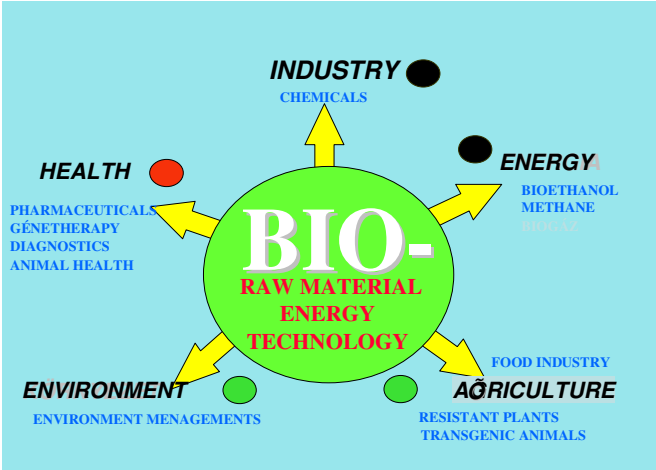

Fig.1.20.: A biosociety

Ereky Károly predicted the necessary coming of a so called biosociety similarly to the industrial revolution. According to the oppinion of many scientists the 21. century will be this age and this era has already started, for the contours of this biosociety has already visible. This means that all the segments of our everyday life are laced with biotechnologies. There are **bio-raw materials** (yearly renewed lignocelluloses and sugars) **bio-energy** (lignocellulose based power stations,bioethanol, biodiesel) and a part of technologies are also **bio** that these are processed by or applied with.

There are a great variety of classic and modern bioprocesses as shown in the tables below.

In the first table directly consumed foods and food indutrial products are listed that are manufactured by bioprocesses.

Table 1.1: Fermented foods

Product	Raw material	Microorganisms
alcoholic beverages beer, vine, spirits	grape, fruits malt, potato, cereals	*Saccharomyces cerevisiae*
non alcoholic food		
vinigar	vine, malt, ethanol	*Acetobacter aceti*
sour cabbage	cabbage	*Leuconostoc mesenteroides* *Lactobacillus plantarum* *Lactobacillus brevis*
olíves	olíves	*Pediococcus, Lactobacillus*
Sour dough	flour of wheat and rye	*Lactobacillus sanfranzisko* *L.fructivorans, L.fermentum* *Torulopsis holmii, S.cerevisiae…*
BAKERY PRODUCTS	…flour	*Saccharomyces cerevisiae*
Milk products		
Sour cream	milk	*Streptococcus lactis ssp cremoris* *Streptococcus lactis ssp diacetylactis*
yoghurt	milk	*Streptococcus salivarius ssp thermophilus* *Lactobacillus delbrueckii ssp bulgaricus*
kefir	milk	*Candida kefir, Lactobacillus kefir,* *Lactobacillus acidophilus,* *Streptococcus lactis*

Soft cheese	milk	*Penicillium caseicolum,* *P.camemberti* *Penicillium roquefortii*
Hard cheese	milk	*Streptococcus salivarius* *thermophilus* *Lactobacillus helveticus* *Propionibacterium freudenreichii*
Meat products		
sausages	meat	*Lactobacillus spp,* *Staphylococcus* *Micrococcus varians*
ham	meat	*Vibrio costicola, Staphylococcus*
Consumer goods		
coffee	Coffee bean	*Enterobacter,* Lactic bacteria, yeasts
tea, tobacco	tealeaves, tobacco leaves	(endogeneus enzymes) *Pediococcus sp*
cacao	Cacao bean	yeasts, lactic and acetic bacteria, bacilli
Soy sauce	rice flour, soya bean	*Aspergillus orizae* *Lactobacillus, Torulopsis sp.* *Zygosaccharomyces rouxii*

Next table shows products that are used as additives in the food industry during the technological steps of production.

Table 1.2: Biotechnological products in the food industry

Product	**Use**	**Producer microorganizm (source)**
Fruit acids		
Citric acid *E330-333*	beverages, jams, syrups Dairy products	*Aspergillus niger*
Itakonic acid	margarine	*Aspergillus terreus*
Gluconic acid *E574-579*	Baking powder, sausages Metal sequestering	*Aspergillus niger*
Fumaric acid *E360-369*	desserts, dairy products, Meat products	*Rhizopus, Mucor*
Malic acid *E350-352*	beverages, jams, geles, candies, oils, breads	*Aspergillus niger* *Penicillium brevicompactum*
Tartaric acid *E335-337*	beverages, jams, desserts, gels	*Penicillium notatum* *Aspergillus griseus*
Succinic acid *E360-369*	Flavour enhancement, K-, Ca-, Mg-salts as NaCl replacements	*Rhizopus, Mucor, Fusarium*
Lactic acid *E270*	juices, mayonnaise, desserts, baked products, dairy products, meat	*Lactobacillus delbrueckii,* *Lactobacillus casei*
Amino acids		
Glu *E620,621*	Flavour enhancement : „umami”	*Corynebacterium glutamicum,* *Brevibacterium flavum*
Lys	Food additives, feed additive	*Corynebacterium glutamicum*
Trp	Antidepressant,animal feed	*Corynebacterium glutamicum*
vitamins		
cobalamin (B_{12})	Dietary complement	*Propionibacterium shermanii*
riboflavin (B_2) *E101*	Dietary complement	*Ashbya gossipii* *Eremothecium ashbii*
β-karotin	Dietary complement	*Blakeslea trispora*

aszkorbinsav *E300*		biotranszformáció (pl. *Gluconobacter*)
Flavouring agents		
IMP *E630-633* GMP *E626-629*	Flavour enhancement, soup powders, Canned products	*Brevibacterium ammoniagenes, Corynebacterium glutamicum*
gélesítő anyagok		
alginate E400	icecream, pudding, foams	*Acetobacter vinelandii* see algae
xanthan E415	beverages, processed cheese, creamy cheese, pudding, dressings emulsion stabilization	*Xantomonas campestris*
pectin E440	jams, ice cream, cheese, mayonnaise	alma, citrusfélék
enzymes		
Glucose isomerase	Fruktose syrup, iso-sugar	*Arthrobacter sp, B. coagulans*
β-glukanase	juice filtering	*Trichoderma harzianum*
β-galaktosidase	Lactose removal	*A. oryzae, Kluyveromyces fragilis*
α-amylase	Starch break down	*B. licheniformis, A. niger*
glükoamylase	Starch break down	*A. niger, Rhizopus oryzae*
pectinase	Fruit and grape juice filtration	*A. niger, A. oryzae, Penicillium simplicissimum*
catalase	H_2O_2 excess removal from e.g. milk	*Micrococcus lysodeicticus*
Glucoseoxydase	O_2 removel from canned food	*A. niger*
rennet	Milk clotting, cheese prod.	*borjúgyomor, Bacillus spp Streptococcus lactis*
proteases	dough, beverages, cheese, meat, soy	*Bacillus cereus, B. subtilis B. licheniformis, A. orizae*
lipases	Chese flavouring, fat removal from protein products, transesterification of fats and oils	*Candida lipolytica, Aspergillus niger, Mucor javanicus*
antocyanase	Vine decolorization	From plants
lyzozim	Cheese production	From egg

Table 1.3: Microbial enzymes produced in large quantities

α-amylase	*Bacillus amyloliquefaciens,* *Thermobacterium sp.*
β-amylase	*B. polymyxa*
amyloglucosidase	*Aspergillus niger*
cellulases	*Trichoderma reesei*
Glucose isomerase	*Streptomyces oligochromogenes* *B. coagulans*
glucoseoxydase	*A. niger*
α-D-glucosidase	*A. niger*
lipases	*A. niger* *Candida cylindraceae* *Geotrychum candidum* *Rhizopus arrhizus, Mucor sp.*
pectinestherase	*A. orizae*
acidic proteinase	*A. saitoi*
alcalic proteinase	*A. orizae B. amyloliquefaciens*
neutral proteinase	*Bacillus stearothermophylus*
pullulanase	*Aerobacter aerogenes*
polygalacturonase	*A. niger*
penicillin acylase	*E. coli*

It is worthy to note that proteolytic enzymes are produced in the largest quantity (about 58% of the total enzyme production), including alcaline proteases (25%) and other proteses (20%), milk clotting rennet 10%.
The amylases and glucose isomerases take about 25% while therapeutic and diagnostic (analytical) enzymes take about 10 % of the total enzyme market.

Table 1.4.: Microbial nonprotein polymers and producing microbes

alginate	*Azotobacter vinelandii*
cellulose	*Acetobacter sp.*
curdlan	*Agrobacterium sp.*
dextran	*Leuconostoc mesenteroides*
phosphomannan	*Hansenula capsulata*
poli-β-hydroxibutirate	*Alcaligenes eutrophus*
scleroglucan	*Sclerotium glucanicum*
xanthan	*Xantomonas campestris*

Table 1.5.: Amino acids and the producing microorganisms

D, L-alanin	*Brevibacterium flavum*
L-arginine	*Brevibacterium flavum*
L-citrulline	*Bacillus subtilis*
L-glutamate	*Brevibacterium flavum* *Corynebacterium glutamicum*
L-histidine	*Corynebacterium glutamicum*
L-isoleucine	*Brevibacterium flavum*
L-leucine	*Brevibacterium lactofermentum* *Corynebacterium glutamicum*
L-methionine	*Brevibacterium flavum*
L-ornithine	*Microbacterium ammoniaphilum*
L-phenylalanine	*Brevibacterium lactofermentum*
L-proline	*Corynebacterium glutamicum*
L-threonine	*Corynebacterium glutamicum*
L-tryptophan	*Brevibacterium flavum*
L-tyrosine	*Corynebacterium glutamicum*
L-valine	*Brevibacterium lactofermentum*
L-serine	*Corynebacterium hydrocarboclastus*

Table 1.6.: Organic acids

Acetic acid	*Acetobacter aceti*
D-arabino-ascorbic acid	*Penicillium notatum*
Citric acid	*Aspergillus niger*
Erythorbic acid	*Penicillium cyaneofulvum*
Fumaric acid	*Rhizopus delemar*
Gluconic acid	*Aspergillus niger*
Itaconic acid	*Aspergillus terreus*
2-keto-gluconic acid	*Serratia marcescens*
α-keto-glutaric acid	*Candida hydrocarbofumarica*
2-keto-L-gulonic acid	*Gluconobacter melanogenus*
Lactic acid	*Lactobacillus lactis...*

L-malic acid	*Brevibacterium ammoniagenes*

Fig 1.7. : Fragrances produced by microbes

Anisol aldehyde	anise	*Trametes sauvolens*
benzaldehyde	mandel	*Trametes sauvolens*
benzyl-alcohol	fruit	*Phellinus igniarius*
citronellol	rose	*Ceratocystis varispora*
γ-deca-lacton	apricot	*Sporobolomyces odorus*
diacetyl	butter	*Streptococcus diacetylactus*
p-methyl-benzyl-alcohol	hyacinth, gardenia	*Mycoacia uda*
Me-p-methoxy-phenylacetate	anise	*Trametes ordorata*
Me-phenylacetate	honey	*Trametes ordorata*
6-pentyl-α-piron	coconut	*Trichoderma viride*
tetramethyl-pyrazine	nut	*Corynebateriumc glutamicum*

Table 1.8.Miscellaneous microbial products

antipain	protease-inhibitor	*Streptomyces sp.*
carotenoids	pigments, provitamins	*Dunaliella bardawil(alga)*
emulsane	Emulgent agent	*Acinetobacter calcoaceticus*
gibberellins	Plant hormones	*Giberella fujikuroi*
herbicidin	herbicide	*Streptomyces saganonensis*
indigo	pigment	*Escherichia coli*
inosin	Flavor enhancement	*Bacillus subtilis*
Lysergic acid	Ergot alkaloid products	*Clariceps paspali*
B_{12}	vitamin	*Propionibacterium shermanii*
shikonin	Medicine, colorant	*Lithospermium sp.*

From the known more thousand antibiotics Fig 1.9 shows the best known items.

Fig 1.9.: Antibiotics

Antibiotic	Type	Producing strain
Penicillin G	lactam	*Penicillium chrysogenum*
Streptomycin	aminoglycoside	*Streptomyces griseus*
Bacitracin	polypeptide	*Bacillus licheniformis*
Cefalosporin C	polypeptide	*Cephalosporium acremonium*
Chlortetracycline	tetracyclines	*Streptomyces aureofaciens*
Griseofulvin	spirocyclohexene	*Penicillium griseofulvum*
Gentamicin	aminoglycoside	*Micromonospora purpurea*
Nystatin	tetraene	*Streptomyces aureus*
Oleandomycin	macrolide	*Streptomyces antibioticus*
Tyrocidine	cyclic polypeptide	*Bacillus brevis*
Vancomycin	glycopeptide	*Streptomyces orientalis*

In Table 1.10. recombinant DNA products are shown. Recently more thousands of such products exist, the examples here are only some representative and historically interesting kinds.

1.10. table: rDNS products

PRODUCTS	APPLICATION
Human insulin	diabetes
Human interferons (α-, β-, χ-IFN)	antiviral/antitumor therapy
HGH	human growth hormone
Hepatitis B virus protein	vaccine against viruses
Urokinase	thrombolytic effect
L-phenylalanine	component of aspartame sweetening
Animal growth hormones	increase milk and meat production
Factor VIII and IX of blood clotting	hemophilia
Erythropoietin (EPO)	anemia
Human serum albumin	blood products
Antigens of herpes, malaria and influenza proteins	vaccines
Immunoglobulins	monoclonal antibodies
Lymphokines: interleukin-2	stimulation of immune system
Tissue Plasminogen Activator (TPA)	thrombolytic effect
Tumor Necrosis Factor (TNF)	antitumor therapy
Rennet	cheese production

Aims and potentials of the modern fermentation processes are the following, grouping on the basis of the kinds of products:

CELL MASS PRODUCTION – Baker's yeast, SCP

PRODUCTION OF CELL COMPONENTS - intracellular enzymes, nucleic acids, polysaccharides, rDNA products…

METABOLITE PRODUCTION - PRIMARY metabolites: etanol, lactic acid...
- SECONDARY metabolites: antibiotics

SIMPLE SUBSTRATE CONVERSION: glucose → fructose
penicillin → 6-NH_2-penicillanic acid

MULTI-SUBSTRATE CONVERSION: biological wastewater treatment

At all types of products raw materials (substrates) are converted by the organism or some part of it during a one- or multistep reaction (series) to the end product. In this sense cells or their active parts can be considered as catalysators that in the case of the first and second types, beside the catalytic action may be multiplied themselves. The process is called de ***novo*** **fermentation** when the organism, growing on a medium, from simple substrates (components of the culture medium) produces the more or less complex product material or **biotransformation** or **bioconversion** when growing or nongrowing organism or a certain part of them (e.g. an enzyme) produce a matter from a given other material (Fig 1.22)

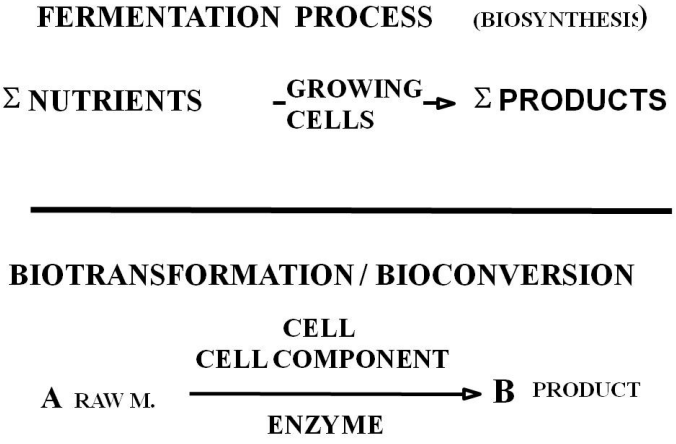

Fig 1.22.: De novo fermentation and bioconversion

Either in case of *de novo* fermentation process or biotransformation the fermentation itself has an important determining central role because directly the product or indirectly the converting enzyme are produced by a fermentation process. (Fig 1.23.).

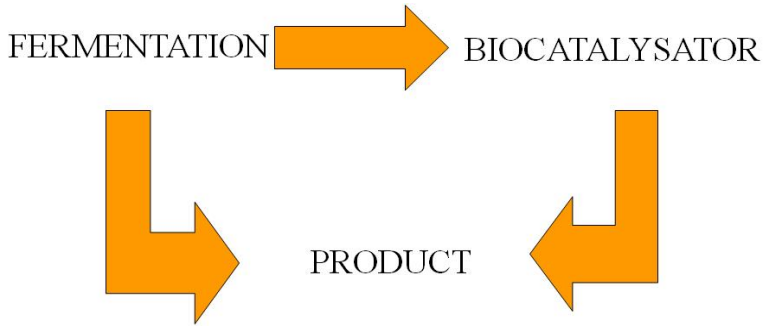

Fig 1.23.: Central role of fermentation

Below we summarize the cases where fermentation presents a good alternative of a synthetic process or where there is no other choice, and it has to be applied exclusively.

WHERE SHOULD WE USE BIOPROCESSES?

- WHEN COMPLEX MOLECULES ARE TO BE SYNTHETIZED, WHEN THERE ARE NO ALTERNATIVES: ANTIBIOTICS, MONOCLONAL ANTIBODIES, PROTEINS..
- At exclusive production of one of isomers, e.g. R os S enantiomer production.
- When the culture is able to realize more than one (a series of) consecutive reactions.
- When cells can produce something with higher yield than a synthetic method, or at least the yield of the bioprocess is comparable with synthetic process.

Bioprocesses often have expressive advantages over the conventional chemical processes. But of course where advantage is surely there are disadvantages, too. Here follows a list of these.

ADVANTAGES OF BIOPROCESSES OVER CONVENTIONAL CHEMICAL METHODS

- Reaction conditions are usually milder (pH, pressure, temperature).
- Bioprocesses use yearly renewed raw materials either form the respect of C-sceleton or the energy source.: SUGAR ← STARCH, SUGAR ← LIGNOCELLULOSE.
- These and the other raw materials (minerals) are cheap and easily attainable in the nature.
- Less dangerous reaction circumstances for the environment and the environmental burden is smaller.
- Biocatalysts (cell, enzyme…) are specific: substrate-, reaction-, stereo- and region specificity are theirs.
- Bioreactors and the other equipment are usually of many purpose.(product and technology change is easy)
- Frequently higher yield and smaller energy requirement.
- Potentials of the rDNA technology are many, almost unforeseeable.
  (foreign proteins, biocatalyst design, metabolic engineering, artificial evolution….),.

DISADVANTAGES OF BIOPROCESS

- Nowadays the productivity and economic feasibility of chemical processes based upon fossil raw materials are yet often higher than of the bioprocesses (the main barrier of the spreading the white biotechnology).
- Complicated product structures are present in diluted solutions, their isolation and purification is complicated and expensive.
- Huge amount and of large BOD containing waste water is formed, but it is usually easily treatable.
- Easy contamination by foreign microorganisms, viruses.
- Contamination hazard. Very strict rules have to be kept because of the biosafety regulations. Special containment rules in the case of GMO-s and pathogens.
- Two side variability:1. renewable materials may change time to time and place to place.2. Special microbes with modified genetics (e.g.: mutants) are inclined to revert (they lose their productivity)
- Social perceptibility is not too high yet. There is a general refusal against microbes and mainly against GMO-s.

2. BASICS OF ENZYME ENGINEERING

2.1. Brief history. Basics of enzymes as biocatalysts

If we want to introduce the evolution of our recent knowledge regarding enzymes in a few sentences, the task is rather difficult because there are so many milestones of its. Nevertheless, some main points have to be mentioned here e.g. 1833 in that year Payen and Persoz, French scientists published a paper in which they submitted the role of breeding barley in the hydrolysis of starch: they observed the appearance of destrins and sugars[2] during the process.

In 1835 Berzelius (1779–1848) stated the fact that the hydrolysis of starch by „diastase" is *catalysis*.

During 1853–1857 two opinions about the essence of enzymes were fighting with each other. As for one the conversions are driven „some N-containing organic matter" that was taken as an unorganized nonliving material, while according the other opinion, a living material is necessary for these transformations. These were taken as lower order plants, some kind of „infusorium".

In 1858 M. Traube (1826–1894) supposed that fermentation is driven by fermentums, he joined the first group, but for example Pasteur belonged to the other group and his enormous scientific prestige prevented the real enzyme-picture (i.e. inorganized N-containing material) spreading for a long time. But this was not a barrier for the foundation of the first enzyme producing firm in 1874 (in Hollande the C. Hansen's Laboratory) for the production of the milk clotting enzyme, rennet.

Following the winner first opinion, Wilhelm Friedrich Kühne (1837–1900) *named these matters enzymes* applying the Greek word ενζημη (enzümé) = in yeast for them. **In** 1897 Edward Buchner (1860–1917) pointed out that in the yeasts there are fermenting enzymes. He got one of the first Nobel Prizes in 1907 for his work regarding the cell free fermentations.

In 1926-ban James Batcheller Sumner (1887–1955) first isolated pure enzyme, the crystallized urease. For his work in the field of enzyme crystallization he got Nobel Prize in 1946 with (J. H. Northroppal and W. M. Stanley).

Enzymes are specific groups of proteins with the task to fasten the many biochemical reactions going on in the living cells. According to the general view all the enzymes are proteins but not all the proteins are enzymes. This later statement is obvious because one knows a series of proteins that does not hold catalytic activity, while there are also proteins holding some catalytic activities, but we do not take them as enzymes. In the list below we see examples of these protein groups.

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[2] Memoir sur la diastase, les principaux produits de ses reactions, et leurs applications aux art industrielles, *Annales de Chimie et dePhysique,1833*, 2me Serie 53, 73–92

Specific groups of proteins and their biological functions

Regulator proteins*	
lac-repressor	RNA-synthesis
interferons	virus-resistance
insulin	glucose-metabolism
growth hormone	
Transport proteins*	
lactose permease	cell membrane transport
myoglobin	O_2 – in muscle
hemoglobin	O_2 – in blood
Protecting proteins*	
antibodies (abzymes)	foreign material - complex
thrombin	blood clotting
Toxins*	
B. thuringiensis	biological insecticide
Cl. botulinum	causing food poisoning
Reserve nutrient-proteins	
ovalbumin	egg white
casein	milk protein
zein	corn germ
Contractile proteins	
dynein	cilia, flagella
myosin	muscle
Structural proteins	
collagen	joints and tendons
glycoproteins	cell wall

Chaperons*

Prions

The groups signed by * also have catalytic activity but do not correspond to the classical definition of enzymes, i.e. they do not catalyze **reactions**. In reality the mode of operation is exactly the same as of the enzymes. This is well known for example in the case of hemoglobin, even if it is not taken as an enzyme, it is the most important prototype of the allosterism: operating of allo enzymes is explained with the mechanism of hemoglobin. Similarly, permeases do not catalyze reactions, but their kinetics show strict similarity with the real enzymes (saturation with substrates). Therefore nowadays a need for redefinition of enzymes arose: the essence of catalysis remains but they fasten not a reaction but *some kind of transformation*.

It is worthy to mention that there are catalytic materials, playing important role in biochemical events, that are not proteins. Ribozymes, ATP NAD, tRNA have catalytic effects. Many RNA-catalysators, that play important role recently, too, fortify the opinion regarding the evolution of life, that first the catalysis were driven by nucleic acids and later the cells turned to the more effective protein catalysis, and the „RNA-world" turned to be the recent „protein-world". Nevertheless there remained some traces of the RNA-world in the examples mentioned above as well as in the form of even mixed catalyst like the example of RnaseP rybozyme in which 377 base pairs of RNA with

~125 kD molecular mass and only a small protein fraction of 119 amino acids (~14 kD) form the whole catalytic entity.

Without enzymes the *thermodynamically possible reactions* (negative Gibbs-energy change) would develop very slow, especially because the reaction circumstances in the living cells are rather mild, at about 30-40 °C and 1 bar pressure and near neutral pH, and these circumstances allow only very small reaction rates of the spontaneous reactions.

In the various cells different proportion of the cell dry material is protein but as a minimum (in case of filamentous fungi) 25% of the dry weight is protein mostly enzyme protein. In an *Escherichia coli* cell as much as 2-3000 different proteins of catalytic effect are present. All of these serve as catalysator of well-defined tasks, mainly fastening of well-defined chemical reactions.

The thermodynamic basis of the catalysis in case of enzymes is the same as in case of other catalysators: they fasten the reaction rate because they lower the activation energy of the reaction.

Let us recall this thermodynamic basis with the following sequence of ideas.

According to the absolute reaction rate theory of **Henry Eyring** (1901–1981) in an A+B reactant system, in order to form a product P – even if the reaction is spontaneous, with negative Gibbs-energy - a certain amount of activation energy has to be introduced to form a higher energy transition state of the system from which the reaction may go further into the direction of the lowest energy final state. (Fig *2.1.*)

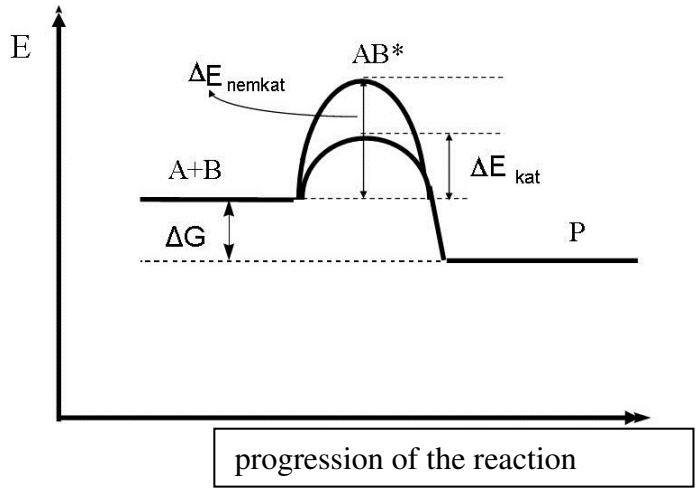


Fig 2.1.: The enzyme lowers the activation energy

For the reaction scheme below, it can be written

$$A + B \Leftrightarrow AB^* \rightarrow P,$$

$$\Delta H^* = \Delta G^* + T.\Delta S^*.$$

The equilibrium constant of the reversible formation of the activated complex is

$$K^* = \frac{C^*_{AB}}{C_A \cdot C_B}.$$

And the product formation rate can be written as follows:

$$\frac{dP}{dt} = k_r C_A C_B = C^*_{AB} \frac{kT}{h},$$

where

T is the absolute temperature (Kelvin degree)
k is the Boltzmann-constant ($1{,}38 \cdot 10^{-23}$ J/K)

h is the Planck-constant ($6{,}62 \cdot 10^{-34}$ J·s)

From the former equation the equilibrium constant can be expressed:

$$K^* = \frac{C^*_{AB}}{C_A C_B} = \frac{k_r h}{kT}$$

Using this, the activation energy change of the reaction will be the following:

$$\Delta G^* = -RT\ln K^* = -RT\ln\frac{k_r h}{kT} = \Delta H^* - T\Delta S^*,$$

From which k_r reaction rate constant is:

$$k_r = \frac{kT}{h} e^{\frac{\Delta S^*}{R}} \cdot e^{-\frac{\Delta H^*}{RT}} \approx konst \cdot e^{-\frac{\Delta E^*}{RT}}.$$

It is obvious that because $\left(\Delta E^*\right)_{nemkat} \rangle \left(\Delta E^*\right)_{kat}$, $\left(k_r\right)_{nemkat} \langle\langle \left(k_r\right)_{kat}$ will be, i.e. the reaction rate of the catalyzed reaction is much higher than of the noncatalyzed.

In the case of enzymatic catalysis, the situation is the same, the thermodynamic basis of the reaction fastening is the lowering of the activation energy. This means the very much increase of the reaction rate constants, sometimes many order of magnitude is this increase as we can see in the Table 2. 1.

An enzyme catalized reaction may million/billion times faster than a non-, or inorganic catalyzed reaction.

Table 2.1.: Comparison of single and enzyme catalyzed reactions

Reaction	**Catalysator**	**Activation energy** kJ/mol	**$k_{relative}$** 25°C
$H_2O_2 \rightarrow H_2O + 1/2O_2$	- I^- catalase	75 56,5 26,8	1 $2{,}1 \times 10^3$ $3{,}5 \times 10^8$
Casein + nH_2O $\rightarrow$(n+1) peptide	H^+ trypsin	86 50	1 $2{,}1 \times 10^6$
Saccharose + $H_2O \rightarrow$ glucose + fructose	H^+ invertase	107 46	1 $5{,}6 \times 10^{10}$
Linoleic acid + $O_2 \rightarrow$ linoleic peroxide	- Cu^{2+} lipoxygenase	150–270 30–50 16,7	1 $\sim 10^2$ $\sim 10^7$

It is generally accepted and assured by several experimental evidences that there exist an activation complex, in our case an enzyme-substrate complex (or complexes).

The former thermodynamic picture will be a bit more understandable if we look at Fig.2.2. that shows the case of a naturally nonexistent enzyme, „stickase" which breaks an iron stick. Well visibly there is great thermodynamic difference between the various modes of formation of the enzyme-substrate activated complexes.

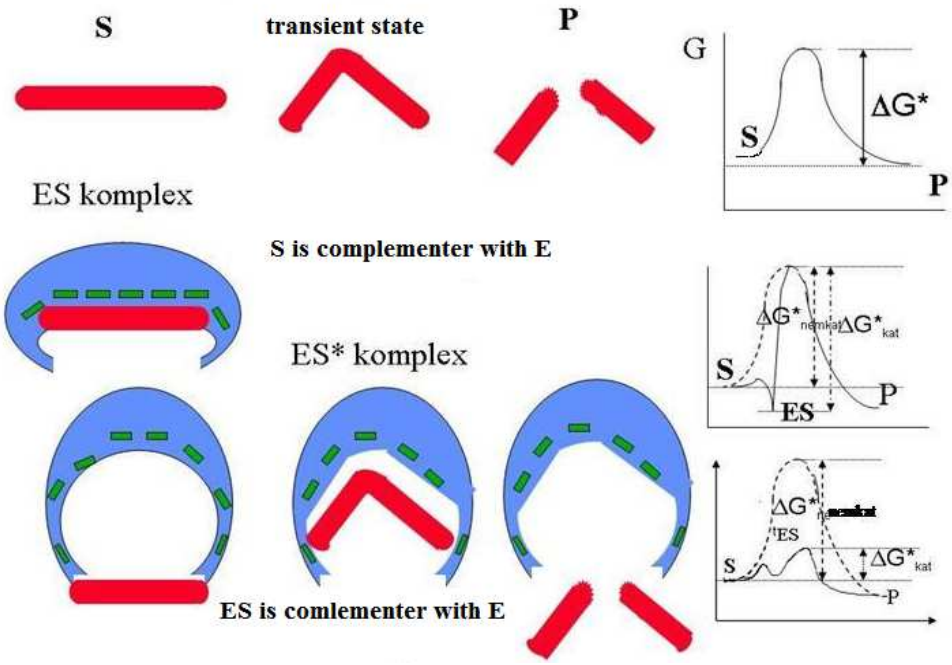
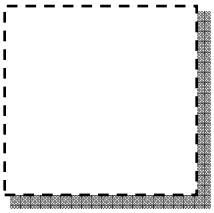


Fig2.2.: It is necessary to introduce activation energy for the formation activated complex Energetically more favored if either the substrate or the enzyme are getting changed during the formation of the activated complex: this is the induced fit.

At its so-called *binding site* the enzyme binds the substrate molecule and then at the *active site* the chemical change happens. Active site and binding site are not necessarily the same, maybe they are identical, maybe not, and in latter case they may be far or next to each other. On the other hand, on the surface of an enzyme molecule not only substrate binding sites but other binding sites may take place for the complex formation of foreign molecules, as activators, inhibitors or prosthetic groups. The site which is responsible for binding the substrate is called *binding site* and the site responsible for the chemical change is called *active site* (formerly it was called active center). In the formation of the enzyme-substrate complexes weak and strong chemical bonds play role, from the Van der Waals forces through the ionic to the covalent bonds. Frequently hydrogen bonds and/or partial electric charges form the binding of the substrate.

The substrate binding and active sites occupy just a relatively small surface of the total protein molecule (Fig 2.3.). Explaining mechanically, enzyme binds the substrate, transform it to an other molecule then the product leaves from the enzyme, this way it is able to accept an other substrate molecule as the simple animation shows (anim 2.1)



2.1. animation: Simple enzymatic reaction

The oldest explanation of the enzyme action was given in 1894 by the lock and key model of E. Fischer (1852–1919) (Fig 2.4.): lock is the enzyme and the exactly fitting key is the substrate. This picture is a good explanation of the substrate specificity of the enzymes.

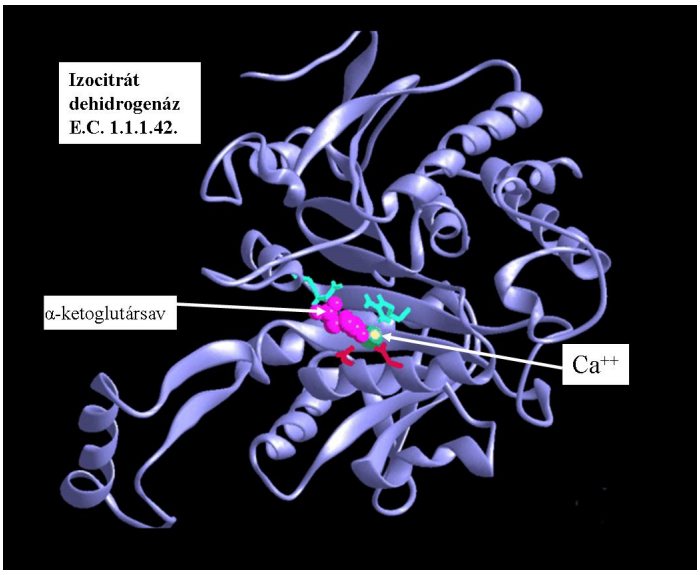


Fig 2.3.: Isocirate-deyidrogenase
Substrate binding and active sites are only relatively small parts of the whole enzyme molecule

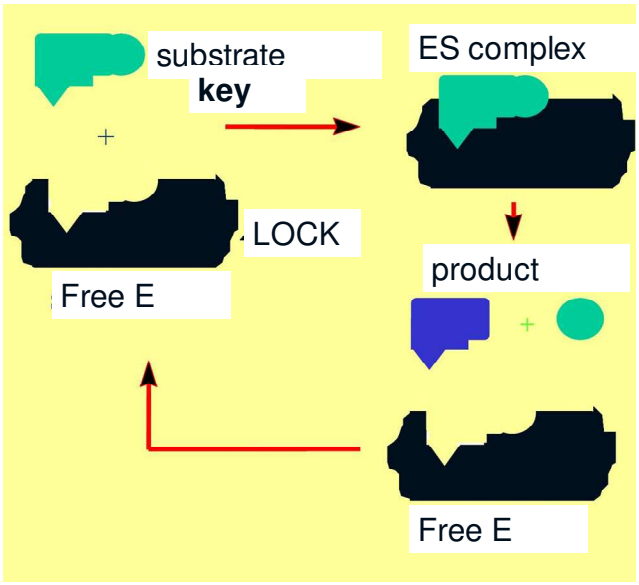


Fig2.4.: Substrate is the key and enzyme is a lock.

Amino acid chain of a protein can be twisted and folded more than one way, but usually only one of these forms a given tertiary structure that holds the enzyme activity by a special spatial arrangement similar to a pocket or sack in which chemically reactive side chains of the amino acids take place. These are as follows:

Asp (COO^-),
Cys (-SH),
Glu (COO^- or $-CONH_2$),
His (imidazole),
Lys ($\varepsilon-NH_3^+$),

Met (CH_3-S),
Ser (-OH), and
Thr (CH_3CHOH-)

These groups and the terminal amino- and carboxyl-groups play the most important role in the enzyme catalysis. Note, that in the formation of the active site only a small part of the building amino acid side chains takes part. Nevertheless, the other amino acid side chains are equally important, i.e. they take part in the formation and stabilization of the active tertiary (and quaternary) structure of the protein.

On large protein molecules often more substrate binding sites and other active domains, binding sites are present for the binding of modulators, prosthetic groups, inhibitors, etc.

The catalytic activity of an enzyme may be caused by various effects, like

acid/base catalysis,
metal ion catalysis and
covalent catalysis.

Lock and key model explains satisfactorily the substrate specificity but leaves more questions open. Daniel Koshland (1920–2007) gave pioneer effect to the understanding of these. He introduced the induced fit theory (mainly regarding the allosteric enzymes…) in 1958. The essence of this theory states that while the substrate reaches the intime vicinity (proximity effect) of the binding and active site of the enzyme, also the structure of the enzyme itself undergoes changes to form even more intimate binding. During this continuous changes the old chemical bonds are getting loosened and at the same time new chemical bond formation possibilities arise. Finally, the whole system fell into the so-called entropy-trap, i.e. the probability of the formation of the product increases compared to the back way to the substrate release (while, of course, the system remains reversible!). An important other effect is the so called orientation effect, that means when substrate approaches the enzyme, it has to turn to the right orientation or position, and – *as the basis of the stereospecificity*- finally at least at three points has to be bound to the surface of the enzyme (=three-point attachment)

.

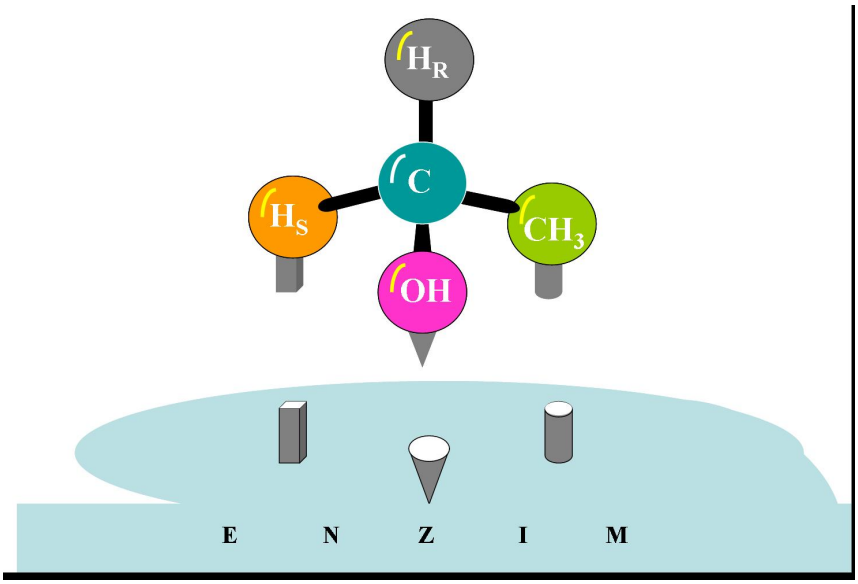


Fig. 2.5.: Orientation effect, three-point attachment: at the active site at least three amino acid (side chains) are responsible to the proper fitting: this is the basis of the stereo-specificity.

Let us look at the mechanism of the phosphorylating enzyme, hexokinase (Fig. 2.6.). Hexokinase enzyme phosphorylates the glucose with ATP during which they form a terner complex. Glucose exactly fits into the active center, meanwhile the enzyme –like a jaw – closes on it. The movement can be measured, it is about 8 Å. The result is a rather intimate proximity of the three reacting partners:

enzyme, glucose, ATP. During the process one molecule of water is excluded from the pocket. Glucose is fixed in the active site by the Lys169, Thr168, Asn204 and Glu256 amino acid side chain functional groups with hydrogen bonds. At this time the free COO^- group of Asp205 – as a basis – abstracts the H of C6 OH of the glucose, initiating a nucleophilic attack against the terminal P of the ATP by the oxygen of this OH. With this attack terminal phosphate releases and glucose takes it up. This mechanism correctly explains that other OH-containing molecules – like cholesterol – why are not phosphorylated: the molecule is too large to fit into the pocket. Or it explains why the water does not hydrolyze ATP by this enzyme: water is too small, thus when it is inside the pocket, the" jaw" does not closes, so it will not be in the necessary proximity of the ATP. This is also proven by the fact, that xylose – not having C6 OH, but its measure approximates glucose –is able to fix water molecule in the active center, and then water pics up phosphate i.e. ATP is hydrolyzed by the enzyme.

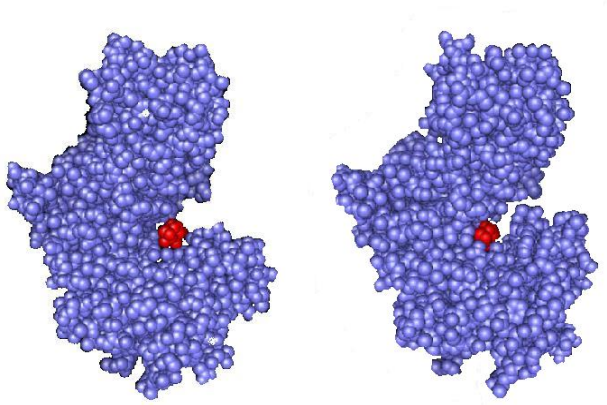


Fig2.6.: The „jaw"of hexokinase closes up on glucose.
Induced fit

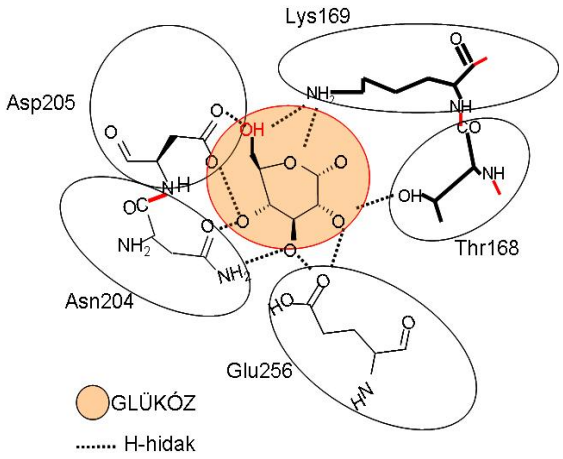


FIG2.7. : Active center of hexokinase with the glucose-binding hydrogen bonds

Summary: During the formation of the activated enzyme-substrate complex, reacting molecules are getting closer and closer to each other (proximity effect). This enhances the probability of the transformation. On the other hand, enzyme can bind the substrate only in a given strict position (orientation effect and three-point attachment). Formation of the transition state has to be imagined as a dynamic process. While the complex is born, the conformation of the enzyme also changes, it moves into the more and more favored state to bind and transform the substrate (induced fit).

2.2. Characteristics of the enzymes. Nomenclature

.

1. Enzymes catalyze only thermodynamically possible reactions = the Gibb-energy change is negative.

2. All the enzymatic reactions are reversible; they are going on until reaching an equilibrium. Enzyme does not affect the equilibrium, just fasten the rate of the reaction, just shorten the time of reaching the equilibrium. A question arises: how is it possible, that enzyme reactions in a cell (metabolic reactions) are going mainly into one direction if all the reactions are reversible? The answer is that the product of a reaction is the substrate of the following, thus removing it from the reaction „mixture", the reaction equilibrium is highly pushed into one direction. With other words: eliminating a reacting agent from the reaction, moves the reaction into the forward direction. Another point is, that the value of the equilibrium constant of the reaction may be under the effect of the environmental circumstances (pH, temperature, ionic strength, etc.).

3. Enzymes are proteins, and their conformation is determined by the tertiary (and quaternary) structure, and these are highly sensible to the environmental conditions. The preferred conformation may change to an unfavored one: this is the **denaturation**. Denaturation means that the protein has a conformation that does not hold the required catalytic activity. The environment caused conformational change may be reversible or irreversible. Denaturation promoting effects can be the following:

increasing temperature

pH change

ionic strength

effects of organic solvents

4. It comes from the proteinaceous nature and the existence of the transition enzyme-substrate complex that enzymes are more or less *specific* from different point of views:

S**ubstrate specificity** means that an enzyme converts only a given substrate. For example, glucose oxidase converts only glucose to glucono-δ-lactone but do not converts fructose. This substrate specificity may be directed to a given molecule or a group of similar molecules (hexokinase phosphorilate glucose as well as fructose, a group of hexoses).

Chemical group specificity means that enzyme converts a special chemical functional group or creates this special group. E.g., α-glycosidase decomposes those disaccharides which hold an α-glycoside bond, but α-amylase is specific to the formed α-glycoside bonds. Group specificity is often called **reaction specificity** they are the same: what a reaction is going on the given chemical functional group.

Stereospecificity is a feature that means, an enzyme – if the substrate or the product holds a chiral center – can change or create a molecule having only one of the antipodes. E.g., L-amino acid-acylase hydrolyses only the acyl-L-amino acid, D-forms remain untouched.

Region-specificity is if there are similar functional groups on a molecule, the enzyme can „choose" a special one at a special region of the molecule. E.g., enzyme act on the hydroxyl group on the second C-atom of a sugar molecule.

The active enzyme often contains other molecules, too, not only protein. The protein part of the enzyme is called **apoenzyme** the bound foreign nonprotein molecule is the **cofactor**. The whole active complex is called **holoenzyme**. The bound cofactor may be a **metal ion** (frequently Mg, Ca, Zn, Fe, Cu, Mo) or an organic molecule that is **coenzyme**. Coenzymes may be of two types: **prosthetic groups** are covalently bound to the protein ($FADH_2$, hem, Pyridoxal-P), while cosubstrate is not strictly bound, as a matter of fact it is a second substrate (NAD,ATP, etc.).

How to name an enzyme? There are different nomenclatures. The name may refer to the substrate:

$$\text{urea + water} \xrightleftharpoons{\text{urease}} CO_2 + 2NH_3$$

After the name of the substrate a suffix **–ase** is given.

An other case is when first we name the substrate then the reaction, using again the suffix –ase.

For example the ethanol → acetaldehyde → acetate reaction is catalyzed by the enzyme alcohol-dehydrogenase.

Protein-degrading enzymes groups have a special trivial naming, all of these are ended by the suffix **–in**: papain, trypsin, etc.

Naturally there has been an old demand to create some informative and systematic nomenclature for the enzymes. And this demand arose together with the demand of systematic grouping of them because since the first active cell extract (1897) today we know many thousands of enzymes. Even the number of industrially applied enzymes is more tens, grouping is understandably reasonable.

