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Biotechnology of Agricultural Wastes Recycling Through Controlled Cultivation of Mushrooms

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1. Introduction

The agricultural wastes recycling with applications in agro-food industry is one of the biological challenging and technically demanding research in the biotechnology domain known to humankind so far. Annually, the accumulation of huge amounts of vineyard and winery wastes causes serious environmental damages nearby winemaking factories. Many of these ligno-cellulose wastes cause serious environmental pollution effects, if they are allowed to accumulate in the vineyards or much worse to be burned on the soil. At the same time, the cereal by-products coming from the cereal processing and bakery industry are produced in significant quantities all over the world (Moser, 1994; Verstraete & Top, 1992).

To solve the environmental troubles raised by the accumulation of these organic wastes, the most efficient way is to recycle them through biological means (Smith, 1998). As a result of other recent studies, the cultivation of edible and medicinal mushrooms was applied using both the solid state cultivation and controlled submerged fermentation of different natural by-products of agro-food industry that provided a fast growth as well as high biomass productivity of the investigated strains (Petre & Teodorescu, 2009; Stamets, 2000).

These plant wastes can be used as the main ingredients to prepare the organic composts for edible and medicinal mushrooms growing in order to get organic food and biological active compounds from the nutritive fungal biomass resulted after solid state cultivation or submerged fermentation of such natural materials (Petre & Petre, 2008; Petre et al., 2010).

Taking into consideration this biological advantage there were tested some variants of biotechnology for agricultural wastes recycling through the controlled cultivation of edible and medicinal mushrooms *Ganoderma lucidum* (Curt.:Fr.) P. Karst (folk name: Reishi or Lingzhi), *Lentinus edodes* (Berkeley) Pegler (folk name: Shiitake) and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (folk name: Oyster Mushroom) on organic composts made of cereal grain by-products as well as winery and vineyard wastes (Petre & Teodorescu, 2010).

2. The solid state cultivation of mushrooms on winery and vineyard wastes

The main aim of this work was focused on screening the optimal biotechnology of edible and medicinal mushrooms growing through the solid-state cultivation by recycling different

kind of agricultural by-products and wastes coming from vineyard farms and winemaking industry (Petre *et al.*, 2011).

Taking into consideration that most of the edible and medicinal mushrooms species requires a specific micro-environment including complex nutrients, the influence of all physical and chemical factors upon fungal pellets production and mushroom fruit bodies formation has been studied by testing new biotechnological procedures (Oei, 2003).

To establish the laboratory biotechnology of recycling the winery and vineyard wastes by using them as a growing source for edible mushrooms, two mushroom species of Basidiomycetes group, namely *L. edodes* (Berkeley) Pegler and *P. ostreatus* (Jacquin ex Fries) Kummer were used as pure mushroom cultures isolated from the natural environment and being preserved in the local collection of the University of Pitesti. The stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar-agar). Slants were incubated at 25°C for 120-168 h and stored at 4°C.

The pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid malt-extract medium at 23°C on rotary shaker incubators at 110 rev min⁻¹ for 72-120 h. After expanding, the pure mushroom cultures were inoculated into 100 ml of 3-5% (v/v) malt-yeast extract liquid medium, previously poured in 250 ml rotary shake flasks and then were maintained at 23-25°C (Petre & Teodorescu, 2010).

The experiments of inoculum preparation were set up under the following conditions: constant temperature, 25°C; agitation speed, 90-120 rev min⁻¹; initial pH, 5.5-6.5. All the seed mushroom cultures were incubated for 120-168 h.

After that, the seed cultures of these mushroom species were inoculated in liquid culture media (20% malt extract, 10% wheat bran, 3% yeast extract, 1% peptone) at pH 6.5 previously distributed into rotary shake flasks of 1,000 ml. During the incubation time, all the spawn cultures were maintained in special culture rooms, designed for optimal incubation at 25°C. Three variants of culture compost were prepared from marc of grapes and vineyard cuttings in the following ratios: 1:1, 1:2, 1:4 (w/w).

The winery and vineyard wastes were mechanically pre-treated by using an electric grinding device to breakdown the lignin and cellulose structures in order to make them more susceptible to the enzyme actions. All the culture compost variants made of winery and vineyard wastes were transferred into 1,000 ml glass jars and disinfected by steam sterilization at 120°C for 60 min. When the jars filled with composts were chilled they were inoculated with the liquid spawn already prepared (Petre *et al.*, 2010).

Each culture compost variant for mushroom growing was inoculated using such liquid spawn having the age of 72-220 h and the volume size ranging between 3-9% (v/w). During the period of time of 18-20 d after this inoculation, the mushroom cultures had developed a significant mycelia biomass on the culture substrates (Carlile & Watkinson, 1996).

According to the registered results of the performed experiments the optimal laboratory-scale biotechnology for edible mushroom cultivation on composts made of marc of grapes and vineyard cuttings was established (Fig. 1).

The effects induced by the composts composition, nitrogen and mineral sources as well as the inoculum amount upon the mycelia growing during the incubation period were investigated. There were made three variants of composts which were tested by comparing them with the control sample made of poplar sawdust (Petre & Teodorescu, 2010).

