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ENVIRONMENTAL SCIENCE - XXII Cycle.

Scale up of a solid state fermentation (SSF) pilot plant for the production
of enzymes by lignocellulose utilization in ecosustainable applications

AGR/13

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PREFACE

The environmental impacts of burning fossil fuels and agricultural activities, the need to find renewable sources of energy, and the need for a sustainable agricultural resource base have been the driving forces for a new evaluation of the role of biomasses.

According to the modern and nowadays common concept of sustainability biomasses are of increasing interest both as an energy source and as a valuable raw material for the production of a large number of compounds, fine chemicals and goods.

The development of biomass conversion technologies is a major goal for applied research and the new market (First Biomass Conference of the Americas, 1993).

The term biomass includes a large number of materials that are biological in nature including agricultural and forestry products and waste products, and animal wastes such as manure.

The development of a public consciousness of the environmental effects of burning fossil fuels has contributed largely to identify biomass as a desirable source to produce goods. There have been many concerned scientists and economists over the years.

Technology advances have increased the rate of growth in the development and adoption of agricultural technologies enough to temporary satisfy the growing demand for food and fibres from agricultural sources. Public opinion and policies have also been deeply affected by global communication enhancing people consciousness on environmental subjects.

Research has identified the negative effects of a broad variety of human activities on air and water quality, depletion of the ozone layer, global climate change, etc. The burning of fossil fuels is supposed to be a major contributor to these problems. Attention has naturally focused on these problems.

Since the carbon accumulated in the biomass of growing plants through photosynthesis is almost entirely derived from atmospheric CO₂, the utilization of that biomass does not cause a net input of CO₂ to the atmosphere if the resource is used in a sustainable way.

Clearly, any technology that can provide goods or energy without adding to the planet's atmospheric carbon budget should be taken in consideration carefully.

1. INTRODUCTION

1.1 General tasks

1.1.1 Environmental short outlines

Some main aspects must be examined keeping attention. The most important of them can be summarised as following:

- the biological sustainability is linked to the atmospheric content of carbon dioxide.
- the content of carbon dioxide in the atmosphere increases continuously making the chemical energy obtained by photosynthesis not sufficient to control its raise to which a quote coming from fossil carbon utilization is added (Fig.1).
- the needs of chemical energy requested by human societies are increasing quickly.
- the availability of water is strictly dependent on environmental conditions and it is decreasing in many countries.
- celluloses, hemicelluloses, lignines, oils and starch could be used by new technologies to avoid their loss through wild uncontrolled biological processes.
- the growth and concentration of the livestock industry has led to the generation of large quantities manure of feedlots, dairies, poultry production plants, animal holding areas and pasturelands.
- wastes produced from these human activities can cause significant harm to both air and water quality, so they must be managed properly.

From the above considerations it results that a better controlled biological utilization of lignocelluloses by environmentally friendly applications can largely contribute to enhance the sustainability.

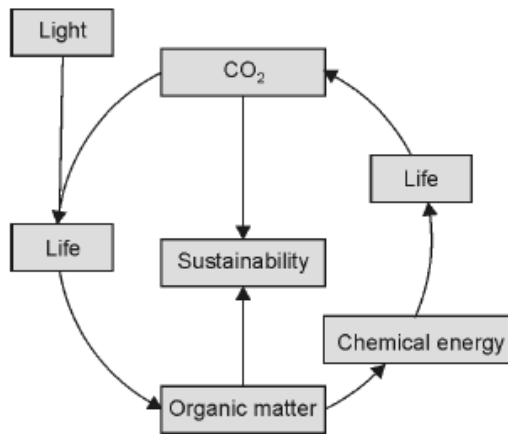


Fig.1 Atmospheric CO₂ levels arising from photosynthesis and biological activities.

1.1.2 Economics short outlines

In the near past the market requests for any industrial production were directly connected to the cash flow, the starting amount of money used and the return of investment. It determined as main goals, when possible but not necessarily, a low consumption of utilities and raw materials, the use of energy as cheap as possible to obtain low investments and the obtainment of high value products a/o in large amount, in order to guarantee a quick return of investment. The technological innovation of the process and the product were obviously already decisive factors in a market competition logic.

Nowadays the rising and the development of new elements such as globalization and an enhanced sensitivity of people respect to environmental problems has forced the market to new trends, in addition to the old needs.

So the energy consumption has to be absent or in the best case it has to be produced. Furthermore the process has to be strictly safe, not polluting and has to guarantee water saving, usage of renewable materials and a very low waste outlet. Finally, to survive, any novel manufacturing production must be extremely flexible and has to hold unoccupied market niches in the complete environmental respect in order to sustain any of the frequent market fluctuations.

All these aspects can be resumed in the term “sustainability” both environmental and economical that is the new “must” for modern industrial productions.

So an accurate evaluation of all the economical aspects involved in the development and run of a new production is fundamental for a successful approach.

It is on evidence that mostly the future market will be occupied by biotechnological and environmental applicable products partly just emerged in the recent past.

Assuming the pharmaceutical applications (also known as “red biotech”) and agricultural applications (so-called “green biotech”) to be respectively the first and second waves of biotechnology, then the emerging industrial application (or “white biotech”) can be considered the third one (Schmid 2001, Burton 2003, Van Beilen & Li 2002). These trends constitutes for sure the most probable prospect for the coming years.

A significant part of such scenario is occupied by enzymes and their applications known and possible either.

Currently, among the ~50 applications of the ~500 enzymes products marketed, only a few of them involve oxidoreductases as industrial commercialized products.

However, many more candidates are being studied, and as a whole, these enzymes will find a wide variety of applications in numerous fields in the next future as we will discuss in chapter 5.

1.1.3 Lignocellulose as a valuable resource

Cellulosic biomass provides a low cost and very abundant resource that has the potential to support large-scale production of chemicals and fuel.

Lignocellulosic biomass includes materials such as agricultural residues (e.g. wheat straw, rice straw and corn stalks), forestry residues (e.g. sawdust and mill wastes) and a considerable part of the urban solid waste (e.g. waste paper). Other industrial wastes and also woody (e.g. poplar trees) and herbaceous (e.g. switchgrass) crops can be considered as biomasses too.

Huge amounts of the materials mentioned above are very simply available all over the planet. It is evident their importance as potential valuable resource but the amount of unutilized lignocellulose can easily reach the 50%. Keeping in mind that more than 3000 million tons of cereal straws roughly are produced per year worldwide and that these residues are mostly disposed of by humification and burning, it results an incredible loss of energy.

Consequently the chemical energy of unused lignocellulosic matter constitute a huge source of energy which could be utilized for numberless applications beside the humification and the burning. Nevertheless novel technologies based upon a more efficient utilization of lignocelluloses require a meaningful engagement to be developed and employed.

Lignocelluloses are to be considered copolymers, characterized by highly complex chemical structure. Celluloses, lignins and hemicelluloses, chemically linked each other, contain large amounts of chemical energy. Composition of lignocellulosic materials can differ depending of kind and provenience, but normally cellulose, the major component of plant cell walls, constitutes up to the 50% of the dry weight of woody materials.

A variety of bacteria and fungi can break down lignocelluloses by using a pool of oxidative and hydrolytic enzymes. It has to be pointed out that few enzymes, necessary for the biosynthesis and biodegradation of the lignocelluloses work at least from 350 millions of years ago, but until now they are scarcely used by means of controlled biological processes. These enzymes belong to oxidative enzymes (polyphenoloxydases, peroxydases, etc.) and hydrolytic ones (cellulases, hemicellulases, polyphenolases, pectinases, etc.).

Cellulose and hemicellulose can be simply hydrolysed to sugars that can be further converted to small size compounds such as organic acids, alcohols, acetone, glycerol, etc.

Cellulose, homopolymer of 1,4-glucose units, hemicellulose, heterogeneous carbohydrate polymer, and lignin, polymer of phenylpropanoidic units interconnected by a great variety of linkages, contain energy which can be utilized by a series of biochemical breakdown reactions, fundamental aspect for any efficient application.

Moreover it is necessary first of all the depolymerization of the water insoluble lignocelluloses to lead to soluble compounds, and this degradation can start by the action of few enzymes which allow the formation of lower molecular weight water soluble copolymers (Crestini C et al., 1998) (Fig.2).

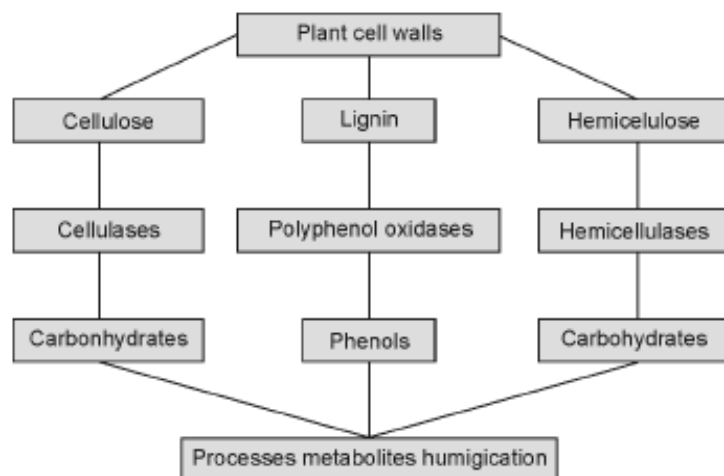


Fig.2 Pathways of biodegradation of lignocelluloses by enzymes.

1.2 Solid Substrate Fermentation

1.2.1 General aspects and definitions

Solid-state fermentation (SSF) processes can be defined as “the growth of microorganisms (mainly fungi) on moist solid materials in the absence” (or near absence) “of free-flowing water” (Cannel and Moo-Young, 1980; Moo-Young et al., 1983). However, substrate must have got enough moisture content to supply growth and metabolism requirements of the fermenting micro-organism (Pandey 1992, Pandey 1994, Pandey et al. 2001).

SSF simulates the natural growth of micro-organisms on moist solids and is considered to be responsible for the birth of fermentation techniques in ancient time, so that almost all the fermentation processes used in the far past were based upon the SSF (Mitchell & Lonsane 1990).

These processes have been used for the production of animal feed, food, agricultural and pharmaceutical products. In particular SSF offers numberless opportunities in processing of agro-industrial residues. Substrates that have been traditionally fermented by solid-state include a broad number of agricultural products such as corn, wheat, rice, grains, millet barley, beans, and soybeans. However, non-traditional substrates which may also be of interest in industrial process development include an abundant variety of agricultural, forest and food-processing wastes such as wheat bran (Pérez-Guerra et al. 2003).

SSF offers several advantages over conventional liquid fermentation. These are:

- The absence of large amounts of water.
- Minimal risk of contamination.
- The use of simple, naturally occurring media.

The requirement of no free water in SSF greatly reduces the volume of fermentation media per mass of substrate as compared to liquid fermentation. This smaller volume gives the some benefits such as the requirement of smaller fermentor for a given amount of product, smaller volume of wastes to dispose off, higher product concentration easier to purify, thus lowering downstream costs.

SSF is less prone to contamination by microorganisms other than molds due to the absence of free water. Most bacteria require a liquid environment and do not thrive in SSF. This brings to less stringent conditions for SSF and to less severe aseptic procedures during operation.

Agricultural materials are mainly used, in contrast to the synthetic media used for liquid fermentation.

These advantages make SSF less capital intensive than SmF. The use of unexpensive waste treatment facilities reduces significantly the investments while the low risk of contamination means that cheaper facilities for providing sterile conditions are allowed.

Solid-state fermentation is therefore an attractive technology for developing countries.

Generally targeting a SSF production for any possible industrial interest, two are the ways to approach the problem:

- the possibility to use available and abundant residues encourages the development of new techniques to produce value-added goods, by enhancing also the biological sustainability.
- the characteristics of the wanted products lead to choose the starting raw material, generally a low-cost agricultural resource simply available.

As it will be shown later, it is of paramount importance the choice of the microorganisms which are dependent on parameters such as temperature, pH, moisture content, etc.

Consequently the development of devoted bioreactors useful to utilize the set of parameters chosen for the process is of major importance.

Also some engineering specifics and physical aspects not directly dependent on the biochemistry of the process must be considered carefully, such as employed materials, economical factors, analytical controls, maintenance, pre- and post-treatments, etc.

1.3. Scale up

1.3.1. General aspects of scaling up

The important role of the scaling up has been pointed out in the present manuscript, according to the concepts of high flexibility, low cost, energy sustainability, low environmental pollution and high innovation.

Once found a process of interest for novel market production purposes, the sensitive procedure to develop and build up a new industrial unit has to be approached with accuracy, considering that hazard, safety, handling of chemical in large scale are ganged (Doble et al. 2004).

The scale-up is particularly complex for biochemical processes because it encompasses many different scientific and technological fields such as biochemistry, biotechnology, chemistry, engineering, physics, process designing, chemical engineering, fluid mechanics, etc.

It also involves other disciplines like economics, marketing, law and authority disposal & restriction management as well.

Furthermore it is fundamental not to forget that large scale activities differ significantly from laboratory scale ones. In facts, when performing laboratory scale activities, we normally use high-grade chemicals and problems due to time and cost of an experiment are not as relevant as in a manufacturing scenario. Instead at large scale industrial grade chemicals are used, leading to pre- or post-purification needs, and any single set of trials might require long time being unpredictably expensive.

The waste management and its influence on production cost in such scenario have also to be examined deeply in accordance to the disposal limits of authority.

The typical activity of process development work remains the pilot plant experiment and it is useful to adopt this approach when one or more of the following reasons occur:

- The operating condition and the influence of the parameters on the process must be studied in a representative unit.

- The passage from laboratory scale to manufacturing unit constitutes a problem in the scale-up process not possible to be solved without carrying out an experiment at intermediate size.
- The investigation on building of by-products, corrosion or other long term effects is required.
- The need to obtain samples in sufficient quantities for various trials and tests emerges (i.e to verify the possibility to use the product in novel large scale application or to show the product(s) to customers).
- It results convenient to convince potential costumers of the value of the process showing an operating pilot plant.

Based on data obtained from laboratory results and literature, it is possible to imagine an industrial unit whose size correspond to the presumed request of the market.

It is necessary to consider and to compare as many different technological alternatives as possible. Identification of constraints and impossibilities is essential as well as the selection of the most interesting flow-sheet obtained during this procedure from the economic and technical standpoints.

It is on evidence that it is quite impossible to proceed before a complete and accurate review of all the know-how on the subject. So all the different existing solutions adopted and the mathematical models used in similar cases have to be compared.

The most important characteristic of a pilot plant is for sure the ability to reproduce to the smallest detail (i.e. product quality, yields, etc.) the behaviour of the manufacturing unit we are planning to build up. Obviously such a procedure force to a series of balanced compromises involving the cooperation of various specialists and somehow it is influenced by the role of the preponderant mechanisms having effect on the overall behaviour of the system.

Nevertheless representativity is not the only main characteristic of a pilot plant. In facts a pilot unit has usually to be transportable or scalable and as small as possible maintaining its ability to be representative.

The most affecting concepts of pilot plant projecting are the design (the machinery used and their operating have to be ideated in order to minimize the complexity of the system) and the dimensioning of the pilot unit. The minimum capacity able to give representative results and to sustain the perspectives (i.e. the need of sufficient quantities of product to be employed for tests and trials) has to be chosen.

Therefore the feed rate of pilot units is generally included between 0.01 and 100 kg/h in continuously working or they have the minimum representative volume if working in batch.

Only the parts of the process that cannot be directly or indirectly investigated otherwise have to be built in the form of a prototype (Euzen et al. 1993).

The interconnections between different parts and the eventual recycle of utilities, raw materials and wastes must be examined with close attention.

Usually the problem connected to recovery is omitted except if it is necessary or interesting to examine it directly.

The utilities involved have to be representative and easy to be used, so conventionally is preferable to use electricity instead of steam or fuel, and water instead of air.

2. PURPOSES OF THE RESEARCH

Nowadays modern society is strictly dependent on a wide variety of products obtained from natural raw materials. Numberless results in biotechnologies and in biochemical engineering have forced the development of many novel applications for enzymes as a valid alternative to the well known usual chemical methods, in particular for their sustainable character (Howard et al. 2003).

Solid state fermentation (SSF) processes have a terrific potential for the production of enzymes (Viniegra-González et al. 2003, Suryanarayan 2003, Rodríguez Couto and Sanromán 2006, Giovannozzi-Sermanni and Porri 1989).

For those reasons our interest have been set on the development and the scale up of processes for the production of enzymes by Solid State Fermentation using a novel prototype demonstration plant at pre-industrial scale, on the optimization of these processes and on the study of new products obtained following dynamics of high economic, environmental and energetic sustainability and their potential for “green” applications (Pandey et al. 2000).

Industrial productions are characterized by a deep and close correlation among the product, the production process and the plant. The contemporaneous development and the strict balance of those three aspects is the main basis for the success of any technologically novel rising industrial activity.

We performed most of the experiments using edible mushrooms strains (mainly *Lentinula edodes* and *Pleurotus ostreatus* strains) setting the major interest on the production of extracts containing a pool of enzymes, almost entirely oxidoreductases.

A considerable part of this study was devoted to the investigation of novel applications based upon these enzymes, focusing especially on laccase which is on our opinion the most promising of them for environmental features.

Consequently the plant, the process, and the applications of the product obtained will be described in the following chapters as deeply as patent restrictions consent.

3. THE PLANT

The pilot plant, whom the description refers, was designed partially based on patent data (Giovannozzi et al. 2001). It was constructed at the Metapontum Agrobios s.r.l. research centre located in Metaponto (Matera, Italy) and it is, at present time, still in the start up period.

Since started, a few months ago, the data collection gave us a large set of opportunities to improve the performance of the process and to modify the plant as current needs require.

3.1. Dimensioning

Once decided to built up a plant, the first major step not to be neglected is the estimation of its size according to some main considerations. In particular the final size is determined by a complex balance among the final production on purpose (calculated on the yield and production rate obtained studying the data collected at laboratory scale), the built plant final cost estimated, the presumed market request of the final product(s), the maintenance and working costs connected to the exercise of the plant.

