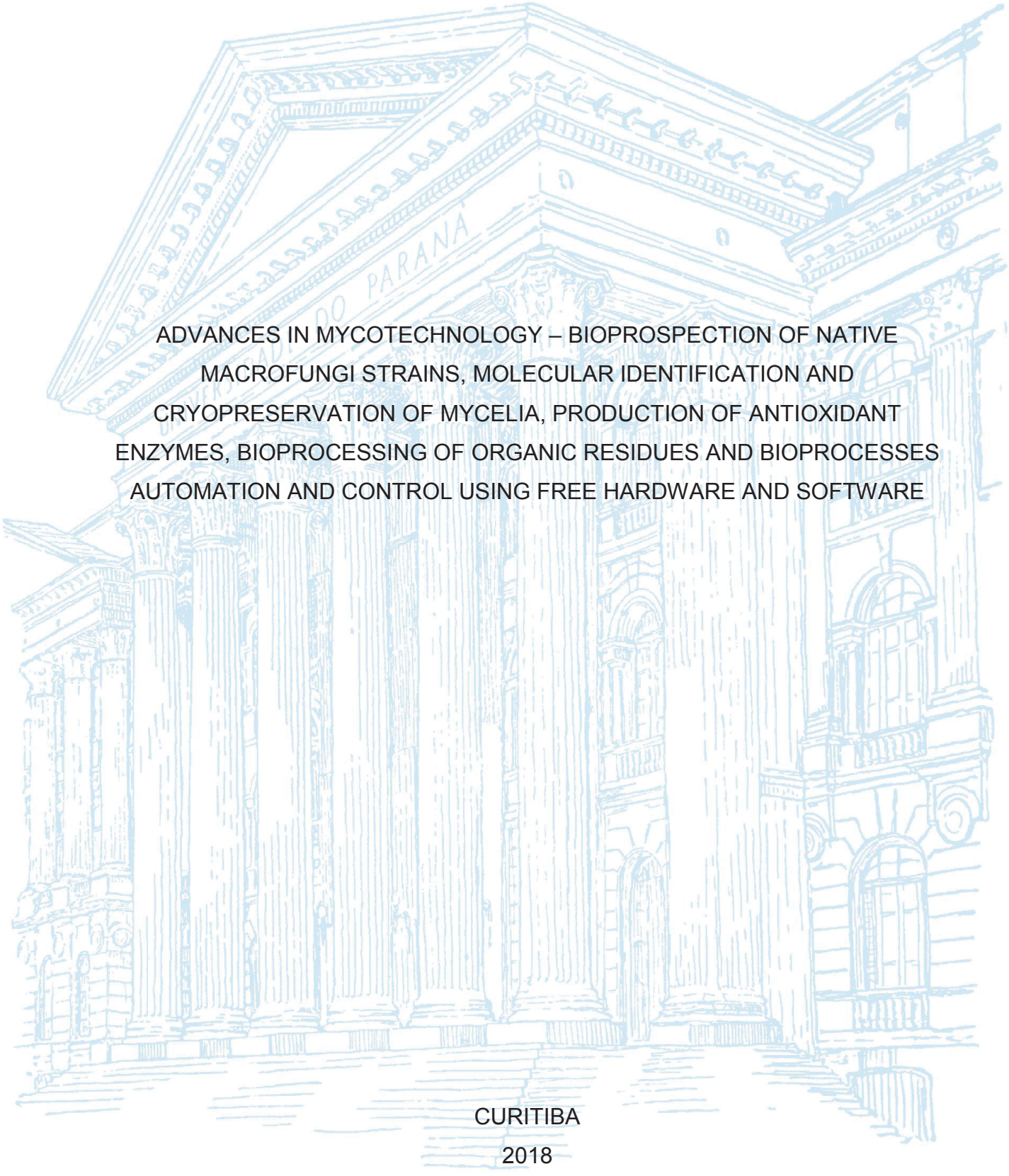
UNIVERSIDADE FEDERAL DO PARANÁ

FRANCISCO MENINO DESTÉFANIS VÍTOLA



ADVANCES IN MYCOTECHNOLOGY – BIOPROSPECTION OF NATIVE MACROFUNGI STRAINS, MOLECULAR IDENTIFICATION AND CRYOPRESERVATION OF MYCELIA, PRODUCTION OF ANTIOXIDANT ENZYMES, BIOPROCESSING OF ORGANIC RESIDUES AND BIOPROCESSES AUTOMATION AND CONTROL USING FREE HARDWARE AND SOFTWARE

CURITIBA

2018

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ADVANCES IN MYCOTECHNOLOGY – BIOPROSPECTION OF NATIVE MACROFUNGI STRAINS, MOLECULAR IDENTIFICATION AND CRYOPRESERVATION OF MYCELIA, PRODUCTION OF ANTIOXIDANT ENZYMES, BIOPROCESSING OF ORGANIC RESIDUES AND BIOPROCESSES AUTOMATION AND CONTROL USING FREE HARDWARE AND SOFTWARE

Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol

CURITIBA

2018

FICHA CATALOGRÁFICA ELABORADA PELO SISTEMA DE BIBLIOTECAS/UFPR
BIBLIOTECA DE CIÊNCIA E TECNOLOGIA

V845a Vítola, Francisco Menino Destéfanis
Advances in mycotechnology: bioprospection of native macrofungi strains, molecular identification and cryopreservation of mycelia, production of antioxidant enzymes, bioprocessing of organic residues and bioprocesses automation and control using free hardware and software / Francisco Menino Destéfanis Vítola. – Curitiba, 2018.
249 p. : il. color.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientador: Carlos Ricardo Soccol.

1. Cogumelos. 2. Antioxidantes. 3. Automação (Engenharia de Bioprocessos e Biotecnologia). I. Universidade Federal do Paraná. II. Soccol, Carlos Ricardo. III. Título.

CDD: 660.6

Bibliotecária: Romilda Santos - CRB-9/1214

Ministério da Educação
Universidade Federal do Paraná
Pró Reitoria de Pesquisa e Pós Graduação
Setor de Tecnologia
Programa Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná, foram convocados para realizar a arguição da Tese de Doutorado de **Francisco Menino Destéfanis Vítola** intitulada: "Advances in Mycotechnology – Bioprospection of Native Macrofungi Strains, Molecular Identification and Cryopreservation of Mycelia, Production of Antioxidant Enzymes, Bioprocessing of Organic Residues and Bioprocesses Automation and Control Using Free Hardware and Software", após terem inquirido o aluno e realizado a avaliação do trabalho são de parecer pela sua APROVAÇÃO no rito de defesa. A outorga do título de doutor está sujeita à homologação do Colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e a pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Dedico esta tese aos mistérios que a motivaram.

ACKNOWLEDGMENTS

This thesis cannot express everything I could learn in this period of study and experimentation. The whole experience gained include the thinkings and feelings shared by all people directly and indirectly connected to this work.

I would like to thank God, nature, my ancestrals and family for the marvelous and unique opportunity of life.

My parents, Paulo and Elizabeth, and grandparents Felipe, Rachel, Humberto and Amenaide, for the excellent education and unconditional love. My brother Bernardo and my sister Paula for the true friendship and companion.

All the teachers and professors that taught me from kindergarten to post-graduation for their patience, knowledge, experience and wisdom.

All great masters, philosophers, poets, scientists, researchers, explorers, discoverers, inventors, creators, cultivators, artisans, engineers and artists whose work and life inspired and motivated my personal journey.

Professor Soccol, for offering the opportunity and structure to produce this thesis and for his vision and advisal.

Professor Cinzia Faraco, for receiving and supervising me in her laboratory, at Universitá degli studi di Napoli.

Professor Luciana, for her serenity and wisdom in the coordination of the post-graduation program.

Professor Vanete, for introducing me to molecular biology techniques and for the wise words in difficult times.

Dr. Herta and Dr. Rosália, for initiating me in scientific practices and in mycotechnology.

Professor Parada, for his patience, creativity and for supporting my return to the laboratory after an hiatus period.

André de Meijer, for sharing his experience and knowledge about mushrooms and nature. Also for the good friendship, the wonderful and educative incursions in Parana's forests and for helping with macromycetes identification.

Fan Leifa for teaching me mushroom cultivation.

Mitiyo Miyaoka, for friendship and for helping with the evaluation of mycelia preservation methods and with antioxidant activities assays.

Gustavo Couto and Ricardo Fendrich for helping with methods to extract, amplify and purificate DNA segments from macromycetes mycelia.

Professor Michele Spier for sharing materials and reviewing this thesis.

Professors Adenise, Júlio, Adriane, Pepe, Paulo, Letti and Miguel for sharing their knowledge and for their attention.

My laboratory colleagues at UFPR for their collaboration and friendship. This includes Denise S., Denise K., Valesca, Ricardo, Daniel, Derek, Camila, Pâmela, Gabriela, Rafaela, Helena, André S., André G., André, Eduardo, Alessandra, Caroline Y., Carolina C., Carol S., Luciana, Sascha, Antônio, Gerardo C., Gerardo T., Luiza, Ana Maria, Maria Rosa, Márcio, Paulo, Fernanda F., Fernanda, Marcelo F., Marcelo B., Bruna, Bruno, Gilberto, Felipe, Darcy, Di Cezar, Rui, Silvana, Ludmilla, Juliana G., Juliana M., Jefferson Coral, Jefferson Costa, Gessiel, Marisa, Carlos, Gisele, Ryu, Francine, Ivo, Sandra, Michele T., Mário, Rafael, Joana, Vanessa, Kely, Otacílio, Tiago, Leandro, Guilherme, Surian, Alejandra, Liceres, Susan R., Susan K.

My friends in Italy and my laboratory colleagues at Universitá degli studi di Napoli for their hospitality and help. This includes: Simona (and Simona's Family), Rossana, Annabel, Lilly (and Lilly's Family), Tiziana, Dr. Sannia, Mr. Marco, Dr. Giardina, Alfredo, Sara M., Sara G., Eugenio, Paula, Giuliana, Mena, Humberto, Francesco, Vincenzo L., Lucia, Gemma, Fortunato, Salvatore and Valério. Thanks also for the support of other important people during my stay at Italy: Luigi, Sidney, Vincenzo, Federica, Federica, Federica, Barlas, Nuno, Christian, Roberto and Lara.

Ilaria, Ricardo and Francesco of Erasmus Point, who helped me finding a nice home in Naples and solving many beaurocratic issues in Italy.

Mrs. Antonieta and Melania for their hospitality and protection, while receiving me in their house in Forcella.

Perditempo, for their good music and library, especially regarding mushrooms. Michele, Trianon and Sorbillo for the best pizzas in the world.

Vesuvius, for not erupting during my stay in Naples.

People at Cogubras, for their help during my post-graduation conclusion.

Denyse Porto, for being at my side all the time, always comprehensive, faithful and lovely.

Thanks CAPES for the financial support.

“Apenas a variedade absorve a variedade”. (BEER, 1979, p.286)

RESUMO

A presente tese de doutorado abrange múltiplas contribuições para o desenvolvimento da micotecnologia. Aproximadamente 70 tentativas de isolamento foram realizadas e pelo menos 15 cepas estáveis foram obtidas. Quatro destas culturas foram identificadas com base no sequenciamento de regiões ITS. Foram desenvolvidos novos métodos para criopreservação de micélios, utilizando vermiculite como suporte. Pelo menos 12 cepas foram reativadas após 12 meses de armazenamento, usando estas técnicas. Duas destas cepas foram avaliadas e mantiveram inalteradas a taxa de crescimento radial (0.58±0.38 cm/ dia) e o rendimento de exopolissacarídeos (250±110 mg/ L). Alguns fatores foram avaliados no cultivo de espécies do gênero *Pleurotus*, utilizando resíduos de pupunha como substrato. A fração externa do resíduo se mostrou excessivamente ácida (pH 3,21±0,15) para o crescimento micelial. *P. ostreatus* (1,38±0,16 cm/dia) e *P. djamor* (1,38±0,1 cm/dia) se adaptaram bem à fração interna do substrato. Taxas de inoculação altas (40%) e maior número de furos (4) produziram aumentos significativos (50%) na concentração protéica dos carpóforos. O processamento de resíduos de pupunha por cultivo micelial melhorou a qualidade deste material como fertilizante para o cultivo de alfaces (até 40% de aumento na produtividade). A cinética de produção das enzimas SOD (superóxido dismutase) e CAT (catalase) foi determinada para o cultivo submerso de *P. ostreatus* (PO). Seis substâncias foram testadas como indutores destas duas enzimas. Sulfonato de lignina 10 g/L resultou em um aumento na atividade de SOD de 700%. A atividade de CAT aumentou em 46% com a adição de H_2O_2 90mM. Duas cepas de *P. ostreatus* (PO e PL24) e três de outras espécies: *P. djamor* (PD), *P. eryngii* (PE) e *P. pulmonarius* (PP) foram comparadas quanto à produção de SOD e CAT em cultivo submerso. PD e PO apresentaram mais que o dobro da atividade de SOD das outras cepas testadas. As atividades de CAT de PE, PL24 e PP foram aproximadamente 59% mais altas que as de PO. PD apresentou atividade de CAT praticamente nula. As mesmas três isoformas de SOD foram encontradas em todas as cepas avaliadas. Técnicas para a recuperação do sulfonato de lignina foram desenvolvidas para purificação parcial de SOD para as análises zimográficas. Este objetivo foi alcançado, mantendo aproximadamente 33% da atividade enzimática inicial. Finalmente, foram desenvolvidos aparatos para cultivo micelial submerso. Hardware e software livres foram utilizados para construir protótipos de biorreatores com sistemas de automação e controle. Um controlador de fotoperíodo, um dispositivo termostático e um sistema de inferência da concentração de biomassa on-line, baseado na absorção de laser, foram projetados, programados e construídos. Espera-se que os avanços propostos contribuam para o desenvolvimento de novos processos, permitindo a obtenção de produtos que tragam benefícios à saúde e à qualidade de vida da população.

Palavras-chave: cogumelos. antioxidantes. SOD. CAT. automação.

ABSTRACT

The present doctorate thesis covers multiple contributions for the development of mycotechnology. Approximately 70 isolation trials were performed and at least 15 stable strains were obtained. Four of these cultures were identified based in ITS regions sequencing. New methods for mycelia cryopreservation were developed, with the use of vermiculite as a carrier material. At least 12 strains were reactivated after twelve months of storage, using these techniques. Two of these strains were evaluated and kept metabolic characteristics unaltered, such as radial growth rate (0.58±0.38 cm/ day) and exopolysaccharide yield (250±110 mg/ L). Several factors were evaluated in the cultivation of some species of the Pleurotus genus, using pejibaye palm sheath residues as substrate. The external fraction of the residue proved excessively acid (pH 3,21±0,15) for mycelial growth. P. ostreatus (1,38±0,16 cm/day) and P. djamor (1,38±0,1 cm/day) adapted well to the internal fraction of the substrate. High levels of both inoculum rate (40%) and number of holes (4) produced significative increases in the protein content (50% increase) of the carpophores. The processing of pejibaye residues by mushrooms cultivation, improved its quality as fertilizer for lettuce cultivation (up to 40% increase in lettuce yield). The production kinetics of the antioxidant enzymes SOD (superoxide dismutase) and CAT (catalase) by the submerged mycelial cultivation of P. ostreatus (PO strain) were determined. Six substances were evaluated as inducers of these two enzymes. Lignin sulfonate was the best SOD inducer and hydrogen peroxide was the best CAT inducer. Lignin sulfonate 10 g/L increased SOD activity in approximately 700%. H2O2 90 mM increased CAT activity in up to 46%. Two P. ostreatus strains (PO and PL24) and three other species - P. djamor (PD), P. eryngii (PE) and P. pulmonarius (PP) - were compared in respect to SOD and CAT production by submerged cultivation. PD and PO presented more than double the SOD activity than the other assayed strains. PE, PL24 and PP CAT activities were about 59% higher than that of PO. PD showed virtually no measurable CAT activity. The same three isoforms were detected for these five strains. Lignin sulfonate recovering techniques were developed in order to partially purificate SOD sufficiently for zymographic methods. This objective was accomplished, maintaining nearly 33% of the enzymatic activity of the product. Finally, apparatuses for macromycetes mycelia submerged cultivation were developed. Free hardware and software were used for building bioreactors prototypes with automation and control systems. A photoperiod controller, a thermostatic device and an on-line biomass concentration estimation system, based in laser absorption were designed, programmed and built. It is expected that the proposed advances can contribute for new processes development, allowing the obtention of products that are intended to bring health benefits and improvements in populations' life quality.

Keywords: mushrooms. antioxidants. SOD. CAT. automation.

FIGURES LIST

GRAPHICS LIST

TABLES LIST

SUMMARY

1 INTRODUCTION

Brazil is notably rich in fungal biodiversity, which stills greatly unexplored in many aspects. Much remains to be studied in respect to native macromycetes, fungi that produce macroscopic fructifications (popularly known as mushrooms). In fact, the traditional Brazilian culture is regarded as mycophobic. That means, great part of the population have fear or repulsion of mushrooms (ARORA, 1986a). In despite of native tribes and colonizers having relatively evolved mycological knowledge, this information was not integrated to the traditional local gastronomy or medicine (FIDALGO, 1985; GÓES NETO; BANDEIRA, 2002; PRANCE, 1984). For a long time, this cultural barrier restricted scientific research of fungi and its results to a little number of individuals, mainly foreign ones. In addition, the pioneer mushroom cultivators in Brazil preferred to protect their knowledge and keep their techniques as a secret. The small production scales forced prices to be excessively high and inaccessible to the lower classes. This also contributed for the maintenance of a mycophobic society.

However, in despite of a great resistance, this scenery is changing, mainly due to cultural globalization. Many new Brazilian research groups focused on multiple aspects of fungi are forming and consolidating. Communities of cultivators are promoting information exchange and technology development, improving quality and reducing prices. Gastronomy courses and communication media are diffusing information about edible mushrooms, including nutritive properties and recipes.

Many cosmopolitan mushrooms' species are found within the Brazilian territory. Some tens of these species are known to be edible and/ or medicinal. However, wild mushrooms consumption is nearly inexistent, due especially to cultural barriers and technical limitations. Little is known about native macromycetes of restricted distribution, however many species present attractive texture and aroma. More studies concerning chemical composition and toxicity should be performed in order to identify potentially edible species (MEIJER, 1999). It is estimated that many edible species exist in the Atlantic Rainforest, taking into account the number of species that are found in mycophilous countries (BOA, 2004). The investment in prospecting new edible species is strategic, because there is a promisor market for wild and cultivated edible mushrooms in the country and more immediately in the U.S., Europe and Asia (BOA, 2004; USDA, 2011).

Nevertheless, much stills need to be explored. These organisms constitute genetic information libraries, devoted to the synthesis of molecules with a wide range of functions and structures. It is possible to affirm that the color and shape diversity in which mushrooms present themselves is reflected in the molecular diversity of their chemical composition. Macromycetes produce enzymes capable to degrade efficiently practically all known classes of biopolymers. They also produce a wide range of molecules with pharmacological and chemical activities and biomaterials with interesting physical properties. Consequently, macromycetes can be applied as tools in biotransformation processes, designed to render highly valued substances from renewable organic resources or even organic residues (PANDEY; SOCCOL, 2000).

Various organic residues are produced in great amounts, in consequence of human activity. Due to a lack of planning or simple negligence, this material is largely discarded and accumulates in the environment. The accumulation of agro-industrial, urban and forest residues constitutes a serious environmental problem, especially for expressive agricultural producer countries such as Brazil.

With the correct management, this enormous amount of organic material can be converted in high value products. Biotechnological processes involving the application of fungi present a great potential, due to the natural adaptation of these organisms to the recycling of organic matter. Biorefinery concepts can be applied in order to obtain multiple bioproducts from the same organic material with virtually zero residue generation. Various organisms should be applied in combination/ sequence in order to accomplish this task. Fungi are fundamental for lignin-cellulose conversion. Fungi based technologies allow the obtaining of a series of products, such as:

- Edible mushrooms, with high nutritional value and functional properties;
- Medicinal mushrooms, with many pharmacological activities;
- Mushrooms extracts and purified substances, to be used as food supplement or as pharmaceuticals and nutraceuticals active principles;
- Cosmetics;
- Biomaterials to substitute Styrofoam and plastics;
- Enzymes for processing biopolymers such as starch, cellulose and lignin;
- Biofuels;
- Mycorrhizal innoculants;
- Products for bioremediation and pests control;

Mushroom cultivation is a promising area, greatly unexplored in Brazil. There are tens of medicinal and edible mushroom species produced in large scale in the world (LEIFA, 2008). Only four among them are commercially produced in Brazil (URBEN, 2004a). Following a trend of healthy nutrition, it is expected that mushroom consumption will rise in the next years, resulting in production growth and lower prices.

Notwithstanding, after composting processes, mushroom spent substrates (residues of mushroom cultivation) can be used as fertilizers for vegetable cultivation, with possible functional properties conferred by the mycelia. This would close an ecological cycle, in which no organic material is discarded: vegetable residues used for mushroom cultivation and mushroom cultivation residues used for soil fertilization.

The present thesis proposes the development of mycotechnologies for the production of antioxidant substances by native macromycetes, using organic residues as substrate. It takes into account the local biodiversity and substrates availability as an extraordinaire opportunity for transformation. It envisions the possibility to generate wealth and improve life quality, while solving a serious environmental issue.

Antioxidants were chosen as interest substances among many other possibilities, due to a social demand for new and better products in this area. Improved health security, stability, low production costs, palatability, aroma and efficacy are some of the desirable characteristics for new antioxidant molecules. There is a wide range of applications for these substances in food, drugs and cosmetics industries.

In despite that there are many other classes of molecules with recognized antioxidant activity, proteins/ peptides were chosen for deeper studies due to the straightforward molecular techniques available for their manipulation and technological application (SON *et al.*, 2010). Proteins/ peptides synthetic pathways and genetic codification and storage mechanisms are well understood. The amino acid sequence can be determined for a given protein/ peptide (SHEVCHENKO *et al.*, 1996). This information can be used for identifying genetic sequences responsible for the codification of the polypeptides in question. Then, those DNA polymers (genes) can be isolated, replicated, sequenced, modified, transferred and expressed using appropriate vectors (AMORE *et al.*, 2012; FARACO *et al.*, 2008).

Well known inducer systems can be exploited for triggering the production of the exogenous protein at a specific growth phase. Also, the expression control mechanisms can be altered, favoring the super expression of the target polypeptide. Genetic sequences can be used for screening databases, searching similar molecules and to make philogenetic analyses (HIBBETT *et al.*, 2007). Rational directed evolution can be applied for enzymatic engineering, aiming the activity/ stability improvement of fungal proteins (MIELE *et al.*, 2010).

Antioxidant enzymes are able to neutralize free radicals, molecules that contribute to aging process and can be responsible for the onset of some diseases. In this sense, they can be the active principles of cosmetics and pharmaceuticals as well as functional components of nutraceuticals. They can also be applied as food and cosmetics additives. Antioxidant substances are useful for maintaining products with fresh characteristics for a longer time. Currently, synthetic antioxidants, such as BHA and BHT are predominantly used for industrial purposes, but new antioxidants from natural sources are desirable for health reasons (MOON *et al.*, 1987). These substances need to be technically effective, safe, stable and the process for their production and purification, economically feasible.

Other possible application is in the field of biosensors. Enzymes such as SOD and CAT, for example, can be used for the detection of superoxide and hydrogen peroxide, respectively (BARTOSZ, 2005).

In parallel to this theme, several contributions were made in related branches of mycotechnology, including cryopreservation, molecular taxonomy, carpophores production, residues bioprocessing and processes automation and control.

Although, in theory, systems developed with tools here described can be designed for industrial-scale applications, they are probably best suited for prototyping applications. Prototypes of interactive, automated hardware and software applications for bioprocesses are highly desirable before investing on the development of tools for end-users. The techniques here described can also be useful for rapidly implementing personalized applications at relatively low costs in laboratories and small factories.

Although intensively studied, macrofungal molecular repertoire still offers a vast universe of largely unexplored biomolecules. Kingdom Fungi is surely replete of useful antioxidant activity, but it is also a great technical challenge to identify and purify the responsible molecules. Only scientific research can bring answers about

which macromycetes' species and derived species can be useful or dangerous, as well as develop cultivation, extraction and purification technologies that will allow humanity to better use these resources. The scientific, environmental, economic and health benefits justify the efforts. The more is known, the more wisely it will be possible to manage the biological patrimony we are conquering and from which we are a part of.

2 OBJECTIVES

The focus of this present thesis consists in the development of processes for the production of antioxidant substances by the mycelial cultivation of native macromycetes, using organic residues as substrate. Due to the complexity and multidisciplinarity of the theme, this technical challenge was decomposed in the following secondary objectives:

- Prospection and isolation of new macromycetes' strains
- Identification and taxonomic classification (including molecular biology techniques)
- Preservation of mycelial cultures (including cryopreservation techniques)
- Evaluation of the growth rate of several mushrooms' strains mycelia, using different pejibaye palm sheath fractions.
- Test of several factors that affect the submerged growth of macromycetes mycelia, using the liquid fraction of pejibaye palm sheath.
- Evaluation of the cultivation of *Pleurotus* spp. edible mushrooms with pejibaye palm sheath as substrate, using an adaptation of the Jun-Cao technique.
- Determination of the influence of inoculation rate and number of package perforations over mushrooms' yield and nutritional composition.
- Production of lettuce, using mushroom spent substrate as soil fertilizer.
- Production of antioxidants by the submerged cultivation of mycelia, using agro-industrial residues as substrate
- Screening of enzymatic activity inducers of SOD (superoxide dismutase) and CAT (catalase) in the submerged cultivation of *Pleurotus* spp.
- Automation and control of bioprocesses for the production of antioxidant substances by the submerged cultivation of mycelia, using free hardware and software as platform

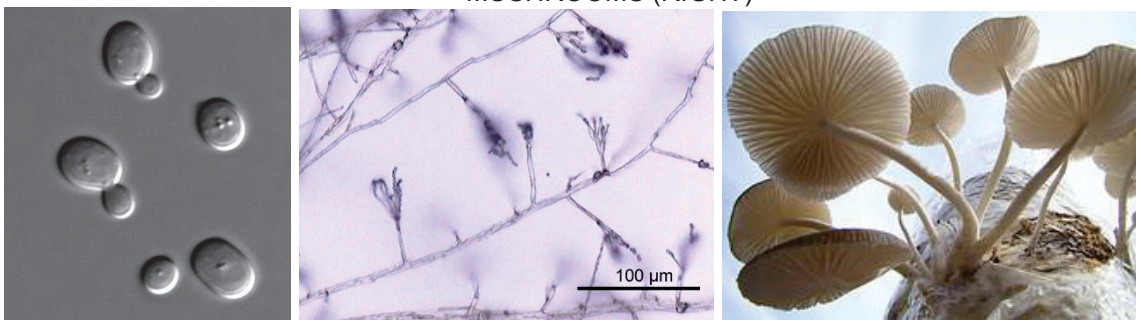
All the research phases were conducted with the central objective of producing substances with antioxidant activity by the processment of organic residues with native Brazilian macromycetes' strains. Notwithstanding, experiments were performed in each step, pursuing the development and improvement of new techniques, useful for other branches of mycotechnology and fungiculture.

3 REVIEW

3.1 FUNGI/ MACROMYCETES

Fungi are organisms with different characteristics from animals and vegetables. They are eukaryotes. They can be aquatic, terrestrial and even aerial. They are achlorophyllous and, consequently, heterotrophic, needing to draw material and energy for their constitution from the decomposition of organic material. They can be saprobes, parasites or symbionts. They are able to perform asexual and, in some cases, sexual reproduction. Some are unicellular, as yeast and others are multicellular, as molds and mushrooms (FIGURE 1). Most of the fungi species are classified as belonging to the Fungi Kingdom, however some are considered by some authors to belong to other kingdoms, such as protozoa and chromista (PUTZKE; PUTZKE, 2013).

FIGURE 1 - YEAST (LEFT) (MASUR, 2010), MOLD (CENTER) (TAMBE, 2005), MUSHROOMS (RIGHT)



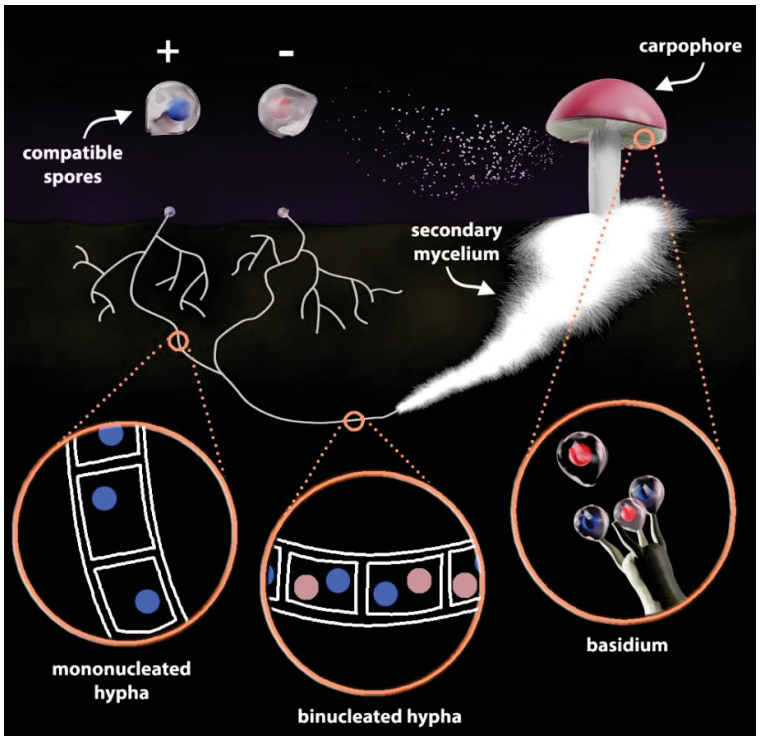
SOURCE: the author (2014).

It is estimated that approximately 1,5 million fungi species exist in our planet. At least 100.000 of these were described (HAWKSWORTH, 1991; HIBBETT *et al.*, 2011) and least than 0,5% of known species are used for industrial processes. At least 4.665 native Brazilian fungi “lato sensu” (4.241 “stricto sensu”) are part of the Species List, organized by the Brazilian Environment Ministery. Among the “stricto sensu” described native species, 206 are endemic (MAIA; CARVALHO JR., 2014).

Macromycetes or macrofungi, popularly known as mushrooms, are fungi that produce fruiting bodies visible to the naked eye. These organisms’ life cycle can be summarized in the following steps: 1. fruiting bodies’ specialized structures produce spores; 2. under adequate nutritional and environmental conditions, spores germinate, originating a web of philamentous haploid mononuclear multicellular

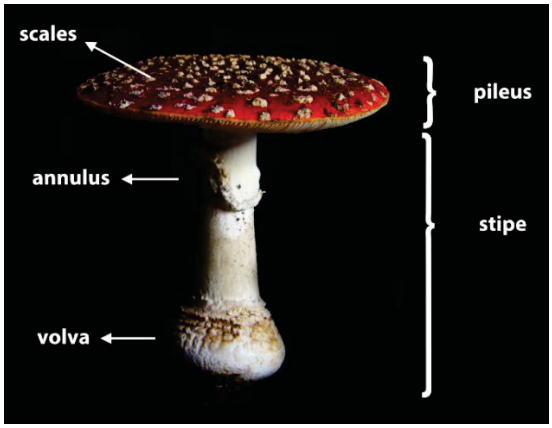
structures called primary mycelium, which secrete digestive enzymes and absorb organic molecules from the substrate; 3. two compatible primary mycelia fuse to form a mycelium with two haploid nuclei per cell, called secondary mycelium; 4. secondary mycelium is able to generate tissues that form fruiting bodies, closing the cycle (FIGURE 2).

FIGURE 2 - SCHEMATIC REPRESENTATION OF MUSHROOMS' LIFE CYCLE.



SOURCE: the author (2014).

FIGURE 3 – BASIC FRUITING BODY'S ANATOMY.



SOURCE: the author (2014).

Mushrooms' morphologies are greatly diversified, but some features can be highlighted as basic anatomic parts of carpophores (FIGURE 3): - pileus (is the hat or head); - stipe (is the stem or foot); - some species present an annulus (ring) around the stipe; - some species present a volva (structure at the base of the stipe); - the region under the pileus is called hymenophore. It can be composed of gills, pores, teeth and other forms (FIGURE 4). Part of the hymenophore, called hymenium, is the place where specialized microscopic structures produce spores.

FIGURE 4 – SOME HYMENOPHORE TYPES. TEETH (UPPER LEFT) (JACOBS, 2009), PORES (UPPER RIGHT), SMOOTH (BOTTOM LEFT), GILLS (BOTTOM RIGHT)

SOURCE: the author (2014).

FIGURE 5 - ASCI (LEFT) AND BASIDIA (RIGHT).

SOURCES: (WERNER, 2008) AND (BROWN; BROTZMAN, 2014).

Macromycetes are classified as basidiomycetes or ascomycetes, depending on the conformation of structures that produce spores. Ascomycetes are fungi that produce spores inside sac structures (asci) and basidiomycetes produce spores over

club-like structures (basidia) (FIGURE 5). Most macrofungi are basidiomycetes (ARORA, 1986b; WRIGHT; ALBERTÓ, 2002).

There are edible and medicinal mushrooms species, as well as toxic and even lethal ones. However, this observation is also valid for all other branches of life, including vegetables, animals and bacteria (LAPA *et al.*, 2007). Although traditional knowledge can give invaluable information related to mushrooms' species characteristics and uses, careful scientific research is required in order to determine edibility, medicinal properties and toxic compounds presence (FDA, 2012).

Tens of mushrooms' species are edible. Others produce hallucinogenic substances, such as psilocybin (*Psilocybe* genus) and muscarin (*Amanita muscaria*) (SATORA *et al.*, 2005; STAMETS, 1996). There are potentially lethal species as well. As an example, some miligrams per kilogram of the substance alfa-amanitin, produced by *Amanita phalloides* (death cap) and *Galerina marginata*, can put an individual's life at risk (KAYA *et al.*, 2013). Among approximately 45.000 described mushroom species, nearly 2.000 are known to be edible, but only about 50 are currently cultivated as food and no more than 10 are expressively popular. At least 4.500 (10%) are considered poisonous and 30 are lethal (BONONI *et al.*, 1995; URBEN, 2004a, 2004b).

Fungi can use amylaceous (OHTA, 1997) and lignocellulosic materials (JIA *et al.*, 1999), as well as chitin (ZHANG *et al.*, 2010) and keratin (EL-GENDY, 2010), just to name a few biological polymers. They can feed on living and dead tissues. They accumulate metals from the environment (HUANG *et al.*, 2012). They can grow both in the absence of free water (solid-state) and submerged in liquid nutrients. Their metabolism support both aerobic and anaerobic routes (NAGPAL *et al.*, 2011).

It is notable that the enzymatic machinery of fungi is adaptable to a large spectrum of substrates and environmental conditions. From an ecological point of view, fungi are possibly the only group of organisms capable of decomposing some organic substances, such as agro-industrial wastes with high cellulose-lignin content (KUBICEK; DRUZHININA, 2007) and recalcitrant molecules (BEHNOOD *et al.*, 2013; SOCCOL *et al.*, 2003; ZEBULUN *et al.*, 2011).

3.2 CARPOPHORES' NUTRITIVE PROPERTIES

Edible mushrooms are a healthy food source (TABLE 1). Their composition depend on the species and on the substrate (BONONI *et al.*, 1995). In general, mushrooms are constituted by 90% water, low sugars and lipids concentration, high protein and fiber content, vitamins (B1 and C), riboflavin, biotin and niacin. They present all the 21 essential amino-acids, are rich in mineral salts and low calory fiber (30 cal/ 100 g of mushroom) (VÁZQUEZ, 1994). It was also discovered that they are able to synthesize vitamin D, when briefly exposed to sunlight (SIMON *et al.*, 2011).

TABLE 1 - PHYSICAL-CHEMICAL COMPOSITION OF MUSHROOMS (DRY WEIGHT).

SPECIES	PROTEIN	TOTAL CARBOHYDRATES	LIPIDS	FIBER	ENERGETIC VALUE
	(%, Nx4,38)	(%)	(%)	(%)	(Kcal/100g)
Agaricus bisporus	23,9-34,8	51,3-62,5	1,7-8,0	8,0-10,4	328-381
Auricularia spp.	4,2-7,7	79,9-87,6	0,8-9,7	11,9-19,8	347-384
Flammulina velutipes	17,6	73,1	1,9	3,7	378
Lentinula edodes	13,4-17,5	67,5-78,0	4,9-8,0	7,3-8,0	387-392
Pleurotus ostrearus	10,5-30,4	57,6-81,8	1,6-2,2	7,5-8,7	345-367
Volvariella volvacea	21,3-43,0	50,9-60,0	0,7-6,4	4,4-13,4	254-374

SOURCE: Trione & Michaels (1977, apud Yokomizo & Bononi, 1985).

Chang & Miles (1989) observe that mushrooms present low lipids concentrations, varying from 1,1 to 8,3%. A greater proportion of insaturated lipids than saturated ones (77,7:22,3) was found in shiitake, from which palmitic, oleic and linoleic acids represent almost the totality of the assayed carboxylic acids (LONGVAH; DEOSTHALE, 1998). Carpophores are rich in mineral salts such as phosphore, potassium, and contain minute amounts of calcium, sodium, zinc and iron. Mushrooms are low calory foods, about 30 cal/ 100 g of fresh mushrooms (BADO, 1994).

TABLE 2 – PHYSICAL-CHEMICAL COMPOSITION OF SEVERAL EDIBLE MACROMYCETES.

SPECIES	PROTEIN (Nx4,38) (% d.w.*)	CARB. (% d.w.*)	LIPIDS (% d.w.*)	FIBER (% d.w.*)	CALORIC CONTENT (% d.w.*)
Agaricus bisporus	23,9-34,8	51,3-62,5	1,7-8,0	8,0-10,4	328-381
Auricularia spp.	4,2-7,7	79,9-87,6	0,8-9,7	11,9-19,8	347-384
Flammulina velutipes	17,6	73,1	1,9	3,7	378
Lentinula edodes	13,4-17,5	67,5-78,0	4,9-8,0	7,3-8,0	387-392
Pleurotus ostreatus	10,4-30,4	57,6-81,8	1,6-2,2	7,5-8,7	345-467
Volvariella volvacea	21,3-43,0	50,9-60,0	0,7-6,4	4,4-13,4	254-374

* dry weight. SOURCE: (AMAZONAS, 1999)

Soluble fiber, such as glucans and insoluble fiber, such as cellulose are found in mushrooms tissues (MANZI; PIZZOFERRATO, 2000) (TABLE 2). Food additives, including flavorizants, aromatizants and vitamins are produced by cultivating the mycelia of macrofungi. For example, niacin (vitamin B3) is produced by the submerged mycelial cultivation of *Pleurotus* spp. Mushrooms are the only non-animal source of vitamin D. Exposing fruiting bodies to UV radiation for several minutes stimulates the production of this substance (FURLANI; GODOY, 2005; MATTILA *et al.*, 2000).

TABLE 3 – COMPARISON BETWEEN THE NUTRITIONAL COMPOSITION OF MUSHROOMS OBTAINED BY DIFFERENT CULTIVATION TECHNIQUES.

	L. edodes			***A. polytricha***			***A. auricula***		
%	***Jun-Cao***	**Sawdust**	**Log**	***Jun-Cao***	**Sawdust**	**Log**	***Jun-Cao***	**Sawdust**	**Log**
Ptn	32,84	28,79	19,65	8,212	7,997	7,376	17,83	9,861	-
Fib.	20,40	17,12	29,81	27,75	19,61	39,80	21,33	13,66	-
Lip.	2,31	2,61	1,710	1,400	0,800	1,200	0,870	0,470	-
Ciz.	9,42	8,02	9,550	9,550	9,620	9,710	9,570	9,480	-
N	5,25	4,61	3,145	1,314	1,280	1,180	2,853	1,578	-
P	0,95	0,86	0,378	0,228	0,195	0,190	0,356	0,36	-
K	1,944	1,45	1,372	1,066	0,829	0,696	1,562	1,690	-
Ca	0,01	0,03	0,023	0,108	0,099	0,249	0,141	0,176	-
Mg	0,14	0,13	0,137	0,15	0,133	0,136	0,128	0,177	-
Cu	15,79	7,10	9,450	2,84	6,720	2,370	2,100	8,680	-
Zn	119,6	74,66	133,2	36,01	39,99	56,96	46,07	69,94	-
Mn	26,88	13,45	16,25	19,43	26,84	26,52	18,75	56,29	-
Fe	101,95	75,12	78,60	98,05	136,37	2480,6	42,09	100,99	-

SOURCE: Lin (1997).

