

Production of Fungal Enzymes by Solid State Bioprocessing



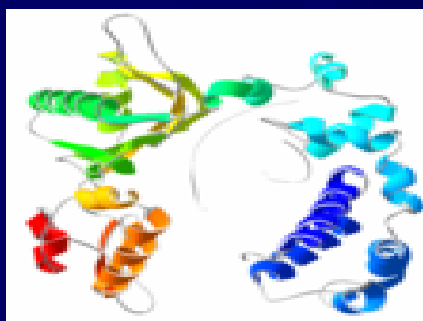
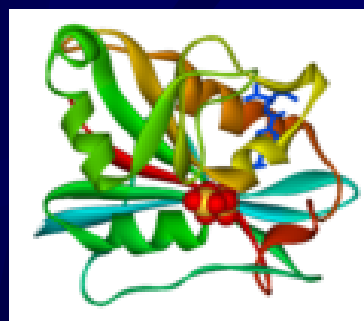
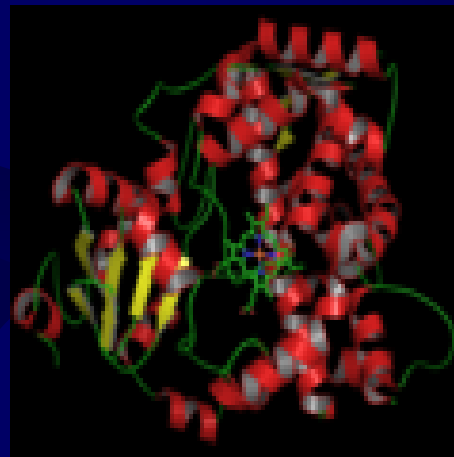
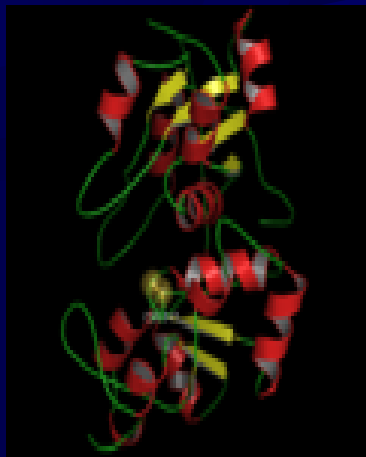
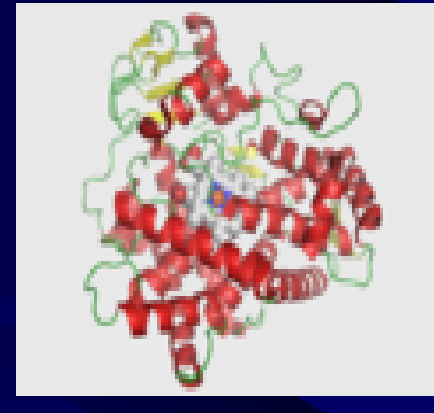
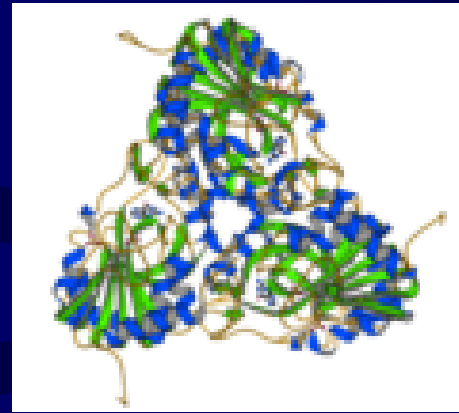
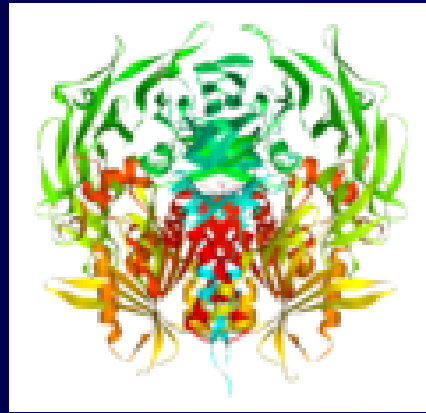
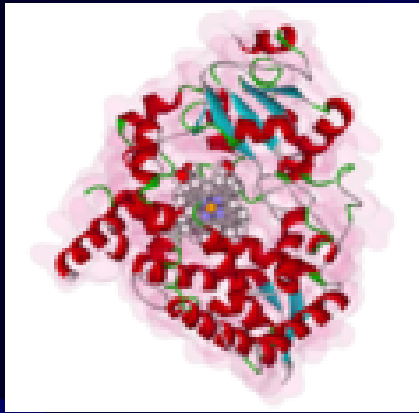
Marin Berovic

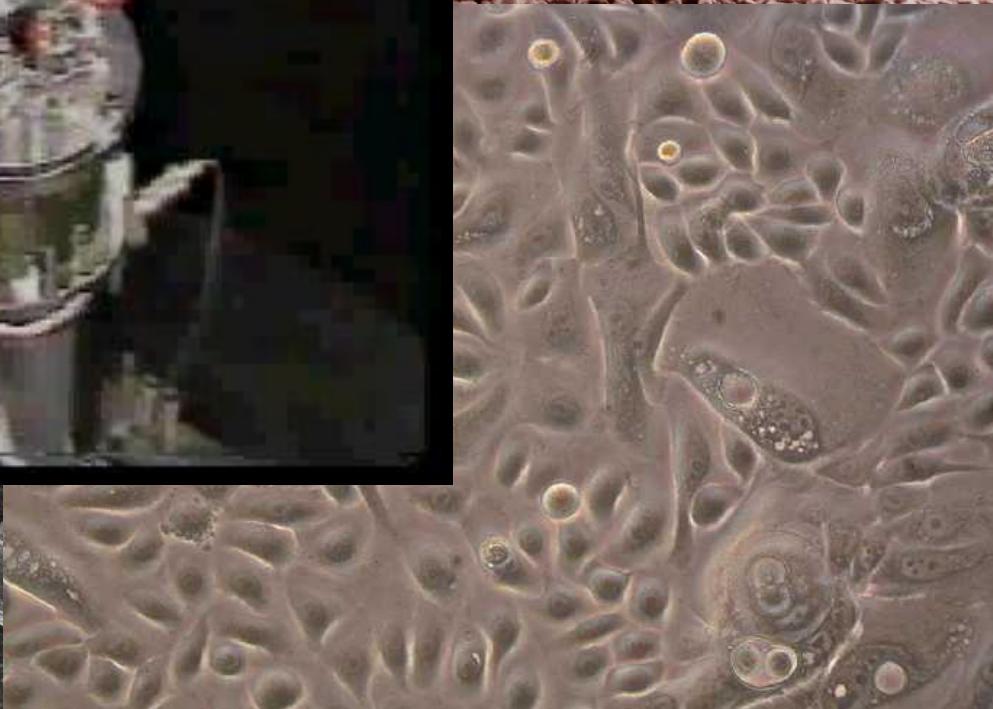
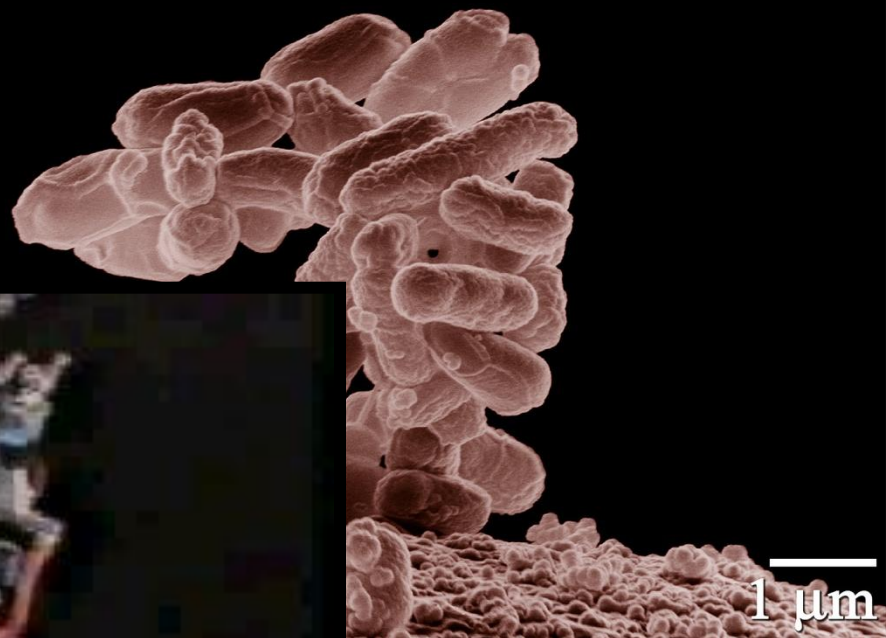
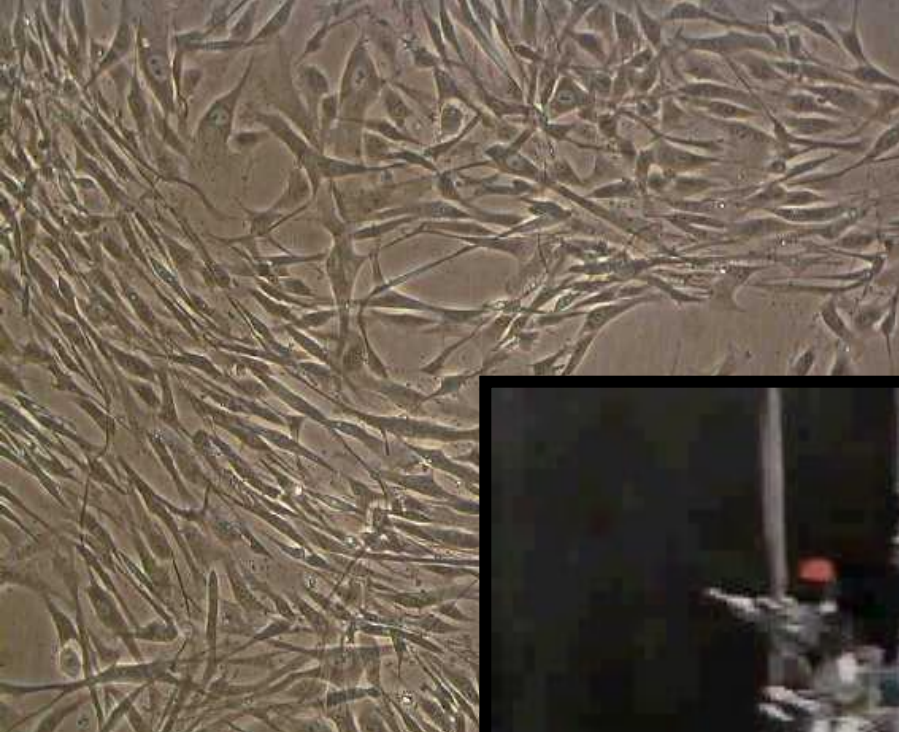
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Slovenia