The nowadays applied system was introduced by IUPAC IUB (=International Union of Biochemistry) (now IUBMB (Int. Union of Biochemistry and Molecular Biology)) in 1955.This is the EC categorization by the Enzyme Commision that has been continuously updated since then (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

According to this, enzymes have 6 groups corresponding the type of the chemical reactions they catalyze, the groups have subgroups and sub-subgroups according to the finer characterization of the reactions. Table 2.2. shows these groups.

This nomenclature is really systematic, a good example is the name of glucose-oxidase: A series of information can be got form the name itself: this enzyme belongs to the oxidoreductases and it is the 49. in the 1.1.1. subgroup:

:

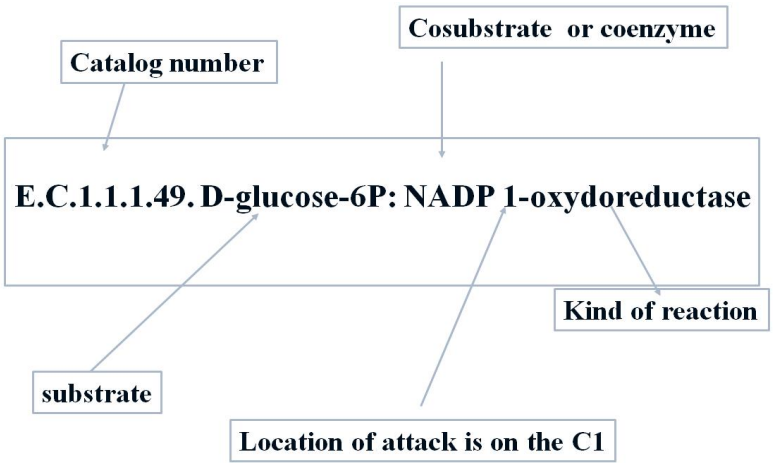


Fig2.12.: Systematic name of glucose-oxidase

The links below we can find very much information regarding the nomenclature as well as from the individual enzymes: their reactions, structures, features, the most relevant literature reference, etc.

IUBMB Enzyme Nomenclature
SWISSPROT *http://www.expasy.org/enzyme*, BRENDA – Comprehensive Enzyme Information system
EMP - Enzymes and Metabolic Pathways database , KEGG – Kyoto Encyclopedia of Genes and Genomes

MetaCyc – Metabolic Encyclopedia of enzymes and metabolic pathways,
BioCarta – Pathways of Life

Table 2.2.: Enzyme groups according to EC

1. Oxido-reductases (oxidation-reduction reactions) (more than 300 groups)
- 1.1. Oxidation of primary -OH group
  - 1.1.1. NAD(+) or NADP(+) acceptor
  - 1.1.2. With cytochrome acceptor
  - …
  - 1.1.99. With another acceptor
- 1.2. Oxidation of keto group: -C=O
- 1.3. Oxidation methylene group: -CH=CH-
- 1.4. Oxidation of primary amino-group
- 1.5. Oxidation of secondary amino group
- 1.6. Oxidation of NADH or NADPH
- 1.7. Oxidation of other N-containing compounds
- 1.8. Oxidation of S-compounds
- 1.9. Oxidation of Hem
- 1.10. Oxidation of Diphenols and similar compounds
- 1.11. Acting on peroxide acceptors (peroxidases)
- 1.12. Acting on Hydrogen donors
- 1.13. Mono- and dioxygenases (oxygen input)
- .....
- 1.19. Oxidation of reduced flavodoxin
- 1.99. Other oxidoreductases

2. Transferases (transfer of functional groups) (more than 300 groups)
- 2.1. C1-group transfer
- 2.2. Aldehyde- or keto-group transfer
- 2.3. Acyl-group transfer
- 2.4. Glucosyl-group transfer
- 2.5. Alkyl- and aryl-group transfer
- 2.6. N-containing group transfer
- 2.7. P- transfer
- 2.8. S- transfer
- 2.9. Se- transfer

3. Hydrolases (hydrolysis reactions) (430 groups)
- 3.1. Ester-hydrolysis
- 3.2. Glycoside-hydrolysis
- 3.3. Ether-hydrolysis
- 3.4. Peptide-hydrolysis
- 3.5. Hydrolysis of other C-N bonds
- 3.6. Hydrolysis of Acidic anhydride
- 3.7. Enzymes acting on C–C bonds
- …
- 3.12. Enzymes acting on S-S bonds

4. Lyases (additions on double bonds and group elimination from substrate thus creatind double bond) (more than 130 groups)
- 4.1. C=C
- 4.2. C=O
- 4.3. N=O
- 4.4. C–S
- 4.5. C–halogen
- 4.6. P–O
- 4.99. Other lyases

5 Isomerases (more than 50 group)
- 5.1. Racemases and epimerases
- 5.2. Cis-trans isomerases
- 5.3. Intramolecular oxidoreductases
- 5.4. Intramolecular transferases (mutases)
- 5.5. Intramolecular lyases
- 5.99. Other isomerases

6. Ligases (creation of new bonds with the energy of ATP) (more than 60 groups)
- 6.1. C–O bond creation
- 6.2. C–S
- 6.3. C–N
- 6.4. C–C
- 6.5. Phospho-ester bond creation

2.3. Kinetic description of simple enzyme reactions

Describing kinetically a system, our goal is to get suitable mathematical formulas to calculate the rate of the reaction, the development of the reaction in time, with parameters characterizing the given enzyme. If the reaction can be described by the scheme

$$S + E \leftrightarrow P + E$$

S , E and P are expressed in terms of molar concentrations. In the real system this is easy in the case of S and P but almost impossible for the E, because enzymes are almost never in pure (crystalline) form, rather they are in some more or less dirty preparations, in which beside the asked enzyme there are many contaminating other proteins, organics and even inorganics present. That is why we do not use molar or g/l concentrations, instead a general, so-called *enzyme UNIT* is used for the expression of the „amount" of the enzyme. This unit is not a mass in reality, rather a rate of the reaction. Definition of the enzyme unit is the following:

> **One unit enzyme is the quantity, that convert exactly 1 μmol substrate or produces 1 μmol product during 1 minute in given reaction circumstances.**

The given circumstances mean the environment of the reaction, temperature, pH, buffer molarity, etc.

In the obligatory SI system the unit „quantity" of an enzyme is Katal:

> **1 Katal is the amount of an enzyme that converts 1 mol substrate or produces 1 mol product during 1 second.**

This is a huge amount; thus it is not too popular in the everyday practice.

Nanokatal, i.e, nKat = 10^{-9} Katal which is much more usable. The conversion between these two enzyme units is as follows:

$$1\ \text{Kat} = 6*10^{7}\ \text{U}, \qquad 1\text{U} = 1.6*10^{-8}\ \text{Kat}, \qquad 1\text{U} = 1/60\ \mu\text{Kat}.$$

We shall apply two different approaches for the description of kinetic behaviour of simple enzyme reactions: first we look at the Michaelis–Menten next the Briggs–Haldane kinetic descriptions.

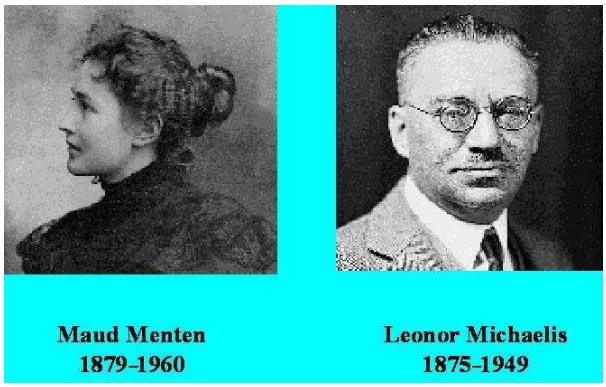


Fig2.13.: Authors of the Michaelis–Menten enzyme kinetics

2.3.1. Michaelis–Menten kinetics

Michaelis and Menten (Fig 2.13.) in 1913 published their approach. The starting point is the scheme of the reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

Let us suppose the irreversibility of the second step ($k_{-2}=0$), and the very fast reach of the equilibrium of the first step – the whole kinetic description is called by this *rapid equilibrium.*

For this equilibrium we may write:

$$k_1 S E = k_{-1} (E S) \tag{2.0}$$

From this K_s dissociation constant of the (ES) enzyme-substrate complex can be expressed:

$$K_s = \frac{k_{-1}}{k_1} = \frac{S.E}{(ES)} \tag{2.1}$$

The rate of the P production

$$V = \frac{dP}{dt} = k_2 (ES) \tag{2.2}$$

Taking into account a material balance equation for the different forms of the enzyme (free enzyme and complexed enzyme), it can be written, that the sum of these is equal to the total amount E_0:

$$E + (E S) = E_o \tag{2.3}$$

Let us divide eq. (2.2) by this material balance:

$$\frac{V}{E_o} = \frac{k_2 (ES)}{E + (ES)} \tag{2.4}$$

Expressing (ES) from (2.1) and putting into eq. (2.4) the non-measurable concentration of the complex can be eliminated:

$$\frac{V}{E_o} = \frac{k_2 \frac{S}{K_s} E}{E + \frac{S}{K_s} E} \tag{2.5}$$

Simplifying with E and rearranging, we get the following

$$\frac{V}{k_2 E_o} = \frac{\frac{S}{K_s}}{1+\frac{S}{K_s}} = \frac{S}{K_s + S} \tag{2.6}$$

It is logical by eq. (2.2) that a maximal reaction rate can be got if all the enzymes are in complex form:

$$V_{max} = k_2 E_o . \tag{2.7}$$

Putting this into eq. (2.6) the Michaelis– Menten enzyme kinetics' well known equations are the results:

$$V = V_{max}\frac{S}{K_s + S} \qquad \frac{V}{V_{max}} = \frac{\frac{S}{K_s}}{1+\frac{S}{K_s}} \tag{2.8}$$

I **II**

In the rapid equilibrium approach of Michaelis and Menten V means *initial reaction rate (V_0),* i.e., it comes from data when the concentration change of substrate and product are going along a straight line. The slope of these extrapolated lines to the zero time gives the initial reaction rate according to the Fig 2.14.

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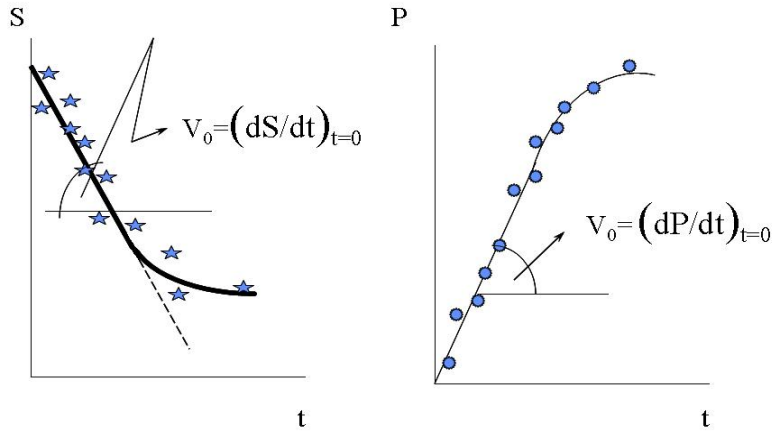


Fig 2.14.: Initial reaction rate

Further assumptions and simplifications of the Michaelis and Menten approach are:

– The enzyme present is in catalytic amount, i.e. its concentration be much more less than that of the substrate:

$$S >> E_o \text{ or } E_o / S << 1$$

– The (ES) complex is stable from a kinetic point of view, and there in no accumulation of an enzyme-product complex (EP).
– Only one S molecule can bind to the only active site of the enzyme.
– Concentrations may be used instead of activities (diluted solutions).

The chain of ideas of the above deduction can be followed in the case of more complicated enzymatic systems, too, supposing the existence of the rapid equilibrium. The II. type general formula can be memorized easily in every case of rapid equilibrium. Furthermore, in these cases the final equation form can be got without any mathematical deduction. The following process is a memory-helping tool with which, based on the formal reaction scheme, the final equation can be written easily.

On the Fig 2.15. in the nominator of the right-hand side formula there are those terms that correspond to the product-forming complexes. The nominator of the first ratio refers to the complex formed with ligand O and the second one refers to the complex with ligand H. According to the imagined (theoretically supposed) scheme the same product will be formed from both complexes. The terms in the denominator represent the various enzyme-forms present (as a matter of fact this is the material balance for the enzyme): here three kinds of enzymes are present, free and three kinds of complexed enzyme, thus four terms are present in the denominator. The number 1 always mean the free enzyme. The ratios correspond to each other complexes. In our example there is also a terner complex from which no product formation is supposed (a useless complex). In the nominator of these ratios there is a product of the complex-forming ligand(s) and the enzyme while the denominator shows the dissociation constant or the product of the dissociation constants.

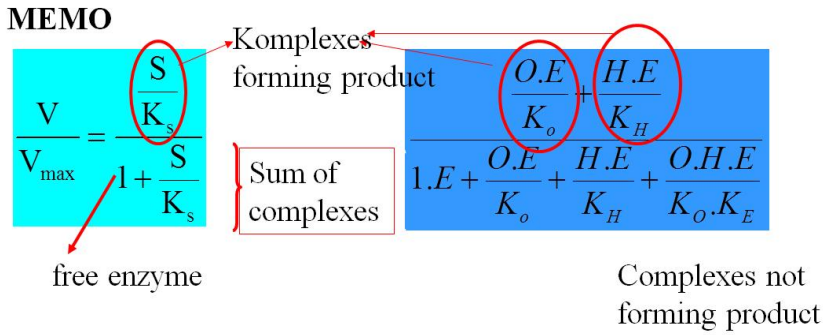


Fig 2.15.: A mnemotechnic aid for writing Michaelis-Menten type kinetic equations

2.3.2. Briggs–Haldane kinetics[3]

The Briggs–Haldane or steady state kinetic approach starts from the same scheme, writing up all the differential equations describing the events:

$$\frac{dS}{dt} = -k_1 ES + k_{-1}(ES) + k_2(ES)$$

$$\frac{d(ES)}{dt} = k_1 ES - k_{-1}(ES) - k_2(ES) \qquad (2.9)$$

$$\frac{dP}{dt} = k_2(ES)$$

A numerical solution of the equation system number (2.9) is shown in Fig2.16. Here we see a very short running up initial period after that the concentration of ES enzyme-substrate complex remains almost constant for a rather long time (it does not change very much). This is called quasi-steady state

[3] Briggs E.B.(1893-1985), Haldane J.B.S.(1892-1964)

in which only a slight decrease of the complex concentration can be observed. The figure demonstrates that the so called *pre steady state* lasts for a very short time, lower than 0,1 s, frequently in the millisecond or microsec order of magnitude. Note, that for this pre-steady state our kinetic approaches are not valid, but there exist other (more complicated) kinetics, that describe also this very short initial phase of an enzyme reaction.

The conditions of existence of the steady state are the same as in the M-M kinetics, but it is a prerequisite that the formation rate of the ES complex must be much faster than its decomposition rate:

$$k_1ES > k_{-1}(ES), \quad \text{and} \quad k_1ES > k_2(ES).$$

In steady state, the concentration of the enzyme-substrate complex does not change

$$d(ES)/dt = 0,$$

which holds

$$k_1ES = (k_{-1} + k_2)(ES)$$

$$(ES) = \frac{k_1ES}{(k_{-1} + k_2)} \qquad (2.10)$$

Collecting the individual reaction rate constants in one group, let us introduce the so called Michaelis constant in the form of $K_m=(k_{-1}+k_2)/k_1$. With this we get an equation formally same as eq (2.8) with the only difference in the constant. Here Km is not a dissociation constant.

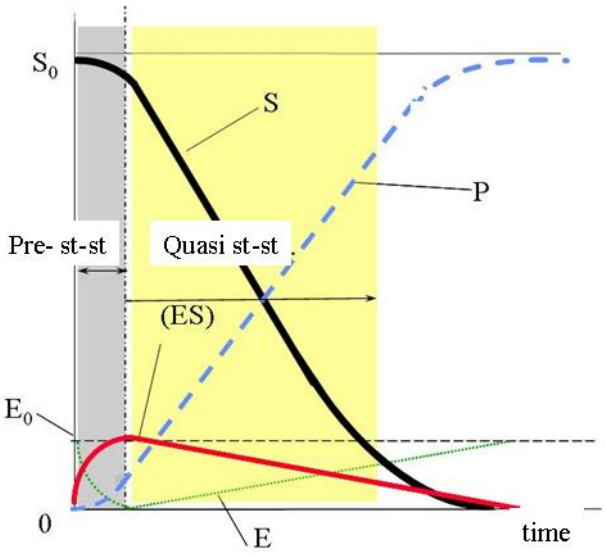


Fig2.16.: Numerical solution of eq. (2.9)

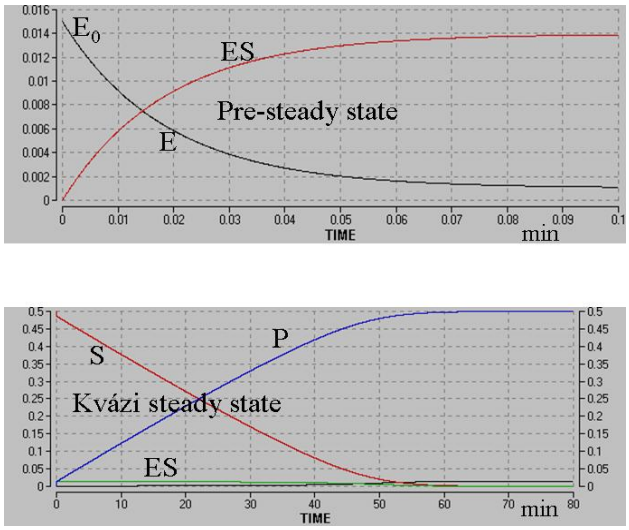
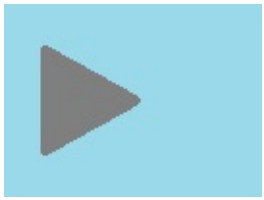


Fig2.17.: Real time solutions of (2.9) for the pre-steady state and the whole reaction

Let us note that d(ES)/dt=0 is just exactly at the maximum point of the time course of (ES). This is why we call this situation quasi-steady state, because in the course of the whole reaction this condition does not hold strictly.

The Briggs–Haldane kinetics and its real-time simulations can be studied in the simulations 2.1. We can study how the individual reaction rate constants influence the time course of the reaction, the pre-steady state, and the steady state.



Simulation 2.1.: Kinetic behavior of simple enzyme reactions

2.3.3. Discussion of the kinetic equation, determination of the parameters and their interpretations

Equations of the two theoretical approaches are formally the same:

Michaelis–Menten	Briggs–Haldane	
$V = V_{max}\frac{S}{K_s + S}$	$V = V_{max}\frac{S}{K_m + S}$,	(2.11)

where $$K_m = \frac{k_{-1} + k_2}{k_1} = \frac{k_{-1}}{k_1} + \frac{k_2}{k_1} = K_s + \frac{k_2}{k_1}.$$

It can be seen from this that $K_m \cong K_S$ is true only if the numerical value of k_1 is much higher than the numerical value of k_2, i.e. k_2/k_1 can be omitted.

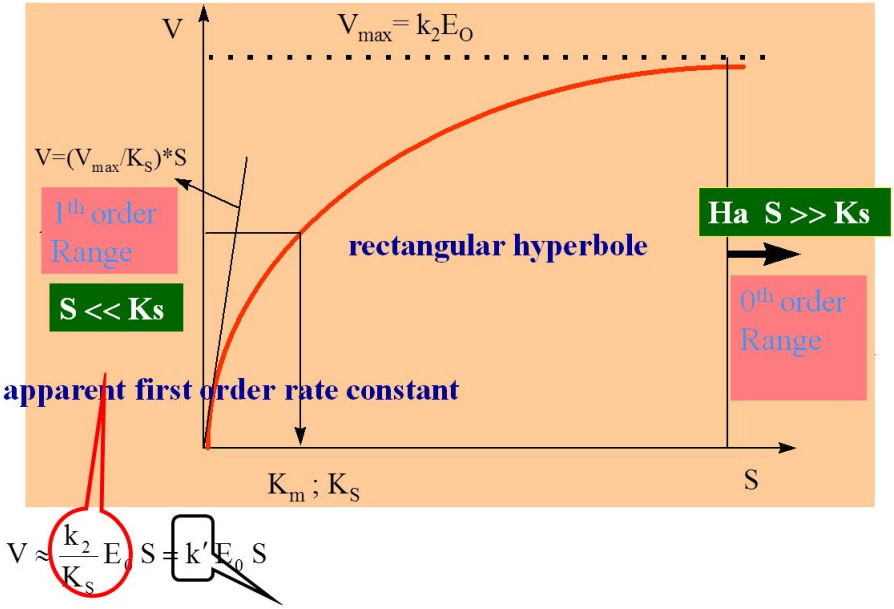


Fig2.18.: Plotting and discussion of M–M és B–H-equations

V-S plot of eq (2.11) is shown on Fig2.18. Two extremes are worth to look at in details:
The asymptote of the rectangular hyperbole is $V_{max} = k_2E_0$.

– for if $S >> K_S$, then K_S can be neglected beside S and then

$$V \cong V_{max},$$

i.e the reaction become of zeroth order regarding S concentration. With other words this means that all molecules of enzyme present are in complex form, enzyme is saturated with substrate.

– At the other extreme, if $S << K_S$, then S can be neglected beside the K_S, and then

$$V \cong \frac{V_{max}}{K_s} S = kS,$$

i.e., at very low S concentrations (near to the origin) the reaction rate is proportional to the S concentration, the reaction is of first order in respect to the S. Do not forget that every points of the curve on Fig 2.18 are initial reaction rates at given S concentrations. To evaluate these points, look at the method on Fig 2.14.

–K_S or K_m value can be got as abscissa coordinate at $V=V_{max}/2$, i.e. these are the substrate concentrations at the half maximum velocity.

The curve is a real rectangular hyperbole that is proved by Fig.2.19, on which the whole hyperbole and the necessary transformations are also seen.

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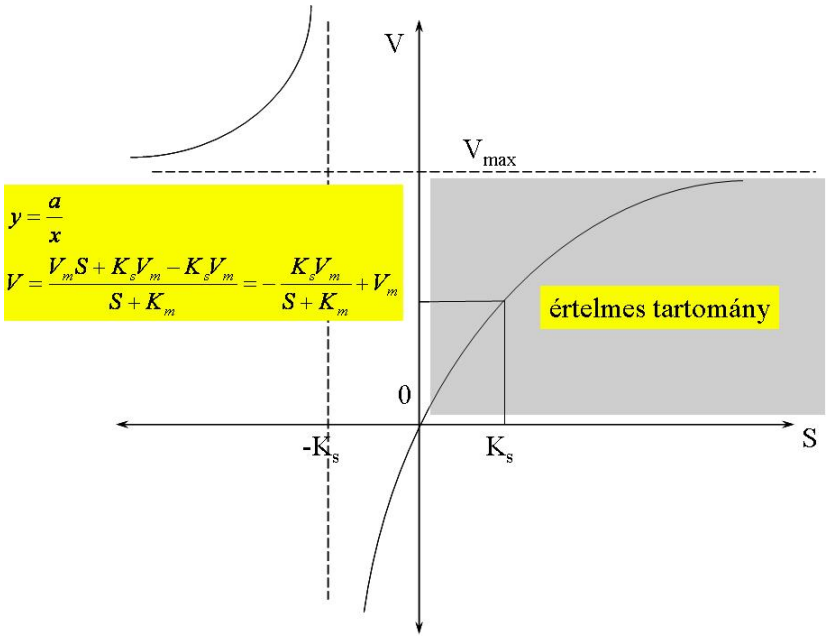


Fig.2.19.: M–M-equation is a transformed hyperbole

Eq (2.11) can be written also in the form of a differential equation

$$\frac{dS}{dt} = -\frac{V_{max} S}{K_m + S}$$

which can be easily solved by separating the variables. At S(o)=S_o initial condition the solution is the following:

$$V_{max} t = S_o - S + K_m \ln \frac{S_o}{S} . \qquad (2.12)$$

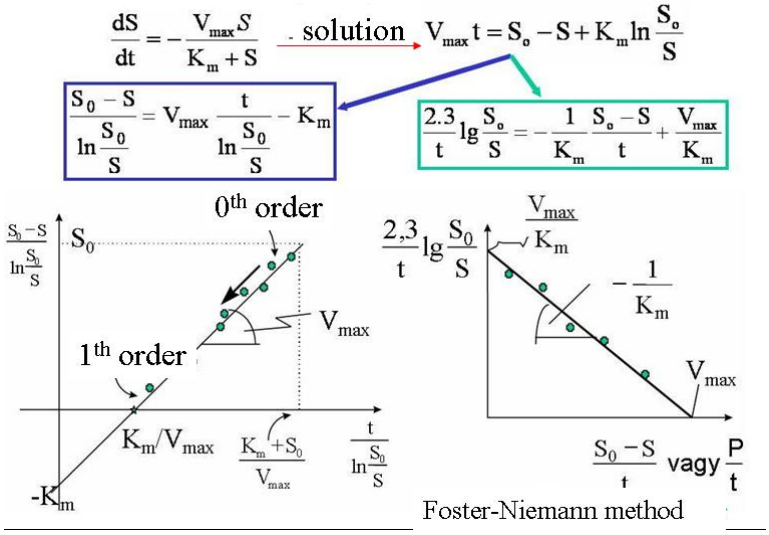


Fig2.20.: Linearization methods of the integrated M–M-equation

Our lab measurements may give such kind of results, if we leave to develop the reaction fully (until it stops), from these results the Fig-given plotting methods serve useful tools to evaluate the constants of the M-M equation. These methods are useful if we follow the time course of an enzymatic reaction.

V_{max} and K_S (or K_m) parameters can be evaluated with other graphical methods as well. These are various linearization methods of the M_M equation. In these cases, our experimental results serve V_0-S values. The best-known linearization method is the Lineweaver–Burk double reciprocal plot. Those linearizations where one of the variables appears on both axes are better from a mathematical point of view, they give more precise results. The three linearization plots are shown on Fig.2.21.

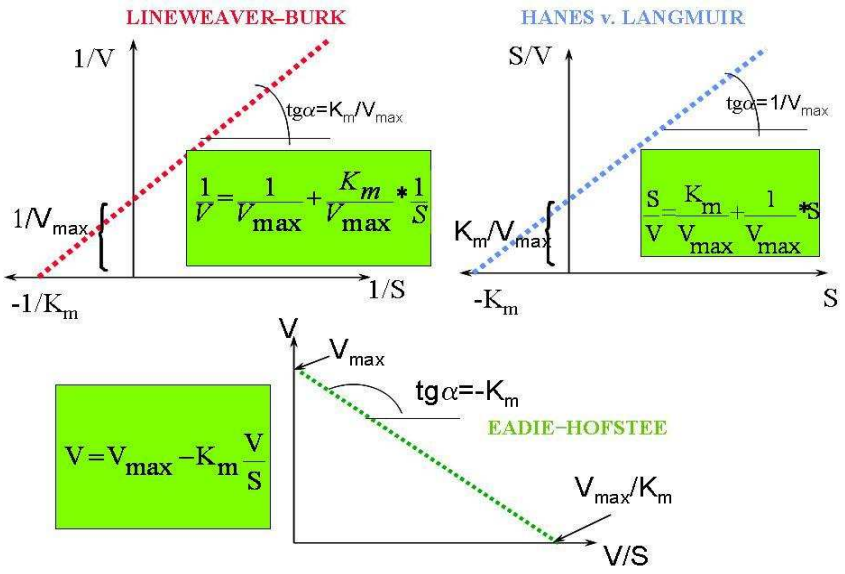
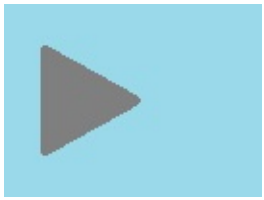
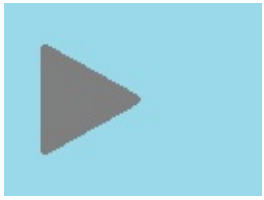


Fig2.21.: Linearizations of M–M-equation

The mathematical function of M–M- and B–H-equation and their shape-changes as a function of the parameters can be followed by the simulations below.



2.2. simulation: M–M-enzyme kinetics



2.3. simulation: Fitting curves to our experimental results

Evaluation of V_{max} is very important. It is proportional to the amount of the enzyme; thus it is the measure of enzyme activity! This is shown on Fig 2.22, the increase of the enzyme input increases the maximal velocity proportionally. At the same time this plot is the basis of the k_2 determination.

It is important to know that Vmax is not enzyme feature, because it does also depend on the amount of the enzyme! But the first order rate constant k_2 is a characteristic feature of the given enzyme its other name is *turnover number*. It has a special meaning: gives the number how many substrate molecules are captured by an enzyme molecule and then converted and released as product during a minute (or s), i.e. it is the frequency of the enzyme action (1/min, 1/s)

Generally speaking always can be defined a $V_{max}=k_{cat}E_0$ relation in which k_{cat} is called catalytic number or turnover number.

In the case of Michaelis–Menten-kinetics kcat is the same as k_2, but at more complicated kinetic cases this is not necessarily true.

k_{cat} values of metabolic enzymes (e.g. glycolytic enzymes) are in the range $1\text{-}10^7$ s^{-1}, while the slower restriction enzymes have values $1\text{-}10^{-3}$,and the slowest are the molecular switches (for example the circadian system) with their range of 10^{-5}–10^{-2} s^{-1}.

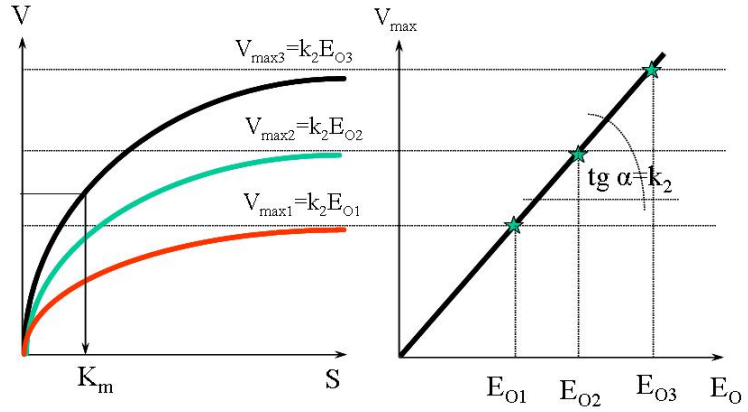


Fig2.22.: V_{max} is proportional to the amount of enzyme present. activity. Determination of k_2.

Interpretation of K_m, or K_S is more complex. (In the nex part of the text K_m and K_S are interchangeable, our statement is valid for both.)

– *K_m gives the approximate substrate concentration in a cell* (see Fig 2.23). It is highly unlikely that S is much less or much higher than K_m. In the former case it would be too sensitive for a small change in S, and at the same time the reaction velocity would be much far from the maximum capacity of the cell ($V << V_{max}$). Similarly meaningless is -from a physiological point of view- if S is much higher than K_m. On the other hand, V is always less (a bit) than V_{max} and at $S=1000K_m$ the velocity is only twice as much as at $S=K_m$. This proves that V is very insensitive on the change in S concentration when S is near the saturation. It is probable consequently, that S in a cell should be at somewhere near to the half saturation where the change caused by S is not too sharp but sensitive enough.

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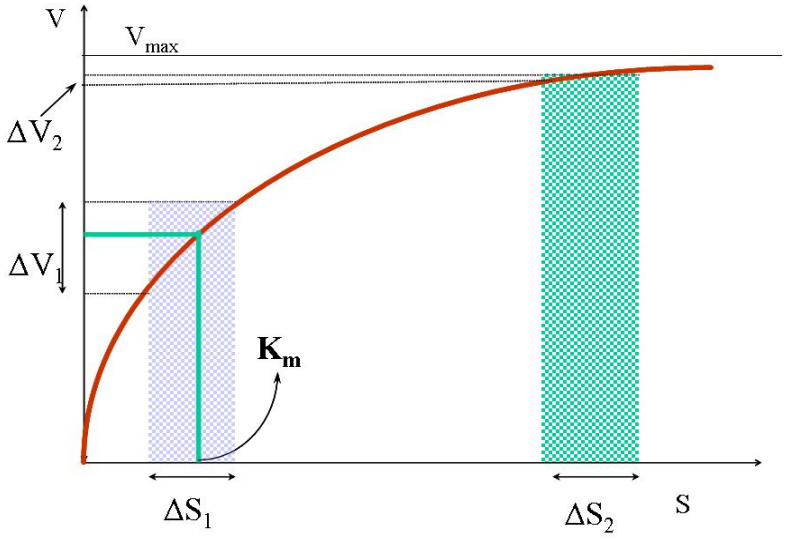

Fig. 2.23.: K_m, or K_S is approximately the substrate concentration the cell.

– K_m means the **affinity** of the enzyme to the substrate. Less the value of the K_m higher the affinity. Best substrate has the least K_m value.

– K_m is a feature of an enzyme, it is characteristic onto it, consequently K_m is a good comparing data considering different enzyme preparation from different sources, from different cells, etc. It is suitable to decide whether a protein A having similar catalytic activity and another protein, B are the same enzymes or not.
– Modification of K_m by an activator or inhibitor may be the principle some enzyme level control. If, for example, an *in vitro* measured K_m is too high compared to a „physiologically probable" value, one can assume that *in vivo* there could have been some activator present that disappeared during the isolation/purification process. Examining the effects of different chemicals on the K_m , it is possible to find compound(s) increasing K_m, thus having some inhibitory effect on the enzyme (possible pharmakon)
– Knowing K_m, it is possible to determine the suitable substrate concentration range for the correct analytical determination of the enzyme activity: if $S >> K_m$ the measured velocity surely will be V_{max}.
– The values of K_S, or K_m are lying in the range of 10^{-6}–10^{-2} mol/dm^3.

Individual rate constants of the Michaelis–Menten and Briggs–Haldane equations have the typical ranges as follows:

The k_1 second order rate constant has the usual range:

$$k_1 = 10^7–10^{10}\ dm^3\ mol^{-1}\ min^{-1}$$

The maximum value is certainly less than 10^{11}, that is the order of magnitude of the diffusion velocity of the small molecules in water solution, it is impossible to have more frequent collision between a substrate and an enzyme molecule than with a rate determined by the diffusion.

The k_{-1} lies between the range of 10^2–10^6 min^{-1}.

Table2.3.: Kinetic constants of some enzymes

ENZYME	SUBSTRATE	K_m (mol/dm^3)	k_{cat} (s^{-1})	k_{cat}/K_m (dm^3/mol.s)
catalase	hydrogen-peroxide	$2{,}5\cdot10^{-2}$	$1{,}0\cdot10^{7}$	$4\cdot10^{8}$
urease	carbamide	$2{,}5\cdot10^{-2}$	$1{,}0\cdot10^{4}$	$4\cdot10^{5}$
fumarase	fumarate	$5{,}0\cdot10^{-6}$	$8{,}0\cdot10^{2}$	$1{,}6\cdot10^{8}$
	malate	$2{,}5\cdot10^{-5}$	$9{,}0\cdot10^{2}$	$3{,}6\cdot10^{7}$
acetylcholinesterase	acetylcholine	$9{,}5\cdot10^{-5}$	$1{,}4\cdot10^{4}$	$1{,}5\cdot10^{8}$

An important combined parameter of an enzyme is the **catalytic effectivity**, or **specificity constant.** Its value can be the same even if the behavior of the enzymes are very different. For instance, it can be 10^7 in two ways:

$$\frac{k_{cat}}{K_m} = 10^7 s^{-1} mol.dm^{-3} = \frac{10^7 s^{-1}}{1 mol.dm^{-3}} = \frac{1 s^{-1}}{10^{-7} mol.dm^{-3}} .$$

One of the enzymes here has very small affinity and at the same time huge k_{cat} and the other just the opposite: high affinity and small turnover rate. The overall reaction velocity is determined by these two parameters at the same time in term of k' (see Fig 2.18). This combined kinetic constant is applied for calculating which substrate will be converted with the highest rate if there are more than one similar substrate present in a reaction mixture= for which of them the enzyme is the most specific.

The k_{cat} values have very broad range. According to the Fig 2.24. the slowest enzymes are the molecular switches while the metabolic enzymes are much more, with even 8-10 order of magnitude faster.

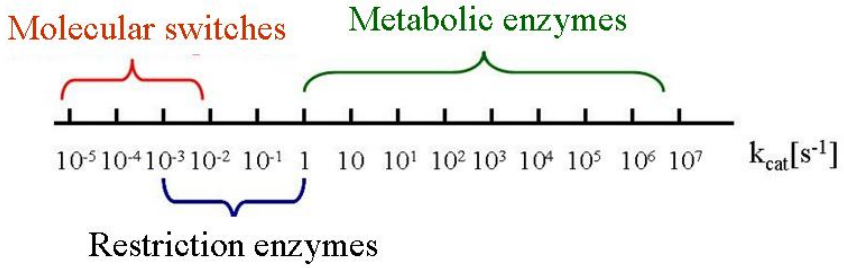

FIG 2.24.: Range of k_{cat} for different types of enzymes

2.3.4. Reversible reactions – equilibrium of an enzymatic reaction

Many enzyme-catalyzed reactions – like biopolymer hydrolyses – are highly pushed into the right hand direction thus the assumption $k_{-2} = 0$ practically holds. But there are many cases – especially the interconversions of small molecules – where a real equilibrium can be observed, substrate and product are present in comparable amount in the reaction mixture. A good example is the glucose → fructose

conversion which is observably reversible. Thus, the kinetic description we introduced so far cannot be simply applied.

$$\mathbf{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathbf{E + P}$$

Theoretically this reaction can be followed into both direction or looking at it as one reaction going into forward and at the same time another reaction going into backward. In both directions let us apply the simple irreversible M-M kinetics and then add them up. Let K_{mS} be the forward reaction K_m value and similarly V_{maxS} the maximum velocity into the forward direction. The same way gives K_{mP} and V_{maxP} parameters for the backward reaction. In this situation we can write:

$$\begin{aligned} K_{mS} &= \frac{k_2 + k_{-1}}{k_1} & V_{maxS} &= k_2 E_o \\ K_{mP} &= \frac{k_2 + k_{-1}}{k_{-2}} & V_{maxP} &= k_{-1} E_o \end{aligned} \qquad (2.13)$$

The equilibrium constant for the overall reversible reaction:
:

this is the reciprocal of K_S — this is K_P itself

$$K_1 = \frac{k_1}{k_{-1}} \quad \text{és} \quad K_2 = \frac{k_2}{k_{-2}}$$

$$K_{eq(uvilibrium)} = K_1 K_2 = \frac{k_1 k_2}{k_{-1} k_{-2}} \qquad (2.14)$$

Performing the following divisions:

$$\frac{V_{maxS}}{K_{mS}} = \frac{k_1 k_2 E_o}{k_2 + k_{-1}} \quad \text{és} \quad \frac{V_{maxP}}{K_{mP}} = \frac{k_{-2} k_{-1} E_o}{k_2 + k_{-1}},$$

then dividing the two expressions by each other, we get the equilibrium constant:

$$\frac{\frac{V_{maxS}}{K_{mS}}}{\frac{V_{maxP}}{K_{mP}}} = \frac{V_{maxS} K_{mP}}{V_{maxP} K_{mS}} = \frac{k_1 k_2}{k_{-1} k_{-2}} = K_{eq} \qquad (2.15)$$

Eq (2.15) is the so called **Haldane relationship**. This is the connection between the individual rate constants and the equilibrium constant.

Let us suppose a reaction mixture containing S and P. Give E_0 enzyme at a given time. A question arises: what will happen? S will convert to P or the opposite? The overall rate of the reaction $S \rightleftharpoons P$ (including the direction) will be determined by K_{eq} and the actual concentrations of P and S.

The overall reaction rate is

$$V_{net} = V_{forward} - V_{backward} = k_2 (ES) - k_{-2}(EP), \qquad (2.16)$$

and

$$V_{netto} = \frac{V_{maxs}\left(S - \frac{P}{K_{eq}}\right)}{K_{ms}\left(1 + \frac{P}{K_{mp}}\right) + S} = \frac{V_{maxs}\frac{S}{K_{ms}} - V_{maxP}\frac{P}{K_{mP}}}{1 + \frac{S}{K_{ms}} + \frac{P}{K_{mP}}} \qquad (2.17)$$

This is the reversible M-M equation.

2.4. Modulation of enzyme activity

There exist chemical compounds that modify the activity of an enzyme if binding to it. These are the modulators or effectors, and the phenomenon itself is called modulation. This can be activation and inhibition depending upon the fact that the bound compound increases or decreases the original activity. In this chapter only the inhibition will be pertracted.

The degree of the activation and the inhibition can be expressed the following way:

degree of inhibition

$$\varepsilon_i = \frac{V_0 - V_i}{V_0}$$

degree of activation

$$\varepsilon_a = \frac{V_a - V_0}{V_0}$$

Binding an effector to the enzyme protein i.e. the formation of an (EI)complex could be totally or partially irreversible or reversible.

Known instances of **irreversible inhibition** ***are the enzyme poisons*** like heavy metals (Hg,Cd) and cyanide ions or the well-known nerve gifts (like Sarine, i.e. di-isopropyl-fluor-phosphate, see Fig 2.25.), which inactivates the acetyl-choline-esterase enzyme which plays an important role in neuro-transmission. Irreversible inhibition actually decreases the amount of the active enzyme.

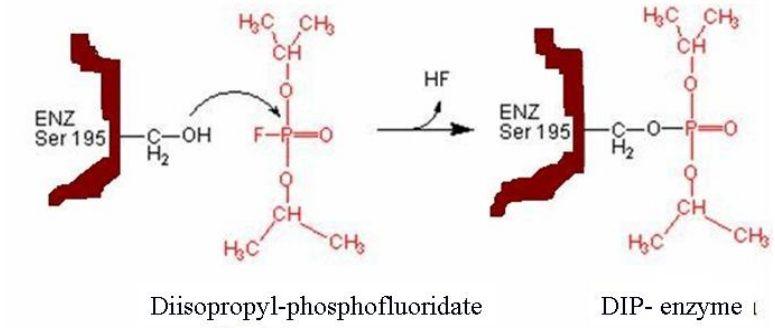

Fig 2.25.: Sarin's effect on the acetyl-choline-esterase

Their effect is a bit similar to the effect of a noncompetitive inhibitor (V_{max} decreases but K_m does not change, see later in details) according to the following:

$$\begin{array}{l} E + S \rightleftharpoons ES \longrightarrow E + P \\ + \\ I \\ \downarrow \\ E\,I \end{array}$$

On the basis of $V_{max} = k_2.E_0$ relation, the presence of an irreversible inhibitor can be proven, and a distinction can be done between reversible and an irreversible inhibitors. If the maximum rate velocity is plotted against the introduced initial enzyme concentration, the picture of the Fig 2.26. will be the result. In the presence of an irreversible inhibitor, we shall get an intercept on the x-axis that gives exactly the amount of the irreversible inhibitor. This is because this inhibitor practically removes a certain part of the enzyme and those inhibitors bound enzyme molecules totally lost their activity, as if they were not present at all.

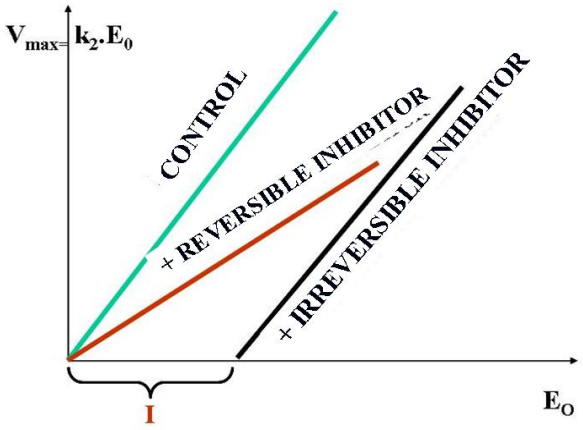

Fig 2.26.: Recognition of an irreversible inhibitor

Reversible inhibitors form dynamic complexes with the enzyme molecules, and the catalytic effect of such a bound enzyme differs from that of not inhibitor bound molecules. The types of the inhibitions may differ from the point of view that the activity is getting zero when an inhibitor molecule forms complex (this is the case of complete inhibition) or there remains a certain fraction of the original activity (= partial inhibition). The former is also called **linear inhibition** because the so-called Dixon-plot gives a straight line (1/V against inhibitor concentration), while the opposite case is called **hyperbolic inhibition = partial inhibition**. Another distinction in case of complete or linear inhibition is based upon the effect of the inhibitor on the kinetic parameters. If V_{max} does not change but K_m increases it is the **competitive inhibition**, if the inhibitor does not touch K_m but decreases V_{max}, we have a **noncompetitive inhibitor**. If both parameters decrease with a constant ratio, the inhibitor is called **uncompetitive** and finally if a certain mixture of effects is observed that is the case of **mixed inhibition**. From the scheme of the latter all the other types can be deduced. From now on we deal only with reversible linear inhibitions.

2.4.1. Competitive inhibition

Competitive inhibitors prevent the binding of a substrate molecule to the enzyme. But a previously bound substrate also prevents the binding of a competitive inhibitor to the enzyme. With other words the substrate and the inhibitor mutually exclude each other from the enzyme. This is a real competition for the binding sites of the enzyme between the substrate and the inhibitor.

Competitive inhibitors can be so called substrate analogues that are chemically similar to the substrate but are unconvertable (non-metabolizable) by the enzyme or they can be alternative substrates or products of the enzyme. These are the case of the classical competitive inhibitions. On the other hand, there exist such competitive inhibitors which are not structurally similar to the substrate but also prevent the substrate binding. Possible mechanisms of the competitive inhibition are shown on Fig 2.28. Let us observe that only the model a) corresponds a real structural similarity when the inhibitor as well as the substrate intend to bind to the same binding site.