Nutritional contents of carpophores vary depending on the composition of the substrate. Lin (1997) observed that that mushrooms cultivated with Jun-Cao technique (the use of grass materials as substrate) present better nutritional quality than those produced using logs or sawdust. Higher protein, fats, phosphore, potassium and magnesium concentrations are obtained by the Jun-Cao technique than those obtained with other methods (TABLE 3).

3.3 HISTORIC ASPECTS

Literature and history documents point that humans had been using mushrooms for nutritive and medicinal purposes for several millennia. The oldest formal records about mushrooms' medicinal properties are traditional Chinese medicine books, written more than 2.000 years ago (NIE *et al.*, 2013). However, there are hypotheses that this history is even older. The highly conserved mortal remainings of a man who lived circa 3.300 a.C. were found between Italy and Austry. This person carried a bag containing fragments of a non-edible mushroom, possibly for a medicinal purpose (KUTSCHERA; ROM, 2000). In Egipt, mushrooms were destinated for noble people only, and romans considered them Gods' food (RAJARATHNAM; SHASHIREKHA, 2003). Some ancient religions, such as those from the Aztec and Greek used hallucinogenic mushrooms in their rituals (STAMETS, 1996).

In despite of the ancient records of mushrooms' knowledge and use, cultivation was viabilized only much more recently, in post-Christ era. The first cultivated mushrooms recorded were from *Auricularia* genus, in China, by nearly 400d.C. and shiitake (*Lentinula edodes*), around 600d.C. The Paris mushroom (*Agaricus bisporus*) is cultivated only since approximately 1.600d.C. (ROBINSON; DAVIDSON, 1959).

Ganoderma lucidum is a medicinal species, known and used in China, Japan and Korea for millennia. It figures in the most ancient traditional Chinese medicine as a superior medicine, prescribed for treating various diseases, without side effects. Not only medicinal properties were attributed to this mushroom, but even happiness and prosperity woud be conferred to those who consumed it. It is relatively rare in nature and was consequently high priced, destinated to the nobles and richer people.

Shen Nong in his *Pen Ts'ao Jing*, a Chinese text about medicine, with more than 2.000 years, includes references to *G. lucidum*. It says that "the flavor is bitter,

its energy is neutral, does not show toxicity. Cures the accumulation of pathogenic factors in the chest. Is good for heads Qi, including mental activities… The long-term consumption makes the body lighter; you will never grow old. Prolongs the years" (YANG, 1998). The Chinese book Bencao Gangmu (XVI century), shows a connection between modern research and popular knowledge, while describing this mushroom: "affects positively heart's Qi, healing and decongesting the chest. Taken for a long time period, keeps body agility and the years are prolonged..." (LI; LUO, 2003).

The millenar therapeutic use of mushrooms and the new production technologies motivated studies for evaluating the bioactivity of substances produced by those organisms. Many scientific articles confirmed properties alledged by the traditional medicine (BOH *et al.*, 2007; PATERSON, 2006; ROUPAS *et al.*, 2012).

G. lucidum cultivation is currently dominated, stimulating a great number of researches around the world about the diverse medicinal properties alledged by the popular culture (BOH *et al.*, 2007). However, the price of this mushroom stills elevated and available for a reduced number of individuals. This is possibly the most studied medicinal mushroom species in the world up to now. It is known that some substances produced by *G. lucidum* present evident pharmacological effects, with highlights to polysaccharides and triterpenes (YU; WANG, 2013).

Techniques were developed for the production of bioactive substances by the mycelial cultivation of this species in solid and liquid media, shortening production time and standardizing the final products (SUÁREZ ARANGO; NIETO, 2013).

Dry and powdered mushrooms, capsules, liquid and powdered extracts rich in polysaccharides and food products such as chocolate and coffee supplemented with *G. lucidum* are currently commercialized in many countries. However, the production and use of this mushroom and derived substances are not regulamented in Brazil yet.

Ascomycete fungi of the *Cordyceps* genus have been used by Chinese people for millennia. Most of these mushrooms are insect parasites, relatively rare and thus expensive. *Ophiocordyceps sinensis* is a species that naturally occurs in the Chinese Tibetan Plateau and marging the elevated fields of Nepal, Bhutan and India, traditionally used for a series of medicinal applications (SHRESTHA *et al.*, 2010). It is believed that the collection of these mushrooms is making this species rarer. Currently, the cultivation of mycelia and stromata of a closely related species, named

Cordyceps militaris, which produces similar active principles. This viabilize the industrial scale production of these substances (DAS *et al.*, 2010; SHRESTHA *et al.*, 2012).

In 2013, ANVISA (sanitary vigilance national agency) conducted a public survey in Brazil concerning the regulamentation of traditional Chinese medicine products (ANVISA, 2012). The original law project did not have references to any medicinal mushroom, however it is expected that the text will be modified to include these important medicinal substances sources. With the pressure of international groups that desire to introduce products in Brazil, along with Brazilian cultivators, physicians and patients already involved with the production and consumption of these mushrooms and derived substances, it is expected that soon we shall have clear laws for normatizing the products from the traditional Chinese medicine.

Agaricus subrufescens is a mushroom species native to Brazil and of controverse scientific nomenclature, popularly known as sun-mushroom. Studies suggest that the species previously identified as *Agaricus blazei* and later as *Agaricus brasiliensis* can be the same species, which occur in North America with the name of *Agaricus subrufescens*. Independently of the classification, a series of pharmacological properties is attributed to this species by the traditional medicine of Sao Paulo state. Because of this, in the 1960's decade, Japanese researchers took samples of this mushroom to Japan, for being cultivated and its medicinal activities studied.

Immunomodulation related activities were detected, initially by Japanese researchers and after by various research groups around the world, such as: antitumor, antiallergic, anti-inflammatory and resistance enhancement against infections (DALLA-SANTA *et al.*, 2013; FIRENZUOLI *et al.*, 2008; ROUPAS *et al.*, 2012). In clinical studies, NK activity increasing and alleviation of chemotherapy side-effects were observed in patients with cervical, endometrial and ovarian cancers (AHN *et al.*, 2004). Polysaccharides linked to A-PBP proteins presented hypolipidemic and weight control effects via a mechanism involving cholesterol absorption (MEE-HYANG *et al.*, 2002).

Extracts of *A. subrufescens* combined with metformin and gliclazide resulted in an increasing in resistance to insulin in patients with diabetes, due to the increasing of adiponectin concentration (HSU *et al.*, 2007). It was discovered that *A. subrufescens* polysaccharides present antiviral activity, acting at the initial stages of

poliomyelitis virus replication (FACCIN *et al.*, 2007). This mushroom's extracts result in a decrease of aspartate aminotransferase and alanine aminotransferase concentrations, normalizing liver functions of patients with hepatitis B (HSU *et al.*, 2008).

Although its culinary utilization is possible, it is currently cultivated almost exclusively for medicinal utilization, commercialized as dried and powdered fruiting bodies, capsules containing dried powdered mushrooms or polysaccharide extracts. However, in despite of a great number of scientific evidences related to the therapeutic properties of this species, Brazilian laws accepts it only as edible. No pharmacological property can be attributed to this mushroom or derived products, in publicitary material, in Brazil. Current tendencies point that, with the volume of available studies and the market that these substances can reach, procedures for regulamentation of medicinal utilization shall be concluded soon. This case would be pioneer in the validation and regulamentation of mushroom derived pharmaceuticals in Brazil.

3.4 KNOWLEDGE AND UTILIZATION OF MACROFUNGI BY NATIVE BRAZILIAN CULTURES

It is known that native Brazilian cultures were non-mycophilic, in general. Some hypotheses for the origin of this behavior involve these peoples' mythologies and the local abundance of edible vegetables. For the "Caiapó" tribe, mushroom consumption dates from a time when agriculture, animal raising and fire control did not exist. Consequently, individuals of this tribe consume mushrooms only when no other food source is available (GÓES NETO; BANDEIRA, 2002). Similarly, the "Txucarramãe" only eat mushrooms in case of extreme hunger (PRANCE, 1984).

In Paraná, only one species (*Pleurotus pulmonarius*) was reported to be consumed, by the remaining individuals of the "Caingangue" tribe (MEIJER, 2008). However, there are other registers of mushroom utilization by some other Brazilian regions' tribes. The "Ianomami", from the Amazonic region used a wide diversity of species for their nutritive and medicinal properties (PRANCE, 1984). The "Tupi-Guarani" had knowledge over the symptoms caused by the ingestion of several toxic mushrooms species and used some other species for their medicinal properties. In "Mawé" tribe, pregnant women include mushrooms in their diet. The "Tucano"

consume at least two mushrooms' species (*Polyporus sapurema, Poliporus indigenus*), known as Indian bread. The "Caiabi" and "Txicão" present knowledge over some edible and non-edible species (MEIJER, 2008).

3.5 MACROFUNGAL BIODIVERSITY EXPLORATION IN THE ATLANTIC RAINFOREST

A relatively small number of researchers had been involved with the study of the Atlantic Rainforest's fungal biodiversity. The first macrofungi collection records in this region date from the XVIII century, however better documentation only started to appear starting from the XIX century. The first recorded mushroom to be collected in the Brazilian territory was a *Pycnoporus sanguineus* specimen, by the French Philibert Commerson, near to Guanabara bay, in 1767 (FIDALGO, 1985).

Among many foreign botanicals that arrived to Brasil past 1816, some collected and described macrofungi, but only a small number of species in the first decades of work. Some outstanding pioneers include K. F. P. Von Martius (german), C. G. Beaupré (french), W. J. Burchell (Britannic), H. A. Weddell (French), H. W. von Fernsee (Austriac) and A. F. M. Glaziou (French). The Swiss amateur mycologist J. S. Blanchet arrived at Bahia in 1828 and, among other realizations, was responsible for the *Lentinula boryana* (the "American shiitake") type collection (MATA *et al.*, 2001).

In the second half of the XIX and beginning of XX centuries, the collection of macrofungi started to be intensified. The Spanish J. I. Puiggari, who arrived at Brazil in 1877, collected many macrofungi in the frontier between São Paulo and Paraná states, from 1881 until 1889. C. A. W. Schwacke (German), A. Puttemans (Belgian) and the germans F.A.G.J. Möller and E.H.G. Ule also explored the Atlantic Rainforest. The collected material was usually sent abroad, to foreign mycologists, for description and classification. These foreign mycologists were: J. P. F. C. Montaigne (French; 1784-1866), J.-H. Leveillé (French; 1796-1870), M.J. Berkeley (Britannic; 1803-1889), P.C. Hennings (German; 1841-1908), C.L. Spegazzini (Italian; 1858-1926) and Möller himself (1860-1922). Other researchers collected fungi in Brazilian territory in the XX century, however not in the Atlantic Rainforest, but mainly in the Amazonic Forest and some regions of Rio Grande do Sul (MEIJER, 2008).

Otero & Cook (1937) published a bibliography about the entire existent mycological literature of South America and Central America up to their time. Since this, many foreign mycologists collected fungi in the Atlantic Rainforest. The most important were R. Singer (German; 1906-1994) and E. J. H. Corner (Britannic; 1906-1996). Even the small Argentine segment of the Atlantic Rainforest received important foreign mycologists, including R. Singer, besides the Argentine taxonomists J. E. Wright and M. Rajchenberg (WRIGHT; ALBERTÓ, 2002).

Brazilian scientists begun to collaborate in Atlantic Rainforest mycobiota studies only in the XX century. The main representative persons of this phase were A.P. Viégas (1906-1986) and A.C. Batista (1916-1967). Some researchers from the Botanical Institute (SP), founded in 1938, performed important studies about fungi collected in the Atlantic Rainforest and various mycology related subjects. A.R. Teixeira (1918-2003), M.E.P. Kauffmann-Fidalgo (1928-1970), O. Fidalgo (1928-) and J.S. Furtado (1934-) were some of the scientists who belonged to the Botanical Institute and developed macromycetes research (FIDALGO, 1968).

The Dutch researcher Andre de Meijer is currently the most active mushroom taxonomist in Parana's Pine Forests. He is performing a comprehensive and detailed work since 1979 in this region (MEIJER, 2001). He is the author of the most complete list of macrofungi from Parana yet published (MEIJER, 2006).

Nevertheless, many macromycetes' species native to the Atlantic Rainforest have not been scientifically described yet. Many more were not isolated, cultivated and properly studied. There is an enormous chemical and genetic library, constituted by innumerable fungi species, with unimaginable metabolic abilities and immensurable biotechnological potential waiting to be discovered.

It is estimated that only a small fraction of what have been the Atlantic Rainforest remains. Many species were extinct because of a lack of ecological and technological vision in the human management of the biological patrimony.

Forest devastation occurred as a direct consequence of development or as a requisite for progress. However, the value of land spaces or of wood extracted from a forest area is, by far, lower than the value of natural services provided by the respective ecosystem and the value of bioprocesses and bioproducts that can be developed from the biological material contained in the same area, including benefits such as the cure of diseases and reduction of pollution.

There is a great group of processes that can be viewed as services performed by ecosystems and biodiversity. Among these, there are: climate stability, hydric fluxes regulation, pollination, biological control and food production. These services can be quantified with reasonable precision and the respective value can be estimated. In the 1990's the value of US$33 trillion per year was calculated as the sum of all these services. In 2000, IBAMA evaluated that only the services performed by the Brazilian biodiversity represent approximately US$4 trillion per year (PRADO, 2010).

Moreover, biotechnology can find an invaluable biochemical and genetic information source in the forest, for the development of various bioproducts (ex.: pharmaceuticals, food, food additives, biofuels, bioplastic, innoculants, biopigments, bioaroma, bioinsecticides) and bioprocesses (ex.: bioremediation, biotransformations, fermentations, effluents treatment, biological control of pests). Great part of the medicines currently produced in commercial scale originated from biological substances, through scientific research (FRANÇA, 2007).

However, the conversion of wild material into economic value through research is not usually a fast process and demands investment. The research of a single substance can take years or even decades until reaching a commercial opportunity. Without proper investments in qualified researchers, structure and materials, it is improbable that significant technological developments are made. Nevertheless, a single useful biomolecule can yield billions of dollars. Thus, biotechnological research financing can be an interesting long-term strategy for solid and robust institutions such as the government or large companies. Nevertheless, it is a healthy practice for all companies to invest part of their budget in research and innovation.

Within this mentality, the remaining part of the Atlantic Forest can be explored in a sustainable mode, in order to be preserved in a useful way.

3.6 NATIVE MACROFUNGI STRAINS PROSPECTION

More than 100.000 species of fungi have already been described, but it has been estimated that there may be from 1,5 to 5,1 million fungi species presently living in our planet. About 1.200 new species have been described in each year over the last decade. Molecular techniques are rapidly evolving, allowing relatively simple, fast and precise analyses (HIBBETT *et al.*, 2011).

The Atlantic Rainforest is regarded as a main conservation area (MELO *et al.*, 2010). In Mittermeier, Myers & Mittermeier (1999) classification, the Atlantic Rain Forest figures as the sixth richer and most endangered biomes. Relatively recent events drastically altered this ecosystem. Since the end of the XIX century, wood exploitation with the installation of wood processing units, as well as the building of a highway connecting Curitiba and Paranagua's seaport had begun (TRANSPORTES, 2007). The forest devastation rhythm was accelerated in the 1930's with the introduction of coffee cultivation. In the beginning of the 1960's the Brazilian wood exporting was leaded by *Araucaria angustifolia*. Forest coverage dropped to least than 40% of the original area in the 1950's and to 24% in 1965 (SEMA, 2009).

Only 740 km^2 of Araucarias' forest currently remain in good preservation condition in Parana. This represents only 0,6% of the original area (CAMPANILI; SCHÄFFER, 2010; PROBIO, 2001). Field burnings promoted by agricultural producers were one of the main devastation causes. In despite of being prohibited, field burnings were intense in the 1960's, particularly 1963, when 5.000 km^2 of primary forest and 15.000 km^2 of secondary forest were destructed in Parana state (MATTOS, 1972).

Most of the reforestation programs adopted exotic trees' species, such as pinus and eucalyptus. Reforestation with Araucarias did not obtain success yet, especially due to technical difficulties. Nevertheless, Bracatinga, is an example of native species successfully cultivated locally (GRAÇA; MENDES, 1987).

All of these environmental changes reflect in alterations in fungi biodiversity composition. Many species are probably already extinct and some might had their populations augmented or diminished. Besides this, new fungi species were introduced carried by exotic trees brought for reforestation programs. Foreign colonizers also brought spores within their bodies, clothes, objects and pets (WRIGHT; ALBERTÓ, 2002). Moreover, mushrooms' spores are carried by the wind for long distances.

Most of the Mixed Ombrofilous Forest macromycetes are saprophytes. However, there are relatively smaller groups of facultative or obligatory ectomycorrhizal and parasite fungi as well. Some fungi species parasitize plants and others parasitize insects. *Ganoderma* is an example of fungi genus that comprises organisms that are both tree parasites and wood decomposers. Those of the *Cordyceps* genus are adapted to parasitize arthropods and decompose their tissues.

Cases of parasitism between fungi and fungi that are capable of attacking and assimilating living nematodes are well reported as well (MEIJER, 2008).

Wood decomposing fungi can be classified as “white rot” or “brown rot”. White rot fungi are able to produce enzymes to decompose cellulose, hemicellulose and lignin. Brown rot fungi selectively degrade cellulose and hemicellulose, but not lignin (PANDEY; PITMAN, 2003). A small number of brown rot fungi species and a great number of white rot ones are found in Araucarias’ Forest (MEIJER, 2008).

At least 135 macrofungi species were found in *Araucaria angustifolia*’s tissues (118 in Parana state) (MEIJER, 2008). Those fungi are interesting from a technological point of view, for the production of enzymes and bioprocessing of residues. Agro-industrial and wood residues can be converted in high aggregated value bioproducts, ranging from edible mushrooms to ethanol, antibiotics and antitumor substances (SELEGHIM; POLIKARPOV, 2009; SOCCOL *et al.*, 2008; STAMETS, 2005).

It is not a simple task to estimate fungal diversity in the Atlantic Rainforest. It is possible that 8.400 fungi species exist in the Mixed Ombrophilous Forest, and it is estimated that at least 2.000 macrofungi species live in Parana state. Only 976 of these species were scientifically described and no more than some tens were researched in pursuit of technological applications (MEIJER, 2008).

Agaricus subrufescens (= *A. blazei*, *A. brasiliensis*) (popularly known as “sun-mushroom” is the most studied Brazilian native mushroom species. Although the Brazilian laws still not allow any medicinal mushrooms or derived substances, this species and derived products are popularly commercialized for various medicinal purposes. *A. subrufescens* is native to the state of Sao Paulo. In the 1970’s specimens were taken to Japan for scientific research. Some pharmacologic properties were detected, including: immunomodulation e antitumor activities (ALMEIDA *et al.*, 2007; DALLA-SANTA *et al.*, 2004a, 2004b; FAN *et al.*, 2003; LIMA *et al.*, 2008).

The study of macromycetes from the Atlantic Rainforest presents high relevancy, taking into account the high diversity, high endemism, and accelerated loss of habitats of this ecosystem (SCARANO *et al.*, 2010).

3.7 *PLEUROTUS* (E. M. FRIES) P. KUMMER GENUS

Pleurotus genus comprises basidiomycetes naturally found in moist tropical and subtropical forests. They are white-rot decomposers of wood and other vegetal residues (BONATTI *et al.*, 2004). This genus belongs to the *Agaricaceae* Fr. family, *Agaricales* order. Most of the species of this genus are recognizedly edible and widespread over the world, including the Brazilian Atlantic Rainforest (PUTZKE; WARTCHOW, 2010). Edible *Pleurotus* species include: *P. ostreatus* (Jacq.) Quelét (the common white to dark grey oyster mushroom), *P. djamor* (Rumph. ex Fr.) Boedijn (pink oyster), *P. pulmonarius* (Fr.) Quelét (popularly known as sajor-caju, it is the brown oyster), *P. eryngii* (known as the king oyster, it has a fleshier stipe and smaller pileus) and *P. citrinopileatus* (golden oyster) (EIRA *et al.*, 1997).

The following taxonomic classification is currently accepted for *Pleurotus* genus, as updated in the index fungorum page (KIRK; COOPER, 2014):

Kingdom: *Fungi*
Subkingdom: *Dikarya*
Phylum: *Basidiomycota*
Division: *Eumycota*
Subdivision: *Agaricomycotina*
Class: *Agaricomycetes*
Subclass: *Agaricomycetidae*
Order: *Agaricales*
Family: *Pleurotaceae*
Genus: *Pleurotus*

At least 15 intersterility groups of *Pleurotus* species were identified by mating tests, performed by Vilgalys *et al.* (1996). DNA sequencing methods confirmed these results. Gonzales & Labarère (2000) showed that *P. sajor-caju* is a synonym of *P. pulmonarius* and that *P. florida* is a synonym of *P. ostreatus*, using molecular methods.

Pleurotus genus mushrooms, called oyster mushrooms for their pileus similarity with oyster shells form are growing in popularity, due to their outstanding colors and aroma (FURLANI; GODOY, 2005). These mushrooms develop well in nutritionally poor substrates and under rustic conditions (SCHMIDT *et al.*, 2003). This substrate versatility and easiness of cultivation is attracting new producers in Brazil (BONATTI *et al.*, 2004; CONDÉ *et al.*, 2013; EIRA, 2000).

The complete cropping cycle of *Pleurotus* spp. takes approximately 30 days, from the vegetative growth initiation until the first harvest. Up to 3 or 4 harvests are

usual. The substrate does not need to be previously composted, making the cultivation easy (STAMETS, 2000a). *Pleurotus* spp. mushrooms present up to three times the productivity of *Agaricus* genus, due to their rusticity and disease resistance (COLAUTO *et al.*, 1998). The Brazilian consumer is easily accepting its soft flavor (DIAS, 2013).

The oyster mushrooms are good fiber and protein sources, with minerals and vitamins (GUNDE-CIMERMAN, 1999). However, additional studies concerning their nutritional and physiological characteristics are needed (SALES-CAMPOS; ANDRADE, 2011). These mushrooms contain the essential L-aminoacids isoleucine, hystidin, phenylalanine, lysine, methionine, tryptophan, threonine and valine, besides riboflavin and folates (Chang & Miles, 2004; Mattila *et. al.,* 2001).

3.8 MYCOTECHNOLOGY

Mycotechnology is a science branch that includes all technological applications of fungi. Ranging from the traditional fermentative processes, such as those used for alcoholic beverages, bread and cheese to the submerged cultivation of genetically modified mycelia for the obtention of pharmaceutical products. Other examples include edible and medicinal mushrooms cultivation and the production of fermented food, such as shoyu, tempeh and miso (SOCCOL, 1986, 1988). Some industrial inputs, like citric acid and enzymes are also produced in large scale by the cultivation of filamentous fungi (MACIEL *et al.*, 2008; RODRIGUES *et al.*, 2013).

Primary decomposers, such as *Lentinula edodes* and *Pleurotus* spp. can be cultivated in a broad range of substrates, including sawdust, cereal brans, cereal straws, grains, some grass species and wood logs. Other possible substrates include bagasses, pomaces, husks, peels and liquid residues such as molasses and corn fiber aqueous extracts (ARAI *et al.*, 2005; IANDOLO *et al.*, 2011; PANDEY; SOCCOL, 2000; ROBINSON; DAVIDSON, 1959; SOCCOL; LEIFA, 2005). Secondary decomposers, such as *Agaricus bisporus* and *A. subrufescens* require previously composted substrates (pre-decomposed by other microorganisms) (ROSS; HARRIS, 1983).

Brazil is a great producer of wood and agro-industrialized products. Consequently, it is a producer of lignocellulosic residues as well. Many agro-industrial residues are produced in enormous quantities and are highly sub-utilized

and wasted in Brazil. These materials are generally burned for energy generation, mixed to fertilizers or animal feed or simply discarded as ordinary organic waste. This currently represents an environmental and economic problem (PANDEY; SOCCOL, 2000).

Not only agro-industrial and wood residues can be used for mushrooms cultivation, but also, following the Chinese example, several grass species are adequate as substrate as well. Tens of edible and medicinal mushrooms species are currently cultivated using this technique, elegantly called “jun-cao”, in Asiatic countries. “Jun-cao” means literally mushroom-grass in Chinese. Embrapa’s (Brazilian Enterprise for Agricultural Research) researchers successfully tested various Brazilian native grass species for cultivating several mushrooms’ species (URBEN, 2004a).

Asiatic, European and North American countries are responsible for most of the world’s mushroom production. Latin America represents only 1,3% of this market (TAVEIRA; NOVAES, 2007). There is still a strong cultural barrier related to mushrooms consumption in Brazil. Although statistics show a considerable increase in production and consumption in recent years, it is still somewhat restricted to wealthier groups and people with favoured access to education and culture (DIAS *et al.*, 2003).

Besides contributing to organic residues’ recycling and the generation of wealth, mushrooms’ cultivation could improve population’s health, due to their nutritive and functional properties, especially in developing countries, with high desnutrition indexes (EIRA *et al.*, 1997). In addition, the residues generated after mushroom cultivation have incremented nutritive properties in relation to substrates previously to cultivation, due to the generated mycelial biomass and to the substrate decomposition promoted by fungi enzymes, rendering better fertilizers and feed complements (BEUX *et al.*, 1997; FAN *et al.*, 2001; STAMETS, 2000a, 2000b). Fungi are capable of synthesizing and biotransforming molecules, generating original structures, with inherent properties and activities (GUPTA *et al.*, 2011; KELLER *et al.*, 2005).

Biofuels production is a highlighted area nowadays. Ethanol is produced by the fermentation of various substrates by yeast. Usual substrates for this process are rich in mono and disaccharides (SIQUEIRA *et al.*, 2008), however, processes for obtaining the so called second generation ethanol are being intensively researched.

With this technology, it will be possible to obtain ethanol from lignocellulosic substrates, through processes involving both filamentous fungi and yeast (KARP *et al.*, 2012; SELEGHIM; POLIKARPOV, 2009). The enzymatic machinery of filamentous fungi will be explored to break up lignin and cellulose polymers, releasing fermentable sugars for yeast to convert in ethanol.

Some other examples of previous studies related to enzymes produced by macromycetes are listed in TABLE 4.

TABLE 4 - EXAMPLES OF ENZYMES PRODUCED BY MACROMYCETES.

ENZYMES	MACROMYCETE SPECIES	SUBSTRATE	REFERENCE
α-L-arabinofuranosidase	*Pleurotus ostreatus*	Tomato pomace (SSF)	**(AMORE *et al.*, 2012)**
Laccase, xylanase, protease	*Pleurotus ostreatus, Trametes versicolor*	Tomato pomace (SSF)	**(IANDOLO *et al.*, 2011)**
Ligninolytic peroxidases	*Bjerkandera adusta, Pleurotus ostreatus*	Linen sheaves+ glucose/peptone	**(KORNEICHIK; KAPICH, 2011)**
Proteases	*Flammulina velutipes, Pleurotus eryngii, Hypsizigus marmoreus, Grifola frondosa*	Synthetic peptides	**(NAKAMURA *et al.*, 2011)**
Endo-xylanase	*Termitomyces clypeatus*	Oat spelt xylan	**(SOREN *et al.*, 2010)**
Proteases, peptidases, lipases and chitinase	*Cordyceps sinensis*		**(ZHANG *et al.*, 2010)**
Laccase, manganese peroxidase	*Phanerochaete chrysosporium, Pleurotus ostreatus*	Acid dye wastewater	**(FARACO *et al.*, 2009)**
Dye-decolorizing peroxidases	*Pleurotus ostreatus*	Acid dye wastewater	**(FARACO *et al.*, 2007)**
Amylase, CMCase, xylanase	*Lentinula edodes*	Corn fiber	**(ARAI *et al.*, 2005)**
Cellulase, cellobiohydrolase	*Volvariella volvacea*	Cellulose rich media	**(JIA *et al.*, 1999)**

SOURCE: the author (2014).

Macrofungal cellulases can be used as coadjuvants in the extraction of vegetable oils, preparation of dry vegetables, agar-agar production from marine algae, recovering of starch and gums from fibrous residues, garlic pre-processment

for preventing gelification, cell wall dissolution for nutritive applications and as digestive auxiliary in feed and vegetable food (URBEN, 2013).

Processes for producing lignases, celobiases, laccases (phenol oxidases), hemicellulases, Mn peroxidases and phytases from macrofungi mycelia cultivation are also being developed (BONATTI *et al.*, 2004; BONONI *et al.*, 2000; CAPELARI, 1996; DALIMOVA; AKHMEDOVA, 2001; EICHLEROVÁ *et al.*, 2000; KOMURA, 2009; ROSADO *et al.*, 2002; SALMON *et al.*, 2012; SPIER, 2012).

Fungi can be also useful for degrading pollutants in effluent treatment stations or even in environmental accidents, by the utilization of adequate inoculums. Industries are more and more responsibilized and pressed to search alternatives for processing their noxious residues, objectivating the transformation of these into useful or at least unharmful to health and the environment. In some cases, the answer can be provided by mycotechnology (SOCCOL *et al.*, 2003; STAMETS, 2005).

Some other potential technological applications of fungi include biological control of pests (insects and nematodes) and the use of symbiotic ectomycorrhizal fungi as innoculants for vegetables cultivation. Both technologies apply natural ecological roles of fungi: parasitism and symbiosis, respectively.

Several fungi species parasitize different insect species, with an astonishing specialization. This will allow the development of bioinsecticides with more specific action than that of chemical insecticides (SANTA *et al.*, 2009). Macromycetes belonging to *Cordyceps* genus, present in the Atlantic Rainforest (MEIJER, 2008), present great potential for this application. With the same logic, nematophagous fungi are being tested for controlling nematodes populations (BRAND *et al.*, 2010).

The development of mycorrhizal innoculants depends on the improvement of mycelial cultivation techniques. Mycorrhizal fungi mycelia have complex and sometimes unknown nutritive necessities. The success in this area would lead both to healthier and fast growing vegetables, and also to the production of valuable edible mycorrhizal mushrooms. Some commercial mycorrhizal innoculants are already available. The highest prized edible fungi are mycorrhizal, such as truffles, matsutakes, morchellas, chanterelles and porcinis (BIOORGANICS, 2014; HALL *et al.*, 2003).

3.9 PHARMACOLOGICAL APPLICATIONS OF FUNGI

The noblest applications for fungi derived substances are probably found in the pharmaceutical area. There are currently at least 126 medicinal functions associated with substances produced by mushrooms (WASSER, 2011).

Among the promising pharmacological activities presented by mushroom derived substances are: antimicrobian, antiparasitary, antiviral, antitumoral, hypocholesterolemic, hypolipidemic, antiaterosclerotic (RUBEL *et al.*, 2011; SANTOS *et al.*, 2013), hepatoprotector, antioxidant, hypothensive, immunomodulator (resulting in antitumoral activity, augmented resistance against infections and relieve of allergic reactions) (EL ENSHASY; HATTI-KAUL, 2013), anti-inflamatory, antidiabetic (ABRAMS *et al.*, 2011; RUBEL *et al.*, 2004a, 2004b, 2005, 2006, 2012; SOCCOL *et al.*, 2008; VITOLA; ADJAM; FERNANDES; FERNANDES; *et al.*, 2008; VITOLA; ADJAM; FERNANDES; MEIJER; *et al.*, 2008) and modulator of hormones production (DUBOST *et al.*, 2007; FREIRE DOS SANTOS *et al.*, 2012).

The classic example is penicillin, produced by fungi of the genus *Penicillium*, discovered in 1928 and still widely used nowadays. A series of other antibiotics produced by fungi was also discovered. For example: cephalosporin, fusidic acid and griseofulvin. Macromycetes are actually also being evaluated for the production of antibiotics (MOORE, 2001; OLIVEIRA-SOUZA *et al.*, 2007).

Fungi produce many other important substances for medicinal applications. Cyclosporine is an immunosuppressant, for inhibiting transplanted organs rejection. Gliotoxin is an immunomodulator, useful for organs transplantation post-operation period. Mevinolin is a hypocholesterolemic agent and its derivatives pravastatin, lovastatin and simvastatin constitute the active principles of drugs that yielded billions of dollars in the 1990’s (MOORE, 2001; WASSER; RESHETNIKOV, 2001).

Scientifically proven medicinal properties found in mushrooms include anti-tumor, anti-hyperthensive, hypoglycemic, hypocholesterolemic, antiallergic, antibiotic, antiviral, antifungic, neuron growth stimulant and cardiac tonic effects (TABLE 5), promoting physical and mental health and improving life quality (URBEN, 2004a).

TABLE 5 - PHARMACOLOGICAL ACTIVITIES OF MACROMYCETES' SUBSTANCES, CONFIRMED BY CLINICAL ASSAYS

(continues)

EFFECT/ DISEASE	SUBSTANCE	SPECIES	MECHANISM/ DETAILS	REF.
Anti-cancer (gastric)	polysaccharide K lentinan	*Lentinus edodes*	Prolongs survival of patients with metastasis.	(OBA *et al.*, 2009)
Anti-cancer (breast)	Aquous extracts	*Agaricus bisporus*	Suppression of aromatase activity and of tumor cells proliferation. Decrease in estrogen production.	(GRUBE *et al.*, 2001)
	Hydroxilated triterpenes	various	Akt/NF-κB signalling supression.	(JIANG *et al.*, 2008)
Anti-cancer (colorectal)	polysaccharide K (PSK)	*Coriolus versicolor* CM-10	Stimulates inate and adaptative immunological responses.	(OBA *et al.*, 2007; SAKAMOTO *et al.*, 2006)
	Lectin	*Agarius bisporus* (ABL)	Inhibits the proliferation of human tumor cells.	(YU *et al.*, 1993)
	Aquous extract	*Inonotus obliquus*	Induction of the expression of pro-apoptotic proteins and inhibition of the expression of anti-apoptotic proteins.	(LEE *et al.*, 2009)
Anti- cancer (cervical, ovarian, endometrial)	Non-especified extract	*Agaricus blazei* Murill Kyowa (AbMK)	Enhacement of NK cells activity. Alleviation of chemotherapy side-effects.	(AHN *et al.*, 2004)
	Lingzhi Lentinan Clytocinet	*Ganoderma lucidium Lentinus edodes Leucopaxillus giganteus*	Anti-proliferative effects via apoptosis induction.	(CHEN *et al.*, 2010; REN *et al.*, 2008)
Anti-cancer (prostate)	Ethanolic extract	*Ganoderma lucidum*	Improves IPSS (International Prostate Symptom Score) of man with symptoms in the inferior urinary tract, via 5-α -redutase inhibition.	(NOGUCHI *et al.*, 2008)
	Non-especified extract	*Ganoderma lucidum*	Apoptosis induction. Angiogenesis inhibition.	(JIANG *et al.*, 2004; STANLEY *et al.*, 2005)
Anti-cancer (pancreatic – solid advanced)	Irofulven (cytotoxin)	*Omphalotus olearius*. (not edible)	Antitumor activity and positive pre-clinical effects.	(ECKHARDT *et al.*, 2000)

TABLE 5 - PHARMACOLOGICAL ACTIVITIES OF MACROMYCETES' SUBSTANCES, CONFIRMED BY CLINICAL ASSAYS

(continues)

EFFECT/ DISEASE	SUBSTANCE	SPECIES	MECHANISM/ DETAILS	REF.
Immunomodulation	Andosan™	*Agaricus blazei* 82% *Hericium erinaceus* 14.7% *Grifola frondosa* 2.9%	Cytokines production enhancement. Reduced expression of IL-1-β (97%), TNF-α (84%), IL-17 (50%) and IL-2 (46%). Antioxidant activity *in vivo.*	(JOHNSON *et al.*, 2009)
Immunomodulation (hypercholesterolemia)	Alfa-glucans	*Agaricus bisporus*	Decrease in TNFα production induced by lipopolysaccharides of 69%. Decrease in IL-12 and IL-10 production *in vivo*.	(VOLMAN *et al.*, 2010)
Immunomodulation (cancer)	Glucan	*Trametes versicolor*	Survival extending and improved immunological functions.	(RAMBERG *et al.*, 2010)
Immunomodulation (various diseases)	Various extracts	Various species	Effects over NK cells, macrophages, T cells, cytokines production. Activation of mitogenic paths by kinases (MAPK).	(KIM *et al.*, 2007)
Diabetes (type II)	AbM extract (with metformin and gliclazide)	*Agaricus blazei* Murill (AbM)	Insulin resistance enhancement, due to the increasing of adiponectin concentration.	(HSU *et al.*, 2007)
Cardiovascular diseases	Non-especified extract	*Pleurotus ostreatus*	Significative reduction in blood pressure, blood glucose, total cholesterol and triglycerids (*in vivo*)	(KHATUN *et al.*, 2007)
	Protein bind polysaccharides (A-PBP e L-PBP)	*Agaricus blazei Lentinus edodes*	Hypolipidemic effect and weight control mechanism, involving cholesterol absorption.	(MEE-HYANG *et al.*, 2002)
Mental health and cognition	Non-especified extract	*Hericium erinaceus*	Increase in cognitive functions score in people with light cognitive disorders.	(MORI *et al.*, 2009)
	Dilinoleoyl-phosphatidylethanolamine (DLPE)	*Hericium erinaceus*	Protects neurons from the beta-amyloid peptide and oxidative stress. Increase in cognitive functions score of people with dementia.	(KAWAGISHI ; ZHUANG, 2008)

TABLE 5- PHARMACOLOGICAL ACTIVITIES OF MACROMYCETES' SUBSTANCES, CONFIRMED BY CLINICAL ASSAYS

(conclusion)

EFFECT/ DISEASE	SUBSTANCE	SPECIES	MECHANISM/ DETAILS	REF.
Mental health and cognition	Hericenones C - H; Erinacines A-I	*Hericium erinaceus*	Induces nerves growth fator (NGF) *in vitro* and *in vivo*.	(KAWAGISHI; ZHUANG, 2008)
Hepatitis B	Non-specified extract	*Agarius blazei* Murill (AbM)	Decreases aspartate aminotransferase and alanine transferase concentrations, normalizing liver functions of patients with hepatitis B.	(HSU *et al.*, 2008)
	Ganopoly®	*Ganoderma lucidum*	Hypoglycemic, anti-viral and liver protection under chronic hepatitis.	(ZHOU *et al.*, 2005)
Anti-viral (HIV)	1-Farnesil hydroquinone, ganomycine I	*Ganoderma colossum*	HIV-1 protease inhibition.	(EL DINE *et al.*, 2009)
Anti-viral (poliomyelite)	Polysaccharide	*Agaricus subrufescens (= Agaricus brasiliensis*, *Agarius blazei*)	Acts in the initial stage of viral replication.	(FACCIN *et al.*, 2007)
Asthma	Non-specified extract	*Cordyceps* sp.	Inhibits the proliferation and differentiation of Th2 cells and reduces the expression of cytokines in peripheral blood's mononuclear cells. Alleviates chronic inflammation by increasing the concentration of IL-10 (*in vivo*).	(SUN *et al.*, 2010)
Constipation	Fiber	*Auricularia* spp.	Attenuates constipation related symptoms without significant side-effects (*in vivo*).	(KIM *et al.*, 2004)

SOURCE: the author (2014).

Ganoderma lucidum is probably the most studied medicinal mushroom in the world. TABLE 6 lists several additional studies showing active substances found in this mushroom species and their respective pharmacological properties.

TABLE 6 - ACTIVE SUBSTANCES FOUND IN *GANODERMA LUCIDUM* AND THE RESPECTIVE EFFECTS.