ENZYMES





LESNE GLIVE



bele trohnobe



rjave trohnobe



ENZYMES PRODUCED BY SOLID STATE BIOPROCESSING

Proteases

<i>Rhizopus oligosporus</i>	rice bran
<i>Aspergillus flavus</i>	wheat bran
<i>Aspergillus oryzae</i>	wheat bran
<i>Aspergillus oryzae</i>	wheat bran
<i>Aspergillus niger</i>	wheat bran
<i>Aspergillus oryzae</i>	wheat bran, rice bran & others

Ikasari & Mitchell (1994)
Malathi & Chakrabort (1995)
Nakadai & Nasuno (1988, 8)
Padmanabhan et al. (1993)
Villegas et al. (1993)
Battaglino et al. (1991)

Amylases

α -amylase

<i>Bacillus licheniformis</i>	wheat bran
<i>Aspergillus oryzae</i>	wheat bran
<i>Aspergillus niger</i>	wheat bran & rice bran
<i>Aspergillus niger</i>	wheat bran
<i>Aspergillus oryzae</i>	impregnated foam
<i>Aspergillus niger</i>	wheat bran & corn flour

Ramesh & Lonsane (1989),
Lonsane & Ramesh (1990)
Shah et al. (1991)
Pandey et al. (1993, 94),
Ghildyal et al. (1985, 91, 92),
Kobayashi et al. (1990)
Pandey (1991)

glucoamylase

ENZYMES PRODUCED BY SOLID STATE BIOPROCESSING

Lipases

Penicillium spp & others

Wheat bran

Rivera-Munoz et al. (1991)

Cellulases

Neurospora crassa
Aspergillus niger
Trichoderma harzianum
Trichoderma viride
Trichoderma reesei
Aspergillus niger
T. reesei + A. niger
(mixed)
Penicillium citrinum

Straw
 Bagasse & others
 Bagasse + wheat bran
 rice straw
 wheat straw & bran,
 wood
 coconut coir
 sweet sorghum silage
 rice husks

Macris et al. (1987)
Madamwar et al. (1989)
Roussos et al. (1992)
Suhartono et al. (1991)
Chahal (1991)
Muniswaran et al. (1994)
Castillo et al. (1994)
Kuhad & Singh (1993)

Other enzymes

Rennet

Endo- β glucanases

Hemicellulases

Pectinases

Pectinases

Chitinase

Tannin acyl hydroalase

Oligosaccharide

oxidase

α -galactosidase

Various others

Mucor meihei
Penicillium capsulatum
Thermonospora
Aspergillus niger
Aspergillus niger
Taralomyces flavus & others
Penicillium crysogenum
Aspergillus niger

Acremonium sp.
Aspergillus niger
Lentinula edodes

Wheat bran
 Wheat bran
 coffee processing waste
 apple pectin
 coffee pulp
 citrus pulp pellets
 spent lignocellulose
 impregnated bagasse

 wheat bran
 wheat bran
 wood

Thakur et al. (1990, 1993)
Connelly & Coughlan (1991)
Srivastava (1993)
Ostroveršnik & Berovič (1997)
Antier et al. (1993)
Siessere & Said (1989)
Sharma et al. (1995)
Lekha & Lonsane (1994)
Lin et al. (1993)
Srinivas et al. (1994)
Leatham et al. (1991)

Table II.

Comparison of solid-state fermentation (SSF) and submerged fermentation (SF).

Solid state fermentation

Some products are only produced well under low moisture conditions. Cannot be used for organisms requiring free water

The medium is relatively simple (eg. a grain) and unrefined. It may contain all nutrients necessary for growth, or only require wetting with a mineral solution. Pretreatment can be as simple as cooking or grinding. However, the substrate can be variable.

The low water availability helps to select against growth of contaminants

Media are concentrated and smaller bioreactors can be used, leading to higher volumetric productivities (even when growth yields and growth rates are lower)

High substrate concentrations can enable high product concentrations

Submerged fermentation

A very wide range of products can be produced from a wide range of microorganisms. Many products are produced best under SF.

The medium often contains more highly processed ingredients and is therefore more expensive. Unprocessed ingredients may need processing to extract and solubilize the nutrients. With defined media good reproducibility is possible

The water activity is usually very high and many contaminants can grow well

Media are dilute and therefore occupy larger volumes, leading to lower volumetric productivities

High substrate concentrations can cause rheological problems. Substrate feeding systems may be required.

Aeration requires less power since pressures are lower. Gas transfer is easier since the particles represent a large surface area

Mixing within particles is not possible and growth can be limited by the diffusion of nutrients

Ability to remove metabolic heat is restricted, leading to overheating problems

Process control can be difficult due to difficulties in making on-line measurements, and in measuring biomass. The addition of substances during the process is difficult

Downstream processing may be simpler since products are more concentrated. However, extracts can be contaminated with substrate components. Large volumes of liquid waste are not produced

Growth kinetics and transport phenomena have received little attention and are poorly characterized

High air pressures can be required. Gas transfer from the gas to liquid phase is slow and can be limiting

Vigorous mixing can be used and diffusion of nutrients is usually not limiting

High water content and more dilute nature makes temperature control easier

Many on-line sensors are available and more are being developed. Additions of substances can be made to control the process

Downstream processing requires removal of large volumes of water and is expensive. However, with defined media, product purification may be easier. Usually large volumes of liquid wastes are produced

Much kinetic and transport information is available which can guide reactor design and operation

World population is expected to reach six billion by the year 2000, and finally to the level of about 8 to 12 billion people in the 21st century.