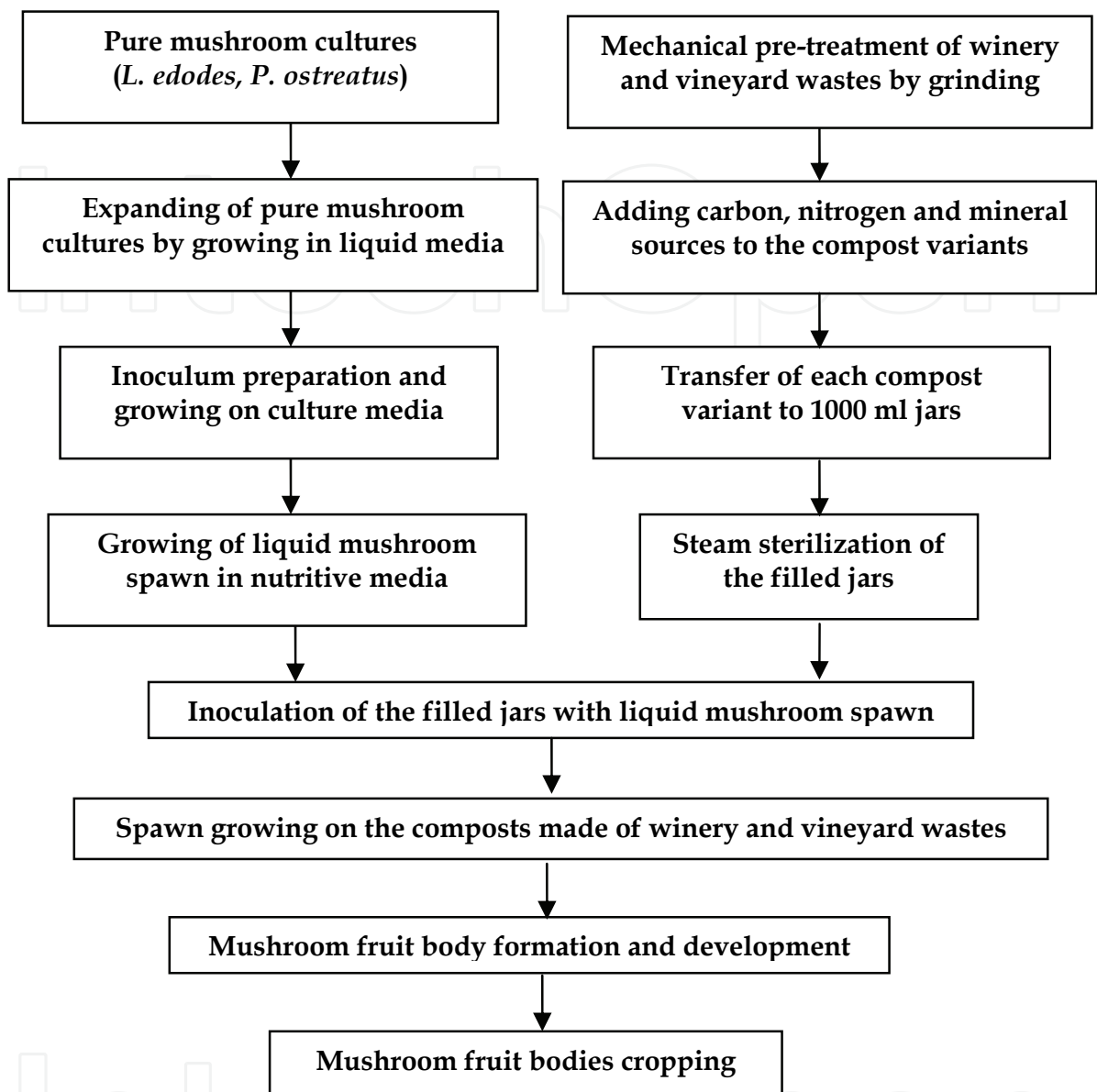


Fig. 1. Scheme of laboratory-scale biotechnology for edible mushroom production by recycling winery and vineyard wastes

The first variant of compost composition was prepared from vineyard cuttings, the second one from a mixture between marc of grapes and vineyard cuttings in equal proportions and the third one was made only from marc of grapes as full compost variant. The experiments were carried out for 288 h at 25°C with the initial pH 6.5 and the incubation period lasted for 168-288 h (Petre *et al.*, 2007).

2.1 Results and discussion

As it can be noticed in figure 2, the registered results show that from all tested compost variants the most suitable substrate for mycelia growing was that one prepared from marc

of grapes, because it showed the highest influence upon the mycelia growing and fresh mushroom production (32–35 g%). All registered data represent the means of triple determinations.

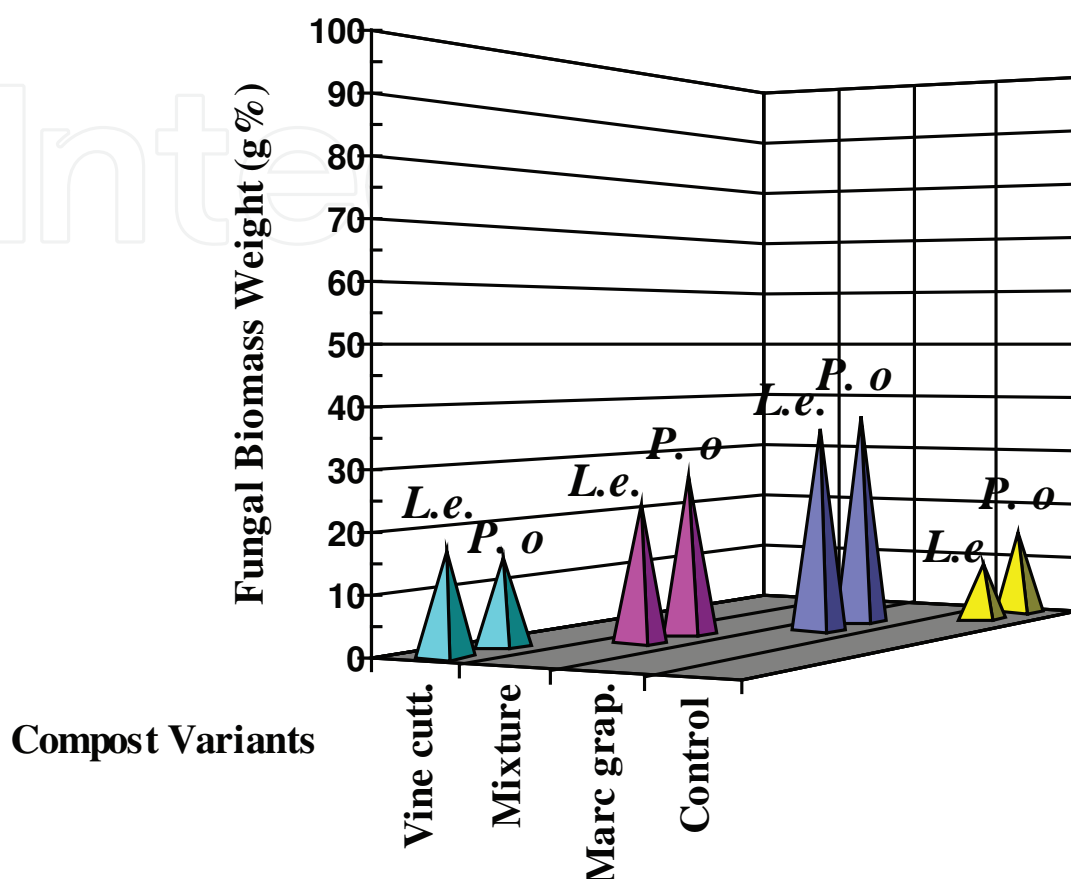


Fig. 2. Comparative effects of composts composition upon mycelia growing of *P. ostreatus* (*P.o.*) and *L. edodes* (*L.e.*)

This compost variant was followed by the mixture prepared from marc of grapes and vineyard cuttings in equal amounts (20–23 g %) and, finally, by the variant made of only vineyard cuttings (12–15 g %). From the tested nitrogen sources, barley bran was the most efficient upon the mycelia growing and fruit mushroom producing at 35–40 g % fresh fungal biomass weight, being closely followed by rice bran at 25–30 g %. Wheat bran is also a well known nitrogen source for fungal biomass synthesis but its efficiency in these experiments was relatively lower than the ones induced by the barley and rice bran added as natural organic nitrogen sources (Stamets, 2000). All registered data are the means of triple determinations. The effects of nitrogen sources were registered as they are presented in figure 3. Among the tested mineral sources, the natural calcium carbonate (CaCO_3) from marine shells yielded the best mycelia growing as well as fungal biomass production at 28–32 g% and, for this reason, it was registered as the most appropriate mineral source, being followed by the natural gypsum ($\text{CaSO}_4 \cdot 2 \text{H}_2\text{O}$) at 20–23 g %, as it is shown in figure 4.