SUBSTANCE	EFFECTS	REFERENCE
mycelium	Immunomodulator, antitumor	(RUBEL *et al.*, 2008)
	antioxidant, hypolipidemic hypocholesterolemic	(RUBEL *et al.*, 2011)
triterpenes	anti-inflammatory	(DUDHGAONKAR *et al.*, 2009)
	Nitric oxide production inhibition	(TUNG *et al.*, 2013)
	Adipogenesis inhibition	(LEE *et al.*, 2010)
	Antitumor	(YUE *et al.*, 2010)
polysaccharides	Antiallergic	(JESENAK *et al.*, 2014)
	antihyperglycemic, antihyperlipidemic	(ZHU *et al.*, 2013)
	anti-HIV	(NIE *et al.*, 2013)
proteoglycan	antioxidant, antidiabetic, nefroprotector	(PAN *et al.*, 2014)
peptide glycan	Hypoglycemic	(TOMODA *et al.*, 1986)
Hydroalchoholic extract (70% ethanol)	antiangiogenic, nitric oxide production inhibition	(SONG *et al.*, 2004)
polysaccharopeptides	apoptosis, suppression of Erk1/2 oxidative stress stimulated phosphorilation, resulting in the inhibition of the expression of c-fos and AP-1 and NF-kappaB transcription factors.	(THYAGARAJAN *et al.*, 2006; WAN *et al.*, 2008)

SOURCE: the author (2014).

Many activities attributed to *G. lucidum*'s polysaccharides are mediated by immunomodulator activity, such as antitumor (RUBEL *et al.*, 2008), anti-inflamatory (DUDHGAONKAR *et al.*, 2009), antiallergic (JESENAK *et al.*, 2014) and enhanced resistance to infections. Other properties include: antioxidant, hypolipidemic (RUBEL *et al.*, 2011), hypocholesterolemic, hypoglycemic, antidiabetic (PAN *et al.*, 2014), antiangiogenic and anti-HIV (NIE *et al.*, 2013; PATERSON, 2006).

The active principles of *Cordyceps spp.* are generally extracted from the carpophores, mycelium or liquid cultivation supernatant. Among these substances are intra and extracellular polysaccharides, cordycepin, adenosine, guanosine, cordymine, lovastatin, gamma aminobutyric acid (GABA), ergosta-4,6,8,22-tetraen-3-one (ergone), 5α,8α-epidioxy-22E-ergosta-6,22-dien-3β-ol; 5α, 8α- epidioxy-22E-ergosta-6,9(11),22-trien-3β-ol; 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol; 5α,8α-

epidioxy-24(R)-methylcholesta-6,22-dien-3β-D-glucopyranoside; 6-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol; miriocine; melanine; cordysinine A-E, sitosterol, ergosterol and serine protease (LO *et al.*, 2013). These products were found to have more than 30 benefical medicinal and nutraceutic activities. The main activities are: immunomodulation, antifatigue, immunossupression, antimicrobian, antitumor, anti-inflammatory, antiglycemic, antioxidant, anti-asthma, hormone production stimulation, anti-depressive and anti cerebral ischemia (LO *et al.*, 2013).

Immunomodulator polysaccharides and antioxidant substances are produced by *C. militaris* by mycelial cultivation (ZHANG, L. X. *et al.*, 2012; ZHONG *et al.*, 2009). This species presented hypolipidemic effect and normalized testosterone levels in mice exposed to high lipids content diets (FREIRE DOS SANTOS *et al.*, 2012).

C. militaris extracts showed promisor results for treating asthma: they inhibit the proliferation and differentiation of Th2 cells and reduce the expression of cytokines, by inhibiting the expression of GATA-3 mRNA and stimulating the expression of Foxp3 mRNA in mononuclear cells of the peripheral blood. These extracts alleviate cronic inflammations, by enhancing IL-10 concentrations (SUN *et al.*, 2010).

Studies with *Pleurotus* spp. show their efficacy for cholesterol levels reduction and antihyperlipidemic activity (ABRAMS *et al.*, 2011), due to the effect of lovastatin-like substances. These species also produce antitumor polysaccharides and pleuromutilin, a substance with antiviral effect. *Pleurotus* spp. extracts also showed antimicrobial activity against *Staphylococcus aureus* (BR-BUSINESS, 2014).

Based in information about the traditional use and rigorous experimental results, new products are being developed. In TABLE 7 are presented some patents of products formulated with mushrooms' bioactive metabolites, for preventing and curing human diseases, illustrating the great industrial interests involved.

Many macromycetes species are known to produce polysaccharides with interesting activities over animal immunological systems (RUBEL *et al.*, 2008; WASSER, 2011). Lentinan (produced by *Lentinula edodes*) (CHENG *et al.*, 2006) and PSK (produced by *Trametes versicolor*) (CHOW; CHU, 2006) are examples of polysaccharides, commercially available and medically prescript for cancer therapies in asian countries. But numerous other polysaccharides produced by macromycetes had shown pharmacological properties, such as schyzophyllan (produced by *Schyzophillum commune*), grifolan (produced by *Grifola frondosa*).

TABLE 7- PATENTS RELATED TO PRODUCTS CONTAINING MACROMYCETES' ACTIVE SUBSTANCES.

YEAR	PRODUCT	REFERENCES
2013	*Ganoderma microsporum* immunomodulator protein with antitumor activity.	(KO, 2011)
2013	*Cyathus striatus* antitumor extracts.	(FARES *et al.*, 2011)
2009	*Lentinus* spp. polysaccharides for pharmaceutical, nutraceutical and cosmetic uses.	(ALES *et al.*, 2009)
2009	Polysaccharides produced by solid-state mycelial cultivation for nutraceutical and pharmaceutical purposes.	(CHEN, 2009)
2003	Anhydric cosmetics containing mushrooms' extracts.	(SANDEWICZ *et al.*, 2003)
2003	*Antrodia camphorata* mycelium extracts, composed mainly by polysaccharides with immunomodulator, antitumor and antiparasitary activities.	(CHEN *et al.*, 2003)
2003	Polysaccharide rich extracts of various mushrooms' species, with anticancer, immunomodulator, antioxidant, hypothensive and hypoglycemic activities, mainly for injectable medicines.	(IKEKAWA *et al.*, 2003)
2002	Nutraceuticals containing mushrooms or extracts.	(DONATINI, 2002)
2002	*Tricholoma conglobatum* e *Scutellariae barbatae* proteins with anticancer activity.	(WONG, 2002, 2003)

SOURCE: the author (2014).

Polysaccharides produced by *Ganoderma lucidum* (LEE *et al.*, 1996; RUBEL *et al.*, 2008) and *Agaricus subrufescens* (=*A. brasiliensis*) (FAN *et al.*, 2003) were also intensively studied. These substances modulate the profile of defense cells and cytokines, helping the defense against tumors, enhancing resistance against infections and controlling allergic reactions. Products such as capsules, syrups, powders and injectable ampoules are currently prescribed and administered in parallel to chemotherapy and radiotherapy in some Asiatic countries (CHANG; MSHIGENI, 2001; KRISTIANSEN, 2009).

Fungi specific metabolic abilities can be explored for the biotransformation of molecules. New steroids are already being produced with this concept. Traditional steroids are added to cultivation the medium and the modified version is obtained at the end of the process. Many reaction steps would be necessary for producing the same structural modifications as those rapidly catalyzed by the fungal enzymes, entirely by synthetic means (MOORE, 2001).

Besides strictly pharmacological substances, mushrooms also produce substances that can be useful as food and cosmetics additives, such as fibers, amino acids, vitamins, immunomodulators, enzymes and antioxidants. Those substances are also some of the main active components of new hybrid products called “functional foods” or “nutraceuticals” (RUBEL *et al.*, 2005; SOCCOL *et al.*, 2008; WASSER, 2011).

3.10 ANTIOXIDANT SUBSTANCES PRODUCED BY MACROMYCETES

Several classes of molecules produced by macrofungi were identified as having antioxidant power, such as phenolics, flavonoids, polysaccharides, peptides and proteins as well as mixed classes molecules.

Olennikov, Tankhaeva & Agafonova (2011) reported phenolic compounds as being the most active antioxidant compounds present in *Laetiporus sulphureus* fruiting bodies, including quercetin and kaempferol. Antioxidant peptides were isolated from *Ganoderma lucidum* fruiting bodies by Girjal, Neelagund & Krishnappa (2012).

Native antioxidant enzymes influence mushrooms shelf life. Enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) are known to present antioxidant roles in mushroom tissues (JIANG *et al.*, 2011; LI; ZHANG, 2010). These enzymes were found to be more active in higher O_2 concentrations (WANG *et al.*, 2011). Those antioxidant enzymes are also involved in heavy metal detoxification processes. It was demonstrated that their activity is up regulated under moderated concentrations of metals (ZHANG, W. *et al.*, 2012). Medium composition was found to greatly influence antioxidant activity of substances produced by submerged cultivation of mushroom mycelium (LIN, 2010).

Additionally, other compounds synthesized by mushrooms were found to enhance antioxidant enzymatic activity. It was shown that *Ganoderma lucidum* polysaccharides can stimulate antioxidant enzymatic activity in animal serum (YOUGUO *et al.*, 2009). *Hericium erinaceum* polysaccharides were shown to enhance dermal antioxidant enzymes activities, with anti-aging effects (XU *et al.*, 2010). It was also observed that PSK (polysaccharide K) from *Trametes versicolor* can modulate the expression of SOD in tumoral tissues, possibly through the

modulation of cytokines (lowering TGF-β and increasing IFN-γ) expression (HABELHAH *et al.*, 1998).

On the other hand, previous studies show that macromycetes also usually produce pro-oxidative enzymes, such as ligninolytic peroxidases (KORNEICHIK; KAPICH, 2011). In addition, substances such as the peptidic toxins amatoxins from *Amanita spp.* mushrooms, besides inhibiting RNA polymerases, possibly also cause liver damage by inducing the generation of free radicals. The most effective treatment for this kind of intoxication consists, indeed, of administering silybin, a potent antioxidant (ZHELEVA *et al.*, 2007).

These examples illustrate the complexity of the fungal molecular constitution and the importance of correctly producing, identifying and purifying active enzymes for each desired application, assuring safety and quality for the final consumer.

3.11 FUNGAL SUPEROXIDE DISMUTASES (SOD)

Superoxide dismutases (SOD) are important antioxidant enzymes, which convert superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) (PIPPENGER *et al.*, 1998). Although catalyzing the same type of reaction, there are many types of SOD enzymes, which can present many specific functions, such as being virulence factors in bacteria and resistance factors to diverse substances, including ethanol by yeast and pesticides by vegetables. They are also involved with freezing tolerance by cellular structures. Knockout-mice for SOD enzymes were found to exhibit various consequences, including some associated with aging processes, such as augmented hearing and hair loss, anemia, neurons degeneration, motor disturbances, cardiac abnormalities and premature obit. Extracellular SOD's altered concentrations were found to affect learning and memory cognitive processes (BARTOSZ, 2005).

Fungal SODs appear in three main isoforms, depending on their metal cofactor: manganese (MnSOD), iron (FeSOD) or copper/zinc (Cu/ZnSOD). Fungi typically have both a mitochondrial MnSOD and a cytosolic Cu/ZnSOD, which also appears at the mitochondrial intermembrane space. MnSODs and FeSODs seem to have a common phylogenetic origin due to their sequence homologies, but are considerably different from the Cu/ZnSODs (FRÉALLE *et al.*, 2006).

SODs have multiple roles in fungi. They are mainly related to the dismutation of endogenous superoxide radicals at mitochondria and endoplasmic reticulum, but also

neutralize toxic compounds and ROS produced by other organisms and some contribute to the virulence of pathogenic fungi. Some relations with the lignin degradation processes are also listed in a following section. Polymorphism of SODs is so great among fungi that their genetic sequences can be used as tags for identification to species level with a good resolution. Also, phylogenetic studies of fungal SODs are in course. It was shown that MnSODs of some fungi have lost their signal sequences, and consequently appear at the cytosol. The activity of known fungal SODs vary between 2 and 7500 $U.mg^{-1}$. SOD genes from at least 19 different fungi species were sequenced. A total of 99 fungal SOD sequences could be retrieved from genetic databases by homology. Some fungal SODs were already cloned and expressed in *Escherichia coli* for diverse assays (FRÉALLE *et al.*, 2005, 2006).

Lavelle, Durosay & Michelson (1974) purified SODs from a luminescent mushroom species called *Pleurotus olearius* at that time (now it is called *Omphalotus olearius*). They have analysed physico-chemical and enzymatic properties and discussed the evolution of this class of enzymes.

Öztürk *et al.* (1999) succeeded in purifying and testing the properties of SOD from *Phanerochaete chrysosporium*. It showed a high activity in comparison to other studied prokaryotic SODs. This enzyme has a molecular mass of 44kD and is a dimer of two 22kD subunits. Each subunit exhibits a manganese atom. This SOD is active in alkaline pH (7.0~8.8) (optimum pH at 8.8), but inactivated out of this range. It remained active even at 45ºC, but not above. The best recovery method involved a freeze and thaw cycle followed by grinding with glass beads. Purification was performed by ion-exchange chromatography, followed by gel filtration chromatography.

Cheng *et al.* (2012) analysed SOD activity of 17 different species of mushrooms. They compared the activities of enzymes present in the pileus and stipe of fruiting bodies. They also tested the thermostability of these enzymes. The conclusions of this article include: -most mushrooms are rich in SODs; -these enzymes are resistant to heat; -pileus and stipe show distinct enzymatic profiles and activities; -*Pleurotus citrinopileatus* was among the species with higher SOD activity. Meng *et al.* (2011) also found SOD-like activity in fruiting body extracts of *P. citrinopileatus*.

3.12 FUNGAL CATALASES (CAT)

Catalases are enzymes that catalyze the conversion of H_2O_2 into H_2O and O_2 (PAN *et al.*, 2006). The first studies of this class of enzymes date from the beginning of the XX century. The researcher Oscar Loew was the pioneer in this area, in 1901, working with tobacco tissues (Loew, 1901 apud NICHOLLS, 2012). After this, many laboratories, mainly in the UK, USA and Germany started researching this class of enzymes in many different organisms and tissues. Nicholls & Schonbaum reviewed the theme in 1963 and proposed at that time that catalases were “fossil enzymes”, with little or no use for higher organisms (NICHOLLS; SCHONBAUM, 1963). Since then, more and more important and diverse roles of catalases had been identified in all branches of the evolutionary tree of life as reviewed by Nicholls (2012) himself. Some of these activities include the maintenance of human health and the degradation of vegetable biopolymers by fungi.

Catalases are involved in various fungal processes, including growth, differentiation and spore germination. Differently from animals, that can relatively control their environment and internal composition, fungi must deal with stressing environmental conditions, including the presence of high concentrations of H_2O_2. For example, parasite fungi must face high H_2O_2 concentrations produced by their hosts’ defenses. A high level of oxidative stress is necessary for germination. Oxidative stress also is a signal at the end of the growth phase, inducing the production of CAT and resistance or reproductive structures (HANSBERG *et al.*, 2012). Some fungal CATs relations with lignin degradation processes are listed in the following section.

In a broad sense, catalases can be divided in two groups: LSC (large subunit catalases) and SSC (small subunit catalases). LSC are present only in bacteria and fungi. Ascomycetes present two types of LSC (L1 and L2), while basidiomycetes present only one (L1). Catalases L1 are involved in spore germination and some L2 contain a signal peptide, being excreted to the extracellular medium. L2 is induced upon stress conditions and is associated with growth.

Not only catalases are capable of dismutating H_2O_2, but at least other three groups of enzymes are capable to do it, each one for different H_2O_2 concentrations. Catalases can metabolize H_2O_2 at higher concentrations than other enzymes, although they also have a limit. Most catalases are resistant to heat, salts, solvents

and even maintaining activity after partial digestion with proteinase K. Catalase tetramers are active, but not its constitutive monomers (HANSBERG *et al.*, 2012).

3.13 RELATIONS OF FUNGAL SODS AND CATS WITH LIGNIN DEGRADATION

Lignin and cellulose are the most abundant renewable organic materials in the world. White rot fungi are able to obtain energy and matter from these biopolymers, including cellulose, hemicelluloses and lignin, using molecular oxygen as the final electron acceptor and generating CO_2 as a final product. Because of this, these organisms play a fundamental role in carbon recycling. Lignin is by far the most recalcitrant of the mentioned chemical structures.

P. chrysosporium has been a model organism for lignin degradation studies. Its genome was sequenced. Nevertheless, other biological systems were also assayed and exhibit distinct pathways for this (KANG *et al.*, 2010; KARHUNEN *et al.*, 1990). Kang *et al.* (2010) observed that the set of fungi species involved in wood degradation under natural conditions depended on the type of wood being decomposed.

The real mechanisms for lignin degradation remained a total mystery for many years of research and their complete understanding still a complex challenge. The two main initial hypotheses were that lignin should be attacked by enzymes, or by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl (OH•) (CRAWFORD; CRAWFORD, 1984).

One of the first to propose mechanisms based in ROS was Hall (1980). After his publication, many researchers dedicated their work to investigate the role of these substances in lignin degradation.

Kirk, Tien & Faison (1984) experimentally investigated the importance of H_2O_2 and O_2^- in microbial lignin degradation processes. Their experiments involved the quantification of lignin degradation within *P. chrysosporium* cultures after the removal of these ROS by using enzymatic scavengers, namely superoxide dismutase (SOD) for removing O_2^- and catalase (CAT) for removing H_2O_2. It was observed that the depletion of either of these ROS resulted in a strong inhibition of lignin degradation. They also evaluated the kinetics of H_2O_2, OH• and O_2^- production and its relation with lignin degradation in *P. chrysosporium* cultures. They observed an increase in all of these substances over time. H_2O_2 and O_2^- concentrations increased dramatically in

the third day of cultivation and remained high during the lignin degradation phase (from day 4 to day 6). OH• concentration peaked at day 4 and then decreased to about half of the peak concentration during the lignin degradation phase. They also noted H_2O_2 and O_2^- production were especially increased when the medium was oxygenated instead of aerated. In addition to this elegant display of ROS role in lignin degradation, this seminal work also described for the first time the purification and characterization of an extracellular enzyme that catalyzed at least some steps of lignin degradation. Its molecular mass was determined by gel filtration and SDS electrophoresis to be 42 kD. They have shown that this enzyme requires H_2O_2 in a specific concentration range (0,2~0,5 mM) for exhibiting its catalytic activity. Also they inferred it should be a metalloenzyme, because of its inhibition by azide.

Later it was shown by many researchers that the wood decaying fungi rely on several enzymatic mechanisms for degrading lignin. One electron oxidation reactions catalyzed by oxidases and peroxidases are probably the main path for them to accomplish this task. This lignin degrading machinery features two widely studied groups of catalysts, which are both H_2O_2 requiring extracellular enzymes containing heme groups, called lignin peroxidase (LIP) (LINKO, 1992) and manganese dependent peroxidase (MNP) (GOLD; GLENN, 1988). Peroxidases (POD) decompose H_2O_2 by the simultaneous oxidation of other compounds such as lignin components and antioxidant molecules (PAN *et al.*, 2006). Also, laccases are known to have an important role on this process (JASZEK *et al.*, 2006).

Many studies confirmed the inhibition of lignin degradation by enzymatic systems upon the addition of CAT and SOD, reinforcing the necessity of ROS for the correct functionality of these ligninolytic pathways (KUTSUKI; GOLD, 1982).

The ligninolytic system of *P. chrysosporium* is part of its secondary metabolism and is only activated after nutrient depletion of the culture medium (especially nitrogen). It was demonstrated that LIP synthesis can be induced by nutrient starvation simultaneous to an excess of O_2. This condition leads to oxidative stress, by the accumulation of reactive oxygen species (ROS), which stimulate an antioxidant enzymatic system, including superoxide dismutase (SOD) and catalase (CAT), besides inducing the expression of LIP (MATITYAHU *et al.*, 2010).

Greene & Gould (1983) also observed a correlation between H_2O_2 production by the mycelium of *P. chrysosporium* and ligninolytic activity. They have noticed that the production of H_2O_2 was stimulated when the mycelium was cultivated in nitrogen

limiting conditions and in the presence of lignocellulosic substrates. These authors also found an increased catalase activity within the described cultivation conditions. They also determined that the optimal pH for H_2O_2 production was nearly 6.0.

In contrast it was shown by Kaal, Field & Joyce (1995) that other fungal species produce lininolytic enzymes better in nitrogen sufficient media or even with high nitrogen concentrations. They tested *Pleurotus ostreatus* and *Lentinula edodes*, for being important commercial edible species.

Jaszek *et al.* (2006) evaluated the effect of paraquat over the production of laccases, SOD and CAT by *Trametes versicolor* and *Abortiporus biennis*. Paraquat is known to catalyze the production of superoxide anion radicals and consequently other ROS, generating a stress condition. It was observed that paraquat induced an enhanced activity of both the enzymes of the antioxidant system as well as laccases. No new isoforms of laccase were produced in this condition. The kinetics of the process was that SOD activity was increased prior to the augmentation of the LAC activity. These authors propose that LAC can be induced when the antioxidant enzymatic system is overwhelmed and also that LAC can be part of this antioxidant system, being able to metabolize some toxic compounds. Also they speculate that oxygen generated by the dismutation of ROS by SOD can act as an electron acceptor for LAC. The other product of dismutation, H_2O_2 possibly induced the production of catalases, and can also contribute to the activity of peroxidases. From a general point of view, oxidative stress can modulate the lignin degrading enzymes activity.

Karhunen, Kantelinen and Niku-Paavola (1990) observed a decrease on the activity of a Mn-peroxidase from *Phlebia radicata* on oxidizing various substrates when CAT or SOD were added, thus concluding the dependence of this enzyme on the presence of O_2^- and H_2O_2. The degree of inhibition depended on the substrate being attacked. This enzyme showed dependence for both O_2^- and H_2O_2 to degrade phenolic compounds. In contrast, the oxidation of NADH was not totally inhibited even under high concentrations of SOD or CAT. It was noted this Mn-peroxidase is able to produce O_2^- by the oxidation of NADH and that this radical is further dismutated into H_2O_2. This suggests an explanation for the independence of the enzymatic system of this organism from exogenous addition of H_2O_2 in the degradation of lignin. This studied Mn-peroxidase is able to generate the necessary H_2O_2 for its own catalytic processes and for other peroxidases at the cost of NADH.

Curreli *et al.* (2004) studied *Pleurotus sajor-caju* [synonym of *P. pulmonarius* as demonstrated by Gonzalez & Labarère (2000)] for its oxidative mechanisms, also involved in lignin degradation. Their conclusions reinforce the hypothesis that compounds such as juglone and lignin are not directly degraded by enzymatic attack but rather through reactive oxygen species, especially hydrogen peroxide, generated by a multitude of enzymatic and non-enzymatic mechanisms. They didn't measure SOD or CAT activities in their mycelial cultures, but they quantified the effect of SOD and CAT purified enzymes addition to the culture medium over juglone degradation. Their results show that SOD addition doesn't affect the process significantly, but CAT addition clearly decreases juglone degradation rates, reaffirming that H_2O_2 should be involved in the mechanisms of oxidation of substrates such as juglone and lignin.

By their turn, Belinky, Flikshtein & Dosoretz (2006) were able to decrease MnSOD activity in mycelial cultures of *Phanerochaete chrysosporum*, by using Mn free media. This resulted in the accumulation of ROS and in the increase in both CAT activity and lignin-peroxidase expression, postulating that ROS can at least partially modulate the expression of LIP enzymes. A similar effect was achieved by increasing O_2 concentrations, reinforcing the role of ROS in the induction of LIP enzymes.

Matityahu, Hadar & Belinki (2010) proposed a mechanism for the induction of the ligninolytic enzymatic complex by hydroxyl radicals (OH•) instead of H_2O_2. According to this article, high levels of OH• repress the expression of the protein kinase C (PKC) and stimulates the expression of MnSOD1, LIP and CAT.

Other enzymatic mechanisms for lignin degradation by litter decomposing fungi, independent of H_2O_2 were shown by Kapich *et al.* (2005). Lignin degradation is better described as a complex process with the intervention of multiple chemical, physical and enzymatic components, as suggested by various articles, such as Karhunen *et al.* (1990), Rothschild *et al.* (2002), Kaal *et al.* (1995) and Reddy (1993).

Zámocký *et al.* (2012) characterized a bifunctional (catalase-peroxidase) from *Magnaporthe grisea* phytopathogenic fungi. This group succeeded in expressing this enzyme in *E. coli* and studied its catalytic activities. It was shown that this same extracellular enzyme is capable of oxidizing a series of substrates and also has a catalase activity. It was observed that catalase activity increased at low pH. Their hypothesis is that this catalase activity is necessary for protecting the mycelium from an oxidative burst produced by the plant host during infection.

3.14 OTHER MISCELLANEOUS INFORMATION ABOUT SODS AND CATS FROM FUNGI

Pathogenic and parasite fungi have evolved special antioxidant systems to face the oxidant defense mechanisms of their hosts. *Aspergillus fumigatus*, for example, is known to produce at least three different catalases and these are encoded by at least seven different regions of its DNA. SODs also appear redundantly in pathogenic fungi: four Cu/ZnSODs and two MnSODs genes were found in the genome of *Candida albicans*, which is currently completely sequenced. Fungi that are able to infect human tissues evolved their enzymatic systems to resist 37ºC temperatures and to face ROS produced by macrophages (FRÉALLE *et al.*, 2005).

Zharare, Kabanda & Poku (2010) tested eight strains of the genus *Pleurotus* (5 different species) for their tolerance to H_2O_2. *P. sajor-caju* (=*P. pulmonarius*) was the species which tolerated the highest H_2O_2 concentrations.

Native antioxidant enzymes influence mushrooms shelf-life. Enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) are known to present antioxidant roles in mushroom tissues (JIANG *et al.*, 2011; LI; ZHANG, 2010). These enzymes were found to be more active in higher O_2 concentrations, leading to the development of O_2 enriched packages for longer conservation of mushrooms (WANG *et al.*, 2011).

Xiong *et al.* (2009) tested the effect of γ-irradiation over the SOD and CAT activities present in *Pleurotus nebrodensis* fruiting bodies during post-harvest storage. There was a natural decrease in both enzymatic activities during time and radiation exposure increased the inactivation rate only slightly.

Those antioxidant enzymes are also involved in heavy metal detoxification processes. It was demonstrated that their activity is up regulated under moderated concentrations of metals (ZHANG, W. *et al.*, 2012).

3.15 MEDIA PREVIOUSLY REPORTED FOR ANTIOXIDANTS PRODUCTION BY FUNGI

Medium composition was found to greatly influence antioxidant activity of substances produced by submerged cultivation of mushroom mycelium (LIN, 2010). Kuforiji & Fasidi (2008) have shown that enzymatic activities of *Pleurotus tuber-regium*, including CAT vary depending on the substrate used for cultivation, which

corroborates the vision of Greene & Gould (1983). Kang *et al.* (2010) noted that the enzymatic profile expressed by ligninolytic fungi under natural conditions also depended on the type of substrate. Some media compositions previously reported are listed in TABLE 8.

TABLE 8 - MEDIA REPORTED FOR THE PRODUCTION OF ANTIOXIDANT SUBSTANCES BY THE MYCELIAL CULTIVATION OF VARIOUS FUNGI SPECIES.

(continues)

SPECIES	MEDIA COMPOSITION	REFERENCES
Pleurotus abalonus and P. geesteranus	% d.w. - asparagus straw (90%), wheat bran (5%), corn meal (3%), gypsum (0,8%), supplemented with maltose or sucrose (1%) and MgSO4 (0,2%)	**(WANG *et al.*, 2012)**
Pleurotus ostreatus, Hericium erinaceus, Agrocybe chaxingu, Auricularia auricula and Cordyceps militaris	5% (w:v) wheat bran solution, 0.1% (w:v) magnesium sulfate, 0.1% (w:v) potassium dihydrogen phosphate and 0.01% VitaminB1.	**(XIE *et al.*, 2010)**
Pleurotus nebrodensis, P. eryngii* and *P. cornucopiae	(g/l): potato, 200; glucose, 20; yeast extract, 3; KH_2PO_4, 1;$MgSO_4 \cdot 7H_2O$, 1, natural pH.	**(LIU *et al.*, 2010)**
Lentinus edodes	Defatted soybean powder	**(MCCUE *et al.*, 2004)**
Agaricus brasiliensis	(g/L): glucose, 40; peptone, 3; yeast extract, 3; $KH_2PO_4 \cdot H_2O$ 0.5; and $MgSO_4 \cdot 7H_2O$, 0.3	**(CARVAJAL *et al.*, 2012)**
Trametes versicolor	(g/L): glucose 20; L-asparagine 2,5; D, L-Phenylalanine 0,15; Adenine 0,0275; Thiamine-HCl 50.10^{-6}; KH_2PO_4 1; $Na2HPO_4 \cdot 2H_2O$ 0,1; $MgSO_4 \cdot 7H_2O$ 0,5; $CaCl_2$ 0,01; $FeSO_4 \cdot 7H_2O$ 0,01; $MnSO_4 \cdot 4H_2O$ 1.10^{-3}; $ZnSO4 \cdot 7H_2O$ 1.10^{-3}; $CuSO_4 \cdot 5H_2O$ 2.10^{-3}; pH5 After 10 days, paraquat was added to 25uM final concentration.	**(JASZEK *et al.*, 2006)**
Phialosimplex sp.	Malt extract broth (MEB) with 15% and 25% NaCl, amended with lysine.	**(RAVINDRAN *et al.*, 2012)**

TABLE 8 - MEDIA REPORTED FOR THE PRODUCTION OF ANTIOXIDANT SUBSTANCES BY THE MYCELIAL CULTIVATION OF VARIOUS FUNGI SPECIES.

(conclusion)

SPECIES	MEDIA COMPOSITION	REFERENCES
Blakeslea trispora	(g/l): glucose (Scharlau, GL 0129) 50; corn steep liquor (Sigma, S-4648) 80; yeast extract (Scharlau, 07-079) 1.0; casein acid hydrolysate (Scharlau, 07-151) 2.0; L-asparagine (Sigma, A-8381) 2.0; KH_2PO_4 (Merck, 4873) 1.5; $MgSO_4.7H_2O$ (Merck, 5882) 0.5; thiamine.HCl (Sigma, T-4625) 0.005; linoleic acid (Sigma, L-1626) 20.0; Span 20 (Sigma, S-6635) 10.0; and butylated hydroxytoluene (BHT) (Sigma, B-1378) 4.4.; pH 7.5	**(NANOU; ROUKAS, 2011)**
Aspergillus phoenicis	modified potato dextrose broth (PDB) (w/w): 15% glucose, 0.25% $MgSO_4 \cdot 7H_2O$, 0.5% KH_2PO_4 and 2% beef extract	**(LAI *et al.*, 2009)**
Aspergillus niger	Immobilization with 3% Na-alginate and then: (%w/v): apple pectin with 75% degree of esterification (Pectin PLC, Pernic, Bulgaria), 1.0; NH_4NO_3, 0.75; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_20$, 0.001; pH 4-8-5.0; $CaCl_2$ (0.01%)	**(ANGELOVA *et al.*, 2000)**
Humicola lutea	(g/L): glucose, 48.0; casein, 3.0; soybean flour, 4.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.0011; $ZnSO_4 \cdot 7H_2O$, 0.0029; $FeSO_4 \cdot 7H_2O$, 0.0043; $MnSO_4 \cdot 7H_2O$, 0.0013. Fed-batch: adjustment to 7,5mg/mL (daily)	**(KRUMOVA *et al.*, 2007)**
Phanerochaete chrysosporium	Ultra-filtered apple pomace sludge, pH 4.5	**(GASSARA *et al.*, 2012)**
Rhyzopus spp.* and *Aspergillus spp.	Black beans, soaked and cooked.	**(LEE *et al.*, 2008)**

SOURCE: the author (2014).

3.16 CULTIVATION OF MUSHROOMS OF THE *PLEUROTUS* GENUS ON *BACTRIS GASIPAES* RESIDUES FOR THE PRODUCTION OF CAT AND SOD

No previous literature is reported within this specific combination of organism-substrate for the production of antioxidant enzymes.

Wang *et al.* (2012) used asparagus straw for the cultivation of two *Pleurotus* species. They observed that the antioxidant activity of mushrooms extracts depended

on the species. They have reported that the addition of some saccharides and salts improved the antioxidant activity of the product (TABLE 8).

Lee *et al.* (2007) found higher antioxidant activities on extracts of *Pleurotus citrinopileatus* fruiting bodies than in the respective mycelium extracts.

McCue, Horii & Shetty (2004) observed a mobilization of antioxidant phenolics from soybean powder within solid-state fermentation by *Lentinula edodes*. This improvement was correlated with a higher activity of laccases. Their hypothesis is that lignin-degrading enzymes are able to release phenolics present in soybeans.

Carvajal *et al.* (2012) analyzed the antioxidant activity of *Agaricus brasiliensis* (=*A. subrufescens*) extracts. They tested both fruiting body and young/ old mycelium extracts and noted that the results depended on the analytic methodology. With DPPH methodology, the activities were: fruiting body> old mycelium> young mycelium. With ABTS, the observed activities were: old mycelium> young mycelium> fruiting body.

Lai *et al.* (2009) optimized the production of SOD by submerged fermentation of fungi. After screening tens of fungi strains, they selected *Aspergillus phoenicis* as the greatest producer of SOD-like activity compounds. Their optimized culture conditions included using a modified potato dextrose broth (PDB) medium, at 30ºC and pH5. They substituted yeast extract and peptone by beef-extract to lower the cost of the medium, with no changes in SOD-like activity. The addition of molasses favored mycelial growth, but didn't affect enzymatic activity. It was noticeable that SOD-like activity was about 20 times higher in bioreactors with aeration (0,4 L/min.) and stirring (400 rpm) than that obtained in Erlenmeyer flasks incubated in shaker (150 rpm). This shows that oxygenation is an important factor for the induction of antioxidant enzymes.

Nanou and Roukas (2011) argument that adequate agitation and aeration help the maintenance of a concentration gradient of substrate, products and byproducts between the interior and exterior of fungal cells. This concentration gradient would facilitate mass transport phenomena, such as the removal of gases and byproducts of fungal catabolism from the microenvironment of cells. They point sufficient aeration as extremely important for the progress of mycelial cultivation. Not only a higher biomass development was reported with sufficiently aerated media, but also the production of carotenes, SOD and CAT were improved. These authors have optimized their culture conditions in Erlenmeyer flasks, varying Va/Vm (air volume/

medium volume) rates. The best Va/Vm for this process was 9,0. Above this, enzymatic activity was inhibited. They noted that at a Va/Vm of 15,7 mycelia formed large pellets with low roughness, possibly in order to difficult oxygen diffusion to the intracellular environment.

Krumova *et al.* (2007) reported higher production of SOD in large bioreactors than in Erlenmeyer flasks. They optimized SOD production by using fed-batch technique, adding glucose daily. Within this they could obtain the highest enzymatic activities within 36~72 hours of fermentation. In batch culture this maximum was only obtained in late stationary phase, nearly after 120 h of fermentation.

Angelova *et al.* (2000) were able to improve SOD (more than 1,5 fold) and CAT (moderately) production by immobilizing *Aspergillus niger* cells. Both enzymes production peaked at about 36 h and then decreased. The effects of adding viscous additives were also assessed. For free-cells, these additives improved SOD activity, but none affected immobilized cells. These authors also tested the inhibition of SOD production by adding antibiotics to the culture medium. Inhibitors of DNA replication didn't affect the process, but inhibitors of transcription and translation totally blocked and significantly decreased (70%) SOD accumulation, respectively.

Ravindran *et al.* (2012) studied a marine pathogenic fungus from the *Phialosimplex* genus for antioxidant enzymes production under saline stress. They were able to achieve up to fifteen fold higher concentrations of SOD using media containing 15% (m/v) NaCl than those reported with sea water or 3,5% NaCl media. They also observed the highest concentrations of CAT using 15% and 25% NaCl containing media in intra and extra-cellular media, respectively. Also they observed that an additional isoform of CAT is expressed under high salt concentrations. In all conditions, the extra-cellular medium showed higher SOD and CAT concentrations than the intracellular material. These authors hypothesized that the enzymes are probably excreted to act against ROS generated by high salt concentrations.

Gassara *et al.* (2012) reported a similar enhancement on available antioxidant phenolics, correlated with ligninolytic enzymes expression, by cultivating the fungus *Phanerochaete chrysosporium* on liquid apple pomace residues. This increasing occurred until 67 hours of fermentation and then both antioxidant phenolics and enzymatic activities decreased. They found a greater activity using this residue than with a synthetic medium previously used by Tien & Kirk (1988).

Lee *et al.* (2008) fermented black beans in solid-state, with fungi from *Rhyzopus* and *Aspergillus* genera. Fermented products showed a higher antioxidant activity than the original substrate. This activity was attributed to a higher content of phenolic substances and anthocianins, which should had been released from the substrate by enzymatically catalyzed reactions.

Other authors attributed enhanced antioxidant activity of substrates after processing with fungi or derived enzymes to diverse substances. These metabolites include quercetin (YANG *et al.*, 2012), ferulic acid (XIE *et al.*, 2010), polysaccharides (LIU *et al.*, 2010) and carotenes (NANOU; ROUKAS, 2011).

Wu *et al.* (2013) noted a higher CAT activity on citrus plants inoculated with the mycorrhizal fungi *Diversispora spurca*.

3.17 MACROMYCETES' STRAINS ISOLATION TECHNIQUES

A new mushroom strain can be isolated starting from virtually any point of its life cycle: mycelium, carpophore's tissues or spores. However, it is usually difficult (although not impossible) to isolate pure cultures from mycelium found in nature, because of its intricate association to the substrate, which is usually plentiful of other microorganisms. Consequently, the most usual techniques for isolating new macromycetes strains are those that start from spores or carpophores' tissues. Each with its own variations and peculiarities.

There is no universal method. The best choice depends greatly on the mushroom's species, size, maturity stage, cleaness and freshness. Nevertheless, it is difficult to write down rules for chosing the best method. Experience makes great difference, but some informations can contribute for increasing chances of success.

New strains are generated by primary mycelium fusions when starting from spores. Differently, clones from the original mushroom are obtained when starting from carpophores' tissues. Viable spores can be stored for long periods, but carpophores' tissues are rapidly decomposed. The spore prints, used for isolation and storage, can be useful for species identification as well (the spore print color and printed hymenium morphology can be distinctive elements).

All available methodologies involve the aseptic transference of the fungal structures to adequate cultivation media.

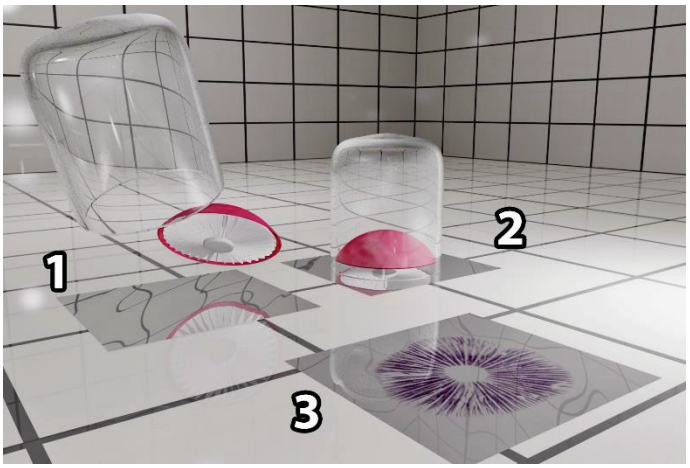
When starting from carpophores' tissues, the healthiest individuals, presenting the most desirable tracts should be chosen, because clones of the original mushroom are obtained. It is better not to wash the material previously to the isolation procedures, because the water can carry contaminations to the inner parts of the fruiting bodies and sanitizing agents can inviabilize fungi cells. When necessary, some parts can be cut and a superficial dry cleaning can be performed to eliminate dirt and possible contaminations. With the superficially clean mushroom, a longitudinal small incison is made with a flamed scalpel, and the carpophore is rip in two by hands. This is made also in order to avoid carrying surface contaminations to the inner tissues. After flaming the scalpel again, a little uncontaminated piece of mushroom's inner tissues is transferred to cultivation medium previously prepared and distributed in Petri dishes.