1. Soy bean protein (above 90% purity) is spun into fibres and combined with meat flavours and fat to produce meat-like products,
2. Soy bean protein is combined with other cereal proteins, meat flavours and fats and extruded to produce chewy-gel meat-like nuggets that can be used to replace meat in many canned products and
3. Edible mould micellium is grown on low-cost starch substrates, collected by filtration and formulated with meat flavours and fats to yield meat-like products. the future

MICROBIAL TYPES

Yeasts

Yeasts generally grow on solid substrates only as minor members of the microflora. This is the case in ensiling and in tape manufacture. Ensiling is an anaerobic SSF process in which agricultural products at 60 to 75% moisture are packed to exclude air and are fermented at ambient temperatures (25 to 30°C) for one to two weeks. Yeasts are only found during the early stages of ensiling, and lactobacilli are the predominant microorganisms.

Bacteria

Bacteria play roles as the major or minor microorganisms in various SSF processes. Lactobacilli are the major microorganisms during ensilage. Thermophilic bacteria predominate early during composting before being succeeded by thermophilic actinomycetes and fungi.

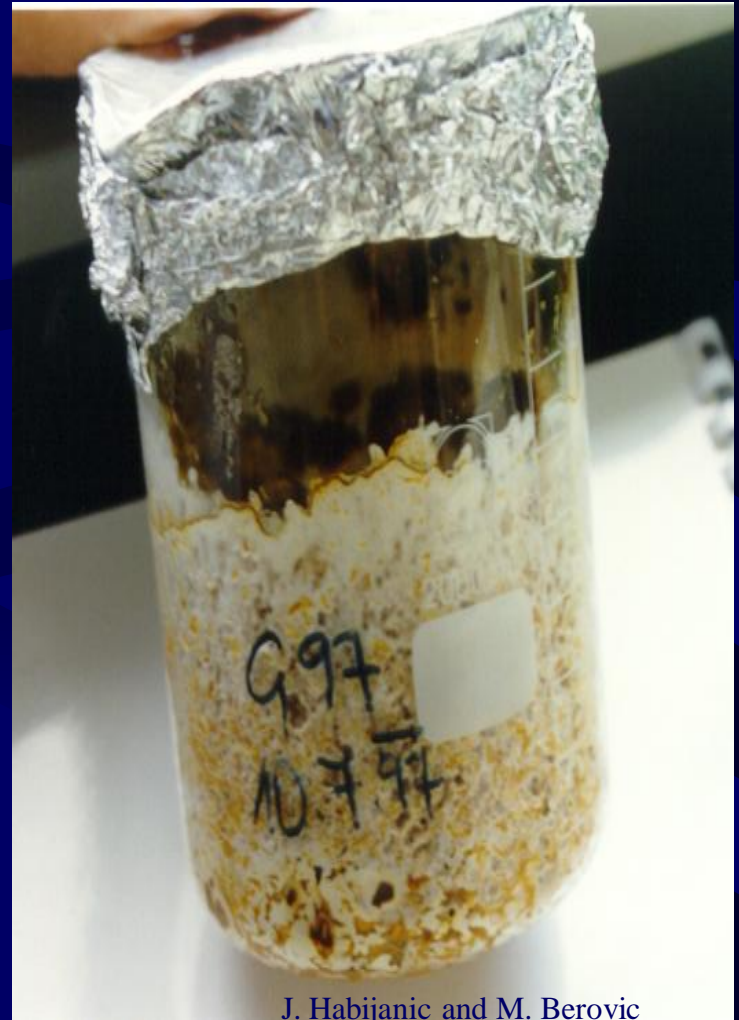
Filamentous fungi

Filamentous fungi are the most important group of microorganisms for SSF and this section will concentrate on them and their properties. Filamentous fungi are ideally suited to SSF due to their hyphal mode of growth and also their physiological capabilities.

INOCULUM



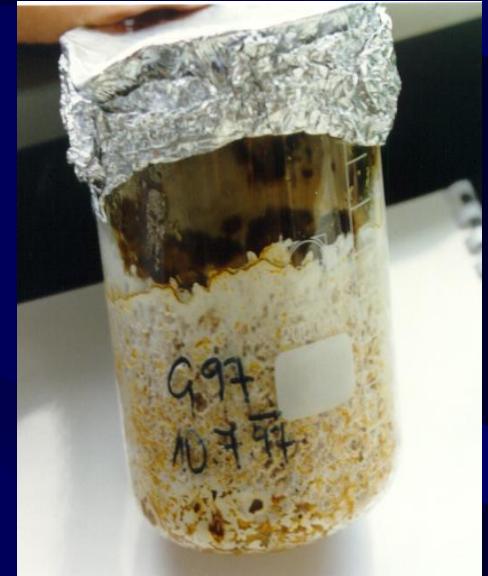
J. Habijanac and M. Berovic



J. Habijanac and M. Berovic

INOCULUM

Inoculum density varies over a wide range, from 10^4 to 10^7 spores per g of substrate, depending on the organism involved. For best results the inoculum size must be large enough and the initial mixing sufficient to ensure that almost all of the substrate particles will be colonized. However, if spore density is too high it can retard germination.



Submerged cultivation

Mushroom farming cultivation

Solid state cultivation

PHYSICAL FACTORS CONTROLLING GROWTH IN SSF

Moisture availability

The availability of moisture in SSF can be expressed as either the water content or the water activity of the solid substrate.

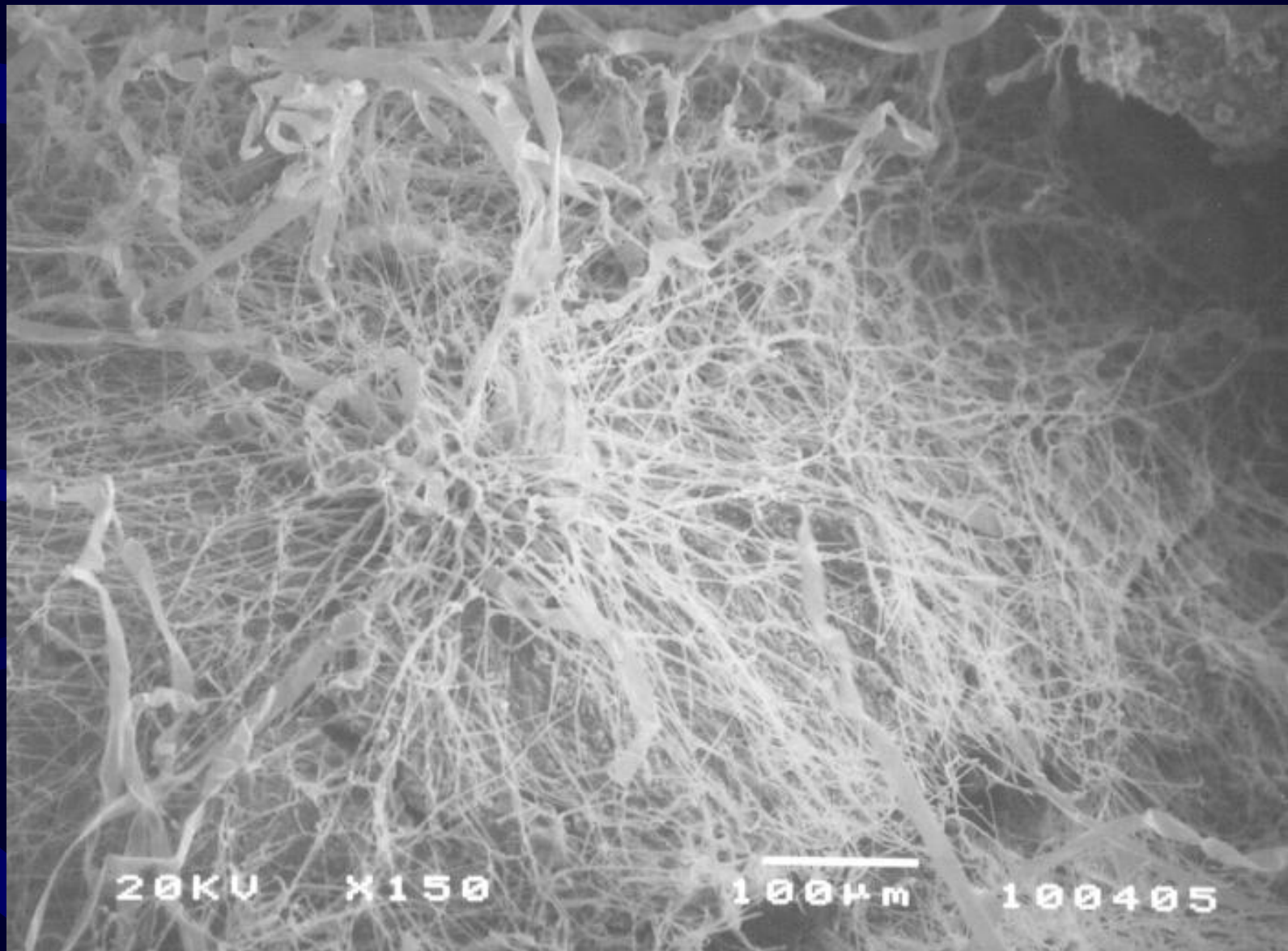
pH

Microbial growth may cause marked changes in the pH of a substrate. Acid production due to incomplete oxidation of the substrate or uptake of ammonium ions will cause the pH to fall, whereas the release of ammonia by deamination of urea or other amines will increase the pH.