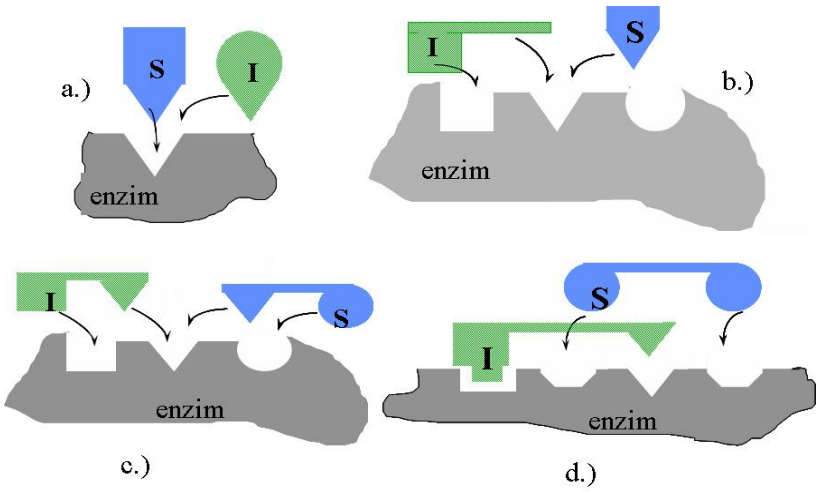

Fig 2.27.: Models of competitive inhibition 1

Case b) is a kind of steric hindrance, here the inhibitor covers the substrate binding site and does not allow binding of the substrate (and vice versa). Model c) and d) are cases of partial or complete overlapping of the binding sites. The situation on the Fig.2.28 is the most important from physiological point of view, because this competitive *feedback inhibition* is one of the enzyme level promptly acting regulation mechanisms. The end product of a series of reactions can prevent the overproduction of the given metabolite because it can combine with the enzyme of the first reaction in the series and competitively stops its conversion step thus ceasing the further unnecessary production of the given metabolite. The mechanism of this model do not need any structural similarity or close binding. The inhibitor can bind even a place far from the substrate binding site, but it causes a conformational change of the tertiary structure of the protein, that prevents the sustrate binding. All these models mean mutual exclusion either the subtrate or the inhibitor.

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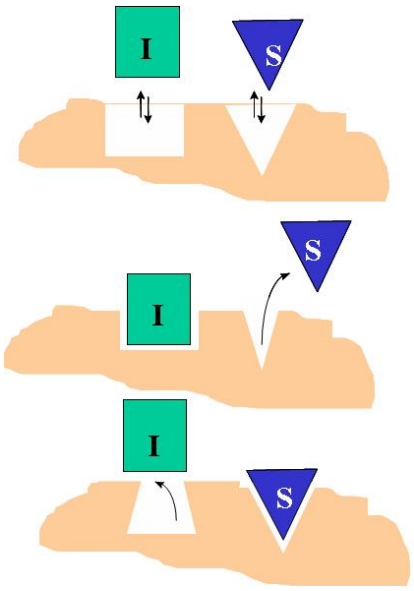
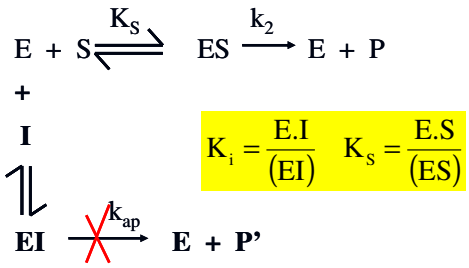


Fig 2.28.: Models of competitive inhibition 2

In order to describe the kinetics of c**ompetitive inhibition let us look at the scheme below:**



If $k_{ap} > 0$, then I is an *alternative substrate of E and*, P' is its alternative product. E.g., in case of hexokinase, glucose is the substrate and fructose may be an alternative substrate, the enzyme equally able to phosphorylate both.

If $k_{ap} = 0$, this is the case of dead-*end competitive inhibition*, i.e EI complex does not form any product.

Applying the mnemotechnic process we have seen in 2.3 it is possible to write a rapid equilibrium kinetics according to the scheme above.

$$\frac{V}{k_2 E_0} = \frac{V}{V_{max}} = \frac{\frac{S}{K_s}}{1 + \frac{S}{K_s} + \frac{I}{K_i}} \qquad (2.18)$$

Considering the non-inhibited case, there is an additional term in the denominator: I/Ki representing the (EI) complex. A more familiar equation of the competitive inhibition can be got from eq (2.18) multiplying by K_S and rearranging:

$$\frac{V}{V_{max}} = \frac{S}{K_s\left(1 + \frac{I}{K_i}\right) + S} \quad \text{or} \quad V = V_{max}\frac{S}{K_s\left(1 + \frac{I}{K_i}\right) + S} \qquad (2.19)$$

(Following the steady state way of thinking, naturally a similar equation would be the result with K_m instead of K_S.)

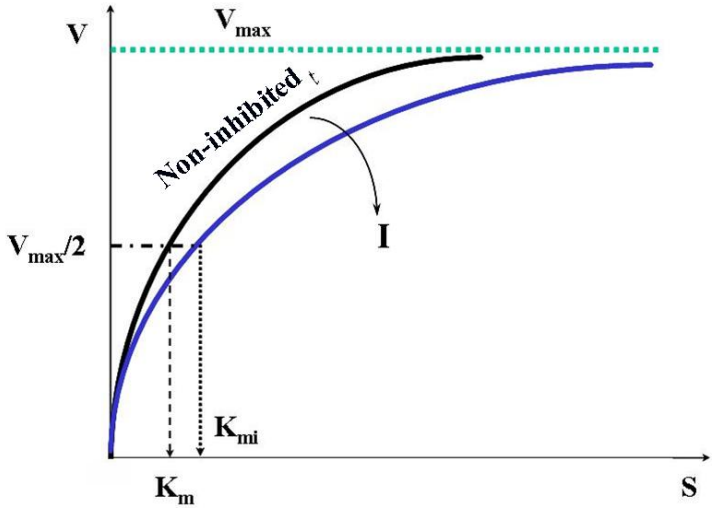


Fig.2.29.: V-S plot of competitive inhibition

The term $K_m(1+I/K_i)$ of the denominator is called K_{mi} apparent Michaelis-constant. As seen on the figure the V_{max} does not change at competitive inhibition, I changes only the apparent K_{mi} value: it is increasing i.e. the inhibitor decreases the affinity of the enzyme to the substrate.

$(1+I/K_i)$ term is worth to note because it will play role at every instance of inhibitions!

On Fig 2.30.the characteristic linearized Lineweaver–Burk plot of the competitive inhibition is shown which gives one of the graphical methods of evaluation of the parameters. It can be seen that the slope of the curves is a linear function of the inhibitor concentration, and this makes possible the K_i determination, too:

$$\mathrm{tg}\alpha = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}K_i}I \qquad (2.20)$$

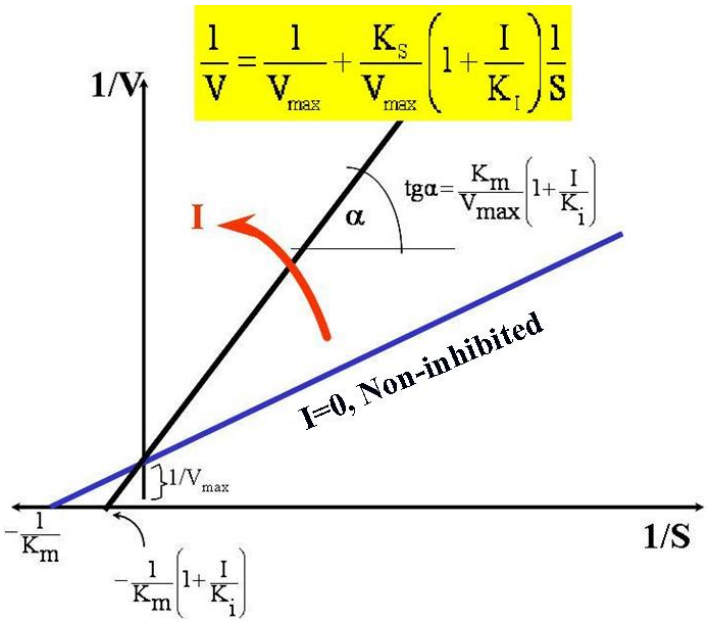


Fig 2.30.: Lineweaver–Burk plot of competitive inhibition

Let us calculate the inhibitor concentration that causes a doubling of the slope of the inhibited L-B curve.:

$$2tg\alpha=\frac{K_m}{V_{max}}\left(1+\frac{I}{K_i}\right)\quad\text{from here}\quad 2=1+\frac{I}{K_i}\ \text{ns}\ \ I=K_i \tag{2.21}$$

At a competitive inhibition K_i gives this concentration that doubles the slope of the L-B strait line. But do not think that in this case the degree of the inhibition is 50%! Latter can be calculated this way:

$$\frac{V_i}{V}=0{,}5=\frac{\dfrac{S}{K_S\left(1+\dfrac{I}{K_I}\right)+S}}{\dfrac{S}{K_S+S}}$$

$$I=K_I\left(\frac{S}{K_S}+1\right) \tag{2.22}$$

Competitive inhibitors play an important role in chemotherapy, we know many pharmaceuticals that act as competitive inhibitors of specific important enzymes of the target cells. For some microbes (causing various contaminations in humans) p-amino-benzoic acid is a vitamin-like compound and it is showing structural similarity with several sulfonamide-drugs (Ultraseptyl, Superseptyl, Sulpha-guanidine etc).

The antibiotic cycloserine is similar to the amino acid alanine. In both cases the drug competes with the substrate of an important key enzyme of the contaminating microorganism, drastically lowers the rate of the enzymatic transformation, that would be vital for the microbes, thus kills them.

Other classical example of competitive inhibition is the succinate-dehydrogenase (EC 1.3.99.1) and its substrate tartrate and its competitive inhibitor substrate analogue malonate (Fig 2.31.). It is interesting that the product of this enzyme - fumarate - is also a competitive inhibitor ($K_i = 1{,}9\cdot 10^{-3}$ Mmol).

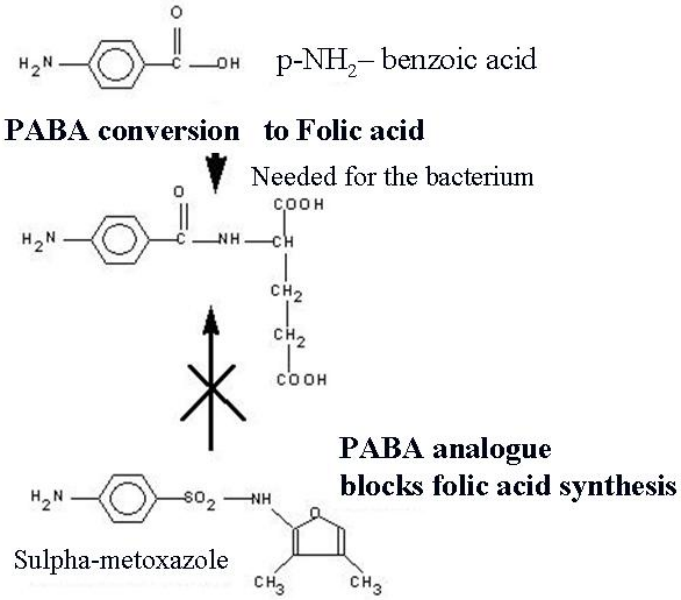


Fig 2.31.: Sulfa drugs are competitive inhibitors

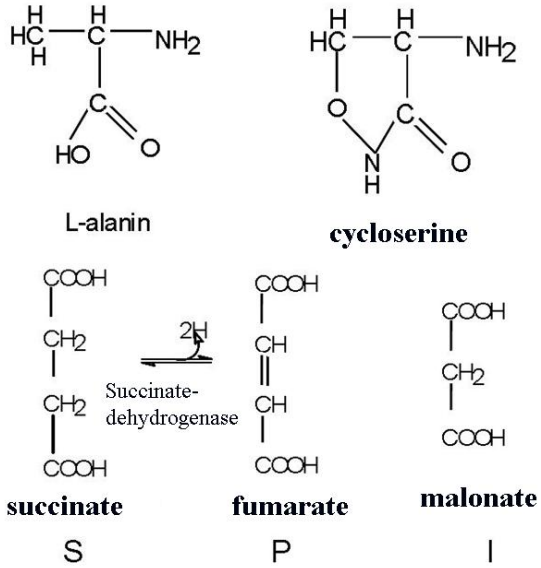


Fig 2.32.: Competitive inhibitor examples

It is not difficult to recognize the similar forms of eq (2.19) in the following analogies:

competitive product inhibition:

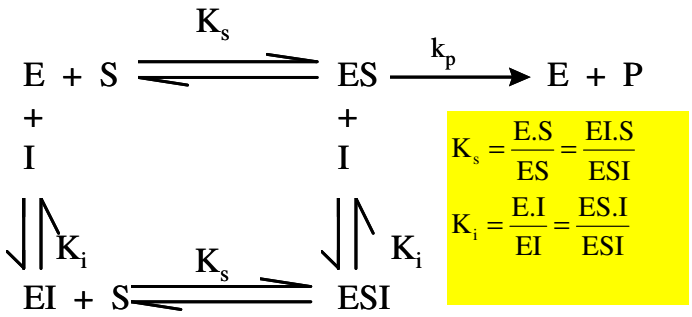
$$V = V_{max} \frac{S}{K_s\left(1+\frac{P}{K_P}\right)+S}$$

Alternative or competing substrates (e.g.: hexokinase: glucose, fructose):

$$V_1 = V_{1max}\frac{S_1}{K_S^1\left(1+\frac{S_2}{K_S^2}\right)+S_1} \qquad V_2 = V_{2max}\frac{S_2}{K_S^2\left(1+\frac{S_1}{K_S^1}\right)+S_2}$$

2.4.2. Non-competitive inhibition

A non-competitive inhibitor does not have influence onto the binding of the substrate and vice versa. I and S incidentally, reversibly and independently bind *to different binding sites* of the enzyme molecule, i.e I and E produce complex (EI), and S and E complex (ES), but at the same time a terner (ESI) complex may also be established as the next scheme shows:.



With a similar way of thinking, followed so far, we can write (supposing here the rapid equilibrium kinetics).

$$\frac{V}{V_{max}} = \frac{ES}{E+ES+EI+ESI},$$

from where, with a bit rearrangement – expressing every complex with E and Ks or Ki, we get the rate equation of the competitive inhibition:

$$\frac{V}{V_{max}} = \frac{\frac{S}{K_s}}{1+\frac{S}{K_s}+\frac{I}{K_i}+\frac{S.I}{K_sK_i}}$$

vagy

$$\frac{V}{V_{max}} = \frac{S}{K_s\left(1+\frac{I}{K_i}\right)+S\left(1+\frac{I}{K_i}\right)} \qquad (2.23)$$

illetve

$$V = V_{max}\frac{1}{\left(1+\frac{I}{K_i}\right)}\frac{S}{K_s+S}$$

Introducing V_{maxi} apparent maximum velocity, (2.23) can be rewritten as follows:

$$V = V_{maxi}\frac{S}{K_s + S} \quad \text{ahol} \quad V_{maxi} = V_{max}\frac{1}{1+\frac{I}{K_i}}$$

In this case inhibitor changes the value of maximum velocity while does not change the value of K_S (or K_m).This means, that inhibitor binds to another binding site and does not influence the binding of the substrate – does not change the affinity of the enzyme to the substrate. It is important that classic noncompetitive inhibition exist only in the case of rapid equilibrium, i.e. $K_S=K_m$.

On the next Fig 2.33 characteristic plots of noncompetitive inhibition are presented.

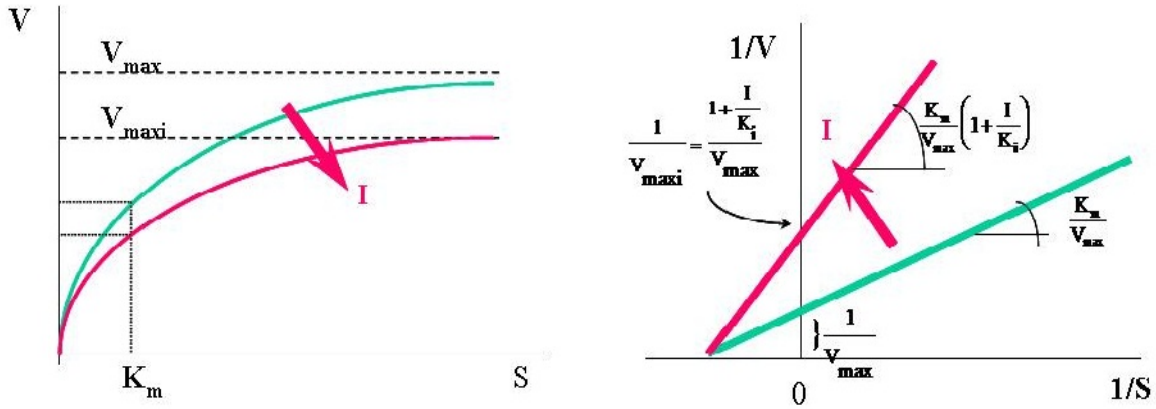


Fig 2.33: M-M and L-B plots of noncompetitive inhibition

Slope of the L-B strait line here is a similar linear function of the inhibitor concentration as in the competitive case, thus plotting the equation gives the same curve.

As an example of the noncompetitive inhibition, see the effect of H^+ ions onto chymotrypsin. Here in the active site, there is a proton acceptor place, which can be inhibited by increasing proton concentration. A L-B plot proves a pure noncompetitive inhibition but remember also to the complex activity influencing effect of pH.

Other examples are the heavy metals (SH-reagents) or the cyanides. With these – as we already have seen – there are always irreversible inhibitory effects, too.

Distinction can be done between a competitive and a noncompetitive inhibitor comparing the L-B plots (or of course the other linearization methods) as the Fig 2.34. shows.

Browning of apple slices on air is caused by a catechol-oxidase (this is an o-diphenol oxidase) enzyme that oxidases catechol to o-chinon. (A similar reaction is catalyzed by tyrosinase that converts tyrosine to melanin.) A competitive inhibitor of this enzyme is the substrate analogue p-hydroxy-benzoic acid while its noncompetitive inhibitor is the phenyl-thiourea. The very different kinetic behavior of these two can assumingly follow in Fig 2.34.

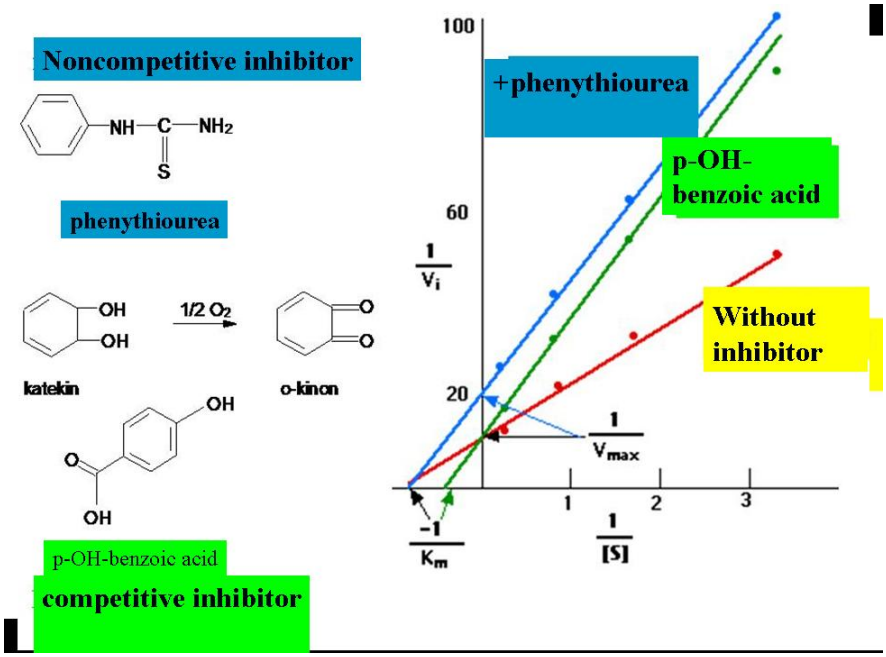
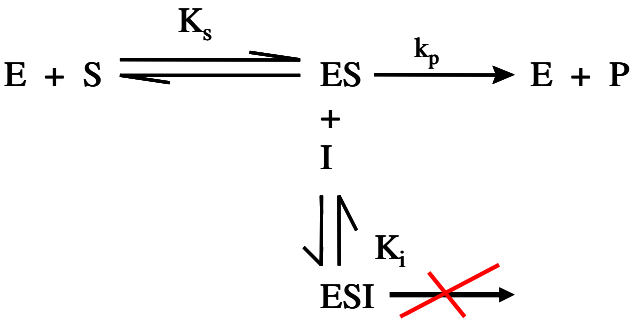


Fig 2.34.: Comp. and noncomp. inhibition of catechol-oxidase

2.4.3. Uncompetitive inhibition

An uncompetitive inhibitor is not able to bind to the free enzyme, merely to the formerly substrate-bound one. Thus an inactive (ESI) complex is formed, from which product does not releases. The simplified scheme is the following:



It strikes one's eye that even infinitely high S concentration cannot stop the effect of I – always there will be – depending on I and KI – nonproductive (ESI) complex. This can be imagined that on the original free enzyme there is no such a domain which is able to accept an inhibitor molecule, an inhibitor binding site is getting formed by a conformational change caused by a substrate molecule binding process (induced fit). But at the same time the active site also undergoes a conformational change, making (ESI) complex not able to form product anymore. Moreover, this ternary complex is more stable than the simple (ES) complex.

The previously good working method here also can be followed, and the resulted kinetic equation is:

$$\frac{V}{V_{max}} = \frac{\frac{S}{K_s}}{1 + \frac{S}{K_s} + \frac{SI}{K_s K_i}} \tag{2.24}$$

or

$$V = V_{max} \frac{S}{K_s + S\left(1 + \frac{I}{K_i}\right)}$$

The Briggs–Haldane approach gives the same form, with K_m. Unfortunately from this equation we cannot see whether V_{max} and/or K_m have been changed. To make this clear, two step rearrangement is necessary:

$$V = V_{max} \frac{1}{1 + \frac{I}{K_i}} \cdot \frac{S}{\frac{K_m}{\left(1 + \frac{I}{K_i}\right)} + S} \tag{2.25}$$

Let us observe that (2.25) is not a mixture of the equations of competitive and noncompetitive inhibition: while the effect on V_{max} is the same as in the case of noncompetitive inhibition but a reverse effect came in the case of K_m. Apparent K_m decreases. This shown on Fig 2.35., an uncompetitive inhibitor decreases both, V_{max} and apparent K_m by the same factor.

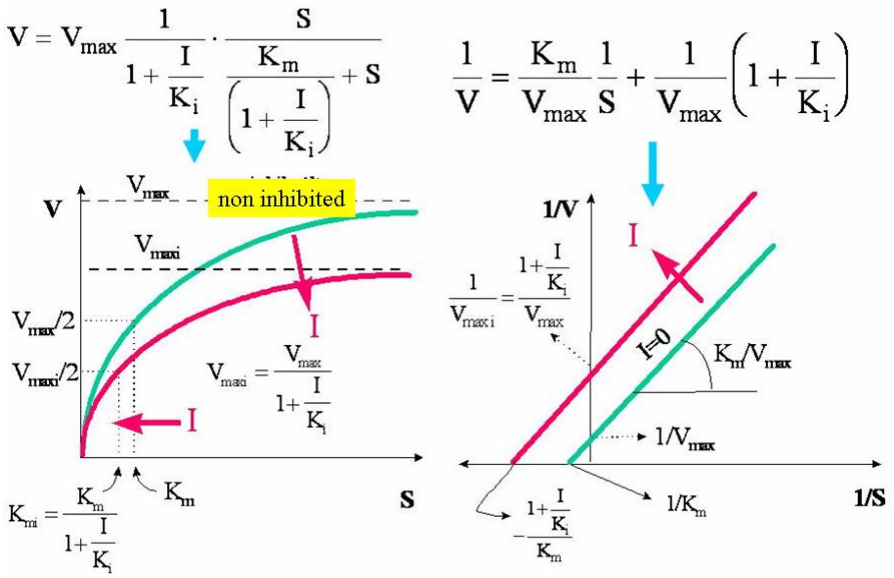


Fig 2.35.: Uncompetitive inhibition

Uncompetitive inhibitors have enormous effect on enzymatic reactions, moreover this effect increases with increasing substrate concentrations. This fact could be the explanation why uncompetitive inhibitors are so rare in the nature (contrary to competitive) and at the same time artificial such compounds are why so efficacious.

A good example is the Glyphosate (Roundup) [N-(phospho-methyl)-glycine (Fig 2.36.)], the well-known herbicide. It is an uncompetitive inhibitor of the 5-enol-pyruvil-shikimate-3-phosphate synthase [(ESPS)-synthase] which plays an important role in the synthesis of aromatic amino acids.

Along the inhibited reaction 5-enolpyruvil-shikimate-3P, a precursor of the essential chorismic acid is not produced consequently the plant will not be able to produce aromatic amino acids.

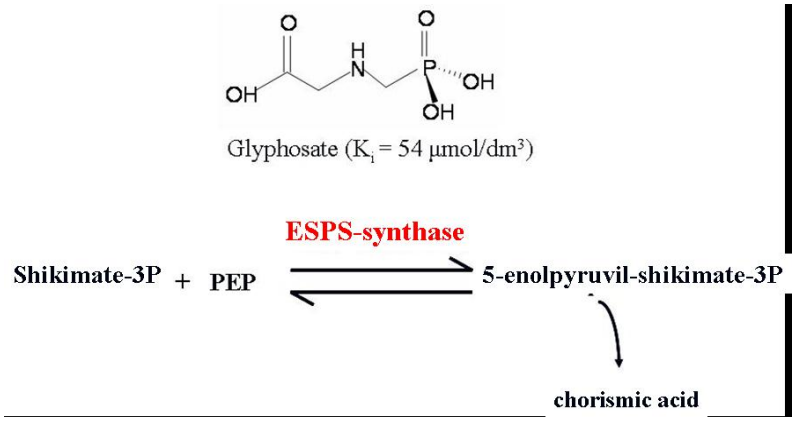
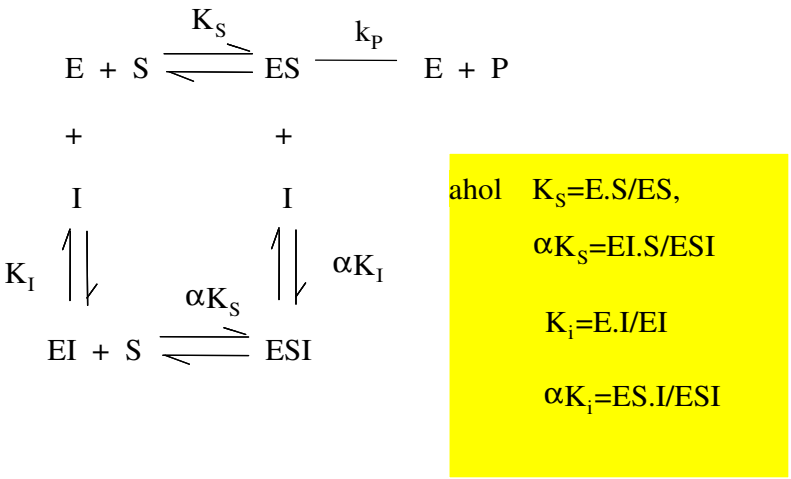


Fig 2.36.: Glyphosate is an uncompetitive inhibitor

2.4.4. Mixed inhibition

The next scheme shows the mechanism of the mixed inhibition, which is a special case of the noncompetitive inhibition. (As a matter of fact, the opposite is right!)



According to the scheme, presence of the inhibitor modifies the dissociation of the substrate from the enzyme, that is why the effective K_s for the step EI+S is αK_S. The same reason modifies the dissociation constant of ESI to αK_i. This means, with other words, that the equilibrium constant of the overall reaction E ⇌ ES ⇌ ESI and E ⇌ EI ⇌ ESI is independent upon the way of the reaction, i.e.

$$K_{eq} = \frac{1}{K_s(\alpha K_i)} = \frac{1}{K_i(\alpha K_s)}.$$

With the ordinary method of deduction as well as applying the mnemotechnical aid we can get the kinetic equation (2.26). Here both, K_S, and V_{max} are modified as a function of the inhibitor concentration. Characteristic plots are in Fig.2.37.

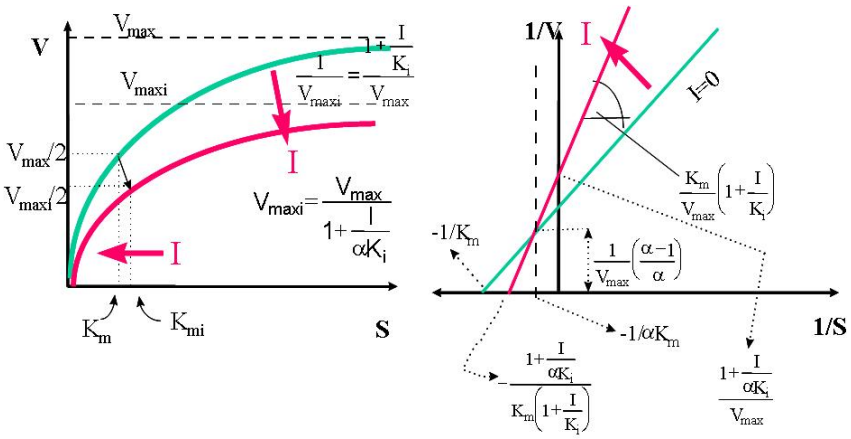


Fig 2.37: Mixed inhibition ($1<\alpha<\infty$) ($K_S = K_m$)

Looking deeply into the starting scheme, even all the submitted types of inhibitions would have been deduced from that scheme. Some textbooks follow that way.

$$\frac{V}{V_{max}} = \frac{\frac{S}{K_s}}{1+\frac{S}{K_s}+\frac{I}{K_i}+\frac{S.I}{\alpha K_s K_i}}$$

or slightly rearranged

$$V = V_{max}\frac{S}{K_s\left(1+\frac{I}{K_i}\right)+S\left(1+\frac{I}{\alpha K_i}\right)}$$

or

$$V=V_{max}\frac{1}{\left(1+\frac{I}{aK_I}\right)}\cdot\frac{S}{K_s\cdot\frac{\left(1+\frac{I}{K_I}\right)}{\left(1+\frac{I}{aK_I}\right)}+S} \qquad (2.26)$$

2.4.5. Summary of linear reversible inhibitions

If in a one-substrate reaction an inhibitor forms a complex with the enzyme and from this there will not be product release, then

- if the S and I mutually exclude each other from the enzyme, **I is a competitive inhibitor**.
- if the binding is not mutually exclusive but they can bind independently to the enzyme and they do not influence the binding of each other, **I is a noncompetitive inhibitor**,
- if inhibitor binds the same way, but I and S influences the binding force of each other, **I is a mixed inhibitor**,
- if I binds only if S has already bound to the enzyme, **I is an uncompetitive inhibitor.**

All the characteristic kinetic plots are summarized in Fig 2.38.–2.42.

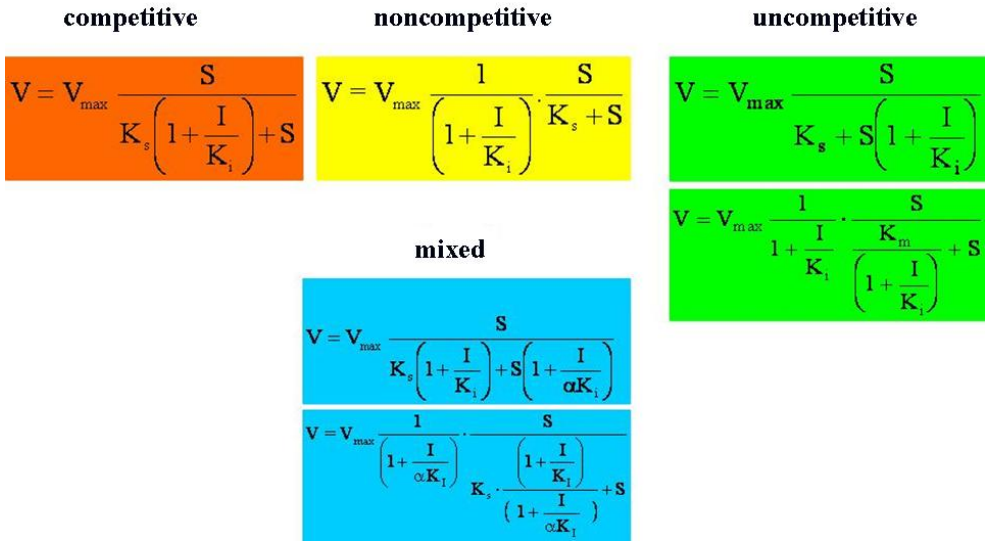


Fig 2.38.: Summary of kinetic equations of linear reversible inhibitions

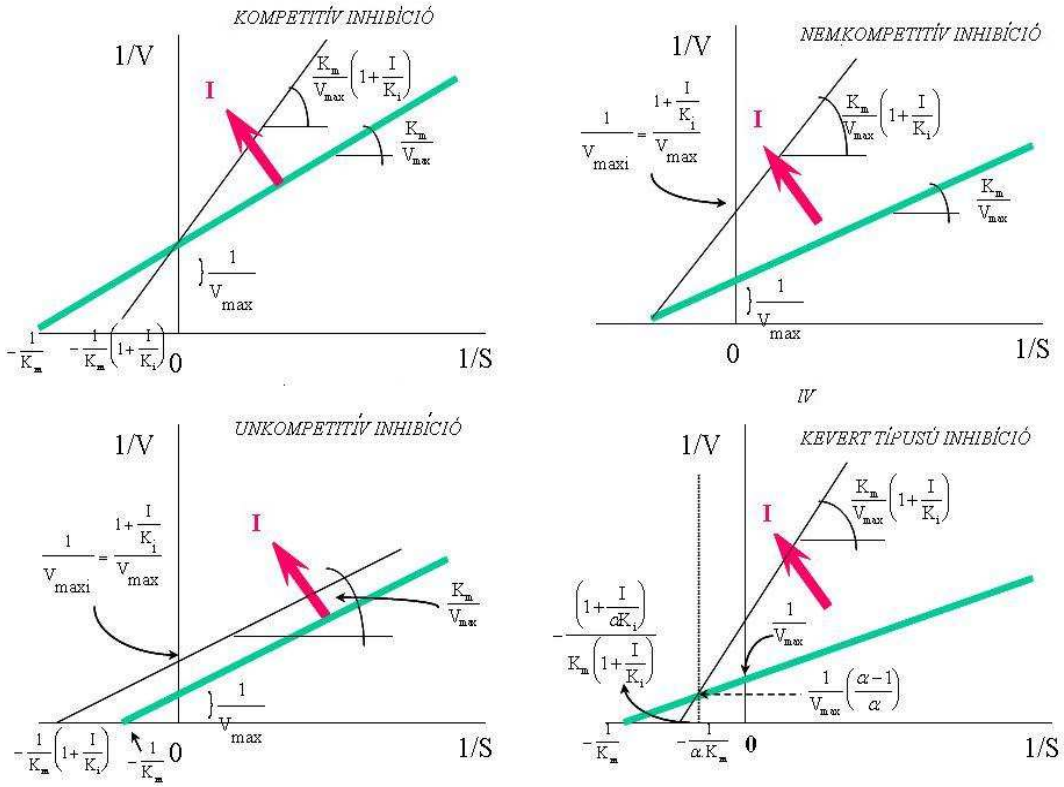


Fig 2.39.: Lineweaver–Burk-plots of linear reversible inhibitions

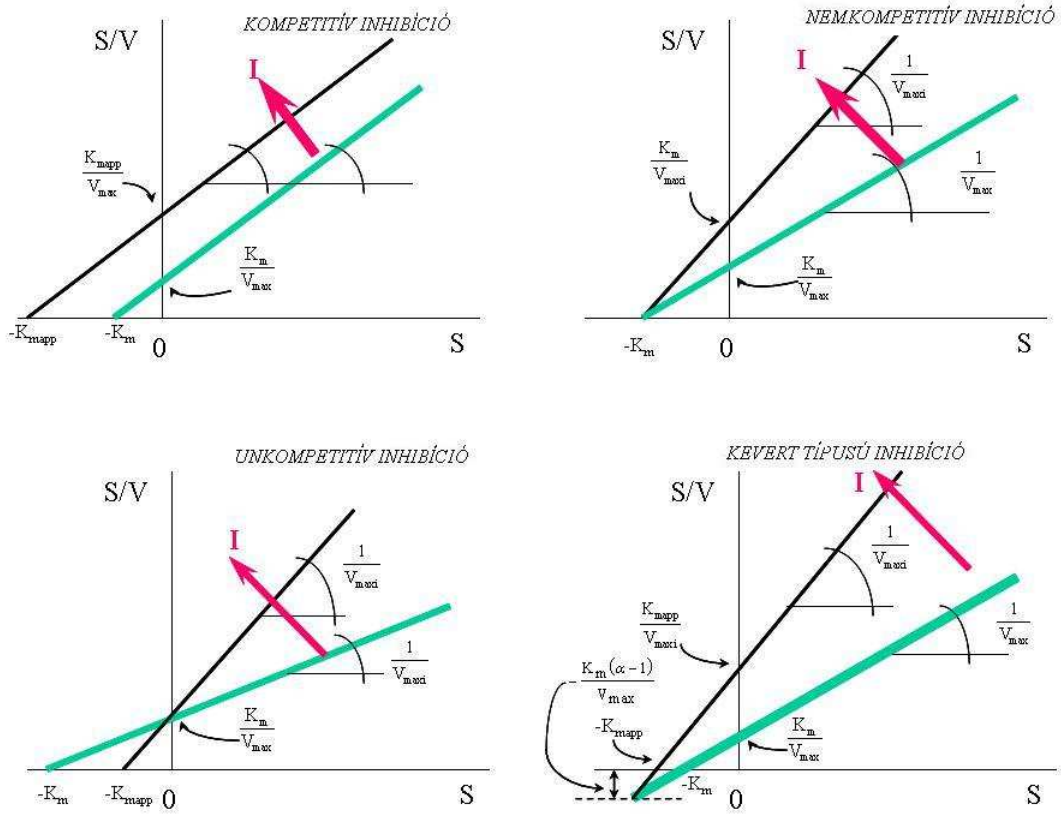


Fig 2.40.: Hanes–Langmuir plots of linear reversible inhibitions

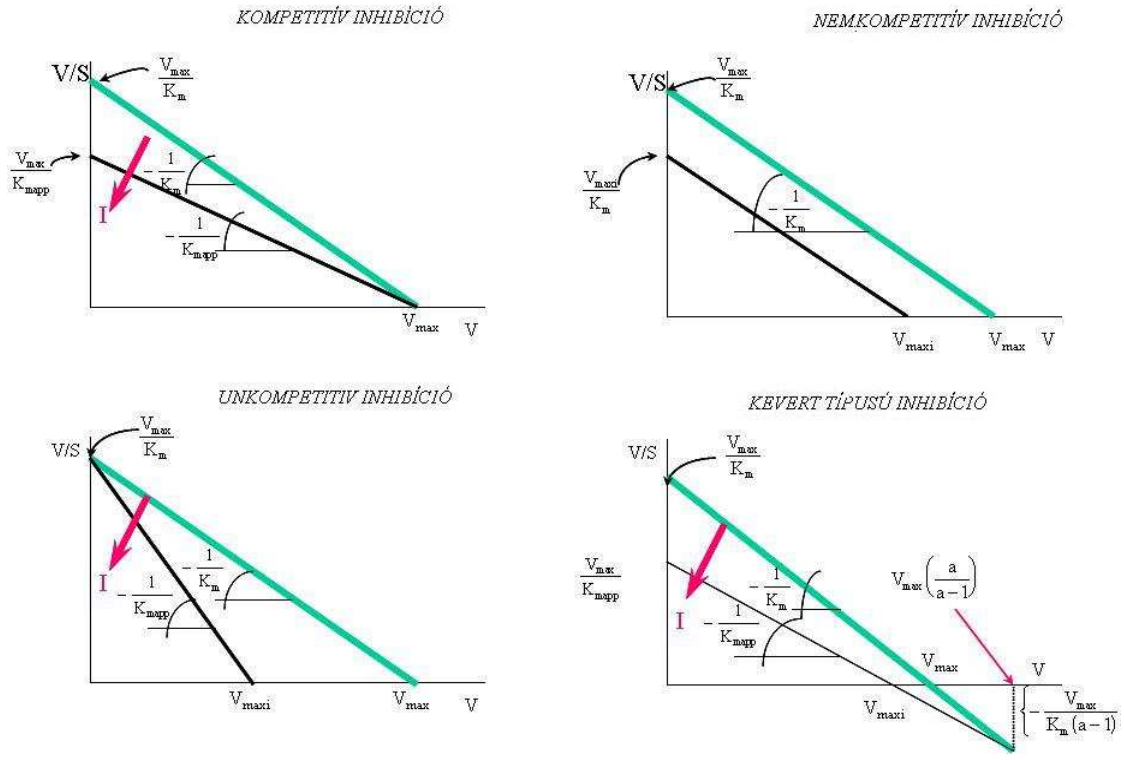


Fig 2.41.: Eadie–Hofstee-plots of linear reversible inhibitions

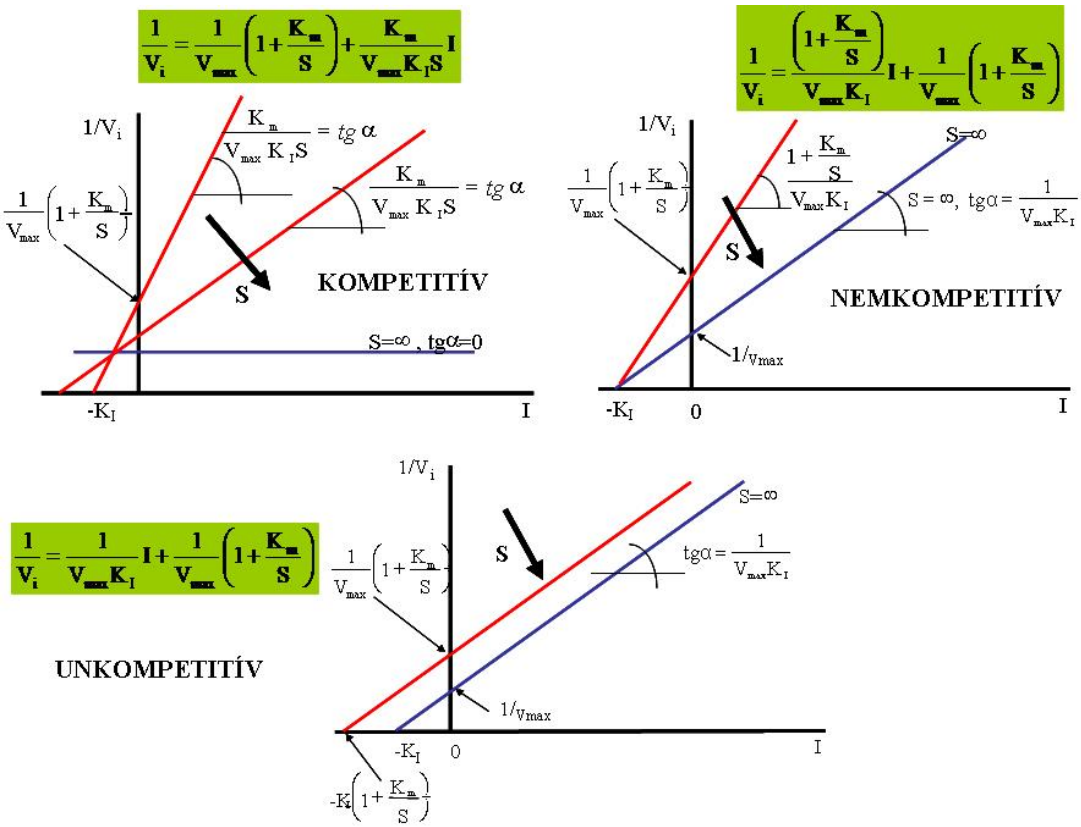


Fig 2.42. : Dixon-plots of linear reversible inhibitions

2.4.6. Substrate inhibition

In numerous instances reaction rate - substrate concentration curve has a maximum. Before the maximum the curve follows the M-M kinetics but with increasing S differs from that with increasing extent and in the range of high S concentrations reaction rate really decreases and goes to zero. This phenomenon is called substrate excess- or simply substrate inhibition. The reason and its mechanism can be the following:

– In order to get a productive complex, substrate must bind to the enzyme at two (or more) different sites (see the example of succinate dehydrogenase on Fig. 2.43). If there are many S molecule present, it is possible that one of the substrates joins the first site, but another S molecule binds to the other and this way inactive complexes are getting formed. This is also a reversible kind of inhibitions.

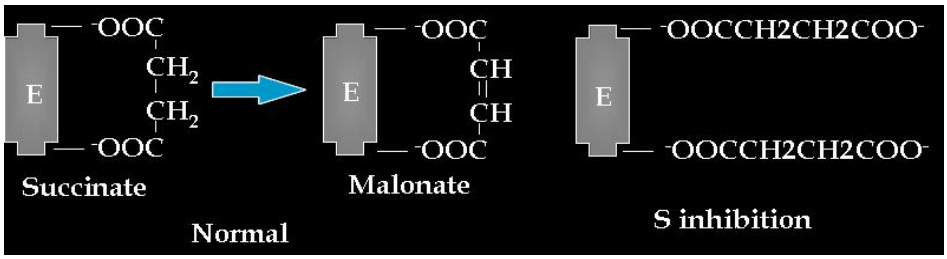


Fig.2.43.: Substrate inhibition of succinate dehydrogenase

– At high S concentrations one of the substrates may bind to a site which is not part of the active site and causes a practically noncompetitive inhibition and prevent the binding of another substrate molecule.

– Sometimes for the proper enzyme operation an activator is needed. If this activator is able to connect to the substrate, the formed complex practically lowers the substrate i.e., removes a part of the enzyme activity.

– In the case of two or more substrate reactions, the excess of one S may occupy the binding site of the other, thus again inactive complexes may be formed.

– Finally, it is possible that high S concentration simply inhibits the enzyme in an aspecific mode, e.g., by the increasing ionic strength.