In our case study, other some aspects were taken in account such as the previous experience on the existing pre-pilot bioreactor, the literature description of similar industrial plants, the availability of the raw material used and its cost.

So, considering a fermentation time of about a week and a confirmed (but actually simply increasable) laccase production yield assessed at about 3 IU, the final dimension of a complete plant has been determined. It was assumed to produce enough product to support the production of biological paper from annual plants of a small-medium size paper mill (5000-25000 tons/year of paper).

The choice to consider this kind of application has been forced by the intent to obtain a good result in the worst possible conditions (large amount of product needed and very low added value possible). Consequently if such approach results successful, similar economical and engineering estimation had to be proper in any other better condition.

The availability of the raw material at reasonable cost (including transport as well) it was also a limiting factor. It has been assumed that the maximum range of raw material availability was about 40 km, both for the enzymes production plant and the paper mill.

The ultimate restriction is due to the physical, chemical, mechanical and technical limits to manage the biochemical process. In particular the heat transfer regulation and the capability to obtain and maintain septic condition were the parameters most affecting the geometry and the final size of the bioreactor.

As final result it was imagined a plant constituted mainly by a battery of 6-8 bioreactors of 9 m³ each, in order to guarantee a sufficient daily production for at least 48 weeks per year (considering 4 weeks per year of stop for festivities or devoted to extraordinary maintenance), and other equipment and machinery of related size.

To construct a pre-industrial pilot plant it was decided to maintain the size of all machineries and equipments as calculated above except for the use of a single bioreactor instead of a complete battery.

The omission of a few down stream processing parts (like a thermoelectric cogeneration equipment) was also chosen.

3.2. Equipments and machineries – choosing and modifying the technology

Knowing the final dimension of the plant, the involved process was studied in detail in all its parts. The operational flow sheet obtained (fig.3) has been used to identify a sufficient number of possible existing machineries candidate to carry out any single part of the process, eventually modifying them at convenience. The only part to necessarily be studied separately was the bioreactor for solid state fermentation.

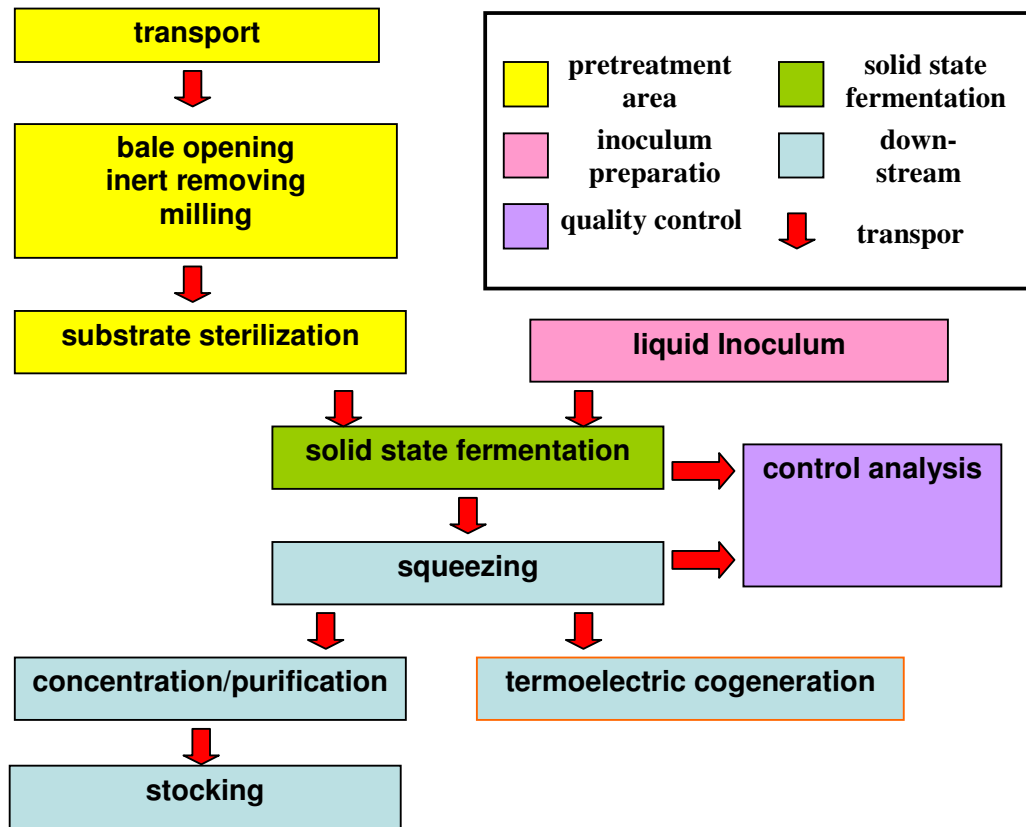


Fig. 3 Schematic of the process flow-sheet.

3.3. Description of the plant

The complete plant can be divided in 7 different sections, as shown in fig.4-6 :

- 1) the pre-treatment section
- 2) the sterilization equipment (it can be considered somehow a pre-treatment machinery)
- 3) the bioreactor
- 4) the liquid fermenter
- 5) the down-stream treatment area
- 6) the utilities management section
- 7) quality control laboratory (external additional supply)

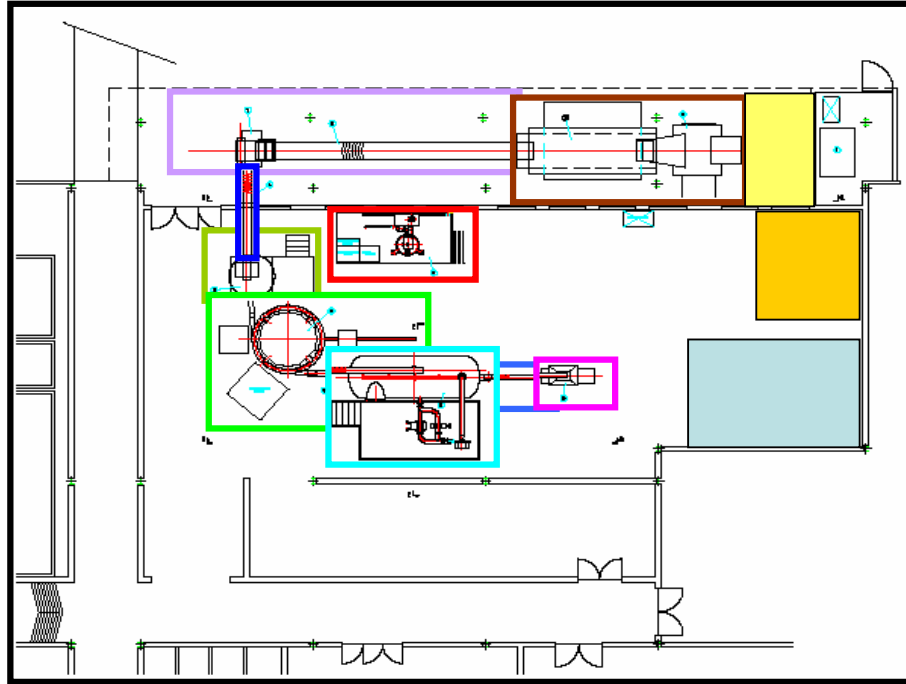


Fig.4 Basic drawings of the pilot plant including: liquid fermentor (red box), sterilization equipment (green box), bioreactor (light blue box), squeezer (purple box), laboratory (light blue area), utilities management area (orange area), raw material stocking area (yellow area), moving systems (blue and violet boxes), pre-treatment machineries (brown box).

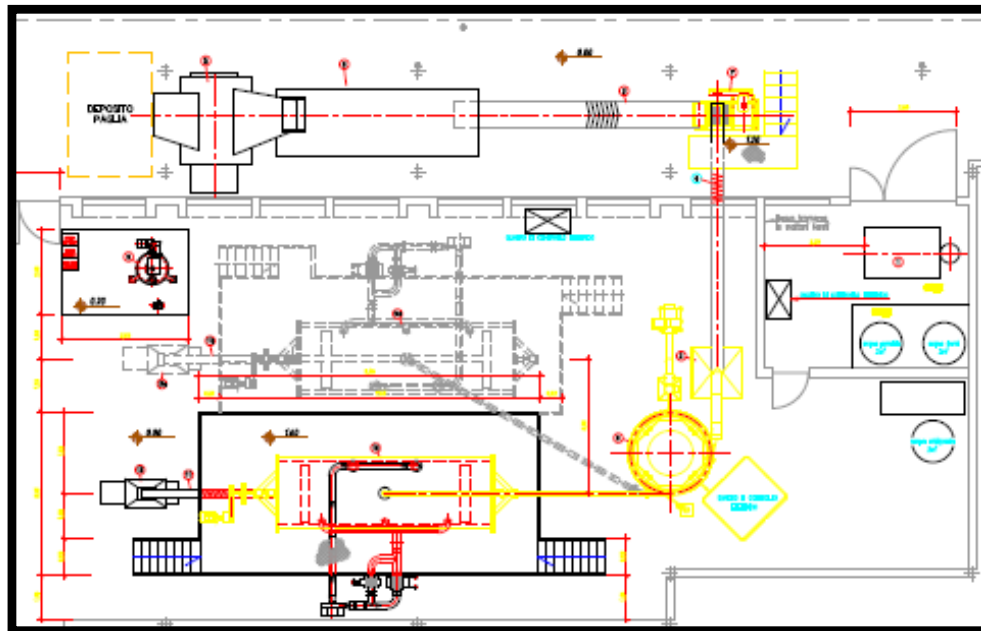


Fig.5 Schematic from the executive project drawings.

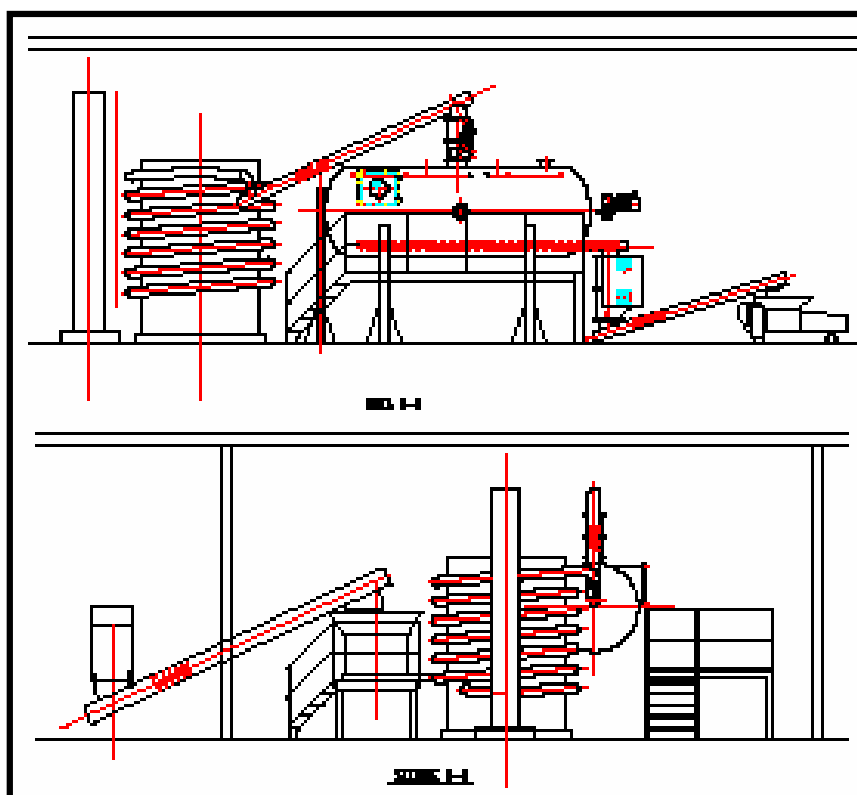


Fig.6 Lateral views of the inner part of the plant from the executive project.

A remote control software equipment to manage the working of the entire process is also part of the plant, but it has to be considered separately because it is not only a mechanical part and it can be excluded at choice.

The pre-treatment area, located outside to minimize problems due to powders, fire risks, raw material moving and stocking, is constituted by a bale opener able to work with different size and geometry of bales, a transport bend, a rotating grid to remove occurring not desired materials such as soil or stones, an hammer mill able to reduce the material to the desired size and connected to a socks filter to remove powders.

The sterilization equipment is a powerful device based on a novel technology chosen to considerably simplify the process in order to reduce power and steam requirement, the time needed for the material charge and sterilization. The reduction of the construction cost of the bioreactors was also a reason for the choice.

The bioreactor will be shown and described in a separate paragraph.

The preparation of the inoculum is provided in a 1 m³ liquid fermentor not too different from the ones conventionally used in other biochemical and pharmaceutical industrial

plants. It is equipped by probes, sensors and gauges to constantly measure liquid level, temperature, pressure, mixing rate, air flow inlet rate, etc. There is also a window located at its top to directly observe the fermenting during the run and to simplify the cleaning. It is also possible to sterilize it by direct steam injection inside the fermenting vessel or by heating the vessel by a steam flow through the external thermostatic chamber.

At the end of the process the SSF bioreactor is emptied and the material is put into a squeezer by a screw. The liquid obtained, full of enzymes, can be then used directly or concentrated a/o purified and eventually stocked. The solid waste is used to feed a cogeneration device (capable to use materials with high humidity contents up to 75%) to produce steam (yield close to 100%) and electricity (yield about 80%). This final procedure grants a dramatic reduction of the waste amount to be treated and a self consistence of the plant (obviously an addition of not processed raw material is needed to let the cogeneration device work continuously).

The utilities used during the process are managed in a separate room.

3.4. The parts of the plant

3.4.1. The pre-treatment section

Description

The pre-treatment area includes a stocking area, capable to hold almost two weeks needed volume of raw materials, usually collected in the form of bales (box bales or cylindrical ones of variable size from 40 up to about 300 kg weight), and a group of machineries shown in fig.7-8.

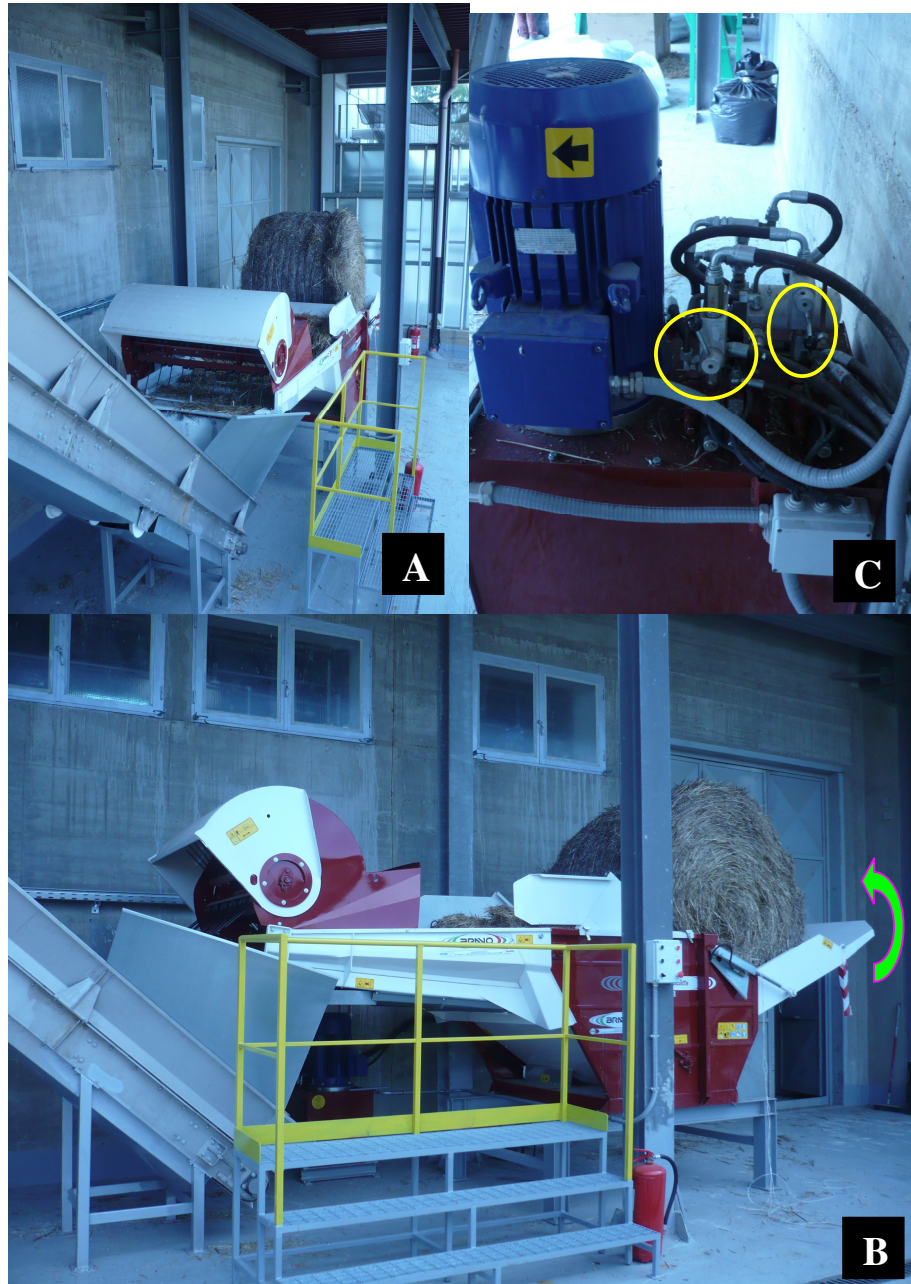


Fig.9 A- bale opener, , B- moving of the hopper in green, C- knobs for manual adjustment of speed in yellow.