Temperature

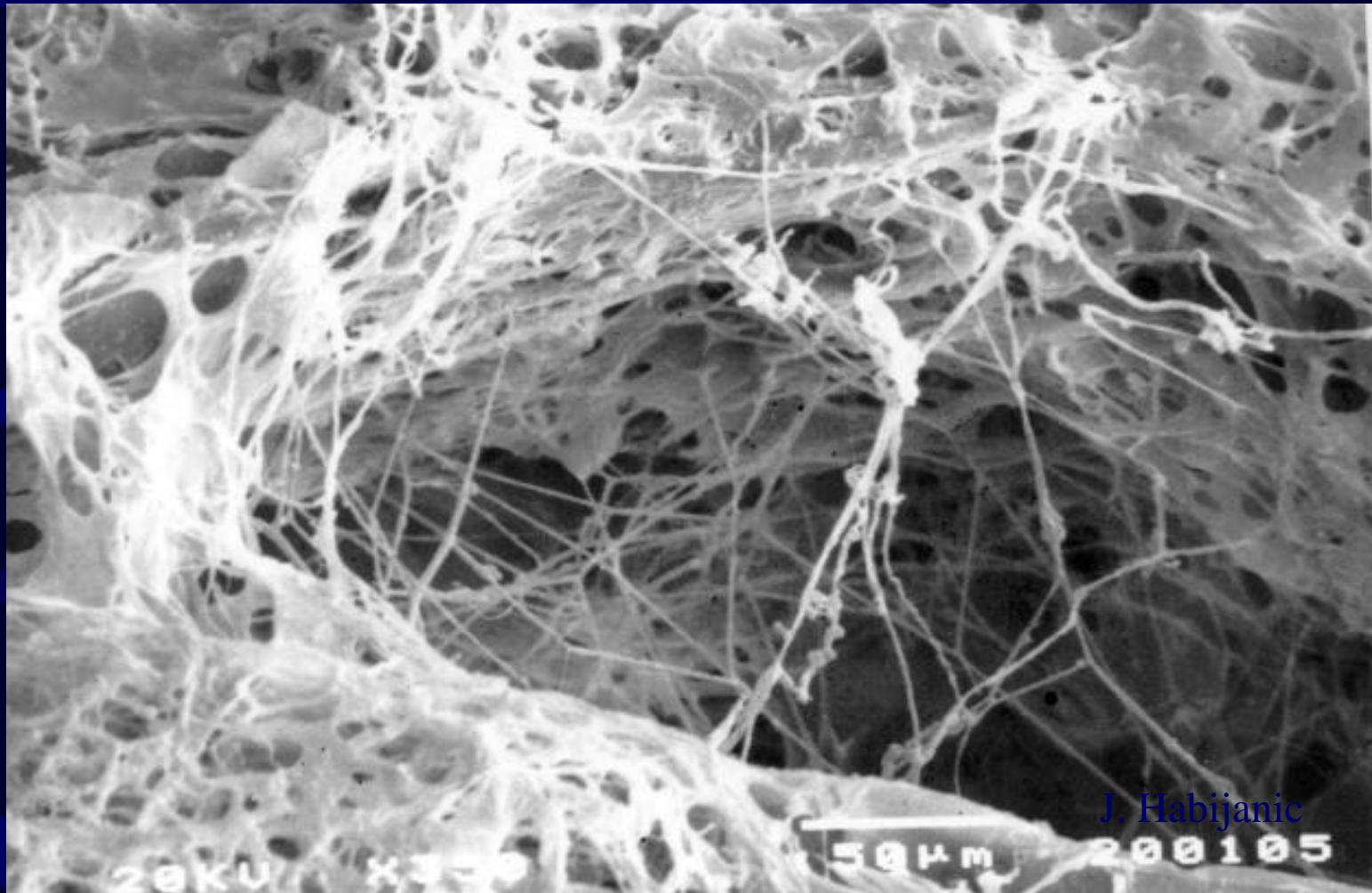
Most microorganisms used in SSF are mesophilic, having optimal temperatures for growth between 20 and 40°C, and maximum temperatures for growth below 50°C.

In undisputed systems, like composting, it was observed that when the temperature of the outermost region of the heap was 37°C, the innermost region recorded was at 60-77°C



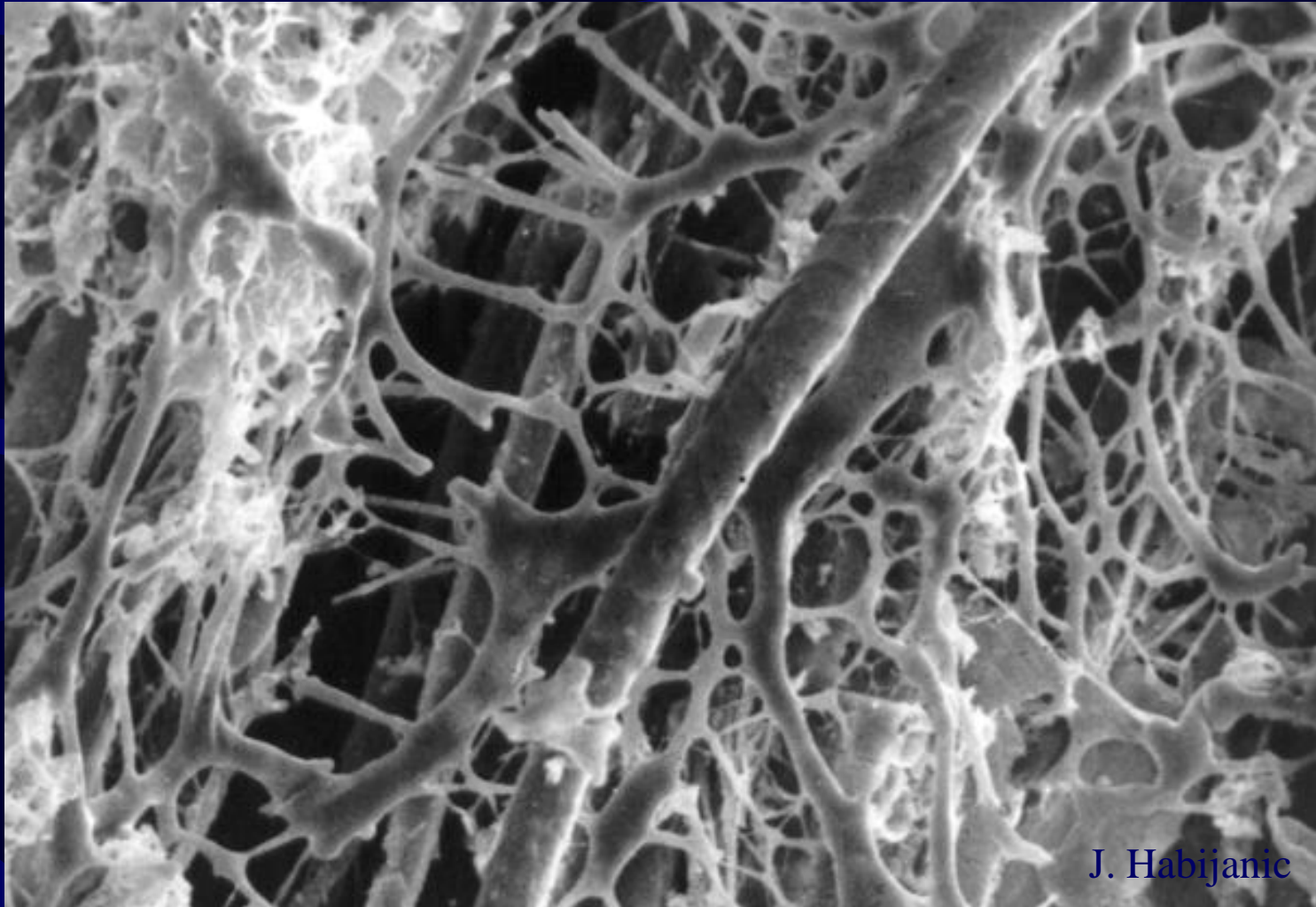
***Ganoderma lucidum* mycelial growth on solid substrate after 7 days of cultivation (350x)**