For spores' collection, a glass campanule and sterilized aluminum foil can be used. Pileus is positioned with the hymenium downwards. Whenever possible, it is advisable to cut the stipe in the form of a support to position the cap so as the lamellae do not touch the aluminum foil. A piece of wire can also be used to help positioning the mushroom. This assemble must be covered with a campanule, to avoid both spores to be dispersed by the airflow and external contaminants to mix with spores. If the fruiting body is relatively dry, a piece of paper soaked with distilled water can be put inside the campanule to keep the humidity high. At the correct maturity phase the mushroom will liberate the spores, making a spore print over the aluminum foil (FIGURE 6).

Some species, such as *Ganoderma lucidum* do not sporulate after being picked. In this case, an adaptation of this apparatus must be installed for collecting spores within fruiting bodies still attached to the substrate.

After spores collection, the system can be disassembled and the carpophore discarded as ordinary organic trash. The campanule and the wire can be washed and used again. The aluminum foil containing the spore print can be stored inside sealed autoclaved test tubes. In order to be reactivated, spores must be transferred aseptically to adequate cultivation media, distributed in Petri dishes and incubated in the dark.

FIGURE 6 - SCHEMATIC REPRESENTATION OF SPORE COLLECTION TECHNIQUE: 1- AUTOCLAVED GLASS CAMPANULE AND ALUMINUM FOIL, PILEUS WITH HYMENIUM POINTING DOWNWARDS, CUT STIPE. 2- ASSEMBLED SYSTEM FOR SPORE COLLECTION. 3- SPORE PRINT.



SOURCE: the author (2014).

At least two different techniques can be used for spreading the spores over the cultivation medium:

1- Wash the aluminum foil surface with distilled water and distribute droplets of the obtained suspension over fresh cultivation medium, with a sterilized pipette.

or

2- Use a platinum loop as following: flame to red and let cool in contact with the semisolid cultivation medium; previously to complete resfriation (approximately 42ºC), pass the loop with some adhered gel, over the surface of the spore print; a great amount of spores stick to the gel; use striation technique to spread the spores across the cultivation medium and separate contaminants from viable mushrooms' spores.

Medium composition and incubation temperature depend on the species. Most macromycetes' species mycelia grow well under 25ºC. Usual cultivation media include PDA (potato dextrose agar) and MEA (malt extract agar) for saprotrophic mushrooms and MMN (Modified Melin Norkrans) for mycorrhizal ones.

Independently from the starting material and chosen technique, some contaminations are likely to appear in the first Petri dish. Pieces of healthy mycelium must be transferred from uncontamined areas to fresh medium in order to completely eliminate contaminants and obtain a pure vigorous mycelial culture. Several transferrences can be necessary before obtaining pure cultures.

3.18 IDENTIFICATION/ MOLECULAR IDENTIFICATION OF MACROMYCETES

Classical macromycetes' identification methods are based in fruiting bodies and spores morphology, ecological habits and biochemical properties. However, mushrooms' cultures are usually maintained in secondary mycelium phase and some of these cultures are not readily fructifying. Mycelia characteristics allow the classification of fungi in large taxonomic groups, though a precise species identification is not possible exclusively based in mycelial morphology/ biochemistry. Molecular biology techniques can help to solve this problem (HIBBETT *et al.*, 2007).

With a small mycelium mass (less than 1 g) it is possible to extract DNA for identification. The polymerase chain reaction (PCR) technique allows the generation of a great number of copies of an interest DNA segment in a few hours, starting from a single DNA molecule, without using cells. It is possible to analyze samples containing small DNA quantities with this technology (KLACZKO; VIEIRA, 2006).

Various techniques can be applied to the extracted DNA. Relations among species in several genera of basidiomycota had been established by RFLP (restriction fragment length polymorphism). Examples include species of the genera *Laccaria* (GARDES, 1990) and *Armillaria* (SMITH; ANDERSON, 1989).

Powerful methodologies for species identification and classification are based in genetic sequencing. It is still not viable to compare complete genomes, due to the high costs and complex and time-consuming work. An alternative consists in sequencing and analyzing small segments. However not all genome regions are equally useful for taxonomic purposes: there are regions that are too conserved, identical in various species; in the other hand, there are regions that are too variable, even among individuals of the same species. Consequently, the ideal regions for taxonomy are those with a variation degree that allow identification to species level.

Gonzales & Labarère (1998) showed that rRNA sequences are useful for macromycetes species identification. These researchers analysed phylogenetic relations among *Agrocybe* genus species. The same researchers also solved ambiguities in *Pleurotus* genus, showing that *P. sajor-caju* is a synonym of *P. pulmonarius* and *P. florida* is a synonym of *P. ostreatus*. They analysed three domains of the mitochondrial small-subunit (SSU) rRNA (GONZALEZ; LABARÈRE, 2000).

Ribosomal DNA (rDNA) regions (DNA sequences that codify the synthesis of ribosomal RNA) had been extensively used for macromycetes identification and phylogenetic analyses. Internal Transcribed Spacers (ITS) regions of rDNA were used for the present studies. These regions are used for taxonomy programs worldwide, for being good species identification tags. Abundant quantities of ITS sequences of various species are available in free access data banks, allowing the identification of organisms to species level with small probabilities of error.

Ganoderma genus species relations were analysed by ITS region sequences comparison (MONCALVO *et al.*, 1995). The same was made with *Pleurotus* genus species by Vilgalys & Sun (1994).

3.18.1 Polymerase Chain Reaction (PCR)

This technique, developed by Kary Mullis in 1983 allows *in vitro* DNA replication. Minimal amounts of genetic material can be amplified millions of times in a few hours (MULLIS, 1990). PCR reaction follows the basic replication steps:

1. Denaturation (opening of the double strands, resulting in single strands)
2. Hybridization (annealing of primers)
3. Extension (addition of deoxynucleotides by DNA polymerase)

Diferently from natural processes, in which enzymes perform all the tasks, some DNA manipulations in PCR are performed by temperature control operations. Hydrogen bonds, responsible for the interaction between strands of double stranded DNA, are broken at high temperatures (>90ºC), in a process called denaturation. Opposedly, complementary DNA strands can hybridize (anneal) at lower temperatures (40~65ºC). When initially developed by Mullis, the reaction was performed by an *E. coli* DNA polymerase. This enzyme is not sufficiently thermoresistant as to resist DNA denaturation temperatures (>90ºC). Consequently, more DNA polymerase was added at each PCR cycle. In 1988, Saiki *et al.* purified a more stable DNA polymerase from *Thermus aquaticus* bacillus. This innovative step allowed PCR reactions to be completely performed without opening the reaction tubes (SAIKI *et al.*, 1988).

DNA replication occurs by the synthesis of new single strands, complementary to each original single strand. The essential molecular tool for this operation is the enzyme DNA polymerase, responsible for catalyzing this synthesis of new complementary DNA strands. For it to function properly, some elements are

necessary: dNTPs, which are the building blocks for constructing new DNA molecules; Mg^{2+}, which is an enzymatic co-factor (added as $MgCl_2$); and primers, which are short DNA fragments which anneal to single strand DNA and allow the DNA polymerase to start synthesizing the complementary strand. Two primers (forward and reverse) are necessary (one for each strand) and the primer pair determines which DNA fragment will be amplified. Primers mark the starting and ending points of the replication.

The PCR reaction, theoretically can be performed using 3 water baths with controlled temperature, but in current practice the process is performed by an automated apparatus called thermocycler. This equipment is capable of precisely controlling the temperature of solutions placed in small polypropylene tubes. Temperature changing algorithms can be programmed to accomplish the necessary heating and cooling cycles.

A typical PCR program includes: 1- an initial denaturation phase, in which the temperature is elevated to 95ºC for a couple of minutes (single strand DNA is obtained); 2- many cycles of: denaturation (at 94ºC), primer annealing (at 50ºC), DNA synthesis (at 72ºC). 3 – final extension (at 72ºC) and 4- reaction stop (at 4ºC).

When successful, this process renders an enormous number of copies of the desired DNA segment. DNA concentration and purity of PCR products can be assessed by agarose electrophoresis, revealed by ethidium bromide staining.

There is a great number of PCR applications, including parasitoses and cancer diagnostics, transgenes identification, forensics, recombinant DNA technologies, besides taxonomic and phylogenetic purposes.

3.18.2 DNA sequencing

DNA samples for sequencing are usually prepared by amplifying a desired sequence, using PCR. However, PCR reaction products need to be cleaned prior to sequencing. Various DNA cleaning kits are commercialized based in the following steps: DNA is bound to a silica matrix (liquid stage or stationary in a spin column) in high salt concentration, washed in EtOH, and eluted in low salt concentration, usually with dH_2O. Cleaning kits allow the purification of DNA from crude stock solutions, removing proteins, polysaccharides, and even pigments and PCR reaction remnants that interfere with the sequencing technique (BINDER; HIBBETT, 2003).

The sequencing reaction, as developed by Sanger *et al.* (1977), is very similar to the PCR reaction, but with important modifications. Fluorescence (or radioactivity) marked dideoxinucleotides (ddNTPs) are included in the reaction mixture. When a ddNTP is added by DNA polymerase, instead of a normal dNTP, the new strand synthesis is prematurely interrupted. After some amplification cycles, random length DNA strands are produced, with fluorescence marked ddNTPs in their extremities. Each of the four ddNTPs are marked with different fluorescence colors. The reaction product is resolved by electrophoresis, and the obtained fluorescence bands pattern indicates the nucleotide sequence in the analyzed DNA molecule. Reading of the electropherogram is automatedly performed with a laser bean and the resulting sequence is outputted as a text file. Sequences are deposited in sequences databanks and comparison analyses are performed by specially designed softwares.

3.19 MACROMYCETES' STRAINS STORAGE METHODS

Spore prints made over aluminum foils can be stored in previously autoclaved sealed test tubes. Spores usually keep viable for many years, in light absence and low humidity conditions, even at room temperature.

Pure mycelial cultures in Petri dishes or in test tubes over inclined semi-solid media can be maintained for weeks and even months at room temperature. Mycelium metabolism can be slown down by refrigeration, extending the storage period. Most strains tolerate well to be stored at 4ºC for several months, but not years.

Although there is no universal method, some of the following techniques are used for long-term preservation of mycelial strains with varying degrees of success, depending on the strain: submersion in sterile water or sterile mineral oil, and cryopreservation techniques such as ultrafreezer (-80ºC) or liquid nitrogen (-196ºC). Cryoprotectant substances (such as glycerol) and/ or adequate supports (such as perlite or vermiculite) are required to cryopreservation. With those low temperature preservation techniques, it is possible to conservate mycelial cultures for many years and, it is thought, even for centuries, maintaining stable genetic features.

3.19.1 Cryopreservation techniques

Humanity had progressively accumulated knowledge about fungi. Many aspects, including their diversity, ecology, biology, life-cycle, nutritive and medicinal properties have attracted the scientific community attention for a long time. Nowadays, whole industries are based on several fungi species. Fermented foods and beverages, mushrooms, mycorrhizal based inoculants, ethanol, enzymes, additives, cosmetics and pharmaceutical substances are some examples of valuable fungal products. Other applications include bioremediation, biopulping, effluents treatment and biological control of plagues (SOCCOL *et al.*, 2008; STAMETS, 2005).

There goes a long way from the discovery of a new fungi species to commercialization of useful and safe derived products. Research and certification processes can take many years or even decades, involving the work of highly qualified people and heavy investment. During all this period, strains must be maintained, with preserved characteristics and productivity (OZERSKAYA *et al.*, 2005; ROBERT *et al.*, 2006; STOYCHEV *et al.*, 1998; TSUTSAEVA *et al.*, 2008; WDCM, 2014).

Numerous are the institutions that maintain microorganisms culture collections. Some of them accept new strains from researchers, publish their protocols and promote training courses. A small fraction of these collections is private, or maintained by industrial corporations. Most of the collections are linked to Universities or governmental entities, mainly located in developed countries (ROBERT *et al.*, 2006).

Usually, for short periods of storage, fungi are maintained in their mycelium phase, being transferred periodically to new cultivation medium, on Petri dishes or tubes. Cultivation medium is commonly PDA or malt extract agar (MEA), for lignivorous species and Modified Melin Norkrans (MMN) or Fries medium for mycorrhizal ones; but there are many other formulations. Some species tolerate hypothermic storage, and can be preserved for some months under 4ºC, but others cannot be reactivated after this procedure (YANG; ROSSINGOL, 1998).

The use of common freezers (-20ºC) for mycelia preservation, was cited as a not recommended alternative by some reviews (HUMBER, 1997). Quantitative studies were performed with mycorrhizal fungi mycelia, stored at -12ºC and -5ºC over semi-solid medium in Petri dishes, without the addition of cryoprotectants. It was

shown that a slow pre-cooling phase before freezing increases significantly survival rates, suggesting that the studied fungi have natural freeze protecting mechanisms, activated by a slowly decaying temperature phase (ADDY *et al.*, 1998).

Storage of any biological material at -20ºC or -80ºC for more than 6~12 months is not recommended, because the commonly used cryoprotective solutions have glass transition temperatures that fall between -90ºC and -115ºC. It means that chemical reactions are not stopped at -80ºC or -20ºC. At these temperatures cells continue to be damaged by free-radicals and salts (BAUST; BAUST, 2007).

Alternative techniques for extending the transfer interval include: covering the mycelium with a layer of sterilized mineral oil or distilled water; drying the mycelium with sand or silica; and freeze-drying, also called lyophillization (HUMBER, 1997; VOYRON *et al.*, 2009). Some species, probably including all non-sporulant mycelia, do not reactivate after drying and the water submersion method also works better for sporulated species. With oil submersion, removing the oil during reactivation can be a difficult procedure (NAKASONE *et al.*, 2004).

Spores can also be stored as a spore-print, in the case of macromycetes. But a specific strain cannot be stored in spore phase, because the spores will generate many new strains when reactivated, by recombination of the monokaryotic mycelia initially generated. If specific traits are desired to be maintained, (i.e. if clones are wanted), dikaryotic mycelium must be the stored phase.

In technical terms, cryopreservation can be defined as the maintenance of viable biological structures at temperatures below -80ºC and typically below -140ºC. Techniques for cryopreservation are on development for more than 50 years, impelled by applications such as personalized medicine, drug discovery, organ transplantation and genetic diversity preservation (BAUST; BAUST, 2007). Cryopreservation is one of the branches of a science field named cryology, which deals with all aspects of phenomena that occur at low temperatures. Cryology originated, within heat science, at the 18th century. The first refrigerating machines were built at the second half of the 19th century (ARKHAROV, 2007). Cryomycology is the application of cryology concepts, especially cryopreservation, to fungi (CHETVERIKOVA, 2009).

Success on mycelium cryopreservation depend on a multitude of factors, including: fungi species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell

composition (water content, lipid content), density at freezing, freezing medium composition, cooling rate, storage temperature, storage duration, warming rate and recovering medium, among other factors (HUBÁLEK, 2003).

Cryoresistance is usually higher at stationary growth phase, with high cell concentrations, but most of the optimal parameters in cryopreservation protocols, mentioned in the previous paragraph, depend on the species and even on the microorganism strain to be cryopreserved. It is possible to find cryoresistant and not-cryoresistant strains of the same species (TSUTSAEVA *et al.*, 2008).

In spite of technical difficulties, some researchers were successful on several attemps of fungi cryopreservation. Methods are evolving and progressively more fungi species are reported to survive the freezing / thaw processes. It is believed that if a biological system maintains viability after being cooled below glass transition temperature (-90ºC~-115ºC), then it can be stored for a virtually unlimited time.

Neurospora crassa spores were maintained viable for more than five years of storage on liquid nitrogen. Tubes containing cultures of this sporulated fungus grown over semi-solid medium were directly submersed in liquid nitrogen without the addition of cryoprotectants (WELLMAN; WALDEN, 1971).

More than 20 sporulated rust fungi species were also successfully cryopreserved at -196ºC for more than 11 years. Tubes containing sporulated cultures were directly submersed on liquid nitrogen. Glycerol and DMSO were demonstrated unnecessary and harmful for spores' cryopreservation. None of those species survived cryopreservation in non-sporulated mycelial phase. This technique is used by the American Type Culture Collection (ATCC) since 1965 (CUNNINGHAM, 1973).

Most fungi species mycelia do not resist freezing without a cryoprotectant, mainly because cells are damaged by the formation of water crystals. But with glycerol as a cryoprotectant, some cultures can be stored under -80ºC or in liquid nitrogen (-195ºC), for a practically unlimited amount of time.

Many substances were tested as cryoprotectant additives (CPAs), but just a few showed satisfactory results. Some examples of CPAs that had shown efficient for various applications include: dimethyl-sulfoxide (Me_2SO), methanol, ethylene glycol, propylene glycol, serum and serum albumin. With a milder cryoprotectant efficiency, there are: glycerol, polyethyleneglycol (PEG), polyvinylpyrrolidone (PVP) and

sucrose. Dextran, hydroxyethyl starch and milk are not very effective (HUBÁLEK, 2003).

Cryoprotectant substances, as glycerol and DMSO, induce water vitrification instead of crystallization. In other words, water molecules form an amorphous solid upon freezing in presence of cryoprotectant substances (WOWK, 2010). Some authors suggest water vitrification allows cells to maintain their shape, instead of being ruptured by crystals, during freezing (DARWIN, 2007). Cell rupturing was experimentally demonstrated for yeast freezing at -196ºC without cryoprotectants (KOMATSU *et al.*, 1987). Radiolabelled DNA was used to measure the extent of cellular rupture. Other authors observe that cell damage during freezing is mainly caused by high local salts concentration due to salt exclusion during water crystallization (LOVELOCK, 1953; MAZUR, 2010; TAKAMATSU; ZAWLODZKA, 2006).

Some cell injuries also occur during freezing because of dehydration due to high extracellular salt concentrations, and more injuries occur during the subsequent rehydration when isotonic conditions are restored, during thaw (YOSHIMURA; TAKAMATSU, 2009). Ice formation involves such molecular reorganization in cells, which can not be reversed simply by thawing. Injuries to cells occur during freezing due to irreversible molecular rearrangements mainly induced by water crystallization (MAZUR, 2010). The main function of cryoprotectants is allowing molecules to solidify with minimum rearrangement. Overall, biological molecular organization is better preserved through vitrification than through crystallization (WOWK, 2010).

Pre-incubation of cells in special conditions was found to greatly influence survival after cryopreservation (REED; UCHENDU, 2008). Exposure of fungi cells to low temperatures (but not freezing) was found to induce the expression of proteins that either have an anti-freeze activity or are responsible for the synthesis of metabolites with this activity, including sugars and polyols (RAYMOND; JANECH, 2009).

Besides contributing to water vitrification, it was shown that some cryoprotectants modulate genetic expression of cells, enhancing multiple repair mechanisms, such as increased expression of genes that code enzymes involved on biosynthesis processes, such as the synthesis of proteins, lipids, nucleic acids and carbohydrates; genes that contribute to the synthesis of cellular structures, such as cellular membranes, cellular wall and ribosomes. Other up-regulated genes include

enzymes responsible for refolding proteins and for accumulating low molecular weight compounds. An example of down-regulation on cells exposed to cryoprotectants is on genes responsible for fatty acids oxidation (MOMOSE *et al.*, 2010).

As cells have external and internal water, simple submersion on a cryoprotectant solution is frequently not sufficient for cryopreservation. Cryoprotectants must also be absorbed by cells (MULDREW; MCGANN, 1998). In case of fungi, this can be achieved by incubation of mycelia in media containing penetrating cryoprotectants, previously to freezing (HOMOLKA *et al.*, 2007). Glycerol and DMSO are examples of penetrating cryoprotectants, easily absorbed through cellular membrane. Non-penetrating cryoprotectants are large molecules, which can not perfuse the cellular membrane, but contribute to vitrification of water in the extracellular space. Some carbohydrates and proteins are examples of non-penetrating cryoprotectants.

Non-penetrant molecules, in general, have hydrophilic groups that are capable of binding to water (BREIEROVA, 1998). Glucose, sucrose and raffinose had shown protective properties for chloroplast thylacoids cryopreservation. Reduction of solutes toxicity and increasing in membranes stability are possible mechanisms for this protection (LINEBERGER; STEPONKUS, 1980). DMSO and glycerol toxicities are also reduced when sucrose is added to the medium (BEST, 2008). Sucrose addition was shown to decrease cell dehydration during freezing and minimize cell damage during rehydration (MAZUR, 2010; YOSHIMURA; TAKAMATSU, 2009).

Many organisms face extremely cold natural conditions and have developed molecular mechanisms for surviving. Anti-freeze proteins (AFP) were found to be produced by a great variety of species, such as fishes, plants, bacteria and fungi, including some macromycetes, such as *Pleurotus eryngii* (king oyster), *Flammulina* spp. (winter mushrooms) and *Lentinula edodes* (shiitake). Those proteins have the property of inducing thermal hysteresis; which is slightly lowering the freezing point, without changing the melting point. Molecular analysis demonstrated a great level of similarity between ice binding proteins produced by different organisms, in spite of their evolutionary distance, suggesting horizontal transfer of the respective genes (KAWAHARA *et al.*, 2006; RAYMOND; JANECH, 2009).

It was shown that some diatom algae strains produce ice binding proteins with a significant similarity to those produced by some fungi strains. Other molecules might

also be involved in cryoprotection, such as polysaccharides (MOCK; JUNGE, 2007). Recombinant DNA technology was successfully applied to transfer a diatom antifreeze protein gene to *E. coli* bacteria for expression (GWAK *et al.*, 2010).

Takemura, Kawahara & Obata (2006) screened antarctic fungi for compounds capable of modifying water crystals structure and found seven out of 40 species that produced ice crystal structure controlling materials (ICSCs). Certain hormones, such as abscisic acid (ABA), were also found to induce low temperature resistance genes expression in plants (REED; UCHENDU, 2008). Those bioproducts are being researched for special commercial applications such as cryopreservation of microorganisms, tissues, organs and also for food cryopreservation.

Interestingly, the expression of cryoprotective proteins by yeast is up-regulated by NaCl stress conditions (BREIEROVA, 1998). This can be used in order to optimize the production of these substances. This evidence also reinforces that high localized salt concentrations are related to mechanisms of cell damage during cryopreservation.

Necrotic mechanisms of cell death at hypothermic conditions are vastly documented, but new paradigms are gradually emerging in cryopreservation as theories incorporate the latest experimental evidences. Baust & Baust (2007) suggest that cell death at hypothermic conditions can also be triggered by apoptotic mechanisms. At low temperatures (0~10ºC), molecules have their kinetic energy reduced. Some metabolic processes practically stop at this temperature range: aerobic synthesis of ATP, for example. It results that adenylates are gradually depleted, further interfering with active transport of ions, such as calcium, sodium and potassium, through membranes. Membranes, cytoskeletons and organelles, as mitochondria are subjected to a set of stresses that can lead to activation of apoptotic cascades.

Based on this theoretic background, scientists are constantly testing new substances for cryoprotection. The best results are often achieved with combinations of substances. For example, polyethilene glycol (PEG) and glucose were shown to lower dimethylsulfoxide (DMSO) toxicity in some plant tissues preservation (REED; UCHENDU, 2008). A combination of DMSO and horse serum showed better results than single cryoprotectants for fungoids preservation (COX *et al.*, 2009). Some products are already commercially available with optimized formulas for specific applications (21ST CENTURY MEDICINE, 2014).

The National Collection of Yeast Cultures (NCYC) maintains their strains both by freeze-drying and by cryopreservation. Common polypropylene straws fragments are used to store yeast cultures, inside cryotubes. Yeasts are cultivated in liquid medium (YM) and, after growth, glycerol is added. Culture aliquots are transferred to straws, which are then heat sealed and put inside cryotubes. No significant changes in cultures were noticed after 10 years of storage at -196ºC (BOND, 2007).

The use of specific solid particulate carriers was shown to drastically improve the efficiency of mycelium cryopreservation procedures. Studies show that cereal grain and perlite are efficient carriers. Perlite utilization for fungi cryopreservation was introduced by Homolka *et al.* (2001) and was firstly tested with success for four different basidiomycete strains, including *Pleurotus ostreatus*. Five years after, the same group extended this technique to 230 more basidiomycete species from 104 different genera, with 100% success (HOMOLKA *et al.*, 2006). Recovery rates reported for the perlite protocol are higher than for any other cryopreservation protocol.

An earlier article, by Addy *et al.* (1998) registered increased survival rates of mycorrhizal fungi grown over soil after storage at -12ºC and -5ºC if compared with the same species stored grown over semi-solid medium in Petri dishes. The authors of this paper suggest that this effect could be due to the insulating properties of soil.

Other experiments suggest that cereal grains can also protect the mycelia during freezing, dispensing the use of cryoprotectants (MATA; PÉREZ-MERLO, 2003). Perlite is even more thermal insulating than cereal grains, but perlite protocol was not previously tested without cryoprotectants. Neither previous studies were found on the utilization of vermiculite or other alternative carriers for this purpose.

The freezing and thaw rates were found to be important factors. Transferring mycelium from incubation temperature directly to cryogenic condition can damage or inviabilize some strains (GLEASON *et al.*, 2007; MULDREW; MCGANN, 1998).

Pre-cooling or controlled freezing can be used to overcome this problem (REED; UCHENDU, 2008). If the cooling rate is too slow, long exposure to toxic conditions, including high intracellular concentrations of salts and cryoprotective agents, due to excessive dehydration, can damage cells. On the other hand, if cooling is too fast, cell dehydration is not allowed, thus favoring damages by intracellular ice formation (BAUST; BAUST, 2007; SMITH; THOMAS, 1997).

Either standard or programmable freezers can be used to cool tissues at a specified cooling rate before plunging cryovials in liquid nitrogen. Strategies with non-programmable freezers include placing cryovials inside thermal insulating containers to slow heat exchange. Styrofoam boxes can be used, but there are also commercially available specifically designed containers with isopropanol that assure cooling rates of about -1ºC/minute (THERMO, 2014). Obviously, programmable freezers offer higher control over the cooling ramp. In either case, controlled cooling usually starts at 0ºC and proceeds at least until the freezing temperature of the cryoprotectant solution, which is usually between -35ºC and -40ºC (REED; UCHENDU, 2008).

Ideal cooling rates can be experimentally determined by using programmable freezers. Different fungi species and even different strains of the same species have different optimal cooling rates. For most fungi species, optimal freezing rates are in a range between -1ºC/min. and -10ºC/min. Direct immersion in liquid nitrogen, without precooling (-200ºC/min.) is rarely recommended. Nevertheless, there are some fungi species that are very cryostable at any chilling rate, as *Aspergillus repens* and some mycorrhyzal fungi (CHETVERIKOVA, 2009).

Special attention should be paid to cryotubes sealing when submerging in liquid nitrogen. Serious problems can arise if liquid nitrogen enters the cryotubes. There is risk of sample contamination with microorganisms present in the cryotank; and cryotubes can even explode during thawing due to nitrogen expansion, which is especially dangerous in case of pathogenic microorganisms (CHEN *et al.*, 2006).

Sandskär & Magalhães (1994) developed a method for the cryopreservation of a zygomycete species. Agar plugs covered by cultures were transferred to cryotubes containing 1 mL of a cryoprotectant solution. Tubes were slowly cooled before being plunged into liquid nitrogen (-196ºC). One of the best cryoprotectant solution compositions tested consisted of 10% glycerol with 0.5M sucrose.

Yang & Rossignol (1998) developed a protocol for mycelia cryopreservation in wich agar plugs are directly submersed on a 5% glycerol solution inside cryovials, left overnight at 4ºC, held at the gaseous phase of liquid nitrogen (-165ºC) for one hour and then plunged at the liquid nitrogen (-195ºC). Reactivation was successful on 503 out of 516 basidiomycete species with this protocol.

Gleason *et al.* (2007) reported better recovery rates after cryopreservation of several Chytridiomycetes species with controlled freeze and thaw rates. They used a

thermal insulating cryocontainer to maintain freezing rate at approximately 1ºC/minute inside a -80ºC ultrafreezer. They obtained similar results with a programmable freezer with 1.5ºC/minute. Both glycerol and DMSO (in a concentration of 10%) were found to be the most efficient cryoprotectants among several other substances tested by this group. Incubation after thawing was more successful in liquid media, than over agar.

Fungoid protists cryopreservation was tested with better results for a mixture of DMSO and horse serum over glycerol, as cryoprotectant. Harvested cells were submersed in cryoprotectant solutions, slow-cooled (1ºC/min.) and stored at -196ºC. For reactivation, fast thaw in a water bath at 50ºC was applied (COX *et al.*, 2009).

In an experiment with mycorrhizal fungi mycelium, a pre-cooling phase applied before freezing at -12ºC increased survival rates from 0% to 60% (ADDY *et al.*, 1998). It is experimentally proved that natural freeze defense mechanisms are activated by incubation at slowly decreasing temperatures.

An experiment by Homma *et al.* (2003) demonstrated gene expression modulation in *Saccharomyces cerevisae* incubated at 4ºC and 25ºC, by means of DNA microarrays. Yeast cells are able to adapt their metabolism and structure to cold conditions, by up and down-regulating hundreds of genes.

Thaw rates are also decisive. Fast thaw rates avoid water recrystallization (REED; UCHENDU, 2008). Reactivation of some rust fungi spores after cryopreservation requires a heat shock (40ºC for 15 seconds) after or during thaw (CUNNINGHAM, 1973). For reactivation of non-sporulated mycelia, fast thaw in a 37ºC~40ºC water bath is recommended (HOMOLKA *et al.*, 2007).

Reactivation after -80ºC storage is usually faster than reactivation after storage in liquid nitrogen. Days and even weeks of incubation may be necessary before reactivation. This lag phase can be shortened if intracellular water crystallization and hyphae compression are minimized (CHETVERIKOVA, 2009).

Not only survival, but also genetic stability is aimed with cryopreservation methods. About a decade and a half ago, Sergei Rozanov formulated the hypothesis of mutagenesis resulting from cryopreservation. Numerous cases of instability of fungi were related after cryopreservation: incapacity to metabolize natural substrates or lowered infectiveness, lowered radial growth rate, alterations in the expression of enzymes and synthesis of secondary metabolites among other mutations. Some scientists are studying the mutagenic effects of cryopreservation, means to minimize

it or stimulate it and its applications on genetic improvement (CHETVERIKOVA, 2009).

Comet assay allows DNA damage assessment. It has been applied to many cell types, including fungi. It can detect DNA damages of various types (PIPERAKIS, 2009). It is a relatively simple, rapid, visual and sensitive method for analyzing DNA lesion and repair in quantitative and qualitative aspects. *Schizosaccharomyces pombe* and *Sordaria macrospora* were fungi species assessed by this technique in order to determine genotoxicity of many different substances (DHAWAN *et al.*, 2009).

Nevertheless, some fungi seem to retain all genetic properties during cryopreservation. Fourteen basidiomycete species had shown unmodified growth rates and enzymatic expression after a short storage period, using either DMSO or glycerol as a cryoprotectant (STOYCHEV *et al.*, 1998).

Experiments with DNA microarrays had shown that *S. cerevisiae* modulates gene expression after cryopreservation. Genes related to cell rescue, defenses, virulence, energy and metabolism are significantly induced. Many of the up-regulated genetic products are specially directed to reconstitution of cell structures such as cell wall and organelles, damaged by the cryopreservation process (ODANI *et al.*, 2003).

Recent developments allow researchers to optimize cryopreservation protocols (freeze / thaw rates and cryoprotective solution composition) with high-throughput technologies. Yu *et al.* (2007) designed a biochip capable of easily and rapidly testing many cryopreservation protocols at the same time, with automated sample preparing and analysis. In addition, cryogenic engineers are constantly testing new materials and equipments for working with low temperatures (ARKHAROV, 2007). Science is quickly progressing to dominate technical aspects of cryopreservation.

3.20 MACROFUNGI MYCELIA AND CARPOPHORES CULTIVATION USING PEJIBAYE PALM (*BACTRIS GASIPAES* KUNTH) SHEATH RESIDUES AS SUBSTRATE

Crescent organic residues production is associated with the human civilization development. Due to an inconsequent expansion of a dated industrial production model and negligent in relation to the rational management of this material, serious harm to the ecological equilibrium is being promoted. The accumulation of damages

in long-term puts life quality and ultimately the survival of humanity at risk. New development models are necessary to solve this problem (MONFORTI *et al.*, 2013).

Macromycetes will play an important role in this process, due to their ability and versatility to decompose a wide range of biological materials. Many edible mushrooms species are already cultivated, using agro-industrial residues and sub-products as substrate. China is a good example of productive model, in which mushrooms are widely applied. Governmental actions aim to stimulate this activity, as means of processing immense residue volumes and to generate economic opportunities for all social classes (TAN; CAO, 2010). However, even the Chinese model stills lack environmental concern (ZHANG, L. X. *et al.*, 2012).

Notwithstanding the complexity of this challenge, this scenery can be viewed as a great opportunity. The development of technologies for recycling and transforming organic residues will not only solve environmental problems, but also present a way for generating employment and wealth (URBEN, 2004a).

The expansion of mushroom culture in Brazil depends on scientific development to improve productivity, quality control, available organic matter utilization, low cost production and international commerce.

Mushroom culture presents one of the highest profitability perspectives per cultivation area, generating up to 150% of the invested capital at the end of the production period. It is a relatively fast culture type, allowing short payback times (MODA, 2003; POPPE, 2000; RAJARATHNAM *et al.*, 1992).

With adequate technical knowledge, it is possible to design low cost production facilities, with low risk and good market perspectives. This already represents an economic and social development wrench for many countries around the world, and could be applied to Brazil, with good success chances, due to the great volumes of agro-industrial production and consequent abundant residues generation.

Examples of organic residues already tested for mushrooms production include: asparagus straw (WANG *et al.*, 2010), handmade paper and cardboard industrial wastes (KULSHRESHTHA *et al.*, 2013), straws, grasses, sawdust, coconut husks, corncobs and sugarcane bagasse (DONINI *et al.*, 2006; SALAS, 2005). In addition, mushroom spent substrates are being tested for soil enrichment and bioremediation (RIBAS *et al.*, 2009). Each different substrate/ mushroom species combination results in particular product qualities and yield (TABLE 9).

TABLE 9 – BIOLOGICAL EFFICIENCY (BE) FOR SOME SUBSTRATES, USING *PLEUROTUS* SPP.

FORMULATIONS	BE (%)
Wheat straw/ lime (1%)	177,41
Fresh coffee pulp	159,68
Dried coffee pulp, stored for 24 months	145,27
Momordica spp.melons straw	139,66
Lemongrass leaves residues	113,01
Cotton residues (75%)/ straw (23%)/ lime (2%)	104,9
Coffee pulp/ barley straw fermented for 5 days	104,9
Cinnamom leaves residues	102,68
Rice straw	81,85
Cotton residues (98%)/ lime (2%)	81,25
Barley straw/ flour (8%)	79,18
Barley pulp (94%)/ feathers flour (4%)	78
Cotton residues (75%)/ tea leaves (23%)/ lime (2%)	74
Dry peas straw	73,22
Barley straw/ alfafa straw (20%)	71,67
Barley straw/ alfafa straw (5%)	69
Beans straw	60,19
Pumpkin straw	56,93
Sugarcane bagasse/ molasses (15%)	54,8
Rice husk	50
Freshly grinded sugarcane	35,17

SOURCE: (EIRA, 2000)

Jun-Cao (Jun = mushroom, Cao = grass) is a widespread mushroom cultivation technique. It uses grass biomass as substrate, instead of wood. This allows great scale production without deforestation. This technique was adapted for using agro-industrial residues as substrate, with evident environmental and economic benefits (CASTRO, 2007; DIAS; GONTIJO, 2000). Jun-Cao has the following advantages over other techniques:

- Abundant and unexplored substrate resources.
- Grasses present short vegetative cycle, with fast growth and can be harvested many times a year.
- 10 to 20% bigger conversion rates than with wood and 20% smaller costs.
- Shorter mushroom crop cycle.
- Practicity and easiness of adaptation to small or large scales.
- Mushrooms cultivated with Jun-Cao technique present better nutritional quality than those produced using logs and sawdust

Brazil is one of the top producers and consumers of palm in the world (SAMPAIO *et al.*, 2007). New cultivation areas, padronized and high quality products desestimulate the illegal clandestine extractivism that have been occurring for decades in the Atlantic Rainforest area (CORRÊA JUNIOR *et al.*, 2011).

Pejibaye palm (*Bactris gasipaes*) presents an important advantage for cultivation in relation to other palmaceae: it perfilates. That is, each plant is capable of generating 1-20 perfiles per plant, and it rarely does not perfilate (VILLACHICA, 1996). This species is native from Amazon, but adapted well to Parana's seacoast. It is a promisor cultivar for small and medium producers, for it is premature, rustic and produces low oxidation rate and high industrial yield palms (CHAIMSOHN, 2001).

Approximately 70% of biomass is usually wasted during industrial processing of vegetables such as banana and pejibaye palm. From a whole plant with 2.600 g, only about 780 g of final product are effectively obtained. That means a small producer that processes around 3.000 palms per day, generates nearly 5,5 t of residues in a single day, 131 t per month. Silva *et al.* (2010) cite that not much had been done for recycling these residues. The environmental problem is aggravated by the absence of public politics for selective collection, recycling of the generated residues and preservating the integrity of ecosystems (ZANIN; MANCINI, 2004).

TABLE 10 – NUTRITIONAL COMPOSITION AND CALORIC VALUE OF PEJIBAYE PALM SHEATH.

COMPONENT	PEJIBAYE PALM FIBER ($G.100G^{-1}$)	STANDARD DEVIATION ($KCAL.100G^{-1}$)
Water	3,3	±0,43
Minerals (ashes)	4,2	±1,52
Protein	4,7	±2,09
Total nitrogen	1,5	±0,71
Non-proteic nitrogen	0,8	±0,52
Lipids	0,9	±0,19
Total fiber	63,2	±2,95
Insoluble fiber	59	±2,51
Soluble fiber	4,2	±0,55
Total carbohydrate	22,3	±4,44
Soluble reducing sugars	10,4	±3,66
Soluble non-reducing sugars	6,3	±1,44
Starch	5,6	±2,19
Energetic content	120,4	±11,83

SOURCE: Tavares *et al.* (2013).

Due to its fibrous composition (TABLE 10), pejibaye palm residues can be included in animal feed, promoting benefic health effects for the animals' intestinal flora. However, it is difficult to process the material correctly for this application.

Mushroom production from ligno-cellulosic waste is expanding in the whole world, including Brazil (RIBAS *et al.*, 2009). According to Bononi *et al.* (1995), there are approximately 2.000 known edible species, however only about 25 are regularly used in human nutrition and a smaller number is commercially cultivated.

TABLE 11 – BRAZILIAN EDIBLE MUSHROOMS' PRODUCTION IN 2011.

SPECIES	**TONS/ YEAR**
Agaricus bisporus	8000
Pleurotus ostreatus	2000
Lentinula edodes	1500
Agaricus blazei	500
Other species	50

SOURCE: Souza (2011)

According to Souza (2011), annual *in natura* mushroom production in Brazil is around 12.050 t/ year, distributed as described in TABLE 11. However, the Brazilian mushroom production stills small and restricted to south and southeast regions of the country. Even with the mentioned production, Brazil imports around 12.000 t/ year of processed *Agaricus bisporus* to supply the internal market demand. In other words, the consumption of mushrooms by Brazilian people is small, but the production is even smaller. In fact, consumption is growing considerably: individual mushroom consumption increased 400% in 30 years (SOUZA, 2011).

Production increase depend on scientific advances on productivity, quality control, available organic matter utilization, costs reduction and international commerce improvement. Mushrooms present one of the greatest payback rates per area, allowing up to 150% profit over the invested capital, with short cycles. Of course, this can vary in function of the mushroom species, substrate, culture conditions and season (MODA, 2003; POPPE, 2000; RAJARATHNAM *et al.*, 1992).