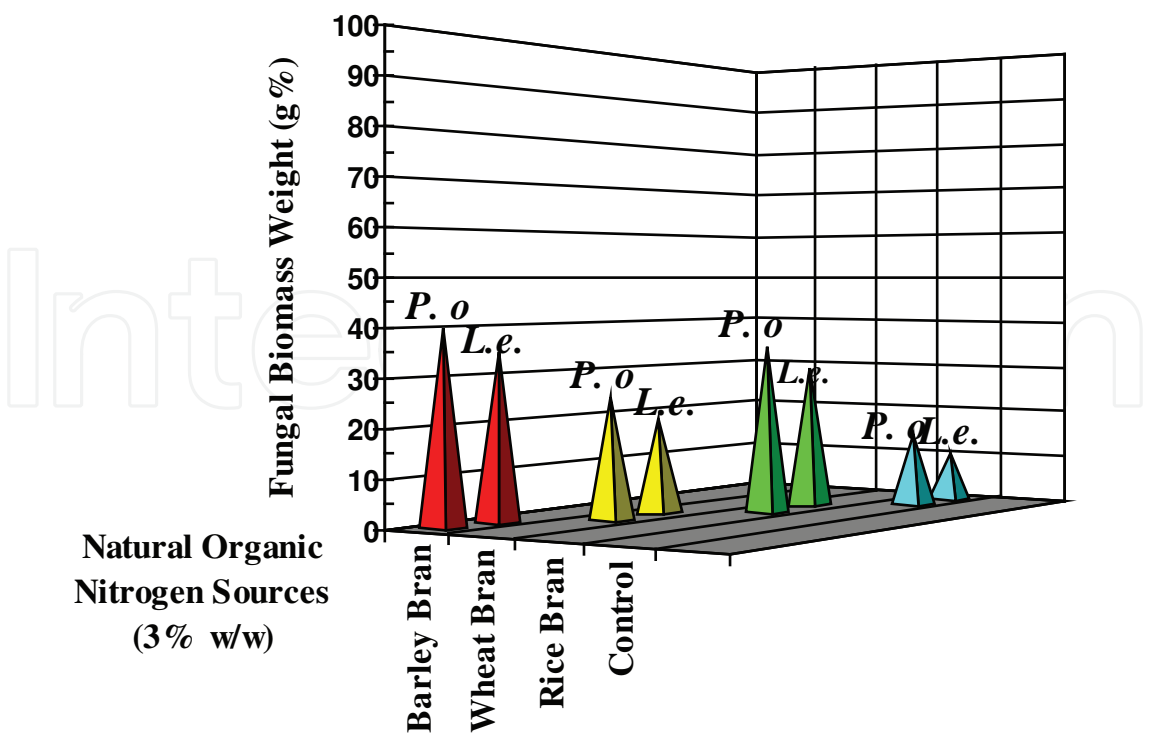


Fig. 3. Comparative effects of organic nitrogen sources upon mycelia growing of *P. ostreatus* (*P.o.*) and *L. edodes* (*L.e.*)

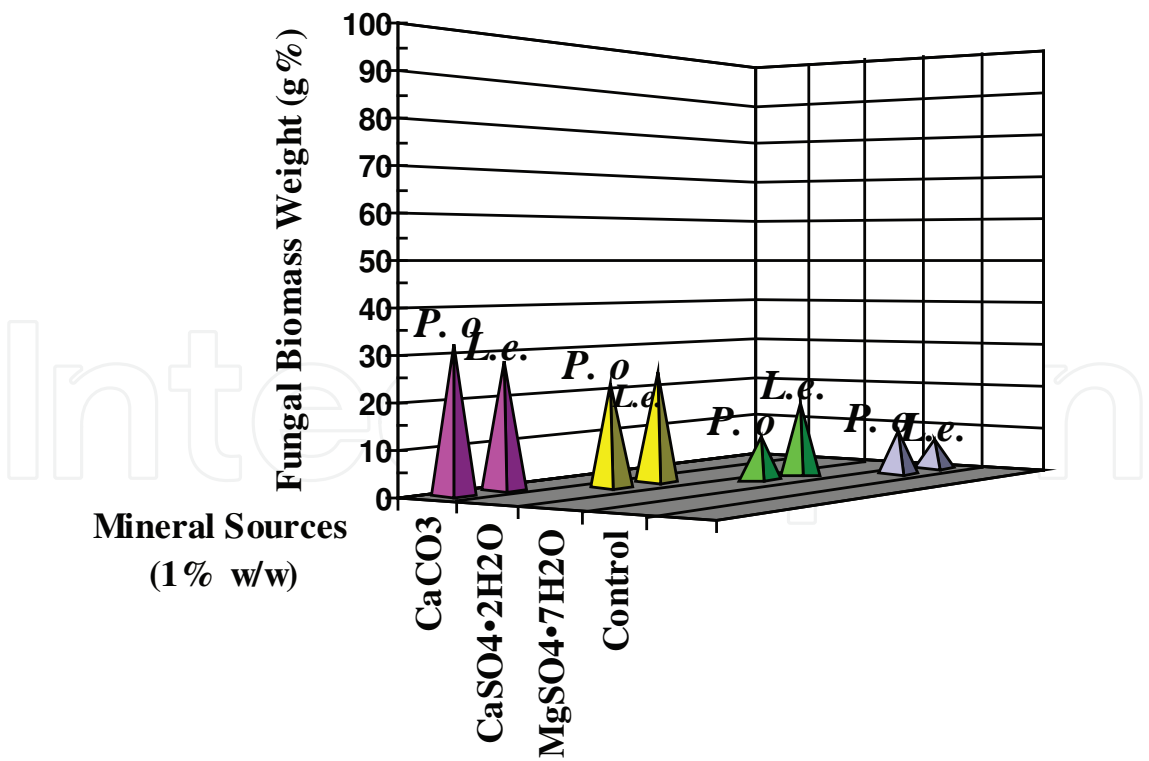


Fig. 4. Comparative effects of mineral sources upon mycelia growing of *P. ostreatus* (*P. o.*) and *L. edodes* (*L.e.*)

The mineral sources like hepta-hydrate magnesium sulfate ($\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$) showed a quite moderate influence upon the fungal biomass growing as other researchers have already reported so far. All data are the means of triple determinations (Stamets, 2000; Chahal, 1994).

The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 25-30 d in case of *P. ostreatus* cultivating and 50-60 d for *L. edodes*, depending on each fungal species used in experiments (Chahal, 1994). However, during the whole period of fruit body formation, the culture parameters were set up and maintained at the following levels, depending on each mushroom species: air temperature, 15–17°C; the air flow volume, 5–6 m³/h; air flow speed, 0.2–0.3 m/s; the relative moisture content, 80–85%, light intensity, 500–1,000 luxes for 8–10 h/d. The final fruit body production of these mushroom species used in experiments was registered between 1.5 kg for *L. edodes* and 2.8 kg for *P. ostreatus*, relative to 10 kg of composts made of vineyard and winery wastes, comparing with 0.7–1.2 kg on 10 kg of poplar sawdust used as control samples.

3. The controlled submerged cultivation of mushrooms on winery wastes

The submerged cultivation of mushroom mycelium is a promising biotechnological procedure which can be used for synthesis of pharmaceutical substances with anticancer, antiviral and immuno-stimulatory effects from the nutritive mushroom biomass (Wasser & Weis, 1994). As result of other recent studies, the continuous cultivation of edible and medicinal mushrooms was applied by using the submerged fermentation of different natural by-products of agro-food industry (Bae, et al., 2000; Jones, 1995; Moo-Joung, 1993). The biotechnology of controlled cultivation of medicinal mushrooms was established and tested in different variants of culture media that were made of different sorts of bran and broken seeds resulted from the industrial food processing of wheat, barley and rye seeds. This biotechnology can influence the faster growth as well as higher biomass productivity of *G. lucidum* and *L. edodes* mushroom species (Petre et al., 2010).

The main stages of biotechnology to get high nutritive fungal biomass by controlled submerged fermentation were the followings:

1. Preparation of culture media and pouring them into the cultivation vessel of the bioreactor.
2. Steam sterilization of bioreactor vessel at 121°C and 1.1 atm. for 20 min.
3. Inoculation of sterilized culture media with mycelium from pure cultures of selected strains inside the bioreactor vessel for submerged cultivation, using the sterile air hood with laminar flow.
4. Running the submerged cultivation cycles under controlled conditions: temperature $23 \pm 2^\circ\text{C}$, speed 70 rpm and continuous aeration at 1.1 atm.
5. Collecting, cleaning and filtering the fungal pellets obtained by the submerged fermentation of substrates made of by-products resulted from cereal grains processing.

Two mushroom species belonging to Basidiomycetes Class, namely *G. lucidum* (Curt.:Fr.) P. Karst and *L. edodes* (Berkeley) Pegler were used as pure cultures in experiments. The stock cultures were maintained on malt-extract agar (MEA) slants. Slants were incubated at 25°C for 5–7 d and then stored at 4°C. The fungal cultures were grown in 250-ml flasks containing 100 ml of MEYE (malt extract 20%, yeast extract 2%) medium at 23°C on rotary shaker incubators at 110 rev min⁻¹ for 5–7 d. The fungal cultures were prepared by aseptically inoculating 100 ml in three variants of culture media by using 3–5% (v/v) of the seed culture and then cultivated at 23–25°C in 250 ml rotary shake flasks. The biotechnological

experiments were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min⁻¹; initial pH, 4.5–5.5. After 10–12 d of incubation the fungal cultures were ready to be inoculated aseptically into the glass vessel of 20 l laboratory-scale bioreactor, that was designed to be used for controlled submerged cultivation of edible and medicinal mushrooms on substrata made of wastes resulted from the industrial processing of cereal grains (Fig. 5).