GROWTH IN SSF



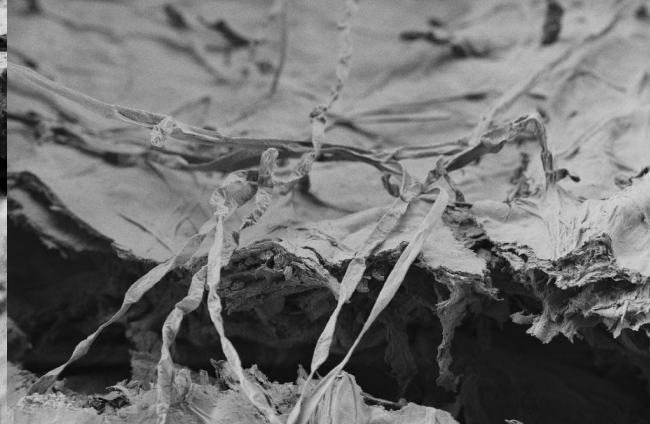
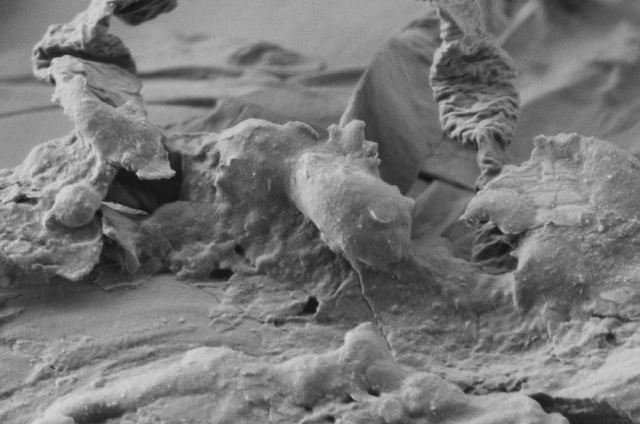
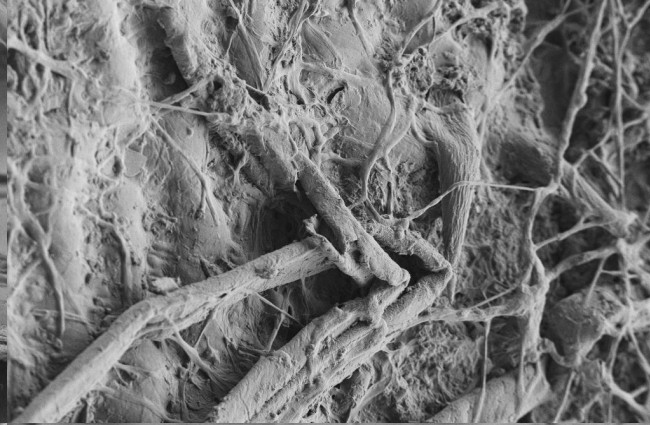
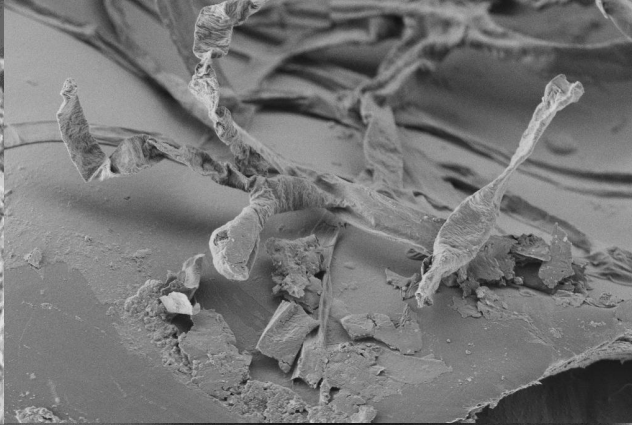
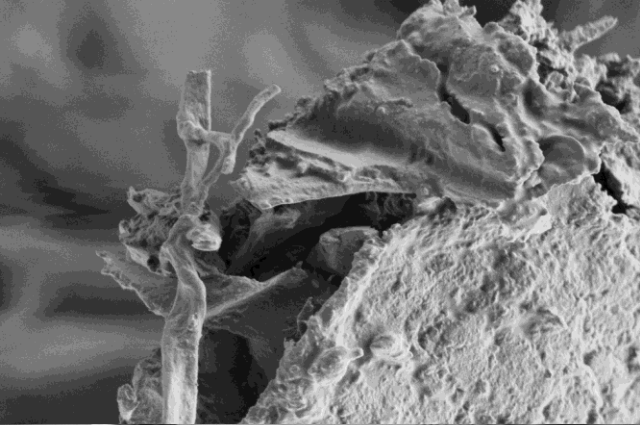
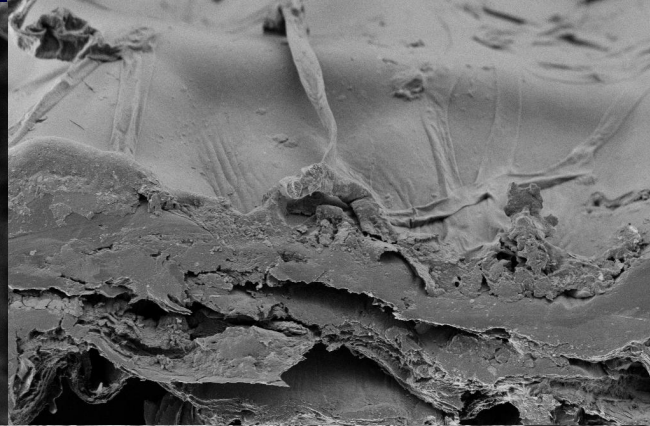
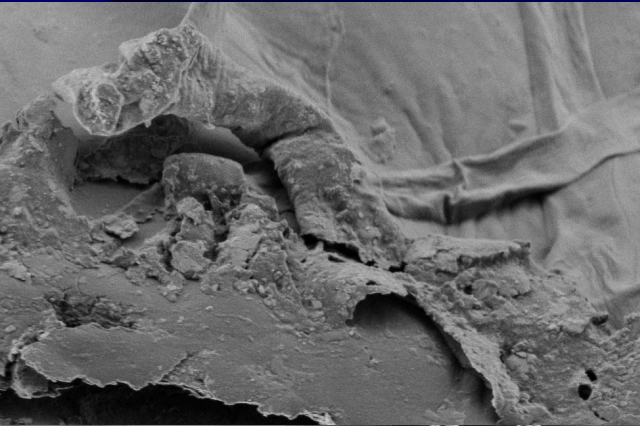
Ganoderma lucidum mycelial growth on solid substrate after 12 days of cultivation (350x)

GROWTH IN SSF



***Ganoderma lucidum* mycelial growth on solid substrate after 14 days of cultivation (350x)**

Grifola frondosa mycelial growth on solid substrate (2000x)

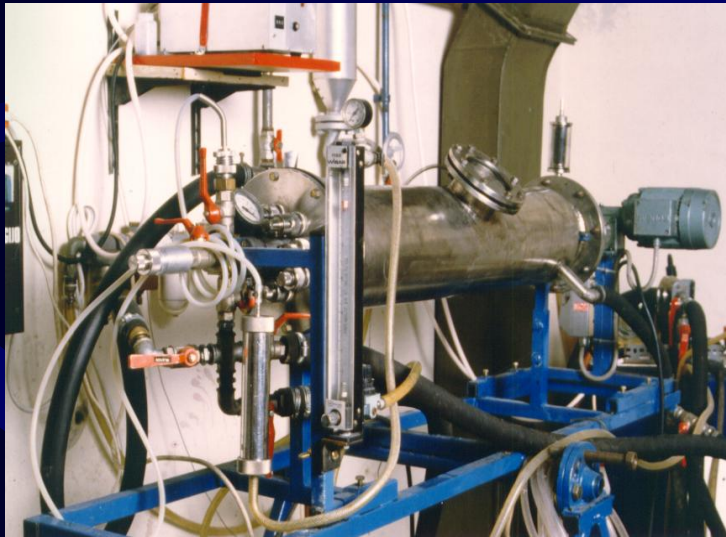


2µm EHT = 1.00 kV Signal A = SE2 Mix Signal = 0.2000 Chamber = 2.88e-003 Pa Date: 27 Feb 2006
WD = 5 mm Aperture Size = 30.00 µm File Name = Grifola_frondosa_13.tif