Paris mushroom (*Agaricus bisporus*), shiitake (*Lentinula edodes*), oyster mushroom (*Pleurotus ostreatus*) and royal sun agaricus (*Agaricus subrufescens*) are

the most produced species in Brazil (URBEN, 2004b). Gradually, new varieties are being introduced, such as portobellos (brown variety of *A. bisporus*), pink oysters (*Pleurotus djamor*) and king pleurotus (*Pleurotus eryngii*), but in very small scales.

3.21 FRUITING BODIES' PRODUCTION PARAMETERS

Mushroom cultivation demands controlling several factors, such as nutritional, chemical and physical properties of the substrate, including water activity (Aw), pH, oxirreductive potential, the presence of antimicrobian agents and the interaction among microrganisms. Chosing an adequate strain is of fundamental importance, for significative intraspecific variations occur. For example, there are both black and white strains of *Pleurotus ostreatus*, as well as white and brown *Agaricus bisporus*. Some strains can be better adapted to hot or cold climates or to different types of substrates.

Environmental factors that influence mushroom cultivation include temperature, humidity and chemical composition of the atmosphere, including O_2 and CO_2 concentrations (EIRA, 2003; MARINO, 2008). The growing mycelium demands carbon, nitrogen, vitamins and minerals (URBEN, 2004b). Fungi are able to synthesize and secrete a great diversity of enzymes for digesting a wide range of substrates and liberating nutrients for absorption (DONINI *et al.*, 2006).

Eira (2000) mentions three substrate groups for mushrooms cultivation:

- *In natura* substrates with higher than 100:1 C:N relations, such as wood logs.
- Agro-industrial residues with C:N relation within the 50~100:1 range, such as non-composted, pasteurized straws.
- Previously composted, pasteurized and conditioned organic substrates, with initial C:N relation between 25 and 50:1 and between 16 and 17:1 after conditioning.

The correct C:N proportion in the substrate favours carpophores production, and inhibits contaminations. Eira (2000) suggests the best proportions are between 15 and 25:1. Urben (2004a) recommends an approximate 28:1 relation. Higher nitrogen concentrations can inhibit the formation of basidiomes (URBEN, 2004a). According to Gowling & Merrill (1966 apud KURTZMAN, 1979), there are several mechanisms that allow macrofungi to grow with low nitrogen levels substrates:

- Physiological adaptations for using the available nitrogen with high efficiency.
- Ability to recycle the available nitrogen, through continuous autolysis systems.

- Ability to use atmospheric nitrogen.

Fungi demand nitrogen in their nutrition for the synthesis of proteins, nucleic acids and some cell wall polysaccharides. Nitrogen supplementation sources include nitrate, ammonia and organic nitrogen (MILES; CHANG, 2004). Other possible nitrogen sources are: ammonium chloride, ammonium sulfide, dibasic ammonium phosphate, ammonium nitrate, sodium nitrate, potassium nitrate, ammonium acetate, ammonium tartarate, urea, hydrolised proteins, aminoacids, corn steep liquor and yeast extract (LIMA *et al.*, 1975).

Based in experimental results, Ortega *et al.* (1992) hypothesized that *Pleurotus* spp. can possibly present metabolic pathways for the fixation of atmospheric nitrogen. They have estimated that the nitrogen content of mushrooms extrapolate the original nitrogen content of the respective substrate they are growing on. Oetterer (1996) verified an increase in the nitrogen content (4~37%) of various substrates during *Pleurotus* spp. cultivation, reinforcing the hypothesis of nitrogen fixation by the mushroom itself or by associated bacterial microflora. However, the mycelium associated to the substrate could have interfered in the results, leading to a superestimation of nitrogen content due to the presence of chitin in the fungal cell wall.

Pleurotus sp. fructification is stimulated by glucose, galactose, manose and fructose presence and inhibited in arabinose and xylose presence. In mycelium growth phase, reducing monosaccharides concentration increases. After fructification, this concentration falls. Bano & Rajarathnam (1988) suggest that reducing sugars are consumed as energy source for the production of basidiomes.

Ideal pH for mycelial growth is within the range from 4,0 to 7,0 and within 3,5 to 5,0 for basidiome formation (URBEN, 2004b). Substrate pH decreases from 6,5~7,0 to nearly 4,0 during colonization, because of organic acids production, mainly oxalic acid before fructification (RAJARATHNAM *et al.*, 1992; ZADRAZIL, 1985).

According to Rainbault & Alazard (1980), the control of gaseous environment for aerobic solid-state cultivation is an important factor for the development of microorganisms. It depends on the oxygen flow through the substrate and the oxygen consumption rate by the cultivated cells. Aeration plays multiple roles, namely O_2 supply for microbial growth and metabolism, moisture control, temperature control, steam, CO_2 and some volatile compounds elimination.

In ideal conditions, microorganisms should obtain O_2 directly from the atmosphere (MURTHY *et al.*, 1993); however in real conditions gases diffusion occur in serial steps of transportation between particles. Lonsane *et al.* (1992) verified that the oxygen transference rate for solid-state processes is higher than that for submerged mycelial cultivation. In this sense, solid-state processes demand less energy for aeration than submerged processes.

Small particle sizes are desirable for solid-state substrates, as larger surface areas are exposed. However, excessively small particles result in a compacted substrate, difficulting the transference of gases. Bigger particles result in more interparticle space, allowing better aeration, but reducing surface area. Thus, the ideal particle size should be intermediate, optimizing both the transport phenomena (heat, nutrients, products and gases) and superficial area (PANDEY *et al.*, 2000).

Minerals such as phosphore, magnesium, sulfur, calcium, iron, potassium, copper, zinc, manganese and cobalt are used in macrofungi cultivation media. Some species require vitamins supplementation of the substrate, but an excessive supplementation can result in undesirable flavor to the food (LIMA *et al.*, 1975).

Supplementation of the substrate with sulfur, phosphore, potassium and magnesium ions, as well as trace amounts of calcium, zinc, manganese, iron, copper and molibdenium stimulates *Pleurotus* spp. growth (MILES; CHANG, 2004).

Silva *et al.* (2007) correlated the composition of *Pleurotus* spp. mushrooms with the medium composition and cultivation conditions. Hyphae can accumulate toxic heavy metals from the substrate, such as mercury, arsenium and lead (KALAČ; SVOBODA, 2000; MOURA, 2008; STURION *et al.*, 2000). Similarly, biotin and tiamin can also be accumulated if incorporated into the substrate (MILES; CHANG, 2004).

Environmental conditions are of great concern for mushroom cultivation incubators design. Control systems are needed for maintaining the conditions within an acceptable range for the development of fruiting bodies. Humidity, temperature, luminosity and air exchange are the main controlled factors for successful crops.

Low moisture levels can inviabilize mycelium growth and halt fructification processes. On the other hand, high humidity inhibits gases exchange and transpiration, difficulting carpophores formation and resulting in the proliferation of undesirable contaminants such as bacteria and nematodes (DEL BIANCHI *et al.*, 2001; URBEN, 2004a). For most fungi species, 20~70% air relative humidity is acceptable, but the ideal range for most mushroom species is 80~90% (URBEN,

2004b). According to Miles & Chang (2004), the best moisture level for *Pleurotus* spp. cultivation is 50~75% for the substrate and 85~95% for the cultivation room air. Nevertheless, water quality is also important. Contaminated water can result in bad taste or even toxic compounds accumulation in the fruiting bodies.

High temperatures can lower carpophores development, as well as favour the development of microorganisms better adapted to hotter climates. Each life cycle stage (mycelial growth and fructification) of the same species can present a different ideal temperature, as shown in TABLE 12 (OEI *et al.*, 2005; URBEN, 2004b).

TABLE 12 – OPTIMAL TEMPERATURE RANGES (^{O}C) FOR MYCELIAL DEVELOPMENT (Tmd) AND FRUCTIFICATION (Tfr) OF SEVERAL MUSHROOM SPECIES, AND THE RESPECTIVE AVAILABLE PRODUCTION TECHNIQUES.

SPECIES/ COMMON NAME	Tmd (ºC)	Tfr (ºC)
Lentinula edodes/Shitake	5-35	8-25
Pleurotus abalonus/ giant mushroom, hiratake	15-35	25-30
Pleurotus cystidiosus/ giant mushroom, hiratake	10-35	25-29
Pleurotus ostreatus/ oyster mushroom, shimeji	5-35	5-25
Pleurotus pulmonarius/ sajor-caju	5-35	13-20
Pleurotus cornucopiae/ trumpet *Pleurotus*	15-35	15-25
Pleurotus djamor/ pink oyster	15-35	20-30
Pleurotus eyngii/ king *Pleurotus*	10-35	15-22
Auricularia polytricha/ wood-ear	20-35	23-28

Techniques for substrate preparation: 1- wood logs, 2- substrate pre-termically treated by various methods including pasteurization; 3- sterilized substrate. SOURCE: Oei *et al.* (2005)

Some macrofungi species develop well in light absence. Others need small light amounts in photoperiod cycles (1.500 to 2.000 lux.hour^{-1} for 12 hours per day). Too much luminosity can cause paleness and deformations (BONONI *et al.*, 2000; URBEN, 2004b). Eira & Bueno (2005) observed that shiny white surfaces of *Pleurotus* spp. pileus could be darkened in light presence, due to the liberation of phenoloxidases, which oxidize phenols, forming melanoidins.

During mycelium running, there is production and liberation of CO_2 and other metabolites, which accumulate in the substrate, inhibiting the growth of competitive microorganisms and stimulating the growth of macrofungi mycelium (ZADRAŽIL, 1975). Mycelia are usually tolerant with high CO_2 concentrations, but carpophores formation can be negatively affected by this condition (URBEN, 2004b).

According to Silva *et al.* (2005) and Sales-Campos & Andrade (2010), abundant, fast and vigorous mycelium growth is correlated with high primordia

formation rate and carpophores' yield. This principle supports tests of mycelium growth for substrates optimization aiming the cultivation of carpophores.

Lack of ventilation in the fructification room can result in poor pileus development, deformations, such as long stipes and rust colored spots, generally caused by contaminations with bacteria and fungi (SOUZA *et al.*, 2005). Rajarathnam *et al.* (1987) suggest that thinner plastic bags and a greater number of holes contribute for a faster mycelial run, early fructifications and a higher productivity, due to improved gaseous exchanges, as shown in TABLE 13.

TABLE 13 – NUMBER OF HOLES EFFECT OVER BIOLOGICAL EFFICIENCY OF *PLEUROTUS* SPP.

NUMBER OF HOLES (1 cm DIAMETER)	FRUCTIFICATION TIME	BIOLOGICAL EFFICIENCY (BE)
	(days)	(%)
0	-	0
8	16-18	7,5
16	5-17	15
24	10-12	35
32	8-10	60
40	8-10	50

SOURCE: Rajarathnam *et al.* (1987).

It is advisable to periodically inspect cultivation rooms for removing contaminated and underdeveloped bags (SOUZA *et al.*, 2005). Contamination sources and causes include inneficient thermal treatment of the substrate, inneficient cleaning and disinfection of the air, objects and tools in the inoculation environment, contaminated spawn, holes in the bags, defective closure of the bags and innadequate personal assepsy and uncareful manipulation. The normal human body microbiota is one of the main sources of contamination in biotechnological processes.

Not all color changes in the substrate are due to contaminations. Some yellowish exsudates composed of alcohol and organic acids are produced by the aging mycelium normal metabolism (BONONI *et al.*, 1995). *Lentinula edodes* mycelium changes from white to brown before fructification, in a process commonly

called “browning”. Other species mycelia can develop colors such as lilac (*Lepista sordida*) and orange (*Pycnoporus sanguineus*) natural pigments.

Some macromycete strains fructificate in the same environmental conditions as myceliation occur. Others may need special signaling, such as an increase in ventilation and humidity, a decrease in CO_2 concentration and temperature, nutrients exhaustion in the substrate, exposition to a light photoperiod, physical shock or a combination of these stimuli. Knowledge of these factors allow the control and relative sincronization of carpophores formation, upon intentional induction. Some of these signals are adverse conditions for mycelial growth and lead to fruiting bodies formation as a survival strategy. Carpophores are functional structures designed to allow spores production and dispersion. Spores are more resistant than mycelium and can carry genetical information through long distances and store it viable for long periods. Spores production also allows the generation of genetical variation, for distinct primary mycelia combine to generate new secondary mycelium with combined genetical traits.

Fruiting bodies growth rate depends on many factors, including genetic factors, inherent to the species or strain and environmental conditions. Some species show short carpophore’s development period. *Pleurotus ostreatus*, for instance, can fully develop in a few days (4~10). *Ganoderma lucidum* takes months until ready for harvest. Excessively cold weather can slow down the process. Extreme environmental conditions can even halt carpophores development.

Some points should be observed at *Pleurotus* spp. harvest:

- The ideal is to pick basidiomata before spores liberation, when the pileus is almost fully open;
- Mushrooms’ surfaces should be dry;
- It is better to avoid snatching mycelium and substrate pieces;
- Stipe pieces should not be let in the substrate, as they become contamination focuses;
- Mushrooms should be handled as little as possible, to avoid oxidation;
- Pick always the younger and better looking individuals;
- Do not touch diseased carpophores while harvesting the healthy ones;
- Throw away diseased carpophores as soon as they are found and wash hands thoroughtfully.

It is advisable to pick the mushrooms by tearing, avoiding cutting instruments. Cell ruptures result in the liberation of intracellular components such as oxidative enzymes and nutrients that can promote the undesirable growth of other microorganisms, contaminating the substrate and perturbating the physiology, especially increasing respiratory and/ or ethylenic activities (SAPATA *et al.*, 2010). This can result in the darkening of the external surfaces, as well as in the hydrolysis of pectins and cellulose.

Basidiomata can be conserved under refrigeration (~4ºC) up to approximately one week, after harvested. The myceliated substrate can fructificate more than once if not contaminated and environmental conditions are kept favorable. It may be necessary to repeat induction procedures previously described. Up to three fluxes are not uncommon; however, the yield is usually lower at late cycles.

3.22 MUSHROOM SPENT SUBSTRATE AS SOIL FERTILIZER

Although using a series of organic residues as substrate, mushroom production also generates a considerable amount of residues. Some authors define spent mushroom substrate (“SMS”) as the exhausted myceliated substrate, obtained at the end of primary decomposers mushrooms cultivation. Spent mushroom compost (“SMC”) is the equivalent, but for secondary decomposers mushrooms, which need their substrate to be previously composted before mushroom cultivation.

For each quilogram of mushroom, approximately five quilograms of residues are generated (WILLIAMS *et al.*, 2001). Considering an annual production of 8 million tons of mushrooms, 40 million tons of residues are generated in this period (CHIU *et al.*, 2000). It is estimated that 70~80% of these residues are simply wasted (SEMPLE *et al.*, 1998), leading to environmental problems, due to its inadequate destination.

This material could be used as fertilizer, especially for floriculture and horticulture. Some producers, mainly in European countries (MAHER *et al.*, 2000), are successfully doing this. The use of SMC as fertilizer allows the supply of nutrients to plants and the enhancement of soil’s physical and chemical properties, reducing water loss by infiltration and increasing aeration and organic matter concentration, improving soil structure (CHANG, 1987; GUO *et al.*, 2001; MAHER *et al.*, 2000). In the case of partially degraded ligno-cellulosic SMS, native soil microbiota contributes to further decompose this material and produce humus, which is fundamental for soil

fertility (CHANG, 1987). SMS presents high digestibility and can be used as animal feed or as biodigestor substrate for biogas production (BUSWELL; CHANG, 1993; FURLANI; GODOY, 2005; MANSUR *et al.*, 1992). Another possibility is to use these residues for the cultivation of other mushroom species. However, research for SMS utilization are still incomplete or unconclusive (CHONG; RINKER, 1994).

Organic matter sources can be classified as organic fertilizers or compost fertilizers. Organic fertilizers are of vegetal or animal origin and contain one or more plant nutrients. Compost fertilizers are obtained by natural or controlled biochemical processing of mixed vegetal or animal residues (VILLAS BÔAS *et al.*, 2004).

Organic fertilization is fundamental for the cultivation of vegetables, especially in tropical climate soils, where organic matter incineration is intensively performed, modifying the soil's physical, chemical and biological properties (ALLISON, 1973).

Mushroom cultivation residue present low density (~300Kg/m^3). It is generally uniform and constituted by stabilized organic matter. Composting processes and mushroom cultivation increase the concentration of some available nutrients in the substrate. Some spent mushroom composts present relatively high pH (7~8). When incorporated to soil, it increases pH as well as cationic exchange capacity and particles aggregation (SUESS; CURTIS, 2006). In this sense, SMC is a fertilizer with interesting agronomic characteristics, mainly for its high organic matter content, water retention capacity and as a source of macro-nutrients, such as N and P (MAGETTE *et al.*, 1998). Some other elements are found in SMC in relatively high concentrations, including nitrogen, potassium, phosphore, calcium and traces of other elements, especially iron (CHONG; RINKER, 1994; GERRITS, 1994).

3.23 SOLID-STATE (SSF) AND SUBMERGED (SMF) CULTIVATION OF MACROMYCETES' MYCELIA

Some metabolites can be obtained directly from mycelial masses, without the necessity of producing fruiting bodies. Mycelium-based processes present sorter production cycles and allow better automation and control, in relation to carpophores-based technologies. Crescently diversified technological applications of mushrooms are contributing to an expressive increase in production of mycelia and derived substances in the last decades (BRAR *et al.*, 2013; KARP *et al.*, 2012;

NAVEGANTES *et al.*, 2013; SALMON *et al.*, 2012; SOCCOL *et al.*, 2013; SPIER, 2012).

The main difference between SSF and SmF is the mixture capacity of each system (GERVAIS; MOLIN, 2003). In submerged cultivation, the reaction mixtures approach perfection, that is, at a given moment each part of the reactor contains the same amount of microorganisms, nutrients and metabolites. In solid-state substrates, the systems are more heterogeneous. According to Murthy *et al.* (1993), wet solid-state substrates are those that are not water-soluble and are not suspended in water. Durand & Chereau (1988) further define submerged cultivation as that in which solid substrates are dissolved or suspended in the cultivation broth. This same author stresses that surface cultivation, in which mycelium grows over the surface of static liquids cannot be defined as a solid-state process. Consequently, mycelial cultures over semi-solid media cannot be classified as solid-state processes either.

Moo-Young *et al.* (1983) define solid-state culture or solid-state fermentation as processes in which microorganisms use insoluble material for their growth and metabolism. Mudgett (1986) describes them as processes in which microorganisms develop in free liquid absence. The liquid for metabolic processes is adsorbed over solid particles or complexed inside solid matrixes.

Solid-state substrates are usually fragmented, of fibrous nature, allowing water retention by hygroscopy and/ or capillarity. Water content vary according to the substrate type. Starchy substrates ideal initial humidity for solid-state processes is between 25 and 60%. Cellulosic substrates such as straw, husks, bagasses and brans require higher moist (60~80%) but without noticeable free water (SOCCOL, 1994).

Solid-state fermentation processes present advantages in relation to submerged cultivation. Del Bianchi *et al.* (2001) presented a comprehensive list:

- Faster reaction rates due to the direct contact microorganism/ substrate;
- Skipping of substrate pre-treatment steps, such as extraction procedures;
- Simple substrate formulation, requiring only water addition in some cases;
- Less water, energy and space demand;
- No need for continuous agitation;
- Relatively low moisture and high inoculation rates minimize microorganism contamination problems;

- Growth conditions are more similar to filamentous fungi natural habitats;
- Lower liquid effluents production;
-In some cases, the yield is bigger than in submerged cultivation processes.

Del Bianchi *et al.* (2001) also highlighted the disadvantages of SSF in relation to submerged processes:

- Scaling up can present problems related to the heterogeneity of the system, such as heat and gases dissipation, as well as measuring and control difficulties;
- When necessary, much more energy is needed for aeration, in relation to submerged processes;
- It is difficult to collect representative samples of the fermented mass, due to the already mentioned heterogeneity;

Although bacteria and yeast can also be used for SSF processes, filamentous fungi are considerably better adapted to solid substrates, due to their physiological and enzymatic properties (SOCCOL; VANDENBERGHE, 2003; SOCCOL, 1994).

Gomez (1990) used sugarcane bagasse and brewers yeast as substrate for *Pleurotus ostreatus* mycelium submerged cultivation. It resulted in a final product rich in proteins and carbohydrates, with good digestibility, based in the low residual lignin levels found. The obtained mycelial mass can be directly used as food, or as a supplement for food products, due to its high proteic content.

Several authors studied the production of biomass and active substances, such as exopolysaccharides by the submerged cultivation of various macrofungi species (GERN *et al.*, 2008; GUTIÉRREZ *et al.*, 1996; ROSADO *et al.*, 2003). Different carbon sources result in variations in biomass and polysaccharides generation. Monosaccharidic composition of polysaccharides synthesized by the mycelium also vary depending on the carbon source (KHONDKAR *et al.*, 2002; KIM *et al.*, 2002).

3.24 BIOREACTORS

At the heart of the majority of biotechnological processes stands this equipment called bioreactor. At its simplest definition, it can be a vessel, inside of which biological processes are carried. It can be a bottle in which beer is fermenting, or a barrel used to brew wine. Erlenmeyer flasks within bacterial cultures, natural yogurt cups, composting tunnels, wastewater treatment lagoons and mushroom growing

bags are all examples of bioreactors. In its most sophisticated forms it can be very complex equipment, for extremely aseptic processes, with sensors and automated functions.

Often, it is necessary to monitor and control processes conditions, like temperature, pH, concentration of gases (especially O_2 and CO_2), humidity (in case of solid state fermentation), cell concentration, substrate utilization and product generation. Instruments can be coupled to the bioreactor for heating/ cooling, stirring, aerating, feeding medium and correcting pH.

The fermenters used for yeast and bacteria can be adapted for submerged cultivation of macromycetes mycelia. Agitation, aeration and temperature control systems are required. All components must resist to multiple cycles of autoclavation (120ºC for 20 minutes). The vessel material can be glass, heat resistant plastic or stainless steel. Agitators are usually constructed of stainless steel. Pipelines can be made of silicon, glass or stainless steel. Air filters need to be adapted in air lines.

Solid-state cultivation of wood-degrading macromycetes (such as *Lentinula edodes* and *Auricularia auricula*) mycelia is traditionally performed using wood logs as substrate and recipient. Wood logs do not need to be autoclavated, because their inner part is uncontaminated. Heavy inoculation rate favours the colonization of the substrate by the mycelium. Log cultivation techniques evolved from almost no control (open air or in the woods) to fully automated environmental conditions controlled rooms.

Agaricus spp. mushrooms cultivation is traditionally performed in beds or trays filled with composted substrate, inoculated with mushroom spawn. After the mycelium run is complete, a thin layer of covering soil is added over the myceliated substrate. This process is conducted in dark and moist places. As well as wood log cultivation, techniques for *Agaricus* spp. evolved from very rustic to fully controlled conditions.

More recently, heat resistant plastic (polypropylene) bags and bottles had been adopted as cultivation recipients for mushrooms production. They allow easier handle of the substrate, keeping it moisted and away from contaminations. These recipients can not be totally closed, because the mycelium growth process is aerobic. Consequently, filters are used for allowing air exchange while avoiding contaminations.

Although rustically developed, this concept of modular solid-state bioreactor stills the most successful production model for mushrooms mycelium and carpophores. The main reasons for the failure of big bulk fermenters are related with mass and energy transferrence difficulties. Even after reasonably well homogenized, static solid substrates tend to rapidly develop heterogeneous distribution of moisture, aeration, heat and particle sizes. Nevertheless, solid substrates demand great amounts of energy for mechanical agitation and aeration. In addition, the agitation of the substrate causes mycelial tearing and heating, affecting negatively the process.

3.25 ON-LINE BIOMASS CONCENTRATION SENSORS

New mechanical, chemical, electric, electronic and informatic devices are currently being devised aiming a better monitoring and control over bioreactors. Biomass concentration is a central variable for most bioprocesses. Off-line biomass quantification methods are time demanding and sample collection procedures present the risk of introducing contaminations in the bioreactor (KIVIHARJU *et al.*, 2008).

Techniques based in electric and optical properties of cultivation brothes can be applied for developing novel on-line sensors for indirectly measuring biomass concentration in bioreactors. Kiviharju *et al.* (2008) evaluated dielectric spectroscopy, optical density, infrared spectroscopy, and fluorescence for in situ measurement of biomass. These same authors proposed software sensors as an economic alternative for this application (KIVIHARJU *et al.*, 2008). Rønnest *et al.* (2011) tested multiple techniques, namely: multi-wavelength fluorescence (MWF) spectroscopy, scanning dielectric (DE) spectroscopy, and turbidity measurements, carbon dioxide evolution rate (CER) and the concentration of dissolved oxygen. They found that sophisticated methods were not more precise as the classical ones. In addition they have observed that combined techniques resulted in more precise measuring.

Other possible approach is to use optical density measurings as an indication of biomass concentration. Some devices were built using optical fibers as a means to autoclave the apparatus apart of the light source (ZHAO *et al.*, 2008).

3.26 HARDWARE/ SOFTWARE

3.26.1 Pure Data

Pure Data (Pd) is an open source programming language, which can be freely downloaded at http://puredata.info/downloads. Pd enables the creation of software through a friendly graphical interface. Pd can process and generate sound, video, 2D/3D graphics, and receive data from sensors, input devices, and MIDI. Pd can work over local and remote networks, integrating wearable technology, motor systems, lighting rigs, and virtually any other equipment, including bioreactors monitoring and control apparatuses. Pd is suitable both for learning basic visual programming methods as well as for building complex systems for large-scale projects (PUCKETTE, 2014).

Pd is a major branch of patcher programming languages family known as Max. Pd expanded the Max paradigm by incorporating video and web interaction. Pd was originally developed by Miller Puckette at IRCAM, in the 1990's.

The structure of a Pd patch is basically composed of objects connected by cords. Data flow among these objects through the connection cords. Objects can transmit sensors signals, process these signals, display them on the screen in numeric or graphic forms and can also activate effectors like turning on lights and machines.

3.26.2 Arduino microcontroller

Arduino is an open-source electronics prototyping platform for creating interactive objects and environments. It is a project supported by many collaborators, coordinated by Massimo Banzi, David Cuartielles, Tom Igoe, Gianluca Martino, and David Mellis. Arduino can receive inputs from a wide variety of available sensors and can control lights, motors and virtually any type of actuators (BANZI, 2014).

It can be used as a standalone device within its own power supply, an internal memory for storing programs and data, and a processor for running applications. While in standalone mode, it requires to be programmed in its native programming language, called *processing*. Otherwise, it can be connected to a computer, taking the full advantage of its processor, memory, softwares and peripherals, such as monitor, printer, keyboard, mouse, speakers, audio inputs, webcams, joysticks, usb

and serial port devices. Many languages can interact with Arduino, while it is connected to a computer, such as Pure Data and Max/MSP (PUCKETTE, 2014).

This board's project is fully available, any person can download it, and there is no restriction to build identical boards, for any purpose. As it is an open source project, there is no need to pay royalties to use this technology, making it affordable. There are many versions of Arduino, covering a broad range of applications, from industrial-scale machines to wearable devices, to far beyond driven vehicles (BANZI, 2014).

Arduino boards suit the requirements of automation and control for innumerable bioprocesses and biotechnological applications. Whole bioreactor control systems can be assembled based in Arduino. It can be used for monitoring and registering sensors data, for actuating thermostatic devices, for controlling motors for agitation, aeration, pH control, and for pumping products outside and fresh culture medium inside the vessel.

This flexibility allowed the development of several example systems described in section 4.7 of this thesis, including a photoperiod timer, a thermostatic device and a biomass concentration indirect measuring system, based in optical density reading, using a red laser as a light source and a phototransistor as a sensor. This system included apparatuses for pumping the samples from the vessel to a reading cell.

3.26.3 Phototransistor

It is a light sensor type with better sensivity than a photodiode. It is a transistor with a transparent cover, created by William Shockley, in 1951. The idea was to optimize a photosensibility effect common to all transistors, in order to create a photo-sensor. Many applications were developed with this component since then, including photo and video cameras, spectrophotometers, CD players, systems for lights switching based in sensed light levels, obstruction checking devices for industrial processes and security systems. Briefly, it outputs current, proportional to the light incidence. In fact, its mechanism relies in the movement of electrons induced by light. It amplifies the sensed signal, by receiving current, generating a high gain and low noise output. Phototransistors are not very sensitive to high frequencies, but for its low price and high sensivity it can be a good choice among light sensors (POOLE, 2014).

3.26.4 R language and rsm (response surface methodology) package

R is an open-source programming language designed for statistical computing, which can be downloaded at http://www.r-project.org/. R language is an expansion of language S, created by John Chambers and colleagues at Bell Laboratories. Ross Ihaka and Robert Gentleman created R at the University of Auckland. The language was named R because of its creators' initials. R currently continues to be improved by the R Core Development Team, featuring John Chambers and R inventors (HORNIK, 2013; R-PROJECT, 2014). R provides a collection of mathematical functions for data manipulation, calculation and graphical display. It can be easily extended via add-on *packages*. *Packages* covering a wide range of modern statistical applications are available from the CRAN family of websites (http://cran.r-project.org/) (CRAN, 2014).

Rsm is an R add-on *package* containing response surface analysis tools. It can be freely downloaded at http://cran.r-project.org/web/packages/rsm/index.html. It provides functions to fit first and second order surface models to experimental data points, to analyse the lack of fit of these models, to plot surface response graphics and to obtain the path of steepest ascent (LENTH, 2009; RSM, 2014).

3.27 MATHEMATICAL TOOLS FOR EXPERIMENTAL PLANNING AND RESULTS ANALYSES

3.27.1 Linear regression by least squares methodology

Linear regression techniques are used to determine the linear tendency of an experimental numbers set dispersed in a space generated by an axis of correlated variables. The obtained line represents a function between the analysed variables. This line is constructed in such a manner spaces between experimental points and the regression line are minimized. This is the concept of least squares methodology. R^2 values indicate the proximity of predicted and experimental points. The highest possible R^2 value is 1 and would indicate a linear function which perfectly fits the experimental results. Low R^2 numbers mean that there is no correlation between variables, or that this correlation is not linear (LANE, 2014). Linear regression by least squares methodology can be currently performed by many computer softwares and graphical calculators.

3.27.2 One way analysis of variance (ANOVA) and Tuckey post-test

This statistics method is useful when analyzing the effect of only one variable (factor) over three or more groups of results, such as in experimental treatment groups and experiments with repetitions. For comparing only two groups of results, Student's T test is applied instead. Software tools are available for performing T test. There is an on-line version of a software for doing this analysis (STUDENTSTTEST, 2011).

One way ANOVA (or one factor ANOVA) is fed with each treatment results mean and standard deviation. It is required that these results are simple independent random samples from normally distributed populations, with equal or at least approximated standard deviations and sample sizes (BROWN, 2013).

One way ANOVA allows the determination wether differences between means are statistically significative or if they are due to experimental error and random variations. However, ANOVA alone can only indicate the presence of different means among the analyzed groups, but to tell which means are different, post-tests are necessary. Post-tests, such as Tuckey are applied for determining which means are different for a given significance level (LOWRY, 2013).

Tuckey compares all means pairs. Results are expressed in such a manner different letters are attributed to significantly different means and equal letters are attributed to statistically equal means. Tuckey's test can be applied for independent observations and groups of results with equal variances (homogeneous) (IBM, 2011).

It is advisable to start by comparing the highest mean with the lowest one. If there is no difference between means, then all the means between are also equal and there is no necessity of comparing them. If differences are detected between extreme mean values, then comparisons should be made between the highest mean and the next lowest one, until two equal means are found. There are specially designed software packages that allow automated ANOVA and Tuckey analyses.

3.27.3 Plackett-Burmann experimental design

This experimental design and data analysis tool was created and published by Plackett & Burmann (1946) and allows the assaying of the main effect of a great number of factors, with a reduced set of runs in comparison to full factorial methods.

This is ideal for screening purposes, for initial experiments destined to choose candidates for deeper studies among a great number of alternatives. It is also useful for situations when a great number of experimental runs is not viable, for economic, environmental or ethic reasons. Briefly, all assayed factors are analyzed in two levels (+1 and -1). Each experimental run is composed by a combination of all factors in either one or other of the levels. Not all the combinations are tested, but a reduced set instead, which stills present meaningful representative results. Some central point run repetitions are needed for the experiment to render statistical analyses, to estimate the experimental error and mathematical model curvatures. Central points are runs for which all the factors are set to a middle value exactly in between the high and low levels (PLACKETT; BURMAN, 1946). Data analyses can be currently performed by several software packages.

Results can be outputted as Pareto's charts, showing graphically the significance level of each assayed factor. ANOVA tables can be constructed for showing the adequateness of the proposed model solution to the experimental data. Tables can be outputted for comparing predicted values and experimental results.

3.27.4 2^k full factorial experimental design

A full factorial experimental design in two levels is the one in which factors are assayed in two levels (+1 and -1) and runs are performed for all possible combinations of factors levels. This type of design is useful for evaluating the main effects and interactions among a little number of factors. It is a richly informative design type, because all factors' levels combinations are evaluated. It should be applied in screening assays with few factors, or in situations in which factors interactions are being tested (NIST, 2014). A design with 2 levels and k factors, presents 2^k runs.

As shown in TABLE 14, for more than 5 factors, the number of runs is significantly high. For these cases, another type of experimental model, such as a fractionated factorial or a Plackett Burmann, is better suited.

As in Plackett-Burmann designs, it is possible to add central point runs to full factorial designs, in order to estimate experimental error and mathematical model curvatures. Full factorial designs are the base for surface response methodologies.

TABLE 14- NUMBER OF RUNS FOR A 2^k FULL FACTORIAL.

NUMBER OF FACTORS	NUMBER OF RUNS
2	4
3	8
4	16
5	32
6	64
7	128

SOURCE: the author (2014).

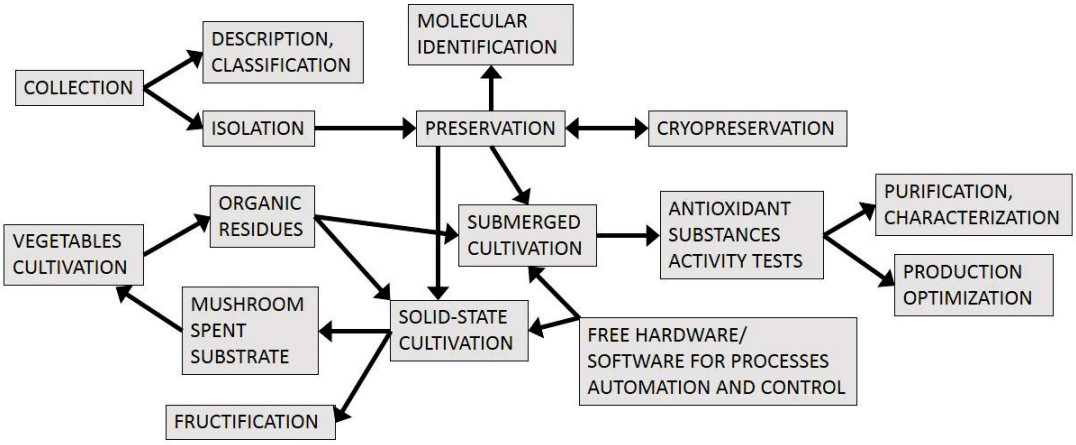
3.27.5 Surface response methodologies and the central composite design

Surface response methodologies are mathematical tools, useful for the optimization of processes. They are better suited for 2-4 variables. Central composite design (CCD) is a popular surface response method. Two data blocks are considered. The first one is called “cube” and comprises points from a full factorial or fractionated factorial design, within center points. First and second order mathematical models are approximated to the experimental values by regression methodologies. Lack of fit of these models are analysed. If a first order model is better suited, it means that the experiments are being conduced outside the optimal range of factors’ levels. New iterations are needed in order to determine the optimal range. First order models point a tendency of steepest ascent, which helps deciding the values for the subsequent experimental iterations. When first order models present significant lack of fit, higher order models need to be tested. Some additional points are required for determining a second order model. These are called “star points” data block and are constituted by axis and center points. When combined, the “cube” block and the “star points” render a full central-composite design. If a second order model fits well to the experimental data, it means that the analyses are being conduced in the optimal range of assayed factors’ levels and that the obtained model can be used to predict the optimum combination of levels for the assayed factors. The second-order function can be displayed graphically. Several parameters of the regression can be evaluated, such as lack of fit, residuals, pure error and values called eigenvalues that indicate wether the curvature indicates a maximum or a minimum point. Various software packages can perform surface response analyses. One free alternative is R programming language rsm library (LENTH, 2009).

4 MATERIALS AND METHODS

Non-linear research dynamics was adopted to optimize time and resources utilization, maximizing results production. Secondary objectives are interrelated as shown in FIGURE 7 diagram. The starting point for all performed experiments was the collection of native carpophores from the Parana portion of the Atlantic Rainforest and Curitiba region. This material was identified based in classical taxonomic methods (morphology and histochemistry) and used for isolating new mycelia strains.

FIGURE 7 – DIAGRAM SHOWING NON-LINEAR RELATIONS BETWEEN EXPERIMENTAL SUB-AREAS.



SOURCE: the author (2014).

Selected isolated cultures were re-identified by a DNA tag sequencing method. Besides being maintained by traditional periodic transfer and refrigeration technique, new long-term preservation methods were evaluated for preserving these isolated mycelial cultures, with highlights for cryopreservation.

The collected, identified, isolated and preserved macromycetes' strains were tested for some technological applications, including the production of carpophores and antioxidant substances, using organic residues as substrate. Several of these strains were screened for the production of two important antioxidant enzymes (superoxide dismutase and catalase) under submerged cultivation. The processes were submitted to optimization iterations in order to enhance the yield of these antioxidant enzymes. Both inducers of enzymatic activity were screened and purification steps were developed in pursuit of efficient bioprocesses for producing these active antioxidant enzymes.

After a starting up phase, all steps were performed in parallel. Collections were not time restricted, allowing new strains to be isolated whenever available, along the whole research period. Conservation techniques tests concentrated in the beginning of the work. After arriving at a functional short-term cryopreservation protocol, it was applied to all isolated strains as soon as they were free of contaminants and vigorous. All stored strains were reactivated within the last six months of research.

Molecular identification procedures and activity analyses were performed in batches, in order to save work time and optimize reagents and equipment utilization. Cultivation of mycelia and carpophores using organic residues, as well as automation/ control apparatuses development were performed throughout the whole experimentation period. Mushroom spent substrate was tested for soil fertilization in the last year of research.

4.1 NATIVE MACROFUNGI STRAINS PROSPECTION

4.1.1 Carpophores collection

Macromycetes fruiting bodies were collected in the regions of Curitiba and Parana's Atlantic Rainforest. Various substrates and environments were explored in order to maximize the diversity of the collections. Forays covered:

- Rural, urban, seacoast and forest environments
- All seasons
- Lignocellulosic and humic substrates
- Parasitic, saprotrophic and ectomycorrhizal fungi

Excessively small, hard, soaked, dirt, dry, rotten or parasitized specimens were avoided due to associated difficulties in isolation procedures. When found in groups, some basidiomata were let in place for spreading spores. In the case of big perennial polypores, only fractions of the fruiting bodies were cut and taken to the laboratory, to aid transportation and to allow spores dispersion by the remaining part.

Whenever possible, all distinctive features of the carpophores were preserved during collection, and photos were taken in the original collection site, to help identifications. Styrofoam, cardboard and waxed paper containers were used for transporting the collected material to the laboratory. Separate packages were used for distinct species. Collections were taken to the laboratory as fast as possible to

avoid degradation and microbial growth. At the laboratory, fruiting bodies were maintained under refrigeration until isolation and identification procedures.

4.1.2 Isolation procedures

New secondary mycelial cultures were obtained from either carpophores tissues or spores. Fungal material was transferred to Petri dishes, containing suitable cultivation media, as aseptically as possible. All successful isolation procedures were performed with potato dextrose agar (PDA) medium, which is adequate to saprotrophic macromycetes. Some trials to isolate mycorrhizal species were performed, using a modified Melin Norkrans (MMN) medium. Innoculated Petri dishes were incubated at 25ºC, in light absence, until mycelial development. In most cases, several transferences were necessary to obtain contamination-free strains.