It can be demonstrated that in these cases the kinetic equation may be written as

$$V=V_{max}\frac{1}{1+\frac{K_s}{S}+\frac{S}{a^*K_s}} \tag{2.27}$$

(Let us note that this equation can also be got from the scheme of uncompetitive inhibition – supposing the inhibitor is the same as the substrate.) The plots of substrate inhibition are shown in Fig.2.45. The Lineweaver–Burk-plot is a superposition of a straight line (that is one of the asymptotes of the hyperbole and it corresponds to the non-inhibited part of the curve at small S concentrations) and a hyperbole (inhibited part at high S concentrations). The extrapolation of the lower asymptote gives real V_{max} value that would be if there were no S inhibition – thus it is a nonexistent V_{max}. The experimentally measurable maximum velocity is lower than that.

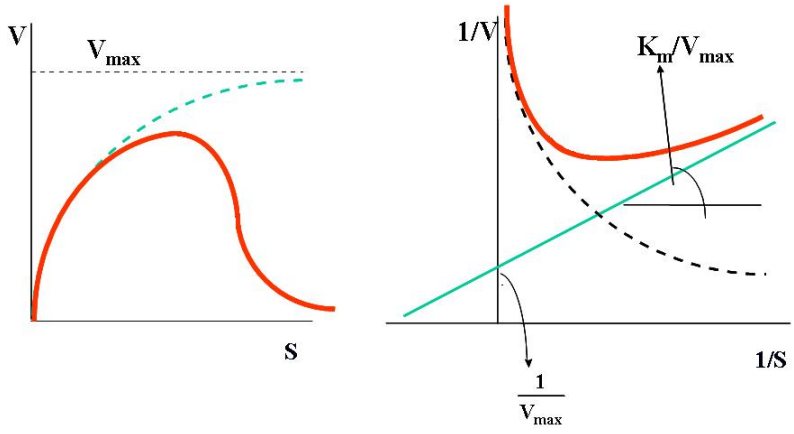


Fig 2.45.: Substrate inhibition

2.6. Other effects on enzyme activity

So far, we have seen the effects of substrate and inhibitor concentrations on the enzyme activity. It is obvious that many other features of the *in vitro* reaction mixture or the natural *in vivo* cell milieu may also and really does influence the catalytic activity of an enzyme. These act through the modification of the enzyme structure (spatial structure, conformation) and the chemical conditions. Such effects are:

Ionic strength (see the exploitation of this at the classical ammonium-sulphate saturation methodology in the first step of protein purification methods), **pH, temperature, hydrodynamic (shear-) forces, hydrostatic pressure, surface tension. Also, there are chemicals influencing enzyme activity: small molecule alcohols** (they are also used protein coagulation methods) **urea,**

hydrogen-peroxide (and other oxidizing agents) **or various waves, like light, or ionizing radiations or ultrasonic vibration.**

The result of these outer effects may cause reversible or irreversible change of enzyme activity. The reversibility or irreversibility often depends on the duration time of the influences, that is understandable if taking into account the structure-activity relations.

Next the two most important environmental effects will be submitted: pH and temperature.

2.6.1. Effect of pH on enzyme activity

The protein building amino acids contain neutral, basic or acidic groups, side chains. Thus, in a solution the native enzyme holds either negatively or positively charged groups at different pH. These ionizable groups take part in the formation of active sites so these active sites hold partially negative or positive charges, furthermore the catalytic activity often based upon simple acid/alkali catalyzes that demands a given ionized state of the active site. Thus, it is easily understandable that pH can change the actual charge of the sites, it has a very strong effect on overall activity. The ionizable groups are: second –COOH of Asp, Cys -SH, Glu -COOH and Gln -$CONH_2$ groups, imidazole group of His,
ε-NH_2 group of Lys, CH_3-S of Met, and -OH groups of Ser as well as terminal amino- and carboxyl-groups. Besides these either the electrostatic interactions and the tertiary structure stabilizing H-bonds are also under the influence of the environmental pH.

To give a more quantitative picture of these rather complicated interactions, let us look at the following very simple **model**: let E^- the active enzyme concentration at a given acid/alkali type catalysis while the E, and the double ionized E^{2-} are inactive forms, but they can go into each other by proton addition or elimination:

$$\mathbf{E} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} \mathbf{E^-} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} \mathbf{E^{2-}}$$

inactive enzyme active enzyme inactive enzyme

$$\mathbf{E} \rightleftharpoons \mathbf{E^- + H^+}$$
$$\mathbf{E^-} \rightleftharpoons \mathbf{E^{2-} + H^+}$$

Since equilibrium constants of these two reactions are $K_1=H^+E^-/E$, and $K_2=H^+E^{2-}/E^-$, furthermore the $E_0=E+E^-+E^{2-}$ material balance equation holds, active ratio of the enzyme ($Y^-=E^-/E_0$) can be given as follows:

$$Y^- = \frac{1}{1 + H^+ / K_1 + K_2 / H^+} \tag{2.34}$$

Similarly, the Y és az Y^{2-} functions can also be expressed. These so called *Michaelis-pH functions* are shown on Fig 2.49.

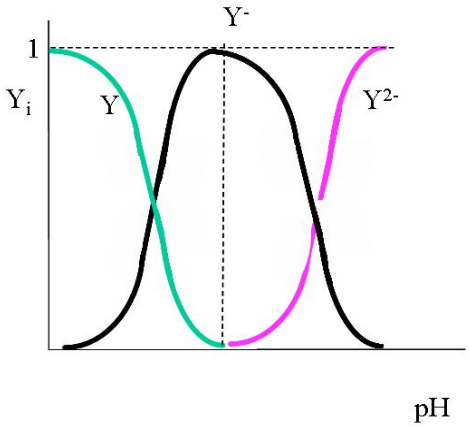


Fig 2.49.: Michaelis pH- functions

The pH optimum is where Y^- has a maximum:

$$H^+_{optimum} = \sqrt{K_1 K_2} \quad \text{vagy} \quad (pH)_{optimum} = \frac{1}{2}(pK_1 + pK_2) \tag{2.35}$$

The Michaelis-function of the active enzyme shows a curve that similar at every enzyme. All the enzymes have one (and only one) pH optimum.

For some enzymes these optimum values are shown in Fig2.50. Enzyme activity at the pH optimum can be got as

$$V_{max} = k_2 E_0 Y^- = k_2 E_0 / \left(1 + H^+/K_1 + K_2/H^+\right). \tag{2.36}$$

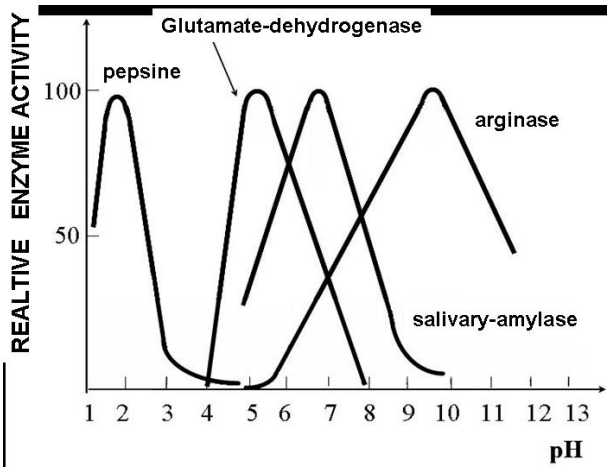


Fig 2.50.: Optimum curves of various enzymes

It is worth to note that pH-optimum of an enzyme is the same as the optimal pH for maintaining the enzyme, here is the highest its stability.

2.6.2. Effect of the temperature on enzyme activity

Temperature has a double effect on enzyme activity. With increasing temperature, the rection rate increases, according to the Arrhenius-equation. At the same time, with increasing temperature the rate of conformational changes =denaturation also increases; thus, the reaction velocity decreases. These two happenings have an overall effect: we get a maximum activity point at a given temperature; this is the optimum temperature. For the most proteins observable denaturation starts at about 45–50 °C, but *both temperature effects are continuously increasing*!
Heat denaturation can be reversible as well as irreversible or partially both at the same time.

Let us suppose that our enzyme is in two forms, an active and inactive:

$$E_a \underset{}{\overset{K_d}{\rightleftharpoons}} E_i.$$

The equilibrium constant is $$\frac{E_i}{E_a} = K_d = \exp\left(\frac{-\Delta G_d}{RT}\right) = \exp\left(\frac{-\Delta H_d}{RT}\right)\exp\left(\frac{\Delta S}{R}\right).$$

A ΔH_d deactivation enthalpy is rather high, e.g. for trypsin and egg white lysozyme are 280 and 310 kJ/mol, respectively (see the same for hydrogen-bonds: only 12,5–29,3 kJ/mol), and the deactivation entropy is 890 kJ/mol·°K. Considering the high ΔH_d the ratio of inactive enzyme strongly increases with the increasing temperature, e.g. in the former examples a 30 °C temperature rise causes almost total inactivation.

Since enzyme is in active and inactive form, the material balance equation holds, $E_0 = E_a + E_i$, thus:

$$E_a = \frac{E_0}{1+K_d} \quad \text{and} \quad V_{max} = k_2(T)E_a$$
$$\text{where} \qquad (2.37)$$
$$k_2(T) = \beta\left(\frac{k_B T}{h}\right)e^{\Delta S^*/R}\cdot e^{-E/RT}$$

In this k_B and h are the *Boltzmann*- and *Planck*-constants, respectively and β is a proportionality constant.

Combining the three equations, the reaction rate simply gives a curve having a maximum

$$V_{max} = \frac{\alpha T e^{-E/RT}}{1 + e^{\Delta S^*/R}\cdot e^{-\Delta H_d/RT}}, \qquad (2.38)$$

where α contains all the parameters β, k_B, h, E_0 és ΔS^*.

On the Fig 2.51.the temperature functions of the catalase enzyme is shown.

Temperature caused denaturation is a time process with a first order reaction velocity:

$$\frac{dE_a}{dt} = -kE_a \qquad (2.39)$$

The solution of this gives an exponential function, i.e. the activity of an enzyme at a given (high) temperature decreases exponentially in respect of the time:

$$E_a(t) = E_{a0}e^{-kt} \qquad (2.40)$$

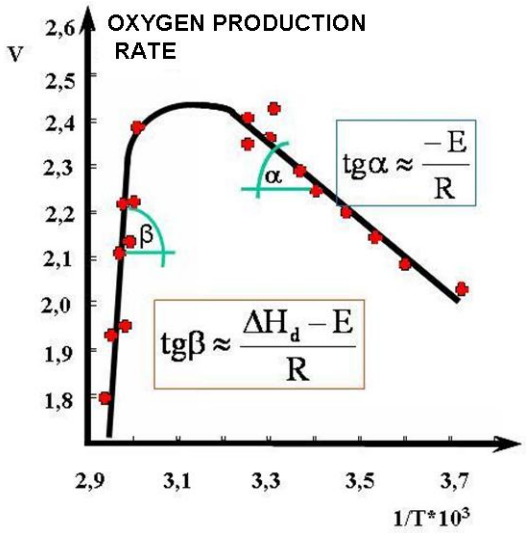


Fig 2.51. Temperature dependence of the activity of an enzyme

Operation optimum and maintenance temperature does not the same, enzymes in buffers are usually kept in 4–5 °C.

2.7. Heterogeneous enzyme systems

Enzymes are globular proteins generally of some ten thousand Dalton molecular weight, thus they are usually water soluble. A homogeneous enzyme system in water phase has an advantage, the homogeneity itself and the enzyme does not need any previous preparation befor use. But these are the only advantages, while there are numerous disadvantages.

Economic disadvantages:

Enzymes are expensive (comparing to common chemicals).
In a homogeneous system they can be used only once, after the reaction they has to be discarded and /or contaminate the products. They isolation from the reaction mixture in order to reuse, is expensive and unworthy.

Technological pitfall:

Because the former, they make unwanted components in the final reaction mixture, making the isolation and purification processes more difficult.

All of these disadvantages can be overcome by immobilizing the enzymes i.e. changing the system inhomogeneous by immobilizing the enzyme *in or onto* some carrier separating it from the homogenous water phase, in which the substrate and the product are present.

Main advantages of immobilized enzyme systems are:

Enzyme particles can easily be separated from the reaction mixture, so isolation and purification of the product becomes easier and cheaper.
Separated enzyme particles can be reused.
Continuous systems can be applyed, enzyme is the load of the reactor and substrate is continuously added to, while product is continuously removed from the reactor.

It is easy to terminate the reaction: ceasing the contact between the particulate enzyme and the homogenous phase.

Frequently a particulate enzyme is more stable than a soluble one, with increased temperature and pH stability.

Naturally there are disadvantages as well:

The immobilization process increases the expenses (process, carrier, etc.)

Most frequently the immobilization decreases the (specific) activity of the enzyme.

Since the carrier separates physically the enzyme from the substrate, access of the substrate may have spatial barriers, i.e diffusional rate limitations frequently occur.

Nelson and *Griffin,* in 1916, accidentally discovered that bakers yeast invertase saved its saccharose hydrolyzing activity when adsorbed to activated charcoal as well as to aluminum-hydroxide precipitate. Immobilization of enzymes became a real practice when *Grubhofer* és *Schleith* immobilized a series of enzymes: carboxypeptidase, diastase, pepsin and ribonuclease. They immobilized these enzymes by diazotation.

The first industrial application of immobilized enzymes was introduced by *Chibata* who immobilized amino acylase enzyme onto DEAE-Sephadex by ionic adsorption in 1969 and applied this particulate enzyme for the resolution of N-acyl-D, L-amino acids.

2.7.1. Methods of enzyme immobilizaton

There are physical and chemical methods of enzyme immobilization. Looking at these methods two questions arise: with what a method and where to immobilize?

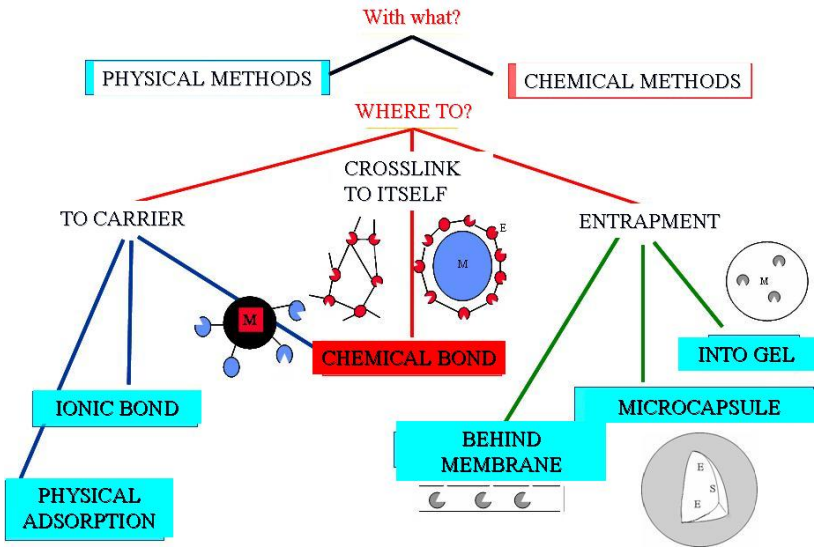


Fig 2.52. What kind of immobilization methods and where to?

There are two groups of chemical immobilizations: onto a carrier and crosslinking of enzyme molecules.

Covalent bonds can be created between a water insoluble matrix with activated functional groups and the non-essential groups of the enzyme. The carrier can be some natural polymers: agar, agarose, chitin, chitosan, cellulose, collagen, etc. or synthetic polymers like polyurethane, polystyrene, nylon etc., or inorganics like glass, aluminum, silica gel, magnetite and so on.

On the protein itself the free α-, β-(Asp) or γ (Glu)-carboxyl groups, α- or other amino groups, and phenyl-, hydroxyl-, sulfhydryl- or imidazole groups may take part in the formation of the covalent bonds.

There are two essential steps of chemical immobilization:

1. activating the functional groups of the carrier, that creates a reactive group-ended arm on the carrier, and then
2. forming the covalent bond between the enzyme and the activated carrier.

Some methods of chemical immobilizations are given in the following figures.

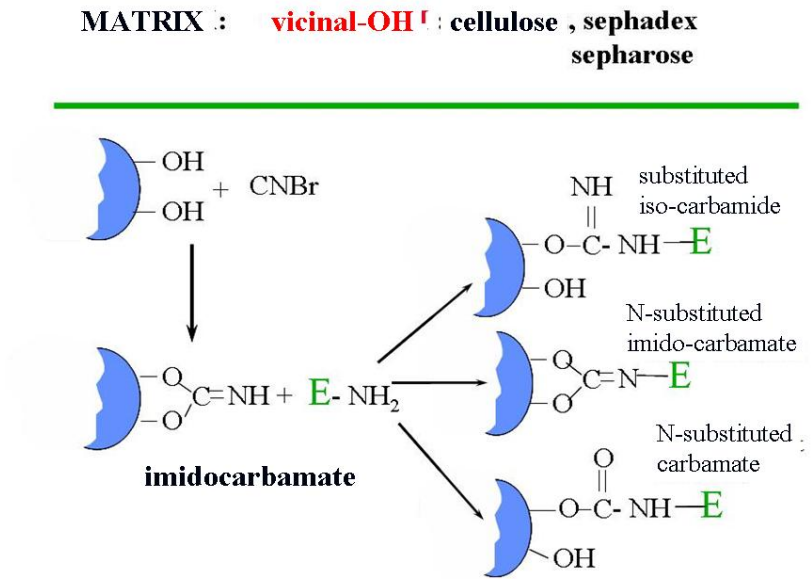


Fig 2.54.: Brome-cyanide immobilization

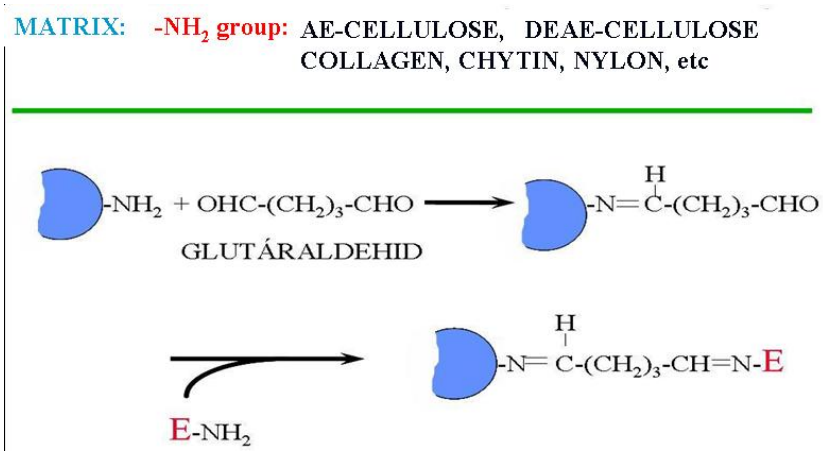


Fig 2.56.: Chemical immobilization with glutarealdehyde

Surprising, but used method is the immobilization onto glass beads with which strong, mechanically stable column loads can be prepared.

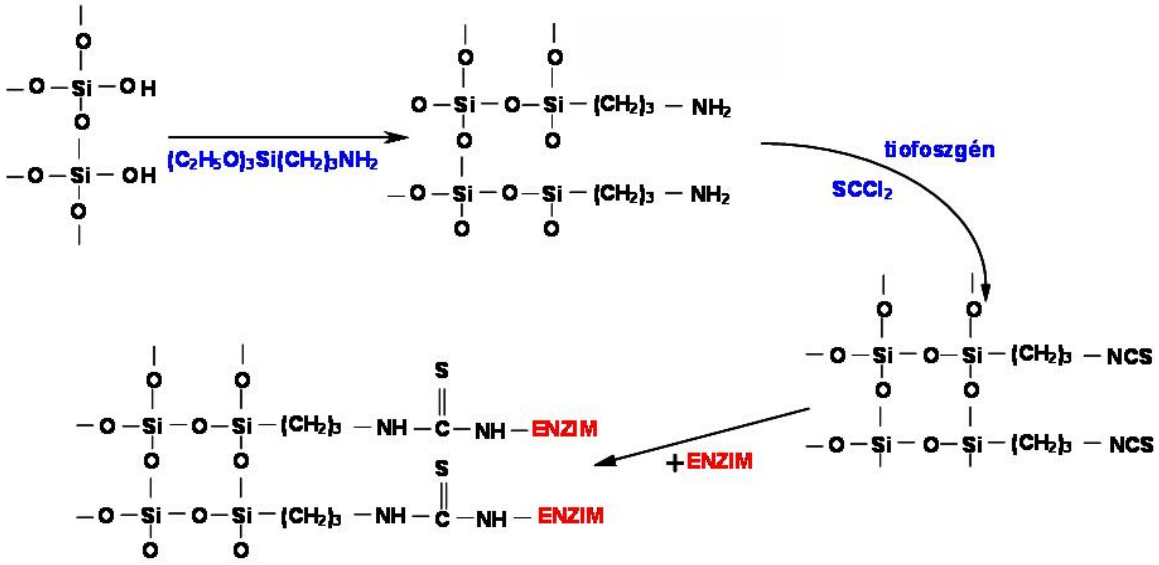


Fig 2.58.: Immobilization onto glass surface

Another chemical method is the crosslinking of enzyme molecules applying two- or many-functional reagents. Among these the most popular is the application of glutaraldehyde as shown in Fig2.59. Here a Schiff-base like covalent bond is formed between the aldehyde and some amino-groups of the enzyme, thus polymerizing the enzyme molecules, and forming a supramolecular enzyme. The huge enzyme polymer becomes water insoluble, thus forms a gel. These are not really applicable in industry, because of their slight mechanical strength. That is the reason that indeed first a physical adsorption is performed onto the surface of some carrier and next the crosslinking of the enzyme molecules creates an immobilized layer on the surface of the carrier.

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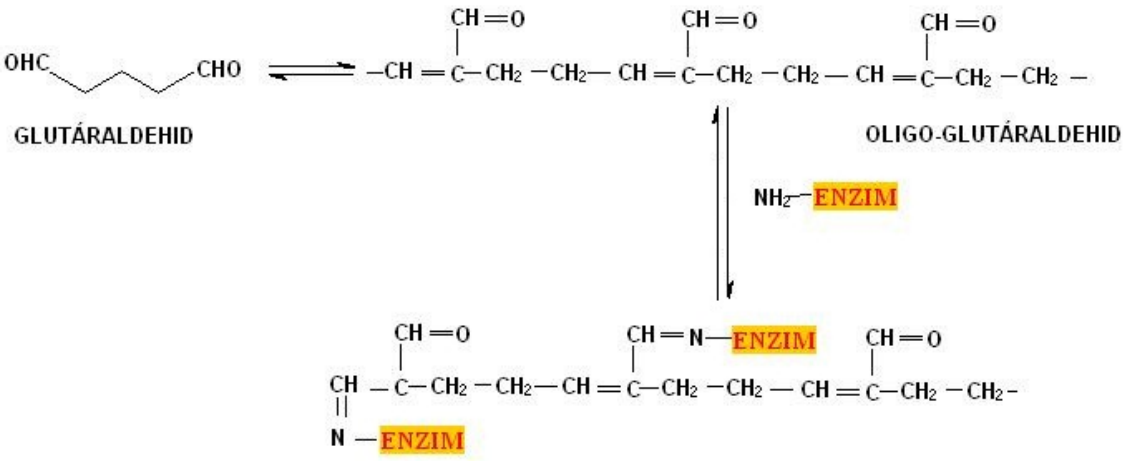


Fig 2.59. Crosslinking as an immobilization method

Usually, the crosslinking is performed together with an inert protein. Such proteins are the gelatin, albumin, collagen and egg white (latter is useful, for it contains lysozyme, a cell wall hydrolyzing enzyme, that prevents the microbial contamination).

Enzyme crystallization is also realizable by crosslinking. The result CLECs (crosslinked enzyme crystals) have very good catalytic features, the enzyme can be a column load itself and the activity is very high, not being too much inert matter present. Fig 2.60 shows such a CLEC (laccase), that have huge surface of 2500 m^2/g [4].

A more recent technology is the CLEA formation (crosslinked enzyme aggregates), where the glutaraldehyde crosslinking is performed *during* precipitation of the enzyme by $(NH_4)_2SO_4$, or butanol. This way the immobilization is combined by a partial purification step of the enzyme. CLEA formation has the advantage that it can be realized either with purified or not purified enzyme solutions, the aggregates have large heat stability, and they are resistant against organic solvents and proteolysis. Thus, they can be applied for biotransformations in organic phase, too.

In the case of combi-CLEA two or even more enzymes are immobilized together, making possible to catalyze even series of reactions at the same time (consecutive reactions).

Chemical methods usually lower the remaining activity of enzymes because it is accidental whether a functionally important or an unimportant chemical group take place in the immobilization reaction. For this the active center needs some protection, which can be done by adding the substrate or preferentially substrate analogue (a competitive inhibitor) to the immobilizing reaction mixture.

Fig 2.60.: Crosslinked enzyme crystals (CLEC)

There are three main groups of physical immobilization methods (Fig 2.52.):

– *adsorption* onto the surface of some carrier,
– Encapsulation of the enzyme into a polymer matrix,
- retention of the (even soluble) enzyme by membranes that are permeable for substrate and product but impermeable for the enzyme.

Adsorption is performed frequently on **ion exchanger** resins. The method is simple, but the enzyme easily comes off the resin when changing pH or ionic strength. Moreover, it is not specific, a series of contaminating matters can also adsorb with the enzyme.

The most widespread and simplest method of encapsulation is the application of **alginate gels**. The enzyme and its buffer solution is mixed with a sodium-alginate solution, then slowly is dripped into an agitated Ca^{++} ion containing buffer solution. An ion exchange will occur between the sodium

[4] Preparation and characterization of cross-linked enzyme crystals of laccase
J. Jegan Roy, T. Emilia Abraham: Journal of Molecular Catalysis B: Enzymatic 38 (2006) 31–36

and calcium ions and since Ca-alginate is water insoluble, the alginate is getting precipitated in the form of small beads encapsulating the enzyme molecules. During the work with an alginate encapsulated enzyme a certain level of Ca-ions has to be maintained to prevent the resolution of the beads. There are no commercial such enzyme preparations, experimenter has to prepare them oneself. As a curiosity we mention that Na-alginate - Ca-alginate transformation is also used in modern gastronomy: e.g. in-mouth-melting „caviars" can be prepared with them.

Other gelforming polymers are also used for enzyme encapsulation purposes: chitosan, carrageenan. The approximate structures of such gel forming polymers can be seen in Fig 2.61

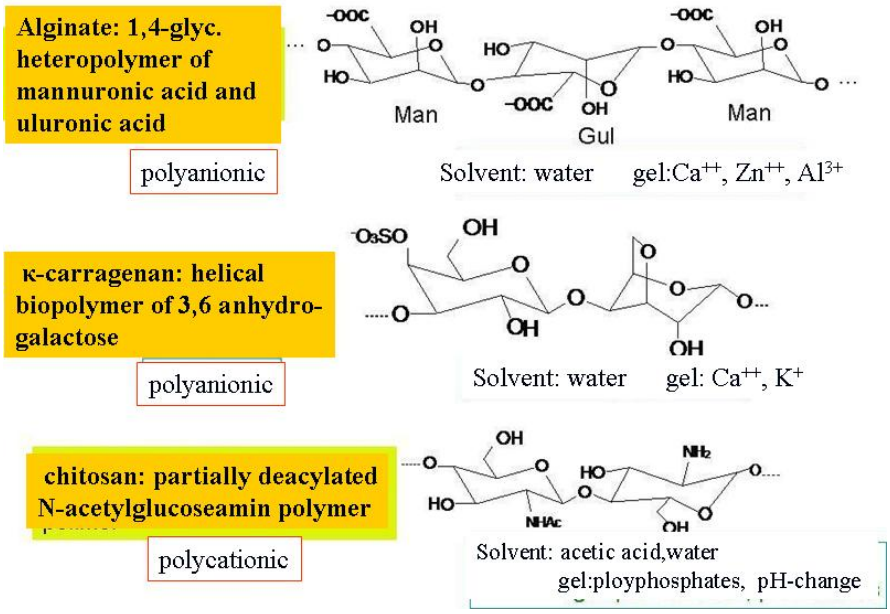


Fig 2.61. Gel forming polymers for enzyme immobilization

Stable, mechanically strong, thus easily applicable immobilized enzymes can be prepared by polyacrylamide–gel formation. The acrylamide and N,N'-methylene-bis-acrylamide monomers are mixed with the enzyme solution and with $K_2S_2O_8$ (potassium-persulphate) polymerization initiator and with β-dimetil-amino-propionitrile fastening agent during vigorous agitation. Agitation-depending smaller or bigger polyacrylamide gel beads are getting formed that contain the enzyme molecules. The measures of the beads-pores (100-400 nm) are much smaller than the measures of the enzyme molecules (300-2000 nm) thus while they remain in the gel particles the substrate and product molecules can more or less freely diffuse in and out the particles. This excellent method cannot be applied for food-industrial purposes because acrylamide is not GRAS (GRAS means „generally regarded as safe").

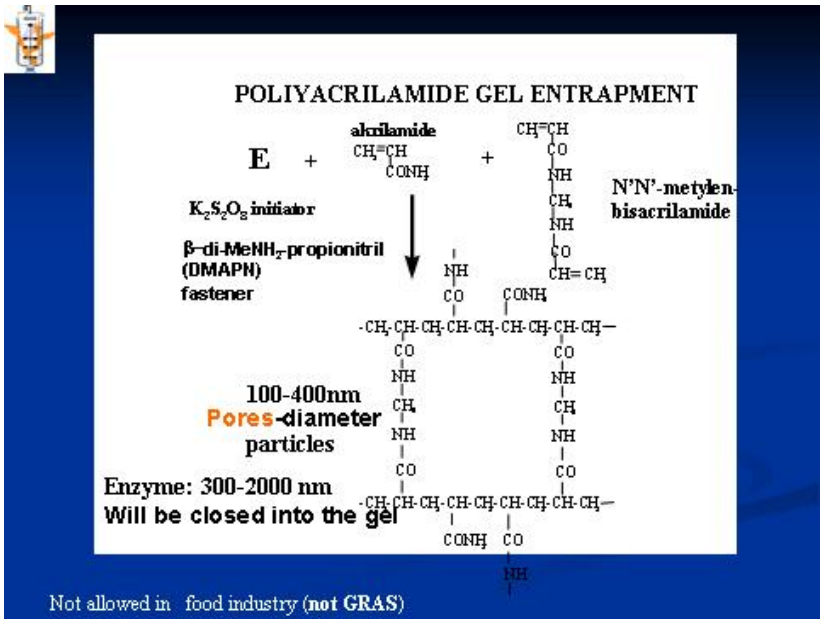


Fig 2.62. Polyacrylamide gel formation

Microencapsulation method of enzyme immobilization means we have very small capsules which are surrounded by a substrate-product transparent membrane that retains the enzyme molecules. Inside the capsules of about 300μm diameter is the buffer and the enzyme. There are two types of these capsules: capsules with a permanent membrane or capsules with non-steady coacervates.

The constant membrane capsule preparation method is the following. A water phase containing the enzyme and the buffers and a monomer that slightly soluble in water is vigorously mixed in an organic phase holding the other monomer to prepare a fine water/organic dispersion. Since there are chemical potential difference between the two phases in respect the two monomers, they start to diffuse opposite to each other. Meeting at the interfacial surface of the small water-phase droplets a polymerization starts and as a result a thin membrane polymer will cover the enzyme solution droplets.

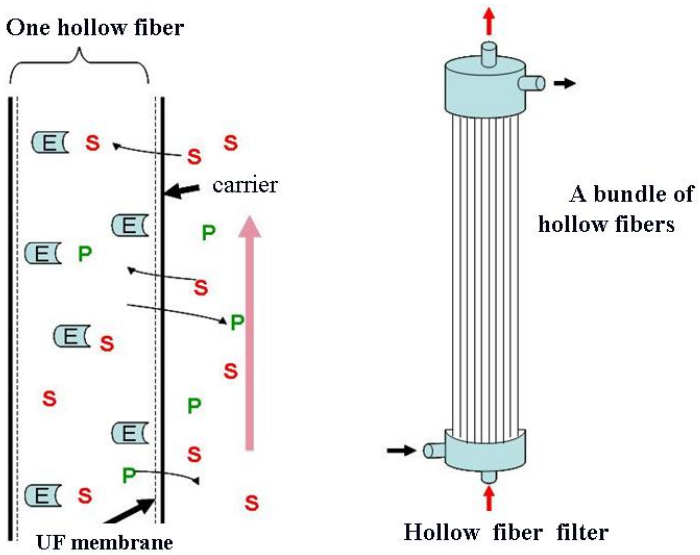


Fig 2.64.: Hollow fiber, UF membrane

Finally, the simplest immobilization method has to be mentioned, the **retention by ultrafiltration membranes**. This is not a real „immobilization", because the enzyme remains is soluble form, but it is separated by an UF membrane that permeable to the substrate and product but cuts off the

macromolecule enzyme. There are many kinds of technical set up of these membrane-immobilization but the most popular and scalable method is the application of hollow fiber units. (Fig 2.64)

2.7.2. Kinetic behavior of immobilized enzymes

Immobilized enzyme systems are special heterogeneous catalytic systems where the substrate to be converted must be transported onto the surface of the carrier particle or into the particle depending upon the applied immobilization method. In both instances there is a concentration gradient in respect the substrate and product between the bulk liquid and the place of the actual reaction. These concentration gradients are the driving forces of the diffusion processes of substrate and product. There are three well distinguishable regions of the transport according to the Fig 2.65.

1. A usually convective transport of the substrate from the bulk liquid to a stagnant liquid film around the particle. Here usually the mixing is perfect, there are no transport resistances.
2. Diffusion through the stagnant liquid film to the surface of the particle (if the immobilization was onto the surface of a carrier), here the transport is ended, then
3. diffusion into the inner side of the particle to the actual place of the reaction, if the enzyme is immobilized inside a particle.

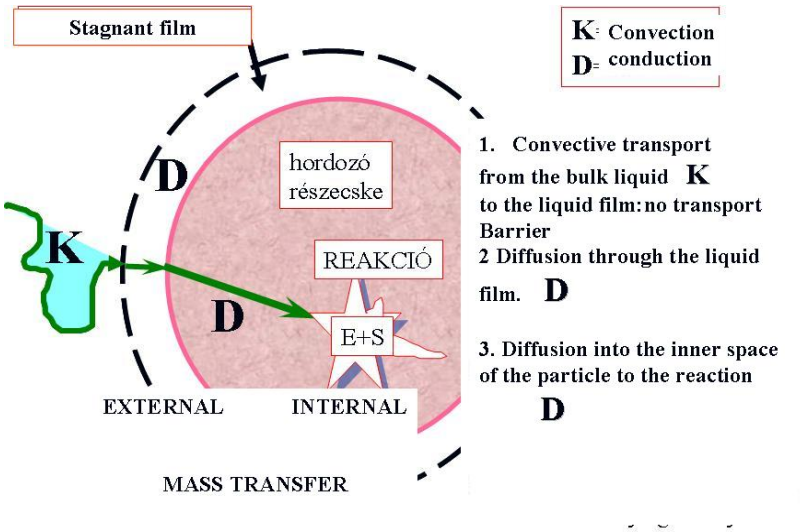


Fig 2.65.: Mass transfer resistances on and in a particle

Transport 1. and 2. represents an external mass transport resistance and 3. means an internal mass transport resistance. Since 2. and 3.are diffusion processes they can slower the reaction comparing to the homogeneous system, which will be shown very shortly and superficially here.

2.7.2.1. External mass transfer

In the diffusion interfacial layer (Nernst diffusion layer) the rate of material transport is

$$N_s = \frac{dS}{dt} = k_s\, a\, (S_o - S) \tag{2.41}$$

Where S_0 and S are the substrate concentration in the bulk liquid and at the surface of the particle, respectively. The mass transfer coefficient is $\mathbf{k_s}$ (cm/s) and **a** is the interfacial area in unit volume (cm^2/cm^3). If the reaction follows M-M kinetics this transport rate has to be equal with the reaction rate:

$$V = k_s a\left(S_o - S\right) = \frac{V_{max} S}{K_m + S} \tag{2.42}$$

The system is determined by 5 parameters: k_S, a, S_0, V_{max} és K_m . This can be reduced to 2, introducing dimensionless variables according to the definitions here:

$$x = S/S_0 \qquad \text{and} \qquad \kappa = K_m/S_0$$

It is useful to group the parameters into a dimensionless criterion **Damköhler-number** (Da), which is also called *reaction number* or *dimensionless reaction rate*

$$Da = V_{max}/ k_S S_0 a = \text{maximal reaction velocity / maximal mass transfer rate}$$

With these introductions eq. (2.42) can brought into a dimensionless form:

$$\frac{1-x}{Da} = \frac{x}{\kappa + x} = \frac{\frac{1}{\kappa}x}{1+\frac{1}{\kappa}x} \tag{2.43}$$

If Damköhler-number is much less than 1, i.e. mass transfer rate is much higher than maximal reaction rate, we have a so-called *reaction limited regime*, and

$$V = V_{max} \frac{S}{K_m + S}.$$

In these circumstances the system can be described with the M-M equation.

If Da>>1, i.e. the mass transfer is the rate limiting we call it diffusion limited regime and then

$$V = k_s a S_o .$$

Here only the mass transfer determines the overall reaction rate.

2.7.2.2. Internal mass transfer

If the enzyme is immobilized inside a particle (by copolymerization or encapsulating) than the substrate transport inside the particle will determine the rate of the reaction. A rather simple picture can be got supposing that inside the particle the dispersion of the enzyme is homogeneous, but the transport of the substrate (and of course that of the product) is going in small channels in which the diffusion happens in free liquid. In this case the so-called effective diffusivity constant will be given by this summarizing equation:

$$D_s = D_{so} \frac{\varepsilon_p}{\tau} \frac{K_p}{K_r}, \tag{2.45}$$

where

D_S is the effective diffusivity of the substrate in the matrix

D_{S0} is the diffusivity in the free liquid phase,

ε_P is the *porosity* of the particle: free liquid volume to the whole volume of the particle.

τ is the tortuosity of the pores: it measures that the effective length of way substrate particles have to go along is higher than a straight line between two points, because of the direction changes in the pores . Understanding tortuosity is easy if looking at the Fig.2.66.

K_P/K_r gives the extent of the diffusion hindrance: the measures of the transported substrate molecule may be close (in a molecular meaning) to the measures of the pores they are moving along, so between the molecules interactions may occur lowering the rate of free diffusion.

The ε_P porosity can be experimentally measured for a given carrier, empirical values of τ is in the range of 1,4_7, and K_P/K_r can be given by the following expression:

$$\frac{K_p}{K_r} \cong \left(1 - \frac{r_s}{r_p}\right)^4 \tag{2.46}$$

where r_S is the equivalent radius of the substrate and r_P is the same for the pores.

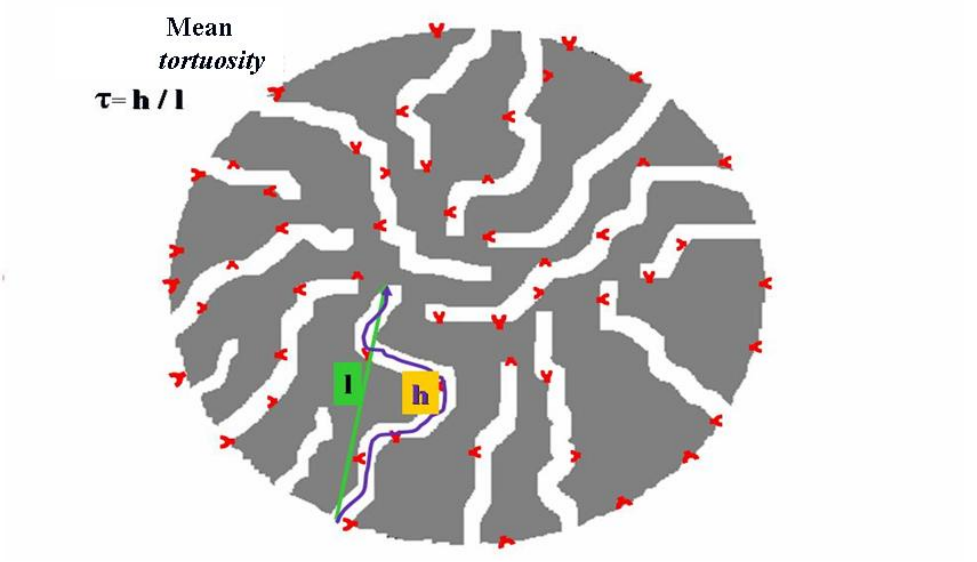


Fig 2.66.: Gel entrapped enzyme. Definition of tortuosity.

2.8. Application of enzymes

2.8.1. General applications

Enzymes have been used in lots of areas of everyday life, science and technology and the number of applications has been continuously increasing. Below some of these application territories are summarized in a series of Tables, not intended to be exhaustive.

Some enzymes are directly applied, i.e. they are used as *end products*.

Table 2.6.: Directly used enzymes.

Application area	Enzyme
Washing powders (detergents)	proteases, lipases, cellulases
Animal feed	β-glucanase, cellulase, phytase, xylanase, lipase
Medical applications/pharmaceuticals	proteases, lipases, amylases, β-lactamase, L-asparaginase, hyaluronidase, lyzozim, collagenase, streptokinase… (see also Table 2.10.)
Analytics and diagnostics	A series of enzymes (see also chapter 2.8.2.)

In many industrial processes enzymes are applied as auxiliary materials, they do not appear in the pruduct but applications of them are inevitable in the production processes. Such enzymes are listed in Table 2.7.

Table 2.7. Enzymes used as auxiliaries

Application area	Enzyme
Textile industry	amylases, hemicellulases, pectinases
Leather industry	proteases
Paper industry	hemicellulases, amylases, laccase
Sugar industry	dextranase, invertase, dextransaccharase, α-galactosidase...
Starch industra	(izo)amylases, amyloglucosidase, glucose isomerase, cyclodextrin-glucano-transferase, xylanase...

Food industry is a great user of enzymes, a series of food products get they final form and quality after enzymatic manipulations. Such applications are listed in Table2.8.

Table 2.8.: Enzymes in food industry

Application area	Enzyme
Dairy industry	proteases, β-galactosidase, lysozyme, lipases, esterases, papain, rennet, glucose oxidase, catalase...
Beer industry	amylases, tannase, β-glucanase, proteases, xylanase...
Wine making, fruit beverages	pectinases, naringinase, cellulase, amylase,
Alcoholic beverages	amylases, amyloglucosidase....
Meat inustry, fisheries	proteases, papain, glucose-oxidase...
Bakery industry	amylases, pentosanases, xylanase, phospholipase, lipoxygenase, protease
Fat- and oil industry	phospholipase, esterases
Coffee, tea, cacao…	pectinase, protease, glucanase, tannase....

Recently the environmental technologies also apply enzymes in increasing extent, for these we can see examples in Table 2.9.

Table 2.9.: Application of microbe origin enzymes in environmental technologies

Enzyme	Producing microbe	Reaction catalyzed
	Bacteria	

dehalogenase	*Pseudomonas* sp	Break down of dichloromethane
benzene-di-oxygenase	*Pseudomonas putida*	Break down of benzene and other aromatics
collagenase	*Streptomyces* sp.	collagen hydrolysis
Several enzymes	*Arthrobacter, Rhodococcus*	detoxification/break down of diff. compounds
	Molds	
cyanide-hydratase	*Stemphylium loti*	cyanide detoxification
tannin acyl hydrolase	*Penicillium* sp.	Hydrolysis of tannins
phytase	*Aspergillus ficuum*	Hydrolysis of phytin
chitinase	Nonpathogen fungi	Hydrolysis of chitin
keratinase		Hydrolysis of keratin
cellulase, xylanase	*Hypocrea* sp., *Aspergillus* sp.	Cellulose hydrolysis
hemicellulase, pectinase	*Chaetomium* sp., *Humicola* sp.	Plant residues and paper degradation
laccase, peroxidase, cytochrome P450	Wood degrading molds	Degradation of lignin, coloring compounds, aromatics

There are spreading applications of enzymes in the chemical industry, too. A part of the so called *white biotechnological processes* are based upon such enzymatic transformations. Some examples are shown in Table 2.10.

Table 2.10.: Enzymes application in chemistry and chemical industry

Reaction type	Enzyme	Product	Annual production volume in 2000, tons
Hydrolysis	Nitrile-hydratase	acrylamide	$100 \cdot 10^3$
	Penicillin-acylase	6-amino-penicillanic acid	$10 \cdot 10^3$
Resolution	Hydantoinase	4-hydroxi-D-phenil-glycine	1200
Oxidation	D-Sorbitol-dehydrogenase	L-sorbose	$80 \cdot 10^3$
Hydroxylation	Niacin hydroxylase	6-hydroxi-nikotinic acid	20
Reduction	β-Keto-reductase	(R)-carnitine	300
Formation of C–C-bond	Pyruvate decarboxylase	(R)-phenyl-acetyl-carbinol	500
Synthases	Aspartate-ammonia-lyase	L-aspartate	400
	Fumarase	(L-DOPA) L-malate	500
Peptide-syntheses	Thermolyzine	α-aspartame	$10 \cdot 10^3$
Glucosyl transfer	Cyclodextrin-glucano--transferase (CGT-ase)	β-cyclodextrin	$10 \cdot 10^3$

Important group of enzyme utilization is their applications for therapeutic purposes. Some therapeutic applications are listed in Table 2.11.