- a transport bend to move the material from the bale opener to the rotating grid.
- a rotating grid (fig. 10) to remove occurring not desired alien materials such as soil or stones. The grid can be substituted by a different mesh number one and it is adjustable in speed rate. Inspections of the equipment are also possible using doors on both sides.



Fig.10 Rotating grid during operation (A) and lateral view (B).

- an hammer mill (fig.11) able to chop the feeding material to an appropriate size and connected to a socks type aspirating filter equipment to remove powders (undesired in the next section of the plant). It is possible to regulate the speed as well as size of the exiting material changing the inner grid.



Fig.11 Hammer mill (left) and socks type aspirating filter (right).

- a second transport bend that brings inside the treated material to the inner part of the plant trough the feeder of the sterilizing apparatus.

All these equipment can be set at different speeds a/o feeding rates according to the temporary needs of the process.

Trials and results

The pre-treatment section has been tested by 8 trials using stocks collected from 4 different lands nearby the plant of 3 different kinds of bales differing in size and geometry as resumed in table.1.

<i>BALE GEOMETRY</i>	<i>SIZE</i>	<i>PROBLEMS NOTICED</i>
<i>BOX</i>	40 Kg	Uncommon in the area
<i>CYLINDRICAL</i>	120 Kg	Apparently none
<i>CYLINDRICAL</i>	300 Kg	Inner residual humidity Uneasy handling High content of powder
<i>CYLINDRICAL</i>	300 Kg	Inner residual humidity Uneasy handling High content of alien material

Tab. 1 Resume of the pre-treatment section trials.

3.4.2. The sterilization equipment

Description

The sterilization apparatus constitutes a fundamental part of the entire plant. Working continuously at ambient pressure, it simplifies considerably the process allowing a lower consumption of energy and steam and a significant time saving.

A successful sterilization of the material has a paramount importance for the process considering that the growing mycelium is particularly sensitive to pollution by any other competitive microorganism.

The sterilization can be conducted directly into the fermenting vessel before the inlet of inoculum step by direct injection of steam. Nevertheless this approach requires a large amount of steam and it takes a long time to reach the proper temperature, maintain it for the necessary interval and get back to a temperature sufficiently low to start the fermentation. It results evident how deeply the chosen equipment makes easier the process. It has to be pointed out that sterilizing not under pressure causes a substantial

reduction of the bioreactor construction cost. This saved amount multiplied to the number of bioreactor contained in a complete battery easily overcome the cost of the sterilization apparatus itself.

The apparatus considered most proper for our purposes appears to be the one shown in fig.12 and provided by Revtech; its advantages can be summarized as following:

- Low steam and electrical consumption (estimated to be close 40 kg/h steam flow rate and 60 kW per ton of treated material).
- Small volume and reduced area occupied.
- Absence of moving mechanical parts (except for the motors), leading to a particularly scarce maintenance both ordinary and extraordinary.
- Great variety of applications (when not used for the process it can be implied in numerous different applications).
- High technological content.

The equipment consists in a feeder, a transport bend and a main structure based on 4 gum fenders to support vibration. A spiral pipe encircles the structure into which the material goes trough from the bottom to the top. The nine spires (six insulated for heating and three for cooling) are heated electrically by connections to an electrical cabinet. Each spire is equipped by a steam inlet regulated by a manual valve and connected to an injection manifold, and an outlet connected to an extraction manifold in the same way. Two motors on opposite sides of the unit generate vibration.

A control panel can regulate feeding rate (up to 100 kg/h solid material flow rate, corresponding to about 1 m³ volume of wheat straw), vibration rate, heating profile (up to 140°C) as well as eventual steam injection and persisting time of the material into the spires.

Actually the apparatus does not perform a real sterilization but an high level pasteurization. However the treatment is intense enough to reach a correct growth of the microorganism inoculated later without a significant condition of competition with the residual microbial flora of the treated substrate.

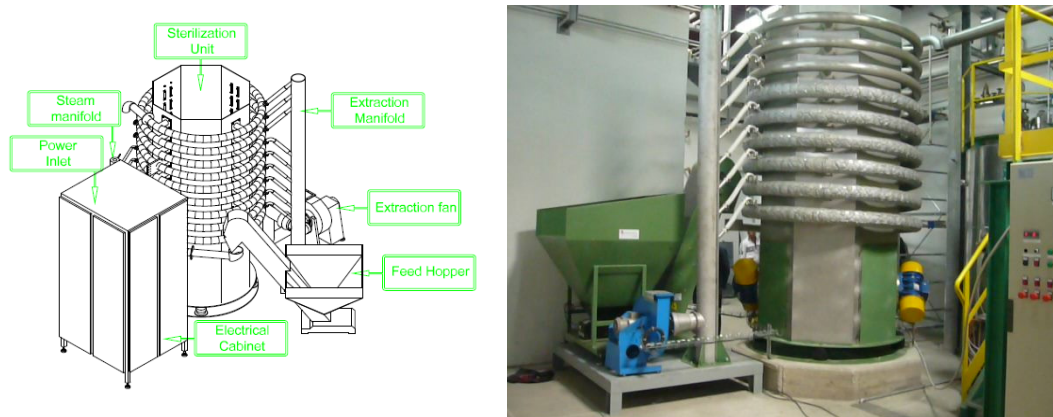


Fig. 12 Sterilization apparatus(right) and its schematic drawing (left).

Trials and results

The equipment has been tested by 18 trials for wheat straw, bran and malt grains leading to quite good results, and the temperatures and steam flow rates investigated are resumed in Tab.2-3.

	<i>untreated</i>	<i>110°C steam 5Kg/h</i>	<i>120°C steam 5Kg/h</i>	<i>130°C steam 5Kg/h</i>	<i>110°C steam 10Kg/h</i>	<i>120°C steam 10Kg/h</i>	<i>130°C steam 10Kg/h</i>
<i>Aerobic mesophilic flora 30°C ufc/g</i>	1.7×10^6	3.2×10^6	1.6×10^6	3.8×10^6	1.6×10^6	0.78×10^6	1.8×10^6
<i>Yests 25°C ufc/g</i>	6300	17000N°	54000N°	8400	720	180	5500
<i>Molds 25°C ufc/g</i>	26000N°	1300	4900	2500	110	900	19000N°
<i>Aerobic mesophilic spores ufc/g</i>	5700N°	4500N°	74000N°	17000N°	990	22000N°	76000N°
<i>Anaerobic mesophilic spores ufc/g</i>	980	5600N°	35000N°	5300N°	520	4300N°	68000N°
<i>Escherichia coli ufc/g</i>	<10	<10	<10	<10	<10	<10	<10
N°=number estimated of micro-organisms.							

Tab. 2 sterilization apparatus trials results for wheat straw.

The results obtained for wheat straw put on evidence that a greater steam injection is required to perform an intimate contact among hot steam and the particles to be sterilized.

	BRAN					MALT	
	<i>untreated</i>	<i>115°C steam 5Kg/h</i>	<i>125°C steam 5Kg/h</i>	<i>115°C steam 10Kg/h</i>	<i>125°C steam 10Kg/h</i>	<i>110°C steam 10Kg/h</i>	<i>115°C steam 5Kg/h</i>
<i>Aerobic mesophilic flora 30°C ufc/g</i>	3.6×10^6	1.8×10^4	6.8×10^3	1.6×10^4	1.5×10^4	1.4×10^6	1.6×10^6
<i>Yeasts 25°C ufc/g</i>	<10	<10	<10	<10	<10	1900	450
<i>Molds 25°C ufc/g</i>	<10	<40(20EP)	<10	<40(10EP)	40NE	60NE	70NE
<i>Aerobic mesophilic spores ufc/g</i>	5700N°	<10	<40(10EP)	<40(20EP)	40NE	40NE	40NE
<i>Anaerobic mesophilic spores ufc/g</i>	<10	<40(20EP)	710	200	110	70NE	<10
<i>Escherichia coli ufc/g</i>	<10	<10	220	<40(30EP)	150	<10	<10
<i>N°= estimated number of colonies. ; EP estimated presence of detected microbs.</i>							

Tab.3 sterilization apparatus trials results for wheat bran and malt grains.

3.4.3. The liquid fermenter

Description

The liquid fermenter (shown in fig.13-14) is quite similar to the ones finable on market. It as a volume of 1 m³. It is equipped by a level gauge, three PT100 probes set into the vessel and in the thermostatic chamber, two flow sensors to measure air and water inlet flow rate, a probe to measure pressure (also to regulated the system to a small overpressure) and one to reveal O₂ content. There is also a window located at the top of the fermenter to directly observe inside the fermenting vessel and to simplify the cleaning and the charge of nutrients to prepare the cultural broth. It is possible to sterilize it by direct steam injection inside the vessel or by heating the vessel using a steam flow trough the external thermostatic chamber. Air and water pass trough groups of filters before entering in the fermenter to improve septic condition and ease sterilization.

The moving paddles are changeable on request as well as the sparger geometry.

The equipment has a remote control to manage operations and register data and all valves of the equipments related to the inner chamber have all a steam jacket in order to be sterilizable and to cut off any possible biological pollution from outside if needed.

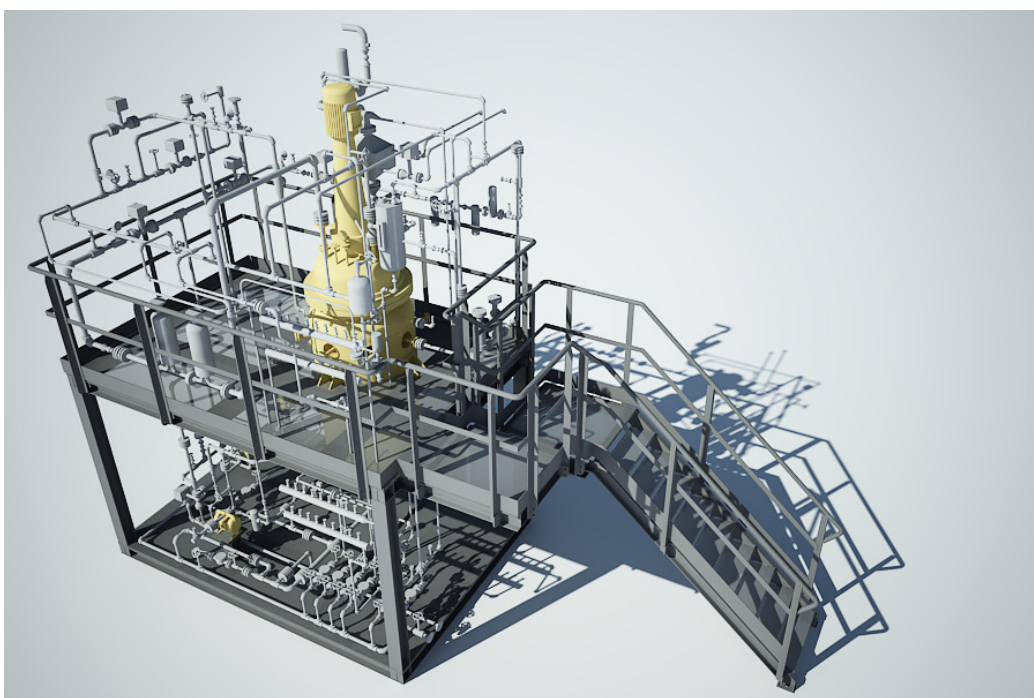


Fig. 13 Rendering of the liquid fermenting unit device.



Fig.14 The liquid fermenter.

Trials and results

The first set of trials was performed to verify the presence of any eventual improper or wrong operating of the system and calibrate it on convenience.

Then it was investigated the real performance of the equipment in order to ensure that it could reach and maintain easily the temperature needed to be sterilized.

This unusual test was necessary because the supplier company was at its first experience in constructing this kind of equipment.

We report in fig.15 the result of one of these trials.

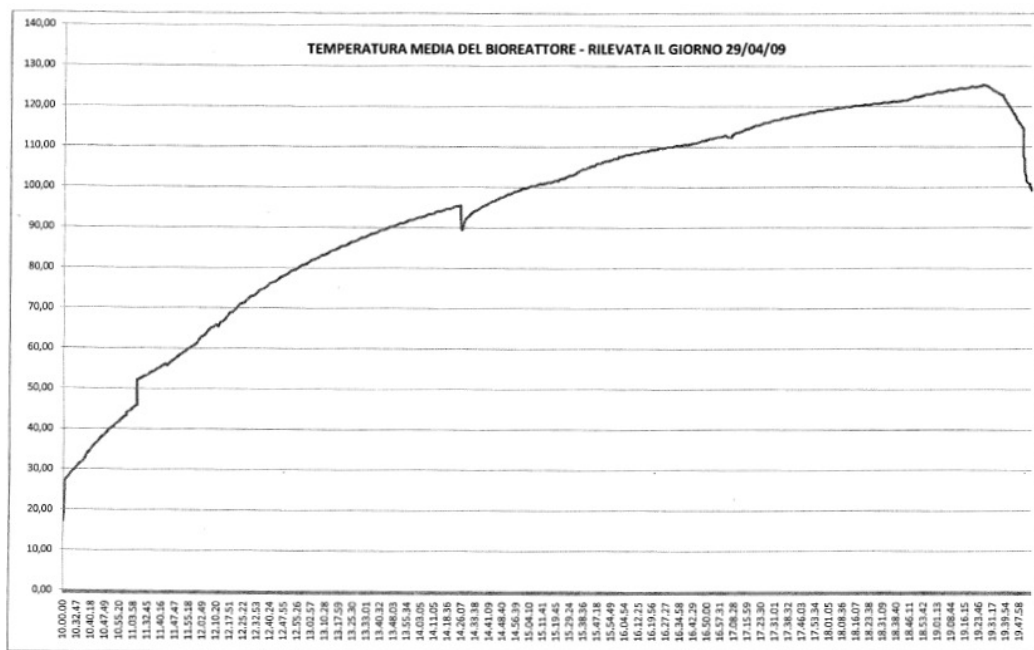


Fig.15 Temperature profile during direct sterilization attempt of the liquid fermenting equipment (maximum temperature reached 126.5°C).

Two running tests have been performed for the liquid fermenter charging the vessel by respectively 400 l of a simple cultural broth (malt extract and commercial sugar) and 375 l broth plus 25 l of inoculum and collecting data for a week.

The results (Tab. 4) shown the need to modify both the process, by changing the content of nutrients adding an antifoam, and the apparatus, by excluding some pipes and connections. Sterilization, fermentation and cleaning protocols have been modified as well in order to solve the malfunction encountered.

<i>EFFECT DETECTED</i>	<i>CAUSE IDENTIFIED</i>	<i>TEMPORARY SOLUTION</i>	<i>LONG TERM SOLUTION</i>
FOAM	LARGE VOLUMES		- USE ANTIFOAM
	HIGH NUTRIENT CONCENTRATION		- USE LOWER CONTENT OF NUTRIENTS
POLLUTION	“COLD” ZONES	- MODIFY STERILIZATION PROTOCOLS	- MODIFY UNUSED PIPES - MODIFY AIR OUTLET
	RESILIENCY	- CUT OUT LEVEL GAUGE	- PERIODICAL CLEANING - CHANGE GROWING CONDITIONS

TABLE.4 *Undesired effects detected during tests on the liquid fermenter and adopted solutions.*

3.4.4. The down-stream processing section

Description

The post treatment section is constituted by a screw squeezer (see fig.16), able to pull out the crude enzymatic extract from the moist colonised substrate up to 80%, and a conventional concentration-purification system, based on ultracentrifugation.

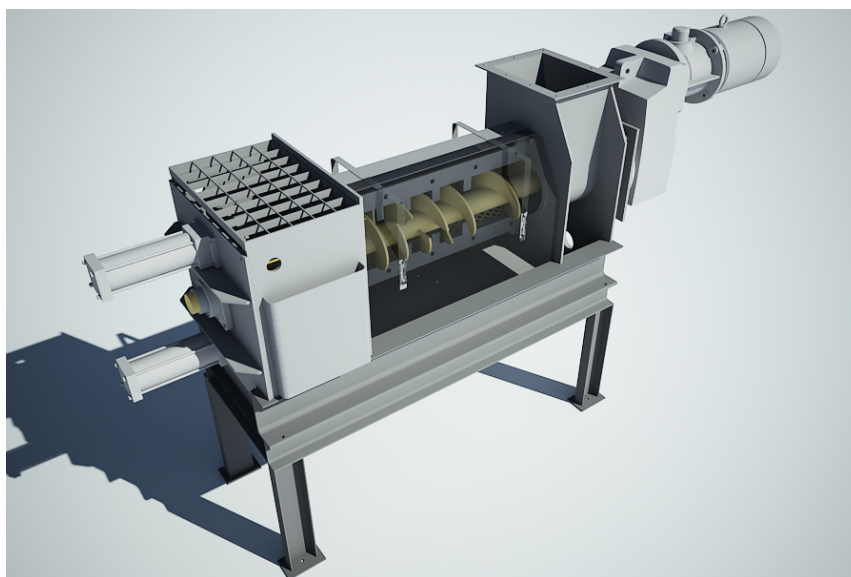


Fig.16 *Rendering of the squeezer, the screw system is shown.*

In the demonstration plant is not present the thermoelectric cogeneration device that is also part of plant hypothesis. This omission is due to the relatively small amount of raw

material treated; in facts it makes such equipment useless and consequently unnecessary for representative purposes because oversized, both for inlet and output.

3.4.5. Utilities

All the utilities are managed separately in a room next to the main structure of the plant. That area (as shown in fig.17) contains a chilling equipment, a gauge tank for water, a tank for demineralised water, a steam generation device and a tank to recovery of sludge water from the plant lines.

