**Budapest University of technology and Economics**

**Faculty of Chemical Technology and Biotechnology**

**Department of Applied Biotechnology and Food Science**

**Biology, Biotechnology: Lecture 3. – (Industrial) Microbiology**

Dr Németh Áron

Senior Lecturer

2013-2014.

 According to the subject description, the 2. lecture is an overview of microbiology and microbial physiology, furthermore types of industrial microbes and their main biochemical characteristics as well as basic metabolism.

Since the 1. lecture was about Cell biology including physiology (like energy production, „cell-organs” etc.) and metabolic pathway were considered detailed in subject of „Biochemistry for chemical engineers”, the focuse of this presentation is on microbiology, especially on industrial microbiology.

*Biotechnology* can be considered as the industrial application of different areas of Biology. While there also exists plant biotechnology (for example improvement of crops with genemodification), in chemical industry mainly microbiology is applied. A bio-based factory is usually divieded into two parts: the main production and its supporting operations together called =upstream processes, and product purification and isolation is called =downstream section.

The production is carried out usually either by microbial *de novo* fermentation or bioconversion, or by enzymatic bioconversion. For the latter one, the enzyms must be prepeared also exclusively by fermentation. This conclude, that there is no biotechnology without fermentation, which plays a key-role (=key step). We call fermentation that processes, in which living organism (or their part) help to convert the given substrate into products. These living organism are most commonly microorganism (ie microbes).

This is usually discussed in two parts: 1) General microbiology, 2) taxonomy. We will see some samples for ranking –with industrial aspects.

**Biotechnology development and/or startup**

During development of a bio-based technology the first step is to find or create the production strain. For this, usually microbes are isolated from soil in a petri dish, or if this was already done by a Strain collection, then have to place an order from them, and microbes will be delivered in lyophilised form. During revive we got again to the petri dish. This latter one is made from glass containing media for cultivation. On the surface of the media colonies or strips will be formed, but sticked/punctured cultivation also exist. The goal on the Petri-dish is to isolate individual, clean clones (colonies), which will go under screening experiments to find the best producer (or best candidate). For these screening either microtiter plates or shaking flasks are used. The cultivation of the chosen isolate will be then optimized, then scaled up, if the microbe tolerate it, since microbes are often sensitive against scale up.

Production has the same process, since every batch of an already developed and existing fermentation plant is always started from either a petri-dish or an ampule, and through continuous scale up they reach the production scale. The reasons are: 1.: microbes are sensitive against changing a scale, because the hydrostatic pressure will increase, which also affect the oxygen solubility, and usually the mixing rate is also different with different shear-force, etc. 2.: every operation must be carried out under sterile conditions to avoid the side reaction of contaminating microbes (i.e. Substrate decrease and by-product formation), however sterilization can only be considered as a statistical operation: if the criteria is 10-3=0,001 it means, that from every 1000 sterilization one will be lost. If we propagate a huge reactor with cells from a petri dish, than cells will be so much diluted, that very long fermentation time will be needed (cells should fill up the media with binary cell division!), and the potentially existing survived contaminations could be in the same magnitude than inoculum, which resulted a questionable competition.

This two example (development and production) could show, that in bioprocesses everything is determined by the microorganism, so this made it necessary to study microorganism in details (=microbiology).

**Microbiology**

Microbiology as a science is usually divided into two main area: *general Microbiology* and *Detailed Microbiology*. The later one is also known as Taxonomy. *General Microbiology* consists also two sub-area: a general Microbiology knowledge, and Microbiology operations. In the following section we discuss about these topics in detailed.