Table 2.11.: Therapeutic use of enzymes

Enzyme	Source	Name of pharmaceutical	What for? What against?

urate oxydase	*Aspergillus flavus*	Uricozyme	gout, hyperurichemia
lipase	*Rhizopus arrhizus*		Digestion enhancing prepatations
Pancreatin: pancreas enzyme mixtures: trypsin, chymotrypsin, lipase, α-amylase	Pig pancreas	Cotazym, Kreon, Nutrizym, Pankreon, Panzytrat…	Digestion enhancing preparations
β-amylase	*Aspergillus oryzae*		Digestion enhancing preparations
β-galactosidase (Lactase)	*Kluyveromyces fragilis*, *A. oryzae*, *A.niger*	Lactaid, Lactrase, SureLac	lactose intolerance
hyaluronidase	rDNS product	Hylase, Vitrase	Myocardial infarct, heart attack
urokinase	human urine or human cell culture	Abbokinase, Actosolv, Alphakinase, Rheothromb	Acute myocardial infarct, heart attack
Factor VIII	recombinant CHO cells	Recombinate, Bioclate	hemophilia A
Tissue plasminogen activator	recombinant CHO cell culture	Activase, Actilyse	Acute myocardial infarct, heart attack acute pulmonary embolism, ischemic stroke
Deoxy-ribonuclease	recombinant CHO cell culture	Pulmozyme	Chronic obstructive tuberculosis

2.8.2. Application as analytical tools

Application of enzymes for analytical purposes should be distinguished from enzyme analytics. In the latter case the enzyme itself is the target of the analysis, we want to determine the activity of the enzyme. In contrast to this, enzymes as analytical tools can be applied to determine the concentration of some other target compound. Here we are speaking about this because the elements of enzyme analytics have already been discussed previously.

For analytical purposes enzymes can be applied in the following areas:

- When the **target matter(analyte) is the substrate** of the enzyme.
- When enzyme is used **as a marker** during an analytical process. (Immune analytics).
- When the target molecule (analyte) is an **enzyme inhibitor**
- When the enzyme is a part of an **enzyme electrode** or other **biosensor.**

When we want to determine the concentration of a substrate the enzymatic reaction has to be developed to its end point. If the ceasing substrate or the developed product cause a measurable change in some features of the rection mixture (e.g., color change, spectral change, pH change, etc.) then from these changes one can conclude to the amount of converted substrate. The essence of such a kind of analytical methods can be understand from the example of uric acid – allantoin transformation when the target is the urate concentration (for instance in the urine for diagnostic purposes) (Fig 2.69). The basis of the method is that uric acid has a characteristic light absorbance at 294 nm while the product allantoin has not. At the end of the reaction, using the specific absorbance value of urate, its starting concentration can be determined. (Why does not go the reaction just to the equilibrium?)

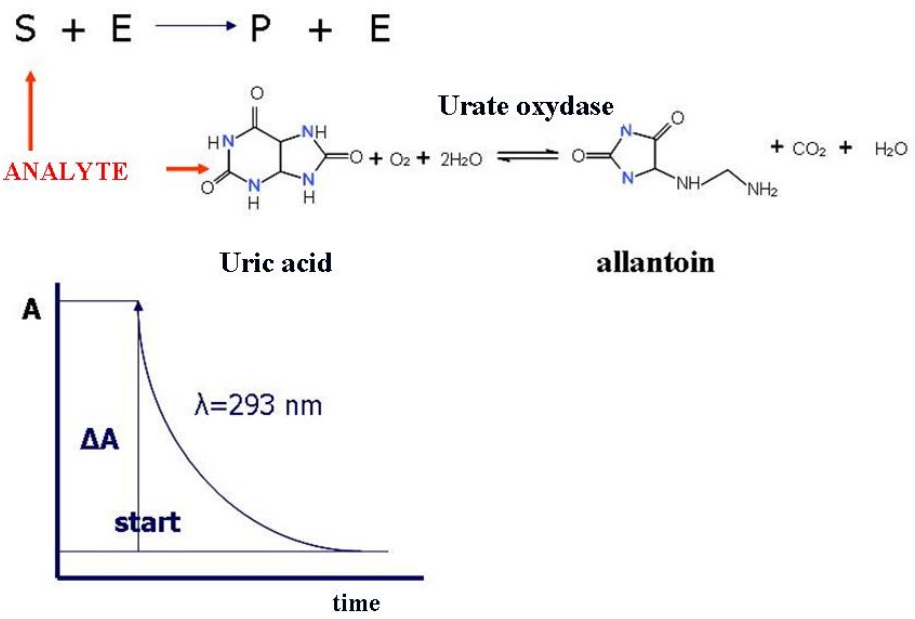


Fig 2.69.: Substrate determination with a reaction run to end point

When there is no directly measurable change along the reaction, an auxiliary reaction is applied. In the Fig 2.70 explains such a situation. Let us note, that the 340 nm determination of NADPH or NADH is a frequently applied method.

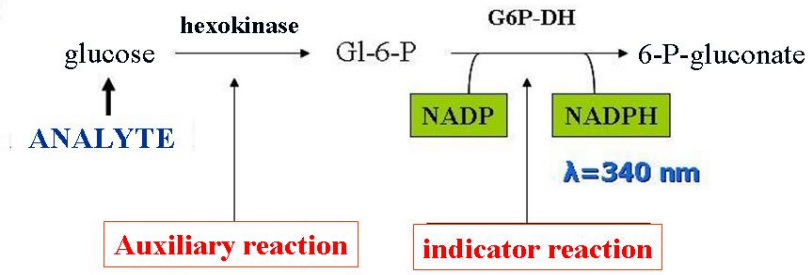


Fig 2.70.: Substrate determination with auxiliary reaction

Expressively as a marker is used the enzyme in the instance of one of the many ELISA methods.

Knowing the kinetic behavior of enzyme, it is obvious that kinetic measurements can be applied directly only if $S<<K_m$, i.e., if we are in the starting (near to origo) region of the M-M hyperbole. There a linear relationship between reaction rate and substrate concentration makes the measurement possible (see Fig 2.72). Here we need a calibration curve. This kind of kinetic method is often applied in automatic analyzers (FIA, flow injection analysis)

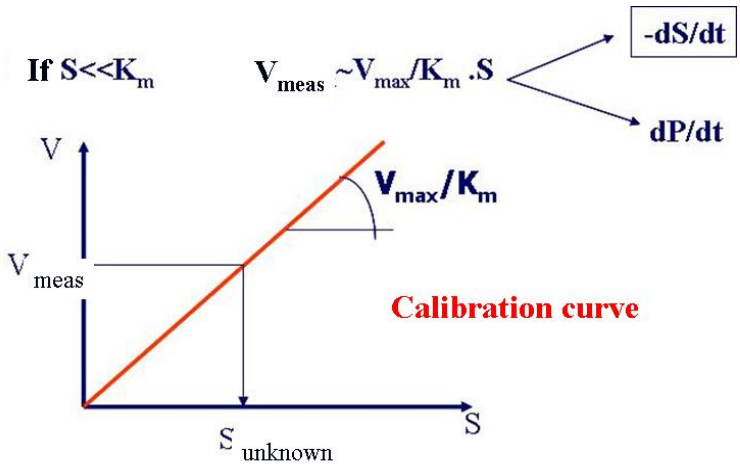


Fig 2.72.: Substrate determination on the basis of kinetic measurement.

In the opposite case, if $S>>K_m$, only inhibitor or activator concentrations can be measured, provided the reciprocal rate of the reaction is a linear function of the inhibitor or activator concentration (=Dixon plot is linear). Such methods are applied for example in the case of herbicide determinations as well as in some human diagnostic methods (e.g., heparin-determination).

In case of *enzyme electrodes* and *biosensors* immobilized enzymes or immobilized cells are used. Recently the application of biosensors and enzyme electrodes are widespread in different areas, e.g., in human diagnostics for the determinations of glucose in blood or alcohol in blood, cholesterol, fats and so on.

4. BASICS OF FERMENTATION UNIT OPERATIONS AND PROCESSES

4.1. Basic rules of microbial growth

If a microbial cell – for the sake of the simplicity, a bacterial cell – is put into an environment where all the necessary nutrients are in high enough concentrations, and the circumstances are also favorable (pH, temperature, osmotic pressure) the cell starts to take up the nutrients and start to grow and after a certain period of time it starts to proliferate, i.e. from a mother cell two equal daughter cells are formed. Look at *animation 4.1.* that starts with a transfer of some cells from a Petri-dish culture colony into a liquid culture medium in a shaking flask and followed by a division at every 30 minutes (this is a fast-growing *Escherichia coli* bacterium). Thus, the first generation makes two, the second, after 60 minutes 2^2=4, the third, 90 minutes after the transfer gives 2^3=8, and the fourth generation results in 2^4=16, and so on and so on while the nth generation will have 2^n daughter cells. It is easy to write the mathematical rule, on the basis of this for a binary fissiparous bacterium:

After n generation from one cell 2^n will be born

After n generations from N_0 cells $N_0 \cdot 2^n$ will be born

This is the exponential growth rule of the binary splitting bacterial cells and it is easy to get that this is a solution of the following differential equation:

$$\frac{dN}{dt} = \nu \cdot N \tag{4.1}$$

Which has the solution form

$$N = N_0 e^{\nu . t} \tag{4.2}$$

In this $\nu=\ln 2/t_g$ that is the specific proliferation rate ad tg is the doubling time.

In the biochemical engineering practice we are much more interested in the mass growth of the culture instead of the number proliferation, thus equation (4.1-4.2) (these are the basic equations of the growth model of Jacques Monod) we apply preferentially in the next form:

$$\frac{dx}{dt} = \mu . x$$

$$x = x_0 e^{\mu t} \tag{4.3}$$

Where $\mu \equiv \frac{1}{x}\frac{dx}{dt}$ is the specific growth rate and **x** is the concentration of the biomass expressed in dry weight and given in the units a g/dm^3 or kg/m^3. According to the eq.4.3, x, plotted against time (look at Fig 4.1.) gives an exponential curve going up to the infinite. Of course, this cannot be the realistic growth curve[5].

[5] If an E.coli cell would grow without any limit with a generation time of 20 minutes then after 43 h 1,09 x 10^{21} m^3 volume cell would be present that is higher than the volume of the Earth, and after waiting an other two hours its mass would be higher than the mass of Her (6,6 x 10^{21} t !)

A realistic picture is on Fig 4.2 that shows a real growth curve of a system in which only a given space and a given amount of nutrients (we call this batch culture) are available.

Development of the curve can be followed in the animated picture, animált görbével (4.2. animation) and let us look at Video 4.1 that shows the growth of *E. coli* under microscope.

Animation4.1:growth of E. coli – generation time

Animation4.2.: A growth curve

Video 4.1: Growth of E. coli under microscope

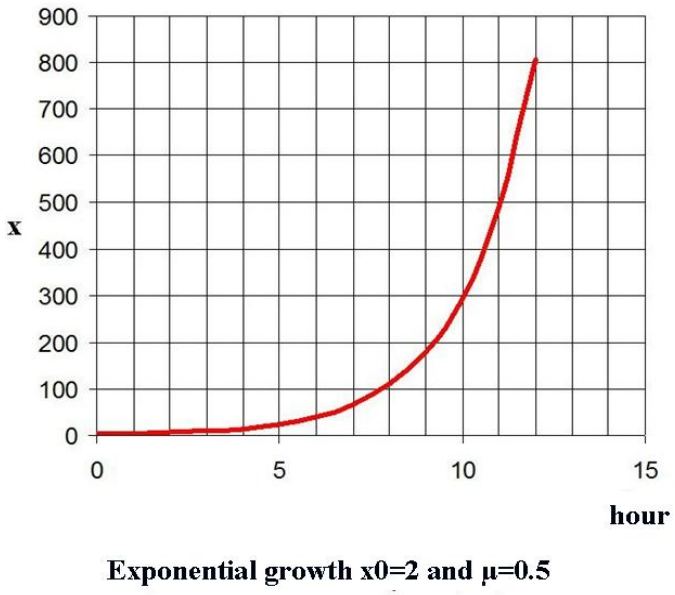


Fig 4.1.: Unlimited exponential growth

Growth curve can be divided into four phases. First is called ***lag phase*** in which there is no visible growth yet, either from numeric or from mass point of view. In this phase the cells are getting accustomed to the new environment . Here μ=0.

Lag phase is followed by the ***accelerating phase***, during which the acclimatization continues but visible growth can already be observed with an increasing rate. In this phase $0<\mu<\mu_{max}$. The lower than maximal growth rate is caused because not all the cells end their adaptation at the same time, more and more cells start to proliferate as the time elapses.

The next phase is called ***exponential phase,*** in this all cells are proliferating with maximal specific growth rate, $\mu=\mu_{max}$

Fourth phase is called ***declining phase***, here again $0<\mu<\mu_{max}$ and goes to not.

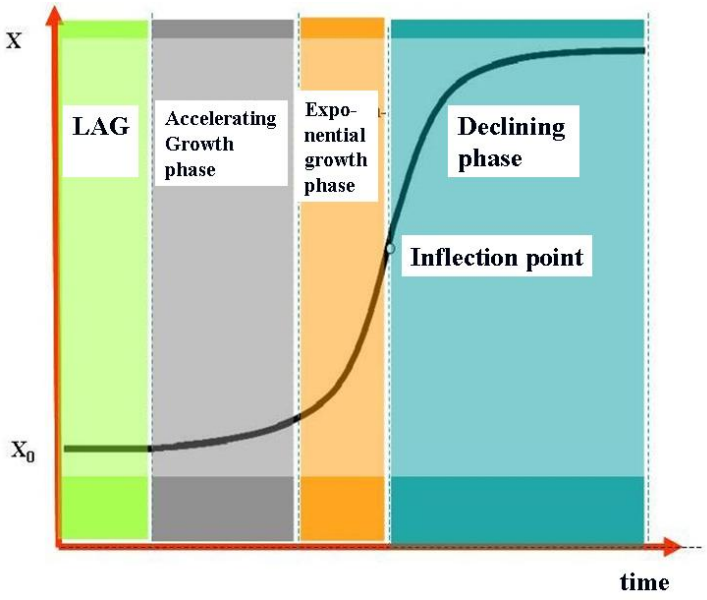


Fig 4.2.: Bach fermentation's real growth curve

On the Fig 4.3. the characteristic basic kinetic curves of a batch fermentation can be seen while the animated growth curve is shown in (4.3. animáció).

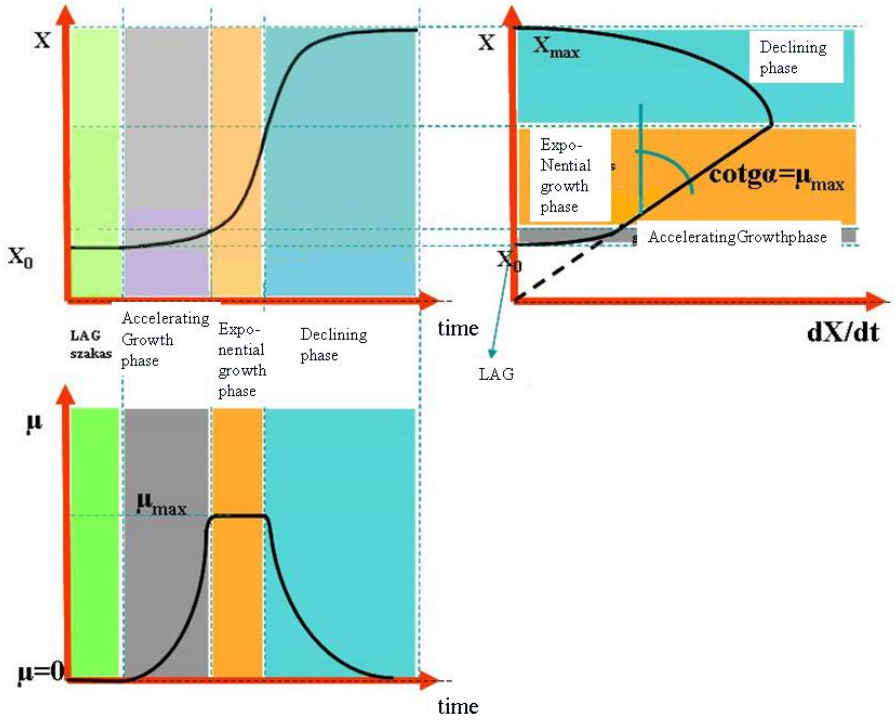
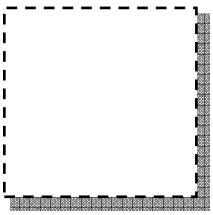


Fig 4.3.: Growth curve of batch fermentation and its primary kinetic representations



Animation4.3.: 3 growth curves

Plotting the number of the **living cells** in time we get a bit differing curve, on which after the declining phase there starts a stationary phase, during which the rate of growth rate is equal with the death rate and this is followed by the pure death phase..

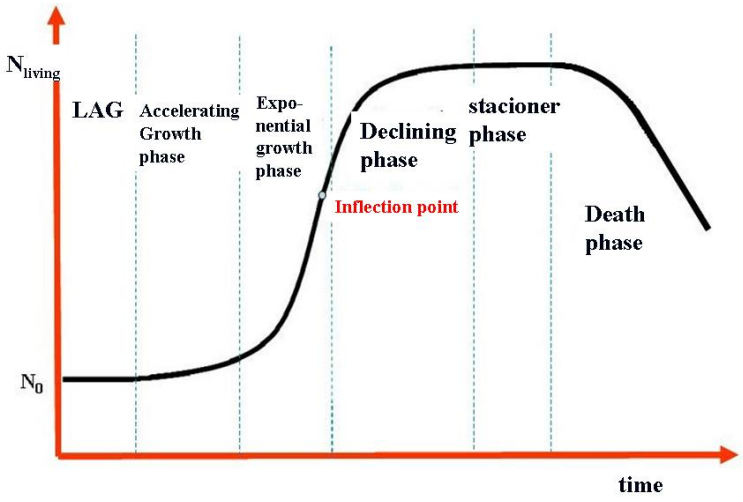


Fig 4.4.: Living cell number versus time

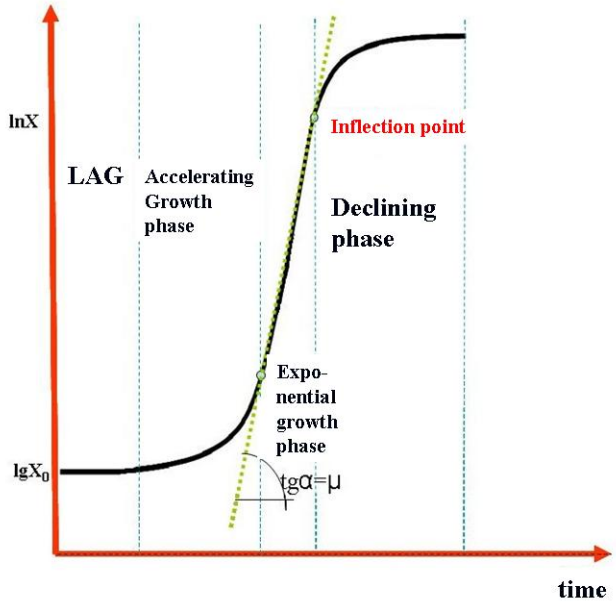


Fig 4.5.: Semilogarithmic plot of the growth curve

In the Fig 4.5. an equally often applied plotting of the growth curve is seen. Here the logarithm of the cell mass is plotted in respect of the time. Naturally on this plot the exponential phase appears as a straight line, this is the reason of the frequently incorrectly used expression – logarithmic phase. Let us observe, that this plotting gives the simplest mode of the calculation of the maximal specific growth rate.

What is the reason of the unavoidable existence of a declining phase? It may have three reasons. It can be imagined that the cells are producing some metabolites that are self-poisoning, inhibiting the further growth. Another reason may be that the population becomes so dense in which the cells are so close to each other, that there is no place anymore to the further growth. (But do not mean this word by word: of course, cells do not touch each other, there are always surrounding water phase!).

The most important and most frequently occurring reason that growth is getting slowed is the substrate limitation. Substrate limitation means that at least one component of the culture medium does not have concentration high enough that would allow maximal specific growth rate. The notion of the limiting substrate was introduced by MONOD in 1942, when he had examined the growth of lactic acid bacteria. He found out that limiting substrate influences growth rate similarly how a substrate affects the rate of a simple enzymatic reaction, and applied the same function

$$\mu=\mu_{max}\frac{S}{K_s+S}, \tag{4.4}$$

where μ_{max} is the maximal specific growth rate (h^{-1}) and K_S is the substrate saturation constant.

Discussion of this equation is similar we had followed in case of M-M kinetics. At small limiting substrate concentrations, the hyperbole starts as a straight line and when S is much higher than K_s it approaches the value of μ_{max}. This (theoretically not) but practically existing concentration is called ***critical substrate concentration*** (Fig 4.6.)

Monod-model well describes the exponential and declining phase of the batch fermentation. The transition between these two phases is at the inflection point of the growth curve. Graphical evaluation of the two parameters of the Monod model is shown on fig 4.3 and 4.7.

$$\mu = \mu_{max} \frac{S}{K_S + S}$$

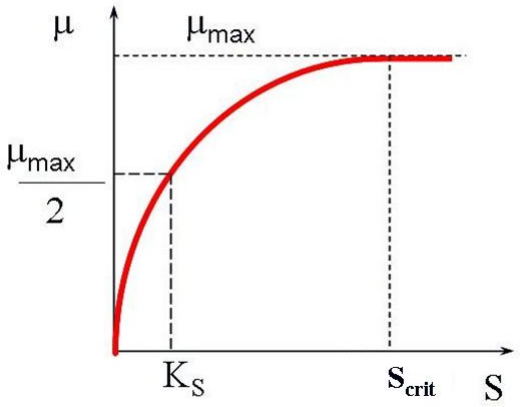


Fig 4.6.: Monod-model

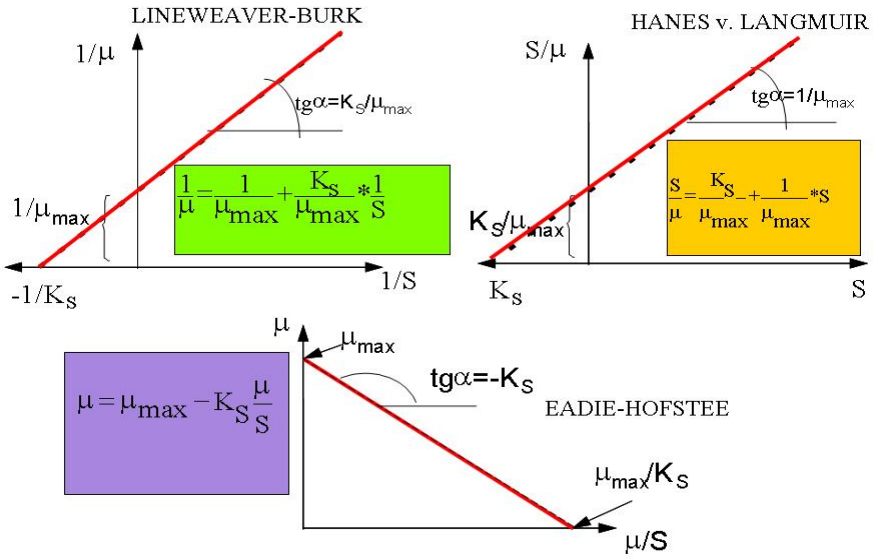


Fig 4.7.: Monod-model, graphical methods to evaluate K_S and μ_{max} .

Thinking about limiting substrate, a natural question arises: which component of the culture medium becomes limiting? To understand this, let us take a look at Fig 4.8. Based on the run and shape of the curves it is impossible to predict the limiting substrate. These are depending on the starting concentration, the maximum specific growth rate and the overall substrate yield (see later), thus the only thing we know that the substrate will be the first limiting one whose concentration will first reach its critical value. As the time elapses second, third… limiting substrates may come to effect.

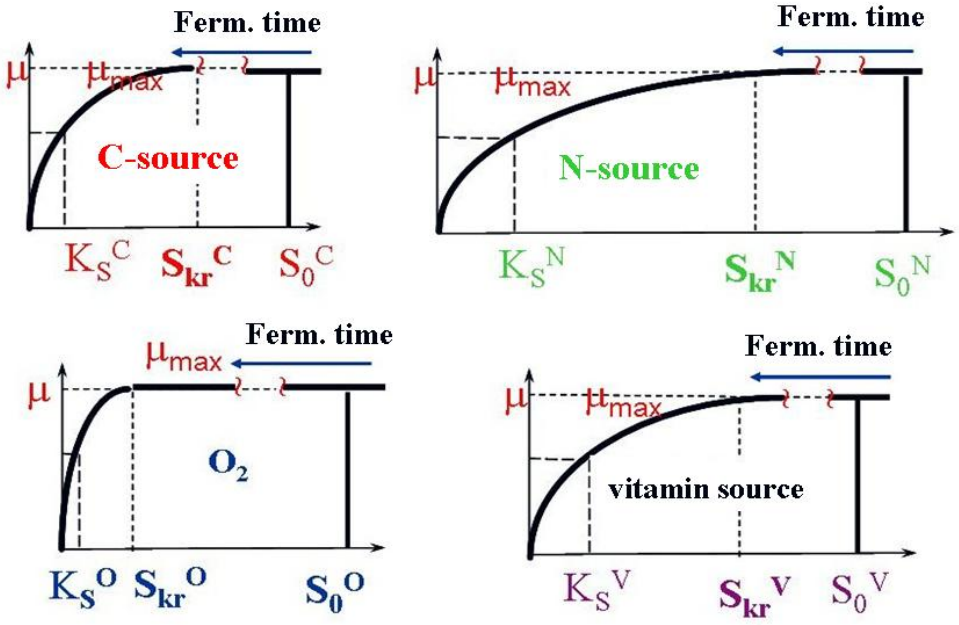


Fig 4.8. ábra: Which will be limiting?

Among the basic rules describing the growth, there is one further relationship that connects quantitatively the substrate consumption and the growth. This is the *overall yield coefficient* that -by definition - describing onto the i^{th} component of the culture medium is

$$Y_{x/S_i} = \frac{dx}{dS_i} \tag{4.5}$$

The overall yield can be considered *differentially,* which is the ratio of the small changes happened during small time interval (as 4.5 shows) or *integrally* i.e. the ratio of grown biomass to the consumed substrate during an arbitrary (shorter or longer) time, according to eq.(4.6)

$$Y_{x/S_i} = \frac{\Delta x}{\Delta S_i}, \tag{4.6}$$

Where Δx is the biomass increase while ΔS_i substrate was consumed.

Note, that without distinguishing notification Y refers to the limiting substrate.

Monod called this notion yield constant – thinking that it is a permanent feature of the substrate utilization, but in 1949 Herbert pointed out that it is not a constant, it depends on different environmental factors and mainly on the specific growth rate, that is why recently we call it overall yield. Yields are very important characteristics of fermentation processes for they refer to the successfulness of substrate utilization of the microbes with a strong economic meaning.

4.2. Nutrients and their use

4.2.1. Nutrient requirements of microbes, culture media

Production capabilities of microorganisms in an industrial process mainly depends upon two factors: genome of the microbe plays a determining role but also very much important is the environment that is responsible for the actual manifestation of microbial behavior. At a given microbe with a given genome environment is the most important.

The notion of environment includes the qualitative and quantitative composition of the culture media and the circumstances like pH, temperature, redox potential of the media, etc.

Microorganisms have varied nutrient requirements and the various microbes can be grouped on the basis of their fundamental nutrient need, first of all according to C-source and energy source requirements (Fig 4.9).

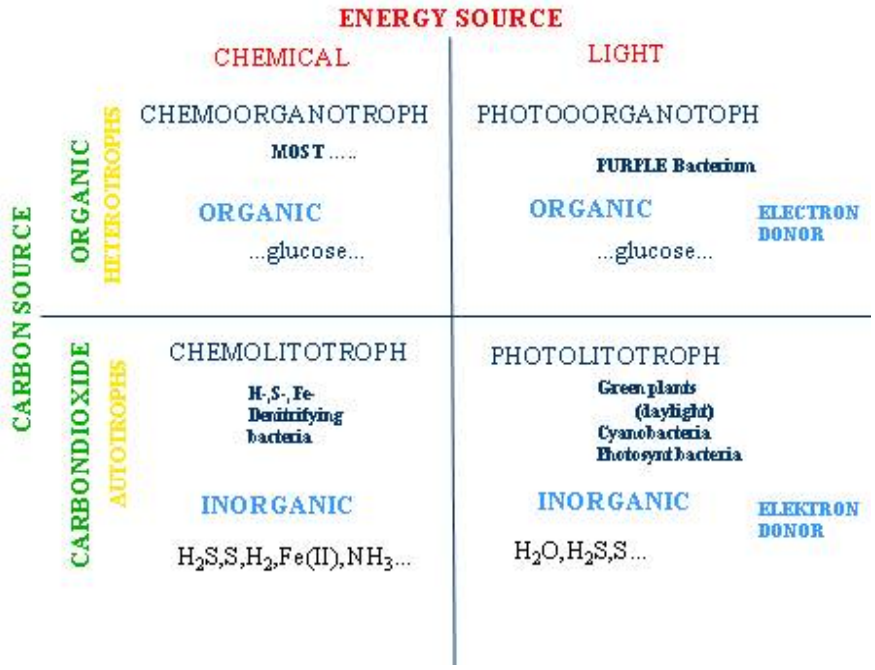


Fig.4.9.: Groups of microbes according to C- and energy source need

In every groups there are microbes which are important from an economic point of view but fermentation industry uses nowadays mostly heterotrophs that use organic matter either as C- or as energy source. But this does not mean there are no industrially important organisms in the other groups like photolithotrophs (algae) or chemolithotrophs (bacterial leaching of ores).

Culture media must contain all the chemical elements that are necessary during the growth process in metabolizable form and in enough quantity. Those materials are needed to build up the new biomass and the extracellular products as well.

Table 4.1. shows average elemental composition of some microbes, while Table 4.2 gives the composition of an „average" bacterium and yeast, Table 4.3 shows the composition of some important fermentation products.

First step of designing a culture medium is to take into account all the elements that are needed to build the intended amount of new biomass and product according to the following formal stoichiometric equation (provided an aerobic fermentation)

C-source + N-source + O_2+ mineral salts + special nutrients (e.g. vitamins)→
→ new biomass + product(s) + CO_2 + H_2O

This formal equation gives only the minimum requirement but it is also necessary to take into account the fact, that the rate of the processes are influenced by the concentrations of the nutrients, to (see Monod model).

Table4.1.: Elemental composition of some microorganisms

Composition in dry weight %					
MICROORGANISM	C	H	O	N	S
Saccharomyces cerevisiae	45	6,8	30,6	30,6	n. a.
Methylomonas methanolica	45,9	7,2	no data	14,0	2,6
Penicillium chrysogenum	43	6,9	35,0	8,0	no data

Table 4.2.: „Average" bacterium and yeast composition

ELEMENT	% in dry weight	% in dry weight	Overall yield[6]
	YEAST	BACTERIUM	
C	47	53	*
N	7,5	12	8–13
P (as $P0_4^{3-}$)	1,5	3,0	33–66
S	1,0	1,0	100
O	30,0	20,0	*
Mg	0,5	0,5	200
H	6,5	7,0	**
Ash[7]	8,0	7,0	**

Table 4.3.: Elemental composition of some fermentation products

Antibiotics		
	Bacitracin	$C_{66}H_{103}N_{17}0_{16}S$
	Cephalosporin	$C_{16}H_{21}N_30_8S$
	Erythromycin	$C_{37}H_{67}N0_{13}$
	Penicillin G	$C_{16}H_{18}N_20_4S$
	Streptomycin	$C_{21}H_{39}N_70_{12}$
Organic acids		
	Citric acid	$C_6H_80_7$
	Gluconic acid	$C_6H_{12}0_7$
	Lactic acid	$C_3H_60_3$
Solvents		
	Acetone	C_3H_6O
	Butanol	$C_4H_{10}0$
	Ethanol	C_2H_60
Vitamins, amino acids		
	B_{12}	$C_{63}H_{88}CoN_{14}0_{14}P$
	Riboflavin	$C_{17}H_{20}N_40_6$

[6] * These yields will be discussed later.
** This yieled is only formal.
[7] Element content of ash: P, Mg, Cu, Co, Fe, Mn, Mo, Zn, Ca, K, Na.

Glutamic acid	$C_5H_9N0_4$
Lysine	$C_6H_{14}N_20_2$
Tryptophan	$C_{11}H_{12}N_20_2$

Among the culture media components especially important is the molecular oxygen, with which later we shall be discussing in more details.

Nitrogen source can be of many kinds, NH_4^+- or NO_3^- salts or organic nitrogen containing compounds or natural N-sources (soya meal, corn steep liquor, meat and yeast extract etc.)

Carbon source is in the most instances the energy source as well, and can be of very much type:

carbohydrates (sugars), alcohols, simple organic acids, fats and oils, etc. these are to be degraded by the anabolic reactions meanwhile energy (in the form of ATP) is produced and at the same time intermediary smaller molecules are formed. From these the carbon content of the new biomass is incorporated. (see Fig.4.10)

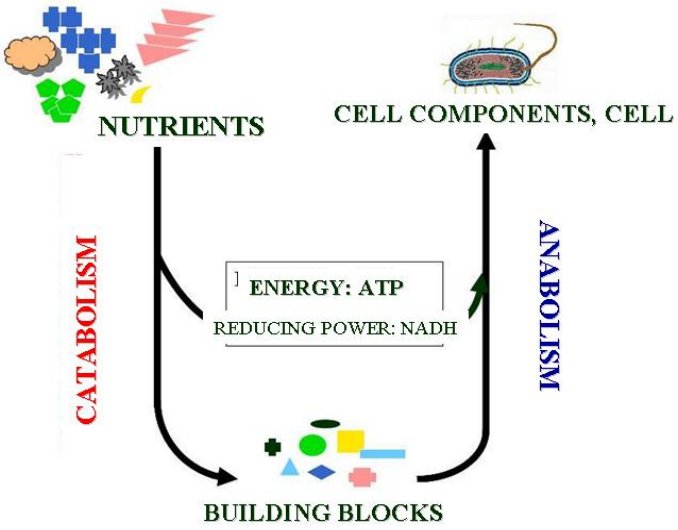


Fig 4.10.:Scheme of nutrient utilization

4.2.2. Utilization of carbon/energy source

Measure of the carbon source utilization is the overall yield coefficient, $Y_{x/s}$, defined by Monod and Herbert. Let us examine, how and what for this carbon/energy source is utilized, and where to it is got.

A certain part of the C/energy source C is getting incorporated into the new biomass in the form of the carbon-containing new organic material. Another part is the source of energy that is formed along the anabolic biochemical reaction routes and reserved in the form of ATP. The total C/energy source uptake consists of these two parts as eq. (4.7) shows:

$$\Delta S=\Delta S_C+\Delta S_E\,. \tag{4.7}$$

Dividing this by the amount of the new biomass we get the reciprocal of the overall yield:

$$\frac{\Delta S}{\Delta x}=\frac{\Delta S_C}{\Delta x}+\frac{\Delta S_E}{\Delta x} \tag{4.8}$$

$$\frac{1}{Y_{x/s}}=\frac{1}{Y_C}+\frac{1}{Y_E} \tag{4.9}$$

where Y_C is the C- or incorporation yield and Y_E is the energy yield.

Is this distinguishing only a formal speculation or are these components really distinguishable and independently measurable? The overall yield itself can be experimentally measured by biomass production and substrate uptake measurements. The $Y_{C,}$ incorporation yield is easily calculable on the basis of a material balance written for the incorporated carbon:

$$\alpha_2\Delta x=\alpha_1\Delta S_C\,, \tag{4.10}$$

where α_2 is the carbon content of the microbial cell dry weight, and as an average it may be taken about 0.46–0.50 (46–50%), but of course, it is possible to measure it experimentally, too.

α_1 is the carbon content of the substrate (for instance for glucose it is 72/180 = 0,4; for ethanol it is 24/46 = 0,52).

From eq. (4.10) with rearranging we get the carbon yield:

$$\frac{\Delta x}{\Delta S_c}=Y_C=\frac{\alpha_1}{\alpha_2} \tag{4.11}$$

Consequently, Y_C can be numerically calculated knowing the carbon content of the microbe and the substrate. It is obvious now; we can also calculate the energy yield from (4.9)

$$Y_E=\frac{Y_{x/s}.\frac{\alpha_1}{\alpha_2}}{\frac{\alpha_1}{\alpha_2}-Y_{x/s}}=\frac{Y_{x/s}.\alpha_1}{\alpha_1-Y_{x/s}.\alpha_2} \tag{4.12}$$

Some fermentations are directly related to the energy production, thus from the amount of the product the energy yield can correctly be calculated. In the case of ethanol fermentation, we know that energy is produced only during the glycolysis, so the ethanol formed is directly proportional to the energy released.

In case of the classical vinegar fermentation *Acetobacter aceti* oxidizes the ethanol and in its terminal oxidation route the removed hydrogen atoms (carried by NADH + H^+) are oxidized by the molecular oxygen, meanwhile a certain amount of ATP is formed.

In these cases, just a smaller part of the C/energy source is utilized to be incorporated and a more significant part is utilized for energy production. This is why in these fermentations the substrate conversion is usually much over 90%.

The ratios of assimilation and dissimilation are shown in the instances of Table 4.9. Data are interesting and typical, and we can conclude that

– effectiveness of the energy production and utilization of aerobic metabolism is higher than the anaerobic, more C can be incorporated,

–on a complete culture medium most part of the C/energy source is utilized for energy production,

–on synthetic culture media the ratio of incorporation and energy production is comparable.

Table 4.9.: Comparison of energy production and incorporation at different microbes and culturing circumstances.

MICROBE	Culture medium	Assimilation %	Dissimilation %
Streptococcus faecalis Anaerobic culture	complete[8]	2	98
Saccharomyces cerevisiae Anaerobic Aerobic	complete	2 10	98 90
Aerobacter cloaceae	minimal[9]	55	45

The substrate utilized for energy production, ΔS_E , can also be divided into two parts, taking into account the goal of energy utilization. A certain part of the energy is used for fulfilling the energy requirement of biochemical processes that are related to the syntheses of new biomass (amino acids, nucleotides, macromolecules: proteins, nucleic acids, fats, etc. of the new biomass).

On the other hand, there are series of processes going on during the microbial life that are not related to the growth of new biomass but requiring much energy. Such activities are the *cell motion*, *osmotic work*, *active transport* of molecules in and out of the cell, etc. Besides these there is a permanent breaking down of the macromolecules during the cell life cycle and these have to be resynthesized continuously. These processes need Gibbs free energy that is not growth related at all. From a thermodynamic point of view, we may say, the cells have to do work on their environment even if apparently, they are in a resting (nongrowing) state. With other words, they have to maintain the low entropy level (for cells are of highly ordered) in order to maintain their viability. This nongrowth associated energy need is called *maintenance energy* and the phenomenon is the maintenance *of viability*.

Because of this, energy yield can be divided into two parts:

$$Y_E = \frac{\Delta x}{\Delta S_E} = \frac{\Delta x}{\Delta S_g + \Delta S_m} \ , \tag{4.13}$$

where ΔS_g is the substrate that was used for energy production for growth of the new biomass, Δx,
ΔS_m is the substrate that was used for energy production for maintenance purposes.

It is known that substrate uptake rate can be expressed in the term of growth rate as

$$\frac{dS}{dt} = -\frac{1}{Y}\frac{dx}{dt} = -\frac{\mu x}{Y} . \tag{4.14}$$

Applying this general relation to the energy production

$$\left(\frac{dS}{dt}\right)_E = \frac{\mu x}{Y_E} = \frac{dS_g}{dt} + \frac{dS_m}{dt} \tag{4.15}$$

and again, for the first term of the latter

[8] Complete medium: contains also natural components besides sugar and minerals
[9] Minimal medium: beside the C-source contains only minerals

$$\frac{dS_g}{dt} = \frac{\mu x}{Y_{EG}}, \tag{4.16}$$

where Y_{EG} is the „maximal growth energy yield", this theoretical maximum would characterize the process if there were not maintenance requirement ($\Delta S_m = 0$)[10].

We can logically suppose that dS_m/dt is proportional with the biomass present (twice as much biomass moves twice as much, it is twice as much ordered, thus needs twice as much energy for the maintenance of its living state, etc):

$$\frac{dS_m}{dt} = mx, \tag{4.17}$$

where m is the specific maintenance coefficient [g substrate/g biomass·hour= h^{-1}].

Putting (4.16) and (4.17) expressions into (4.15) we get the following:

$$\frac{\mu x}{Y_E} = \frac{\mu x}{Y_{EG}} + mx \tag{4.18}$$

or

$$\frac{1}{Y_E} = \frac{1}{Y_{EG}} + \frac{m}{\mu}. \tag{4.19}$$

And finally, if the incorporation is also taken into account for the overall yield

$$\boxed{\frac{1}{Y_{x/s}} = \frac{1}{Y_c} + \frac{1}{Y_{EG}} + \frac{m}{\mu}} \tag{4.20}$$

One can see $Yx_{/s}$ is not a constant it varies with the specific growth rate!

The question again arises, whether this whole way of thinking is only a speculation or a real, measurable distinction can be made between the different parts of the substrate utilization. The answer is on the Fig.4.19. If we can measure the overall yield at different specific growth rates and we know the carbon content of the microbe and the substrate, then the distinguishing is real and all the components and parameters can be evaluated.

[10] This is a not reachable theoretical maximum.

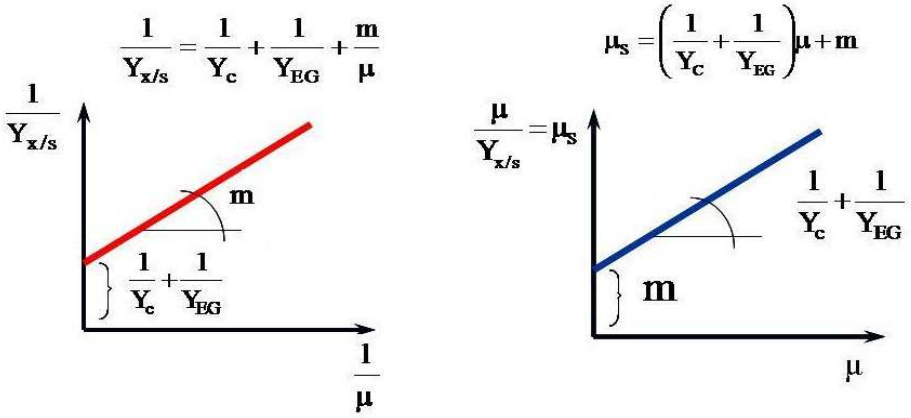


Fig 4.19.: Graphical determination of maintenance coefficient

Maintenance energy requirement and m specific maintenance coefficients for some microbes at several culture conditions are shown in Table 4.10. Let us observe that in case of baker's yeast a tenfold increase in NaCl concentration of the culture medium (this means a tenfold osmotic pressure increase) goes together with an also tenfold increase of maintenance coefficient, m.

Naturally if there is no external carbon source present in a medium, the cells will try to maintain their viability and, in this situation, internal C-sources (if they are available) will be used for maintenance purposes. This phenomenon is called *endogenous metabolism*. The internal C may be a reserved carbon source or partly the cell mass itself. The rate of endogenous metabolism is also proportional to the biomass present, so $(dS/dt)_{endmet} = k_e.x$, in which k_e is the *specific endogenous metabolism rate*. Utilization of the internal reserved energy is of less significance in the case of fermentation processes, it is more significant in the case of biological wastewater treatment.

Table 4.10.: m and mATP values of some microorganisms

	culture conditions	specific maintenance coefficients	
		*m	**m_{ATP}
Aerobacter cloaceae	aerobic, glucose	0,094	14
Saccharomyces cerevisiae	anaerobic, glucose + 0,1 mol/dm^3 NaCl	0,036	0,52
Saccharomyces cerevisiae	anaerobic, glucose + 1,0 mol/dm^3 NaCl	0,360	2,2
Penicillium chrysogenum	aerobic, glucose	0,022	3,2
Lactobacillus casei	aerobic, glucose	0,135	1,5

* g energy source/g cell mass
**mmol ATP/g cell mass·h

Another important metabolic feature is the ATP-yield, per definitionem

$$Y_{ATP} = \frac{\Delta x}{\Delta ATP} = \frac{Y'_{x/s}}{Y_{ATP/s}}, \qquad (4.21)$$

where $Y'_{x/s} = MY_{x/s}$ (substrate molecular wiight)·(overall yield) $\left[\frac{g}{mol}\right]\cdot\left[\frac{g}{g}\right]=\left[\frac{g}{mol}\right]$

$Y_{ATP/S}$ = number of ATP moles produced from one mole of energy source, which can be calculated considering all the ATP producing matabolic paths of the given organism: it gives how many ATP can be formed utilizing a mole substrate. For example, it is 2 in the case of glycolysis.
According to many practical experiences an average of ATP yield is 10,5 g biomass/one mole ATP.

ATP utilization can also be divided into two parts, one for the utilization for growth and one for utilization for maintenance purposes.

$$\Delta ATP = \left(\Delta ATP\right)_g + \left(\Delta ATP\right)_m \tag{4.22}$$

$$\frac{1}{Y_{ATP}} = \frac{1}{Y_{ATP}^{max}} + \frac{m_{ATP}}{\mu} \tag{4.22a}$$

Naturally there are many other yield-like expressions representing the metabolic activity of a microbial culture, some of these are as follows here.

Y_H is the so-called *heat yield* that is the ratio of the new biomass to the energy dissipation during the growth (heat evolution)

$$Y_H = \frac{\Delta x}{-\Delta H_X.\Delta x + \Delta H_S \Delta S} = \frac{\Delta x}{\Delta Q} \tag{4.23}$$

In this definition ΔH_X and ΔH_S mean the specific enthalpy of the biomass and the substrate, respectively [KJ/g], and ΔQ is the evolved metabolic heat during the growth. Equation 4.23 divided by ΔS gives a relation between overall yield and the heat yield.

$$Y_H = Y_{kcal} = \frac{\frac{\Delta X}{\Delta S}}{-\frac{\Delta X}{\Delta S}\Delta H_X + \Delta H_S \frac{\Delta S}{\Delta S}} = \frac{Y_{X/S}}{\Delta H_S - Y_{X/S}\Delta H_X} \tag{4.24}$$

Another important measure – another metabolic quotient – of the effectiveness of substrate utilization of aerobic cultures is the *respiration quotient*, defined either in differential or integral way:

$$RQ = \frac{\Delta CO_2}{\Delta O_2} \quad \text{vagy} \quad RQ = \frac{dCO_2}{dO_2} = \frac{\frac{dCO_2}{dt}}{\frac{dO_2}{dt}} = \frac{q_{CO_2}}{q_{O_2}} \tag{4.25}$$

These are expressed strictly in mol/mol.