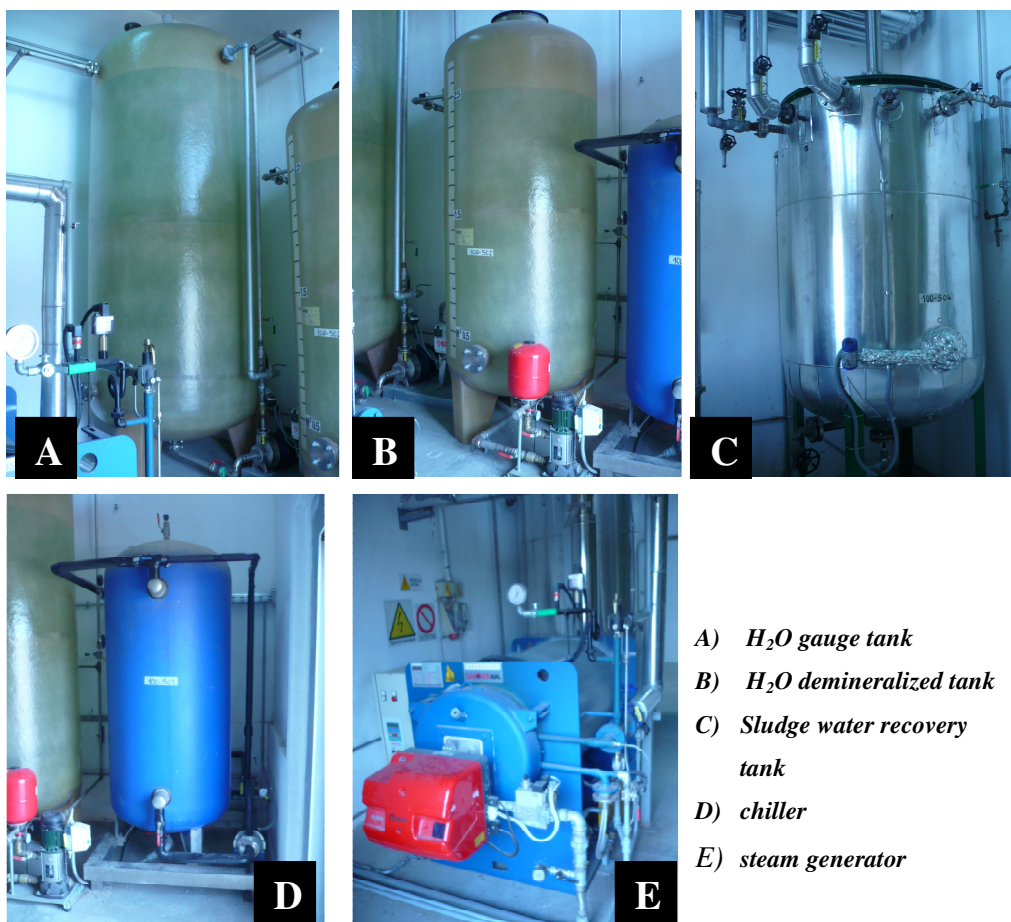


Fig.17 Utilities & auxiliary supplies.

3.4.6. The SSF Bioreactor

This part of the plant requires some more lines than the others to be discussed. In fact, after a detailed study onto the existing know-how reported in literature, resulted necessary to imagine, ideate, draw and build the bioreactor as a complex novel prototype.

It was also impossible to size up the existing 4 m³ rolling drum pre-pilot unit, held at dep. I.B.A.F. of the National Research Council (sited in Montelibretti, Roma).

This constraint is due mainly to the heating transfer problems occurring when large amount of substrate have to be fermented and as shown before to the upgrade of the sterilizing method.

General aspects of SSF bioreactor design

The use of solid matrix, either as an inert support or substrate support, has considerable implications on the engineering aspect of bioreactor design and operation (Robinson & Nigam 2003).

Unlikely SSF bioreactor systems have not yet reached a high degree of development, mainly due to the problems associated to the solid beds like poor mixing, heat transfer and material handling. Some of the desired features of a solid-state bioreactor system are the following (Raghavarao et al., 2003):

- the uniformity of biomass must be guaranteed.
- the system has to be preferably microbiological contamination-free to prevent accidental hazardous due to biological pollution.
- if necessary the system has to be able to avoid any eventual release of dangerous matter when using biological unsafe compounds.
- the control and regulation of the operational parameters must be as efficient as possible.
- the constitutive material has to be cheap, inert and resistant to abrasion and corrosion.
- the bioreactor design has to simplify as much as possible the maintenance, the loading-unloading operations and the product recovery (according to the labour cost of the countries where the SSF process is developed).

Scaling-up the process, the availability of bioreactors designs decreases considerably.

SSF scaling-up, essential for uses at industrial scale, raises severe engineering problems due to the increase of temperature, pH, O₂, substrate and moisture gradients. Hence,

most published reviews also focus on progress towards industrial engineering (Giovannozzi and Tiso 2007).

Only a few designs of bioreactors have been studied and applied to pilot scale or industrial level. The limited application of SSF for large scale is due to some further important parameters present for the scaling-up.

First of all the solid medium can agglomerate during the process causing air channelling, shrinkage and other problems during the operations. Furthermore it can make difficult the heat removal and the gas transfer and severely restricts the design strategies available.

The maintenance of uniformity can result difficult for large volumes of biomass.

Then mechanical stirring, often used, can damage the mycelium hyphae, in particular if they have no septa, allowing only few possible designs to respect the aeration and heat removal requirements.

Inoculation, control and sterilization or pasteurization of large volumes can limit the choices as well. In fact beyond 3 days of mycelial growth it could be prohibitive working in non-sterile conditions.

Moreover a bioreactor versatile is hopeful for a flexible usage in different conditions.

The maintenance and the procedures of filling, emptying, cleaning of the bioreactor must be taken in account too.

Very few bioreactors have been used to develop industrial and pilot plants that can be grouped in typologies based on the mixing and aeration strategies adopted (Table 4) (Mitchell et al., 2000):

- static, pulsed mixed, continuously mixed.
- unforced or forced aeration (through the biomass).

	<i>Static</i>	<i>Intermittently mixed</i>	<i>Continuously mixed</i>
<i>Without forced aeration</i>	- Tray bioreactor (Koj type)	- Rotating drum	- Rotating drum
<i>With forced aeration</i>	- Packed-bed - Zymotis	- Intermittently-stirred bed - Rocking drum	- Rotating drum - Continuously stirred aerated bed - Gas-solid fluidized bed

Tab.5 Summary of most used bioreactors typologies.

The scale up of bioreactors is often based on empirical criteria, connected to transport processes (Hsu & Wu 2002), but the application of mathematical modelling techniques to describe the phenomena within the system is the basis of the most significant improvements in designing and scaling up SSF. Lenz et al 2004 and Pandey 2003 have shown to be a powerful aid for the designing and the defining of large scale bioreactors (Raghavarao et al., 2003).

An important concept in design a bioreactor is the possibility to let take place in one single vessel as many major operations as possible. As example we report the pilot plant described by Grant et al., in 1978. In this bioreactor it was possible to hydrolyze (121°C, 30min, 0.5N H₂SO₄, 7:3 liquid:solid) the substrate (100kg of ryegrass straw per batch), to treat it with ammonia to raise the pH (to 5.0), to inoculate it (with *Candida utilis*) and to conduct the fermentation holding the material stationary with air blowing up through it, as shown in Fig.18.

On the same concept a novel efficient design of integrated matrix bioreactor, consisting in a computer controlled device, using complex fermentation control algorithms, has been patented (Suryanarayan & Mazumdar 1999, Suryanarayan 2001). The operations are all done in one single equipment, maintaining all the advantages of SSF.

Maximization of the rate of formation and the yield of product within the bioreactor are a key of the optimizing the bioprocess.

Description

The SSF prototypal bioreactor (fig.18-20) consists in a stainless steel horizontal cylindrical vessel.

It has a volume of 9 m³, 7 of them are actually usable, once counted the dead volumes and the real volume occupied by the inner moving system.

The vessel has an external cooling-heating jacket fed by an independent water tank to aid reaching and maintaining the desired temperature during the process. It also provides the sterilization of the vessel by direct steam injection before the filling operations.

Temperature and heat removal are managed by up to 7 different temperature exchange mechanisms working in synergy. In addition to the external thermostatic jacket there is an other one covering the main shaft that supports the mixing paddles. The use of the second jacket was suggested by literature study and by results on experiments conducted using the pre-pilot bioreactor. In fact for wheat straw in the range of

conditions investigated the heat removal by contact with the walls of the vessel seems to be extremely poor beyond a distance of 15-20 cm from the exchange surfaces. Using a second exchange surface means quite doubling the thickness of the material involved in the thermal exchange.

A poor additional contribution is due to the mixing mechanism. Furthermore the bioreactor is equipped by a forced air recycling device. The air is removed from two openings located onto the top (in which is located an O₂/CO₂ probe) and recycled inside from other three openings located in the bottom of the bioreactor. The recycling air line consent to eventual aid the temperature management because it is split in two separate pipes passing through heat exchange batteries (one for cooling and one for heating) that can be chosen on need.

That line has also an other important function, in facts it regulates the gas exchange by eventual input of fresh air as requested to maintain the desired O₂/CO₂ content.

Five PT100 temperature probes are situated on both sides at two different heights through the full length of the vessel symmetrically located on opposite sides in order to have a complete map of the temperature profile.

On one side there are two openings closed by steam valves used for sampling. A screw like sterile sampling system is used for that purpose.

Two windows are situated onto the top of the vessel to directly observe inside.

There are also two series of openings for the input alternatively of water and inoculum and a large opening in the middle top for the substrate charging and two other ones for eventual use (i.e. addition of nutrients or pH correction).

Finally three large cutting valves are located on the bottom for the emptying and cleaning operations.

Two gauges provide a continuous measurement of the pressure in the inner chamber and into the thermostatic jacket.

The upper part of the bioreactor is surrounded by a platform reachable by stairs.

Moreover the mixing is guaranteed by a motor able to move on both verses, to run in a wide range of speeds and equipped by a safety cushion clutch.

A safety valve for the jacket and one for the inner chamber, and a water seal system (equipped with two sealing barrels) are provided.

All the operations can be managed by a remote control system equipped by a computer device.

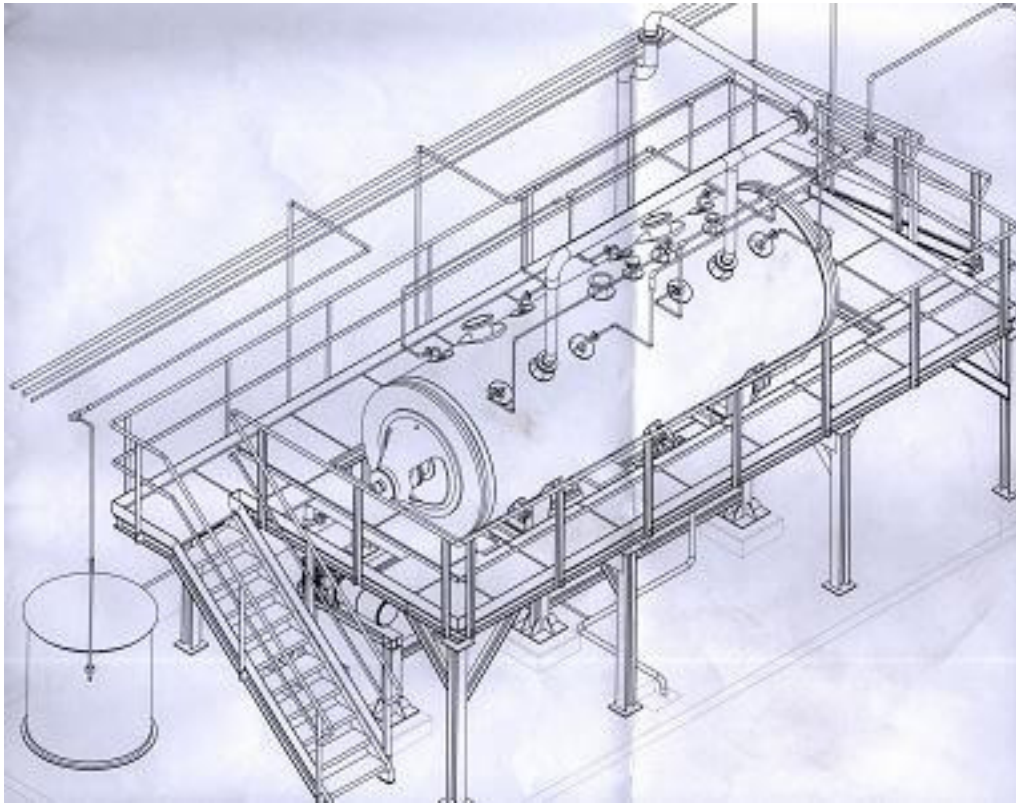


Fig.18 Schematic drawing of the bioreactor.



Fig.19 Rendering of the bioreactor.



Fig.20 SSF prototypal 9m³ bioreactor during construction (left) and refining by insulation covering of the vessel (right).

4. THE PROCESS

Lentinula edodes is a good producer of laccase and Mn dependent peroxidase (MnP), while apparently lacks lignin peroxidase (LiP) (Silva et al., 2008).

Laccase shows a great potential for numerous applications as it will be discussed in chapter 5. For these reason it was selected as main candidate to study the bioprocess of interest.

The degradation pathways of lignin by white-rot fungi are shown in fig.21.

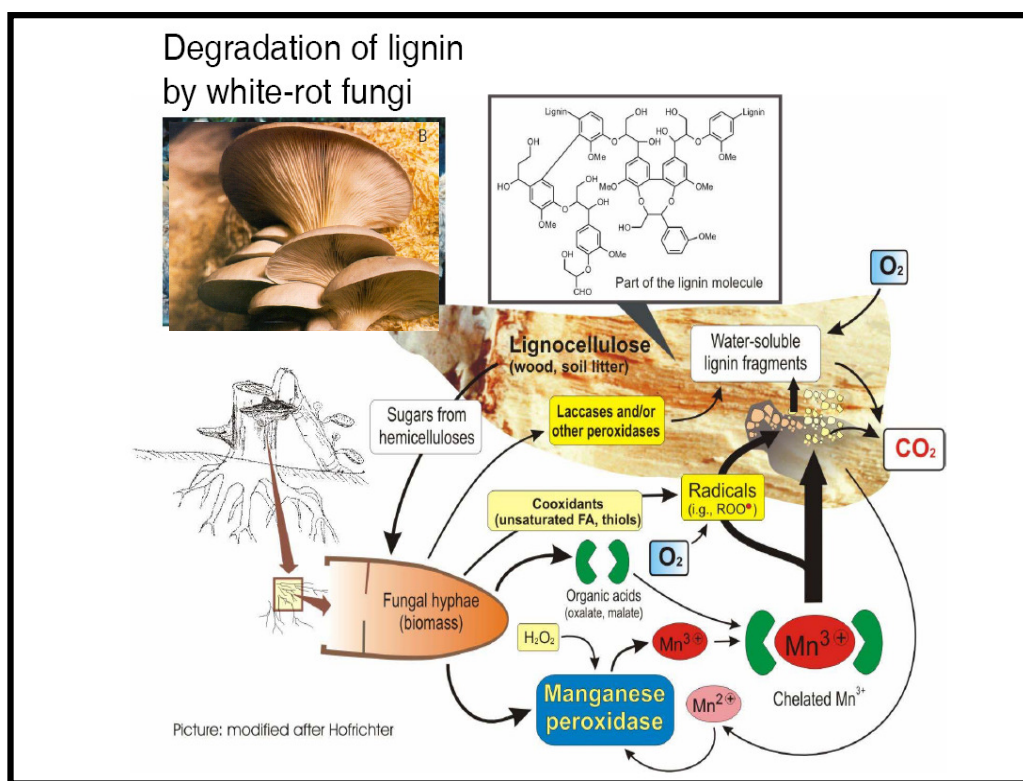


Fig.21 Mechanisms of degradation of lignin by white-rot fungi.

4.1. Parameters affecting the bioprocess

The most important parameters characterizing an SSF process are the following:

- water activity and moisture content of the substrate.
- temperature and heat transfer.
- pH.
- aeration.

- nutrient diffusion.
- mixing.
- septic conditions.
- particle size (density).
- physico-mechanical properties of the solid matrix.
- microbiological inoculum.
- biomass uniformity.
- morphology of the microorganism.
- pressure.

Most of the above parameters are strictly interconnected, but few of them appears to be of major importance in the development of new processes such as heat transfer, pH, gas exchange and water content.

We will briefly examine them one by one in the following paragraphs pointing out the data acquired during laboratory experiments.

4.1.1. Water activity and moisture content

This variable has a deep influence on biodegradation processes, biosynthesis and secretion of metabolites (Perez-Guerra et al., 2003).

Water has a solvent function providing nutrients and scavenges wastes. It also holds a structural role involved in the stability and the function of the biological structures (Gervais & Molin 2003).

Water in SSF systems is present as a thin layer either absorbed on the surface of the particles or in the capillary region of the solid or as a complexed form within the solid matrix.

An exceed of moisture content can perturb the transport phenomena at macroscopical and cellular level, but good diffusion of nutrient and gas exchange are partly granted by free water. The excessive lack of it can also cause a loss of the functional properties of some enzymes and a disequilibrium on the metabolic chain of the cells.

Water requirements should be defined in terms of water activity (A_w), a thermodynamic parameter related to the chemical potential of water. A_w is well correlated to the relative humidity (RH) as follows:

$$A_w = RH/100 = p/p_0$$

where p = vapour pressure of water in the substrate and p_0 = vapour pressure of pure water at the corresponding temperature (Raimbault 1998).

A reduction in A_w normally extends the lag phase of the microbial growth, causing a low biomass production.

4.1.2. Temperature and heat transfer

This is a particularly important parameter in SSF because it affects directly bioprocesses characteristics, spores germination, cells growth and the efficiency of the process.

It has to be pointed out that the most used substrates for SSF are definitely adverse to heat transfer making temperature a critical parameter to control.

Respiration during growth, which is dependent to the oxygen consumption and CO_2 formation, is highly exothermic and heat generation depends directly to the level of metabolic activities of the microorganisms.