1µm EHT = 1.00 kV Signal A = SE2 Mix Signal = 0.2000 Chamber = 2.96e-004 Pa Date: 27 Feb 2006
WD = 5 mm Aperture Size = 30.00 µm File Name = Grifola_frondosa_32.tif

10µm EHT = 1.00 kV Signal A = SE2 Mix Signal = 0.2000 Chamber = 5.80e-004 Pa Date: 27 Feb 2006
WD = 5 mm Aperture Size = 30.00 µm File Name = Grifola_frondosa_24.tif

Cultivation of *Ganoderma lucidum* biomass



Solid state cultivation

Farming

Ganoderma lucidum mycelial growth on solid state substrate in horizontal stirred tank reactor



Cultivation of *Ganoderma lucidum* mycelium growth on sawdust (40x) after 6 weeks

PHYSICAL FACTORS CONTROLLING GROWTH IN SSF

Gaseous environment

The gases of interest are oxygen and carbon dioxide. Oxygen must diffuse from the interparticle space to the biomass, and carbon dioxide must diffuse from the biomass to the interparticle space.

Effects of substrates and products in SSF

Nitrogen is an important nutrient in SSF. Many solid substrates are supplemented with soluble sources of nitrogen during substrate preparation. the nitrogen source can play an important role in affecting the pH changes in the substrate during the fermentation, and should be selected with this in mind..

ESTIMATION OF BIOMASS

Direct separation of the biomass from the solid matrix

**with an infrared analyzer for CO₂ and with a
paramagnetic analyzer for O₂**

**samples can also be removed with a syringe and analyzed
by gas chromatography**

relatively simple method can be used for CO₂ uptake

ESTIMATION OF BIOMASS II

MEASURING BIOMASS COMPONENTS

Khjeldahl Nitrogen Determination

The method estimates total nitrogen given sample and will therefore measure both soluble and insoluble nitrogen.

Infra-red-photoacoustic spectroscopy

Methods which detect amide bonds in proteins, such as infrared-photoacoustic spectroscopy

ESTIMATION OF BIOMASS



J. Habijanac & M. Berovic

Ganoderma lucidum mycelial growth in
horizontal bioreactor for solid state cultivation
after 6 weeks

ESTIMATION OF BIOMASS III

INDIRECT METHODS OF BIOMASS ESTIMATION

Adenosine Triphosphate (ATP)

ATP estimation can be used to determine fungal biomass in solid substrates in the absence of the other living organisms. It gives an estimate of the quantity of viable hyphae but not dead hyphae

Chitin Estimation

Chitin is an insoluble linear polymer of alpha-1,4 linked N-acetylglucosamine units produced by most fungi and insects. Chitin assay also gives an estimate of total fungal material but does not distinguish between fungal species in substrate or between living or dead hyphae.

ESTIMATION OF BIOMASS IV

INDIRECT METHODS OF BIOMASS ESTIMATION

Ergosterol

Ergosterol is predominant sterol in most fungi and is probably component of fungal cell membranes. It can be quantitatively measured by gas chromatography, HPLC, or UV spectrometry.

Fluorescent Antibodies

Recent work has attempted to use fluorescent antibodies to measure the biomass of specific fungi in complex environments..

Enzyme Linked Immunosorbent Assay (ELISA) and Radioimmunoassay (RIA)

The benefits of this assay methods are that by linking enzymes or radiolabels to specific antibodies..

ESTIMATION OF BIOMASS V

Growth linked enzymes

In certain special cases fungal extracellular enzymes can be used to measure biomass. The extracellular enzymes, **laccase** (polyphenoloxidase), could be used for biomass estimation of the mycelium when edible mushroom

BIOREACTORS

Bioreactor design

A reactor for SSF must fulfill the following functions :

Sterilization and prevention of contamination

Heat removal to prevent the fermenting material from overheating

Mixing of the substrate to maintain homogeneity and aid in heat and mass transfer

Aeration to supply sufficient oxygen and prevent accumulation of carbon dioxide

Measurement and control of key process parameters

Solids handling during inoculation, sampling and processing

BIOREACTORS I

Mixing

- maintaining homogeneity within the reactor
 - promoting heat and mass transfer

Static fermentations,

- periodically agitated fermentations,
- continuously agitated fermentations.

Mixing helps to bring the fermenting solids into contact with heat transfer surfaces and also helps to replenish the gas phase in the interparticle spaces

AERATION

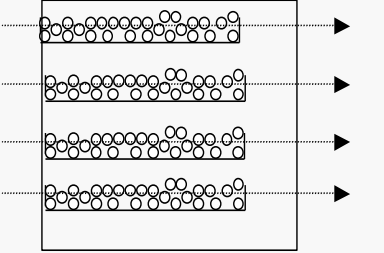
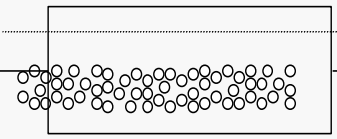
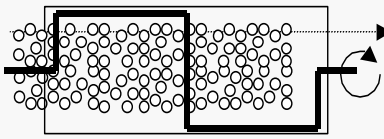
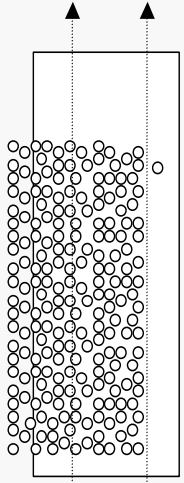
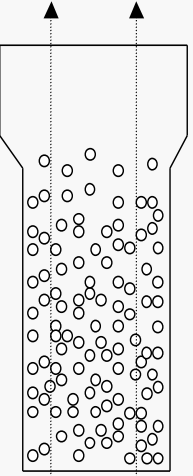
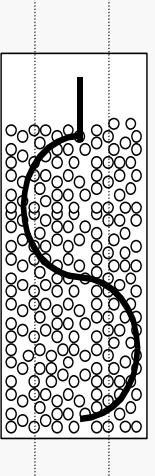
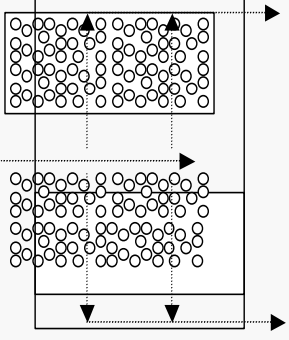
- transfer of oxygen from the bulk oxygen phase in the interparticle spaces across the stagnant gas film at the surface of the particle.
- transfer of oxygen across the interface between the stagnant gas film and the liquid film at the substrate surface
- uptake of oxygen by the microorganism, either directly from the stagnant gas film or from the liquid film at the substrate surface, depending on the location of the mycelium.
- diffusion of oxygen through the liquid phase of the substrate into the interior of the particle

BIOREACTORS TYPES

Bioreactors used in SSF:

- trays,
- covered pan fermenters ,
- inclined incubation cell,
- butler-type corn storage bin fermenter,
 - packed beds,
 - rotating drums,
 - stirred bioreactors
- air-solid fluidized beds.