To interpret RQ let us look at an aerobic fermentation with glucose C/energy source. How much the RQ could be? We can get the answer if burn the glucose in oxygen and then from the stoichiometric equation

$$\mathbf{C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6\,H_2O}$$

we get an RQ=1 value. This is a maximum, and corresponds a situation when glucose used only for energy production and not for incorporation. This means that in a real fermentation RQ has to be less than 1, and higher the incorporation less the RQ will be.

There is another efficiency measure of an aerobic fermentation, the **P/O ratio**. It gives the number of ATP moles formed during utilization half a mole (16 g) oxygen by the respiratory chain. Theoretical values of P/O are 2 or 3 taking into account the terminal oxidation route.

4.4.3. Models of growth and metabolit production

4.4.3.1. Monod –model family

The most widely known and used mathematical description of the microbial growth is the Monod-model.

$$r_x = \frac{dx}{dt} = \mu_x x \quad , \tag{4.62}$$

according to which the growth is an autonomous process, the rate of which is proportional to the actual biomass concentration. The differential equation can be solved with constant μ_x and gives an equation for the exponential phase. Exponential phase is characterized by an *unlimited balanced growth.* Providing that the reason of the exponential phase following declining phase is that one of the nutrients is not enough to guarantee the maximal specific growth rate (limiting substrate), the extended Monod model says

$$\mu_x = \mu_{x\,max} \frac{S}{K_S + S} \tag{4.4}$$

The whole differential equation system of the growth model is using the overall yield, too:

$$r_x = \frac{dx}{dt} = \mu_{x\,max} \frac{S}{K_S + S} x$$
$$r_S = \frac{dS}{dt} = -\frac{1}{Y_{x/S}} \mu_{x\,max} \frac{S}{K_S + S} x \tag{4.63, 4.64}$$

In certain cases, S is not only limiting but may be inhibiting, too. In those situations, a slight modification of the original model can describe the growth:

$$r_x = \frac{dx}{dt} = \mu_{x\,max} \frac{S}{aS + \frac{S^2}{K_i} + K_S} x \tag{4.67}$$

where **a** is often 1 and K_i a characteristic constant of the substrate inhibition. Substrate inhibition can be easily recognized on the basis of its graphical representations shown in Fig. 4.27.

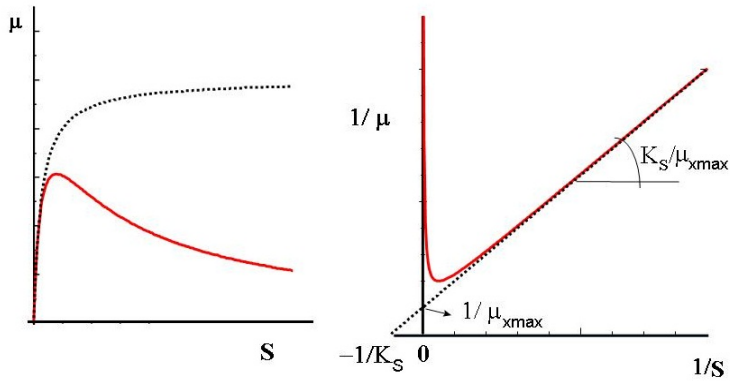


Fig.4.27.: Substrate inhibition

A further extension of the Monod model is the description of the growth when more than one substrate limits the growth rate. This multiple substrate limitation for n limiting substrates has been modelled in three different ways

a) interactive or multiplicative model:

$$\mu_x = \mu_{x\,max} \frac{S_1}{K_{s1} + S_1} \frac{S_2}{K_{s2} + S_2} \cdots \frac{S_n}{K_{sn} + S_n} \qquad \mu_x = \mu_{xmax} \prod_{i=1}^{n} \frac{S_i}{K_{si} + S_i} \tag{4.68}$$

b) additive description

$$\mu_x = \mu_{xmax} \cdot \left(w_1 \frac{S_1}{K_{s1} + S_1} + w_2 \frac{S_2}{K_{s2} + S_2} \cdots + w_n \frac{S}{K_{sn} + S_n} \right) \tag{4.69}$$

Where w_j is a weight function of the j^{th} limiting substrate

$$w_j = \frac{\frac{K_j}{S_j}}{\sum_{i=1}^{n} \frac{K_i}{S_i}} \tag{4.70}$$

c) non interactive description

$$\mu = \mu(S_1) \quad \text{vagy} \quad \mu = \mu(S_2) \quad \text{vagy} \quad \ldots \quad \mu = \mu(S_n) \tag{4.71}$$

In the latter, each function corresponds to a simple Monod model, and that is used, which predicts the smallest specific growth rate.

Describing the effect of the limiting substrate onto the specific growth rate, sometimes the original Monod relation did not give good results. With other mathematical approaches of the rectangular μ –S hyperbole many other descriptions were introduced by several authors. Among those the following three are the most accepted:

Teissier-equation: $$\mu = \mu_{x\,max} \left(1 - e^{-KS} \right) \tag{4.76}$$

Moser-equation: $$\mu = \mu_{x\,max} \frac{S^n}{K_s + S^n} = \mu_{x\,max} \left(1 + K_s S^{-n} \right)^{-1} \tag{4.77}$$

Contois-equation: $$\mu = \mu_{x\,max} \frac{S}{K_{sx} x + S} \tag{4.78}$$

So far only the growth behavior of fermentation processes was exposed though frequently we are equally interested in the rate of the *extracellular production*, too.

The first systematic approach was published by Elmer Gaden Jr.[11] in 1959. He had put the fermentations into three groups and stated the following:

[11] Elmer L. Gaden, Jr.: Fermentation Process Kinetics, J. of Biochem. and Microb. Technol and Eng. 1(4) 413–429 (1959).

„Experience has shown that fermentation processes fall more or less into three kinetic groups, which may be designated 'types I to III' for convenience… Type I: processes in which the main product appears as a result of primary energy metabolism. Examples of this type of system are most common in the older branches of fermentation technology, for instance: (1) aerobic yeast propagation (mass propagation of cells in general), (2) alcoholic fermentation, (3) oxidation of glucose to gluconic acid, and (4) dissimilation of sugar to lactic acid.
Type II: processes in which the main product arises indirectly from reactions of energy metabolism. In systems of this type the product is not a direct residue of oxidation of the carbon source but the result of some side-reaction or subsequent interaction between these direct metabolic products. Examples are: (1) formation of citric and itaconic acids, and (2) formation of certain amino acids.
Type III: processes in which the main product does not arise from energy metabolism at all but is independently elaborated or accumulated by the cells. It is perfectly true that carbon, nitrogen, etc., provided in essential metabolites appear in product molecules but the major products of energy metabolism are CO2 and water. Antibiotic synthesis is a prime example of this type."

These types can be well distinguished from each other examining the time course of the growth and production as well as the specific growth and production rates (Fig 4.28.).
In the **Type I** both are running parallel, in case of **Type III** growth and production are fully separated from each other (production starts only in the *idiophase*) in time, and the **Type II** is a kind of a mixture or transient between the two previous types.

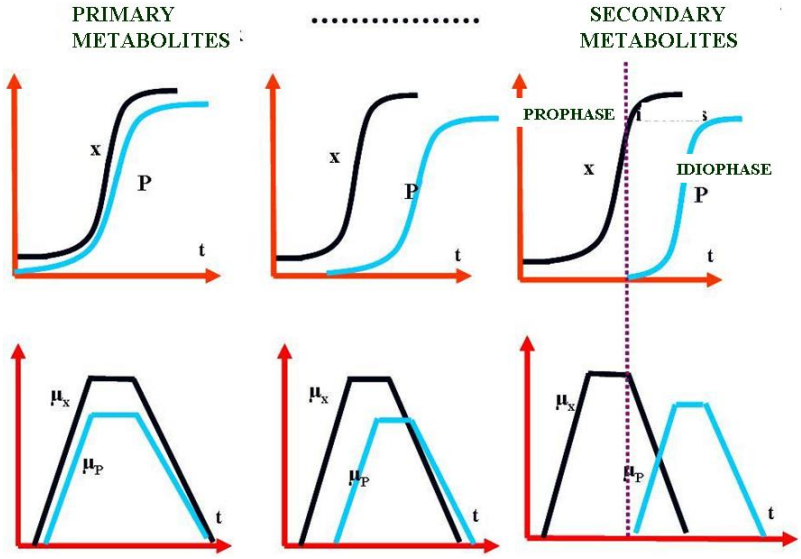


Fig 4.28.: Production types according Gaden

The most known and simplest kinetic description of the production is more or less in harmony with the types of Gaden and was introduced by Luedeking és Piret[12] :

[12] Luedeking R., Piret E. L.: Kinetic study of the lactic acid fermentation batch process at controled pH. J. Biochem. and Microbiol. Technol. and Eng. 1(4), 393(1959)

$$r_P = \frac{dP}{dt} = \alpha \frac{dx}{dt} + \beta x$$
$$\frac{1}{x}\frac{dP}{dt} = \mu_P = \alpha \mu_x + \beta \qquad (4.80, 4.81)$$

According to the values of α and β fermentations can be grouped again into three types, namely:

TypeI. : $\alpha > 0$	és $\beta = 0$	growth associated production
TypeII. : $\alpha = 0$	és $\beta > 0$	nongrowth associated production
TypeIII. : $\alpha > 0$	és $\beta > 0$	mixed type fermentation

Characteristic graphical plot of the Luedeking–Piret production kinetics is shown on Fig.4.29. that also gives the method of calculation of the parameters of the model.

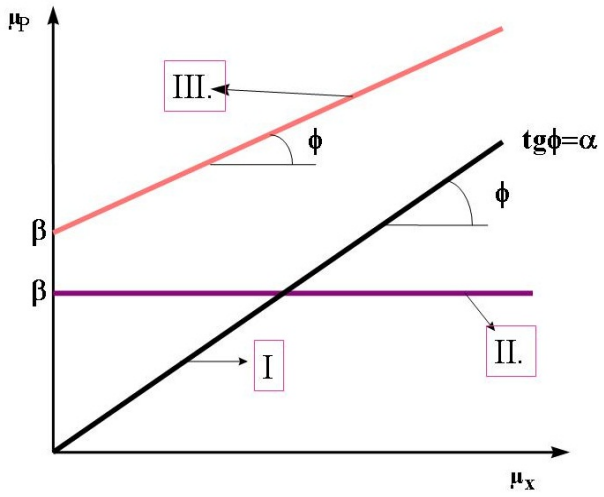


Fig 4.29.: Luedeking–Piret model of production

4.4.5. Continuous fermentation systems

4.4.5.1. Chemostat

Among continuous fermentation systems the most widely used is that of chemostat principle. It is a significant research tool but has also practical, even industrial applications as well, and biological wastewater treatment systems are based upon chemostat continuous fermentations.
Its name refers to the steady state of the microbial population in a constant chemically (and physically) constant environment. Chemostat was introduced into the fermentation practice in 1950 by Monod and at the same time by Novick and Szilárd. The „original" chemostat is shown on Fig.4.41.[13], and it was an aerated continuously fed glass equipment.

For easier understanding of this principle, let us look at the simplest necessary set up of a continuous fermentation in Fig.4.42. It consists of a perfectly mixed reactor (CSTR = continuous stirred tank reactor) of constant volume (V) that is fed with a constant fresh culture medium flow (f) by a pump and the broth is taken off by another pump with the same constant flow rate (f).

[13] From the reprint of Novick A., Szilárd L.: Science 111, Dec 15 (1950)

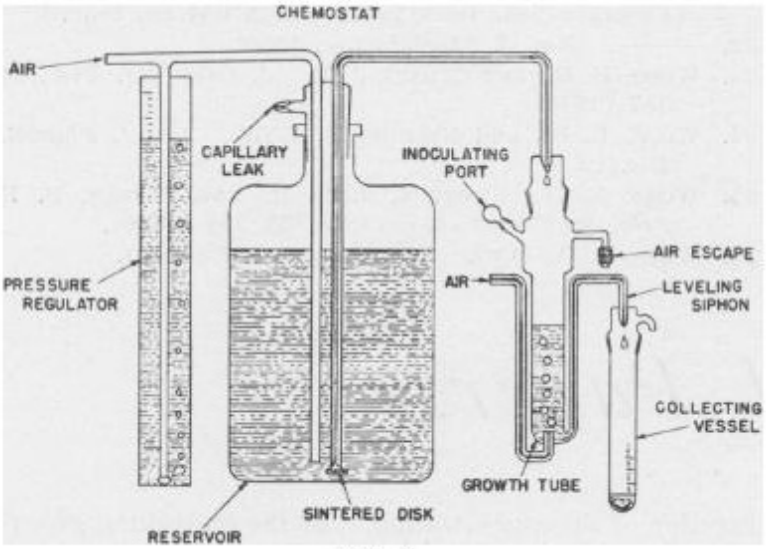


Fig. 4.41.: The „original chemostat" of Novick and Szilárd

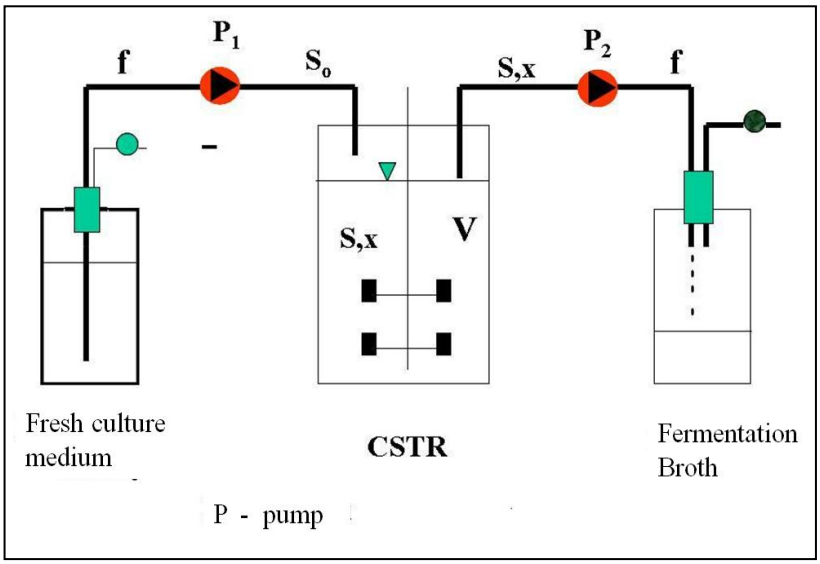


Fig 4.42.: Chemostat continuous fermentation setup

Material balance equations can be written either on the biomass or the substrates of the culture medium:

Biomass material balance:

$$V\frac{dx}{dt} = V\left(\frac{dx}{dt}\right)_{growth} - f.x \tag{4.172}$$

Material balance on the i-th substrate:

$$V\frac{dS_i}{dt} = fS_{i,0} - fS_i - V\frac{1}{Y_{x/S_i}}\left(\frac{dx}{dt}\right)_{growth} \tag{4.173}$$

Introducing the notion of **dilution rate**, which is per definition **D=f/V**, and turning to the simplest case, when one of the substrates is the limiting, and all the others are in high excess, as well as providing that the Monod model holds[14], we can write

$$\frac{dx}{dt} = \mu x - Dx = (\mu - D)x = \left(\mu_{max}\frac{S}{K_S + S} - D\right)x \tag{4.174}$$

$$\frac{dS}{dt} = D(S_0 - S) - \frac{\mu x}{Y}. \tag{4.175}$$

These kinds of continuous systems are tending to reach a **steady state**. In steady state all parameters characterizing the system will be constant, thus

$$\frac{dx}{dt} = 0 \quad \text{és} \quad \frac{dS}{dt} = 0 \ ,$$

from which μ=D comes. The microbe concentration and substrate concentration in the reactor (and in the outlet) can be got if we put Monod relation into μ=D. So, in steady state:

$$D = \mu_{max}\frac{S}{K_S + S} \qquad \text{and} \qquad \bar{S} = \frac{K_S D}{\mu_{max} - D} \tag{4.176}$$

From (4.175) we get

$$D(S_0 - \bar{S}) = \frac{\mu x}{Y}, \tag{4.177}$$

and this will give the steady state concentration of the biomass:

$$\bar{x} = Y(S_0 - \bar{S}) = Y\left(S_0 - \frac{K_S D}{\mu_{max} - D}\right). \tag{4.178}$$

In a chemostat the inlet limiting substrate concentration S_0 and the dilution rate value that is chosen by us unambiguously determine the steady environment of the microorganisms (of course beside its kinetic parameters).
As eq.4.178 shows μ has an upper limit because the denominator must not be 0 or negative.
$\mu \cong \mu_{max}$ if $S >> K_S$, this is true in our case if $S = S_0$. Then

$$\mu = D_{critical} = \mu_{max}\frac{S_0}{S_0 + K_S} \cong \mu_{max} \tag{4.179}$$

This situation is called *washing out* when the substrate flows through the system untouched and its outlet concentration is S_0 as well, and the biomass concentration becomes zero.
Consequently the chemostat system may be operated only with dilution rates $\mu = D < \mu_{max}(S_0/(S_0+K_S))$.
This means that chemostat system **always operates in substrate limit** that is a limited balanced growth, μ is always less than specific growth rate in a corresponding exponential phase of a batch fermentation.

[14] It is an important condition, for applying other models other steady state values will be resulted.

The **productivity** of a chemostat system is higher than that of a batch one. Productivity is defined as

$$J = D.x \quad [g/l.h] \quad \text{vagy} \left[kg/m^3h \right]. \tag{4.180}$$

Let us try to calculate the possible maximal productivity of a chemostat continuous fermentation system. Putting 4.178 into 4.180 we get

$$J = D.\bar{x} = D.Y\left(S_0 - \frac{K_S D}{\mu_{max} - D} \right) \tag{4.181}$$

This is a function depending upon D, and its maximum will be where its first derivative becomes not:

$$\frac{\partial J}{\partial D} = 0. \tag{4.182}$$

In this case the dilution rate, which exactly gives a maximum productivity, will be the following:

$$D_{max} = \mu_{max}.\left(1 - \left(\frac{K_S}{S_0 + K_S} \right)^{1/2} \right) \tag{4.183}$$

And the outlet biomass concentration will be

$$\bar{x}_{max} = Y\left[S_0 + K_S - \sqrt{K_S\left(S_0 + K_S\right)} \right]. \tag{4.184}$$

As a result, the maximum productivity of a chemostat is

$$\begin{aligned} J_{max} &= D_{max}\bar{x}_{max} = \mu_{max} Y\left[1 - \left(\frac{K_S}{K_S + S_0} \right)^{1/2} \right].\left[K_S + S_0 - \sqrt{K_S\left(S_0 + K_S\right)} \right] = \\ &= Y\mu_{max} S_0 \left(\sqrt{\frac{K_S + S_0}{S_0}} - \sqrt{\frac{K_S}{S_0}} \right)^2 \end{aligned} \tag{4.185}$$

If $S_0 >> K_S$, then (4.185) simplifies to $J_{max} \cong Y.\mu_{max}.S_0$, that gives a theoretical maximum productivity that cannot really reach but is usually used as a design parameter.

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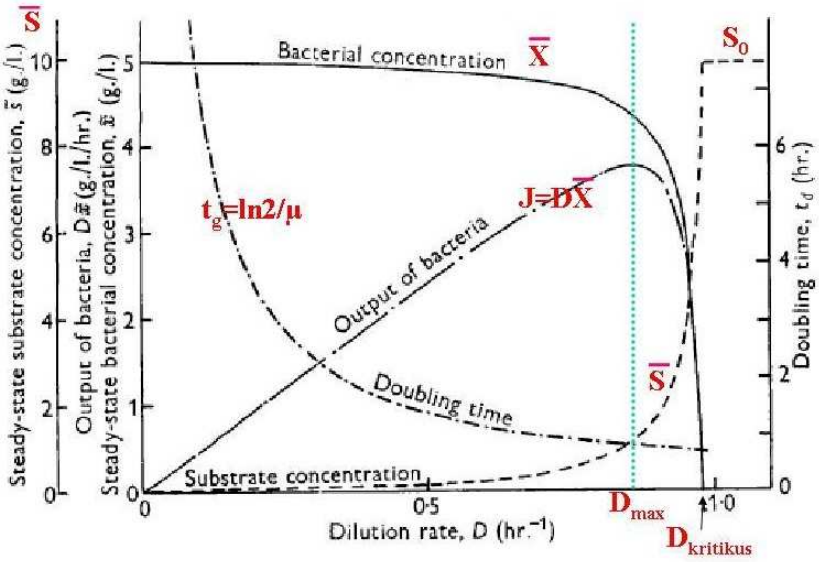


HERBERT, ELSWORTRH, TELLING (1956). The Continuous Culture of Bacteria; a Theoretical and Experimental Study , *J . gen. Microbiol.* 14, 601–622

Fig 4.43.: Dependence of steady states on dilution rate

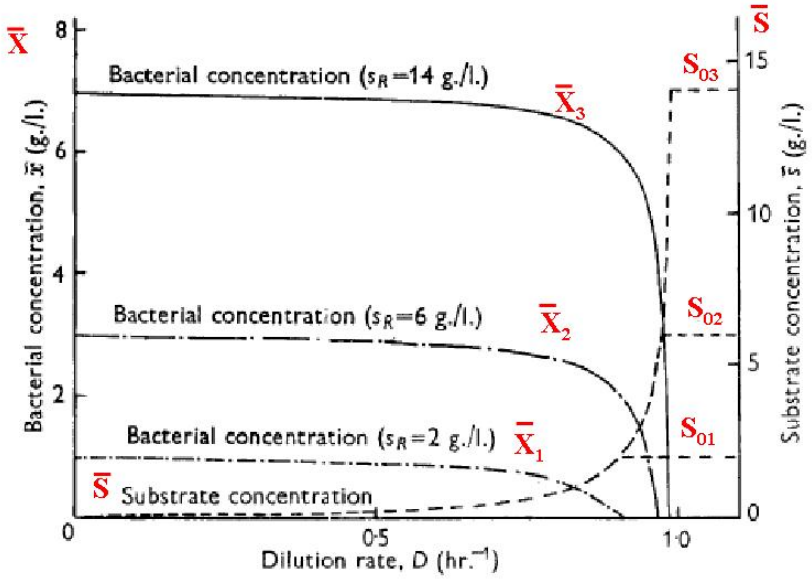


Fig 4.44.: Dependence of steady states on inlet substrate concentration.

Dependence of the steady states on dilution rates is shown on the Fig 4.43 that is taken from the original paper of Herbert and coworkers[15]. It can be seen that along a relatively long range of dilution rates, the outlet x does not change very much but nearing the critical dilution rate it sharply decreases to the washing out. Similarly the outlet S concentration remains near to the zero but going nearer to D_{crit} , it sharply increases to the S_0 value (mathematically it is infinite!).

Fig 4.44 shows how the performance depends on the inlet S_0 concentration. Naturally x strongly depends upon the inlet substrate but the outlet S does not depend at all! Note that this is a common feature of the continuously operating reactors (not only fermentors!). This means that let the inlet

[15] Herbert D., Elsworth, R., Telling R. C. (1956): The Continuous Culture of Bacteria; a Theoretical and Experimental Study *J . Gen. Microbiol.* 14, 601–622.

concentration even very high be, it falls near to zero instantaneously as the culture medium flows in, so it is almost impossible to examine substrate inhibition in a chemostat.

4.4.5.1.1. Transients in chemostat

So far, the steady behavior of the chemostat has been shown, but of course there are instances of transient behavior as well:

- When we start the continuous system – it starts as a batch one and at a given time one can start the inlet and outlet pumps, thus the continuous run. Every continuous fermentation starts as a batch!
- At any jump, like changes of the control parameters: dilution rates, substrate concentration, temperature, pH, etc.

  On the Fig 4.45 this startup situation is seen.

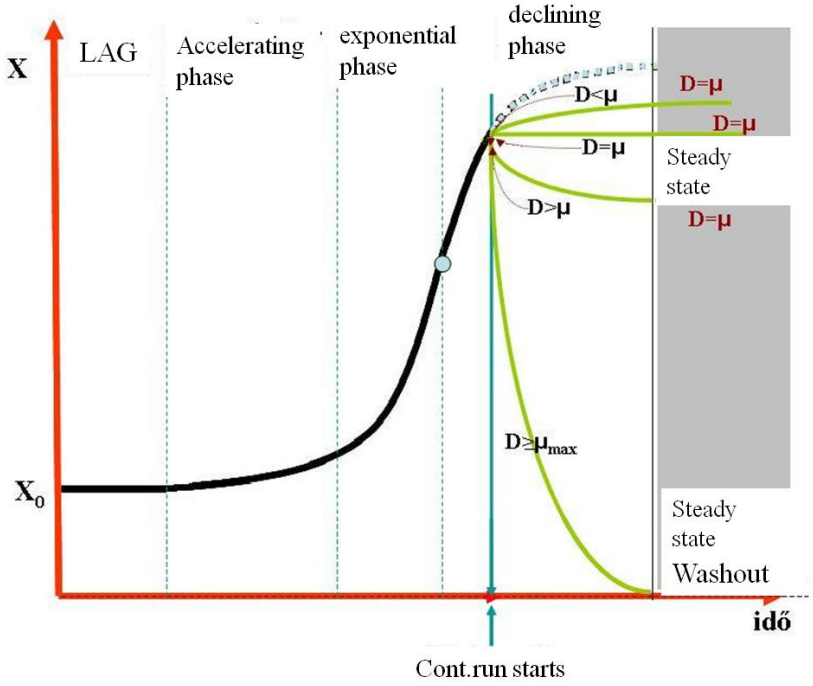


Fig 4.45.: Continuous fermentation starts as a batch one.

4.4.5.1.4. Designing a chemostat

The (4.176) and (4.178) steady state equations create the possibility of one of the designing methods. If the culture medium and all the other environmental conditions are the same in the designed continuous system as in a corresponding batch one, based on the previously determined kinetic parameters and planned dilution rate, the outlet concentrations can be calculated.

If we do not know the kinetic parameters, the graphical method shown in Fig 4.8. can be applied.

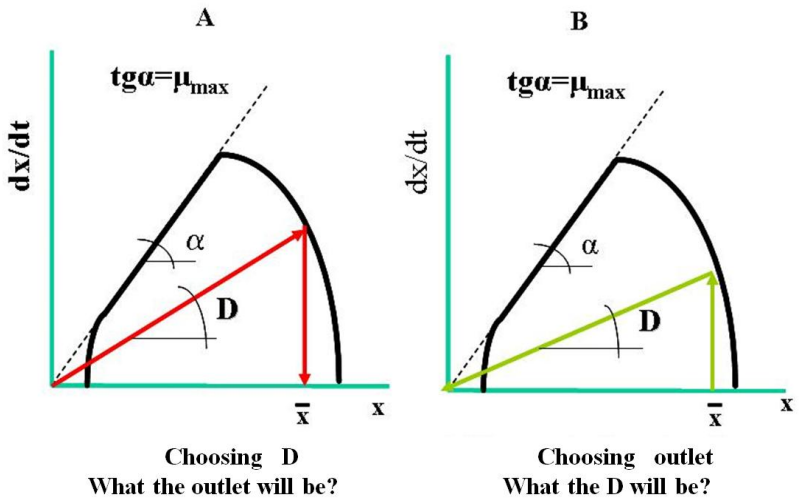


Fig 4.52.: Designing a chemostat with a graphical method

4.4.5.1.5. Application of the chemostat continuous fermentation

Chemostat have several advantages over the batch culture:

- it is of higher productivity, in steady states microbes are growing in homeostasis, so a constant broth can be taken off the bioreactor for an arbitrarily long time.
- in a continuous system steady state makes a better possibility for measurement and control tasks.

It is used first of all for biomass production purposes: SCP, baker's yeast, fodder yeast, beer, intracellular products).
There are also a series of research use. With the help of it the physiological responses of a strain given on environmental changes may be examined thoroughly. With responses in the steady states, stepwise optimization processes (examining the effect of pH, temperature, culture media composition, etc) can be realized, too.(Fig 4.53.)

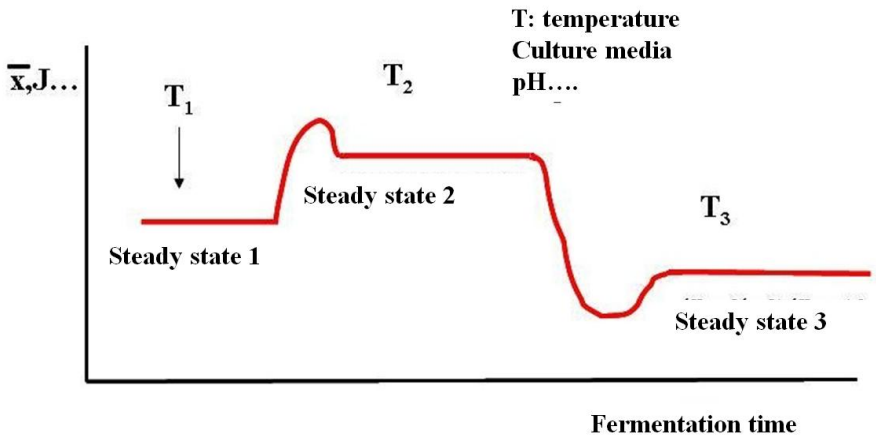


Fig. 4.53. Using chemostat for optimization

Application of a single, one stage continuous system is not suitable for the „product" fermentations. But in several cases, it is used: alcoholic fermentation and beer production. Other primary and mainly secondary productions are not known so far.

Nevertheless, in biological wastewater treatment (aerobic as well as anaerobic) chemostat is exclusive.

4.4.5.2. Other special chemostat systems

Chemostat can be realized in more than one stage, too. This means the connection of subsequent more bioreactors after each other. According to the mode of the substrate inlet there are different possibilities:

– single sream, multistage systems (Fig. 4.54.),
– multiple stream, multistage systems (Fig.4.55.).

Fig 4.52 shows the graphical design method applicable to single stream multistage systems

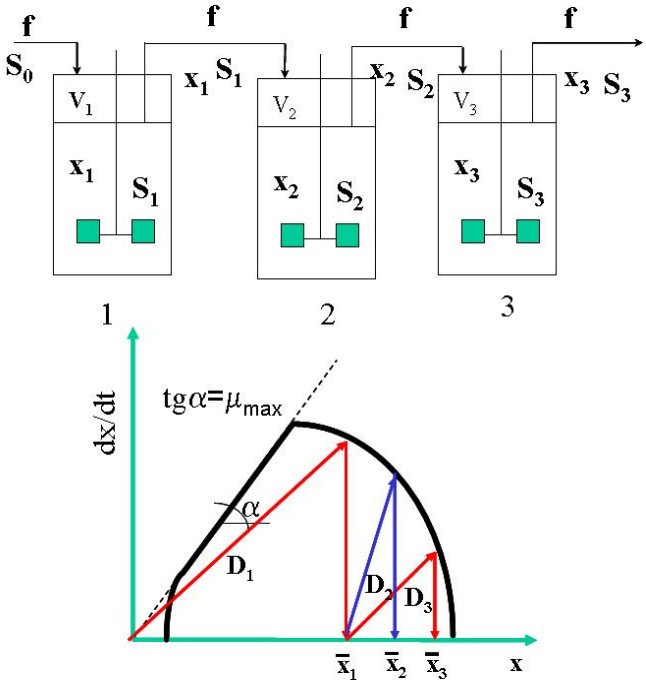


Fig.4.54.: Single stream multiple stage continuous system

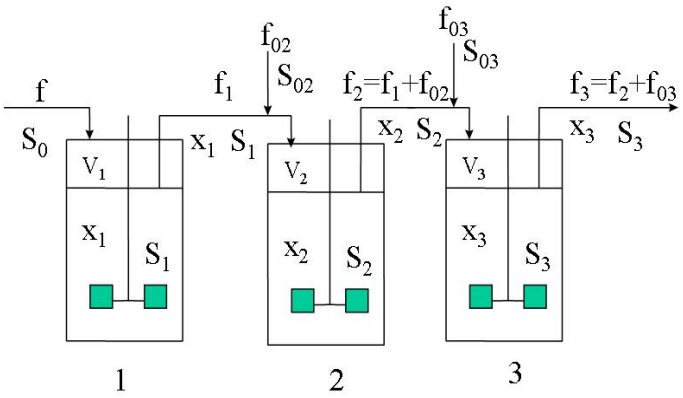


Fig 4.55.: Multistream multistage chemostat

An often applied method – especially in biological water treatment – is that the outlet broth is introduced into a cell separation device (a settling tank, a centrifuge, a filter) in which the biomass is separated from the medium and the whole biomass or a part of it is led back to the bioreactor. These, so called cell retention or recycling systems are of highly effective.

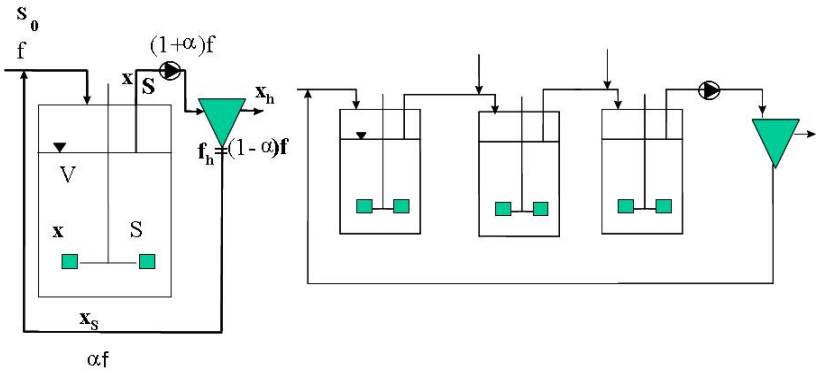


Fig 4.56: Cell retention and recycle

4.4.5.3. Other continuous fermentation systems

Turbidostat is a special fermentation technic. Look at the diagram on Fig. 4.60., where the technical set up is shown. This fermentation also starts with a batch run. Fermentation broth is continuously circulated through a loop by pump 1. In this loop there is a photometer cell in which the turbidity (optical density) of the broth can be continuously monitored.

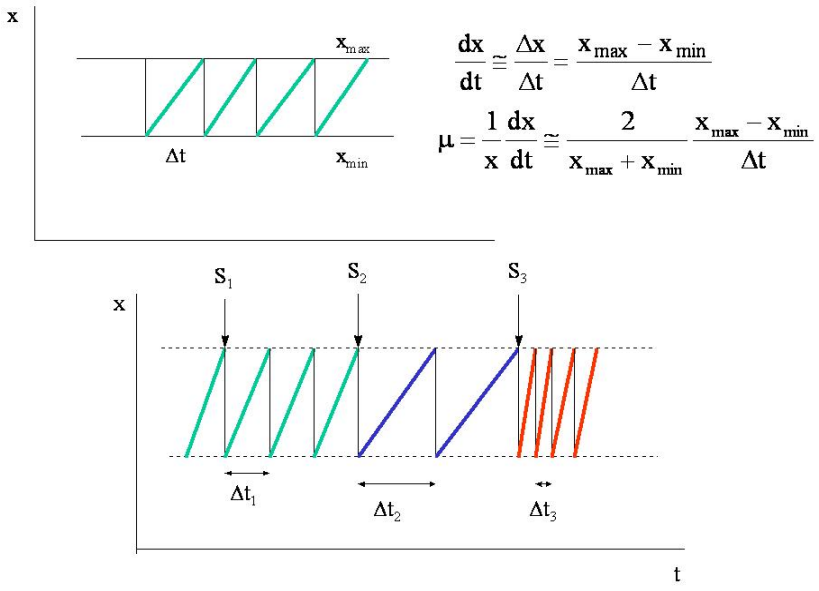


Fig. 4.59.: Turbidostat continuous fermentation and its use for optimization

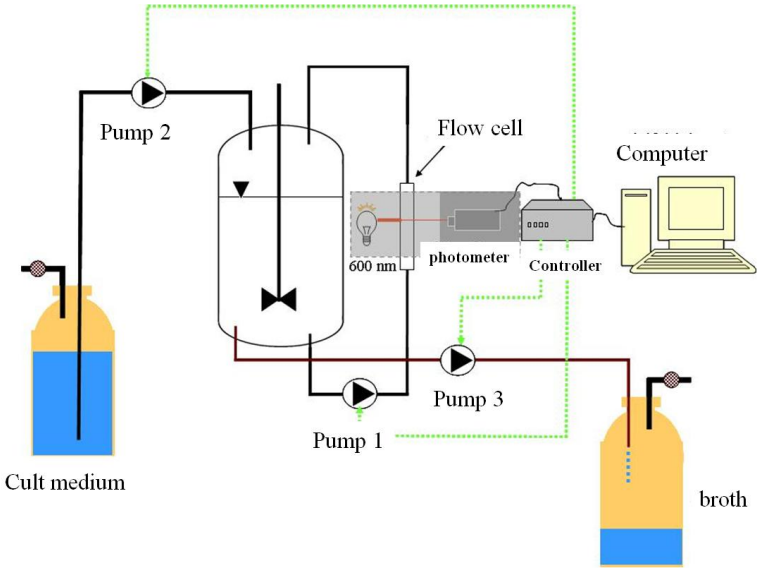


Fig 4.60.: Setup of a turbidostat

When biomass concentration, i.e. the turbidity, which is proportional with it, reaches an arbitrarily chosen value, x_{max} , pump3 starts to take off broth and at the same time another pump (pump2) starts to introduce fresh media into the bioreactor. This corresponds a dilution of the broth, and when the optical density decreases to a certain value of x_{min} , these two pumps stop. In the system the growth continues, the optical density increases, and the cycle starts again. The difference between the maximum and the minimum biomass concentrations is so small that this is a real continuous system with practically constant x biomass concentration. In a turbidostat the growth rate and specific growth rate can easily be determined according to the following relations:

$$\frac{dx}{dt} \cong \frac{\Delta x}{\Delta t} = \frac{x_{max} - x_{min}}{\Delta t} \tag{4.213}$$

and

$$\mu = \frac{1}{x}\frac{dx}{dt} \cong \frac{1}{x}\frac{\Delta x}{\Delta t} = \frac{2}{x_{max} + x_{min}}\frac{x_{max} - x_{min}}{\Delta t} \tag{4.214}$$

As one can see, these determinations are based upon time measurements. The time intervals of the nonoperation of the pumps are to be measured. An advantage of this system is, that it can be operated either in the exponential or the declining growth phases, a disadvantage of it is, that this system can be used only with clear culture media and with rather diluted broths of bacterial or yeast (unicellular) cells.

4.4.6. Special fermentation technics

4.4.6.1. Fed batch fermentation

Similarly, to the chemostat the fed batch system also operates in the declining growth phase. practically it is an extension of that phase. In a fed batch system fresh culture medium is fed into the bioreactor but broth is not taken off, so the volume of the broth increases according to the feed. The feed can be run with a constant volumetric rate or periodically according to a predetermined time program as well as according to the need of the biomass, automatically.

This is a spreading technic, nowadays almost the most frequently used fermentations belong to this type. It is applied when a constant low substrate concentration is to be kept (e.g., baker's yeast

fermentation, for the so-called Crabtree effect), or when, as an opposite, high concentration of a substrate would be favorable (e.g. citric acid fermentation with glucose feed) or when some kind of precursor has to be added continuously (penicillin fermentation: phenylacetic acid, tryptophan fermentation, indole)

Fed batch system can be considered as a special case of a chemostat, realized in a CSTR, which is run by changing volume and with some fresh medium addition. The rate of volume change is a function that can be constant, time dependent or even periodic:

$$\frac{dV}{dt} = f(t) \tag{4.215}$$

Dilution rate also can be defined, but now it will be a variable:

$$D(t) = \frac{f(t)}{V(t)} \tag{4.216}$$

If we mark the total biomass in the system with X, then the biomass concentration is x = X/V, thus the rate of change of the biomass concentration become a total derivative:

$$\frac{dx}{dt} = \frac{V\frac{dX}{dt} - X\frac{dV}{dt}}{V^2}. \tag{4.217}$$

and

$$\frac{dx}{dt} = \frac{1}{V}\frac{dX}{dt} - \frac{1}{V}D.x \tag{4.218}$$

It is true furthermore, that

$$\frac{d(Vx)}{dt} = \mu(Vx). \tag{4.219}$$

With eq(4.218) and (4.219) we get the following expression for the time change of x:

$$\frac{dx}{dt} = \frac{1}{V}\mu(V.x) - \frac{1}{V}D(V.x) = (\mu - D)x, \tag{4.220}$$

that is formally the same as the chemostat expression was. Here again a steady state will form but with a changing dilution rate thus, with a changing specific growth rate, and $\mu = D$.

For the substrate, the same material balance equation will give also a steady state concentration of the substrate and biomass, and these are the same as in the case of the chemostat:

$$\frac{dS}{dt} = (S_{be} - S)D - \frac{1}{Y}\mu_{max}\frac{S}{K_S + S}x \tag{4.221}$$

$$\bar{S} = \frac{DK_S}{\mu_{max} - D} \qquad \bar{x} = Y(S_{be} - \bar{S}) \tag{4.222, 4.223}$$

Thus, biomass concentration is constant while the total biomass, X continuously increases. E.g., providing a constant medium feed rate it will increase along a straight line:

$$\frac{dV}{dt} = f \text{ is constant, so} \rightarrow \int_{V_0}^{V} dV = f \int_{0}^{t} dt \rightarrow V = V_0 + ft \,. \tag{4.224}$$

and

$$X = V\bar{x} = V_0\bar{x} + f\ \bar{x}\ t \cong x_0 + fYS_{be}t \tag{4.225}$$

Performance of a fed batch fermentation with constant feed is shown in Fig4.62.

In a fed batch system, the duration of the feed is determined by the possible volume difference between the feed start and its stop. Usually $(0{,}5–0{,}6)V_{total}$ is the starting volume and it goes up to about to $(0{,}7–0{,}85)V_{total}$.

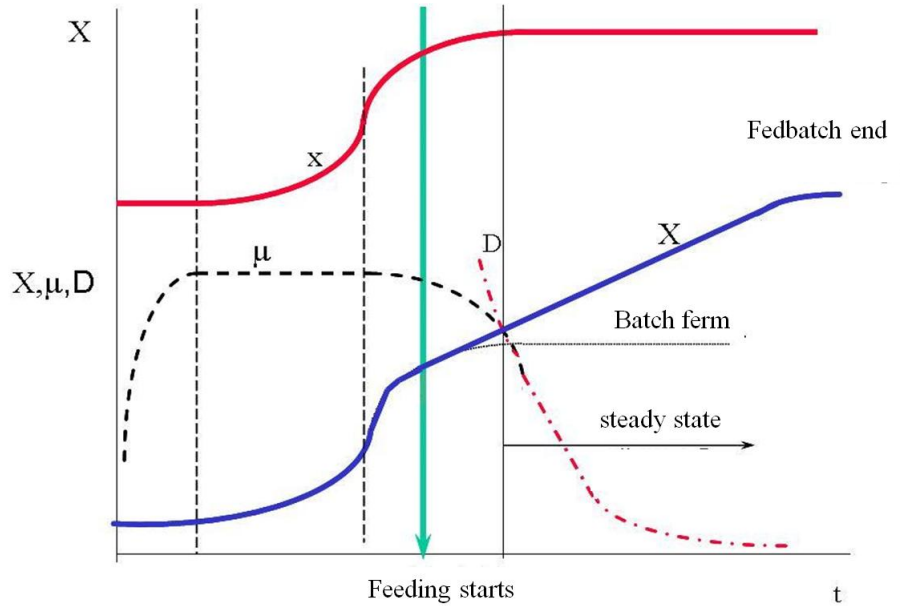


Fig 4.62.: A constant fed fermentation

4.5. Aeration and agitation of fermentation systems

4.5.1. Introduction, role of the oxygen

Energy releasing mechanisms of living systems are of multiple kinds. There are organisms that need, others do not need molecular oxygen for the energy production. In both cases the metabolic process along which the energy is produced in a metabolizable form, is called respiration. *RESPIRATION is an energy yielding metabolic process in that an energy source (organic or inorganic) is oxidized by an inorganic compound*. If the oxidizing agent is the molecular oxygen the process is an **aerobic respiration** but in the opposite case it is called **anaerobic respiration.** For both types we can see examples in the Table 4.21. (the *-signed examples are anaerobic respirations)

Table 4.21.: Types of the respirations

Energy source (reducing=substrate to be oxidized)	Oxidant (Terminal electron acceptor)	Product of the respiration	Example
H_2	O_2	H_2O	Hydrogen bacteria
*H_2	SO_4^{2-}	H_2O+S^{2-}	*Desulfovibrio*
NH_3	O_2	$NO_2^- + H_2O$	Nitrifying bacteria
NO_2^-	O_2	$NO_3^-+H_2O$	Nitrifying bacteria
*organic compound	NO_3^-	N_2+CO_2	Denitrifying bacteria
Fe^{2+}	O_2	Fe^{3+}	*Ferrobacillus*
S^{2-}	O_2	SO_2+H_2O	*Thiobacillus*
organic compound	O_2	CO_2+H_2O	Most of microbes, and plants and animals

Most industrial fermentations are operated by aerobes, but there are important anaerobic examples, too.

If we examine what is the role of the oxygen in the life of aerobes, the simplest answer is that **oxygen is the final electron acceptor** in the terminal oxidation metabolic route (the details are given in biochemistry textbooks).

4.5.2. Oxygen demand of microbes

There are two different possibilities of culturing an aerobic microorganism. One possible culturing way is the so called *surface culture*, when the microbe is growing on the surface of a solid (solidified) or liquid culture medium, the other is the *submerged culture* when the microorganism is living inside the broth. Let it be this or that, the microbes take up the oxygen in dissolved form in both cases.