*Microbiology – Structure of microbial cells*

This is the main topic of the 1st (previous) lecture

*Microbiology – Microscopic observations*

For microscopic observations broth containing microbes will be dropped onto a glass subject plate (slide), and will be covered by another glass plate (cover), and will be put onto the stage. We set up the correct distance between the sample and the objective with the rough and fine adjustment, and through the eyepiece lens we make observations. The way of light follows: lamp (and/or mirror), condenser, slide, objective, tubular, eyepiece lens, eyes. By multiplication of the magnification of objective and eyepiece lens we receive the nominal enlargement. The size of different microbes can differ even in more orders of magnitude, that’s while different objectives can be applied. To see the smaller microbes (bacteria 1-2m) so called oil-immersion should be used, because the oil droplets between the sample and the objective has the same refractive index then the glass, thus the light will not be broken and scattered on the surface of sample-air and air-objective borders, which results larger magnification.

Yeasts are somewhat larger than bacteria, so they do not need oil-immersion, and since they are eukaryotic cells, the corn of the cell can often be detected.

Fungi and moulds can detected even with eyes, their size is ca 10m-1mm.

Since microbes are not visible with eyes and observation on them need special devices, the measurements of microorganism is also a challenge.

Among others the next 6 methods are widely used resulting almost 6 different units of measurement:

-OD or optical density is carried out at 600-660nm with UV-vis spectrophotometer, on the basis of light scattering by cells, without dimension.

-turbidimetric devices are usually builded into the fermenter making possible in-line measurmets. Since in this case it is not possible to dilute the sample this method differ from OD measurement in wavelength (1000nm, without dimesion).

-Cellcounting with microscope (application of Buerker chamber) or electronic counter resulting a unit of pieces/ml.

-CDW or cell dry weight can be determined by mass measurement of a dried filtercake after filtering a given amount (ml) of sample through a pre-weighted membrane filter disc (g/L).

-Diluting-dispering method results CFU/ml (Colony Forming Unit). Sample should be diluted to several dilution and these have to be poored onto agar surfaces and incubated. After incubation the best dilution resulted separate (individual) and easily visible colonies (every colony originated from one mother cell). This unit is correlating with the viable cell number, in contrary to the previous methods measuring the total cell numbers (both living and dead cells).

*Microbiology – Micro ecology*

We can better understand the behaviour of “our little friends” if we study and consider their living conditions in Nature.

Microbes can be found everywhere in the biosphere (air, water, soil). They have very different living methods like saprophytic, symbiotic, commensalism, parasitism etc. The relations of a microbe to other living organism determine its “toolbox” like degrading enzymes, secondary metabolites etc.

Natural living places also determine pH and temperature optimum. Optimum is definitely the temperature (or pH) where the growing rate the highest is. If this optimum is very low in temperature, then the microbe is psychrophilic; if around room temperature than mesophilic; or above 45°C it is termophylic (sometimes hyper-thermophilic). In term of pH we differ acidophilic, neutrophilic and alkalophilic strains. There are also some special behaviour like osmotolerancy (which are able to tolerate very high component concentration) or salt concentration (i.e. halophiles)

From ecological point of view some microbes pay special role in global material cycles, for examples via photosynthesis, or N2 fixing, and phosphorous recycling.

*Microbiology operations – Media preparation*

Most of the microbes are able to grow both in fluid and on solid media. The later often differs only in agar content of it. Independently from the phase state, both have to serve the microbes with carbon-sources (usually with carbohydrates), organic (proteins or protein hydrolysates usually for bacteria) or inorganic (ammonium salt usually for yeasts and fungi) nitrogen sources, and phosphorous source (salts of phosphoric acid). Sometimes special media component have to be added like vitamins precursors and minerals. Anaerobes may also need reduced components (for example DiThioThreitol, NaSH) and oxygen indicators (like resazurin).

The prepared media have to be sterilized commonly by thermal handling, but in case of heat-sensitive components or large scale also filtration may applied.

 *Microbiology operations – (Strain) Isolation*

Isolation methods differ depending on source: most frequently soil samples (solid) are used because of largest diversity. Samples can be put onto petri dish filled with agar, or previously suspended in water and then put onto petri dish before incubating. From solid surfaces can samples be taken with sterile cotton-wool, and then onto agar-plates which should be incubated.

Fluid sources are more dilute, thus pre-concentration is often needed. For this pre-incubation or filtration followed by filter disk incubation on agar-plates should be applied. Finally air samples are most dilute, thus filtration or exposition can be applied.