The microbial growth is particularly sensitive to the rise of temperature and to the thermal gradients produced by heat generation. For this reason the removal of metabolic heat during large-scale SSF is one of the most critical issues (Schutyser et al. 2003, Saucedo-Castañeda et al. 1990).

A variety of different approaches have been attempted using different cooling techniques to control the microbial growth enhancing the heat transfer.

Such methods as utilization of cooling jackets a/o additional cooling surfaces, mixing, forced aeration, evaporative cooling, that are just the most used systems to achieve the best thermal conditions are applied case by case forced by experimental experience.

These approaches have a main importance in designing a bioreactor.

4.1.3. pH

It determines the enzyme activities, given that each of them can be dependent on different optimal pH values.

The acidity of both medium and substrate has great importance in the bioprocess, not only because the pH partly gives a right habitat for the microorganism but also because partly screens the microbial growth from a large number of competitors.

In fact the metabolic regulation routes, among other factors, may be controlled by pH (Fig.22) (Giovannozzi Sermanni et al.,1978).

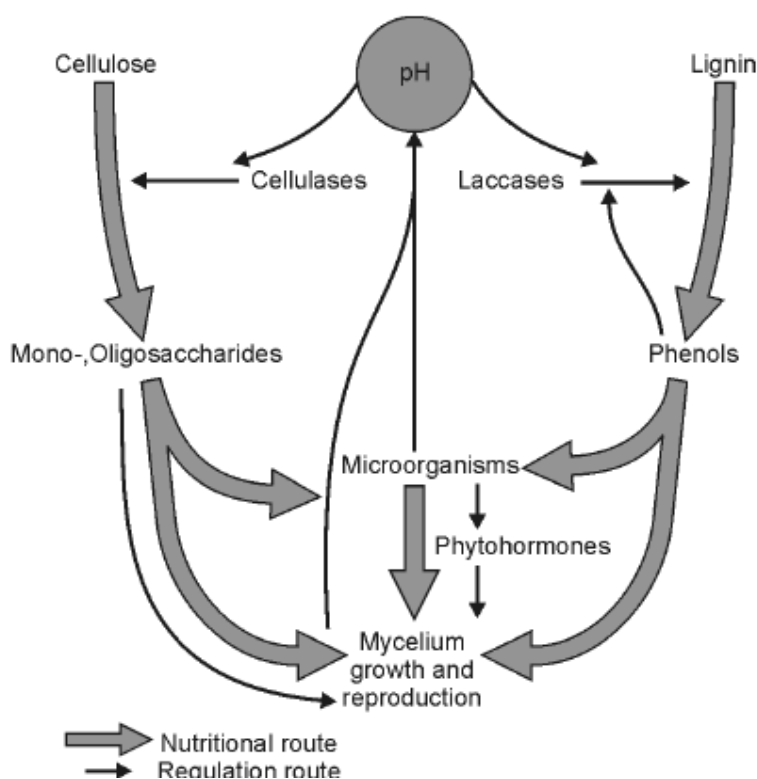


Fig.22 Influence of pH on the possible regulation of cellulose and lignin degradation by mycelial and microbial metabolism (pH stat) (redraw from Giovannozzi et al., 1978).

4.1.4. Aeration

It establishes the O_2 and CO_2 concentration inside the bioreactor, regulating the removal of CO_2 and volatile compounds (such as ethylene), the relative humidity, and it also can improve the heat transfer.

The major consideration in the design and scale up of bioreactors for submerged liquid fermentations is to guarantee a sufficiently high rate of O_2 transfer into the liquid medium. That is due to the fact that the rate limiting step for aerobic submerged liquid fermentation (SmF) is normally the O_2 transfer into the liquid phase.

In the SSF processes other parameters have a large influence especially heat and mass transfer. Overheating results often a major problem (Saucedo-Castaneda et al. 1992, Gowthaman et al. 1993), due to unidirectional flow of air in the case of packed beds. The air warms up and loses its cooling efficiency after its passage through the column and heat removal from the substrate. The temperature gradient established that way along the column cause an higher temperature at the outlet end of the bed.

In a rotating drum bioreactor the gas exchange is reached by circulating air through the biomass. In this way it is possible to maintain a constant chosen O_2 and CO_2 value as shown in the diagrams in fig.23, and the total fluxed air during the fermentation and the gas exchange can be recorded continuously to maintain the reproducibility of the process. For example after few days of fermentation the CO_2 and the O_2 levels can be modified and maintained at a chosen value.

Fung and Mitchell (1995) demonstrated that maximum O_2 uptake rates are higher in a baffled rotating drum than in an unbaffled one, so that O_2 limitation can occur in the unbaffled drum too (Marsh et al. 1998).

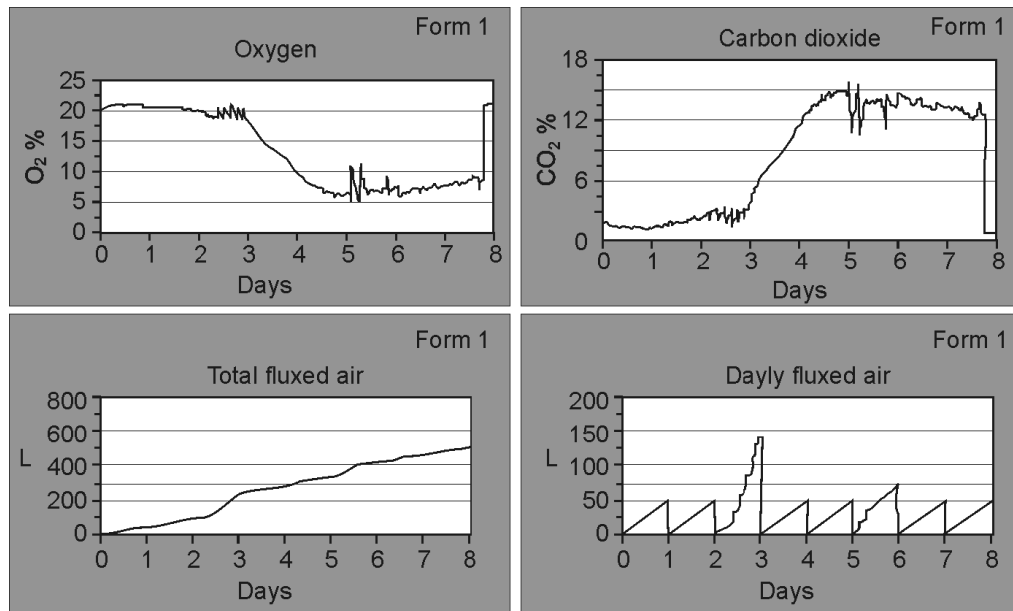


Fig.23 Control of O_2 and CO_2 levels in the pre-pilot rotating drum bioreactor by using fresh sterile air fluxes.

4.1.5. Nutrient diffusion

It affects the nutrient concentration and regulates the actions of enzymes over the solid substrate (normally water insoluble). Consequently it has a great influence on the microorganism growth rate and metabolic pathways.

4.1.6. Mixing

Mixing is a crucial parameter in SSF because it aids to obtain uniformity of the substrate and enhance heat removal and gas exchange. It also influences the process conditions and in particular the water content.

It can also influence the colonization rate of the substrate and it has to be managed carefully because many microorganism are very sensitive to mechanical stirring, particularly fungi for the eventual breaking of the hyphae.

4.1.7. Septic conditions

It is usually needed to avoid the release of the process organism in the environment and the entry of polluting microorganisms (it is dramatically hard to manage competition against other microorganisms).

4.1.8. Particle size (density)

It regulates the utilization of the substrate by the microorganism at a molecular level, the gas-liquid interfacial area and the thickness of the wet fungal layer. It makes the oxygen utilization dependent on this parameter (a too small particle size may cause interferences with microbial respiration, causing poor growth; a large particle size provides only a modest surface area limiting the microbial attack) (Pandey et al. 1999).

4.1.9. Physico-mechanical properties of the solid matrix

The matrix should have wide surface area and should stand gentle stirring or compression if required by the chosen fermentation to avoid sticking behaviour during the process.

4.1.10. Biomass uniformity

It is of fundamental importance to maintain uniform biochemical reactions all over the biomass.

4.1.11. Microbiological inoculum

Its type, amount, relative humidity, etc. influence deeply the growth rate.

4.1.12. Morphology of the microorganism

The most important characteristics are to penetrate into a solid substrate with a low water activity, assimilating complex and variable mixtures of nutrients, the capability of adherence and i.e. the presence or not of septum in the hyphae for mechanical resistance.

4.2. Materials and Methods

The experiments to investigate the bioprocess were performed partly at dep. ABAC of Tuscia University (Viterbo), partly at I.B.A.F. of C.N.R. (Montelibretti, Roma) and partly at Metapontum Agrobios s.r.l. (Metaponto, Matera).

Some of the equipment used are shown in fig.24.

The medium size experiments on liquid were performed by 2.5 l and 25 l fermenters.

The study of the solid state fermentations was performed mainly using an automated rolling bottle bioreactor device (shown in fig. 24).



Fig.24 2.5 l laboratory scale liquid fermenter (left); 25 l liquid fermenter (middle) laboratory scale SSF fermenter (right).

The large scale experiments were performed by using a 4 m³ pre-pilot scale rotating drum bioreactor (fig. 25-26). It is equipped by an external cooling-heating jacket, a CO₂/O₂ control device and an inoculation system device.

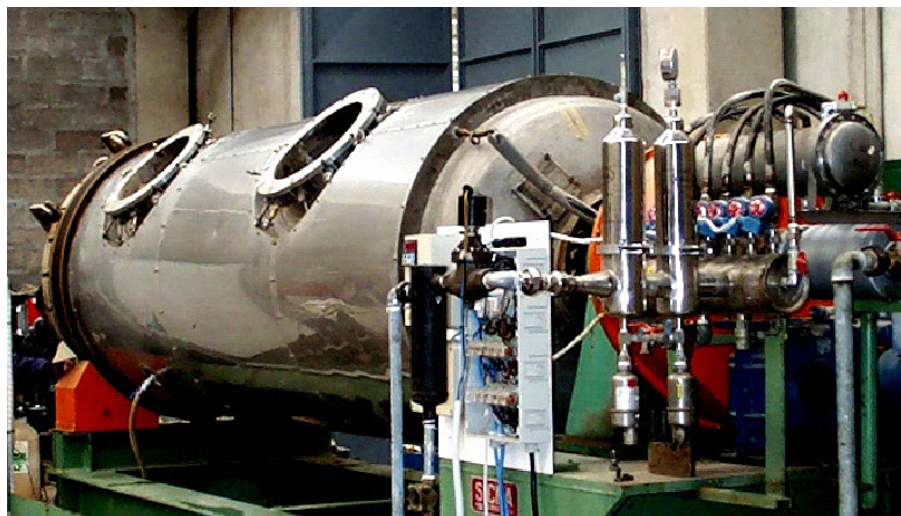


Fig.25 Pre-pilot SSF rotating drum bioreactor.

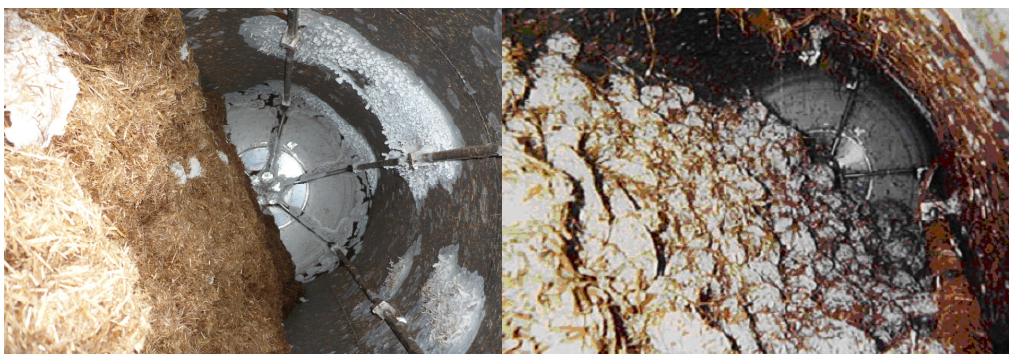


Fig.26 Fermentation of wheat straw into the pre-pilot unit during the colonization of the substrate (left) and at the end of the fermentation process before unloading of the vessel (right).

The experimental data collected were used to study the effect of parameters on the process and to evaluate corrections to the pilot plant design and operations.

The influence of various additives on growth rate, enzyme production, stability and reproducibility of the product was also investigated.

5. APPLICATIONS

5.1. General tasks

As natural catalysts, enzymes possess extremely various biological, catalytic, and physicochemical properties such as reactivity, specificity. Many of their characteristics are highly perspective for many industrial applications. Nowadays, enzymes are being largely and systematically developed, as environment-friendly catalysts for industrial economic purposes (Aehle 2004, Burk 2003, Bommarius & Riebel 2004). Currently the market slice devoted to industrial enzyme involves around two billions of US dollars per year, with more than 500 products for more than 50 major applications 5,6. However, at present time they hold an exiguous but quickly growing part in the whole specialty chemicals market in particular for biotechnological features.

Based on application, ~65% of the commercial enzymes are technical enzymes (for applications such as textile or detergent), ~25% are food enzymes, and ~10% are feed enzymes (Xu 2005). In the next few years it presumed that enzymes will increase considerably their occurrence in the market.

As catalysts for industrial applications, they may have considerable advantages over chemical- or living cell-based catalysts (Ahuja et al. 2004, Xu 1999, Van Beilen et al. 2003).

The largest part of the market slice occupied by the industrial enzymes is made of hydrolases (including proteases, carbohydrases, and esterases). Only a few Oxidoreductases are at present commercialized in industrial market occupying very small niches for limited applications in contrast to their wide presence of in nature (May 1999, Kirk et al. 2002). That generates the possibility to enlarge their commercial occurrence by developing new future applications based on oxidoreductase biocatalytic properties.

The products based on oxidoreductases may be grouped into some main application fields as shown in fig.27 :

- specialty chemical synthesis.
- industrial-technical.
- food and feed.
- environmental.
- medicals and personal care fields.
- biosensors.

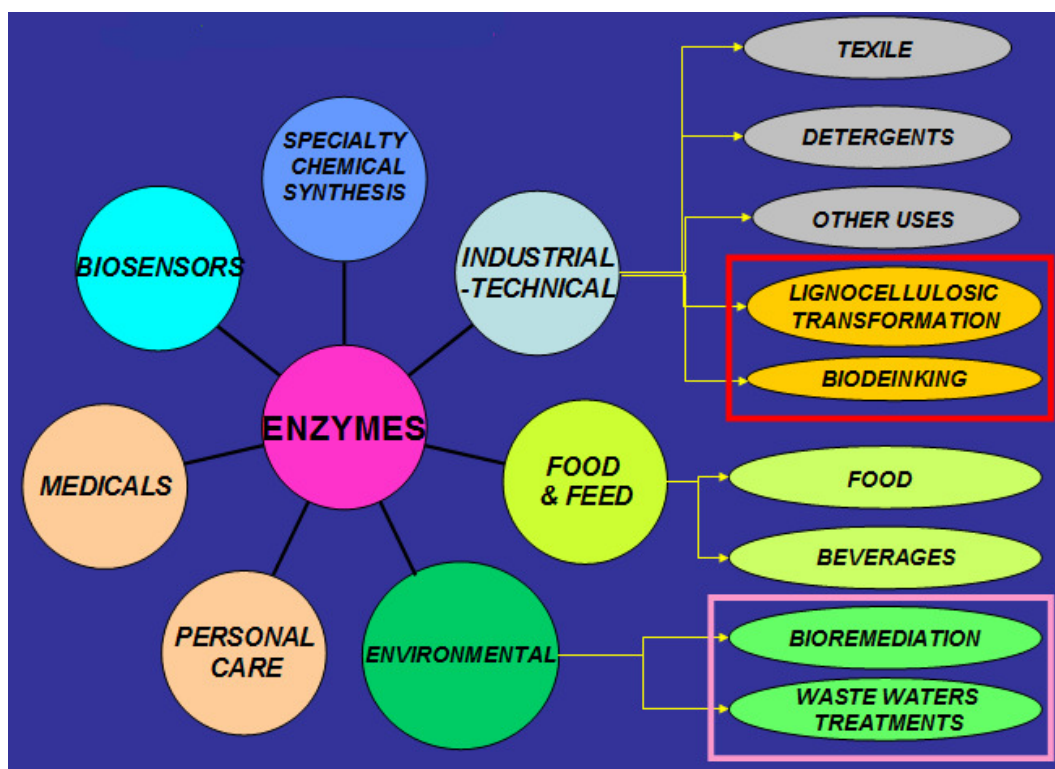


Fig.27 Main fields of application for oxidoreductases.

Being highly specific, biodegradable, energy saving, the reactions based upon oxidoreductases find industrial application for environmentally-friendly, high efficient, sustainable developments.

Many expensive a/o hazardous chemicals can be profitably replaced by oxidoreductases to enhance energy saving and reduce environmental impacts as well.

Laccases (EC 1.10.3.2) are polyphenoloxidases which reduce oxygen while oxidizing organic substrates by a one-electron redox process. Laccases are mainly produced by filamentous fungi (ascomycetes and mainly wood-decaying basidiomycetes) and they are involved in several biological processes such as lignin degradation (Eggert et al. 1997), morphogenesis and pathogenesis (Mayer & Staples 2002). In addition to lignocellulosic substrates, an extensive list of xenobiotics (pesticides, polycyclic aromatic hydrocarbons derivatives, dyes, etc.) often containing phenolic or aromatic amino groups are transformed by laccases (Gianfreda et al. 1999, Jolivald et al. 1999) either by bond cleavage or by oxidative coupling. When the oxidation of non-phenolic substrates is carried out by laccase, the presence of natural oxidizable low-molecular weight compounds (laccase mediator system), some of which are produced by the

mycelium, is requested. Because of the great variety of reactions which this enzyme catalyses, it has attracted considerable interest in various fields of research, e.g. development of oxygen cathodes in biofuel cells, green biodegradation of xenobiotics, biosensors, organic synthesis, labelling immunoassays (Madzak et al. 2006, Mayer and Staples 2002, Kunamneni 2008).