4.2 IDENTIFICATION/ MOLECULAR IDENTIFICATION OF ISOLATED MACROMYCETES

The renowned mycologist André de Meijer kindly performed morphological and histochemical identification of carpophores. He used an Ernst Leitz Wetzlar ® monocular microscope (with 10X, 50X and 100X objectives and 10X ocular with micrometric wheel), a microcomputer and a personal macrofungi taxonomy library, besides some colorants and reagents.

TABLE 15 – MINIMAL LIQUID MEDIUM COMPOSITION FOR MYCELIA CULTIVATION.

SUBSTANCE	CONCENTRATION (G/L)
Glucose	10,0
Yeast extract	4,0
$MgSO_4$	0,3
KH_2PO_4	0,3

SOURCE: the author (2014).

For molecular analyses, the DNA extraction method was adapted from Lee and Taylor (LEE; TAYLOR, 1990), as described by Binder & Hibbett (BINDER; HIBBETT, 2003). Protocols similar to those described by Ko *et al.* (2001) and Menolli Jr. et al.

(2010) were applied for samples identification. Steps are following descripted in more detail.

Isolated strains mycelia were cultivated in minimal liquid medium (TABLE 15) prior to DNA extraction. Mycelial cultivation was performed in Erlenmeyer flasks incubated in an orbital agitator at 25ºC, 120 rpm for ten days. Then, mycelium pellets were washed with sterile distilled water by the following steps: -suspend the mycelium pellet in 500µL sterile distilled water, in a microcentrifuge tube; -vortex for five seconds; -centrifuge for 15 minutes at 7500 rpm; -discard supernatant.

Alternatively, mycelium cultivated in Petri dishes, over PDA medium was harvested with an autoclaved toothpick and transferred to a 2 mL microcentrifuge tube. In this case, the mycelial mass was washed by adding 0,5 mL of miliQ water, gently agitating, centrifuging at 10.000 rpm for 15 minutes and discarding the suppernatant.

After this step, the small pellet (~200mg) of washed mycelium was transferred to a cryovial containing approximately 0,3 mL of quartz beads. The cryotube was submitted to vigorous shaking for one minute with a bead-beater, in order to break fungal cells. Mycelial debris was resuspended in 0,5 mL of extraction buffer with 100µg/mL proteinase-k (SIGMA-ALDRICH, 2014a) (TABLE 16), the tube was gently homogenized and incubated at 37ºC for 2 hours.

TABLE 16 – COMPOSITION OF DNA EXTRACTION BUFFER.

SUBSTANCE	CONCENTRATION
Tris-acetate	40mmol/L
EDTA	1mmol/L
sodium acetate	20mmol/L
SDS pH7·8	1% w/v

SOURCE: Lerner and Model (1981 apud Al-Samarrai & Schmid, 2000).

The deproteinated lysate was transferred to a microcentrifuge tube and centrifuged at 10.000 rpm, 4ºC for 15 minutes. The recovered supernatant was transferred to a fresh tube and submitted to a DNA extraction procedure by phenol-chloroform protocol as previously described (SAMBROOK; RUSSELL, 2006).

DNA extraction protocol consisted of: - transferring the sample and an equal amount of phenol:chloroform (1:1) to a microcentrifuge polypropylene tube; - gently

mixing to form an emulsion; - centrifuging at 10.000 rpm for one minute in order to separate phases; - using a micropipette to transfer the aqueous phase to a fresh tube (organic phase and the interface were discarded); - repeating the whole procedure until no protein was visible at the interface; - repeating the whole procedure but with chloroform-isoamyl alcohol 24:1 instead of phenol:chloroform.

The extracted DNA was recovered by ethanol precipitation as described elsewhere (BINDER; HIBBETT, 2003; SHAPIRO, 1981). Basically, the sample, 0,1 volumes of 3M sodium acetate and 1,8 volumes of cold ethanol 95% were added to a conical polypropylene tube; the tube was gently shaken and stored at -20ºC for 20 minutes; the tube was centrifuged at 10000rpm, -2ºC, for 15 minutes; the supernatant was carefully removed with a micropipette until 10-30µL of the ethanolic solution was left in the tubes; Precipitated nucleic acids were dried at 37ºC. Ethanol:water solution (2,5:1), 0,1M NaCl at -20ºC was added (same volume as ethanol 95% was previously added); the precipitate was dried at 37ºC, redissolved in miliQ water and stored at -20ºC for further analyses.

Internal transcribed spacers (ITS) regions of ribosomal DNA (ITS 1 and ITS 2) were amplified, by using ITS 5 (forward) and ITS 4 (reverse) primers. The PCR mix composition used for ITS segments amplification is detailed in TABLE 17:

TABLE 17 – COMPOSITION OF PCR MIX UNITARY REACTION.

SUBSTANCE	VOLUME (µL)
dH_2O	15
10x PCR buffer	2,5
DNA solution	5
dNTP mix	0,5
ITS 4 primer	1,25 (=12,5 pmol)
ITS 5 primer	1,25 (=12,5 pmol)
Taq DNA polymerase (5U/µL)	0,125

SOURCE: the author (2014).

Multiple DNA solution dilutions in the 1:10-1:3000 range were tested for each extracted sample. The PCR cycle described in TABLE 18 protocol was used:

TABLE 18 – PCR PROGRAM FOR AMPLIFICATION OF ITS SEGMENTS OF rDNA.

CYCLES	STEP	TEMPERATURE (ºC)	TIME (MINUTES)
1	1 – initial denaturation	95	2
30	2 – denaturation	94	1
	3 – annealing	50	30
	4 – extension	72	1
1	5 – final extension	72	5
1	6 – reaction stop	4	indefinite

SOURCE: the author (2014).

PCR products were purified by a protocol adapted from Binder & Hibbett (2003). The following steps were performed: transfer the PCR reaction in a 1.5 ml tube, add 3 volumes NaI and mix; add 5 ul glassmilk, mix and allow the DNA to bind for 5 min.; centrifuge at 10000 rpm for 5 sec, remove and keep supernatant separately; wash the DNA/glassmilk pellet 2x with 500 ul NewWash, use a micro-pipet to dissolve the pellet completely, centrifuge at 10000 rpm for 5 sec each washing step; dissolve pellet in 20~35 µl H_2O for 5 min.; centrifuge at 10000rpm, 30 sec, transfer supernatant (clean PCR product) in a new tube and freeze until used for sequencing.

The amplified product was sent for sequencing. The resulting nucleotide sequences were cut based in electropherogram quality, using BioEdit software (BIOEDIT, 2014). Edited sequences were compared with those previously deposited in NCBI GenBank (GENBANK, 2014) and AFTOL (Assembling the Fungal Tree of Life) (AFTOL, 2014) data banks, aided by BLAST software algorithm (ZHANG *et al.*, 2000).

4.3 VERMICULITE AS A SUPPORT FOR MYCELIA CRYOPRESERVATION

4.3.1 Introduction

The storage of viable and stable fungal strains for long periods is a requirement for many germplasm banks, research institutions and bioprocesses industries. Some authors have described techniques for this purpose, but there is no universal best method. Choice depends greatly on the strain to be preserved, the desired time of storage and available resources. Although general guidelines for establishment and

operation of microbiological collections are supplied by institutions such as WFCC (World Federation for Culture Collections), more practical resources are needed to assist biotechnologists in choosing among preservation methods (HUMBER, 1997; NAKASONE *et al.*, 2004; RYAN *et al.*, 2000).

Presently there is a global trend towards isolating a vast number of new microorganism strains in specialized niches. Molecular based identification of strains became relatively cheap, easy and reliable, allowing the comparison of sequencings with huge databases that are being fed by scientists worldwide. Efficient means for conserving these isolates can provide scientists and companies an invaluable source to prospect and develop biological based technology (ROBERT *et al.*, 2006).

There are worldwide efforts to unify information databases about culture collections. The WFCC maintains a database of culture collections: the World Data Centre for Microorganisms (WDCM). Today, 578 culture collections are registered at WDCM. Almost 3.000 people work to maintain more than 1.600.000 microbial strains, including almost 500.000 fungi strains, of more than 25.000 species. Brazil is one of the countries with greatest numbers of culture collections and strains. According to WDCM, at least 60 institutions maintain nearly 150.000 strains in Brazil. Many culture collections accept deposits and maintain catalogs for requests. Some also offer patent deposit and strain identification services (WDCM, 2014).

Probably the most effective mean for long time storage of mycelium previously described is the perlite protocol (PP), in which fungal strains are stored in liquid nitrogen (-195ºC) after being cultivated over perlite particles moistened with liquid cultivation medium containing 5% glycerol (HOMOLKA *et al.*, 2007).

Although extremely efficient, PP still presents some technical challenges. Specially, adjusting the moisture and avoiding contaminations during incubation.

The present experiment evaluates new preservation techniques derived from the perlite protocol. Differences from the original protocol include: pre-culturing of mycelia over semi-solid medium containing the cryoprotectant and a carrier material, in Petri dishes; transference of mycelia within pieces of the described medium to cryotubes for a second incubation before freezing; potato dextrose agar (PDA) was used instead of wort based media; vermiculite was tested as an alternative carrier material for substituting perlite; absence of a freezing ramp; and ultrafreezer (-80ºC) or common freezer (-20ºC) instead of liquid nitrogen for final storage.

Vermiculite was tested as a carrier material, for being a common and cheap granulated material, besides having a low thermal conductivity coefficient and low density, similarly to perlite. Carrier material and/ or cryoprotectant absence were also evaluated as negative controls.

Agaricus subrufescens (= *A. brasiliensis, A. blazei*) is a mushroom species native to Brazil, popularly known as “cogumelo do sol”, “royal sun agaricus” or “himematsutake”. Some medicinal properties of biomolecules produced by this macromycete were already scientifically proven, including antitumoral and immunomodulatory activities. Several products based in this species, including dry mushrooms, powders, capsules, extracts, supplemented food and cosmetics are commercialized in many countries.

A. subrufescens was chosen for this experiment because of its economic importance and storage difficulty relatively to other mushroom species. It is remarkable that *A. subrufescens* mycelium does not tolerate well simple storage at 4ºC for long periods. Frequent subculturing is necessary for maintaining cultures at room temperature, which demands much work, is relatively expensive and highly vulnerable to contaminations and undesirable mutations.

Polysaccharide yield was selected as a genetic stability marker, because many researches show that this class of biomolecules is probably responsible for the most notable medicinal properties of these mushrooms. Several industries are based on the production and purification of mushrooms polysaccharides.

Some methods for preservation of this species were already published. The purpose of this experiment is to reevaluate central assumptions of previously described mycelia cryopreservation protocols and to propose new efficient alternative techniques, for maintaining *A. subrufescens* mycelium under low temperatures.

4.3.2 Materials and Methods

New techniques for the preservation of viable *Agaricus subrufescens* (= *A. brasiliensis*, *A. blazei*) mycelium at low temperatures were evaluated. Storage at common freezer (-20ºC), ultra-freezer (-80ºC) and liquid nitrogen (-195ºC) were tested for three distinct strains (AbSC51, AbCG31 and Ab3). Before being transferred to cryovials for storage, mycelia were pre-cultivated in potato dextrose agar medium supplemented with 5% (m/v) glycerol and a carrier material. Two alternative carrier

materials were tested: perlite and vermiculite. The absence of cryoprotectant and/or carrier material were also tested. All strains were reactivated after twelve months of storage. Radial growth rate was evaluated in PDA medium and exopolysaccharide production under submerged cultivation was assayed by the difference between total sugars (phenol-sulfuric method) and reducing sugars (DNS method).

Petri dishes containing a thin (~0,5cm) layer of dry carrier material were autoclaved at 121ºC for 30 minutes. Potato dextrose agar (PDA) supplemented with 5% (m/v) glycerol was autoclaved separately and poured while melt into autoclaved plates containing carrier materials, under aseptic conditions. Perlite and vermiculite were evaluated. Cryoprotectant and/ or carrier absence were also tested.

Each plate was inoculated with pure cultures of *A. subrufescens* and incubated at 25ºC for about 10 days. Then, agar plugs (2~4mm diameter) with cultured mycelia and carrier material were transferred to 1.8mL cryovials. In both cases, 2~3 plugs were transferred to each cryovial under aseptic conditions (filling about ¾ of the tube). Closed cryovials were incubated at 25ºC for 10 additional days, and then maintained at 4ºC, in a refrigerator, for 24 hours before being transferred to either: a freezer at -20ºC, an ultra-freezer at -80ºC or liquid nitrogen at -195ºC. One important difference between the original PP and present variations is that in the original protocol, freezing is performed with a programmable freezer ($1ºC.s^{-1}$), and in the proposed methods, a simple step of pre-refrigeration (4ºC for 24 hours) was applied instead.

The 18 different conditions were tested for three *A. subrufescens* strains (AbSC51, AbCG31 and Ab3). Experimental runs are listed on TABLES 19, 20 and 21. Each condition was tested in triplicate for better statistical analysis of data.

All strains were reactivated after twelve months of storage. For mycelia reactivation, the cryovials were thawed at room temperature and colonized carrier grains were transferred to Petri dishes, containing simple PDA medium. Thus, 18 conservation protocols were evaluated for three different strains and compared based on strain survival and radial growth rate before and after storage at low temperatures.

For radial growth rate evaluation, Petri dishes were incubated at 25ºC and each colony diameter was measured and registered once a day for 14 days. Radial growth rate results were statistically analyzed to detect differences between treatments, to determine variables significance and interaction between variables.

TABLE 19 - EXPERIMENTAL RUNS CONDITIONS AND RESPECTIVE CODES IN FREEZER (-20ºC), APPLIED TO THREE DIFFERENT *AGARICUS SUBRUFESCENS* STRAINS.

CODE	STRAIN	SUPPORT	CRYOPROTECTANT
1	AbSC51	Perlite	glycerol
2	AbCG31	Perlite	glycerol
3	Ab3	Perlite	glycerol
4	AbSC51	Vermiculite	glycerol
5	AbCG31	Vermiculite	glycerol
6	Ab3	Vermiculite	glycerol
7	AbSC51	-	glycerol
8	AbCG31	-	glycerol
9	Ab3	-	glycerol
10	AbSC51	Perlite	-
11	AbCG31	Perlite	-
12	Ab3	Perlite	-
13	AbSC51	Vermiculite	-
14	AbCG31	Vermiculite	-
15	Ab3	Vermiculite	-
16	AbSC51	-	-
17	AbCG31	-	-
18	Ab3	-	-

SOURCE: the author (2014).

TABLE 20 - EXPERIMENTAL RUNS CONDITIONS AND RESPECTIVE CODES IN ULTRA FREEZER (-80ºC), APPLIED TO THREE DIFFERENT *AGARICUS SUBRUFESCENS* STRAINS.

CODE	STRAIN	SUPPORT	CRYOPROTECTANT
19	AbSC51	Perlite	glycerol
20	AbCG31	Perlite	glycerol
21	Ab3	Perlite	glycerol
22	AbSC51	Vermiculite	glycerol
23	AbCG31	Vermiculite	glycerol
24	Ab3	Vermiculite	glycerol
25	AbSC51	-	glycerol
26	AbCG31	-	glycerol
27	Ab3	-	glycerol
28	AbSC51	Perlite	-
29	AbCG31	Perlite	-
30	Ab3	Perlite	-
31	AbSC51	Vermiculite	-
32	AbCG31	Vermiculite	-
33	Ab3	Vermiculite	-
34	AbSC51	-	-
35	AbCG31	-	-
36	Ab3	-	-

SOURCE: the author (2014).

TABLE 21 - EXPERIMENTAL RUNS CONDITIONS AND RESPECTIVE CODES IN LIQUID NITROGEN (-195ºC), APPLIED TO THREE DIFFERENT *AGARICUS SUBRUFESCENS* STRAINS.

CODE	STRAIN	SUPPORT	CRYOPROTECTANT
37	AbSC51	Perlite	glycerol
38	AbCG31	Perlite	glycerol
39	Ab3	Perlite	glycerol
40	AbSC51	Vermiculite	glycerol
41	AbCG31	Vermiculite	glycerol
42	Ab3	Vermiculite	glycerol
43	AbSC51	-	glycerol
44	AbCG31	-	glycerol
45	Ab3	-	glycerol
46	AbSC51	Perlite	-
47	AbCG31	Perlite	-
48	Ab3	Perlite	-
49	AbSC51	Vermiculite	-
50	AbCG31	Vermiculite	-
51	Ab3	Vermiculite	-
52	AbSC51	-	-
53	AbCG31	-	-
54	Ab3	-	-

SOURCE: the author (2014).

For exopolysaccharide productivity quantification, the cultured broths were assayed for reducing carbohydrates (DNS method) and total carbohydrates (phenol-sulfuric method); the polysaccharides concentration in the cultivation broth was estimated to be equal to the concentration of total sugars minus the concentration of reducing sugars (EQUATION 1):

Polysaccharides = total carbohydrates – reducing carbohydrates (1)

Results were compared by ANOVA and Tukey post-test for $p<0,05$.

4.4 MACROFUNGI MYCELIA AND CARPOPHORES CULTIVATION USING PEJIBAYE PALM (*BACTRIS GASIPAES* KUNTH) SHEATH RESIDUES AS SUBSTRATE

4.4.1 Spawn production

Corn grains were used as substrate for spawn production. Although least productive, when compared to other cereal grains, corn is readily available in our region and consequently cheaper. The method here presented proven efficient and

economic. General procedures were adapted from previously published methods (CASSOU *et al.*, 2001; SOCCOL, 1994; STAMETS, 2000c).

Corn grains were washed in tap water and then boiled until turgid and soft. The cooked corn was sieved and water was discarded. The hot corn was washed with cool tap water for stoping cooking processes and water evaporation.

After driping the excess water, 2% (w/ w) calcium carbonate and 2% (w/ w) calcium sulphate were added. The mixture was homogenized and distributed in polypropylene bags, closed with polyurethane foam pieces, adapted with rubber bands. These polyurethane pieces functioned for both avoiding contaminations and allowing fungal respiration. The whole system was autoclaved for 20 minutes, at 120ºC. The final substrate humidity was within the 40~60% range.

The bags were let cool down to room temperature and transferred to a laminar flow hood. The material was submitted to UV light for 15 minutes and superficially sprayed with alcohol 70%. Three approximately 5 mm diameter pieces of mycelium pre-cultivated over PDA medium were aseptically transferred to each corn substrate bag. Mycelium matrices were obtained as described in section 4.1.2 of this thesis.

Innoculated bags were incubated in the dark, at 25ºC for 30 days, until complete myceliation. The cultivation room was periodically inspected and bags with any sign of contamination were immediately discarded. Those bags with abundant and vigorous mycelial colonization were selected for being used as spawn.

Spawn bags were maintained at 4ºC for a maximum of 2 months before usage, with the exception of *Pleurotus djamor* spawn, which was stored at room temperature for being more sensitive to cold weather.

4.4.2 Substrate elaboration

Pejibaye palm (*Bactris gasipaes* Kunth) sheaths were gently provided by a commercial producer, located at Alexandra-Matinhos highway, Km 12, Paranagua-PR, under the coordinates 25 º 09’ 19,2” S and 48 º 35’ 15,0’ W. These sheaths were removed for pickled palm production. The usual destination of these residues would be common landfill. The residues were grinded in a knives mill to approximately 5 cm pieces (FIGURE 8) and the grinded material was pressed for excess water draining. This excess water was collected and stored at -18ºC. The solid fraction was dried in an

air circulation stove, at 55ºC for 3 days (FAN *et al.*, 2001). The dried residues were stored in polyethylene bags at room temperature and were stable for at least 6 months.

FIGURE 8 - MILLED PEJIBAYE PALM SHEATH RESIDUES(LEFT).
DRIED PEJIBAYE PALM SHEATH MILLED RESIDUES (RIGHT).

SOURCE: the author (2014).

4.4.3 Screening of macromycete strains for adaptation to pejibaye palm (*Bactris gasipaes* Kunth) sheath residues utilization as substrate

Five distinct edible macromycetes strains were evaluated: *Agaricus subrufescens*, *Coprinus comatus*, *Pleurotus djamor*, *P. eryngii* and *P. ostreatus*. *A. subrufescens* LPB3 and *P. ostreatus* PL-24 were gently supplied by the Bioprocesses Engineering and Biotechnology Laboratory Strain Collection. *P. djamor* was isolated from a wild specimen and *P. eryngii* was isolated from a commercial fruiting body.

A thin layer of dry milled pejibaye palm sheath residues was distributed in glass Petri dishes and autoclaved at 121ºC, for 40 minutes. In a laminar flow hood, agar-agar, separately prepared and autoclaved (at 121ºC, for 20 minutes) was poured inside the dishes, as to almost completely cover the residue's fibers.

Still inside the laminar flow hood, after agar-agar solidification, small (~10mm diameter) pieces of mycelium previously cultivated over PDA medium were used to inoculate the Petri dishes prepared with residues and agar-agar. Innoculated dishes were incubated at 25ºC until complete myceliation. The colonies were measured in two directions (bigger diameter and smaller diameter) every 24 hours.

The experiment was performed in triplicate. Instant diameters means (in cm) were plotted against time (in days) and a model equation was obtained by linear regression. The angular coefficient was taken as an estimative of radial growth rate

(in cm/ day). Differences between strains growth rates were analysed by ANOVA and Tuckey post-test for a 5% significance level (P<0,05).

4.4.4 Effect of inoculation rate and number of holes over biological efficiency

Dried and milled pejibaye palm sheath residues, obtained and processed as described in section 4.4.2. were kept immersed in water (60g/ 2L) for 24 hours. Excess water was drained by press to a final humidity of approximately 70%. This hydratation water was recovered and tested as substrate in a subsequent experiment for submerged cultivation of *Pleurotus* spp. mycelia.

After humidity adjustment, the substrate was distributed in polypropylene bags (20x30cm.0,12mm). Approximately 500g of the rehydrated residue (~150g dry weight) were acconditioned in each bag, and a piece of polyurethane foam was adapted to close the bag aperture with a rubber band and function as an air filter. The filled and closed bags were autoclaved at 121ºC for 40 minutes.

Bags were let cool down to room temperature and inoculated in laminar flow hood. Innoculation rate varied as described in TABLE 16. Innoculated bags were incubated at 20~25ºC, air humidity of approximately 70%, in light absence (figure 7), for approximately 30 days, until complete myceliation of the substrate (figure 8).

Fully myceliated bags were transferred to the fructification room and submitted to fruiting induction conditions and procedures (BONATTI *et al.*, 2004; SANTOS *et al.*, 2009). A flamed scalpel was used for cutting holes in the bags. Each hole consisted of two cuts, in X form, with nearly 2,5 cm diameter. The number of holes per bag varied according to table 16. The frutification room received indirect sun light (1.500 to 2.000 lux/ hour), in a photoperiod of 12 hours light/ day. Temperature was not controlled and varied from 18ºC to 25ºC. Air relative humidity was maintained at 80~90% with a water spray system, programmed to operate for one minute in three hours intervals.

A 2^2 factorial experimental design was applied for this experiment. The experimental factors were inoculation rate (%) and number of holes per bag. Factors' levels are described in TABLE 22. The experiment consisted of 4 runs plus 3 central point repetitions. Response variables were: biological efficiency (%), productivity (%), physic-chemical composition (g.100 g^{-1}) (humidity, gross proteins, lipids, ashes and total carbohydrates), and production aspects, including time lag before fructification,

carpophores dimensions, number of holes that fructified and total cultivation time. Empirical equations were adjusted to experimental results and the significancy of each polynomial term was statistically evaluated by Tuckey post-test (P<0,05).

TABLE 22 – 2^2 FACTORIAL EXPERIMENTAL DESIGN FOR EVALUATING THE EFFECTS OF INOCULATION RATE AND NUMBER OF HOLES PER BAG OVER *P. OSTREATUS* CULTIVATION.

FACTORS	LEVELS	
	-	+
Inoculation rate	10	40
Number of holes	2	4
RUNS	INNOCULATION RATE	NUMBER OF HOLES
	(%)	(units)
1	10	4
2	10	2
3	40	4
4	40	2
5	25	3
6	25	3
7	25	3

SOURCE: the author (2014).

4.4.4.1 Time lag before fructification

Period (days) between making holes in the bags and the formation of the first primordia, was taken into account for the first cycle. For the second cycle, time between the first harvest and the appearance of new primordia was considered.

4.4.4.2 Carpophores dimensions

Basidiocarps were measured as soon as harvested. **Longitudinal diameter** refers to the distance between the stipe and the pileus margin, **width** is the pileus diameter, perpendicular to the longitudinal diameter and **height** is the distance between the stipe base and the most distant portion of the pileus.

4.4.4.3 Biological efficiency (BE) and productivity (P)

Both biological efficiency (BE) and productivity (P) are indicatives of the mushroom productive process adequation. They are the main response variables

analysed for strain selection, substrate composition and incubation conditions optimization, new substrates and cultivation methods evaluation. Although similar, they express different measures. Both expressions are concerned with the yield of fresh mushrooms at the end of the process. However, **BE** represents a ratio between **fresh mushrooms mass** harvested and the respective **dry substrate** mass used for cultivation. Slighthly different, **P** represents a ratio between **fresh mushrooms** and **moist substrate** (DAS; MUKHERJEE, 2007; YILDIZ *et al.*, 2002; ZHANG *et al.*, 2002). Equations for BE and P, respectively named eq. 2 and eq. 3 are written down following.

$$BE\ (\%) = \frac{\text{fresh mushrooms mass}}{\text{dry substrate mass}} \times 100 \qquad (2)$$

$$P\ (\%) = \frac{\text{fresh mushrooms mass}}{\text{fresh substrate mass}} \times 100 \qquad (3)$$

4.4.4.4 Moisture

Determined by gravimetry, as described by (A.O.A.C., 1990). Moisture (%) was defined as the ratio between the fresh and dry weights of the sample times 100. Dry weight being the mass of the sample maintained at 105 ºC until constant weight.

4.4.4.5 Ashes

Calculated by the sample's mass after incineration, following the (A.O.A.C., 1990) method. One gram (1,0 g) of sample was put in porcelain capsules, previously dry in muffle furnace and calcinated at 550 ºC during 4 hours. Capsules containing samples were then cooled down inside a dessicator and weighted with an analytical balance. Ashes (%) were defined as the ratio between the mass of the calcinated sample and the respective fresh sample initial weight, times 100.

4.4.4.6 Total lipids

Gross fats content was determined by gravimetry, after continuous extraction of the samples with petroleum ether in a Soxhlet equipment, according to the (A.O.A.C., 1990) method. 250 mL flat bottom glass flasks were washed, dried at 105 ºC, let cool

down inside a desicator and weighted with an analytical balance. The cellulose extraction catridge containing 3,0 g of sample was acconditioned inside the reflux tube of the Soxhlet apparatus. Approximately 100 mL of petroleum ether were added and the system was heaten until volatilization of the ether. The system was maintained at 40 ºC for 5 hours, extracting the lipidic fraction of the sample and other ether-soluble substances. The extracted lipids were dried at 105 ºC for 12 hours. Lipidic content was measured by the mass difference before and after extraction.

4.4.4.7 Gross protein content

Determination performed by macro-Kjeldahl, according to (A.O.A.C., 1990) method. 0,6 g of sample were submitted to an acid digestion at 350 ºC, with 4,0 g of catalyzer (33,3% cupper sulfate and 66,6 % potassium sulfate) and 25 mL of sulfuric acid (H_2SO_4) PA, for 3 hours and a half. This procedure converts all nitrogen into ammonium $(NH_4)^+$. After cooling down, the digestion product was mixed with 150 mL of water, 80 mL of sodium hydroxide (NaOH) 40% and a metallic zinc piece. This mixture was heaten and the distilled fraction was recovered in a 250 mL Erlenmeyer flask containing 25 mL of 0,1M sulfuric acid solution with 4 drops (approximately 0,7 mL) of methyl red indicator (CECHI, 2003). Samples nitrogen content was determined by titration of the recovered fraction with 0,1 N NaOH (eq. 4). All procedures were performed with a standard sample reference, for correcting the interference of any nitrogen present within reactives.

$$N\ (\%) = \frac{(V_1 - V_2) \times N_1 \times mmN}{P_{sample}} \times 100 \qquad (4)$$

Where:

V_1 = H_2SO_4 volume used for titration of macromycetes containing samples
V_2 = NaOH volume used in the titration of the standard sample
N1 = normality of the NaOH used
mmN = nitrogen molar mass
P_{sample} = sample mass analysed

Protein content was estimated by a correction factor applied to the measured nitrogen content. The usual correction factor for foods is 6,25. However it does not take into account non-proteic nitrogenated compounds, which is acceptable for most food samples. Notwithstanding, macrofungi samples present a significative content of

non-proteic nitrogenated compounds in the form of chitin in their cellular walls. This material is not absorbable by digestion. For not superestimating proteic content, 4,38 was the adopted correction factor (considering only 70% of the nitrogenated compounds to be digestible by the human organism (MILES; CH, 1997) (eq. 5).

$$P\ (\%) = N\ (\%) \times 4{,}38 \qquad (5)$$

4.4.4.8 Total carbohydrates

Total carbohydrates content, or nifext fraction (nitrogen-free fraction) was estimated as the difference between the total dry mass (100%) and the sum of protein, ether extract, ashes and humidity. Nifext fraction does not include fibers (SILVA *et al.*, 2008; SNIFFEN *et al.*, 1992; VÉRAS *et al.*, 2005). Equation 6 presents the calculation of total carbohydrates (MELLO *et al.*, 2012).

$$NIFEXT = CHO + fiber = 100 - (\%\ moisture + \%\ ashes + \%\ lipids + \%\ protein) \qquad (6)$$

4.5. MUSHROOM SPENT SUBSTRATE AS SOIL FERTILIZER FOR LETTUCE (*LACTUCA SATIVA* VAR. VERÔNICA) CULTIVATION

The pejibaye palm sheath residues were obtained and processed as described in section 4.4.2 of this thesis and used as substrate for the cultivation of *Pleurotus ostreatus*, as described in section 4.4.4. Mushroom spent substrate was collected immediately after the second cropping cycle. Substrate blocks were grinded and mixed in varied proportions to soil, as described in TABLE 23. Fresh dried and milled pejibaye palm sheath residues were also assayed as control.

Soil was collected in Paranagua-PR, and consisted of haplic cambisol, of medium texture (EMBRAPA, 2005). It was sieved (0,5 cm mesh), for eliminating big fragments of organic matter and homogenized. Commercial compost from “Salto” brand was used for minimal necessary soil enrichment. Six different treatments (T2-T7) plus a control (T1) were defined. Each condition was performed in twenty repetitions, totalizing 140 vases. Plastic 1L vases containing 700g of substrate were prepared as described in TABLE 23.

TABLE 23 – TREATMENTS FOR SOIL ENRICHMENT WITH PEJIBAYE PALM SHEATH RESIDUES (P_R) AND MUSHROOM SPENT SUBSTRATE (M_R).

TREATMENTS	FORMULATION	% EQUIVALENCE
T1	500 g soil 200 g comercial compost	soil
T2	465 g soil 35 g pejibaye sheath residues 200 g comercial compost	soil + 5% P_R[1]
T3	430 g soil 70 g pejibaye sheath residues 200 g comercial compost	soil + 10% P_R[1]
T4	395 g soil 105 g pejibaye sheath residues 200 g comercial compost	soil + 15% P_R[1]
T5	465 g soil 35 g spent mushroom substrate 200 g comercial compost	soil + 5% de M_R[2]
T6	430 g soil 70 g spent mushroom substrate 200 g comercial compost	soil + 10% M_R[2]
T7	395 g soil 105 g spent mushroom substrate 200 g comercial compost	soil + 15% M_R[2]

[1]Percentage in dry weight of P_R in relation to soil mass.
[2]Percentage in fresh weight of M_R in relation to soil mass.
SOURCE: the author (2014).

After preparing and distributing soil mixtures, vases were let in a greenhouse (FIGURE 9) for 40 days (RIBAS *et al.*, 2009) and watered daily to maximum water retention. This greenhouse is located 25º26´55˝ west and 49º13´50˝ south, 923,9 m height. Local weather type is subtropical moist (Cfb by Köppen classification), moist mesothermic, characterized by fresh summer and relatively cold winter. The greenhouse is semi-arc type, with polyethylene covering (1,5 mm) and the following dimensions: 5 m width; 10 m depth; 3,6 m height and 5,1 m arc concavity height.

Lettuce (*Lactuca sativa*) seedlings of “Veronica” crop were obtained from Agro-Horta Produtos Agropecuários Ltda (Colombo-PR). The seedlings were kept in 200 cell trays containing commercial substrate (Plantmax® HT) and vermiculite.

Plants were automatically watered every hour, during the day and once during the whole night period. This crop was chosen based in season (autumn-winter)

temperatures, for being the market leader and standard, with big light-green leaves and high resistance to premature bolting.

FIGURE 9 – LETTUCE VASES IN GREENHOUSE 5 DAYS AFTER THE TRANSPLANTATION.

SOURCE: the author (2014).

After pre-developed in trays for 20~25 days, seedlings, presenting 2~3 leaves and approximately 4~6 cm height, were transplanted to the previously prepared vases and incubated in the same greenhouse, for 63 days, until maximum plant growth (FIGUEIRA, 1982). Vegetal development was evaluated at the end of this period.

Evaluated response variables were: fresh weight, fresh weight of aerial parts and roots, total dry weight, dry weight of aerial parts and roots, number of leaves, length and height of aerial parts and roots height (DOS SANTOS *et al.*, 2013).

Fresh weight was individually determined with a semi-analytical balance. Plants height and width were measured with a common ruler. Leaves bigger than 3 cm were detached and counted. Dry mass was quantified by drying plants at 65ºC, for 72 hours and weighting with an analytical balance (LIMA, 2007).

Differences between treatments were determined by ANOVA and Tuckey post-test for a 5% significance level ($P<0,05$).

4.5.1 Evaluation of soil fertility before and after *Lactuca sativa* cultivation

Routine soil analysis were performed in the Soils and Agricultural Engineering Department of Parana's Federal University. The following parameters were analysed: pH in 0,025 M $CaCl_2$ solution (1:2,5 relation) – relative acidity; available phosphore and potassium extracted by H_2SO_4 0,025 N + HCl 0,05 N; organic carbon; exchangeable calcium, magnesium and aluminum, extracted by 1N KCl; hydrogen + exchangeable aluminum (potencial acidity), according to the methodology described

by Embrapa (1999). For organic matter (OM) estimative calculation, carbon value multiplied by 1,724 was used (SEGNINI *et al.*, 2008).

4.6 ANTIOXIDANTS PRODUCTION BY SUBMERGED CULTIVATION OF MYCELIA, USING AGRO-INDUSTRIAL RESIDUES AS SUBSTRATE

4.6.1 Preliminar Plackett-Burman experimental design for evaluation of significative factors affecting the process

Pleurotus djamor was selected among three strains of the same genus (the other two were *P. ostreatus* and *P. eryngii*) for its better adaptation to *Bactris gasipaes* residues substrate (higher radial growth rate).

An aqueous extract of *Bactris gasipaes* residues was used for formulating the semi-solid media for radial growth evaluation and for the submerged cultivations following described. This extract was obtained by submerging 400g of the fresh grinded residue in 2L of distilled water and letting at room temperature for 24 hours. The liquid fraction was recovered by press-filtration.

The semi-solid medium was obtained by simply adding 4g/L of agar-agar to this aqueous extract and sterilizing at 120ºC for 15 minutes, before plating.

For the initial optimization of submerged cultivation parameters, *Pleurotus djamor* mycelium was cultivated in 125mL Erlenmeyer flasks, containing 60mL of culture medium. After formulation and autoclavation at 120ºC for 15 minutes, the substrates were left at room temperature until thermal equilibrium and inoculated, aseptically, with mycelium previously cultured over BDA medium. The inoculated flasks were incubated in an orbital shaker, at 25ºC, 120rpm.

A Plackett-Burman experimental design (for 12 factors) was applied for assaying 6 factors, with 4 response variables.

The factors and respective levels were:

- Substrate concentration [*B. gasipaes* extract] (20% and 80% v/v)
- Glucose (0 and 10 g/L)
- Yeast extract (YE) (0 and 4 g/L)
- CuSO4 (0 and 100 uM)
- ZnSO4 (0 and 100 uM)
- Days of cultivation (7 and 14)

Three central point experiments were also performed, for estimating the experimental error. The chosen response variables and respective analytic methodologies applied were the following:

- **Biomass** - mycelial pellets were recovered by filtration, dried in a stove at 50ºC and measured by gravimetry
- **Protein content** – broth assayed by Lowry method (FRYER *et al.*, 1986).
- **Antioxidant activity** - Trolox Equivalent Antioxidant Capacity (TEAC), adapted from Re *et al.* (RE *et al.*, 1999).
- **Fructification** – after incubation, the Erlenmeyer flasks were removed from the shaker and left at room temperature, under indirect natural illumination. After a week, the primordia formation over the media surface was evaluated (5 level comparative scale).

4.6.2 Plackett-Burman experimental design for evaluation of inducers of SOD and CAT activities

Five different factors were screened for the induction of SOD and CAT activities in mycelial cultivation broths of *Pleurotus ostreatus*, namely volume/ surface ratio and the addition of: CuSO4, lignin sulfonate, H_2O_2 and aniline.

TABLE 24 - DETAILMENT ABOUT THE COMBINATION OF FACTORS LEVELS FOR EACH EXPERIMENTAL RUN.

	CUSO4 (mM)	LIGNIN SULFONATE (G/L)	H_2O_2 (%)	ANILINE (mG/L)	VOLUME (mL)
1	0,50	0,0	0,050	0	125,00
2	0,50	15,0	0,000	80	125,00
3	0,00	15,0	0,050	0	62,50
4	0,50	0,0	0,050	80	125,00
5	0,50	15,0	0,000	80	62,50
6	0,50	15,0	0,050	0	62,50
7	0,00	15,0	0,050	80	125,00
8	0,00	0,0	0,050	80	62,50
9	0,00	0,0	0,000	80	62,50
10	0,50	0,0	0,000	0	62,50
11	0,00	15,0	0,000	0	125,00
12	0,00	0,0	0,000	0	125,00
13 (C)	0,25	7,5	0,025	40	93,75
14 (C)	0,25	7,5	0,025	40	93,75
15 (C)	0,25	7,5	0,025	40	93,75

SOURCE: the author (2014).

FIGURE 10 - ERLENMEYERS FOR EACH OF THE 15 RUNS, READY FOR INNOCULATION.

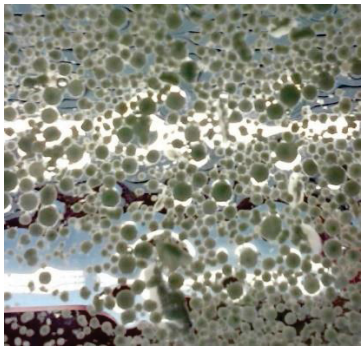
SOURCE: the author (2014).

All factors were assayed in two levels plus a central point level. The low levels of substances added to the culture medium were always zero (no addition). High levels values were selected based in reviewed literature. Center point levels were, logically, half of the concentration of the respective high levels. Levels for culture volume were 125mL, 62,5mL and the center point (TABLE 24).

The pre-inoculum for this experiment was obtained by the following method: Erlenmeyers containing PDY broth were inoculated with mycelium of *P. ostreatus* previously grown in Petri dishes containing PDY agar medium (FIGURE 10). These flasks were incubated in orbital agitators, under 120rpm and 27ºC for 12 days.

The obtained liquid spawn was used to inoculate each of the Erlenmeyers of the experimental design. The procedure included processing the pre-inoculum with an autoclaved homogenizer and pipetting defined volumes for inoculating the flasks, containing autoclaved medium. Inoculation taxa of 10% v/v was adopted, in manner that the total volume after inoculation is expressed in table 24. The inoculated flasks were incubated in orbital agitators (120rpm and 27ºC for 15 days) (FIGURE 11).

FIGURE 11 - MYCELIUM OF *P. OSTREATUS* GROWING IN MEDIUM CONTAINING ANILINE.



SOURCE: the author (2014).

Each of the runs were sampled 6 times, in order to evaluate CAT and SOD enzymatic activities, such as described in sections 4.6.2.1 and 4.6.2.2.