Fig. 5. General view of the Laboratory scale bioreactor (15 L)

After a period of submerged fermentation lasting up to 120 h, small mushroom pellets developed inside the nutritive broth (Fig. 6, 7).

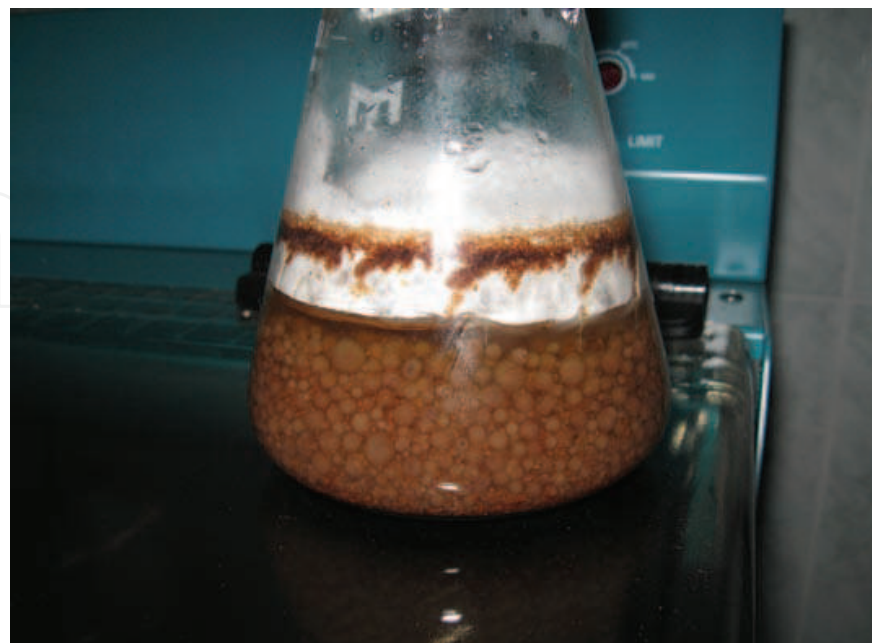


Fig. 6. Mycelial biomass of *G. lucidum* collected after submerged fermentation

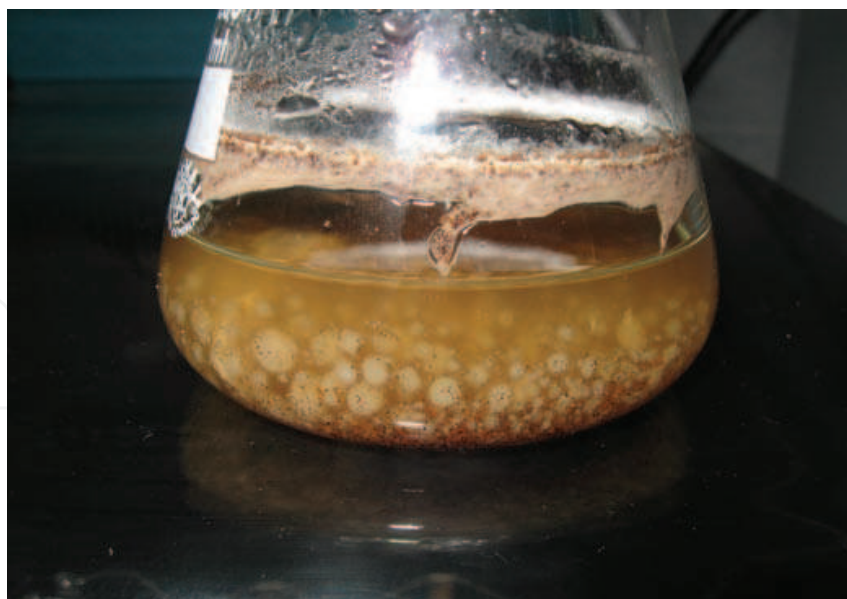


Fig. 7. Mycelial biomass in the shape of fungal pellets of *L. edodes*, collected after submerged fermentation

The fermentation process was carried out by inoculating the growing medium volume (10,000 ml) with mycelia inside the culture vessel of the laboratory-scale bioreactor. The whole process of growing lasts for a single cycle between 5-7 days in case of *L. edodes* and between 3 to 5 days for *G. lucidum*. The strains of these fungal species were characterized by morphological and cultural stability, proven by their ability to maintain the phenotypic and taxonomic identities. The experiments were carried out in three repetitions. Observations on morphological and physiological characters of these two tested species of fungi were made after each culture cycle, highlighting the following aspects:

- sphere-shaped structure of fungal pellets, sometimes elongated, irregular, with various sizes (from 2 to 5 mm in diameter), reddish-brown colour – *G. lucidum* culture (Fig. 8).

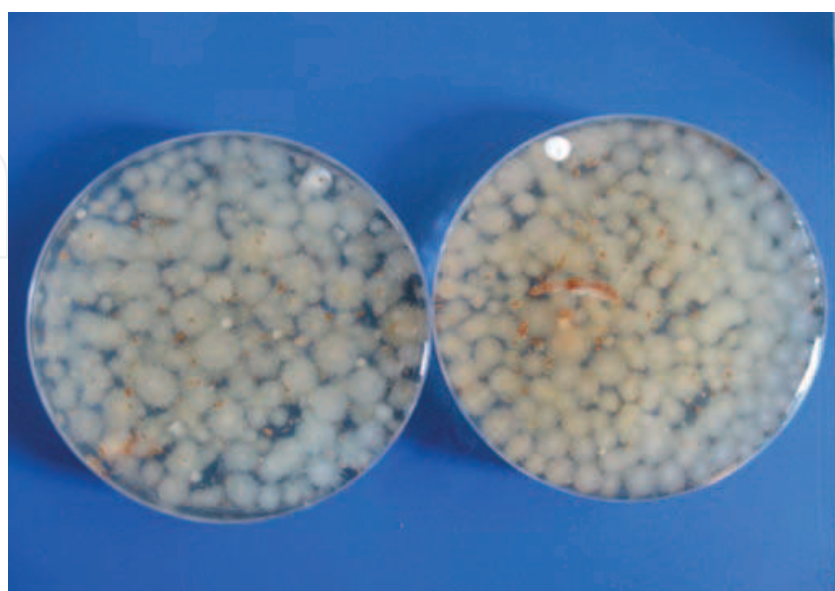


Fig. 8. Stereomicroscopic view of *G. lucidum* pellets after controlled submerged fermentation

- elliptically-shaped structures of fungal pellets, with irregular diameters of 4 up to 7 mm showing mycelia congestion, which developed specific hyphae of *L. edodes* (Fig. 9).