5.1.1. Specialty chemical syntheses

Oxidoreductase-based biocatalysts show high potentiality for efficient, asymmetric synthesis applications (Liese et al 2001, Hummel 1999, Roberts 2004). This is mainly because of their wide versatility, high specificity, high stereo-selectivity and for their environmentally friendly character. They do not demand for particularly stringent/extreme conditions, making them an interesting option for organic or medicinal syntheses.

Oxidoreductases are able to modify a broad number of aliphatic/aromatic molecules; transform inert hydrocarbons (by hydroxylation, sulfoxidation, epoxidation, etc.) by the introduction of functionalities. They are able to carry out various chemo-selective reactions and build interesting synthons from renewable and cheap biomaterials with extremely low environment impact.

Nevertheless currently only few oxidoreductases have been commercialized in that field. However applying oxidoreductases may lead to new industrial synthetic methods in the next few decades.

In example laccase can be used to synthesize several complex medicinal agents including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, penicillin X dimer, cephalosporin antibiotics, and dimerized vindoline.

Laccase can also be used to synthesize various functional organic compounds including polymers with specific chemical/physical properties, dyes, pigments, flavour agents, and pesticides.

5.1.2. Utilization of lignocelluloses

Conventional delignification of lignocellulose in paper industry uses either chlorine- or oxygen-based chemical oxidants, eventually causing loss of structure of the fibres. The use of laccase, naturally capable to degrade lignin, might solve such drawback and also simplify waste treatments.

Based on its lignin-oxidation property, laccase could be used as a bioadhesive to replace hazardous chemicals (urea, chemical resins, etc.) by starting the cross-linking and enhancing its efficiency.

By the use of laccase for that kind of application wastes such as lignin from paper industry can be converted into value-added products.

5.1.3. Textile applications

Oxidoreductases find many potential applications for the textile industry including cotton fibre whitening, dye finishing, waste treatment and recently biostoning (Tzanov et al. 2003, Zhu et al. 2005).

Potential benefits of the application of oxidoreductases in the textile industry include chemical, energy, and water saving.

Laccase is currently commercialized as alternative to the conventional chemical oxidants (ipochlorite, etc.) for dye-bleaching processes. It can increase the efficiency of dye finishing processes as well. Laccase can also enhance dye fixing onto fibres minimizing the use of chemical fixing agents.

5.1.4. Detergent applications

Laccase, peroxidase, and other enzymes have been studied for potential cleansing applications in normal washing conditions, such as cloth- and dishwashing (Galante & Formantici 2003).

Oxidoreductase may also be included in a cleansing formulation to eliminate the odor on fabrics or generated during cloth washing (Hiramoto & Abe 2004).

5.1.5. Feed, food and beverages

It is easy to find carbohydrates, unsaturated fatty acids, phenolic compounds, and thiol-containing proteins as important components of many foods and beverages.

Oxidoreductases may modify them guaranteeing quality improvement, or cost reduction (Minussi et al. 2002). Oxidases may be used as O₂-scavengers to improve food packaging and reduce the detrimental effect of O₂ to the quality of food/beverage (Andersson et al. 2002). In fact by their utilization it is also possible to reduce the eventual O₂ negative effect onto the quality or the storage of food/beverage because of unwanted oxidation.

These enzymes are also able to alter flour components either directly or indirectly through produced active oxygen species.

Laccase may be used to increase or alter the colour appearance of food or beverage.

During the industrial processing or storage of beverages (i.e. fruit juice or fermented alcohol beverages) can occur some major undesired chemical or physical modifications like browning, haze formation, and turbidity development.

Phenolic compounds are supposed to be involved in such processes. So they are normally adsorbed and removed by various common but usually low specific fining agents. That procedure may affect colour or aroma. The utilization of laccase and other oxidases is predictable to replace such fining agents in order to remove or eventually modify phenolic compounds. Their use may alter positively fruit juice, beer or wine characteristics (e.g. clarity, colour appearance, aroma, flavour, taste, stability) (Descenzo & Irelan 2003).

Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents. Laccases are also used to improve storage life of beer. The reduction of odours with laccase is documented in the patent literature (Tsuchiya 2000).

As example of laccase potentiality we report the recent commercialization of one kind of its to treat cork stoppers for wine bottles (Conrad et al. 2000). Laccase oxidatively reduces the characteristic cork taint/astringency which frequently occurs in the bottled wine.

5.1.6. Biosensors and bioreporters

Many oxidoreductases have co-substrates that are either chemiluminescent, fluorogenic, chromogenic, or electroactive. They may generate compounds that are reactants/substrates for coupled chemical or enzymatic reactions usable for optic, electric, or other measurements. For that reason many oxidoreductases can be applied as biosensors or bioreporters.

A discrete number of examples of the oxidases include (i.e. laccase, horseradish peroxidase, and glucose oxidase) can be found in literature for application in that field.

As example laccase catalysis, coupled with various physical transducers, could be suitable as biosensors for detecting O₂ and a wide variety of reducing substrates, particularly phenols or anilines.

5.1.7. *Disinfection and pharmaceutical-medical applications*

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their bio-based nature and specificity, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc.

One potential application is laccase-based *in situ* generation of iodine, a reagent widely used as disinfectant.

The system may be used in various industrial, medical, domestic, and personal-care applications such as sterilization of drinking water and swimming pools, as well as disinfection of minor wounds.

Urushiol, a catechol derived toxin can cause poison ivy dermatitis. Oxidized urushiol (an o-quinone derivative), however, is nontoxic. Laccase has been shown to oxidize, polymerize, and detoxify urushiol, thus reducing the effect of poison ivy dermatitis.

5.1.8. *Application in cosmetics, hygiene and personal care*

A laccase-based system may overcome the drawbacks (malodor, eventual irritation, or uneasy handling) of current hair-dyeing or waving processes by replacing harsh chemicals and operating at milder conditions (in terms of pH and solvent). Laccase-catalyzed oxidation, transformation, and cross-linking of various precursors (mostly phenols and anilines) have been reported to result in satisfactory hair dyeing or waving. In addition to providing an easier-to-handle hair-care procedure, a laccase-based system may also improve or complement the cosmetic effect achieved by conventional chemical methods.

Laccases may find interesting uses as deodorants for personal-hygiene products, including mouthwash, toothpaste, detergent, soap, and chewing gum.

A large number of current or possible applications of oxidoreductases have great economical potential.

As catalysts for synthesis, oxidoreductases may have some advantages over other enzymes (e.g., hydrolases, lyases, or transferases). Oxidoreductases may show higher stereoselectivity, be able to act on substances (such as hydrocarbons) that are often inert to other enzymes, and use bio-based/renewable starting materials.

However, many oxidoreductases suffer from the eventual need for cofactors and lower specific activity.

The technical and food application fields, as well as the emerging synthesis and biodegradation fields, should grow sensibly for oxidoreductases due to performance improvement and cost reduction (Holland & Weber 2000, Yoshida et al. 2003, Jaeger 2004, Zhao & van der Donk 2003). In the next future, the development of bio-enhanced energy sources (i.e. bio-fuel cell), high-value bio-based materials (i.e. polymers, adhesives), or protein pharmaceuticals based on oxidoreductases might increase their market request.

On our opinion, the most interesting environmental fields for further application are:

- paper mill application (i.e. annual plant paper production, waste paper betterment, biodeinking).
- Biodecontamination and biodecontamination of soils, manure and waste waters.

5.1.9. Paper industry applications

Nowadays paper production and manufacturing represents an industrial sector of major importance. Its relevance is due to the wide utilization of paper in numerous fields of life and its continuous increase.

Many different environmental questions are deeply linked to that industry and they have indisputable relevance.

We have concerned, as case studies, about the possibility to transfer the production of biological papers from annual plants to industrial scale and the biodeinking of wastepapers.

5.3. Production of biological paper from annual plants

Natural fibres used to produce paper derive mostly from wood, but they can be obtained also from annual plants such as wheat or rice straw, kenaf, flax, hemp, etc.

The industrial utilization of those plant was quite abandoned in the recent past due to the waste waters management problems and their seasonal character. Besides at present it rouses new interest if a biochemical and environmentally-friendly approach is adopted.

Such biological approach, if carefully managed, reduces considerably the polluting charge of waste waters and the consumption of energy and chemicals normally used during the productive process. The physico-mechanical properties of papers made in

that way are significantly enhanced as previously demonstrated by many studies at laboratory scale (Giovannozzi et al. 1994; Giovannozzi et al. 1990).

The use of the just mentioned method grants a quality betterment that can be resumed as following:

Burst index increment: up to +50%.

Tensile index increment: up to +36%.

Tearing index decrement: down to -16%.

Gurley porosity index increment: up to +20%

Schopper-Riegler number: +8°

Considering that annual plants for their seasonal growing character are able to reduce up to 20% the utilization of celluloses obtained from trees, the importance of developing new technologies for the production of biological paper from annual plants results evident.

5.3.1. Laboratory equipments

The paper production was firstly performed on laboratory scale using some equipments; the most important of them are the boiler (Lorentzen & Wettre) for cellulosic paper pulp preparing, the Hollander Valley refiner, the disks refiner (Bauer), the laboratory scale pulper (Toniolo) and the standard Rapid Koethen paper sheets preparation equipment (Toniolo).

The standard paper sheets were tested each time by a burst index tester (Toniolo), an Elmendorf dynamometer for tensile index, an equipment to determine the pulp Schopper-Riegler grade (Toniolo) and few other equipments of less relevance.

5.3.2. Trials and results

In order to verify the transferability at industrial-market level of such approach, a small paper mill (12 ton/day paper production) was individuated. This manufacturing plant was small enough to consent tests and trial.

This paper mill unlikely is devoted to waste paper recycling, so the pulp production section is quite absent and some other equipments are required. Moreover the plant was unable to supply the right process conditions (in particular a sufficiently high temperature).

So after a few attempts to use anyway the equipments and conditions granted by the plant, we determined the impossibility to reach good results unless a few addictions and modifications to the plant were done or an other paper mill with the needed equipments was found.

Nevertheless a few trials reproducing a complete operating paper mill behaviour shown the concrete possibility to transfer the process to large scale.

5.4. Biodeinking of wastepaper

An important slice of the paper market is held by the recycled paper manufacturing. Environmental concerns and diminishing landfill space have created a growing demand for recycled paper on the world market. Deinked wastepaper can be recycled into newsprint, high quality white papers, tissues and paper towels.

The value and applicability of paper obtained from waste is extremely poor, that leads to the enhance of its quality as a major goal for the market.

The most effective treatment to reach this purpose is the deinking of the wastepaper pulp, usually obtained from wasted newspapers and magazines.

Deinking wastepaper, or secondary fiber, is essentially a laundering and bleaching process. Chemicals, heat and mechanical energy combine to dislodge oil-based printing inks from the paper fibers and disperse them in an aqueous medium. Ink and other contaminant particles are then separated from the stock by washing, flotation, dispersion or a combination of the three.

As shown in fig.28 a typical deinking operation consists of three main steps:

- Repulping
- Ink removal
- Postbleaching

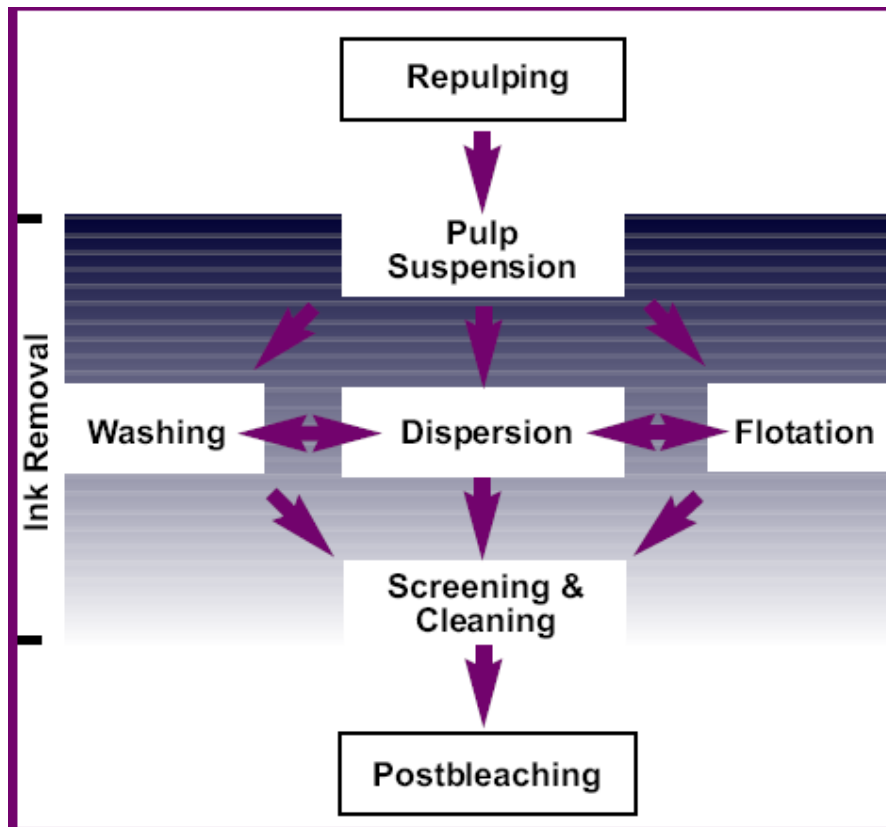


Fig.28 Typical deinking operation.

5.4.1. Repulping

Paper is mechanically broken down into fibers in the repulping step. Agitation and the combined action of sodium silicate, soaps, alkalis, surfactants and other deinking chemicals lift and remove ink and other contaminants from fiber surfaces.

During repulping, sodium silicate contributes detergency. That is, it functions as a dispersant and contributes buffering, emulsification and anti-redeposition properties. Sodium silicate keeps inks and other particles suspended in solution, rather than allowing them to redeposit on the fibers, where even trace amounts will discolor recycled stock. The suspended particles can be removed later by washing or flotation.

Hydrogen Peroxide is often used to reverse the effect of alkali darkening on pulp brightness. In deinking, as well as in peroxide bleaching of virgin mechanical fibers, optimum alkalinity is a function of both peroxide and sodium silicate addition.

Hydrogen and sodium peroxides improve brightness and aid in the dispersion of inks, coatings and sizing agents.

5.4.2. Washing

One of the most used deinking process is washing (fig.29). During such procedure the pulp is washed several times, then it is moved into a cylindrical condenser and finally mechanically removed. The operation is often repeated several times.

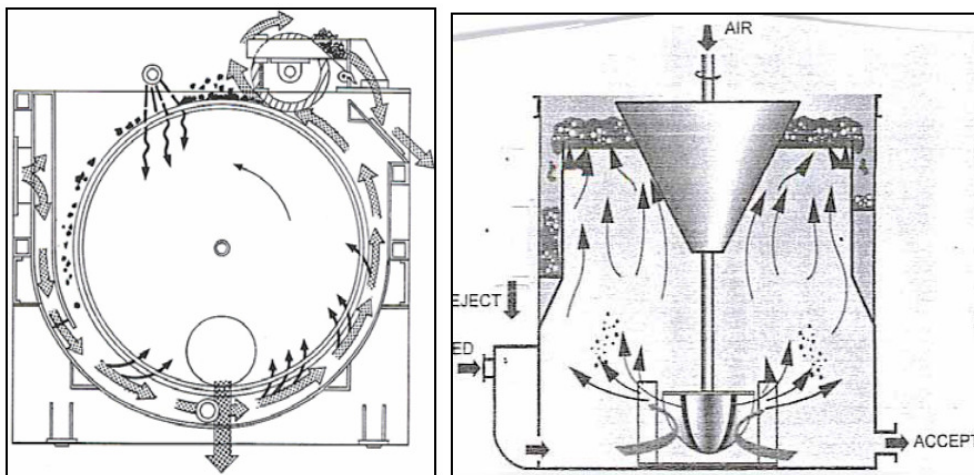


Fig.29 Deinking process schematic by washing (left) and flotation (right).

5.4.3. Flotation

Flotation is a chemical-mechanical process that selectively removes ink particles from a dilute suspension of pulp (fig.29). The pulp is aerated in the presence of sodic soap to form a froth. Ink and other particles attach to the microscopic air bubbles in the froth (fig.30).

Finally the froth containing the ink particles collected by the air bubbles is mechanically removed.

Ink removal by flotation is often superior to that of washing. The total number of ink particles remaining after flotation deinking is typically half that after washing deinking.