At the end of the cultivation, dry biomass content was determined for each run. Biomasses were harvested by filtration and dried (FIGURE 12) (60ºC, 15 hours) over tared Petri dishes. Mycelium masses were determined by gravimetry.

FIGURE 12 - FRESH BIOMASSES RECOVERED BY FILTRATION (LEFT) AND AFTER DRYING (RIGHT).

SOURCE: the author (2014).

Kinetic graphics were built for SOD and CAT activities of each run. Results for the Plackett-Burman method were computed, using the software Statistica v.7. Kinetic data of each run were combined in multiple ways to render the results. Simple mean, sum and the area under curves were tried, with similar conclusions. Consequently, only the results obtained with the areas under curves are shown in the results section.

4.6.2.1 CAT activity determination

a) Principle of the assay

This method is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalatic pathway) and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining by a colorimetric method. The colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-

chloro-5-sulfonate-p-benzoquinone-monoimine) that absorbs at 520 nm (SIGMA-ALDRICH, 2014b).

b) Materials

- Catalase Assay Kit (Sigma-Aldrich product catalog number CAT100)

This kit includes:

- Assay Buffer 10 x 100 ml (Catalog Number A9725)

500 mM potassium phosphate buffer, pH 7.0

- Chromogen Reagent 1 vial (Catalog Number C5237)

Chromogen Reagent Components:

4-aminoantipyrine (06800-25G) [Fluka/ Sigma-Aldrich]

3,5-dichloro-2-hydroxybenzenesulfonic acid (D4645-5G)

Chromogen solution: **150 mM potassium phosphate buffer, pH 7.0, containing 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid.**

To prepare 200 ml of the Chromogen Solution, mix 60 ml of Assay Buffer 10 x with 140 ml of water in a 250 ml beaker. Add 10 ml of diluted buffer to the Chromogen Reagent vial (Catalog Number C5237) and mix until completely dissolved. Transfer the chromogen solution from the vial into the buffer-containing beaker and mix well. **Divide into suitable aliquots and store at –20 °C. The solution is stable for 12 months**. Avoid multiple freeze-thaw cycles. Before use prepare the Color Reagent by adding 30 µl of the Peroxidase Solution to each 30 ml of Chromogen Solution. **The Color Reagent may be kept at 4 °C for three days if necessary.**

- Stop Solution 100 ml (Catalog Number S5691)

15 mM sodium azide in water.

- **Catalase Positive Control 0.25 ml (Catalog Number C8362)**

from bovine liver (EC 1.11.16) in Crystalline suspension in water containing 0.1% thymol, 30–50 mg protein per ml. 3–10 x 106 units per ml (as measured using the colorimetric assay in this kit)

Unit definition: One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM hydrogen peroxide.

Catalase (C8362) is a crystalline suspension in water and the crystals precipitate to the bottom of the tube. Vortex the tube of catalase vigorously to obtain a homogenous suspension and immediately remove 20 µl of the suspension. It is recommended to pipette up and down several times before removing the suspension. Serially dilute the 20 µl of the enzyme suspension 10,000-fold [An example of this dilution is to dilute the 20 µl of the enzyme suspension with Enzyme Dilution Buffer to 400 µl (1:20), then dilute 20 µl of the first diluted solution to 400 µl (1:400), and finally, dilute 20 µl of the second solution to 500 µl (1:10,000)]. Use between 2–5 µl of the final dilution per reaction. Vortex well before adding to the reaction mixture. **Prepare the Catalase Control fresh each day.** The assay is linear in the range of 0.25–3 units per reaction mixture depending on the length of the reaction.

- 3% (w/w) Hydrogen Peroxide Solution 10 ml (Catalog Number 323381)

The preparing of Colorimetric Assay Substrate Solution (200 mM H_2O_2) is following described. The concentration of 3% H_2O_2 (Catalog N. 323381) is in the range of 3–4%. Thus, it is vital to determine the exact concentration spectrophotometrically and correct it to 200 mM before using the Colorimetric Assay Substrate Solution in the assay. Dilute 200 µl of the 3% H_2O_2 to 1 ml with 1 x Assay Buffer. In order to determine the exact concentration of the Substrate Solution, dilute 50 µl of the above solution to 1 ml (20-fold) with 1 x Assay Buffer. The expected concentration is in the range of 10–15 mM. Determine the actual concentration by UV absorbance by measuring the absorbance at 240 nm (1 x Assay Buffer is used as the blank). Calculate the actual H_2O_2 concentration using Beer's Law (εmM = 0.0436) (Eq. 7):

$$[H_2O_2]\ (mM) = \frac{A_{240}}{0.0436} \qquad (7)$$

Adjust the final concentration of the Colorimetric Assay Substrate Solution to exactly 200 mM with 1 x Assay Buffer. **The standardized Colorimetric Assay Substrate Solution may be stored for 6 days at 4 °C**. The final concentration of H_2O_2 in the assay mixture is 50 mM.

10 mM H_2O_2 Solution: This solution is for obtaining a standard curve of the absorbance of the red quinoneimine dye versus H_2O_2 concentration. Dilute 200 µl of the standardized Colorimetric Assay Substrate Solution (200 mM H_2O_2) to 4 ml with 1× Assay Buffer. **This solution may be stored for 6 days at 4 °C**.

UV Assay Substrate Solution (20 mM H_2O_2): Preparation of volume sufficient for 20 Direct UV assays. Dilute 200 µl of the 3% H_2O_2 (Catalog Number 323381) to 10 ml with 1 x Assay Buffer. Determine the actual concentration spectrophotometrically (use Beer's Law). Adjust the final concentration of the UV Assay Substrate Solution to exactly 20 mM with 1 x Assay Buffer. **The standardized UV Assay Substrate Solution may be stored for 6 days at 4 °C**. The final concentration of H_2O_2 in the assay mixture is 10 mM.

- **Peroxidase 5 mg** (P6782) from horseradish (EC 1.11.1.7). Essentially salt free. 800–1,200 units per mg solid as measured with ABTS at 25 °C at pH 5.0. Weigh 1 mg of solid Peroxidase (Catalog Number P6782) and dissolve in 1.45 ml of 1 x Assay Buffer. **The solution can be stored at 4 °C for up to 2 weeks**.

- Enzyme Dilution Buffer 100 ml (Catalog Number E5779) **50 mM potassium phosphate buffer, pH 7.0,** containing 0.1% Triton™ X-100.

- **Sample** preparation: **1 x Assay Buffer** can be used to dilute the samples. If there is no information available for a source, it is recommended to prepare several dilutions (1, 10, 20, and 50-fold dilutions) and run a 1 minute reaction with 10 µl of each dilution. The dilution recommended should decrease the concentration of H_2O_2 in the reaction by 30–50% in 1–5 minutes.

c) Equipment and other materials needed

- Spectrophotometer (UV-vis)
- 1 ml cuvettes (quartz)
- Analytical balance
- Ultrapure water
- Beaker (250 ml)

d) Methods

- Allow the 1 x Assay Buffer, Colorimetric Assay Substrate Solution (200 mM H_2O_2), and Color Reagent to equilibrate to room temperature.
- Add (x µl) of sample (volume depends on sample type) to a microcentrifuge tube.
- Add (75–x) µl of 1 x Assay Buffer to the microcentrifuge tube (TABLE 25).
- Start the reaction by addition of 25 µl of the Colorimetric Assay Substrate Solution.
- Mix by inversion and incubate 1–5 minutes.
- Add 900 µl of the Stop Solution and invert the tube.

TABLE 25 - REFERENCE FOR PIPETTING VOLUMES.

	Sample Volume	**1x Assay Buffer**	**200 mM H2O2 Solution**
Blank	0	75 µl	25 µl
Sample	x µl	75 – x µl	25 µl

SOURCE: Sigma-Aldrich (2014b).

Colorimetric Reaction

Remove a 10 µl aliquot of the catalase enzymatic reaction mixture and add to another microcentrifuge tube. Add 1 ml of the Color Reagent. Mix by inversion.
Note: Perform this step within 15 minutes of stopping the enzymatic reaction.
Wait at least 15 minutes at room temperature for color development and measure the absorbance at 520 nm.

Calculations

1. Determine the amount of H_2O_2 (µmoles) remaining in the Colorimetric Reaction mixture using a H_2O_2 standard curve.
A520(Blank) = µmoles of H_2O_2 in Blank
A520(Sample) = µmoles of H_2O_2 in Sample
Δµmoles (H_2O_2) = µmoles of H_2O_2 (Blank) – µmoles of H_2O_2 (Sample)
Δµmoles (H_2O_2) is the difference in amount of the remaining H_2O_2 between the Blank and a given Sample.

2. The value from calculation 1 can be used to determine the catalase activity (Eq. 8):

$$\text{Activity} = \frac{\Delta\mu\text{moles } (H_2O_2) \times d \times 100}{V \times t} \quad (8)$$

(μmoles/min/ml)

Where:

$\Delta\mu$moles (H_2O_2) = difference in amount of H_2O_2 added to the Colorimetric Reaction between the Blank and a given Sample.

d = dilution of original sample for Catalase Reaction

t = Catalase Reaction duration (minutes)

V = sample volume in Catalase Reaction (x μl = 0.00x ml)

100 = dilution of aliquot from Catalase Reaction in Colorimetric Reaction (10 μl : 1 ml)

Unit definition: One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM hydrogen peroxide.

Preparation of Standard Curve

Absorbance of Quinoneimine Dye versus H_2O_2 concentration (0.0125–0.075 μmole)

1. Prepare a series of standard solutions of H_2O_2 by placing 0, 125, 250, 500, and 750 μl of 10 mM H_2O_2 solution in microcentrifuge tubes and adding 1 x Assay Buffer to a final volume of 1.0 ml (see TABLE 26). Mix by inversion.

TABLE 26 - DILUTIONS FOR PREPARATION OF THE HYDROGEN PEROXIDE STANDARD CURVE.

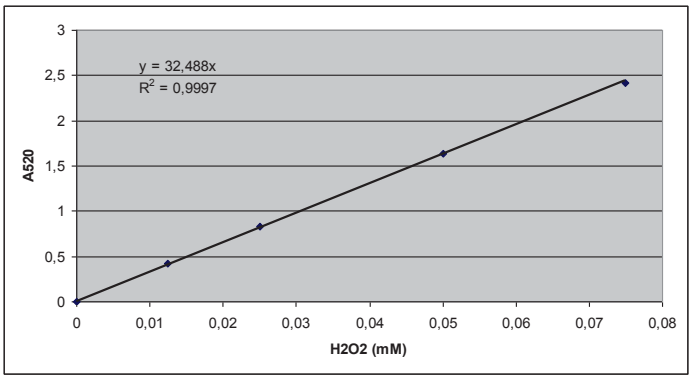
Volume of 10 mM H_2O_2 (µl)	1x Assay Buffer (µl)	H_2O_2 in standard solution (mM)	H_2O_2 in Reaction Mixture* (mM)
0	1000	0	0
125	875	1.25	0.0125
250	750	2.5	0.0250
500	500	5.0	0.0500
750	250	7.5	0.0750

*Note: The H_2O_2 concentrations shown in Table 2 are based on an exact 10 mM concentration of the starting solution. The values should be corrected for the actual concentration of H_2O_2 found spectrophotometrically (see 10 mM H_2O_2 Solution preparation). SOURCE: Sigma-Aldrich (2014b).

2. Transfer 10 μl of each solution to a tube and add 1 ml of the Color Reagent.
Wait 15 minutes and then read the absorbance at 520 nm.
Note: The series of standard solutions of H_2O_2 should be prepared fresh each day.

4. Plot a standard curve of the absorbance at 520 nm versus the final amount of H_2O_2 in the reaction mixture (see GRAPHIC 1).

GRAPHIC 1 - H_2O_2 CONCENTRATION VERSUS ABSORBANCE OF THE RED QUINONEIMINE DYE STANDARD CURVE, EXPERIMENTALLY DETERMINED.



SOURCE: the author (2014).

UV assay (alternative protocol)

Add (x μl) of sample to a quartz cuvette (the volume depends on the type of sample).
Add (500–x) μml of 1x Assay Buffer to the quartz cuvette and mix by inversion.
Start the reaction by adding of 0.5 ml of UV Assay Substrate Solution (20 mM H_2O_2) and mix by inversion (TABLE 27).
Follow the decrease of A240 for 30 seconds with the kinetic program.

Notes:
The initial A240 should be ~0.500.
Use the buffer in which the sample was diluted as the blank.
The concentration of Triton X-100 in the assay should not exceed 0.02%.
The reliable detection limit is 0.025 DA240/minute, which is equal to 0.575 mmole/minute.

TABLE 27 - REFERENCE FOR PIPPETING VOLUMES.

	Sample Volume (µl)	**1x Assay Buffer (µl)**	**20 mM H_2O_2 Solution (µl)**
Blank	0	500	500
Sample	x	500 - x	500

SOURCE: Sigma-Aldrich (2014b)

Calculations

$$\text{Units/ml} = \frac{[\Delta A/\text{min(Blank)} - \Delta A/\text{min(Sample)}] \times d \times 1}{V \times 0.0436} \quad (9)$$

d = dilution of original sample for Catalase Reaction

V = sample volume in Catalase Reaction, (x µl = 0.00x ml)

0.0436 = εmM for hydrogen peroxide

1 = reaction volume in ml

Unit definition: One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 10 mM hydrogen peroxide.

4.6.2.2 SOD activity determination

a) Materials

- Kit for SOD activity determination (500 tests) (Sigma-Aldrich Product Number , 19160)
- SOD standard (Sigma-Aldrich Product Number , S7571)

b) Equipment

- Plate reader (450nm filter)
- 96-well microplate
- 10µl & 100-200µl pipettes and a multi-channel pipette
- Incubator (37°C)

c) Method [as described by Sigma-Aldrich (2014c)]

Preparation of working solutions

- WST working solution: 1ml of WST Solution + 19ml of Buffer Solution.
- Enzyme working solution: centrifuge the Enzyme Solution tube for 5 sec. Mix by pipeting, and dilute 15µl of Enzyme Solution with 2,5ml of Dilution Buffer.
- SOD Solution (for assay monitoring): dilute SOD with Dilution Buffer to prepare SOD standard curve as follows: 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml

Concentrated protein assay

1) Add 20µl of sample solution to each sample and blank 2 well, and add 20µl of ddH2O (double distilled water) to each blank 1 and blank 3 well.

2) Add 200µl of WST Working Solution to each well, and mix.

3) Add 20µl of Dilution Buffer to each blank 2 and blank 3 well.

4) Add 20µl of Enzyme Working Solution to each sample and blank 1 well, and then mix thoroughly (TABLE 28).

5) Incubate the plate at 37°C for 20 min.

6) Read the absorbance at 450nm using a microplate reader.

7) Calculate the SOD activity (inhibition rate %) using the following equation:

SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) - Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100

TABLE 28 - REFERENCE FOR PIPPETING VOLUMES. BLANK 2 IS NECESSARY WHEN ASSAYING SOLUTIONS WITH VISIBLE COLORS.

	Sample (µl)	**Blank 1 (µl)**	**Blank 2* (µl)**	**Blank 3 (µl)**
Sample solution	20		20	
ddH_2O		20		
WST working solution	200	200	200	200
Enzyme working solution	20	20		
Dilution buffer			20	20

SOURCE: Sigma-Aldrich (2014b)

Principle of the assay

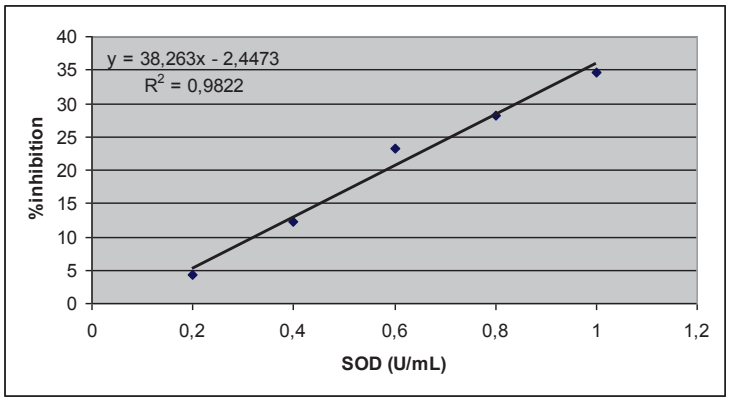
Superoxide radicals are produced by an enzymatic reaction involving xanthine and xanthine oxidase. The reagent WST-1 is converted to a WST-1 formazan derivative, with yellow color. SOD activity is measured by the inhibition rate of the WST-1 reagent conversion into the respective formazan product (FIGURE 14). The reactions are performed in microscale, using microplates and micropipetes.

FIGURE 13 - SCHEMATICS OF THE ASSAY PRINCIPLE (LEFT).
MICROPLATE READY FOR READING (RIGHT).

SOURCES: (Sigma-Aldrich, 2014c) and the author (2014).

Standard curves are prepared using a SOD standard from Sigma, in several concentrations. GRAPHIC 2 shows the standard curve obtained experimentally and used for all SOD activity analyses in this thesis. Microplates (FIGURE 13) are read in a microplate reader at 450nm. Two blanks are needed for colored solutions, in order to discount the liquid inherent absorption.

GRAPHIC 2 - SUPEROXIDE DISMUTASE ACTIVITY STANDARD CURVE EXPERIMENTALLY OBTAINED.



SOURCE: the author (2014).

4.6.3 Central composite, non-factorial, surface response experimental design for optimization of inducers of SOD and CAT activities

The following step of this optimization process was to evaluate in more depth the most effective inducers evidenced in the preliminary study. This was accomplished by applying an experimental planning for evaluating fewer variables, but in more levels, namely surface response methodologies.

Relations between antioxidant and ligninolytic enzymes were explored by applying a laccase activity assay in parallel, to the same samples analyzed for SOD and CAT. Briefly, the addition of lignin sulfonate and H_2O_2 over CAT, SOD and LAC production was evaluated, by applying a central composite non-factorial surface design, with 2 factors and 8 runs plus 5 central points.

The other factors assayed previously, were maintained constant in their respective best levels, as better results were obtained this way.

This surface response methodology experiment was performed in order to optimize CAT and SOD production, and to elucidate their relations with LAC production. The optimized production technique was applied to evaluate SOD and CAT production by different *Pleurotus* species, in section 4.6.4. Further, SOD isoforms from different species were analyzed as described in section 4.6.4.1.

4.6.3.1 Phase I – “cube”

For an initial step of optimization, only the “cube” part of the central composite design was carried, for evaluating the effect of the two chosen factors, their interactions and to find the path of steepest ascent for the next optimization iterations.

For this phase of the experiments, cultivations were performed in multiwell microplates. Autoclaved media were added to 24-well microplates. Hydrogen peroxide solution was prepared based on spectrophotometrical measuring, in the UV range. Before added to the media, this H_2O_2 solution was microfiltered through a membrane presenting 0.02µm diameter pores.

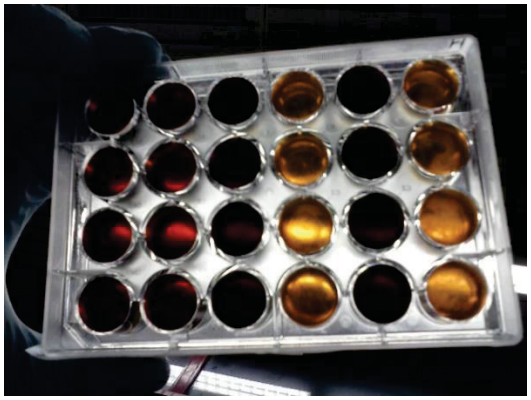
TABLE 29 - MEDIA COMPOSITION AND INOCULATION RATE FOR EACH RUN OF THE EXPERIMENT.

COMPONENT \ WELL	**1**	**2**	**3**	**4**	**5**	**6**
PDY (µL)	1000	0	1000	0	500	**500**
PDY + Lignin Sulfonate 30g/L (µL)	250	1250	250	1250	750	**750**
H_2O_2 750mM (µL)	20	20	100	100	60	**60**
H_2O (µL)	80	80	0	0	40	**40**
Inoculum (µL)	**150**	**150**	**150**	**150**	**150**	**150**

SOURCE: the author (2014).

P. ostreatus pre-inoculum was prepared in Erlen-Meyer flasks, with PDY medium, incubated for 10 days, at 28ºC, 150 rpm. The resulting mycelial suspension was homogenized with an autoclaved blender, and standardized aliquots were pippeted, for inoculating the multiwell plates (FIGURE 14).

FIGURE 14 - MULTIWELL PLATE PREPARED FOR THE EXPERIMENT.



SOURCE: the author (2014).

Media formulations and inoculation rate are expressed in TABLE 29. Inoculated plates were incubated in orbital agitator, at 150 rpm, 28ºC, in the dark, for 15 days. Samples were collected in days 3, 5 and 10, for CAT, SOD and LAC activity evaluation. Data was statistically analyzed with the R software.

4.6.3.2 Phase II – induction curves

Based in phase I results, a second iteration of optimization was designed to evaluate the production of the assayed enzymes in gradients of concentration of both

tested inducers. This was performed aiming the approximation of the optimal concentrations for each of the inducers.

This phase was conducted in Erlenmeyer flasks, due to the better results observed in these containers, in previous experiments. These results are probably effect of the better aeration of this bioreactor system, according to the reviewed literature. Media compositions are detailed in TABLES 30 and 31:

TABLE 30 - MEDIA COMPOSITION FOR EACH RUN OF THE EXPERIMENT (LIGNIN SULFONATE GRADIENT).

RUN	PDY + LIGNIN SULFONATE 50G/L (ML)	PDY (ML)	FINAL LIGNIN SULFONATE CONCENTRATION(G/L)
1	0	100	**0**
2	20	80	**10**
3	40	60	**20**
4	80	20	**40**
5	**100**	**0**	**50**

SOURCE: the author (2014).

TABLE 31 - MEDIA COMPOSITION FOR EACH RUN OF THE EXPERIMENT (H_2O_2 GRADIENT).

RUN	10M H_2O_2 (µL)	PDY (ML)	H_2O (µL)	FINAL H_2O_2 CONCENTRATION (mM)
1	0	99	1000	**0**
2	300	99	700	**30**
3	500	99	500	**50**
4	700	99	300	**70**
5	**900**	**99**	**100**	**90**

SOURCE: the author (2014).

Incubations were conducted in orbital agitators, at 150 rpm, 28ºC, for 10 days. Samples were collected in days 7, 8 and 10 for CAT, SOD and LAC activities assays.

4.6.3.3 Laccase activity assay

A colorimetric method, based in ABTS oxidation was applied, as described by Palmieri *et al.* (PALMIERI *et al.*, 1997): laccase activity was assayed at 25°C using 2,29-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate, as follows: the assay mixture contained 2mM ABTS and 0.1M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420nm (ε = 36,000 M^{-1} cm^{-1}). Enzyme activity was expressed in international units (IU).

4.6.4 Characterization of antioxidant enzymes produced by four different species of the *Pleurotus* genus

4.6.4.1 Identification of SOD enzymes isoforms in electrophoresis gels

This technique was applied in order to characterize enzymatic isoforms produced by different species and media compositions. Four species from the *Pleurotus* genus were assayed: *P. ostreatus*, *P. pulmonarius*, *P. eryngii* and *P. djamor*.

Cultivation media compositions were chosen based in the previous studies of enzymatic inducers. Three cultivation media were tested: simple PDY, as control; PDY containing 10g/L lignin sulfonate, as a SOD inducer; and PDY containing 90mM H_2O_2, as a CAT inducer. Pure mycelial cultures were conduced in 250mL Erlen-Meyer flasks, containing 125mL of total volume broth. The inoculation rate was 10%. The cultures were incubated in orbital agitators, at 150 rpm, in a room with controlled temperature, at 28ºC, for 12 days. Samples were taken in days 7 and 12 for preparing zymograms.

This assay is based in the following principle:

- Photochemically reduced flavins generate O_2^- radicals by reoxidation in the air.
- O_2^- radicals reduce NBT to a blue formazan dye.
- SODs inhibit the formation of the blue formazan.
- When these reactions are performed in acrylamide gels, SODs signal their location by causing achromatic zones on otherwise blue stained gels

a) Materials

- NBT (Sigma-Aldrich Product Number , N5514)
- Riboflavin (Sigma-Aldrich Product Number , 47861)
- TEMED (Sigma-Aldrich Product Number , T9281)
- SOD standard

Other recommended reagents:

- $K_2HPO_4 \cdot 3H_2O$ (Sigma-Aldrich Product Number , P5504)
- KH_2PO_4 (Sigma-Aldrich Stock Number , P9791)
- EDTA-Na_2 (Sigma-Aldrich Stock Number , ED2SS)

Equipment:

- Protein electrophoresis apparatus;
- Staining recipients;
- Test tubes;
- Incubator at 25ºC;
- Fluorescent light bulb;
- Photo camera (within red filter if possible);

b) Method [as an adaptation made by Weisiger and Fridovich (1973) of a method originally described by Beauchamp and Fridovich (1971)].

1) Polymerize both resolving and concentrating gels with riboflavin $2{,}8.10^{-2}$mM;

2) Apply samples in the gel within 20% glycerol;

3) Perform electrophoresis at 80~120V;

4) Soak gel in NBT solution (2,45mM) for 20 min.;

5) Immerse gel in a solution containing 28mM TEMED, $2{,}8.10^{-2}$mM riboflavin and 36mM potassium phosphate at pH 7.8 for 15 min.;

6) Place the gel in a small test tube within 0,05M potassium phosphate, 1.10^{-4}M EDTA at pH7.8 and 25ºC, and illuminate for 5 to 15 min. (until maximum contrast);

7) Photograph (with red filter).

4.6.4.2 Sample preparation

Lignin sulfonate was selected in a previous phase of the study, as an inducer for superoxide dismutase production. Though, this substance imposes some problems for the zymograms techniques. Because lignin sulfonate consists of a mixture of molecules presenting a wide range of molecular sizes and a very dark color, it produces a dark smear when electrophoresed in polyacrilamide, masking enzymatic activities and even rendering coomassie staining impossible.

Further, some molecules present in lignin sulfonate have molecular weights similar to the known superoxide dismutases isoforms, difficulting their separation by techniques based in molecular size, such as ultra-filtration, dialyzing and preparative gel-filtration chromatography. After some initial assays, precipitation of lignin sulfonate with CaOH was found to be a viable step for sufficiently eliminating this compound of the samples, prior to electrophoretic analyzes. A precipitation curve with increasing concentrations of CaOH was performed to determine the best CaOH concentration for this process. The following concentrations were assayed (TABLE 32):

TABLE 32 - PRECIPITATION CURVE OF LIGNIN SULFONATE WITH CaOH.

Lignin sulfonate solution 10g/L (µL)	1000	998	995	990	980	950	900	800	600	400
CaOH 0,5M (µL)	0	2	5	10	20	50	100	200	400	600

SOURCE: the author (2014).

As SOD loses activity when stored in high pH, and the enzyme is diluted after lignin sulfonate precipitation, a step of dialysis was tested for both neutralize the pH and concentrate the samples. The fluxogram presented in FIGURE 15 was adopted, with samples being collected at multiple stages for assaying SOD activity.

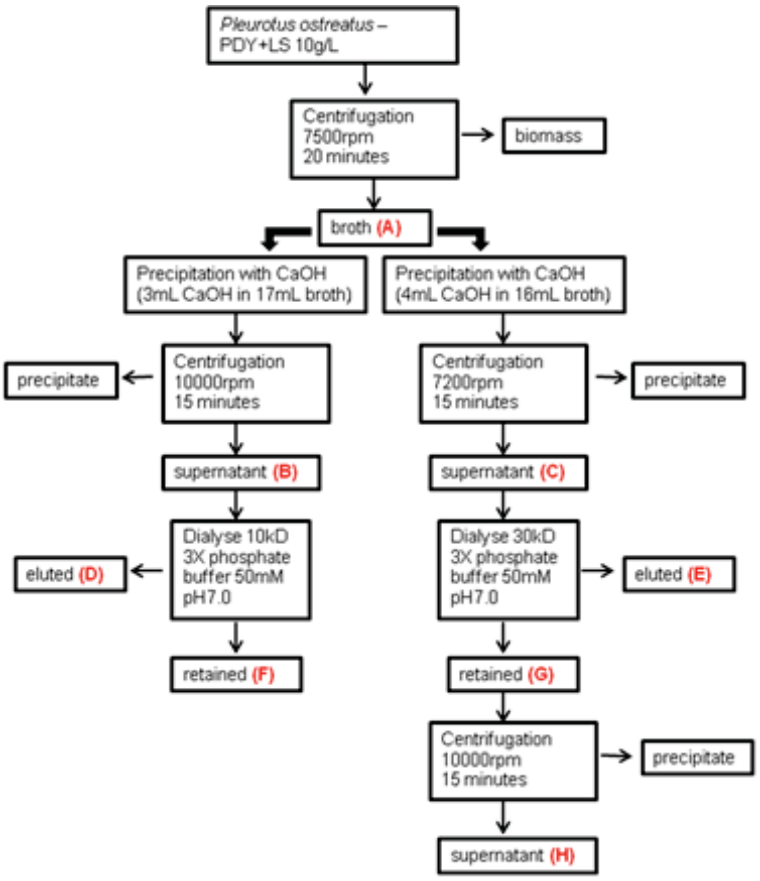
In general lines, *P. ostreatus* was cultivated using PDY + 10g/L lignin sulfonate as a SOD activity inducer. It was incubated in an orbital agitator, at 25ºC for 10 days. The resulting mycelial cultivation broth (A) was centrifuged at 7500 rpm for 20 minutes in order to decant biomass. The supernatant was submitted to precipitation with CaOH.

The centrifugation process to decant lignin sulfonate was attempted both in microcentrifuge tubes (2mL) and, also in 25mL Falcon tubes. The centrifugation speed for the microtubes was 10.000 rpm and only 7.500 for falcon tubes. 15 minutes of centrifugation was applied for both treatments.

The resulting supernatants (B and C) were dyalised with 3 volumes of a 50 mM phosphate buffer solution, pH 7. Microtubes content was dyalised with 10 kD membranes, while Falcons' content was filtered with 30 kD membranes.

The eluted (D and E) and retained (F and G) fractions were assayed for SOD activity. The product obtained with Falcon scale process was further centrifuged at 10.000 rpm for 15 minutes. The resulting supernatant was also assayed.

FIGURE 15 - FLOWCHART OF SAMPLE PREPARATION STEPS. LETTERS FROM A TO H DENOTE SAMPLES EVALUATED FOR SOD ACTIVITY. LS = LIGNIN SULFONATE.



SOURCE: the author (2014).

4.6.4.3 Electrophoresis

Native protein polyacrylamide electrophoresis technique was applied, for maintaining enzymatic activity until the end of the run, objectivating the revelation of superoxide dismutase isoforms in the gels. Samples, prepared as described in the previous section, were mixed with a sample buffer, in a 75% proportion (sample/ buffer), prior to application in a polyacrylamide gel. This sample buffer contained glycerol and bromophenol blue. The separating gels contained 15% acrylamide and the stacking gels, 10%. No SDS or mercaptoethanol were used, neither in the buffer nor in the gels, to avoid protein denaturation. 100µL wells were chosen, to allow the greatest amount possible of sample to be added, due to the diluted nature of the obtained product. The running buffer was potassium phosphate 50mM, pH7.8.

One lane per gel was dedicated to an enzymatic control, which consisted of purified SOD enzyme, acquired from Sigma-Aldrich.

While the samples were in the upper gel, 80V were applied, and while in the lower gel, the voltage was raised to 120V.

4.6.4.4 Zymogram revealing

When finished the electrophoresis, the gel was carefully removed from inside the glass apparatus and immersed in a solution of nitroblue tetrazolium (NBT). The gel remained submerged for 20 minutes under gentle agitation, in the dark, with chilled reagent. After, this solution was eluted and the gel was immersed in a second solution consisting of riboflavin, dissolved in potassium phosphate buffer. While immersed, the gel was illuminated over a light box until the desired contrast of the activity bands was achieved. Then, this solution was discarded, the gel was briefly rinsed with distilled water and the zymogram was scanned. Activity bands appeared as discolored spots against a blue background. Different positions of spots between the assayed strains should point different isoforms of the enzyme, or polymorphisms.

In parallel to this zymogram technique, coomassie blue was used to color all protein bands in the gel, along with a molecular mass marker, for the estimation of enzyme molecular mass (as in native electrophoresis, molecules are separated based in their conformation and charges, besides mass, only an approximation of the molecular weight is possible) and mainly to evaluate sample purity.

Besides the analysis of enzymes isoforms and sample purities, these zymograms are intended to be the first step towards the sequencing of the most active enzymes, aiming molecular technological applications.

4.7 AUTOMATION AND CONTROL OF BIOPROCESSES USING FREE HARDWARE AND SOFTWARE

Bioprocesses engineering and biotechnology are multidisciplinary fields. They bring together biological sciences and engineering. Disciplines as distant as molecular biology, microbiology, informatics, electronics, calculus and transport phenomena contribute to describe biological systems and help to control them at some level.

Control and automation is an essential discipline for this technological area. It allows the application of machines for doing precise, aseptic and repetitive tasks. Although mechatronic engineering consists of an independent field, it is highly desirable for biotechnologists and bioprocess engineers to achieve some practical skills at programming machines. This section of the present thesis discusses the potential of applying a programming language (Pure Data) and an electronics prototyping board (Arduino) on the development of automated systems for bioreactors monitoring and control. Pure Data is free software and Arduino is free hardware, which means any application developed with them can even be sold without the need of paying royalties or copyright fees. Arduino boards are relatively cheap and PD software can be downloaded for free. Both are formidable tools, which allow programming a computer to sense the environment and act upon it. Also, both have good documentation and provide a relatively fast learning curve.

Pure Data and Arduino are flexible tools that can be used to rapidly implement monitoring and control systems. PD not only can access and control Arduino boards, but also virtually any instrument plugged in a computer, including webcams, keyboards and mouse. Arduino makes it possible to plug sensors and actuators to a computer. Sensors include: thermometers, level sensors, pH probes, gas sensors, photocells and pressure sensors. Actuators can be theoretically any electricity controlled device, including LEDs, lamps, motors, valves, pumps, coolers and heaters.

Those sensors and actuators cover the most needed instruments for bioreactor monitoring and automation. With PD, data can be collected from any of these equipment and stored in a hard disk or outputted to a monitor, printer or even through the internet. Data from virtually any sensor, raw or processed, can be used to control any actuator. Operations can also be programmed in a timeline-like manner, with a linear or cyclic set of instructions. Data from different sensors can be combined in control algorithms.

Arduino and PD are totally independent. PD can work without any Arduino, or with more than one. In addition, Aduino can be programmed in other environments and languages, like maxMSP, MatLab and Processor. Programs in Processor language can even be uploaded to the board, so it can work without being connected to a computer, only needing a power supply source. On the other hand, it will be easier to use PD to communicate directly with any computer device (such as keyboard, display or mouse), than to connect those devices to an Arduino. There is no doubt that Arduino and PD can be combined in powerful interactive applications. This article proposes and exemplifies applications of those combined tools in bioreactor control systems.

4.7.1 Materials

- PC (with internet connection)
- Arduino UNO board (with USB cable)
- LED (any color)
- Red laser source
- Resistors (1KΩ)
- LM35 temperature sensor
- Phototransistor
- Solenoid valve (Actuated by 110V, normally open)
- 2 inline air filters (0.22μm pores)
- Electric heater
- Relay (Actuated by 5V. To drive 110V~220V devices. Must also be adequate to the chosen electric heater potency.)
- Breadboard
- Colored coated copper wires

4.7.2 Methods

4.7.2.1 Setting up the environment

Some preliminary steps are needed in order to use Pure Data for controlling Arduino. These are described in the following lines:

Download and install Pure Data software. An actualized version should be available at http://puredata.info/community/projects/software. For this work, version 0.40.3-extended was used.

Download and install Arduino software. An actualized version should be available at http://arduino.cc/en/Main/Software. Version 0022 of Arduino software was used for the present work.

For communication between Arduino and a PC, it is necessary to upload a protocol called Firmata to the Arduino. It is included in Arduino software package since version 0012. For doing this, run Arduino software, connect the board to an USB port and then, under the menu file/examples, find and open the StandardFirmata file. Click verify (the play button). Wait for the verification. Click upload. Wait for the upload.

The standard PD libraries do not include objects for communicating with Arduino. Pduino objects allow PD to communicate with Arduino through the Firmata protocol. Pduino is available at:

http://puredata.info/community/projects/software/pduino.

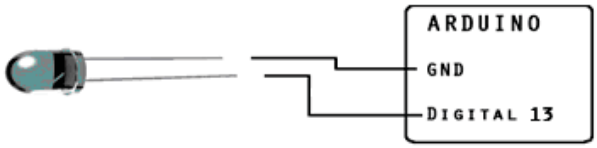
Simply put Pduino folder inside a PD folder called extra and it is ready to use. Version 0.5 beta 8 of Pduino was used in the present study.

4.7.2.2 Testing

Connect the Arduino board to the PC via USB. Run arduino-help.pd from Pduino folder. A Pure Data patch will open. It is necessary to specify the serial port to which the board is connected. It can be done by editing the patch (ctrl+E) to send a message “open” with the correct port number. It is possible to discover the port number in Arduino software in tools/serial port menu. After editing the patch, it is required to exit edition mode (ctrl+E) and click the message “open (port number)”. If everything is working, then clicking the toggle at the right of the patch to on should turn on a little LED on the Arduino board. Also, if a LED is plugged in Arduino digital

pin 13 and GND (with the long electrode and short electrode, respectively) it should light with the same toggle (FIGURE 16).

FIGURE 16 - SCHEME SHOWING CONNECTION OF A LED TO ARDUINO PIN 13 AND GND.



SOURCE: the author (2014).

4.7.2.3 Examples

a) Photoperiod timer

This example can be adapted to any other application that involves the execution of tasks based on a timeline. In the present example, the task is to turn on and off a light source at specific intervals. The same program structure can be used to turn on and off other equipments, like aerators, agitators, pumps and heaters in a cyclic manner. Also, many devices can be controlled in group, or independently in fully customizable intervals. For example, with these tools it is possible to set up a photoperiod of 12 hours, to a process with intermittent aeration and agitation (cycles of 3 minutes on and 20 minutes off). Finally, time based applications can be linear instead of cyclic and randomness can be added to periods. This should cover a broad range of applications.

Some examples will be presented in crescent complexity. With the next few lines we simply want to program our equipment to turn on and off light sources.

Arduino boards can only send up to 5V DC signals. This can be adequate to directly power little LEDs, but for lamps that demand more power, a device named relay is needed. For simplicity sake, let’s start with a little LED.

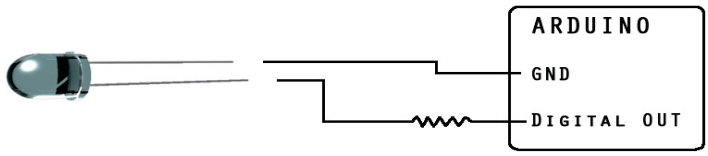
A simple LED has two pins. For it to be turned on, a potential difference between 1,6 and 3,3V and current between 6 and 30mA must be applied to these pins. These values depend on the color and size of the LED. As Arduino can only send 5V signals, adequate resistors must be connected in series in order to prevent damages to LEDs. Digital port 13 is already equipped with an 1KΩ resistor, for

plugging a LED directly, as explained in the testing subsection of the materials and method section.

The relation R=(Vsource-VLED)/iLED can be used to dimension the resistor. In this expression, R is resistance in ohms, Vsource is 5V in case of Arduino digital outputs, and iLED is the current supported by the LED.

The correct way of connecting a LED to an Arduino, using a resistor is shown in the following drawing (FIGURE 17):

FIGURE 17 - CONNECTION OF A LED TO ARDUINO, USING A RESISTOR IN LINE (THE RESISTOR IS REPRESENTED BY THE ZIG-ZAG LINE).



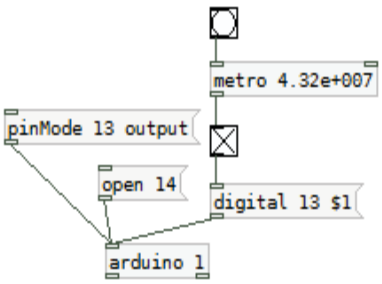
SOURCE: the author (2014).