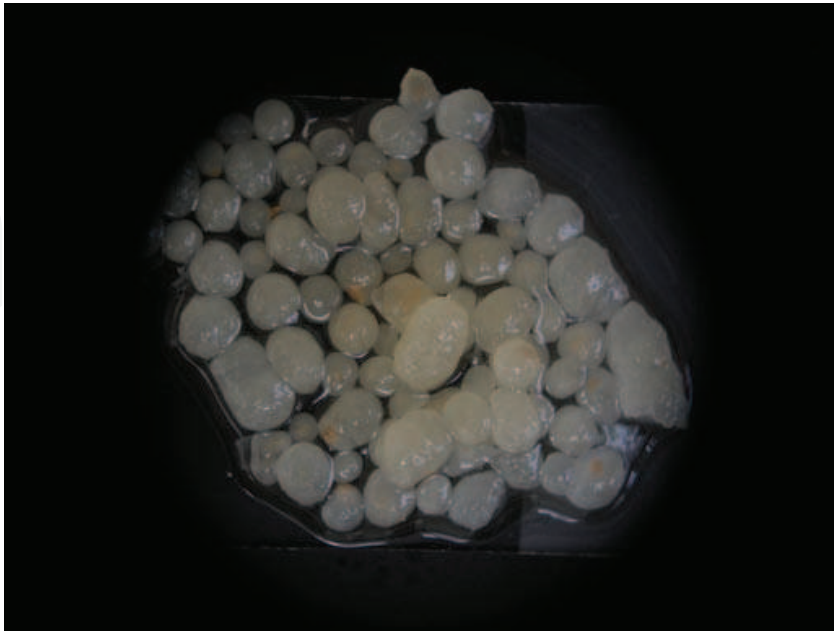


Fig. 9. Stereomicroscopic view of *L. edodes* pellets after controlled submerged fermentation

Samples for analysis were collected at the end of the fermentation process, when pellets formed specific shapes and characteristic sizes. The fungal biomass was washed repeatedly with double distilled water in a sieve with 2 mm diameter eye, to remove the remained bran in each culture medium.

3.1 Results and discussion

Biochemical analyses of fungal biomass samples obtained by submerged cultivation of mushrooms were carried out separately for the solid fraction and liquid medium remained after the separation of fungal biomass by filtering. The percentage distribution of solid substrate and liquid fraction in the samples of fungal biomass are shown in table 1.

Mushroom species	Total volume of separated liquid per sample (ml)	Total biomass weight per sample (g)	Water content after separation (%)
<i>L. edodes</i>	83	5.81	83.35
<i>L. edodes</i>	105	7.83	82.50
<i>L. edodes</i>	95	7.75	82.15
<i>L. edodes</i>	80	5.70	79.55
<i>G. lucidum</i>	75	7.95	83.70
<i>G. lucidum</i>	115	6.70	82.95
<i>G. lucidum</i>	97	5.45	80.75
<i>G. lucidum</i>	110	6.30	77.70

Table 1. Percentage distribution of solid substrate and liquid fraction in the preliminary samples of fungal biomass

In each experimental variant the amount of fresh biomass mycelia was determined. The percentage amount of dry biomass was determined by dehydration at 70°C, up to constant weight. Total protein content was determined by biuret method, whose principle is similar to the Lowry method, this method being recommended for the protein content ranging from 0.5 to 20 mg/100 mg sample. In addition, this method required only one sample incubation period (20 min) and by using them was eliminated the interference with various chemical agents (ammonium salts, for example).

The principle method is based on reaction that takes place between copper salts and compounds with two or more peptides in the composition in alkali, which results in a red-purple complex, whose absorbance is read in a spectrophotometer in the visible domain (λ - 550 nm). The registered results are presented as the amounts of fresh and dry biomass as well as protein contents for each fungal species and variants of culture media (Tables 2, 3).

Culture variants	Fresh biomass (g)	Dry biomass (%)	Total protein (g % d.w.)
I	20.30	5.23	0.55
II	23.95	6.10	0.53
III	22.27	4.79	0.73
IV	20.10	4.21	0.49
Control	4.7	0.5	0.2

Table 2. Fresh and dry biomass and protein content of *L. edodes* after submerged fermentation

Culture variants	Fresh biomass (g)	Dry biomass (%)	Total protein (g % d.w.)
I	25.94	9.03	0.67
II	22.45	10.70	0.55
III	23.47	9.95	0.73
IV	21.97	9.15	0.51
Control	5.9	0.7	0.3

Table 3. Fresh and dry biomass and protein content of *G. lucidum* after submerged fermentation

According to the registered data, using wheat bran strains the growth of *G. lucidum* biomass was favoured, while the barley bran led to the increased growth of *L. edodes* mycelium and *G. lucidum* as well. In contrast, dry matter content was significantly higher when using barley bran for both species used. Protein accumulation was more intense in case of using barley bran compared with those of wheat and rye, at both species of mushrooms.

The sugar content of dried mushroom pellets collected at the end of experiments was determined by using Dubois method (Wasser & Weis, 1994). The mushroom extracts were prepared by immersion of dried pellets inside a solution of NaOH pH 9, in the ratio 1:5.

All dispersed solutions containing the dried pellets were maintained 24 h at a precise temperature of 25°C, in full darkness, with continuous homogenization to avoid the oxidation reactions. After removal of solid residues by filtration, the samples were analyzed by the previous mention method. The nitrogen content of mushroom pellets was analyzed by Kjeldahl method (Table 4).

Mushroom species	Culture variant	Sugar content (mg/ml)	Kjeldahl nitrogen (%)	Total protein (g % d.w.)
<i>L. edodes</i>	I	5.15	6.30	0.55
<i>L. edodes</i>	II	4.93	5.35	0.53
<i>L. edodes</i>	III	4.50	5.70	0.73
<i>L. edodes</i>	IV	4.35	5.75	0.49
	Control	0.55	0.30	0.2
<i>G. lucidum</i>	I	4.95	5.95	0.67
<i>G. lucidum</i>	II	5.05	6.15	0.55
<i>G. lucidum</i>	III	5.55	6.53	0.73
<i>G. lucidum</i>	IV	4.70	5.05	0.51
	Control	0.45	0.35	0.3

Table 4. The sugar, total nitrogen and total protein contents of dried mushroom pellets

Comparing all registered data resulted from triple determinations, it can be noticed that the biochemical correlation between dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushrooms (Stamets, 2000).

Among all mushroom samples that were tested in biotechnological experiments *G. lucidum* G-3 showed the best values of their composition in sugars, total nitrogen and total protein contents. In this stage, 70-80% of the former fungal pellets were separated by collecting them from the culture vessel of the bioreactor and separating from the broth by slow vacuum filtration. On the base of these results, the optimal values of physical and chemical factors which influence the mushroom biomass synthesis were taken into consideration in order to established the following schematic flow of the biotechnology for mushroom biomass producing by submerged fermentation, as it is shown in figure 10.

The main advantages of the submerged fermentation of winery wastes under the metabolic activity of selected mushrooms, by comparison with the solid state cultivation are the followings:

- a. the shortening of the biological cycle and cellular development in average from 8-10 weeks to at mostly one week per cellular culture cycle;
- b. the ensuring of the optimal control of physical and chemical parameters which are essential for producing important amounts of mushroom pellets in a very short time;
- c. 20-30% reduction of energy and work expenses as well as the volume of the volume of raw materials materials which are manipulated during each culture cycle;
- d. 15-20% increasing of fungal biomass amount per medium volume unit for each mushrooms species;
- e. the whole removing of any pollutant sources during the biotechnological flux;
- f. the culture media for mushroom growing are integrally natural without using of artificial additives as it is used in classical cultivating procedures;
- g. the mushroom pellets produced by applying this biotechnology for ecological treatment of agricultural wastes was 100% made by natural means and will be used for food supplements production with therapeutic properties which will contribute to the increasing of health level of human consumers having nutritional metabolic deficiencies.
- h. the biochemical correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.