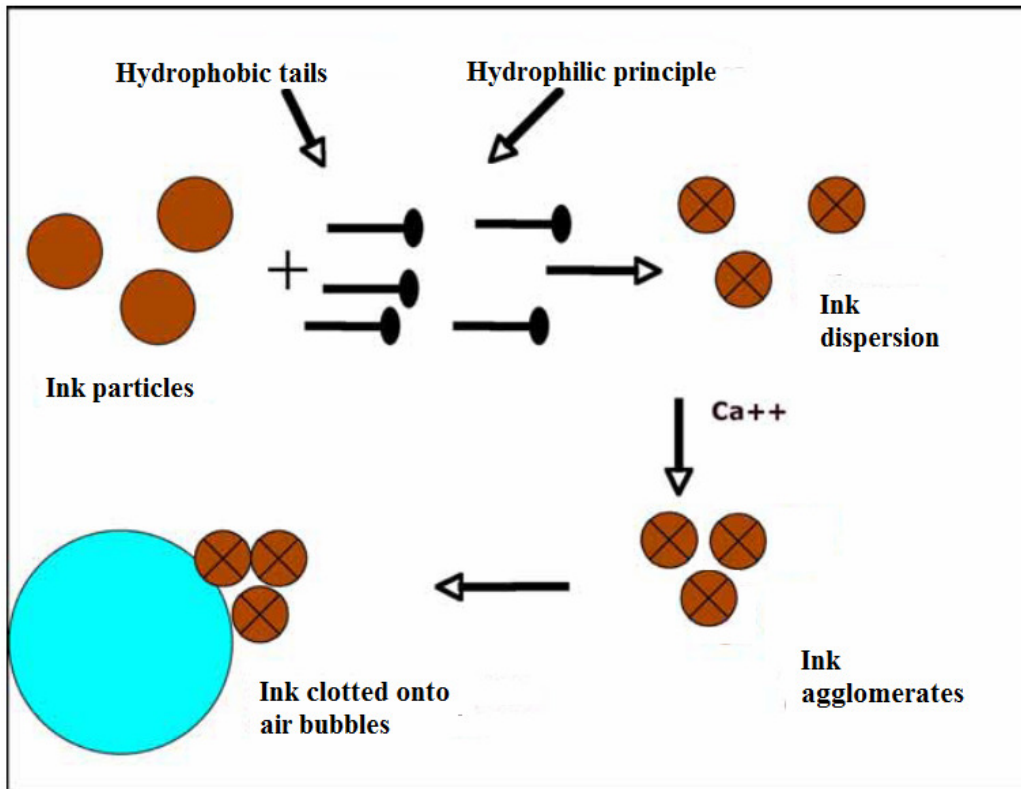


Fig.30 Ink particles are separated from fibrous material during flotation process.

5.4.4. Postbleaching

Some recycling plants add a postbleaching or brightening step to compensate for lack of brightness development during the repulping and ink removal stages. The postbleach can be either oxidative (peroxide) or reductive (hydrosulfite).

Typically few chemicals are required to perform the repulping and the deinking operations. Their amount is variable as well as the characteristics of the wastepaper to be treated as shown in table 6.

However, considering the huge amounts of wastepaper annually treated in this way, it is simply to understand the importance of an eventual reduction of these chemicals for environmental safety or economical growing purposes. On the other hand an higher quality of the recycled paper obtained by using not larger quantities of chemicals will obviously lead to similar interest.

	WASHING	FLOTATION
NaOH	1-1.5%	1-1.5%
Tensioactive	0.3%	0-0.1%
Sodium Silicates	1-1.5%	1-1.5%
Hydrogen Peroxide	0.7-0.9%	0.7-0.9%
Complexing compounds	0.2%	0.2%
Fat Acids		0.4-0.6%

TABLE.6 *Typical % of chemicals used for deinking.*

5.4.5. Materials and methods

In addition to the equipment described in the previous paragraph 5.3.1 a laboratory scale FORMAX Flotation DeInk Cell (28l) has been used (fig.31).



Fig.31 *Laboratory scale flotation de-inking cell during operation (left) and standard Rapid Koethen paper sheets preparation equipment (right).*

Three samples were prepared as following: 8.4 kg of newspaper were pulped for 1h, subsequently split in 3 quotes of 2.8 kg each.

The first was added by 100 ml of concentrated enzymatic crude extract from *lentinula edodes* strain SC495 in order to reach 3 IU/g of dry pulp and a 100 ml quote of liquid mycelium of the same fungal strain. The second one was added by 100 ml of the extract just described and 100 ml of water. The third one, used as control sample, was added by 200 ml of water.

After 24h any sample was put into the deink cell and added by water till reaching the maximum volume of 28l. The pulp (10% of consistence) was deinked for ½ h once the additives (at the maximum rate reported in table 6) were put into the cell.

The deinked pulped was then used to prepare 3 standard 80g paper sheets.

5.4.6. Results

The paper sheets obtained in this way were tested. The burst index and tensile index measured didn't show significant different among the 3 samples.

Instead the appearance of the paper sheets was consistently different.

The paper sheets obtained from the pulp enzymatic treatment shown a lower number of resilient ink spots per cm² and they resulted of a well evident higher whiteness if compared to the control as shown in fig.32.

Instead the paper sheets obtained from the pulp treated by mycelium shown an higher whiteness if compared to the control, but they shown also a significant number of large brown spots, probably due to dry mycelium particles entrapped into the paper matrix and browned during the paper drying process.

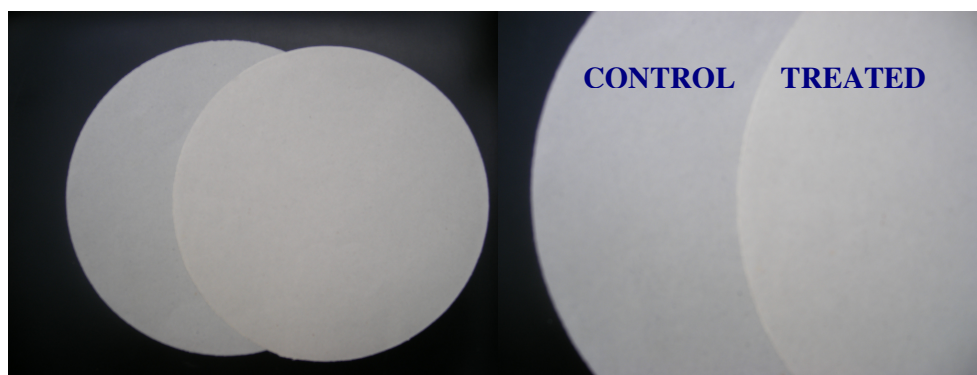


Fig.32 Standard paper sheets obtained from newspaper waste pulp: control and enzymatic treated before deinking process.

5.5. Degradation of antibiotics

Veterinary antibiotics are broadly utilized in intensive farming to treat diseases protect animal health, and enhance growth rate (De Liguoro et al. 2003). One of the most commonly and widely used in pig farming is Oxytetracycline. It has been demonstrated to partly persist in animal faeces, due to scarce intestinal absorption (Blackwell et al. 2004, Brambilla et al. 2007). Although a significant reduction over time is noticed in manure and bedding, if used as a soil amendant before maturation time (at least 5 months), they can still contain quantities greater than the disposal limits of authority (CVMP/VICH/52/98). Oxytetracycline persistence is due to a strong bond with organic matter that makes it difficult to remove a/o degrade from the top layer soil, where it is

normally located. Environmental occurring OTC is often in equilibrium with the isomer epi-oxytetracycline.

Its presence can cause severe environmental problems and may induce ecosystem alterations, due to due to eventual modification of soil microbial resistance and composition (Migliore et al. 2009).

If OTC is present in soil, as shown by Thiele-Bruhn and Beck (2005), fungi dominated the microbial biomass while the number of bacteria was reduced. The changes in soil microbial community unluckily stop once started. These changes are able to persist for long periods, probably due to the effect of the metabolites generated.

White-rot fungi and their enzymatic activities are capable to degrade a wide number of persistent organic pollutants dangerous for the environment.

The direct use of enzymes in practical application seems to be a preferable approach than the use of fungal mycelium, thus excluding the problems connected to fungal growth.

For these reasons we decided to investigate the possibility to degrade these compounds in pig slurry and/or in soil to prevent or reduce hazardous effects on environment as a novel potential application of interest for the enzymes produced in the pilot plant.

It was demonstrated by experiments performed with *P. ostreatus* the possibility to degrade up to 95% the OTC present in a liquid medium after a few days (Galli et al. 2008b).

Then it appeared reasonable to try a degradation performed by direct use of fungal non-specific ligninolytic enzymes such as laccases, which are able to oxidize a large range of substrates by a non-specific free radical mechanisms (Xu et al. 1999).

A few experiments have been performed to demonstrate that such enzymes (obtained from white-rot fungi) can degrade Oxytetracycline (OTC) and its epimer (epi- OTC) (Fig. 33) in aqueous solution and to explore the possibility of applying this approach to the treatment of pig slurry (Donati et al.).

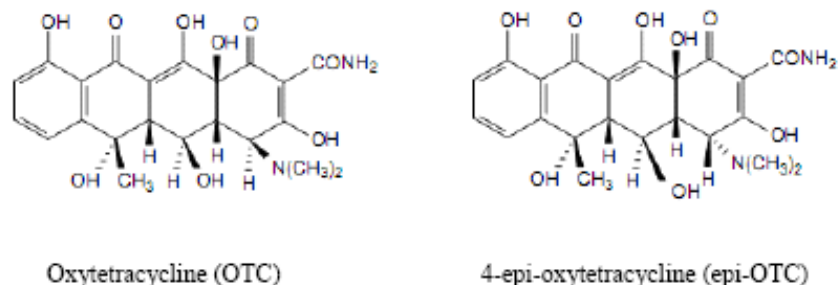


Fig.33 *Oxytetracycline (OTC) and 4-epi-oxytetracycline (epi-OTC) structural formulas.*

5.5.1. Materials

Oxytetracycline (OTC), Oxalic acid dihydrate, Methylenediamine tetraacetic acid disodium salt dihydrate and Terbutalin were obtained by Sigma. Epi-oxytetracycline (epi-OTC) was obtained by Acros organics (Geel, Belgium). HPLC grade Methyl alcohol and Acetonitrile and Acetic and Formic acid were purchased by Carlo Erba. HPLC grade Water was prepared with a Milli-Q system by Waters.

5.5.2. Enzymatic crude extract preparation

Mycelium of *Lentinula edodes*, previously grinded by omnimixer homogenizer, was inoculated (about 80 mg dry weight) in a flask containing 200 ml liquid culture (3% malt extract), incubated at 25 °C and stirred at 95 rpm for 4 days. Then the total quantity was divided into four flasks with 450 ml of liquid medium containing 50 g/l D(+)-glucose and 5 g/l yeast extract. The flasks were incubated at 25 °C and stirred at 95 rpm. After 3 days of incubation, 10 ml of a CuSO₄ 0.5 mM water solution have been added in each flask to enhance laccase production.

Laccase and Mn dependent Peroxidase activities have been tested daily by UV spectrometric assay at 525 nm (Galli et al., 2008a). At 10th day of incubation the four solutions were put together, filtered and the crude extract treated with ammonium sulphate (30%) at 0 °C, in order to obtain a solid precipitate, and centrifuged at 10,000 g for 15 min. Supernatant was treated with ammonium sulphate (85%) and centrifuged at 13,000 g for 15 min with a Du Pont Sorvall® RC 5B plus Centrifuge. The solid stocks obtained were dissolved in a 8 ml sodium acetate-acetic acid buffer solution (0.2 M, pH 4.5).

5.5.3. Standard solutions

OTC and epi-OTC stock solutions were prepared in methanol (500 g ml⁻¹) and stored under dark at -18 °C. Terbutaline (internal standard) stock solution was prepared in water (23 g ml⁻¹) and stored at 4 °C.

5.5.4. Sample preparation

The crude enzyme extract (1 ml) and OTC stock solution (1 ml) were added to a 18 ml of sodium acetate-acetic acid buffer solution (0.2 M, pH 4.5). The same procedure was followed in the case of epi-OTC samples. At the same time corresponding samples of the OTCs in 19 ml buffer solution without any enzymatic addition were prepared as control. All experiments were carried out under dark at 25 °C in a rotatory shaker.

Experiments were performed twice and each sample was in triplicate.

5.5.5. Analysis

Samples treated with enzymatic extracts and controls were analyzed by Capillary Zone Electrophoresis (CZE) and HPLC. Each sample was analysed thrice. Before analysing they were purified by solid phase extraction (SPE) on SDB (200 mg) Bakerbond SPE™ columns, sequentially preconditioned by 5 ml of MeOH and 5 ml of a Na₂EDTA solution (2 g L⁻¹). 1 ml of sample was eluted through the cartridge and washed by 5 ml of water, dried and eluted with 4 ml of MeOH:ACN:oxalic acid 0.01 M (30:30:40) solution (Samanidou et al. 2007). The eluate was concentrated with rotavapor and then 100 l of terbutaline aqueous solution (0.023 mg ml⁻¹) was added as internal standard. The obtained solution was diluted up to 1 ml with water and then injected in the CE or HPLC instrument.

Standard samples for the calibration graph were prepared in 0.016 M oxalic acid in the range 3.5-27 g ml⁻¹ ($R_{\text{OTC}} = 0.9999$; $R_{\text{epi-OTC}} = 0.9989$).

CZE analysis was performed by a Hewlett Packard 3DCE instrument (Agilent Technologies, USA) equipped with a diode array detector. Data were acquired and stored by a ChemStation software. Separation were carried out in an uncoated fusedsilica capillary (total length 33 cm, effective length 24.5 cm, 75 µm id) purchased from Composite Metal Services (Hallow, Worcestershire, UK). The analyses were carried out using 25 mM phosphoric acid adjusted at pH 2.5 with NaOH 1M, as running buffer.

Every morning the capillary was rinsed with NaOH 1M for 5 min, NaOH 0.1M and water for 15 min and then running buffer for 10 min. Between analyses the capillary was washed by flushing with water, NaOH 1M, NaOH 0.1M , water and buffer, each washing step lasting for 5 min.

The sample was introduced in the capillary by hydrodynamic injection at a pressure of 50 mbar for 5 sec; the capillary temperature was 20 °C and the applied voltage was 15 kV. The detector was set at 205 nm.

HPLC analysis was performed on a C18 Synergy Fusion-RP80A (Phenomenex) 150x4.6 mm, 4 μ m) column eluted with a gradient of CH₃OH-0.03% formic acid (solvent B) in water-0.03% formic acid (solvent A), starting from 10% (isocratic elution for 10 min) and then reaching 90% of B in 30 min. HPLC apparatus was composed by a LabFlow 3000 HPLC solvent delivery system (LabService Analytica, Anzola Emilia, Italy) equipped with a Knauer solvent mixing chamber and a series 7125 Rheodyne valve as injector. Chromatographic elution was detected by a photodiode-array SPDM6A (Shimadzu, Kyoto, Japan) detector.

5.5.6. Enzymatic extract

The use of crude extracts offers some advantages such as its economical convenience and it is quickly becoming a usual practice in the environmental field to overcome the cost of purification treatments. Moreover the use of a mixture containing a highly concentrated specific enzyme being part of an enzymatic pool, often leads to synergic effects.

The mycelium was grown in the presence of CuSO₄ to rouse the production of laccase activity in the extracellular enzymatic system. The enzymatic activity of the culture medium was tested during the mycelium growth to select the best moment for the precipitation with ammonium sulphate.

Laccase activity increased with time and reached a maximum at 9th- 10th days as shown in fig.34 .

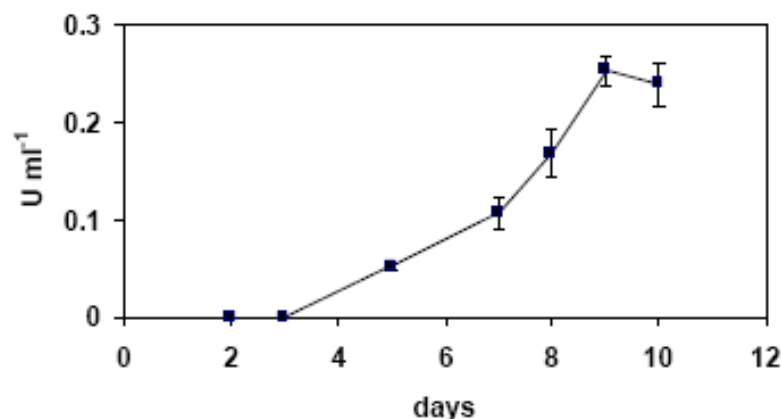


Fig.34 Laccase content in *Lentinula edodes* culture medium during mycelium growth.

Then the enzymatic crude extract was obtained containing a laccase activity of about 3.7 U ml⁻¹ after concentration with ammonium sulphate, from a starting average activity of about 0.25 U ml⁻¹ in the culture medium,. During the 10 day period of mycelium growth no significant Mn peroxidase activity was detected.

In a recently published study on the degradation of Tetracycline and OTC by lignin peroxidase, obtained from *Phanerochaete chrysosporium*, the authors observed a very rapid oxidation of both antibiotics, which however took place only when H₂O₂ and veratryl alcohol were added (Wen et al., 2009). Otherwise in that case the laccase catalyzed oxidation takes place directly, as both isomers of OTC have a phenolic group. The experiments have been carried out in acetate buffer at pH 4.5, because laccase is particularly effective at this pH.

5.5.7. Sample analysis

To verify the OTCs degradation in the reaction mixture containing the crude enzymatic extract, a specific analytical procedure was performed forced by the possible contamination of Cu⁺⁺ ions and the high protein sample content. Therefore several different methods described in the literature for the analysis of tetracyclines in environmental samples and in serum or animal tissues were considered (Anderson et al. 2005).

The samples were pre-cleaned by SPE on a styrene-DVB cartridge, as free silyanol groups in C18 stationary phase interact with the amino group of OTC, and eluted with a

mixture of methanol, ACN and oxalic acid, which is able to complex residual Cu^{++} ions if present (Himmelsbach and Buchberger 2005, Samanidou et al. 2007).

The extraction gave a 100% recovery both for OTC and epi-OTC. CZE with diode-array detection was considered the more suitable analytical technique as it allows the OTCs detection at their maximum absorbance, 205 nm.

Tetracyclines CZE analysis can be accomplished both in acidic or alkaline medium, but tetracyclines are not very stable at high pHs, so an acidic Back Ground Electrolyte (BGE) was chosen. As the oxalic acid lower constant is 1.25, it was assumed that an oxalate buffer was not appropriate for the CZE separation: in facts a BGE pH lower than 2.5 hinders syanols dissociation on the capillary wall and consequently the formation of the electro-osmotic flow. So a pH 2.5 phosphate buffer, which allowed a good separation of both OTC isomers, was chosen (Penido Maia et al. 2007).

As reported, OTC hydrolysis is pH and temperature dependent, while ionic strength does not appear to influence the reaction rate (Loftin et al. 2008). As literature data does not correspond completely with the operating conditions used in these set of experiments, the behaviour of OTC and epi-OTC in the buffer were further investigated.