BIOREACTORS TYPES

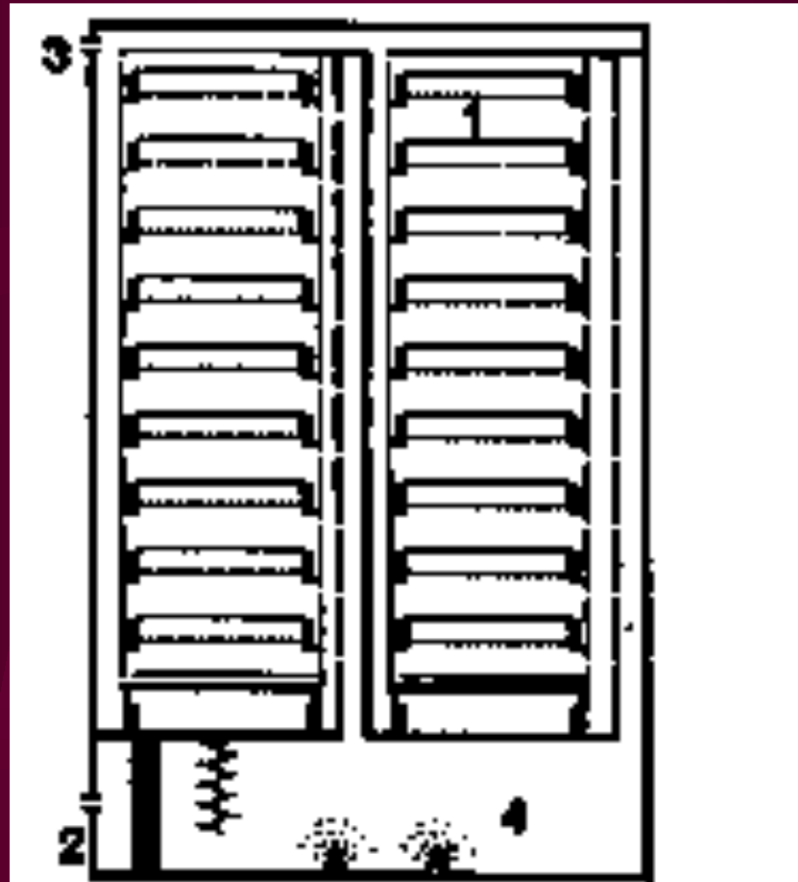
Mixing → ↓Aeration	No mixing (or very infrequent)	Continuous mixing, or frequent intermittent mixing		
No forced aeration (air passes around bed)	 <p data-bbox="569 635 763 678">Tray chamber</p>	 <p data-bbox="1014 635 1207 678">Rotating drum</p>	 <p data-bbox="1439 635 1632 678">Stirred drum</p>	
Forced aeration (air blown forcefully through the bed)	 <p data-bbox="560 1242 724 1278">Packed bed</p>	 <p data-bbox="966 1249 1139 1313">Gas-solid fluidized bed</p>	 <p data-bbox="1197 1249 1352 1278">Stirred bed</p>	 <p data-bbox="1449 1249 1642 1278">Rocking drum</p>

TRAY BIOREACTORS

The characteristics of tray bioreactors are

- a relatively thin layer of substrate contained in a tray often constructed of wood, bamboo or metal.
- the bottom and sides of the tray may be perforated to promote oxygen transfer
- the substrate is usually not mixed, although in some cases the substrate is turned intermittently

TRAY BIOREACTORS



STIRRED BIOREACTORS

→ Stirred bioreactors fall into one of two categories depending on the orientation of the reactor: horizontal stirred bioreactors and vertical stirred bioreactors.

→ Horizontal stirred bioreactors are in contrast to rotating drum bioreactors are mixed by scrapers or paddles attached to a central shaft. Agitation may be continuous or intermittent..

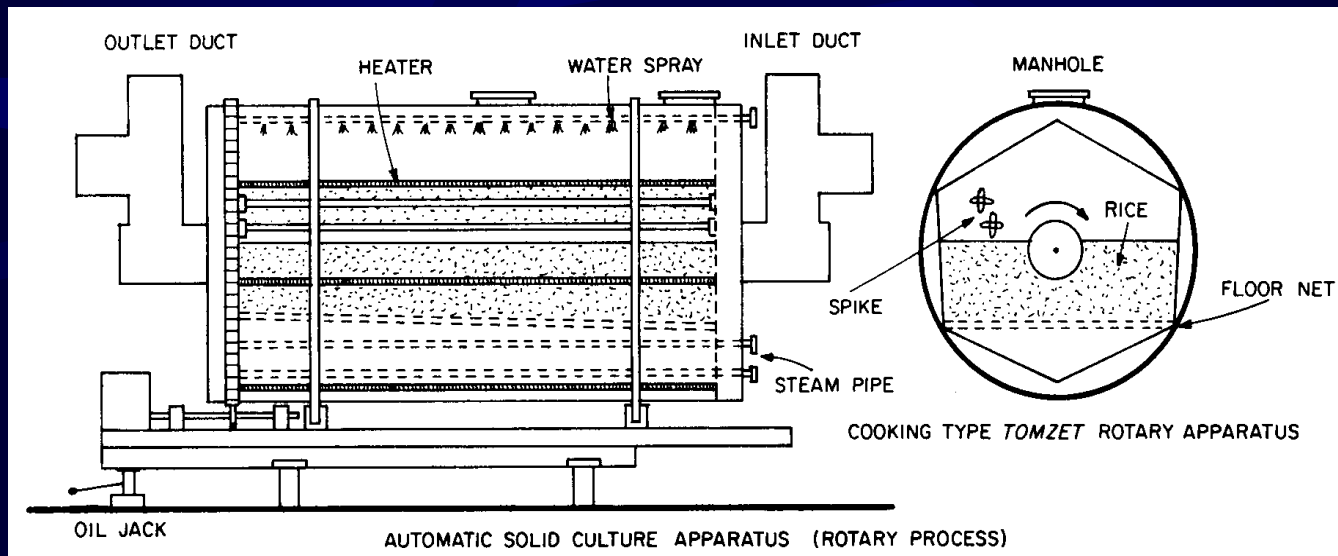
AIR-SOLID FLUIDIZED BED BIOREACTORS

→ Air solid fluidized bed bioreactors are similar to packed bed bioreactors, except that the gas is passed upwards through the bed at a velocity sufficient to fluidize the substrate particles

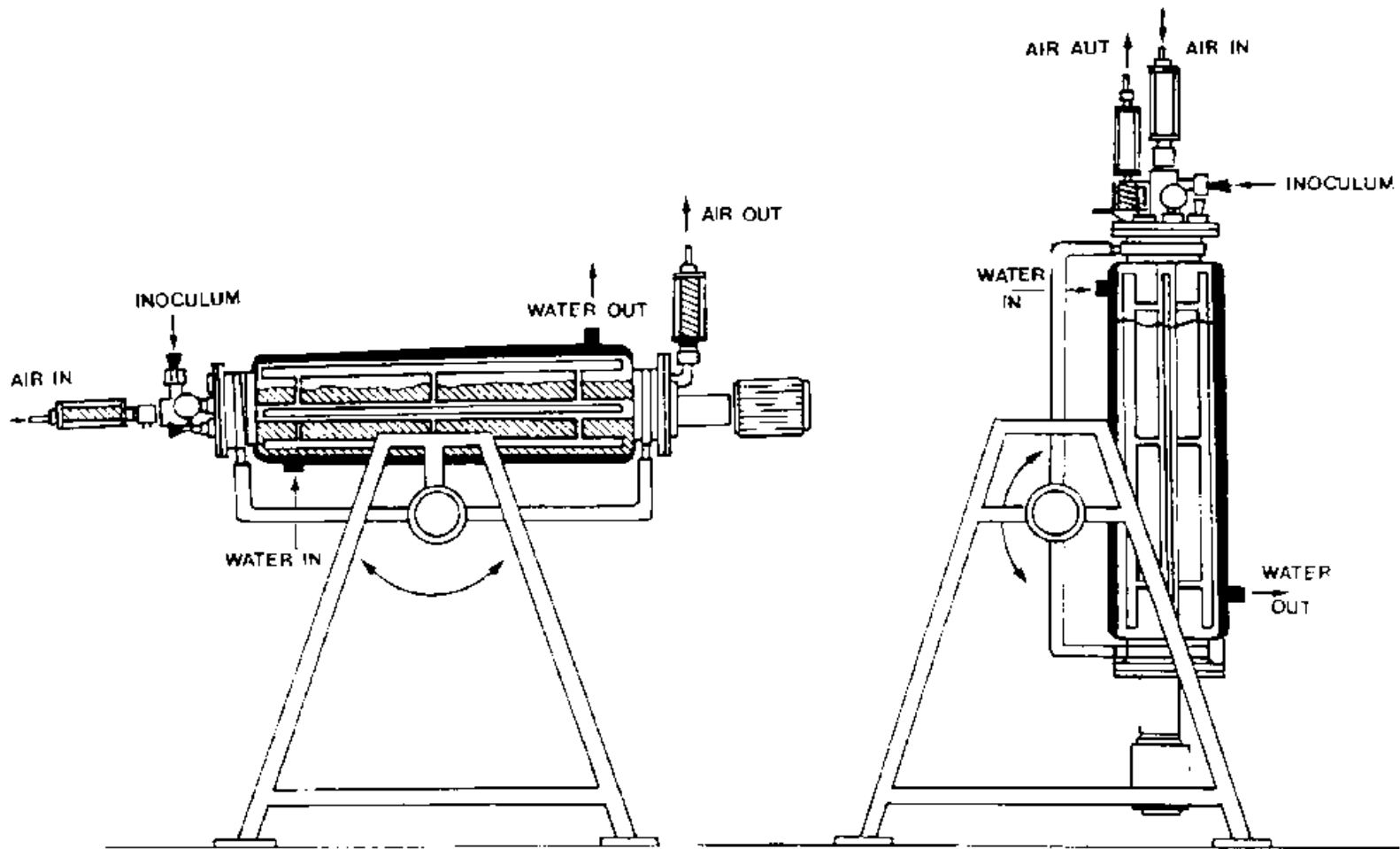
→ The high air-flow in air-solid fluidized beds provides good mixing, and ensures good oxygenation and heat removal. However, much larger volumes of air are required than for packed-bed bioreactors.