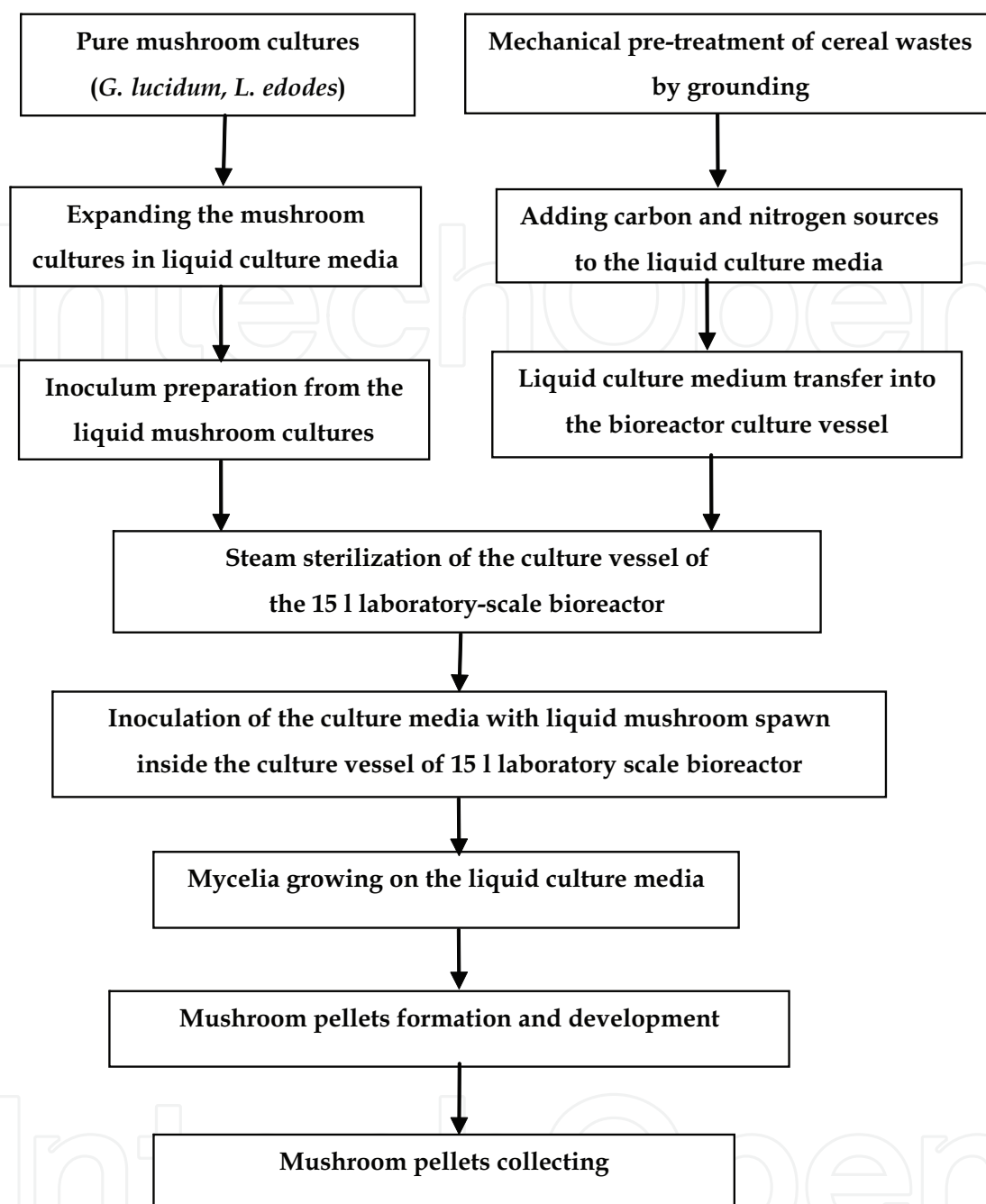


Fig. 10. Schematic flow of the biotechnology for mushroom biomass producing by submerged fermentation.

4. The controlled cultivation of mushrooms in modular robotic system

The agricultural works as well as industrial activities related to plant crops and their processing have generally been matched by a huge formation of wide range of lignocellulose wastes. All these vegetal wastes cause serious environmental troubles if they accumulate in the agro-ecosystems or much worse to be burned on the soil. For the human-operational farms, all processes are made by human personnel exclusively, starting from filling of cultivation beds with compost, up to fruit-bodies harvesting (Reed *et al.*, 2001).

In this respect, a strong tendency for increasing the number of researches in the field of mushroom's automated cultivation, harvesting and processing technologies as well as for continuously development of new robotic equipments can be noticed (Reed *et al.*, 2001).

The solid state cultivation of edible and medicinal mushrooms *Lentinula edodes* and *Pleurotus ostreatus* could be performed by using a modular robotic system that provides the following fully automatic operations: sterilization of composts, inoculation in aseptic chamber by controlled injection device containing liquid mycelia as inoculum, incubation as well as mushroom fruit bodies formation in special growing chambers with controlled atmosphere and the picking up of edible and medicinal mushroom fruit bodies (Petre *et al.*, 2009).

The biotechnology concerning the controlled cultivation of edible mushrooms in continuous flow depends on the strictly maintaining of biotic as well as physical and chemical factors that could influence the bioprocess evolution. The proceeding of edible mushroom cultivation consists in a continuous biotechnological flow, having a chain of successive stages that are working in the non-sterile zone and mostly in the sterile zone of the modular robotic system. In this way, there is provided the technological security both from the structural and functional points of view in order to produce organic foods in highest security and food quality. The functional biotechnological model of the modular robotic system was designed for controlled cultivation and integrated processing of edible mushrooms to get ecological food in highest safety conditions (Petre *et al.*, 2009).

The modular robotic system designed for edible mushroom cultivation provides the automatic sterilization of composts, the automatic inoculation inside the aseptic room by a special device of controlled injection of liquid mycelia, the incubation and fruit bodies formation in special chambers under controlled atmosphere as well as the automatic harvesting of mushroom fruit bodies (Petre *et al.*, 2011).

This system includes three major zones, respectively, the non-sterile zone, the sterile zone and the fruit-body processing zone (Fig. 11).

Thus, during the first stage of the biotechnological flow, in the non-sterile zone of the cultivation system, a natural and nutritive compost is prepared from sawdust or shavings of deciduous woody species in the ratio of 30-40 parts per weight (p.p.w.), marc of grapes chemically untreated, in 20-30 p.p.w., brans of organic cereal seeds (wheat, barley, oat, rye, rice), in 10-20 p.p.w., yeasts, in 3-5 p.p.w., and powder of marine shells, in 1-3 p.p.w., for pH adjustment, which then, it is hydrated with demineralized water, in 20-30 p.p.w. In the next stage, such prepared compost is decanting in polyethylene thermoserilizable bags, which have round orifices of 0,3-0,5 mm in diameter, uniform distributed between them, at 10-15 cm distance, each one of them having a working volume of 10-20 kg (Petre *et al.*, 2011).

Beforehand, special devices for uniform distribution of mycelia as liquid inoculum are mounted inside of these bags. Then, these bags are fitted out with supporting devices on the transfer and transport systems and special devices for coupling to the automatic inoculation subdivision by controlled injection of liquid mycelia (Fig. 11).

Each one of these zones is linked with next one by an interfacing zone. In this way, the non-sterile zone is linked with the sterile zone through the first interfacing zone and this one is connected with the fruit body processing zone by the second interfacing area, as it is shown in figure 11.