The electropherograms obtained from the injection of a standards mixture (internal standard, Terbutaline, OTC, and epi-OTC) (a), and of OTC (Fig. 35b) and epi-OTC (Fig. 36b) samples dissolved in acetate buffer without enzymatic extract, and with enzymatic extract addition (Fig. 35c and Fig. 36c) after a 72h incubation are shown.

As it is possible to observe in fig. 35b and 36b, there is an interconversion of the two isomeric forms, which is more pronounced in the case of epi-OTC. After 72 h the 72% of the original epi-OTC is present, while, at the same time, the residual OTC is 88 %. As the 21.5% of epi-OTC is converted in OTC and the 8% of OTC is converted in epi-OTC (fig.37a and 38a), in both cases less then a 6% of hydrolysis takes place, even if degradation compounds are not detectable , as they are formed in a very low amount.

In the electropherograms of enzyme-treated antibiotics solutions (Fig.35c and 36c) a trace of the other isomeric form is present, which is however degraded by the enzyme.

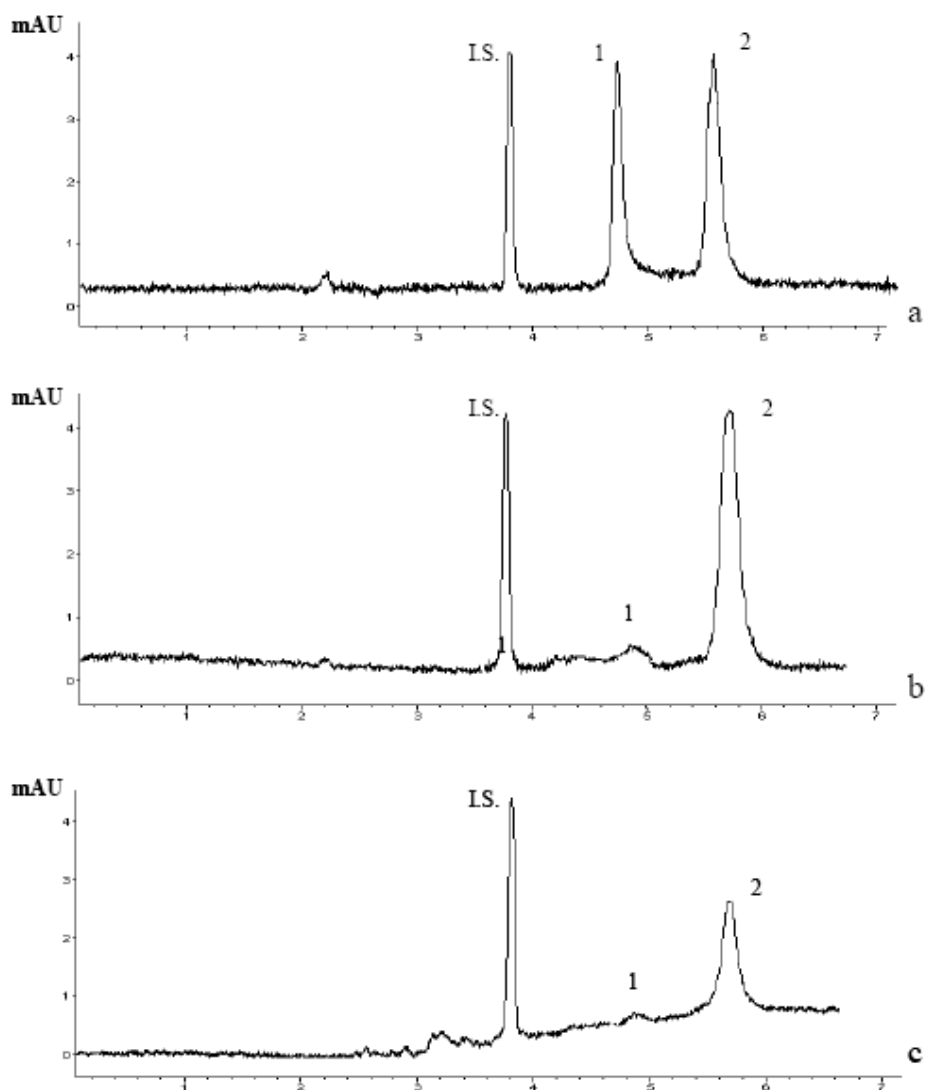


Fig.35 Electropherograms obtained from: a) standard mixture containing Terbutaline ($2.3 \mu\text{g mL}^{-1}$; i.s.= internal standard), epi- OTC ($12.5 \mu\text{g mL}^{-1}$; peak 1) and OTC ($12.5 \mu\text{g mL}^{-1}$; peak 2), in 16 mM oxalic acid; b) SPE purified fraction of a $25 \mu\text{g mL}^{-1}$ OTC solution in acetate buffer (pH 4.5) after 72h at 25°C , without enzymatic extract addition; c) SPE purified fraction of a $25 \mu\text{g mL}^{-1}$ OTC acetate buffer (pH 4.5) solution, incubated in the presence of 1 ml of enzymatic extract, after 72h at 25°C .

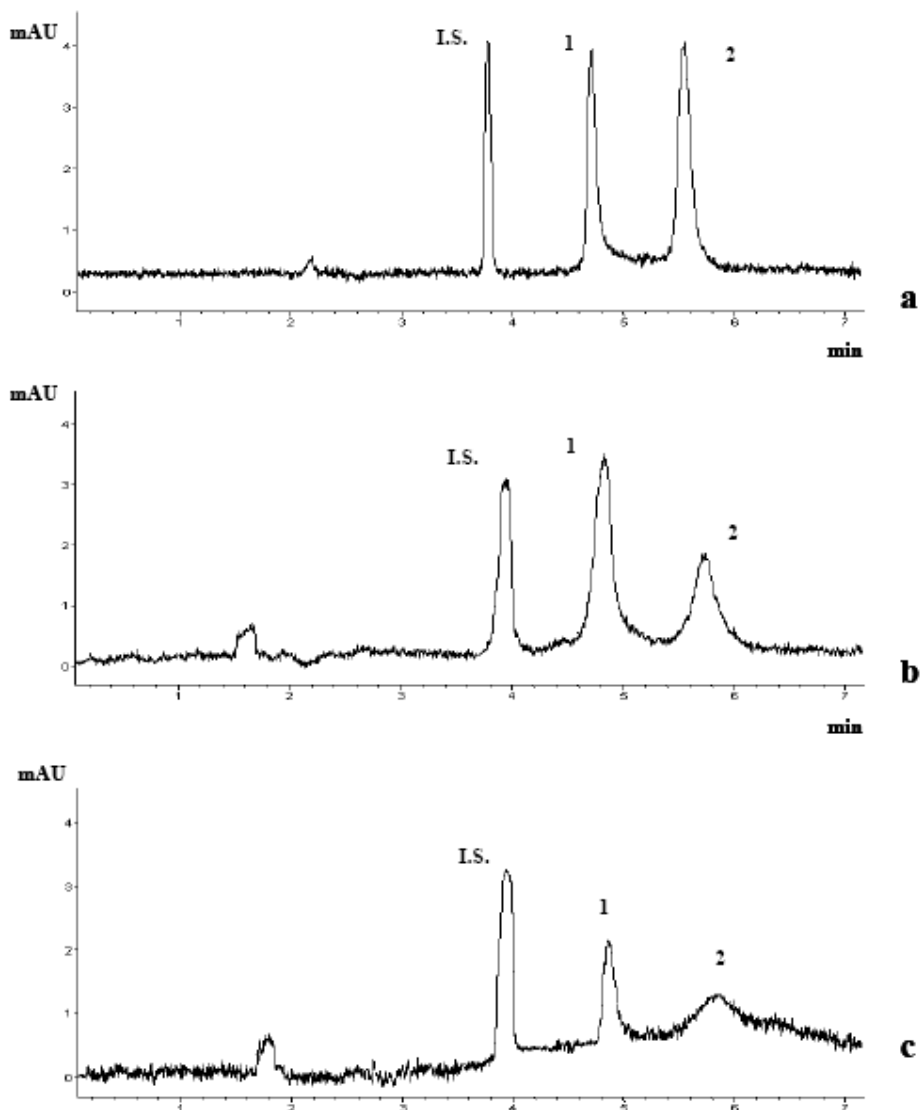


Fig.36 Electropherograms obtained from: a) standard mixture containing Terbutaline ($2.3 \mu\text{g mL}^{-1}$; i.s.= internal standard), epi- OTC ($12.5 \mu\text{g mL}^{-1}$; peak 1) and OTC ($12.5 \mu\text{g mL}^{-1}$; peak 2), in 16 mM oxalic acid; b) SPE purified fraction of a $25 \mu\text{g mL}^{-1}$ epi-OTC solution in acetate buffer (pH 4.5) after 72h at 25 °C, without enzymatic extract addition; c) SPE purified fraction of a $25 \mu\text{g mL}^{-1}$ epi-OTC acetate buffer (pH 4.5) solution, incubated in the presence of 1 ml of enzymatic extract, after 72h at 25°C.

In fact, the enzymatic degradation of the two isomers has a similar trend.

In Fig. 37b (OTC) and 38b (epi-OTC) the amount of reagent in solution versus time is reported: the two curves have a logarithmic shape, corresponding to a pseudo-first order

reaction. The degradation is more rapid in the first 24 hours (the half life times being 30 h), then it continues slowly up to 74-75 % in 72 h.

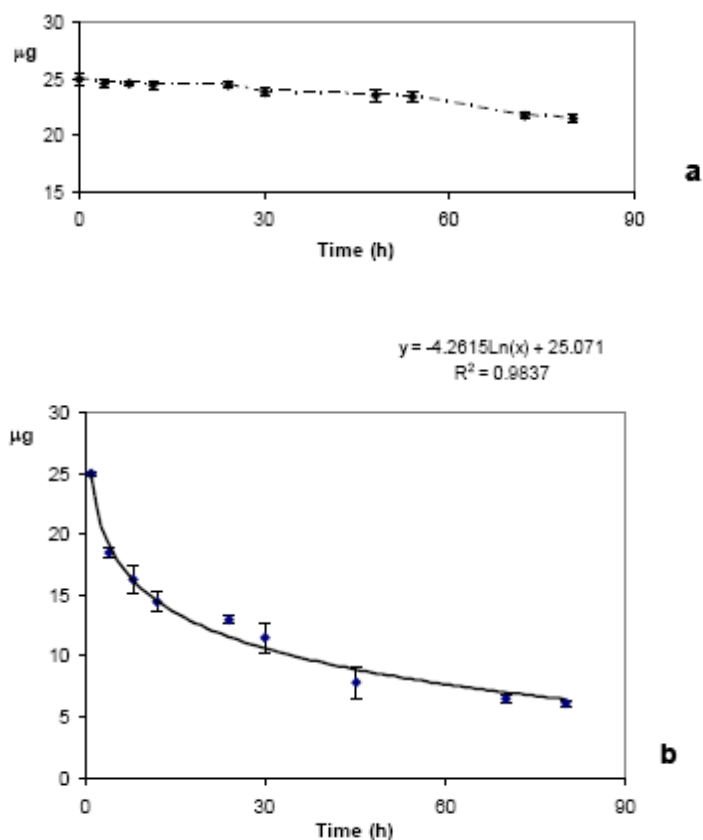


Fig.37 a) Concentration decrease of OTC versus time in a 25 $\mu\text{g mL}^{-1}$ OTC solution in acetate buffer (pH 4.5) at 25°C; b) Concentration decrease of OTC versus time of a 25 $\mu\text{g mL}^{-1}$ OTC acetate buffer (pH 4.5) solution, incubated in the presence of 1 ml of enzymatic extract at 25°C.

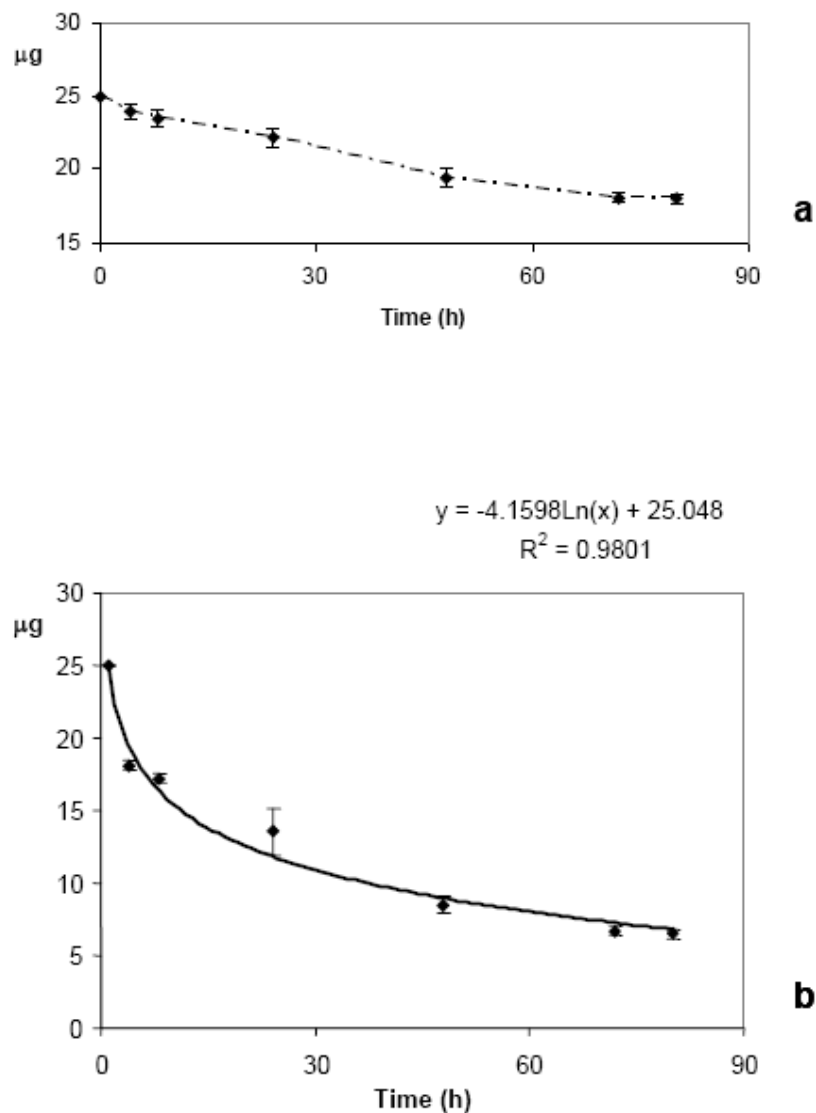


Fig.38 a) Concentration decrease of epi-OTC versus time in a 25 μg ml⁻¹ epi-OTC solution in acetate buffer (pH 4.5) at 25°C; b) Concentration decrease of epi-OTC versus time of a 25 μg ml⁻¹ epi-OTC acetate buffer (pH 4.5) solution, incubated in the presence of 1 ml of enzymatic extract at 25°C.

With the aim of checking the presence of eventually formed metabolites, α-apo- OTC, β-apo-OTC (Halling-Sørensen et al., 2003), the 24 h sample of enzymatically treated OTC was analyzed by HPLC.

The chromatographic analysis did not evidence the formation of other compounds, apart from epi-OTC, in detectable amounts.

On the basis of the exposed results some considerations can be done: as the degradation of the two isomeric OTC is very similar, probably the enzymatic attack begins from the phenolic ring, so the steric disposition of the C4 substituents is ineffective. Even if a more detailed study about metabolites formation at various stages of OTC and epi-OTC degradation would be necessary, the fact that no relevant peak, a part from the two isomers, has been detected both in CZE and HPLC analysis could mean that, after the phenolic ring cleavage the reaction continues quite rapidly. This consideration would be in accord with the well known activity of the laccase towards small aromatic amines and phenols, which could be formed during the OTC molecule degradation.

6. CONCLUSIONS

A large number of solid materials can be used for SSF such as soils, animal residues, hides, but without any doubt the most attractive solid state substrates for new processes are the lignocelluloses, present all over the Planet.

By keeping in mind that the amounts of agriculture residues can reach up to the 50% of totally produced biomass, it is obvious the paramount importance of the SSF for utilizing the huge amount of available chemical energy improving at the same time the biological sustainability.

The practical approach for the production of enzymes and other metabolites can be related to different areas (paper pulp production, paper deinking, paper recycling, agricultural residues utilization, pollutants biodegradation, olive and seeds oils residues, fuels, etc.) and each of them can require some different sets of biotechnological conditions.

As it results from the data of the scientific literature, in order to obtain profitable results it is necessary to develop specific plants and dedicated bioreactors which can take into account some particular characteristics of the new processes such as dissipation of heat due to the metabolic respiration and/or hyphen fragility if filamentous fungi are used.

Hence the CO_2/O_2 ratios of gas phase inside the bioreactor are important to judge and to define the more useful bioconversion conditions. In fact these ratios can describe the bioreactor like a living cell, where the biochemical activity can determine different respiratory quotients.

Nevertheless the description in the literature of many different solid state bioreactors suggests that a bioreactor suitable for all the bioconversion conditions seems to represent a major goal, being perhaps the working flexibility the best aspect to achieve.

The novel prototypal plant, built up in the last few months and still in the start up period, appears to be close to this ambitious purpose and shows high potentiality.

The high flexibility and the low utilities requirement are the most valuable qualities of the plant design obtained.

The simultaneous and well balanced development of the product, the production process and the plant has guaranteed good prospects for this technologically novel rising industrial activity.

Some novel promising applications in the environmental field have been investigated.

The results of the experiments shown the concrete possibility to apply laccases to the biodeinking treatment of wastepapers and suggest that ligninolytic enzymes can be useful utilized in novel remediation processes for recalcitrant pollutants removal. However, the study of better reaction conditions (temperature, enzyme concentration, etc.) in order to increase performance of the enzymatic pool need further investigation.

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