To make isolation more specific, special additives may be used. Antibiotics are applied to decrease the number of bacteria which increase the number of growing fungi, while anti-fungal compounds support bacterial isolation. Mild acidification is preferred by yeasts.

 *Microbiology operations – Screening*

After successful isolation we may look after the best candidates among many isolates. This process is called *screening* and has two main goals: 1) either to find a producer strain among isolates for a given task, or 2) to find a better producer then recently applied (either among different isolates, or during strain improvement among mutants). In every case large numbers of samples (isolates) have to be tested requiring automated methods, BUT every subject have different and unique goal thus requiring different techniques, which are usually manual methods. To satisfy both needs usually automatic and manual methods are combined. Automatic screening methods are usually applying HTS (HihghThroughputSystem) which are miniaturized large number test (like microtiter plates evaluated by microtiterplate-readers). These tests also often apply similar visible methods like manual techniques. For screening of acid producers CaCO3 is often mixed into the agar-media, and around the acid producer colony a clean-ring zone can be visible. Similar theory is applied for looking antibiotic producers against a given microbe (this is called sensitive): the sensitive microbe is suspended into agar media, and after transferring isolates onto this agar surface incubation should be done resulting clean-zones around antibiotic producer colonies (around which the sensitive microbe was inhibited while every other place become turbid). In case of lipase producer screening also the same theory is applied, but turbidity is caused by oil emulsion. Around lipase producer colonies cleaned zones will be resulted. In case of protease producers turbidity is induced by protein aggregates.

 *Microbiology operations – Identifications*

After finding a new producer strain it should be examined whether a new type or only a related was found. During isolation the goal is to rank the unknown microbe into the taxonomy system. Earlier phenotypes was exclusively compared and used for ranking, but recently genotype is more emphasis. While phenotypes based relationship can be demonstrated on geneology (taxonomy) tree, genotype based relationship will be illustrated on evolution tree (on the basis of different conserved part of genome). These “trees” are discussed more detailed in *Specific Microbiology.*

The characterisation also can be carried out by HTS systems or manual methods. Manual methods starts with macroscopic observations (colour, smell, and shape of the colony), then continued with microscopic observations (cell shape, grouping of cells, motion-organs, cell-corn, cell-wall (Gram staining etc.), and finally biochemical tests are carried out (oxidase probe, aerob/anaerob dextrose consumption, urease, hydrogen sulphide etc.)

 *Microbiology operations – Strain storage and maintenance*

There are some possibilities for storing microbes in their natural inactive forms (like spores), but usually they are stored in active form, either in lyophilised form, or on agar slants. The cultures on the agars have to be grown up during incubation, and after it the bioreactions in the cells are slowed down in fridge. For storing anaerobes either punctured (sticked) agar used to be applied, or an oil layer above the agar to eliminate oxygen.

 *Microbiology operations – Transfers, subculters*

The only place where cells can be safely manipulated is a laminar box. The front of it is opened, but laminar air flow hinder mixing of the room air with sterile air in the box, produced by filtration. Sub-cultivation is often necessary, since the cultures are slowly aged in the fridge, or if starting a production with inoculum preparation, then also transfer of cells have to be carried out. After the cell transfer, the subculture in the fresh media have to be incubated. For this operation sterile place, sterile loop, Bunsen flame, autoclave, sterile water and sterile pipettes are needed. It is very important to apply the corresponding box for a given microbes, since microorganism differ in their risk. WHO suggest to rank microbes into 4 groups, where 1st Biosafety level include only secure microbes, and 4th level the very harmful ones having the risk of epidemics and causing severe infections.

 *Microbiology operations – Strain improvement*

Capabilities of microbes are determined by the genome, thus every improvement require to change the DNA. If not direct genetic modifications are planned and applied, than random mutagenesis is induced by mutagenic agents (physical: UV or -radiation; chemical: base-analogous etc.). The most important question is the applied dosage: if someone apply low dosage, than only a few mutants will be generated beside lot of survival cell, but application of high dosage can result zero survival because of too much (and lethal) mutations. Strain improvement is a cyclic process starting with random mutagenesis, then mutant incubation followed by screening, and finally starting again with re-mutation.