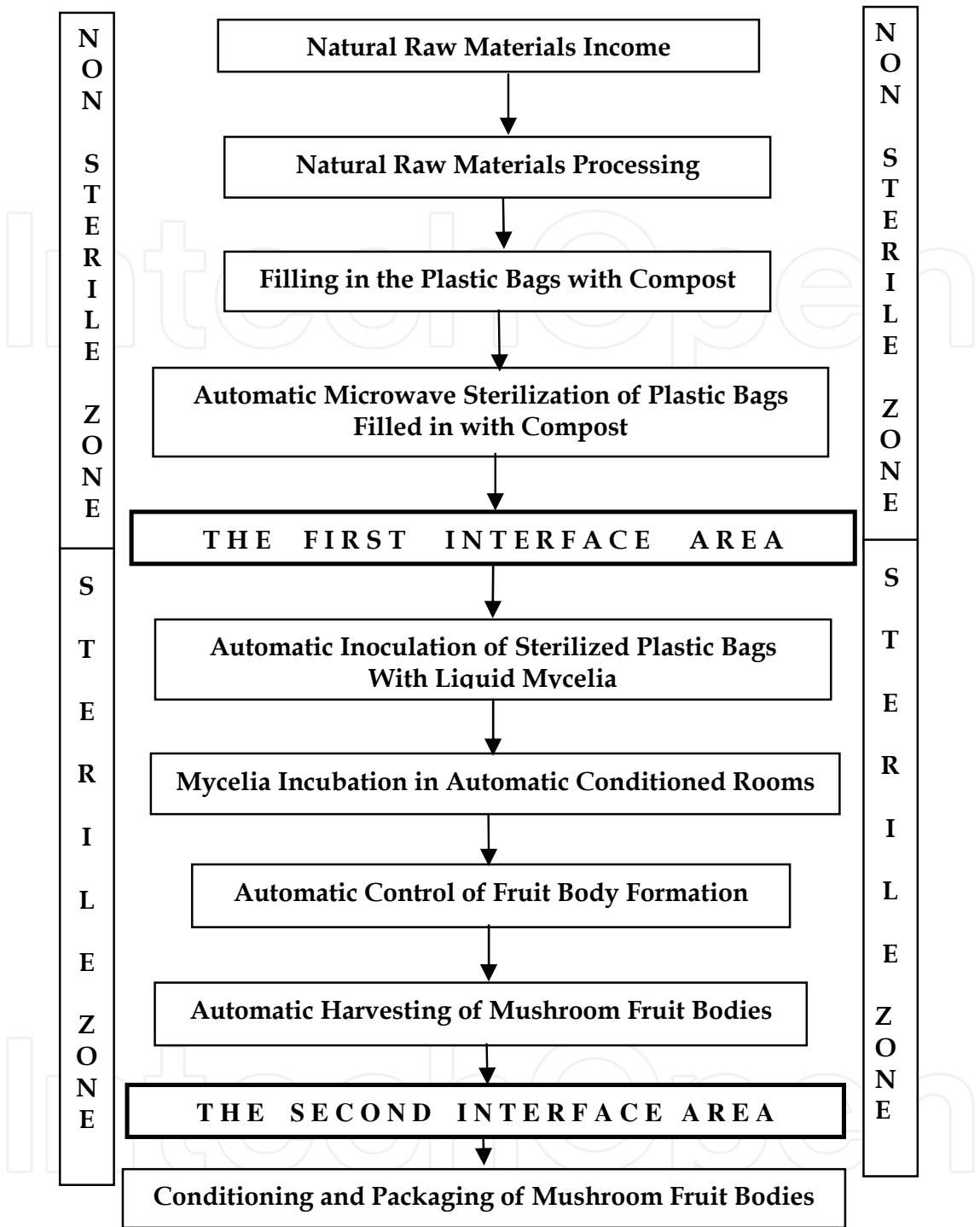


Fig. 11. Schematic flow of the modular robotic system for controlled cultivation of edible mushrooms

Inside the non-sterile zone, the bags filled with composts are placed on the supporting devices, mounted on the transfer pallets, which are inserted in the first part of the sterile zone, respectively, in the module of the automatic sterilization with microwave at 120-125°C, and the pallets with bags are automatically chilled in the zone of controlled cooling of sterilized composts up to the room temperature. These pallets with sterilized bags are

automatically transferred into the aseptic room to make the inoculation with liquid mycelia by using a robotic device of controlled injection. Further on, the pallets with the inoculated bags either are evacuated from the sterile zone or they are automatically transferred to the incubation and fruit body formation rooms. In these rooms of incubation and fruit body formation, both the optimal temperature of mycelia growing and the relative air humidity are provided as well as a constant steril air flow introduced under pressure by using an automatic device and an adequate lighting level (Petre *et al.*, 2011; Petre *et al.*, 2009).

In this way, the bags are maintained from 15 up to 30 days, during this time a mycelial net being formed from the hypha anastomosis having a compact structure and a white-yellowish color, that covers the whole surface of compost and from which the mushroom fruit bodies will emerge and develop soon as specific morphological structures of the origin species. These mushroom fruit bodies were grown and maturated in almost 3-10 days, depending on the cultivated mushroom species, at constant temperature of 18-21⁰C, air relative humidity 90-95% and controlled aeration at 3-5 air volume exchanges per hour and the suitable lighting at 2.000-3.000 luxes per hour, for 12 h daily. For the fruit bodies picking-up, the pallets are automatically discharged by the same robotic system and transferred to the automatic harvesting zone, where another robotic system automatically collects all the mushroom fruit bodies by a special designed device to be conditioned and packaged aseptically (Fig. 11). The modular robotic system designed for edible mushroom cultivation provides the automatic sterilization of composts, the automatic inoculation inside the aseptic room by a special device of controlled injection of liquid mycelia, the incubation and fruit bodies formation in special chambers under controlled atmosphere as well as the automatic picking-up of mushroom fruit bodies (Reed *et al.*, 2001).

Both interfacing zones were designed to keep the sterile zone at the highest level of food safety against the microbial contamination. Using this robotic biotechnological model of mushroom cultivation, the economical efficiency can be significantly increased comparing to the actual conventional technologies, by shorting the total time of mushroom cultivation cycles in average with 5-10 days, depending on the mushroom strains that were grown and providing high quality mushroom fruit bodies produced in complete safety cultivation system (Petre *et al.*, 2009).

4.1 Results and discussion

To increase the specific processes of cellulose biodegradation of winery and vineyard wastes and finally induce their bioconversion into protein of fungal biomass, there were performed experiments to cultivate the mushroom species of *P. ostreatus* and *L. edodes* on the following variants of culture substrata (see Table 5).

Variants of culture substrata	Composition
S1	Winery wastes
S2	Mixture of winery wastes and rye bran 2.5%
S3	Mixture of winery wastes and rise bran 2%
S4	Mixture of vine cuttings and wheat bran 1%
S5	Mixture of vine cuttings and barley bran 1.5%
Control	Pure cellulose

Table 5. The composition of five compost variants used in mushroom culture

The fungal cultures were grown by inoculating 100 ml of culture medium with 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in 250 ml rotary shake flasks. The experiments were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min⁻¹; initial pH, 4.5-5.5. After 10-12 d of incubation the fungal cultures were inoculated aseptically into glass vessels containing sterilized liquid culture media in order to produce the spawn necessary for the inoculation of 10 kg plastic bags filled with compost made of winery and vineyard wastes (Petre *et al.*, 2011; Petre *et al.*, 2009).

These compost variants were mixed with other natural ingredients in order to improve the enzymatic activity of mushroom mycelia and convert the cellulose content of winery and vineyard wastes into protein biomass. Until this stage, all the technological operations were handmade. In the next production phases, all the operations were designed to be carried out automatically by using a robotic modular system, which makes feasible the safety culture of edible mushrooms in continuous flow using as composts the winery and vineyard wastes.

The modular robotic system designed for edible mushrooms cultivation provides the automatic sterilization of composts, the automatic inoculation inside the aseptic room by a special device of controlled injection of liquid mycelia, the incubation and fruit bodies formation in special chambers under controlled atmosphere and the automatic picking-up of mushroom fruit bodies. In this way, the whole bags filled with compost have to be sterilized at 90-100°C, by introducing them in a microwave sterilizer. In the next stage, all the sterilized bags must be inoculated with liquid mycelia, which have to be pumped through an aseptic injection device (Fig. 12).