Respiration rate may be given in two ways:

1. respiration rate = $\frac{dc}{dt}$ [mmol $O_2/dm^3 \cdot h$], [kg $O_2/m^3 \cdot h$] (4.253)

2. specific respiration rate [h^{-1}] (4.254)

Dissolved oxygen (DO) is a substrate for the cells thus it acts similarly to the other substrates. If all the other substrates of the culture medium are in enough excess, then only the dissolved oxygen will limit the growth. Its effect can be described by the Monod model

$$\frac{dx}{dt} = \mu_{max} \frac{c}{K_{O_2} + c} x \,, \tag{4.255}$$

where c is the concentration of the dissolved oxygen [mg·dm^{-3} vagy mmol·dm^{-3}].
We shall use the notion of the overall yield for oxygen, defined as follows

$$Y_O = \frac{\Delta x}{\Delta c}, \tag{4.256}$$

with introducing it, the *respiration rate* can be described as

$$\frac{dc}{dt} = -\frac{1}{Y_O}\frac{dx}{dc} = -\frac{1}{Y_O}\mu_{max}\frac{c}{K_{O_2} + c} x \tag{4.257}$$

Dividing this by x, it refers to unit quantity of biomass, so it is the *specific respiration rate*:

$$Q = \frac{1}{x}\frac{dc}{dt} = -\frac{1}{Y_O}\mu_{max}\frac{c}{K_{O_2} + c} \tag{4.258}$$

Plotting (4.258) is shown in Fig 4.72. This is a rectangular hyperbole with an assymptote of μ_{max}/Y_O, thus it is the maximum specific respiration rate, Q_{max}. If $C >> K_{O2}$, denominator becomes negligible and

$$Q \cong Q_{max} \,. \tag{4.259}$$

This means that there is a *practica*l oxygen level, C_{kr}, *critical dissolved oxygen concentration*, above which the specific respiration rate does not depend on the dissolved oxygen level any more, respiration enzyme system of the microbe becomes saturated with oxygen.

If $C << K_{O2}$ (near to the origin), C becomes negligible to K_{O2} and the

$$Q \cong Q_{max}\frac{c}{K_{O_2}} \tag{4.260}$$

relationship holds, showing that at low dissolved oxygen levels the respiration is a linear function of the dissolved oxygen concentration. From Fig. 4.72. also comes that the numerical value of K_{O2}, cc of the dissolved oxygen is equal to the oxygen level at the half maximum of the specific respiration rate.

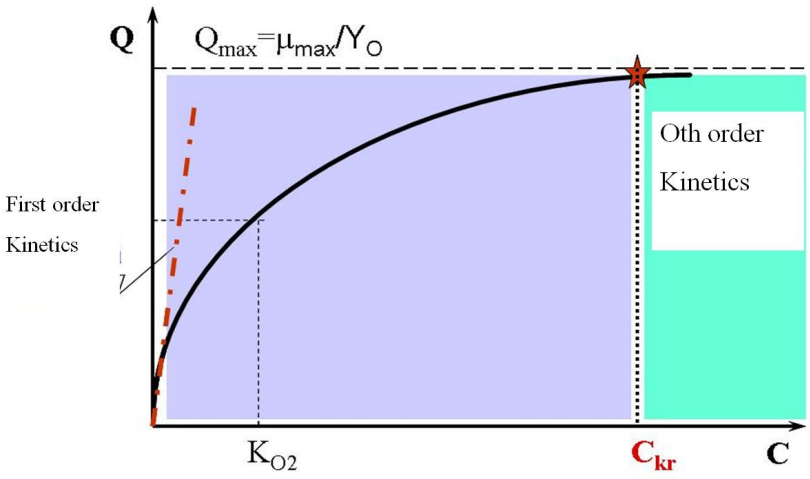


Fig 4.72.: Specific respiration rate as a function of dissolved oxygen concentration.

The overall yield coefficient for oxygen depends on the specific growth rate as the equation below shows

$$\frac{1}{Y_O} = \frac{1}{Y_{OG}^{max}} + \frac{m_O}{\mu} \tag{4.261}$$

This equation does not contain an incorporation term, despite the fact, that there are several biochemical reactions going together with direct oxygen incorporation. But the contribution of these to the whole oxygen consumption is so small that they are negligible (taken involved into Y_{OG}^{max}.)

Summarizing the important parameters describing the respiration, we get the following table:

μ_{max}	– specific growth rate
Y_O	– overall yield for oxygen
m_O	– Specific maintenance coefficient for oxygen [g O_2/g cell·h]
Y_{OG}^{max}	– (theoretical)maximal growth yield for oxygen
Q_{max}	– maximal specific respiration rate or specific oxygen demand
K_{O2}	– substrate saturation constant (Monod-constant) for oxygen.
C_{kr}	– critical dissolved oxygen concentration

In the Table 4.25. there is a comparison of the energy source glucose and oxygen regarding the available concentrations in the culture medium, their critical concentrations, and their specific consumption rate in a yeast culture of 1 g/dm^3 biomass concentration. One can follow, that while the available concentrations for the microbes are higher with several order of magnitude in case of glucose (because oxygen is a slightly soluble gas), the consumption rates are comparable. It is the proof that only a continuous supply can assure the necessary oxygen level for the growth. If not – in the example - the glucose would be enough for 17 hours but the oxygen only for less than two minutes.

This simple way of thinking may be convincing of the outstanding importance of the oxygen transfer as a necessary unit operation of fermentation systems. This mass transfer will be discussed in the following part **as aeration and agitation**.

Table 4.25.: Comparison of glucose and dissolved oxygen as substrates

(Saccharomyces cerevisiae, 1 g/dm^3)

	Glucose	Oxygen
Concentration in the fermentation broth	1% ≈10^4 mg/dm^3	7 mg/dm^3
Critical concentration	50 mg/dm^3	0,7 mg/dm^3
Specific consumption rate	580 mg/g.h	208 mg/g.h

4.5.3. Basic correlations of oxygen mass transfer

The oxygen transfer into the dissolved state and its uptake by the microorganism cells are basically determined by the diffusion of the oxygen through not or only slightly mixed fluid and gas layers. This complex process can be followed on the Fig. 4.74. The molecules of the oxygen have to go through the following barriers or otherwise the following primary mass transfer steps characterize this process:

1. Mass transfer of the oxygen from the bulk gas (the bubbles) with diffusion through the gas film around the gas/liquid interface. This can be characterized by $1/k_g$ resistance or k_g mass transfer coefficient of the gas film.
2. Next transfer step is going on through the liquid film of δ_l thickness around the bubble. The resistance of this unmixed, stagnant liquid film is $1/k_l$ and its mass transfer coefficient is k_l.[16]
3. Often the bulk liquid also has a given resistance, though through this the mass transfer is driven by convection. If the mixing here is not perfect, this transfer also may be rate limiting.
4. The next unmixed region is the liquid film around the microbes. The resistance of this sometimes is not negligible. Here starts the chain of mass transfer into the cells by the uptake and consumption mechanism of the oxygen, that
5. continues into the different forms of the biomass (individual cells: bacterium and yeast and fungi mycelia) or a mass of microbial cells (flocs) or into a fungi pellet.
6. Finally there exists a resistance of the utilization „reaction" of the oxygen. It means that the enzymatic utilization of the oxygen (the respiration) is also a time process, going on with a rate of finite velocity.

Among these six elements of the mass transfer usually the first two are the slowest, i.e. rate limiting.

For the description of these process steps more theoretical approaches have been born. Only the so called *two film theory* will be discussed here, the other two approaches will only be briefly mentioned.

[16] Let us observe that the interface itself is a physical fiction, without thickness and resistancy. Its just an imagined line between the two masses of different properties: liquid and gas.

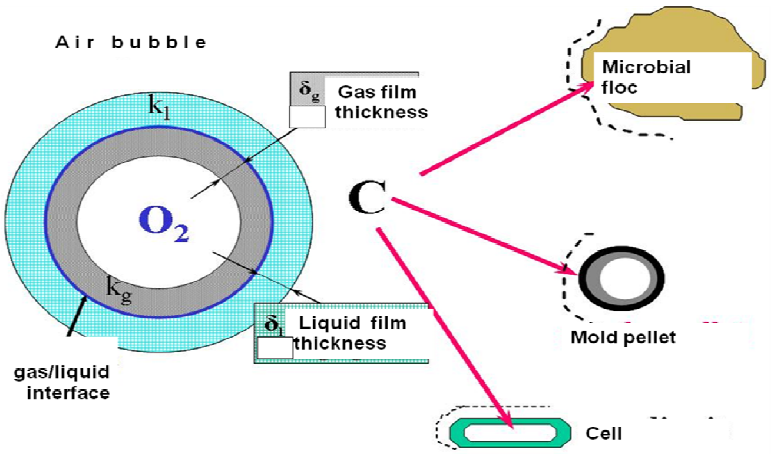


Fig 4.74.: Process steps of the oxygen absorption from a gas bubble and the uptake by the microbes.

According to the two-film theory a stagnant gas and fluid layer can be assigned to the inner and outer surface of the bubble with a resistance $1/k_g$, and $1/k_l$ respectively.

The reciprocal of these – mass transfer coefficients of the gas and the liquid films – are proportional to the diffusivity of the oxygen in the gas and the liquid phases:

$$k_g = \frac{D_{O_2}^{gas}}{\delta_g} \quad \text{és} \quad k_l = \frac{D_{O_2}^{liquid}}{\delta_l},$$

where δ_g and δ_l are the film thickness of the gas and liquid film, respectively

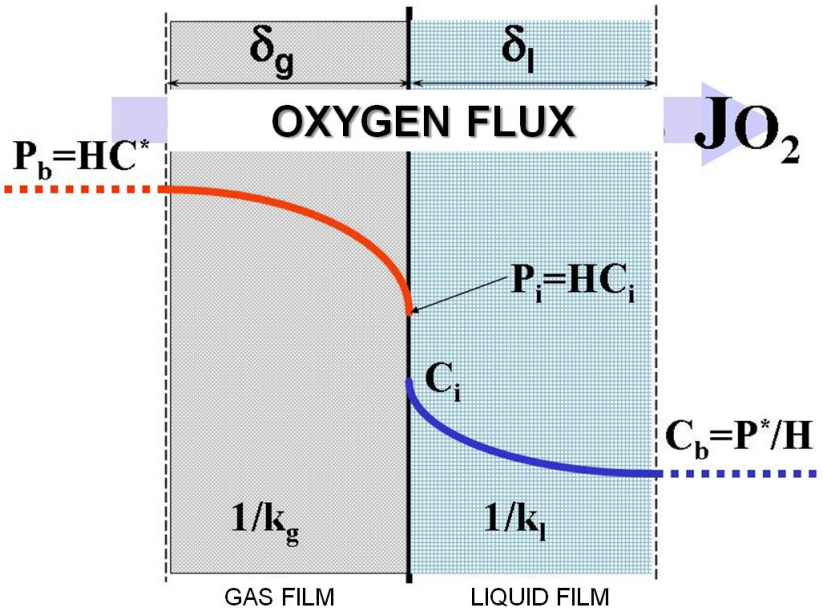


Fig.4.75.: Scheme of the two-film theory

Consider a system - shown on Fig.4.75. - of mass transfer from bubbles into a well-mixed liquid phase, having a uniform dissolved oxygen concentration of (C_b). Let us suppose steady state for the interface, i.e., there is no oxygen accumulation on the interface.[17]

Oxygen flux can be written as:

$$J_{O_2} = \frac{\text{transferred } O_2 \text{ (mol or g)}}{\text{time.surface}} = \frac{\text{driving force}}{\text{resisi tan ce}}$$ [18]

In our case this flux can be written in two ways, applying concentrations or partial pressures for the driving force.

GAS BUBBLE $\rightarrow$ INTERFACE = INTERFACE $\rightarrow$ LIQUID

$$J_{O_2} = \frac{p_b - p_i}{\frac{1}{k_g}} = \frac{C_i - C_b}{\frac{1}{k_l}}$$

$$J_{O_2} = Hk_g\left(C^* - C_i\right) = \frac{\frac{p_i}{H} - \frac{p^*}{H}}{\frac{1}{k_l}} \qquad (4.262)$$

where: **H** is the Henry-constant

p_b partial pressure of the oxygen in the gas bubble and **C*** is the oxygen concentration in a (hypothetical) liquid that would be in equilibrium with it.

C_b concentration of the dissolved oxygen in the bulk liquid, **p*** would be the partial pressure of the oxygen in a (hypothetical) gas phase in equilibrium.

C_i and **p_i** the concentration and partial pressure at the interface.

Crosswise is also possible to write these equations, one with concentrations and one with partial pressures:

$$J_{O_2} = k_g\left(p_b - p_i\right) = \frac{k_l}{H}\left(p_i - p^*\right)$$
$$J_{O_2} = Hk_g\left(C^* - C_i\right) = k_l\left(C_i - C_b\right) \qquad (4.263)$$

Since the partial pressure and concentration at the interface cannot be measured, let us express them from (4.263).

---

[17] Obviously an oxygen molecule arriving to the fictive interface will instantenously go further – accumulation is impossible!

[18] See.: Ohm's law: $I = \frac{\Delta U}{\sum R_i}$

$$p_i = \frac{\frac{p^*}{k_g} + \frac{H}{k_l} p_b}{\frac{H}{k_l} + \frac{1}{k_g}}, \tag{4.264}$$

$$C_i = \frac{Hk_g C^* + k_l C_b}{k_l + Hk_g} \tag{4.265}$$

Putting latter into (4.263) we get the flux expressed with concentrations as follows:

$$J_{O_2} = \frac{C^* - C_b}{\frac{1}{Hk_g} + \frac{1}{k_l}} \tag{4.266}$$

Or with partial pressures:

$$J_{O_2} = \frac{p_b - p^*}{\frac{H}{k_l} + \frac{1}{k_g}} \tag{4.267}$$

Introducing the overall liquid phase and gas phase mass transfer coefficients

$$\frac{1}{K_L} = \frac{1}{Hk_g} + \frac{1}{k_l} \qquad \frac{1}{K_g} = \frac{H}{k_l} + \frac{1}{k_g} \tag{4.268}$$

the oxygen flux written for the liquid phase and for the gas phase will be:

$$J_{O_2} = K_L \left(C^* - C_b \right) \qquad J_{O_2} = K_g \left(p_b - p^* \right) \tag{4.269}$$

From now on we shall use the equation written for the liquid phase.

Since the Henry-constant is a number of fourth order of magnitude (see Table 4.26) and the diffusivity of the oxygen in the gas phase is again forth order of magnitude higher than in the liquid phase, i.e.

$$\frac{k_g}{k_l} \cong \frac{D_{O_2}^{\text{gáz}}}{D_{O_2}^{\text{folyadék}}} \approx 10^4, \tag{4.270}$$

$K_L \cong k_l$, thus really the rate limiting step of the mass transfer is the 2. process, the transfer across the liquid film around the bubble.

Note, that beside the two film theory (that was introduced by Nernst in 1904), other descriptions of the oxygen transfer are also known, that in some cases more or less better describe the processes.

The two-film theory itself supposes that mass transfer coefficient is proportional to the diffusivity:

$$k_l = \frac{D_{O_2}^{\text{liquid}}}{\delta} \tag{4.271}$$

Many times instead of (4.271) the following empirical correlation gives a better approach:

$$k_l = \frac{(D_{O_2}^{liquid})^n}{\delta} \tag{4.272}$$

where n is only between 0,8-0,9.

Higbie's (1935) „liquid penetration model" gives another approach:

$$k_l = 2\sqrt{\frac{D_{O_2}^{liquid}}{\pi t_{displacement\ time}}} \tag{4.273}$$

In this relation $t_{displacementtime}$ means the residence time of a fluid sack on the interfacial surface of the bubble.

Dankwerts's (1951) „surface renewal model" supposes that on the bubble surface there are small fluid packages and when such a package is saturated with oxygen it will be exchanged by a package that is emptier regarding oxygen. The **s** (1/sec) in this model is the frequency of the fluid exchange:

$$k_l = \sqrt{D_{O_2} s} \tag{4.274}$$

All these models are of *one parameter-models*, and the parameter – beside the diffusivity – depends on the hydrodynamic behavior of the given system.

If we multiply the flux by the mass transfer area, the overall transfer rate is given as

$$\boxed{\frac{dC}{dt} = K_L a\left(C^* - C\right)}, \tag{4.275}$$

where

K_L – overall liquid-side oxygen mass transfer coefficient [cm.s^{-1}],
a – interfacial area in unit volume [cm^2.cm $^{-3}$= cm $^{-1}$],
K_La – overall liquid-side volumetric oxygen absorption coefficient [s^{-1}],
(In the fermentation practice we rather use [h^{-1}]),
C* – saturation dissolved oxygen concentration or solubility of the oxygen (mg/dm^3),
C – actual dissolved oxygen concentration in the liquid (mg/dm^3).

With constant K_La and C* (4.275) can be solved analytically with separation of the variables:

$$\int_0^C \frac{dC}{C^* - C} = \int_0^C -d\ln\left(C^* - C\right) = \int_0^t K_L a \cdot dt \tag{4.276}$$

$$\boxed{C = C^*\left(1 - e^{-K_L a.t}\right)} \tag{4.277}$$

Graphical representation of eq. (4.275) and (4.277) is shown on the Fig.4.76. $K_La.C^*$ is the maximal value of the absorption rate, and it is called *OTR (oxygen transfer rate)* (kgO_2 /m^3.h). that is used for characterization of oxygen mass transfer conditions of bioreactors.

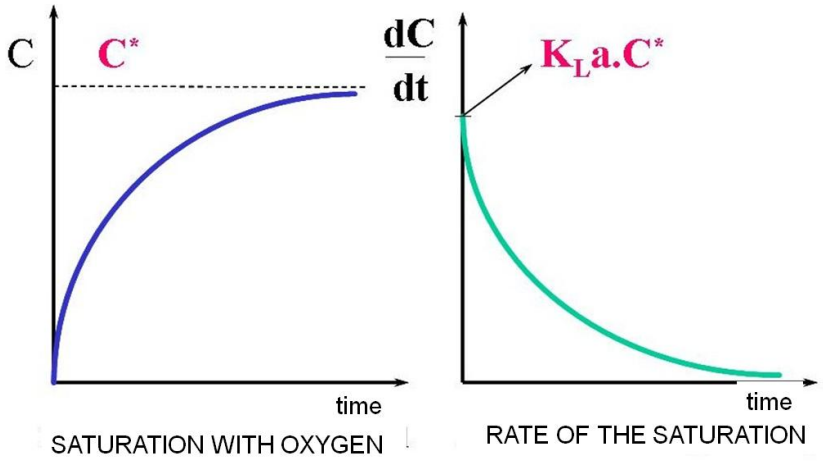


Fig 4.76.: Oxygen absorption: saturation and absorption rate

So far, the mass transfer conditions in a reactor without oxygen consuming organisms was discussed. If we take into account the oxygen consumption by microorganisms present in a real fermentation system, the picture is getting modified: beside the rate of the absorption the rate of consumption has to be considered as well:

absorption rate consumption rate

$$\frac{dC}{dt} = K_L a\left(C^* - C\right) \quad - \quad xQ$$

It is reasonable that a steady state will develope between absorption and consumption:

$$\frac{dC}{dt} = 0 \qquad \text{és} \qquad K_L a\left(C^* - C\right) = xQ \tag{4.278}$$

Let us suppose that this steady state does not hold. Let be $K_La(C^*–C)>xQ$. Then $dC/dt>0$, i.e C would increase and at the same time the driving force $C^*–C$ would decrease until the steady state would be reached. Similarly, if $K_La(C^*–C)<xQ$ at a given time, the driving force would increase, and the steady state would be reached again. Consequently, by all means there is an equality between oxygen absorption and consumption rates!

Nevertheless during a fermentation course the equilibrium oxygen concentration C shows a strict changing profile in respect of the time. It is shown in Fig. 4.77. The steady state and the changing profile is not a contradiction because all the parameters x, Q, as well as K_La and C* are changing during the fermentation, thus the every time steady state means a continuously changing equilibrium dissolved oxygen concentration.

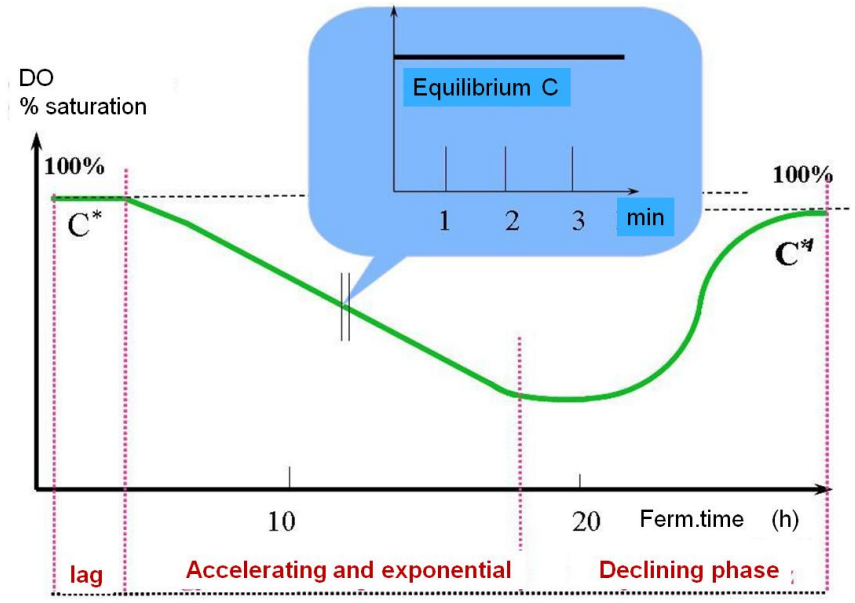


Fig. 4.77.: Equilibrium dissolved oxygen concentration in the time course of a batch fermentation.

4.5.4. What and how solubility of the oxygen depends on?

Oxygen is a slightly soluble gas in water solutions. Its solubility depends on the partial pressure of the oxygen according Henry's law:

$$C^* = \frac{1}{H} p_{O_2} \tag{4.279}$$

where

H – is the Henry-constant [bar/molfraction; bar.dm^3/mol; bar.dm^3/mg] (its values are in the Table 4.26.),

p_{O2} – partial pressure of the oxygen (that would be in equilibrium with a liquid phase of C^* concentration) [bar],

C^* –Saturation dissolved oxygen concentration, solubility of the oxygen [mol/dm^3; mg/dm^3].

Temperature dependence of C^* can be described by the temperature function of the Henry-constant:

$$\frac{d \ln H}{d\left(\frac{1}{T}\right)} = \frac{\Delta G}{R}, \tag{4.280}$$

where: T – absolute temperature (^{O}K),
R – gas constant,
ΔG – absorption heat of the oxygen (it has negative value).

Table 4.26.: Henry-constants of different gases at various temperature (in water)

Temperature	**Henry-constant *10^{-4} [bar/molfraction]**		
^{O}C	N_2	CO_2	O_2
0	5,29	0,073	2,55
10	6,68	0,104	3,27
20	8,04	0,142	4,01
30	9,24	0,186	4,75
40	10,40	0,233	5,35
50	11,30	0,283	5,88
60	12,0	0,341	6,29

Solubility values of oxygen in water at different temperatures are given in Table 4.27.

There are different approaching solutions of eq. (4.280). Such an approach was introduced by Wilhelm (1977), whose equation gives good approximations at 1 bar pressure:

$$R \ln X = A + \frac{B}{T} + C \ln T + DT \tag{4.281}$$

where X is the molfraction of the oxygen or CO_2, and the parameters are given in the Table 4.28 below.

Table 4.27.: Oxygen solubility in water for air and pure oxygen

Temperature **^{O}C**	Henry-constant x10^2 bar.m^3.kg^{-1}	C* (air) mg/dm^3	C* (pure oxygen) mg/dm^3
0	7,03	14,8	70,3
10	5,49	11,5	54,9
15	4,95	10,4	49,5
20	4,5	9,45	45,0
25	4,14	8,69	41,4
26	4,07	8,55	40,7
28	4,01	8,29	39,5
30	3,84	8,05	38,4
35	3,58	7,52	35,8
40	3,37	7,07	33,7

Table 4.28.t: Parameters of the Wilhelm's approach for oxygen and carbon-dioxide

	Temp. range	A	B	C	D
OXYGEN	274–348 ^{o}K	–286,94	15450,6	36,5593	0,0187662
CARBON-DIOXIDE	273–353 ^{o}K	–317,66	17371,2	43,0607	–0,00219107

Also a good approximation is given by the Antoine-equation

$$C^* \cong \frac{A}{B + t} \tag{4.282}$$

in which the constants in the range of 4–33 °C are as follow:

C^* – solubility (mg/dm^3),

A – 468,

B – 31,6 and

t – temperature in ^{O}C.

Finally a third approximation of the eq. (4.280) is the following mathematical expansion that is also suitable for predicting solubility:

$$C^* \cong 14{,}16 - 0{,}3943.t + 0{,}007714.t^2 - 0{,}0000646.t^3 \tag{4.283}$$

where C^* is in a (mg/dm^3), and t is the temperature in ^{O}C.

All the three approximating functions give solubility values for pure water. Let us recognize that the solubility of oxygen (as gas) is decreasing with increasing temperatures.

Oxygen solubility also depends on the composition of the culture media, it is determined first by the kinds and concentrations of the dissolved electrolytes:

$$\lg \frac{C_0^*}{C^*} = \sum_i H_i I_i \,, \tag{4.284}$$

where C_0^* – oxygen solubility in pure water,

C^* – oxygen solubility in the given electrolyte solution

H_i – ion specific salting out constants,

I_i – ionic strength for the az i-th ion, that can be calculated from the concentration and charge of the ions:

$$I_i = 0{,}5 c_i z_i^2 \tag{4.285}$$

am c_i – molarity of the i-th ion (g-ion[19]/dm^3), z_i – charge of the i-th ion.

For demonstrating the salting out effect of inorganic salts, stands here the Table 4.29. , in which we see the effect of sodium chloride on the oxygen solubility:

Table 4.29.: Oxygen solubilities in sodium chloride solutions at 25 °C and 1 bar oxygen pressure

NaCl-concentration mol/dm^3	C* mg/dm^3
0	41,4
0,5	34,3
1,0	29,1
2,0	20,7

Table 4.30. contains ion specific salting out constants of oxygen and CO_2 for several ions that may be components of culture media:

[19]g-ion = mass equal with ionic mass.

Table 4.30.: Ionspecific constants for CO_2 és O_2 (25 °C)

Cations	H_i (l.g-ion$^{-1)}$		Anions	H_i (l.g ion^{-1})	
	O_2	CO_2		O_2	CO_2
H^+	-0,774	-0,311	Cl^-	0,844	0,340
Na^+	-0,550	-0,129	Br^-	0,820	0,324
K^+	-0,596	-0,198	I^-	0,821	0,311
NH_4^+	-0,720	-0,264	OH-	0,941	
Mg^{2+}	-0,314	-0,079	NO_3^-	0,802	0,291
Ca^{2+}	-0,303	-0,071	SO_3^{2-}	0,453	0,213
Mn^{2+}	-0,311		CO_3^{2-}	0,485	
			PO_4^{3-}	0,320	0,147

Fermentation culture media usually contain organic matters beside inorganics, and those also have decreasing effect on the oxygen solubility. A similar Setchenov-equation can be applied for these:

$$\lg \frac{C_o^*}{C_{org}^*} = kC_{org}, \tag{4.286}$$

where K is Setchenov constant and C_{org} the concentration of the given organic material in the culture medium. This logarithmic equation is often approximated by the following

$$C_{szerv}^* = C_o^*\left(1 - mC_{szerv}\right) \tag{4.287}$$

Approximations become possible with the data for glucose, lactose and saccharose, where m = 0,0012 dm^3/g in the range of 150–200 g/dm^3 sugar concentration.

4.5.5. Aeration and agitation in fermentation systems

Oxygen transfer in fermentation bioreactors is basically realized in two manners according to the Fig 4.79. The simplest way is when compressed air is flown through an air sparger, put at near the bottom of the reactor. In the case of the other basic type, beside the former a mechanical mixing device is also operating in the reactor. Any real bioreactor types belong to these two basic aeration/agitation modes.

In the following chapter the performance of these basic types will be examined, through the parameters $\mathbf{K_L}$ and **a**. We shall examine what physical and hydrodynamical features affect these parameters and how can we predict their values.

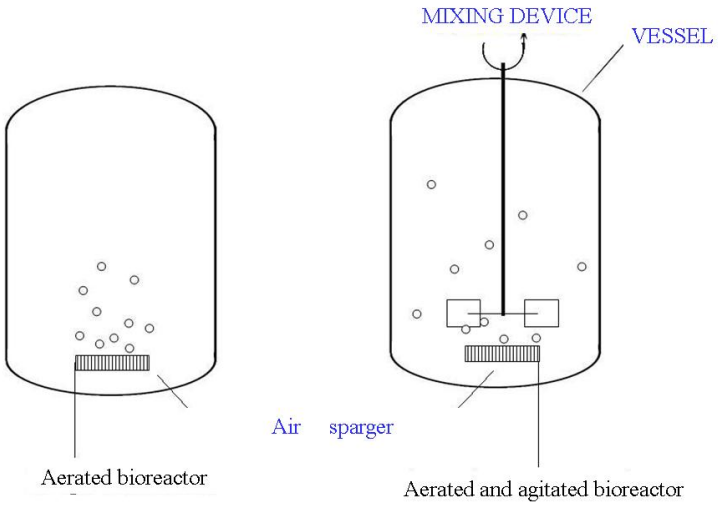


Fig 4.79.: Technical realization of aeration in bioreactors

4.5.5.1. Oxygen transfer from bubbles. Estimation of K_L and a (non-mixed reactors)

For the g/l interface we can write the oxygen flux with the Fick's law using the notations of the Fig 4.80., where z is the coordinate in the direction of bulk liquid and the driving force of the diffusion (concentration gradient) is calculated on the bubble surface (at the interface).[20]

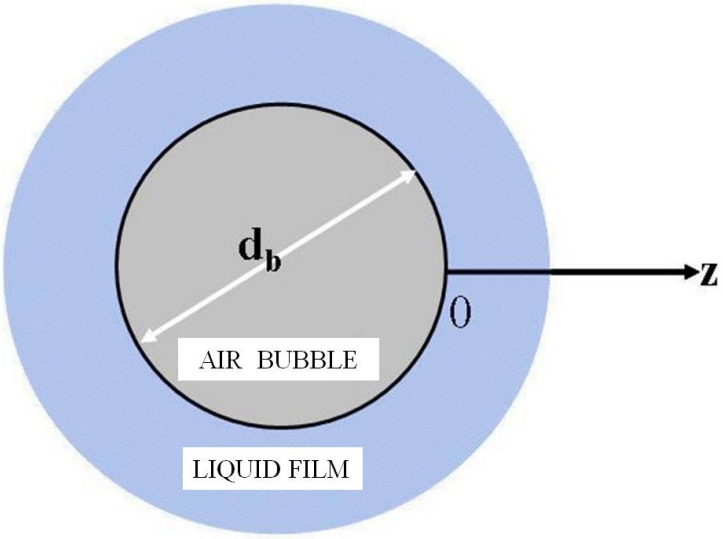


Fig 4.80.: One dimensional model of mass transfer from bubble.

The rate of oxygen transfer according to this model is

[20] I.e. here the one dimensional Fick's law is applied.

$$\frac{dC}{dt} = -D_{O_2}\left(\frac{\partial C}{\partial z}\right)_{z=0}, \tag{4.288}$$

on the other hand, the oxygen flux for unit surface is $J = k_L (C^* - C)$. Putting these equal, k_L can be expressed in the form

$$k_L = -\frac{1}{C^* - C} D_{O_2}\left(\frac{\partial C}{\partial z}\right)_{z=0} \tag{4.289}$$

Introducing the following definitions of dimensionless variables for the oxygen concentration and the for the distance from the surface

$$\overline{C} = \frac{C}{C^*} \quad \text{és} \quad \overline{z} = \frac{z}{d_b},$$

where d_b is the diameter of the bubble. According to this we get a dimensionless expression of the mass transfer coefficient, called Sherwood-number:

$$Sh = \frac{k_L d_b}{D_{O_2}} = -\frac{1}{1-\overline{C}}\left(\frac{\partial \overline{C}}{\partial z}\right)_{\overline{z}=0} \tag{4.290}$$

At the near vicinity of the interface the form of the solution of this differential equation is

$$\overline{C} = f\left(\overline{z}, Sh, Sc, Gr\right) \tag{4.291}$$

and for the Sherwood number the solution is

$$Sh = g(Sc, Gr) \tag{4.292}$$

In the Table 4.31 one can find a series of dimensionless criterions that are frequently found in the different empirical correlations used for describing various cases of mass transfer. In the Table the criterions are defined by a given way[21]. In the literature there are hundreds of empirical correlations applicable to various aeration types and hydrodynamic features, here we show *only one example*.

[21] According to an other type of definitions these are ratios of special time constants of the system.

Table 4.31.: Dimensionless criterions in mass transfer related calculations

REYNOLDS $\mathrm{Re}=\frac{\text{inertial forces}}{\text{viscous forces}}$	$\frac{dv\rho}{\mu}$	$\frac{d_b v_b \rho_l}{\mu_l}$
PECLET $\mathrm{Pe}=\frac{\text{convective component flow}}{\text{conductice component flow}}$	$\frac{dv}{D}$	$\frac{d_b v_b}{D_{O_2}}$
SCHMIDT $\mathrm{Sc}=\frac{\text{momemtum diffusivity}}{\text{mass diffusivity}}$	$\frac{\mu}{\rho D}$	$\frac{\mu_l}{\rho_l D_{O_2}}$
FROUDE $\mathrm{Fr}=\frac{\text{centrifugal force}}{\text{gravitational force}}$	$\frac{v^2}{gL}$	–
GRASHOF (Archimedes) $\mathrm{Gr}=\frac{\text{buoyant force}}{\text{viscous force}}$	$\frac{d^3\rho g\Delta\rho}{\mu^2}$	$\frac{d_b^3\rho_l g(\rho_l-\rho_g)}{\mu_l^2}$
SHERWOOD $\mathrm{Sh}=\frac{\text{bubble diameter}}{\text{film diameter}}$	$\frac{kd}{D}$	$\frac{k_l d_b}{D_{O_2}}$

d, L – characteristic geometrical measures
v – characteristic velocity
μ – dynamic viscosity
D – diffusion constant
ρ – specific density
g – gravitational acceleration
k – mass transfer coefficient
b index refers to the bubble, **1** to the liquid phase

In the most laboratory and producing aerated bioreactors bubbles are moving in clusters and the bubbles are in loose or more strict connection with each other (affecting the motion of each other). CALDERBANK and MOO-YOUNG introduced two correlations depending upon the mean bubble diameter range. They stated, that there is a critical bubble diameter d_b=2,5 mm, below which the first and above that, the second correlation hold, respectively:

$$\mathrm{Sh}=\frac{k_L d_b}{D_{O_2}}=0{,}31\mathrm{Gr}^{\frac{1}{3}}\mathrm{Sc}^{\frac{1}{3}} \tag{4.300}$$

$$\mathrm{Sh}=\frac{k_L d_b}{D_{O_2}}=0{,}42\mathrm{Gr}^{\frac{1}{3}}\mathrm{Sc}^{\frac{1}{2}} \tag{4.301}$$

Small bubbles are formed:

- in aerated tanks in which hydrophile materials are present,
- when aeration is forced through very small holes,
- in the cases of aeration through sintered ceramic or metal filters and in the case of bubble columns.

Big bubbles are probable in

- bioreactors filled with pure water, or

– in the case of sieve tray bubble columns.

Let us observe that (4.300) and (4.301) differ from each other not only in the value of the constant but also in the exponent of the Sc-number, and this refers to the different hydrodynamic features of the two cases that is caused by the change of shape and movement of the bubbles when their diameter increases (Fig.4.81.). Another important fact is, that there is no strict turnover at diameter 2.5 mm, the change is continuous, there is a transient between the two extreme behaviors. This means that the equations are valid only at sufficiently far from this 2.5 mm.

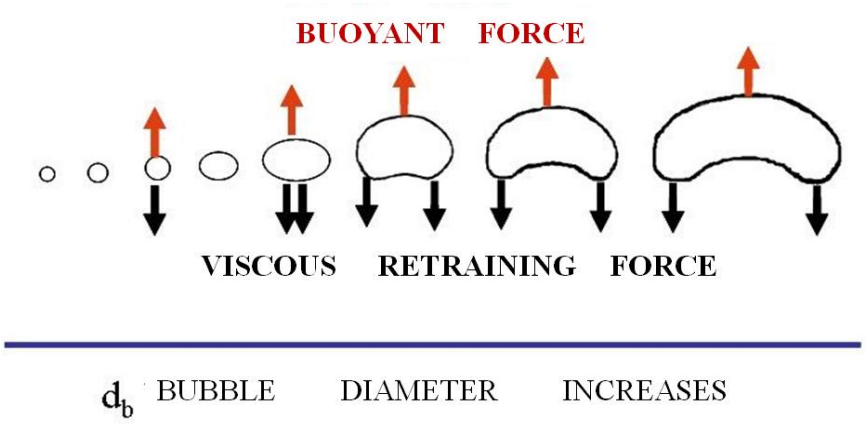


Fig 4.81.: Deformation of the bubble shape with the increasing diameter

Turning back to small bubbles, supposing they do not move (floating at a given point of the liquid space), from eq.(4.300) k_L=0 is given. Naturally this cannot be true if anyway there are concentration gradient between the liquid and the bubbles. Therefore the form of above-like equations had to be modified to the form

$$Sh = \frac{k_L d_b}{D_{O_2}} = 2 + 0{,}31 Gr^{\frac{1}{3}} Sc^{\frac{1}{3}} \qquad (4.302)$$

Thus if the bubbles are stagnant, Sh=2 will remain, so $k_L = \frac{2D_{O_2}}{d_b} \rangle 0$ value will be got for the mass transfer coefficient, that just corresponds to the simple diffusion without stream.(Let us observe that here $k_L \propto D_{O_2}$, similarly to the classic two film theory).

If the local k_L (and by this the overall K_L) is estimated somehow, we further need to estimate the mass transfer area, too. How is this possible with calculation?

Let us start from the simple picture of Fig. 4.82. Air is pumped through one small hole tube of d_0 diameter, with a very small velocity (almost not) and bubbles are getting formed on the orifice. At the moment when bubble leaves the orifice, there is (was) an equilibrium between the buoyant force acting on the bubble and the retraining force that is expounded by the surface tension on the circumference of the orifice:

$$\frac{d_b^3 \pi \Delta \rho g}{6} = \pi d_o \sigma, \qquad (4.303)$$

where σ is the surface tension.

Bubble diameter can be expressed from this equation; thus its surface can also be calculated:

$$d_b = \left(\frac{6\sigma d_o}{g\Delta\rho} \right)^{\frac{1}{3}} \qquad f_{\text{egy buborék}} = \pi d_b^2 . \tag{4.304}$$

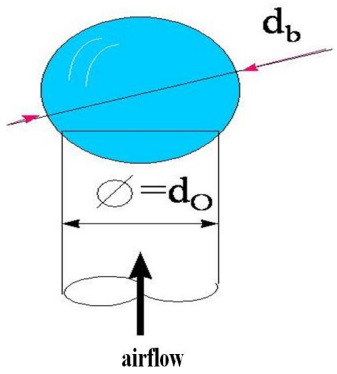


Fig.4.82.: Bubble evolution on an orifice

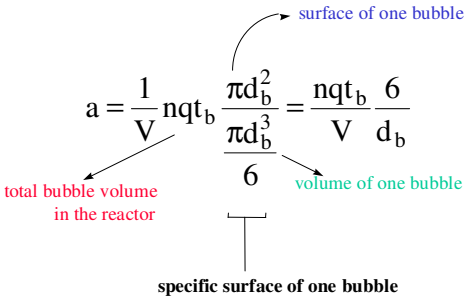
To get the total mass transfer area, we have to know how much bubbles are present in the system at a given time, for this the residence time of the bubbles is also necessary. The residence time is determined by the liquid height and the bubble rising velocity. For rising bubbles

$$t_b = \frac{H_L}{v_b}, \tag{4.305}$$

where t_b – bubble residence time,
H_L – liquid height,
v_b – bubble velocity.

Latter is not a constant, while the bubble is moving up, it varies. Usually, as an approximation, the terminal velocity of the bubble (at the surface of the fluid, when bubble is getting exploded into the air space) is taken into account.

If there are n pieces of aerating holes through that q is the volumetric rate of air one by one, it is easy to describe the overall mass transfer area in unit volume



(4.306)

where V = total volume (liquid and bubbles) and nq=Q aeration volumetric rate. nqt_b/V is the so called hold up, thus finally the following relation is got for the spacific mass transfer area:

$$a = H_o \frac{6}{d_b}, \tag{4.307}$$

In this H_o = volume of the gas/total volume i.e., the ***hold up***.[22] This expression can be generally applied for calculating mass transfer area.

Based on eq. (4.307) we can give answer on the practical question: how can we increase mass transfer area in an aerated bioreactor? Naturally by increasing the hold up. But how to do this? First, by increasing the rate of the aeration, second, increasing the residence time of the bubbles and third, decreasing the diameter of the bubbles. In an only aerated reactor latter is realized through decreasing the orifice diameters, but in an agitated/aerated reactor a more effective mode is to increase the intensity of the mixing.

4.5.5.2. Oxygen transfer in aerated/agitated reactors

It would be an oversimplification to consider mixing only from the point of view of mass transfer. It has more functions as follows:

-energy input into the liquid,
-dispersing gas in the liquid phase,
-separation of the gas and liquid phase and
-good mixing of the dissolved and solid components of the fermentation broth.

[22] For the hold up literature uses an other definition, too. a H'_0 = gas volume/liquid volume. In this case eq. (4.307) becomes $a = \frac{H'_0}{H'_0 + 1} \frac{6}{d_b}$.

– *Energy input* is the most important and fundamental function. It means, the liquid has to be kept in continuous motion not least for fulfilling the other three roles. Energy, taken up by the fluid converts into heat, its continuous retrieval is inevitable.
– Dispersion of gases in the fluid is also an (less) energy consuming process: forming the bubbles and dispersing them equally in the entire bulk liquid. This is a twostep process, first is the formation and distribution of bubbles, and second is the renewal of the coalesced bubbles. We shall see, that oxygen absorption is determined by the energy input in unit volume of broth.

– Gas separation is also important, it is the formation of carbon-dioxide containing bubbles, coalescing them to form bigger bubbles and removing them and the „used" air bubbles from the fermentation broth. This process is an opposite direction driven mass transfer.

- The good mixing function is a general mixing effect in order to avoid concentration gradients and reach the perfect mixing state as far as possible.

In lab fermentors and pharmaceutical ones the most frequently used agitator type is the Rushton-turbine or flat blade turbine as shown in Fig.4.83. where some other types are also shown.

In the Fig. 4.84. the most characteristic geometrical ratios of the Rushton-turbine impeller and the aerated/agitated reactors are given. The main ratios are similar from some liters to about 100 m^3 volume reactors. In the case of industrial reactors the H_L/D_T ratio becomes higher, up to 2-3:1.

If $H_L/D_T > 1$, more than one impellers are mounted onto the agitator shaft, according to the empirical rules below (Fig 4.85):

$$\text{distance between impellers: } D_i < H_i < 2D_i \tag{4.308}$$

$$\text{number of impellers: } \frac{H_L}{D_i} - 1 \rangle n \rangle \frac{H_L}{D_i} - 2 \tag{4.309}$$

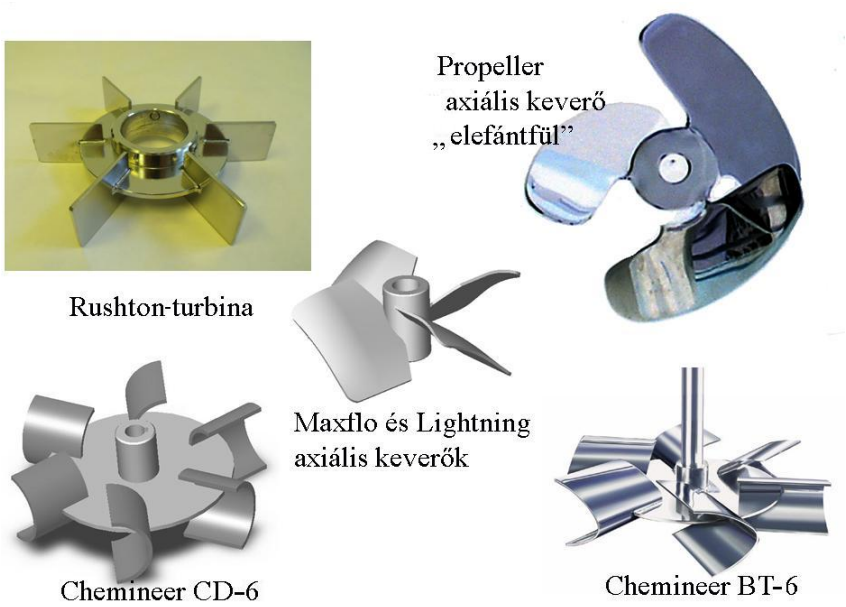


Fig 4.83.: Agitator types

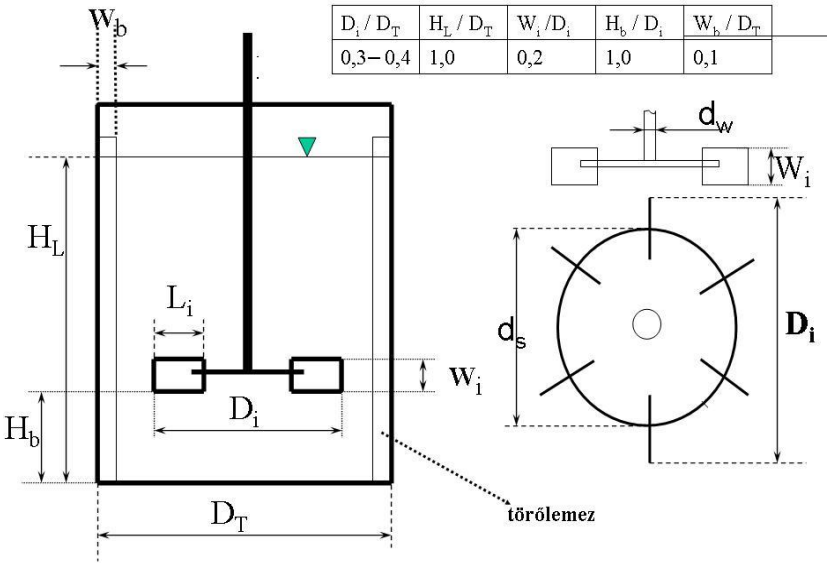


Fig 4.84.: Main geometrical ratios of the standard fermentor

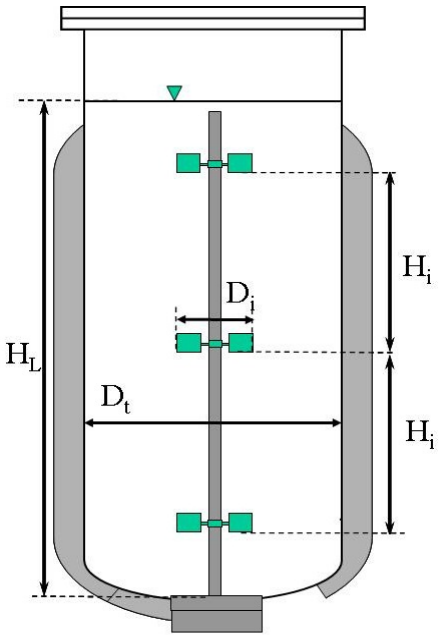


Fig 4.85.: Bioreactor with more mixing devices

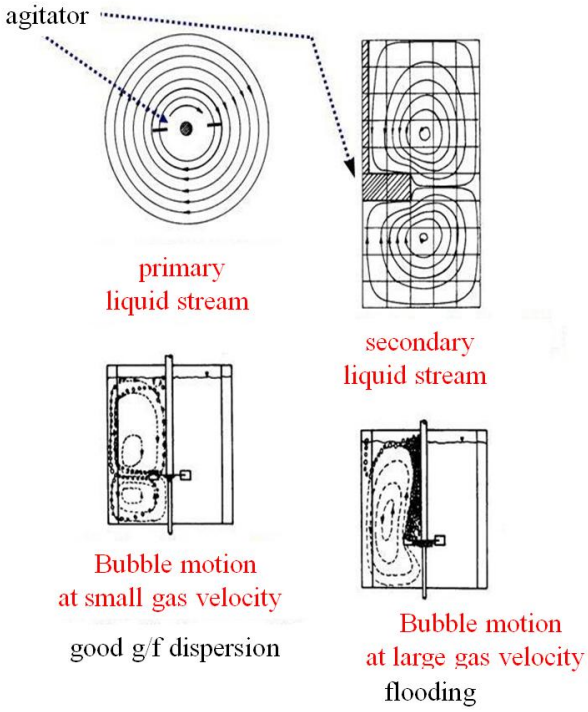


Fig 4.86.: Primary and secondary fluid streams and bubble movement in agitated reactors

For the bubble formation at smaller aeration volumetric rates, the *screw vortices* formed in the lower pressure space behind the impeller paddle are responsible, while at higher air flow rates the similarly formed *sheet vortices* are responsible. The bubbles formed this way are distributed by and along the primary and secondary streamlines (Fig 4.86) all over the entire reactor. At higher aeration rates the impeller will not be able to break up the air into bubbles, the air will flood the impeller (see later).