**Detailed Microbiology**

The “Tree of life” can represent the relations between every living organism. It represents 6 different Kingdoms (Archeabacteria, Eubacteria, Protista, Fungi, Animalia, Plantae) among them the first 4 include microorganism.

*Prokaryotes*

The taxonomy of the known microbes is described in *Bergey’s manual*. In its first edition (around 1980-90) 4 division was mentioned on the basis of bacterial cell-wall structure: Gram negatives, Gram positives, Bacteria without hard cell-wall, and Archeabacteria. The ranking was based on classic phenotype and biochemical methods. The sub-ranks also indicated some characters like anaerobes or aerobes, or the shape of the cells. Thus if one microorganism could be found among Gram negative, rod shaped facultative anaerobes then the taxonomy rank have had information content. In contrary, the second edition of Bergey’s manual is based on genotype, and only has numbers, so the ranking do not provide any informations on the character of a microbe.

 *Eukaryotes*

In industry the widely applied eukaryotic microorganism are fungi. They are classically ranked on the basis of reproduction cycle: Basidomycetes, Ascomycetes,and Zygomycetes.

Basidomycetes include also mushrooms, having 4 spores at the end of basidiums on the bottom of the cap. Ascomycetes have ascus with usually 8 ascospore at the end of mycelium, and Zygomycetes have conidiospores in condiums like some moulds (*Mucor sp.*). For a long time, fungi taxonomy was divided into perfect and imperfect fungi, but later several imperfect fungi was observed as identical to a given perfect fungi (its perfect pair). The same is the situation with yeasts and some filamentous fungi: recently the same genome was found in totally different fungus.

**Potential of Biotechnology**

Shortly we should give some examples why microorganism are important “biocatalysts”, what they can do or produce? The products of microorganism are usually grouped into 5 groups: 1) primer metabolites, 2) seconder metabolites, 3) Bioconversions product, 4) recombinant proteins/enzymes, 5) biomass.

1. Primer metabolite are produced during normal energy production of the cells in glycolysis and tri-carboxylic acid cycle. ATP –the energy mediator molecule can formed directly in these processes as well as NADH2, which is also converted to ATP in Terminal oxidations, where the oxygen (from air) will be reduced into water. In absence of oxygen NADH2 have to be regenerated in other reactions, like pyruvate->lactic acid reduction or Acetyl-coenyzmeA->acetic acid production. Which metabolites to produce is strongly depending on the applied strain.

In case of lactic acid more pathway can be applied: in homo-fermentative way from 1 glucose 2 lactic acid will be formed as a single product, but in case of hetero-fermentative way after an early decarboxylation (resulting in CO2) the resting xilulose will divided into three and 2 carbon atoms resulting finally lactic acid, ethanol and acetic acid. The ratio of these products determine the flavour of the product (like yoghurt etc).

Some researchers predestine, that in the future instead of the use of the degradation metabolites produced by microorganism, people will use assimilation metabolites of plants (having Calvin cycle very similar to TCA)

1. Secondary metabolites: these molecules are used to make connections with the microecology: inhibit concurrent with antibiotics, and support symbiotic hosts with siderophores etc.

The primary and secondary metabolites are collected on a map to represent real “Biotechnology potential”.

1. Bioconversion products like steroids – Steroids have large skeletons, thus their synthesis is really difficult, especially if only one function-group have to be changed. However microbes are able to modify the basic compounds with good yield, and stereospecific way.
2. Proteins are produced for bioconversions, detergents washing, for food industry, for therapeutic purposes, organic catalysis, for organic catalysis, or for agricultural. The main challenge is to control whether the product have to be released otside of the cells or only inside.
3. Biomass can also be a product for feed and food industry (i.e. probiotics etc.).