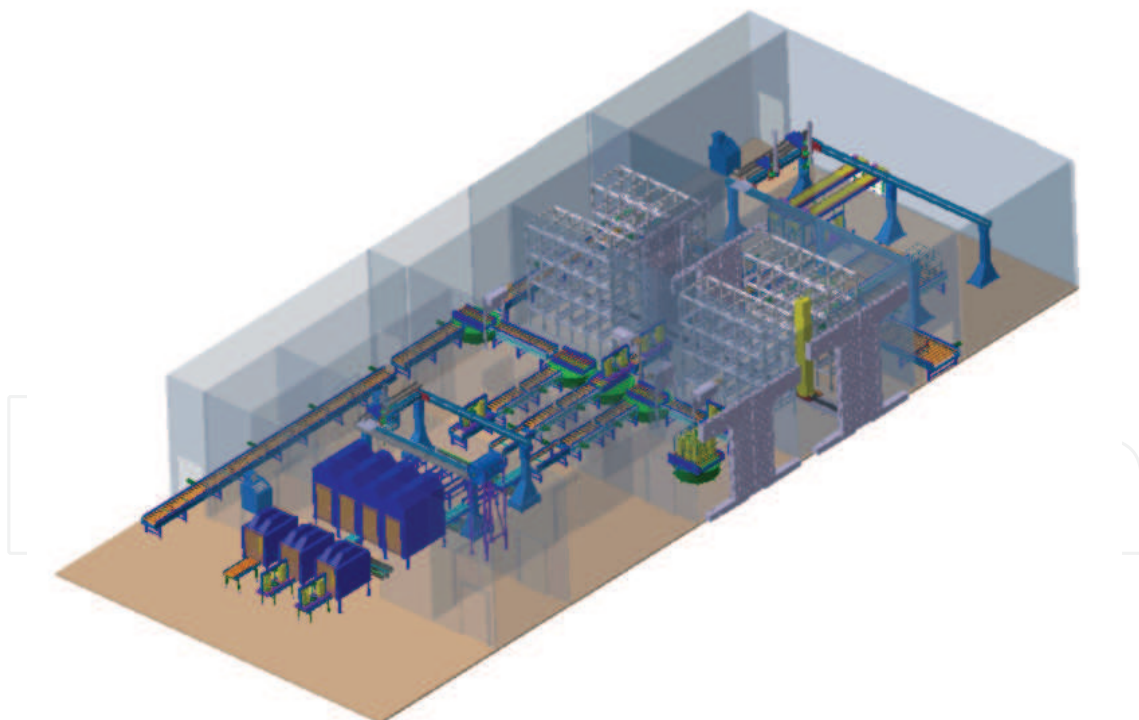


Fig. 12. General overview of the modular robotic system for controlled cultivating of mushrooms

Then, all the inoculated bags have to be transferred inside the growing chambers for incubation. After a time period of 10-15 d from the sterilized plastic bags filled with compost, the first buttons of the mushroom fruit bodies emerged.

For a period of 20-30 d there were harvested between 1.5 – 3.5 kg of mushroom fruit bodies per 10 kg compost bag. The specific rates of cellulose biodegradation were determined using the direct method of biomass weighing the results being expressed as percentage of dry weight (d.w.) before and after their cultivation. The registered data are presented in Table 6.

Variants of culture substrata	Before cultivation (g% d.w.)		After cultivation (g% d.w.)	
	L. edodes	P. ostreatus	L. edodes	P. ostreatus
S1	2.6-2.7	2.7-2.9	0.5	0.9
S2	2.3-2.5	2.5-2.8	0.4	0.7
S3	2.3-2.5	2.3-2.5	0.5	0.4
S4	2.5 -2.7	2.5 -2.7	0.7	0.8
S5	2.7-2.9	2.5-2.7	0.5	0.7
Control	3.0	3.0	1.4	1.5

Table 6. The rate of cellulose degradation of culture substrata during the growing cycles of *L. edodes* and *P. ostreatus*

The registered data revealed that by applying this biotechnology, the winery and vineyard wastes can be recycled as useful raw materials for mushroom compost preparation in order to get significant production of mushrooms.

In this respect, the final fruit body production during the cultivation of these two mushroom species was registered as being between 20–28 kg relative to 100 kg of composts made of winery wastes.

Significant bioconversion increasing of the winery and vineyard wastes by using the modular robotic system of continuous controlled cultivation of edible mushrooms can be achieved by:

- a. using pure strains of the mushroom species *P. ostreatus* and *L. edodes* whose biomass has got nutritive and functional properties proved by the research results of some achieved projects or others that are running now;
- b. excluding any potential contamination sources for the edible mushrooms by using total sterilization or filtration equipments in each production module, by controlling all raw and auxiliary materials, water and air;
- c. keeping the high precision and accuracy of the inoculation operations, incubation and fruit body formation of edible mushrooms which induce constant biomass composition of either fungal mycelia or mushroom fruit bodies;
- d. avoiding all errors in the sterile zone of production flow as well as the potential risk of edible mushroom contamination by the human operators.

5. Conclusions

According to the previous mentioned results, the following conclusions can be drawn:

1. Most suitable organic compost for mycelia growing was prepared from marc of grapes, showing the highest influence upon the mycelia growing and fresh mushroom production of 32–35 g%.

2. From the tested nitrogen sources, barley bran was the most efficient upon the mycelia growing and fruit mushroom producing at 35-40 g%, being closely followed by rice bran at 25-30 g% both in case of *P. ostreatus* and *L. edodes*, all data being reported as fresh biomass.
3. Among the tested mineral sources, the natural calcium carbonate (CaCO_3) yielded the best mycelia growing as well as fungal biomass production at 28-32 g%; for this reason it was registered as the most appropriate mineral source being followed by natural gypsum ($\text{CaSO}_4 \cdot 2 \text{H}_2\text{O}$) at 20-23 g%.
4. The originality and novelty of this biotechnology of winery and vineyard wastes recycling was confirmed by the Patents no 121717/2008 and 121718/2008 issued by the Romanian Office of Patents and Trade Marks
5. The mushroom pellets produced by applying the controlled cultivation of mushrooms as biotechnology for ecological treatment of winery wastes was 100% made by natural means and will be used for food supplements production with therapeutic properties which will contribute to the increasing of health level of human consumers with nutritional metabolic deficiencies.
6. The biochemical correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents was kept at a balanced ratio for each tested mushroom species.

Among all mushroom samples that were tested in biotechnological experiments *G. lucidum* G-3 had shown the best values of its composition in sugars, total nitrogen and total protein content.

7. The originality and novelty of these biotechnological procedures to recycle the cereal wastes in order to get high nutritive biomass of mushroom pellets were confirmed through the Patents no 121677/2008, 121678/2008 and 121679/2008 issued by the Romanian Office of Patents and Trade Marks
8. By applying the biotechnology of controlled cultivation of edible mushrooms in modular robotic system, the final fruit body productions of both mushroom species *P. ostreatus* as well as *L. edodes* were registered as being between 20-28 kg relative to 100 kg of composts made of winery wastes.
9. The continuous controlled cultivation of edible mushrooms by using the modular robotic system can be achieved by:
 - a. using pure strains of the mushroom species *P. ostreatus* and *L. edodes* whose biomass has got nutritive and functional properties proved by the research results of some achieved projects or others that are running now;
 - b. excluding any potential contamination sources for the edible mushrooms by using total sterilization or filtration equipments in each production module, by controlling all raw and auxiliary materials, water and air;
 - c. keeping the high precision and accuracy of the inoculation operations, incubation and fruit body formation of edible mushrooms which induce constant biomass composition of either fungal mycelia or mushroom fruit bodies;
 - d. avoiding all errors in the sterile zone of production flow as well as the potential risk of edible mushroom contamination by the human operators.

10. The originality and novelty of this biotechnology of controlled cultivation of edible mushrooms in modular robotic system were confirmed by the Patent no 123132/20010, issued by the Romanian Office of Patents and Trade Marks.

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