STIRRED BIOREACTORS

ROTATING DRUM REACTOR



STIRRED BIOREACTORS

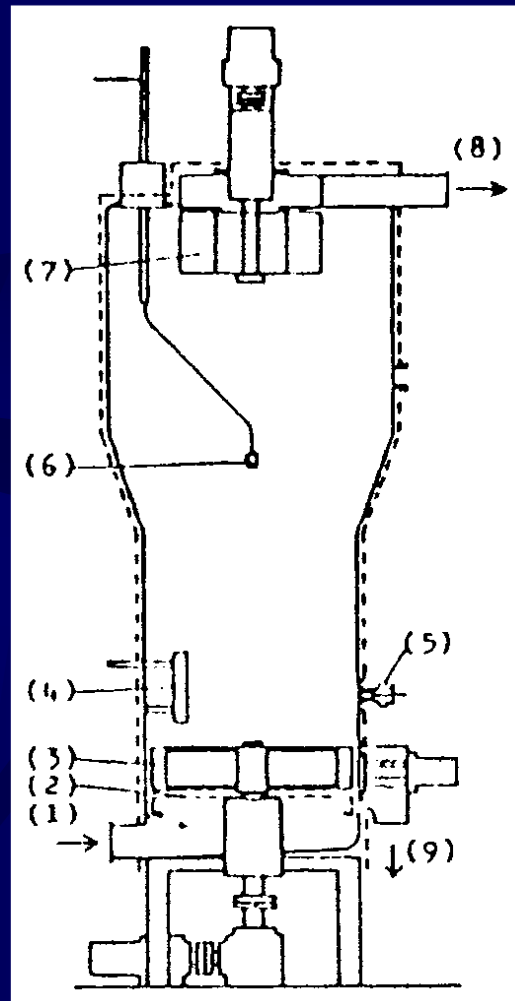


STIRRED BIOREACTORS

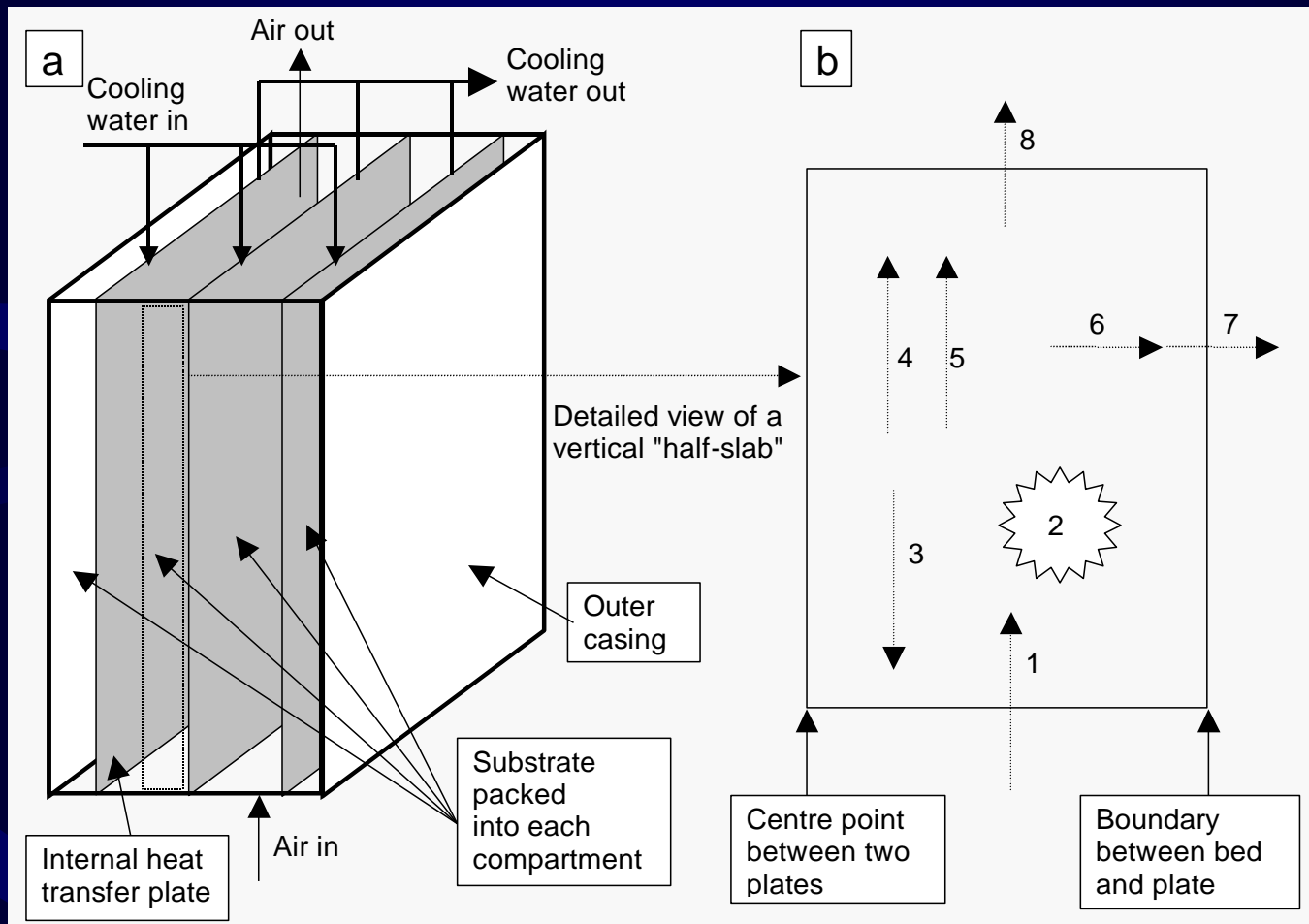


Horizontally Stirred Tank Bioreactor
(M.Berovic)

AIR-SOLID FLUIDIZED BED BIOREACTORS



ZYMOTIS PACKED BED BIOREACTOR



CONCLUSIONS

Advantages of solid state fermentations

- relatively simple and inexpensive substrates
- less water is used and substrate is concentrated
- since spores are used directly in fermentation, neither seed tanks nor preformed inoculum necessary
- low moisture required to get maximum yields of the product with fungi excludes any problem of bacterial contamination
- the conditions under which the fungus grows are more like those in nature
- when constant agitation is used, sporulation is almost completely inhibited

CONCLUSIONS

Advantages of solid state fermentations

- aeration is easily obtained because of void spaces between the particles
- if the product has to be extracted, less solvent is needed
- if the product needs to be dried, there is minimum moisture to be removed
- in many cases the yields are at least the same or higher than in submerged cultures
 - high reproducibility of results
 - useful wastes as animal feed
- there are no enormous amounts of liquid waste to present a disposal treatment problem

DISADVANTAGES OF SOLID STATE FERMENTATIONS

- continuously mixed processes demands high power requirements
- the amount of spore inoculum must be quite large presumably
- the substrate has to be pretreated by pearling or cracking
- relatively humble possibilities for effective process control
- relatively complicated scale-up, especially for continuously mixed processes

Death is not a problem, only a life it is. To be in life it means to look for the problems.....

Zorba The Greek