As the most important function of the mixing is the energy input, let us look at this energy requirement now. The power consumption of a mixing device is the following:

$$P = AD_i^5 N^3 \rho \, Re^m \, Fr^n \left(\frac{W_i}{D_i}\right)^{\alpha} \left(\frac{D_T}{D_i}\right)^{\beta} \left(\frac{H_L}{D_i}\right)^{\gamma} \ldots , \qquad (4.310)$$

where ρ – is the density of the mixed liquid,
N – revolution rate of the impeller.
In this equation Re means the so-called mixing-Reynolds,

$$Re = \frac{D_i . N D_i \rho}{\mu} = \frac{N D_i^2 \rho}{\mu} \qquad \left(\text{vö.: } Re = \frac{dv\rho}{\mu}\right) \qquad (4.311)$$

and Fr means the mixing Froude-number:

$$Fr = \frac{(D_i N)^2}{g D_i} = \frac{D_i N^2}{g} \qquad \left(\text{vö.: } Fr = \frac{v^2}{gL}\right) \qquad (4.312)$$

In case of a given geometry power consumption expression becomes simpler:

$$P = A' D_i^5 N^3 \rho \, Re^m \, Fr^n \tag{4.313}$$

Collecting the dimension holding terms to the left hand side, we get a new dimensionless criterion, power number (or Ne=Newton-number or Eu=Euler-number):

$$N_P = \frac{P}{D_i^5 N^3 \rho} = A' \, Re^m \, Fr^n \tag{4.314}$$

Dependence on the Fr can be eliminated if baffles are mounted on the inner surface of the bioreactor, (i.e., Fr measures the ratio of centrifugal and gravitational forces), and in a fully baffled system there will not be vortex-formation at the top of the liquid.

In Fig 4.87 N_P-Re plots are shown for systems with and without baffles. There are three distinguished ranges of mixing:

Laminar range: $0 < Re \leq 10$.

$$N_P = A' Re^{-1} \qquad P = A' N^3 D_i^5 \rho \frac{\mu}{N D_i^2 \rho} = A' \mu N^2 D_i^3 \tag{4.315}$$

Transient range: $10 < Re < 10^2\text{-}10^3$:

$$N_P = A \, Re^{-1} - \text{tõl} \qquad N_P = A \, Re^0 - \text{ig változik.} \tag{4.316}$$

Turbulent zone: $10^2\text{-}10^3 \leq Re$.

$$N_P = A', \qquad \text{azaz} \quad P = A' D_i^5 N^3 \rho \tag{4.317}$$

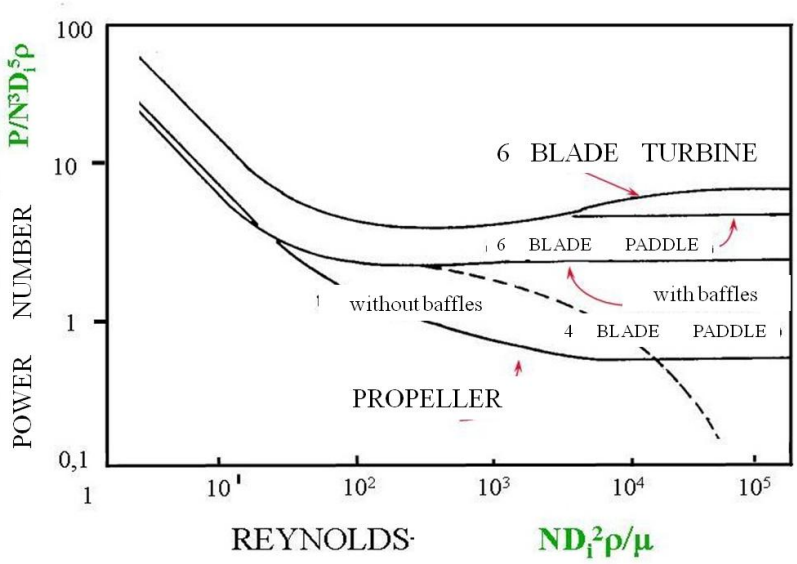


Fig 4.87.: N_P-Re plot for different impellers (Newtonian fluids)

If the system is aerated, this will modify the power consumption of the impeller, as the composite specific density of the gas/liquid mixture is less than that of pure liquid, thus the power consumption decreases. This effect can be well correlated with a new dimensionless criterion: aeration number **(Na),** which is defined as follows:

$$\mathrm{Na} = \frac{\text{apparent superficial(linear) air velocity}}{\text{circumferential rate of the impeller}} = \frac{\dfrac{\mathrm{F\ m^3/s}}{\dfrac{\mathrm{D_i^2}\pi}{4}\mathrm{m^2}}}{\mathrm{ND_i}\pi\ \mathrm{m/s}} = \frac{\mathrm{F}}{\mathrm{ND_i^3}} \tag{4.318}$$

It was found that the plot

$$\frac{P_g}{P} = f(\mathrm{Na}), \tag{4.319}$$

is characteristic for a given impeller type and at given revolution rate (Fig.4.89). These curves are actually transients from the non-aerated situation to the fully flooded case. It is also understandable now, why the flat blade turbine is the most favored type, i.e., it has a wide range where we can change the aeration rate while reaching good gas/liquid dispersion.

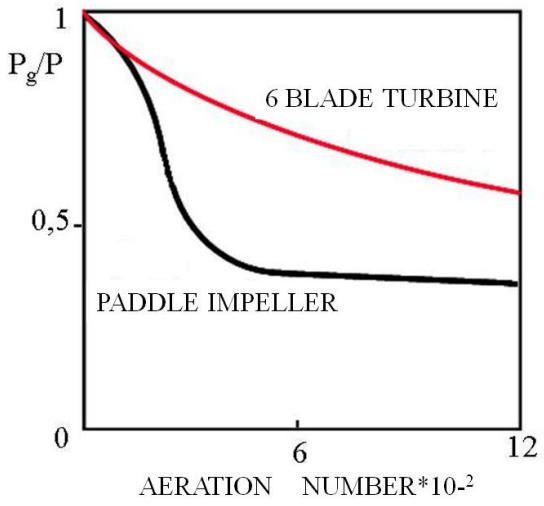


Fig.4.89.: P_g/P depends on the aeration number

Summarizing the knowledge, we got regarding aeration and agitation in bioreactors, we can write a rather general equation that describes oxygen transfer. The volumetric overall oxygen absorption coefficient is a function of the power input in unit volume and the aeration rate as shown below:

$$K_L a \propto \left(\frac{P_g}{V}\right)^{0,4} v_s^{0,4} N^{0,5} \tag{4.326}$$

This expression holds for lab size fermentors, while a more general form is

$$K_L a \propto \left(\frac{P_g}{V}\right)^{\alpha} v_s^{\beta} N^{0,5} \qquad (4.327)$$

in which α and β are size dependent constants between 0,3 and 0,95 and 0,5–0,67 respectively.

4.7. Sterilization

4.7.1. Introduction

Sterilization is one of the most important unit operation of biotechnological processes and it has a significant importance on the fields of everyday life, too. Contamination free food products (milk, beer, vine, canned food, etc), pharmaceuticals and medical instruments and aids (injections, infusions, prostheses, oxygen, etc) as well as cosmetics products and tap water, treated water, etc all need to be inflectionless. Furthermore, the final waste of a biotech. plant also have to be sterilized before taken to the waste water treatment plant. And of course, fermentation culture media also has to be sterilized before inoculation to avoid the possible growth of useless or even harmful foreign microorganisms. Thus culture medium sterilization is one of the most important operation of upstream processes of a bioprocess. This is important either from biological or from economical point of views.

In the following text we shall introduce the sterilization through the medium sterilization but of course, the basic statements are equally applicable at other sterilization tasks, too.

For sterilization, theoretically the following methods are suitable:

physical methods:

mechanical methods: filtration (for gases and fluids) It is not a sterilization in a classical meaning, because it does not kill contaminations, instead remove them.

electromagnetic irradiation: UV, X-ray, gamma-ray (for rooms and fluids)

heat.

chemical methods: disinfection.

There are three notions regarding the contamination free state. *Sterilizing or sterilization is a unit operation* with that we kill (or remove) the contaminating microorganisms. *Asepticity* means the maintaining the sterile state, keeping off contamination. And the third notion is as important as the formers, that is the *containment*, that means we keep off our (useful) microorganisms from the environment. It is very important not to contaminate the surrounding places with the producing microbe. There are special strict rules to keep pathogens and GMO-s inside the producing system and not to allow them to get out. These questions belong to the tasks of biosafety.

4.7.2. Rules of thermal death of microorganisms

It is well known that microbial species have a wide temperature range in which they can grow (Fig. 4.137). Above the optimal temperature the generation time increases and sooner or later the death will overcome the birth rate, the number of living cells start to decrease. Even the thermotolerant microbes are getting die above 80–90 °C.
The reasons of this thermal death are caused by the *denaturation* of at least one of the vital enzyme proteins that are needed for the metabolic processes and/or by the *irreversible disintegration* of the highly organized cytoplasmic- and other membrane systems of the cells.

Some basic statements are worth to note concerning thermal death:

- Sensitivity against heat is depending on the kind of the microbe (see Fig.4.138).
– In the case of a given species, vegetative cells are much more sensitive than bacterial endospores (that are of „condensed life form" (with small free water content)).
– In case of vegetative cells, thermal resistance depends on several factors, e.g., antecedents of the cell, its age. Cells from the exponential growth phase are more sensitive that cells from the declining or stationary phase.
– Every cell is more sensitive against moist heat (steam) than dry heat (in an oven).

– Thermal sensitivity increases with increasing temperature.
– Sensitivity of the microbes depends also on the medium, that holds the cell (culture medium, a dust particle, surface of a vessel, etc.), on the features of these media, their pH, viscosity, osmolality, and the presence of defending colloidal materials.

At a given temperature with constant other circumstances the death rate of a given species follows a first order kinetics:

$$\frac{dN}{dt} = -kN, \tag{4.360}$$

where **N** is the number of living cells [cfu/cm^3], and **k** is the thermal decay constant [min^{-1}].
At constant k (at a given temperature) (4.360) can be integrated:

$$\int_{N_0}^{N} \frac{dN}{N} = \int_{N_0}^{N} d\ln N = -\int_{0}^{t} k\,dt \quad \rightarrow \quad \begin{cases} \ln\dfrac{N}{N_0} = -kt \\ N = N_0 e^{-kt} \end{cases} \tag{4.361, 4.362}$$

Plotting the number of the survivors against the treating time, an exponential curve is got, that is suitable to the evaluation of k at a constant temperature according to Fig 4.139. Let us observe, that the exponential curve never touches zero that means that absolute sterility cannot be reached, the survivors' concentration cannot be zero.

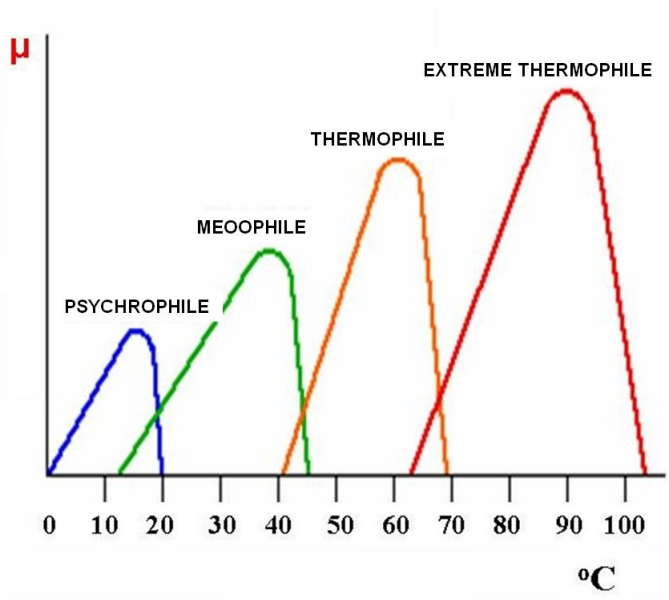


Fig 4.137.: Temperature range of microbial growth

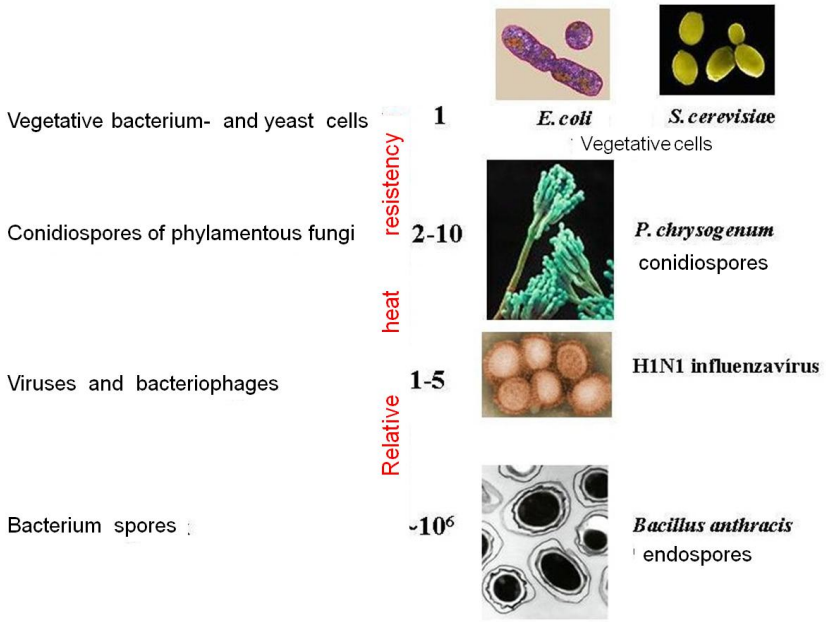


Fig. 4.138.: Relative heat resistance of various microbial groups

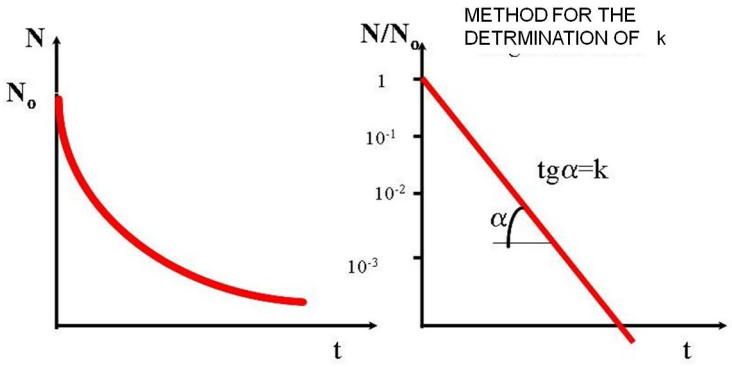


Fig 4.139.: Exponential heat decay. Determination of decay constant

Thermal decay constant is a strong function of the temperature, according to the Arrhenius-equation:

$$k = A.e^{-\frac{E_a}{RT}} \qquad (4.363)$$

$$\ln k = \ln A - \frac{E_a}{R}\frac{1}{T}$$

where A is an empirical constant,
E_a is the apparent activation energy of the thermal death process [KJ/mol].

Eq. (4.363) in the usual lnk–1/T plot gives a straight line with the help of that the constants of the Arrhenius equation can be determined (Fig.4.140).

For some microbes Table 4.7 serves data of k and E_a values.

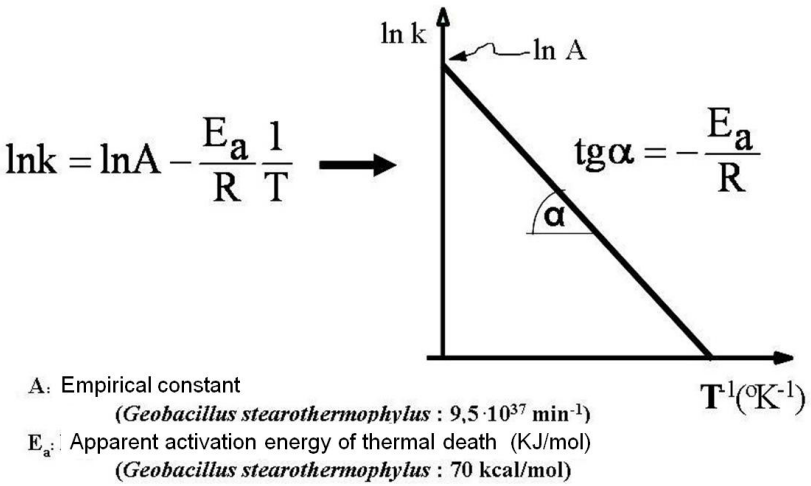


Fig. 4.140.: Temperature dependence of thermal decay constant

Based on the Arrhenius-equation, if we measure the k values at two temperatures, other k-s at different temperatures *between* these two temperature values can be interpolated. But be careful, because either A or E_a are depending upon the temperature, thus extrapolation is not allowed!

Table 4.7.: Arrhenius-constants for some microbes

MICROBE	T [^{O}C]	k [min^{-1}]	E_a[KJ/mol]
Bacillus subtilis (vegetative cell)	110	27	310
Bacillus subtilis (spores)	121,1	3	-
Bacillus stearothermophilus (spores)	104	0,051	283
	125	6,06	283
	130	17,52	283
Clostridium botulinum (spores)	104	0,42	344
Hemoglobin (heat denaturation)	68	6,3.10-3	312

Table 4.8. Thermal decay of culture medium components: apparent activation energies [kJ/mol]

Maillard-reaction of carbohydrates and proteins)	130,6
B_1-vitamin decomposition	87,9
B_2-vitamin decomposition	98,8

Reciprocal of the thermal decay constant is the average life span. Notion of the decimal reduction time is explained by the Fig 4.141. it is the time during the number of the living cells reduces to its one tenth.

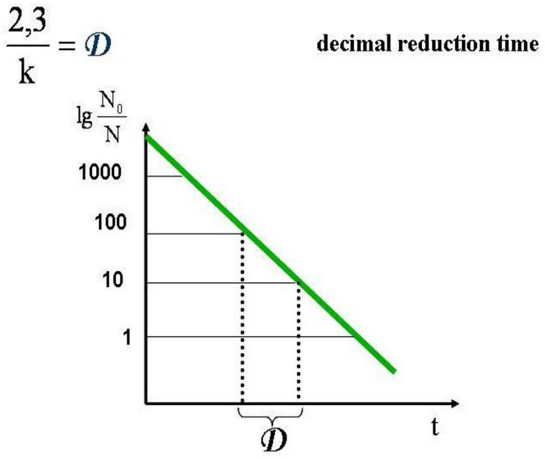


Fig.4.142.: Decimal reduction time

4.7.3. Probability approach of thermal death

The kinetic description above is valid, when the number of the microbes (spores) are very high. But less this number higher the alterations will be between the reality and the prediction. The reason of this is that the heat decay is a probabilistic (stochastic) process in which the individual spores behave differently, and just the average can be described by the above given kinetics, the whole population behavior can be described with distribution functions.

Definition: the life span of a cell (spore) is the time during that the cell(spore) still remains alive.

Every individual cell, bacterium spores have their own life span in a given environment (temperature) and the whole population can only be described with a distribution of these life spans. The distribution can also be characterized by its mean value, the mean life span:

$$\bar{t} = \frac{1}{N_0}\sum_{1}^{\infty} N_i t_i \tag{4.367}$$

where: $\bar{t}$ is the mean life span of population
N_0 the number of living cells at time zero,
N_i is the number of cells of t_i life span.

It is easily understandable that the reciprocal of the mean life span gives the mean thermal decay constant:

$$\frac{1}{\bar{t}} = \bar{k} \tag{4.368}$$

Let us look at a thermal killing process when the temperature is the same all over the entire vessel, naturally there is no growth (surely not, because we are at a high temperature!), the fate of the individual cells is independent on each other. Here, based upon probability considerations, it can be deduced that the probability, that a given t time, the number of the survivors is just N (where N = 0,1,2,...N_O) follows a binomial distribution and can be expressed as:

$$P_N(t) = \binom{N_0}{N} \left[p(t)\right]^N \left[1-p(t)\right]^{(N_0-N)} \tag{4.369}$$

where p(t) is the probability of that ***one*** cell just survives at the given time, t.

Probability p(t), using $\bar{k}$ reaction rate constant can be expressed as $p(t) = e^{-\bar{k}t}$. Putting this into (4.369), the following expression will be got for the above probability, $P_N(t)$:

$$P_N(t) = \frac{N_0!}{(N_0-N)!N!}\left(e^{-\bar{k}t}\right)^N \left(1-e^{-\bar{k}t}\right)^{(N_0-N)} \tag{4.370}$$

What is the probability of that, all the cells are dead in a given time? So we look for the probability $P_N(t)$ when N=0. This is on the basis of (4.370):

$$P_0(t) = \left(1 - e^{-\bar{k}t}\right)^{N_0} \lhd 1, \tag{4.371}$$

i.e., always smaller than 1, with other words, the *probability that at least one survivor remains is always higher than 0*:

$$1\text{-}P_0(t) = 1 - \left(1 - e^{-\bar{k}t}\right)^{N_0} \rhd 0 \tag{4.372}$$

If N>>1 (that naturally the case at a sterilization task), then an approximation of (4.372) will be

$$1 - P_0(t) \cong 1 - e^{-N}$$
$$\text{in which } N = N_0 e^{-\bar{k}t}. \tag{4.373}$$

It has a strong emphasis that there is no perfect sterilization, even if applying very long sterilization time, as can be observed on the basis of equations (4.371–4.373). Therefore, when sterilizing, we have to reach a *high enough probability of the zero surviving*. $1 - P_0(t)$ is called *criterion of the sterilization* or *criterion of the sterility* and is usually 10^{-2}–10^{-4} in the general biotechnology.
If it is for example $1 - P_0(t) = 10^{-3}$ it means that $1–10^{-3}=0{,}999$ is the probability, that of our sterilization is successful, there did not remain living cells in the system. With other interpretation it means, that it is very likely, that among 1000 sterilization one will not be successful, there will be survivors. Furthermore, let us observe that the approximation below holds

$$1 - P_0(t) = 1 - e^{-N_0 e^{-\bar{k}t}} \approx N_0 e^{-\bar{k}t}\ ^{23}, \tag{4.374}$$

According to this, the criterion of the sterilization also means the number of the survivors in the total system.

4.7.4. Sterilization as a unit operation

In labs dry heat sterilization is taken in ovens at temperatures 140–160 ^{O}C. This method is applied for empty glass vessels, test tubes, pipettes, several metal equipment. The moist heat is more generally applied for the same lab vessels, but exclusively for culture media and media containing equipment (tubes, Petri dishes, shaking flasks and fermentors). This sterilization is realized at 121-123 ^{O}C, typically for 20–45 minutes. Such laboratory autoclaves are shown on Fig 4.144. and 4.145.

[23] (4.374) can be approximated by the serie $e^{-x} \sim 1\text{-}x+....$

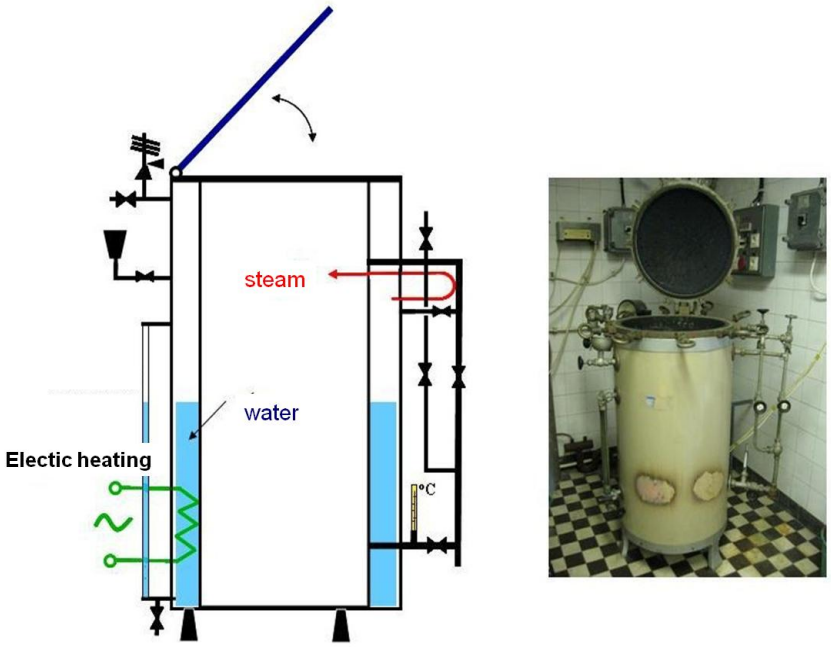


Fig 4.144: Conventional laboratory sterilizing autoclave

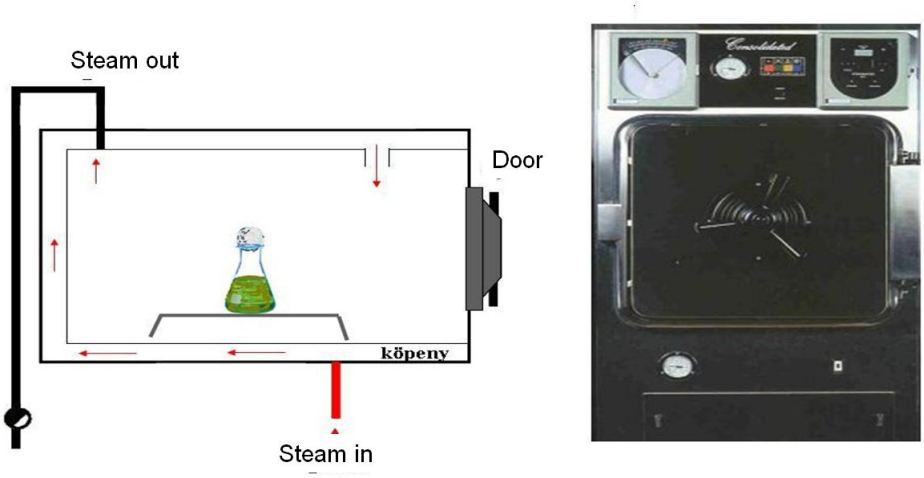


Fig.4.145.: Recently used sterilizer autoclave

4.7.4.1. Batch sterilization of culture media

Sterilization of fermentation culture media of some ten liters to some ten cubic meters volume, is usually performed in the bioreactor *in situ*, and at the same time the vessel and all the auxiliary part of it are sterilized together with the medium. The culture medium is prepared in the bioreactor itself, then it is warmed up to the sterilization temperature (conventionally 121 or 123 °C), kept there for a certain time, and finally cooled down to the temperature of the subsequent fermentation (Fig 4.146.) Heating, temperature keeping, and cooling is depending upon the construction of the fermentor, some methods are shown in Fig 4.147.

Because the time duration of the heating and cooling are comparable to the holding time (these are depending upon the measures of the bioreactor, the realization of heating and cooling and the culture medium), we have to take into account these when calculating the necessary holding time. Thus, a

design of a batch culture medium sterilization means the estimation of the holding time that is necessary in order to fulfill the requirement of the sterilization criterion.

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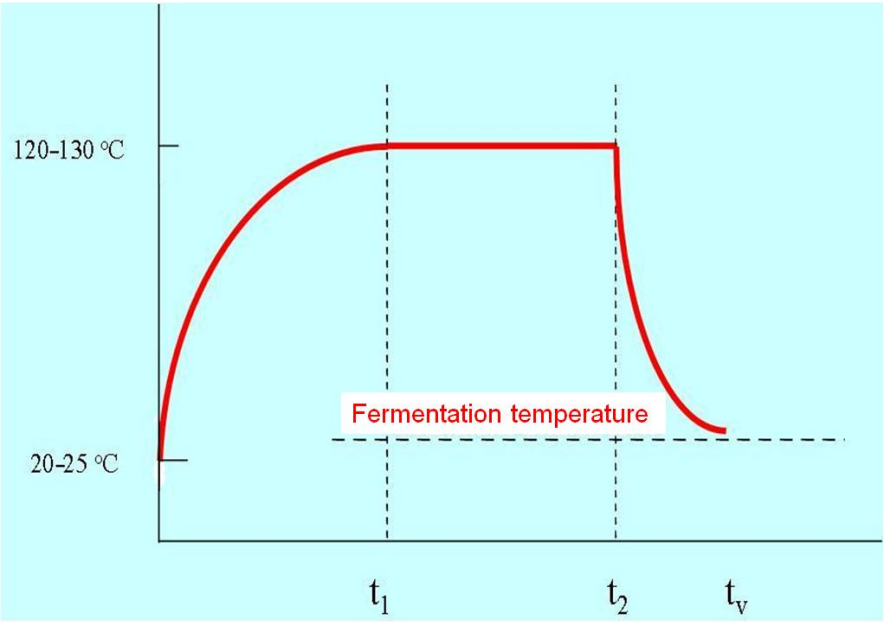

Fig.4.146.: Heat penetration curve

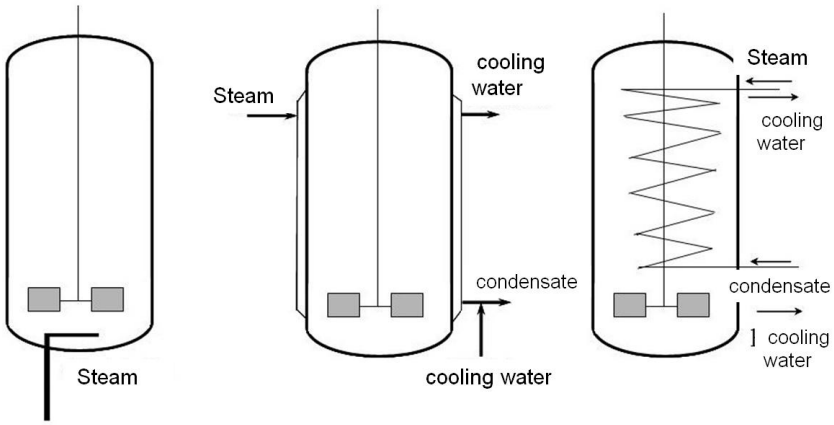

Fig. 4.147: Heating, cooling solutions

An excellent measure of the heat decay is the logarithm of the ratio of initial and final cell numbers; thus we can write for the heating, holding and cooling phases:

heat decay during the heating phase: $$\ln\frac{N_0}{N_1} = \int_{t_0}^{t_1} k\,dt = \nabla_f$$

heat decay during the holding phase: $$\ln\frac{N_1}{N_2}=k_{holding}\cdot\left(t_2-t_1\right)=\nabla_{holding}$$

heat decay during cooling phase: $$\ln\frac{N_2}{N_v}=\int_{t_2}^{t_v}k\,dt=\nabla_h$$

These logarithms can be summed for the three periods:

$$\nabla=\nabla_f+\nabla_{tartás}+\nabla_h$$

$$\ln\frac{N_0}{N_v}=\ln\left(\frac{N_0}{N_1}\cdot\frac{N_1}{N_2}\cdot\frac{N_2}{N_v}\right)=\ln\frac{N_0}{N_1}+\ln\frac{N_1}{N_2}+\ln\frac{N_2}{N_v} \qquad (4.375)$$

The contributions of heating, holding and cooling thermal decay to the total sterilization are comparable. As an example, let it stand here the following:

$$\frac{\nabla_f}{\nabla}=0{,}2 \qquad \frac{\nabla_{tartás}}{\nabla}=0{,}75 \qquad \frac{\nabla_h}{\nabla}=0{,}05$$

According to the eqs. (4.375) a batch sterilization task can be solved if we know the heat penetration curve (shown on Fig 4.146) of the fermentor and the thermal decay constants (as a function of the temperature) of the most resistant bacterium spore that may be present in our system. In the general biotechnology the spores of the *Geobacillus stearothermophylus* is this test organism, while in the food industry the *Clostridium botulinum*. In the design calculations we suppose that all the microbe present are these.

4.7.4.2. Continuous sterilization of culture media

Above we have seen that at a batch sterilization, the length and the contribution of each phase are comparable, moreover their lengths are very much dependent on the size of the bioreactor. The batch sterilization time takes 60-180 mins, and it increases with the increase of the size. Fermentor volume increases with the 3rd power of the diameter while the surface of the bioreactor only with the 2nd power (as a measure of the heat transfer surface). This means that beyond a certain volume it is not feasible to perform batch culture media sterilization.
In the case of large volumes, continuous sterilization may be the solution. The advantages of the continuous operational mode are

- Continuous sterilization applies higher temperatures (130-150 ºC) that makes it possible to apply shorter contact time, and consequently, the other components of the culture medium do not decay so much.
- Continuous process can be reproducible, it results in constant quality sterile medium that assumingly enhances the yield of the fermentation.
- It is not necessary to agitate the culture medium during the sterilization, thus the energy requirement is less.
- It is possible to separately sterilize the heat sensitive component of the culture medium: sugars and proteins.
- The continuously operating sterilizing equipment are easier controlled and the process can be automatized.

The most widely used continuous sterilizing systems are shown on Fig.4.148–4.151. Main differences between the systems are in the mode of the heating and cooling, the main holding parts are the same.

The system on Fig 4.148. heats up the medium instantaneously as it pumped through a mixing valve into which steam is introduced together with the medium. Here the medium temperature increases very fast to the sterilization temperature (130–140°C). After this the medium goes through an insulated tube for 2-3 min., and then is led through an expansion valve into a vessel of vacuum decreased pressure. There the temperature instantaneously falls to about 80 °C because of the removal of the evaporation enthalpy. Direct steam inlet and the expansion cause change the volume of the medium that has to be taken into account (a dilution and a concentration occurs). Finally, a simple heat exchanger cools down the medium to the fermentation temperature.

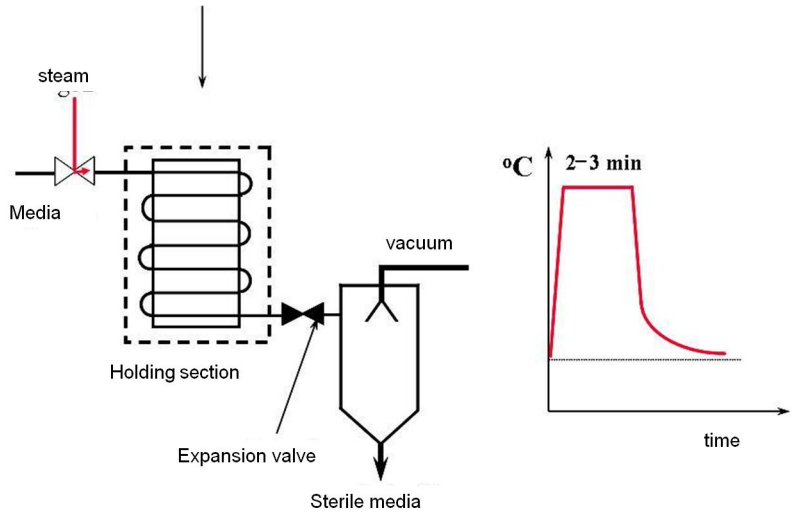

Fig. 4.148: Steam injecting continuous culture media sterilizer

The system in Fig 4.149 uses sheet and plate heat exchangers for the preheating, heating, and cooling down. Here a very much energy saving mode of heating and cooling are realized, and the time durations of these are also very short. The plate and sheet exchangers are shown in the Fig 4.150. This system is not the best from the respect of the fermentation media that frequently contains easily sedimenting particles. It is better applicable in case of pure homogeneous fluid (beer, milk, water, etc).
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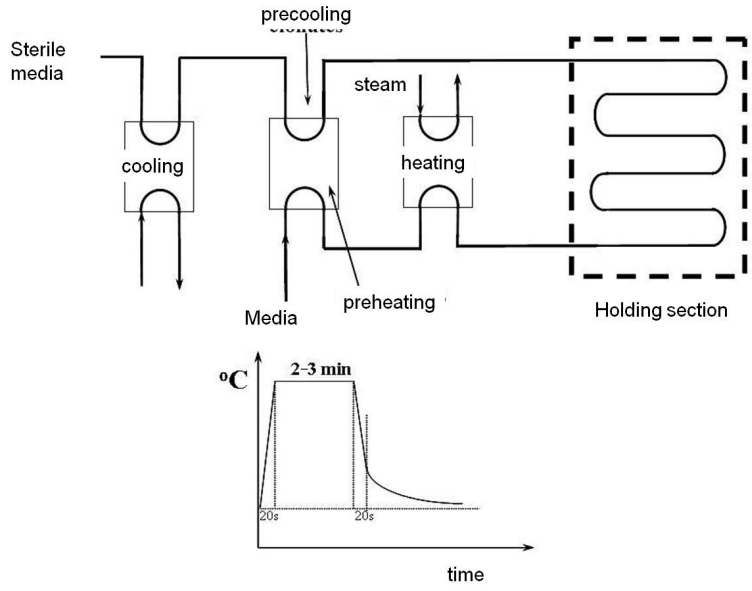


Fig. 4.149.: Sheet and plate heat exchanger continuous sterilization system

Fig. 4.150: Sheet and plate heat exchanger

On the Fig. 4.151 a similarly set up system is shown but instead of the former heat exchangers special spiral exchangers are used in a similar connection. In a spiral heat exchanger the stream lines are breakless and thus the harm of sedimentation and being get stopped is much less.

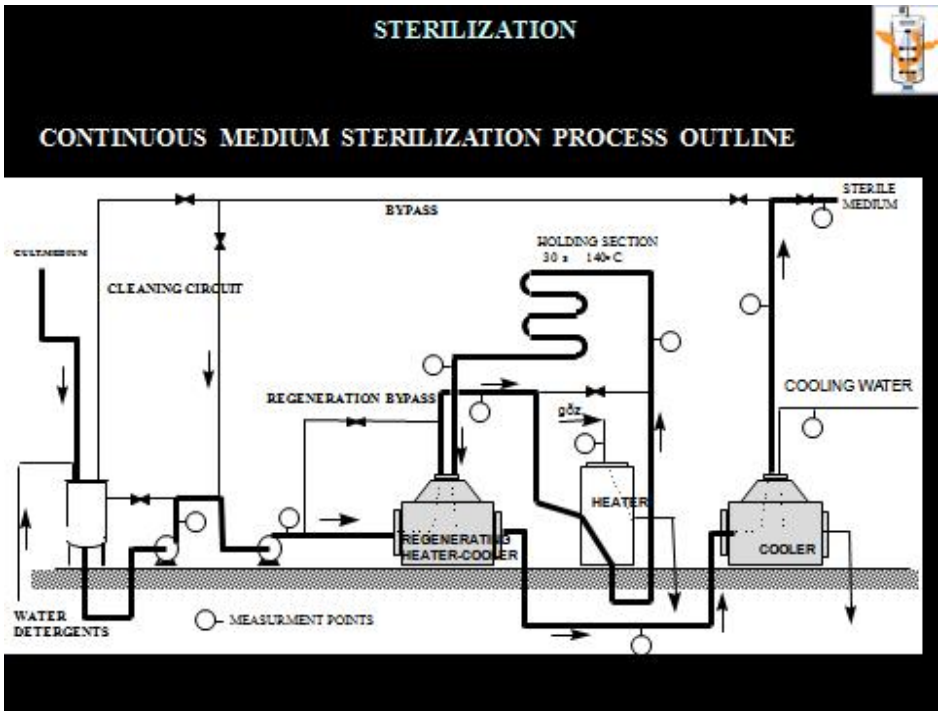
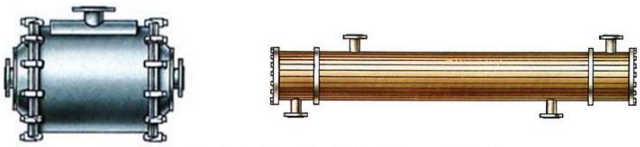
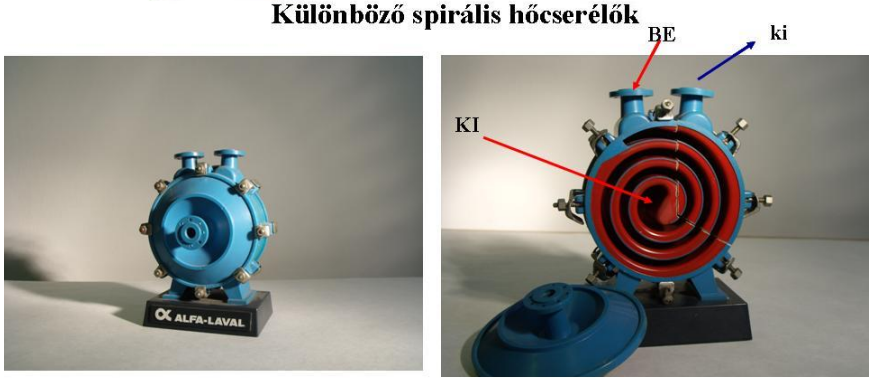


Fig. 4.151.: Siral heat exchanger continuous sterilization system



Különböző spirális hőcserélők



Spirális hőcserélő modellje és a szétszerelt modell

Fig 4.152.: Spiral heat exchanger

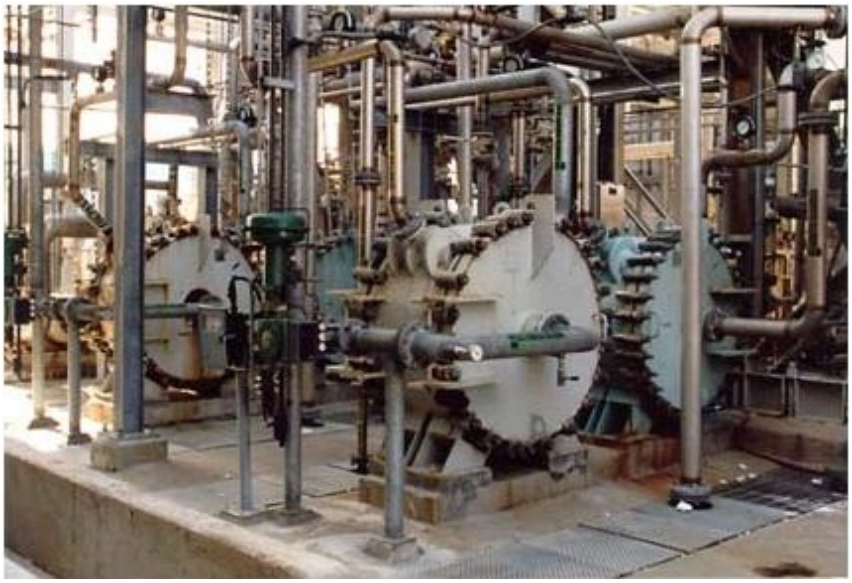


Fig. 4.153.: Sterilization station with spiral heat exchangers.

From the temperature profiles of these continuous systems it is obvious that the time duration of the heating and cooling phase are so short (only 1-2 % of the total time), that only the holding section time has to be taken into account when designing such a system. Thus the heat decay is:

$$\ln \frac{N_0}{N_v} = k\Delta t = k \frac{L}{\frac{w}{q}} \tag{4.376}$$

where: L is the length of the holding section [m],
w is the volumetric rate of the culture medium stream [m^3/min],
q cross sectional area of the pipe of the holding section [m^2].

Nevertheless, this simple way of calculation may lead to mistakes. The real calculation method is much more complicated because the streamline characteristics in the holding section has to be taken into account. The stream can be laminar, turbulent or piston like. Just the latter is good, only that assures the equal residence time of all fluid particles and this way a sure uniform sterility in the culture medium. In practice piston like stream can only be approached according the Re and Pe numbers of the streaming liquid.