It should be observed that common LEDs only work with the right polarity of wiring. The shortest LED electrode is the cathode. At the base of the bulb there is a chamfer, which indicates the side where the cathode is localized. Reversing polarity can lead to damages, but not instantaneously. It is possible to test an inverted LED for a few seconds without any risk. Each digital port of Arduino can be set to light individual LEDs and multiple LEDs can be connected to the same GND of Arduino.

Following there is a simple program example showing how PD can be used to control Arduino to turn LEDs on and off. Assuming all steps of setting up the environment section are accomplished and PD is communicating properly with Aruino, through Pduino and Firmata, a new PD file can be open.

The following patch structure will do the work (FIGURE 18):

FIGURE 18 - PURE DATA PATCH FOR CONTROLLING A PHOTOPERIOD OF 12 HOURS.



SOURCE: the author (2014).

The square boxes are called objects. The central object is that called arduino. To create this, press crtrl+1 and write the box content. The boxes with “chamfered” upper right corner are called messages. To create messages, press crtrl+2 and write the box content. The message open must be followed by the port number to which the Arduino board is connected. It is possible to discover the port number in Arduino software in tools/serial port menu. The pinMode and digital messages must refer to the digital pin number of the Arduino board to which the LED is connected (in this example we are using port number 13).

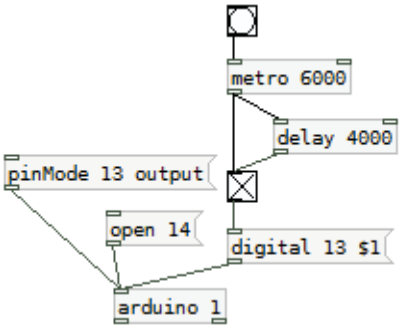
The toggle box can be created by pressing ctrl+shift+t, and the bang button, by ctrl+shift+b. To link boxes, simply drag and drop between boxes outlets and inlets. To run this program, it is required to exit edit mode (ctrl+E) and click the messages open, pinMode and the bang (above the metro object).

The message digital, sends an electric signal to digital pin 13 while the toggle box is checked. The object metro sends bangs at the interval specified by the imputed number, in milliseconds. In this case, $4.32*10^7$ is the number of milliseconds in 12 hours, half of the photoperiod time. Consequently, this patch results the LED will be on for 12 hours and then off for 12 hours.

For testing, shorter intervals can be used. For example, ten seconds are 10000 milliseconds. Boxes can only be edited in edit mode (ctrl+E). Entered intervals only will have effect after being banged.

For irregular intervals, the delay object can be used (FIGURE 19):

FIGURE 19 - PURE DATA PATCH FOR A CYCLE OF 4s ON AND 2s OFF.

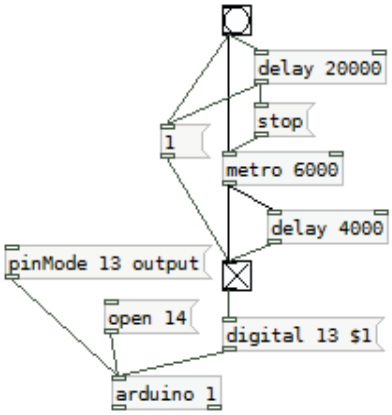


SOURCE: the author (2014).

In the patch above, the LED is lit for 4 seconds and then turned off for 2 seconds. Technically, the delay object sends a new signal to the toggle after a specified time. Multiple delay objects can be used in parallel, for programs with

multiple steps with varied times. This same delay object can also be used to specify an end time to the process, as shown in the next example:

FIGURE 20 - PURE DATA PATCH FOR A CYCLE OF 4s ON AND 2s OFF. THIS PROGRAM IS SET TO START THE LAST CYCLE 20s AFTER STARTING THE FIRST ONE.



SOURCE: the author (2014).

The patch above (FIGURE 20) controls cycles of on (4 seconds) and off (2 seconds) for 20 seconds and then stops with the LED turned off. Observe that the LED will turn off 4 seconds after the 20 seconds programmed in this patch, because the message stop starts the last cycle. A mechanism was also introduced by the message 1 to secure that the program starts with on and ends with off.

Remember that not only LEDs, but all types of electrical devices can be controlled with similar programs. In the next example, the use of relays, which allow controlling 110~220V devices will be explained.

b) Simple thermostatic device

This example can be adapted to any other application that involves the execution of tasks based on data inputted by sensors. In the present example, the task is to turn on and off a heat source based on temperature values given by a thermometer. The same program structure can be used to turn on and off other equipments, like aerators, agitators, pumps and lamps based on data collected by sensors like photocells, O_2 and CO_2, humidity and pH sensors.

Many parallel control tasks based on data collected by sensors can be implemented simultaneously. For example, aerators and impellers can be controlled

based on dissolved O_2 values while heaters and pumps are set to maintain temperature and pH based in thermometers and pH sensors.

As in time-based applications, randomness can be added to control algorithms. Data from different sources can also be combined in expressions to control devices (for example, the mean value of different thermometers can be used to control heating devices). Finally, time-based and sensor-based operations can be combined in the same program, as will be demonstrated with the example in section 4.7.2.3.

For didactic reasons, the following described system consists of a simple thermostatic controller. In this system, switching of an electric resistance depends on temperature measured by an analogical thermometer.

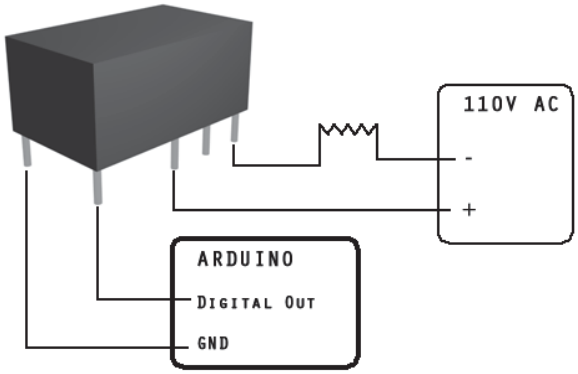
As the power needed by the heater highly surpasses that which Arduino can directly supply, a device named relay must be used. The relay works like a simple power switch, but operated by electric current. There are a number of available relays that can be actuated by Arduino signals. For example, some relays can close 110V circuits while receiving 5V signals from Arduino.

Power threshold of relays must be observed to avoid damage. Some relays can only operate devices with less than 60W, for example.

A relay consists of an electronics component provided of at least 4 pins: two that will receive Arduino signals and two that will be connected while the relay is receiving signals. For the present example, a relay controls the closure of a circuit containing an electric resistance-based heater.

Following there is a graphical representation of components interconnection for turning a heater on and off with Arduino and a relay (FIGURE 21):

FIGURE 21 - SCHEMATIC REPRESENTATION OF RELAY WIRING TO ARDUINO AND 110 AC POWER SOURCE. THE HEATER RESISTANCE IS REPRESENTED BY A ZIG-ZAG LINE.

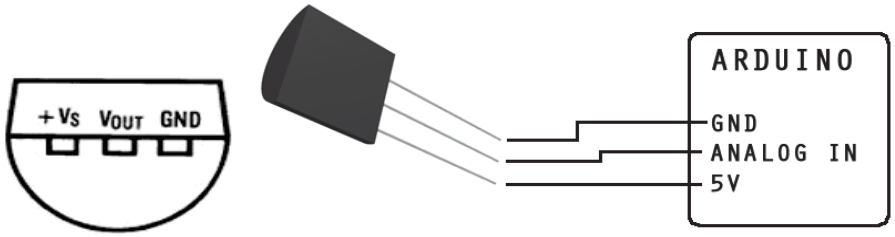


SOURCE: the author (2014).

If the relay is connected to digital pin 5, it is possible to turn the heater on and off by sending signals through this pin. It is done by sending “pinMode 5 output” to the “arduino 1” object to define pin 5 function. Then, sending “digital 5 1” message to “arduino 1” object will switch on the relay and the message “digital 5 0” will turn it off.

For a thermostatic system, relay operation depends on temperature measuring. An analog thermometer named LM35 was used for the present study. It is efficient and easy to use, at a low price. It consists of a very small device, with three pins. One of these pins needs to be fed with 5V, other must be connected to GND and the third pin outputs a signal between 0 and 5V, proportional to the environment temperature. Voltage values can be converted to temperature units with an empiric mathematical correlation. FIGURE 22 shows pins scheme for connecting LM35 thermometer to Arduino.

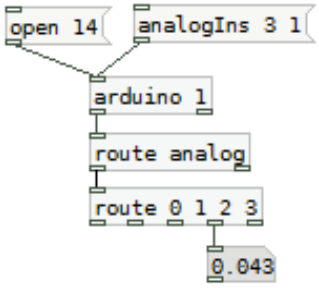
FIGURE 22 - LEFT: PIN SCHEME FOR LM35 (BOTTOM VIEW). RIGHT: SCHEME FOR CONNECTING LM35 TO ARDUINO. +Vs SHOULD BE CONNECTED TO 5V, GND TO GND AND VOUT TO AN ANALOGICAL INPUT.



SOURCE: the author (2014).

Assuming that all steps of setting up the environment section were successfully accomplished and LM35 thermometer is correctly connected to an Arduino, it is now possible to access temperature values with Pure Data. The PD patch shown in FIGURE 23 allows the display of direct values outputted by the sensor.

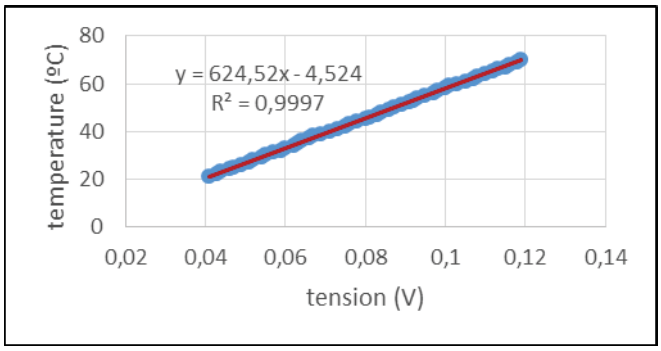
FIGURE 23 - PD PATCH FOR DISPLAYING THE LM35 RAW READING VALUE.



SOURCE: the author (2014).

A linear correlation can be used to convert values given by the sensor to usual temperature units. This can be accomplished with a thermostatic bath. The water temperature should be stabilized at different values and the corresponding raw values read by the LM35, written down. When the obtained values are plotted in a scatter graphic, it is possible to fit a first order equation to the points (GRAPHIC 3). This equation can be used for transforming LM35 raw reading into ºC scale, for example.

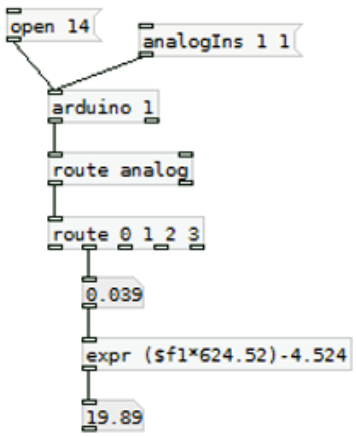
GRAPHIC 3 - LM35 TEMPERATURE SENSOR CALIBRATION.



SOURCE: the author (2014).

Now, inserting the obtained function in the patch, using the object “expr”, as shown in FIGURE 24, the temperature reading from the LM35 sensor is automatically converted from volts (V) to celsius degrees (ºC).

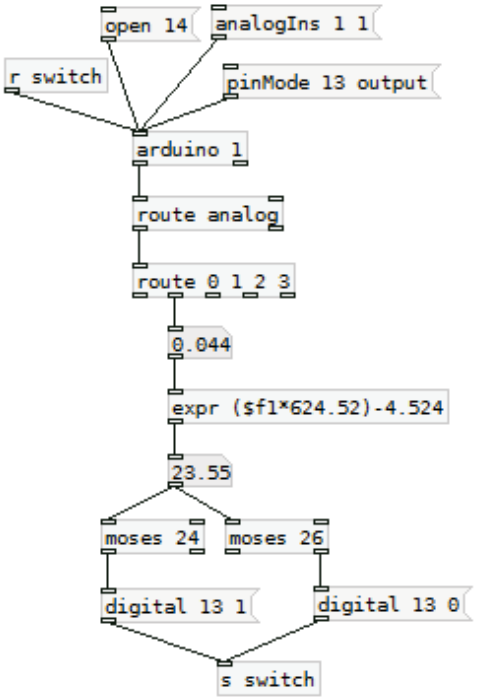
FIGURE 24 - CONVERSION OF THE LM35 SIGNAL FROM V TO ºC.



SOURCE: the author (2014).

With all preliminary settings accomplished and both a LM35 thermometer and a relay for switching an electric resistance correctly plugged in an Arduino, it is possible to use Pure Data for programming a thermostatic bath, that could be, theoretically, a bioreactor temperature control system. An obvious variation of this system involves the utilization of air heaters and fans, for controlling the temperature of incubation chambers, cultivation rooms and solid-state bioreactors. In all cases, the following Pure Data patch should work as a thermostatic controller (FIGURE 25).

FIGURE 25 - THERMOSTATE CONTROL PATCH.



SOURCE: the author (2014).

“Moses” objects output bangs at the left outlet while receiving values lower than those specified in the object; and bangs at the right outlet while receiving values higher than those specified in the object. Two “moses” objects were used in order to turn on the resistor when the temperatures are lower than 24ºC and to turn it off when temperatures are higher than 26ºC. In this manner, temperatures are controlled in the 25ºC±1 range. Other values can be inserted in “moses” objects in order to maintain the temperatures in other ranges. The “s switch” object sends all received signals to the “r switch” object, just for a better organization and display of the connections.

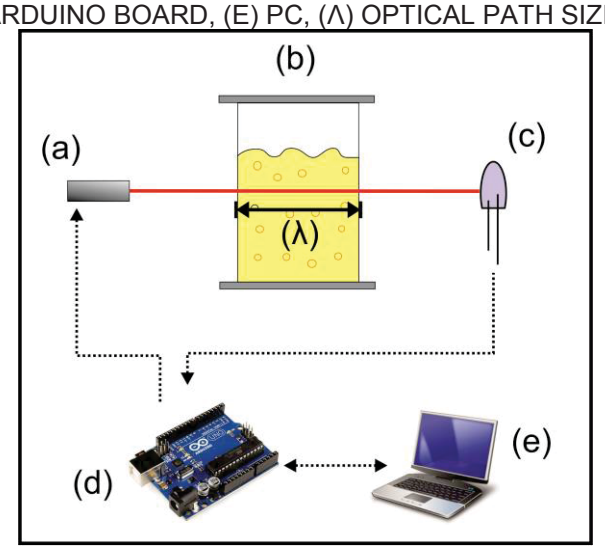
c) Online biomass concentration indirect measuring system for SmF bioreactors, based in laser absorption

As an additional example of application for Pure Data/ Arduino control systems, an online biomass estimation system based in laser absorption was designed and a full functioning prototype was built. This system includes Pure Data algorithms to turn devices on and off periodically, to register the collected data and to present results graphically. The core of the system consists of a phototransistor and a laser source, used for measuring the absorbance of culture broths online in bioreactors.

The core of this technology is a laser based photometry system. This same principle would be applied for building a simple turbidimeter or a spectrophotometer.

Diagram in FIGURE 26 shows the system's components and connections.

FIGURE 26 - APPARATUS FOR LASER ABSORBANCE DETERMINATION.
(A): LASER SOURCE, (B): READING CELL OR BIOREACTOR, (C) PHOTOTRANSISTOR, (D) ARDUINO BOARD, (E) PC, (Λ) OPTICAL PATH SIZE.



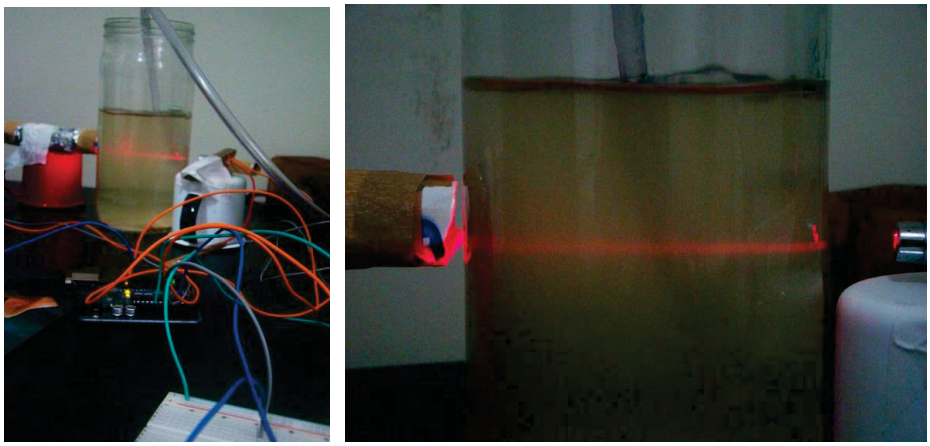
SOURCE: the author (2014).

Briefly, a laser source is positioned in order to cross a sample of the cultivation broth and hit a phototransistor. The vessel can be a spectrophotometer cuvette, a bioreactor or a separate reading cell. The optical path size (λ) need to be adjusted adequately to the sensitivity of the system. If it is too small, the cell growth cannot be sensed. In the other hand, if the path is too large, the absorbance surpasses the threshold (that is, the laser light is entirely absorbed before hitting the sensor). For small glass bioreactors, it is possible to position the laser source and sensor directly on the bioreactors' walls, as suggested in FIGURE 26. The whole apparatus need to

be placed in a container with controlled illumination (mycelium grows well in the dark), to avoid external light interferences. Alternatively, the phototransistor can be placed at the end of a long opaque tube. As laser light propagates in a straight line, it reaches the sensor more intensively than external light, minimizing the interference.

A simple bioreactor was built equipped with a biomass sensor within this concept. Yeast cells were used, due to their fast growth rate, in order to evaluate the adequateness and stability of the measuring system (FIGURE 27).

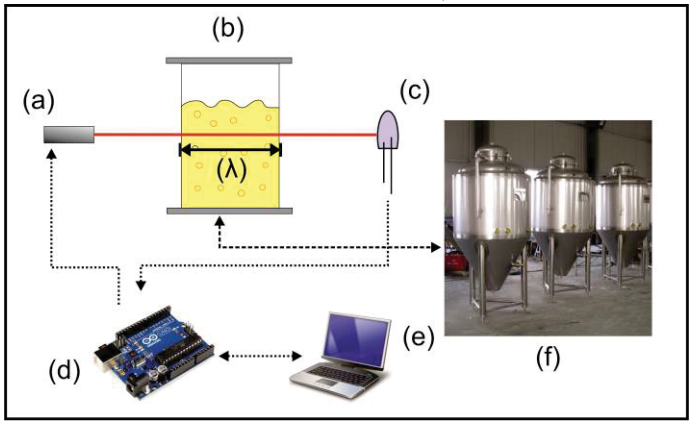
FIGURE 27 - BIOMASS CONCENTRATION SENSOR, BASED IN LASER ABSORPTION, FOR MONITORING YEAST GROWTH.



SOURCE: the author (2014).

For big and/or opaque bioreactors, a separate photometer cell can allow readings to be within the sensitivity range of the system, independently of the reactor model and dimensions. An automated system for periodically pumping samples to a separate reading cell was designed, as shown in FIGURE 28.

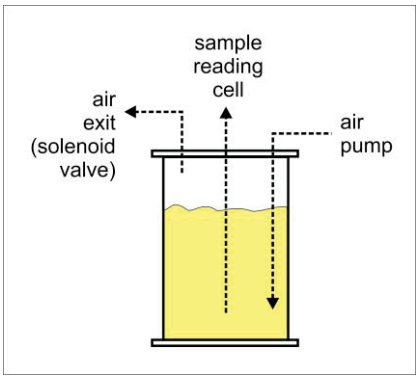
FIGURE 28 - SEPARATE CELL FOR READING ABSORBANCE OF SAMPLES PUMPED FROM A BIG AND OPAQUE BIOREACTOR.



SOURCE: the author (2014).

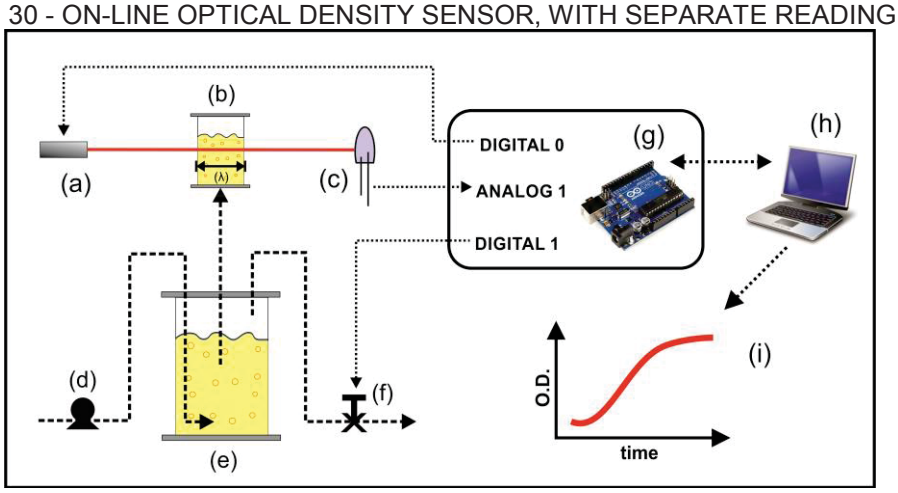
A system with a separate aborbance reading cell was assembled, based in the following mechanism: a well closed bioreactor vessel with three connections as shown in FIGURE 29 receives pumped filtered air constantly. Sample pumping is controlled by opening and closing the solenoid valve.

FIGURE 29 - BIOREACTOR'S AUTOMATED SAMPLE WITHDRAWER SYSTEM.



SOURCE: the author (2014).

FIGURE 30 - ON-LINE OPTICAL DENSITY SENSOR, WITH SEPARATE READING CELL.



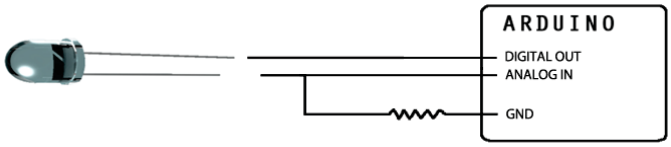
SOURCE: the author (2014).

The system is more completely presented in FIGURE 30. The pump (d) feeds the bioreactor (e) with filtered air. The exceeding pressure is relieved by an air escape, controlled by a solenoid valve (f). When the solenoid valve is closed, the exceeding pressure inside the bioreactor is relieved by pumping its liquid content to the separate reading cell (b). The amount of light emitted by a laser source (a) absorbed by the sample is measured by a phototransistor (c). The cell should be positioned higher than the reactor, so the sample can return by gravity after the reading. The sample returns to the bioreactor as the solenoid valve opens. The reading cell should receive liquid from a bottom entrance for solid residues not to

settle. The cell should also have an air exit, closed by a filter. An Arduino board (g), controlled by a computer (h) is used to send signals for closing the solenoid valve and turning on the laser source. The Arduino board also receives signals from the phototransistor, proportional to the light transmitted through the sample. Signals are transmitted to the computer, registered and presented in graphic form (i) by a software programmed in Pure Data.

Briefly, there is one analog input and two digital outputs in this Arduino system. The analog input deals with signals coming from the phototransistor. One of the two digital outputs controls the solenoid valve and the other digital output controls the laser source. The laser source can be actuated directly by the Arduino's 5V output. The (normally open) solenoid should be actuated by 110 or 220V, using an adequate relay. The phototransistor must be wired as shown in FIGURE 31.

FIGURE 31 – SCHEME FOR CONNECTING A PHOTOTRANSISTOR TO ARDUINO.
THE ZIG-ZAG LINE IS A 2 KΩ RESISTOR.



SOURCE: the author (2014).

This system was programmed to perform the following algorithm:

1- The air pump is constantly turned on.
2- The open solenoid valve allows the flow of air pumped to the bioreactor to escape, maintaining the vessel's internal pressure.
3- Periodically, the solenoid valve is closed in order to use the pressure generated by the pump to pull samples from the bioreactor vessel to the absorbance-reading cell.
4- The sample flow washes the cell for some seconds.
5- Both the phototransistor and the laser source are turned on.
6- The values outputted by the phototransistor are processed, registered and the result is graphically presented in real time.
7- The phototransistor and laser source are turned off.
8- The solenoid valve is turned off (open).

Which was translated into electric signals and analog/ digital ports (TABLE 33):

TABLE 33 - CONTROL ALGORITHM FOR PERIODICAL ABSORBANCE READING BASED IN ARDUINO'S PORTS ACTIVITIES.

ALGORITHM STEPS	DIGITAL OUT 1 (SOLENOID)	DIGITAL OUT 2 (LASER)	ANALOG IN 1 (PHOTOTRANSISTOR)
1 and 2	off	off	off
3 and 4	on	off	off
5 and 6	on	on	on
7 and 8	off	off	off

SOURCE: the author (2014).

TABLE 34 shows the algorithm loop in a simplified manner, with three steps. This table includes steps durations for a 15 minutes cycle.

TABLE 34 - SIMPLIFIED ABSORBANCE READING ALGORITHM.

ALGORITHM STEP	DIGITAL OUT 1 (SOLENOID)	DIGITAL OUT 2 (LASER)	ANALOG IN 1 (PHOTOTRANSISTOR)	TIME (MINUTES:SECONDS)
1	on	off	Off	00:05
2	on	on	On	00:05
3	off	off	Off	14:50

SOURCE: the author (2014).

Data collection is performed at step 2, for 5 seconds. This program is set to collect data every 15 minutes. Step 1 washes the reading cell for 5 seconds and step 3 is the interval between readings, with the sampling/ reading apparatus turned off.

A Pure Data patch was developed, with an object-oriented design, aiming to turn on and off the solenoid valve, the laser source and the phototransistor, to register the collected data and to display it graphically. The main patch contains sub-patches (the boxes started by pd) that function as objects. "Lamp" defines the port for turning on and off the laser source and features a switch for testing the device. "Sensor" defines the digital port for feeding the phototransistor and the analog port for receiving its signals; it also features a switch for testing the device. "Sensor" sends data collected by the phototransistor to the "processor" object. The "processor", by its turn outputs "sensor" data to the "recorder", tared and converted to absorbance values; the "processor" also tares readings when receives bangs from zero_A (tares absorbance) or zero_T (tares transmittance). The "recorder" receives

the processed sensor data and outputs it graphically. The “program” object contains the algorithm for turning on and off the devices in the correct sequence for a single reading cycle. One reading cycle is started everytime the “program” object is banged. “Timer” sends crescent integers to the “recorder” and bangs to the “program” objects. Data is recorded and displayed each time the “recorder” object receives an integer from “timer”.

Delay objects are used to implement the sequence of operations in Pure Data. Steps 1 and 2 should have a fixed time, but step 3 duration can be defined by sending numbers (in miliseconds) to the “s interval” object.

With this system, it is possible to monitor absorbance increasing during the cultivation even in big and opaque bioreactors. This absorbance increasing can be correlated with cell growth or metabolites production. These values can be further used to control other apparatuses, in order to pump the product out of the bioreactor and to pump fresh medium into the vessel, allowing the design of fed-batch or continuous systems. In addition, the evolution of absorbance can be used as a quality control parameter. When there is a subtle rising in turbidity in mycelial cultivation, it can be interpreted as an indication of bacterial or yeast contamination. This can aid the early interruption of contaminated cultivations, saving time and money.

5 RESULTS AND DISCUSSION

5.1 NATIVE MACROFUNGI STRAINS PROSPECTION

At least 100 isolation trials were performed, resulting in about 30 successfully isolated macromycetes strains. Approximately half of those strains were lost due to inadequate manutention. This had lead to experiments for finding better preservation methods, which resulted in a new cryopreservation protocol, described in section 4.3 of this thesis. After the adoption of those cryopreservation strategies, it is believed that the remaining 15 new isolated strains will be indefinitely available for future research.

TABLE 35 – ISOLATED SPECIES, STRAIN CODES, ORIGIN/ ENVIRONMENT AND SUBSTRATE OF THE COLLECTED MUSHROOMS WHICH WERE SUCCESSFULLY ISOLATED AND TEMPORARILY MAINTAINED BY PERIODICAL TRANSFERS AND REFRIGERATION (4 ºC).

(continues)

SPECIES	CODE	ORIGIN/ ENVIRONMENT	SUBSTRATE
Agaricus mediofuscus	AM	Urban	grassy soil
Agaricus volvatulus	AV	Forest	humic soil
Agaricus sylvaticus	AS	Forest	humic soil
Agaricus bisporus	AB	market	compost
Lepista sordida	LS	Urban	grassy soil
Gymnopilus imperialis	GI	Forest	decomposing wood
Pycnoporus sanguineus	PS	Forest	decomposing wood
Coprinus comatus	CC	Urban	grassy soil
Macrolepiota bonaerensis	MB	Rural	soil - pasture
Chlorophyllum molybdites	CM	Urban	grassy soil
Lycoperdon marginatum	LM	Urban	grassy soil
Trametes cubensis	TC	Forest	decomposing wood
Oudemansiella canarii	OC	Forest	decomposing wood and living trees
Pleurotus djamor	PD	Forest	decomposing wood
Pleurotus pulmonarius	PP	Forest	decomposing wood
Pleurotus ostreatus	PO	market	decomposing wood
Pleurotus eryngii	PE	market	decomposing wood

TABLE 35 – ISOLATED SPECIES, STRAIN CODES, ORIGIN/ ENVIRONMENT AND SUBSTRATE OF THE COLLECTED MUSHROOMS WHICH WERE SUCCESSFULLY ISOLATED AND TEMPORARILY MAINTAINED BY PERIODICAL TRANSFERS AND REFRIGERATION (4 ºC).
(conclusion)

SPECIES	CODE	ORIGIN/ ENVIRONMENT	SUBSTRATE
Ganoderma australe	GAus	Urban	decomposing wood and living trees
Ganoderma applanatum	GA	Urban	decomposing wood and living trees
Ganoderma stipitatum	GS	Forest	decomposing wood and living trees
Ganoderma lucidum	GL	market	decomposing wood and living trees
Psylocibe cubensis	PC	Rural	soil – pasture
Auricularia fuscosuccinea	AF	Urban	decomposing wood and living trees
Laetiporus sulphureus	LSUL	Forest	living trees
Lentinula edodes	LE	market	decomposing wood
Xyllaria berteri	PM	Forest	*Perenniporia martiusii* specimen

SOURCE: the author (2014).

Mycelia of many species could not develop in the tested substrates and/ or cultivation conditions. These species include *Russula spp*., *Suillus spp*., *Amanita spp*., *Lactarius deliciosus*, *Phallus sp*., *Ramaria sp*., *Cookeina sp*., *Hygrocibe sp*., *Entoloma bloxamii*, *Scleroderma citrinum*, *Pysolithus tinctorus*.

TABLE 35 lists the successfully isolated strains that could develop mycelium under the mentioned artificial conditions. These strains were temporarily maintained by periodical transfers and refrigeration (4ºC), at the Bioprocesses Engineering and Biotechnlogy Laboratory in UFPR. Some of these strains lost their viability or got heavily contaminated due to inadequate storage techniques. TABLE 36 lists the isolated species that are being conserved under cryopreservation techniques.

TABLE 36 – ISOLATED SPECIES, STRAIN CODES, ORIGIN/ ENVIRONMENT AND SUBSTRATE OF THE COLLECTED MUSHROOMS WHICH WERE DEPOSITED IN THE UFPR BIOPROCESSES ENGINEERING AND BIOTECHNOLOGY LABORATORY AND ARE BEING MAINTAINED BY CRYOPRESERVATION TECHNIQUES.

SPECIES	CODE	ORIGIN/ ENVIRONMENT	SUBSTRATE
Agaricus bisporus	AB	market	compost
Lepista sordida	LS	Urban	grassy soil
Pycnoporus sanguineus	PS	Forest	decomposing wood
Coprinus comatus	CC	Urban	grassy soil
Oudemansiella canarii	OC	Forest	decomposing wood and living trees
Pleurotus djamor	PD	Forest	decomposing wood
Pleurotus pulmonarius	PP	Forest	decomposing wood
Pleurotus ostreatus	PO	market	decomposing wood
Pleurotus eryngii	PE	market	decomposing wood
Ganoderma applanatum	GA	Urban	decomposing wood and living trees
Ganoderma stipitatum	GS	Forest	decomposing wood and living trees
Ganoderma lucidum	GL	market	decomposing wood and living trees
Psylocibe cubensis	PC	Rural	soil – pasture
Lentinula edodes	LE	market	decomposing wood
Xyllaria berteri	PM	Forest	Appeared as a contamination while isolating *Perenniporia martiusii* specimen

SOURCE: the author (2014).

5.2 IDENTIFICATION/ MOLECULAR IDENTIFICATION OF ISOLATED MACROMYCETES

The proposed DNA extraction methodology was practical and efficient, rendering sufficiently pure DNA for amplification purposes. All analysed samples scored 100% of identity with sequences previously deposited in NCBI GenBank and AFTOL, by BLAST algorithm. All sequence analyses confirmed classical identification results, with the exception of *Xylaria berteri*, which was isolated starting from a *Perenniporia martiusii* fruiting body. *P. martiusii* is a very rigid species, extremely

difficult to cut. Possibly, spores of *X. berteri* contaminated Petri dishes during the isolation procedure. Contaminating spores could be present over *P. martiusii* carpophore and accidentally fell over the isolation medium, due to the necessary vigorous cutting. Sequences were deposited in GenBank and their respective accession numbers are written in TABLE 37.

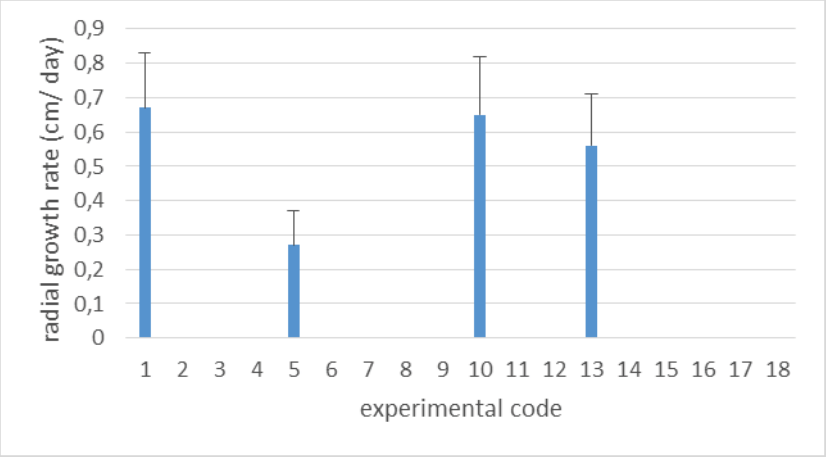
TABLE 37 - rDNA ITS1 AND ITS2 INCOMPLETE SEQUENCES DEPOSITED IN GENBANK AND THEIR RESPECTIVE ACCESSION NUMBERS.

SPECIES	GENBANK ACCESSION NUMBER	SEQUENCE
Oudemansiella canarii	KJ620018	**GCTTGCACAGAGTCGCATGTGTATCGCTTAGAGCGAACGCAG AAGAGCCAGAAGCCCTTCAAAACCCGACCTCTGTCAACACTC TGATCTAGCTAGCTTAGATATTATCACAGCTTAGCGTAGCCAA GTAATGGTTGTTATCGCTAATGCATTTCAGAGGAGCCGAACGT TCATCGTCCGGCAAGCCTCCACTATCCAACCTCTGACCTTGCC AATAAAGCAAGGAGAGTTGAGAGTTTACTGACACTCAAACAG GCATGCCCTTCGGAATACCAAAGGGCGCAAGGTGCGTTCAAA GACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGC ATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGT TGTTGAAAGTTGTATAA**
Pleurotus djamor	KJ620019	**GCTGGTCTCTAGGGACATTGTGCACGCTTCATTAGTTTCCACT TCATACCCCTGTGCACCTTTGATAGATTTCGGTTTGGGTTATCC TTTGGTTTTTTTTTCTTAATTGAAAGGCCTTTGGTTTCCTTAAAA CGACTTCTATACTATACCACACACCAAATGTATGTTTTATAATG AATGGTTTATAATGACAAGGCCATGAGCCTTATAAACTTAATA CAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG TGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATT CCGAAGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTC TATGACTTTATTGTTGTAGCTGTTTGGATTGTTGGGGGTTGCTG GCTTCTTTCTTTGAAGTCGGCTCCTCTTAAATGCATTAGCGGG ACTTTGTTGCCTCTGCGCATAGTGTGATAATTATCTACGCTAGA**
Lepista sordida	KJ620020	**TTTGTCCAAGTCAATGGACTGTTAGAAGCTGAACCCCATGTTA AAGCTGCTTCACAACCATGGCGTAGATAATTATCACACCAAAA GCTGGTCCACAAAGGTTCCGCTAATGCATTTAAGAGGAGCCG ACTTCTAGAGAAGCCCGCAATAACCTCCACATCCAAGCCAAT CCAACTTGCAAAAGCTGAAAAGGTTGAGAATTTAATGACACTC AAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCG TTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACT TATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAG ATCCGTTGTTGAAAGTTGTATTAATTTAAAGGCATAAAGCCCA TTAATAACATTCTATTACATTCTTATG**
Xylaria berteri	KJ620021	**AGGGGTTTTACGGCGGGAGACCGGTCCAACTAATAGGCGAGA TAAAATTACTACGTCTAGAGTGTGAACCGACTCCGCCACTAAC TTTGAGGAGCTACAGGTGCCGTAGGCTCCCAACGCTAAGCAA CAGAGGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCC ACTAGAATACTAATGGGCGCAATGTGCGTTCAAAGATTCGATG ATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTG CGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG TTTTAACTTATTTAGTTATATGTTCAGAATTCAATATTAAACAGA GTTTCGTGGGCCGCCGGCAGGCTTACCCGCGTCTCCCGGGTA GGCCCTACAGGGTAGGGTGCTACTAGGTAGGCGCGACCTGCC GAGGCAACGTAAGGTATGTTCACA**

SOURCE: GenBank (2014).

5.3 VERMICULITE AS A SUPPORT FOR MYCELIA CRYOPRESERVATION

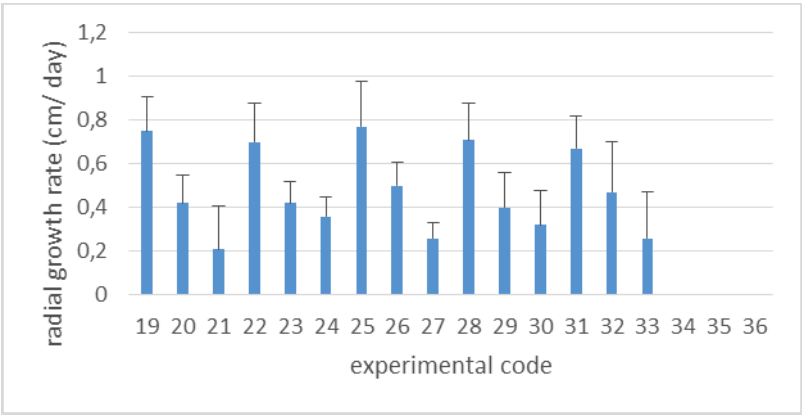
GRAPHIC 4 - RADIAL GROWTH RATE OF *A. SUBRUFESCENS* MYCELIA, STORED AT FREEZER (-20ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 19.



SOURCE: the author (2014).

Storage at -20ºC was greatly harmful to the mycelia. Even glycerol and carrier materials were not sufficient for preserving the viability of most of the material at this condition. This temperature range probably favored the crystallization of water, damaging the cells. However, at least one strain (AbSC51) survived with glycerol and perlite and one strain (AbCG31), survived with glycerol and vermiculite.

GRAPHIC 5 - RADIAL GROWTH RATE OF *A. SUBRUFESCENS* MYCELIA, STORED AT ULTRA FREEZER (-80ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 20.

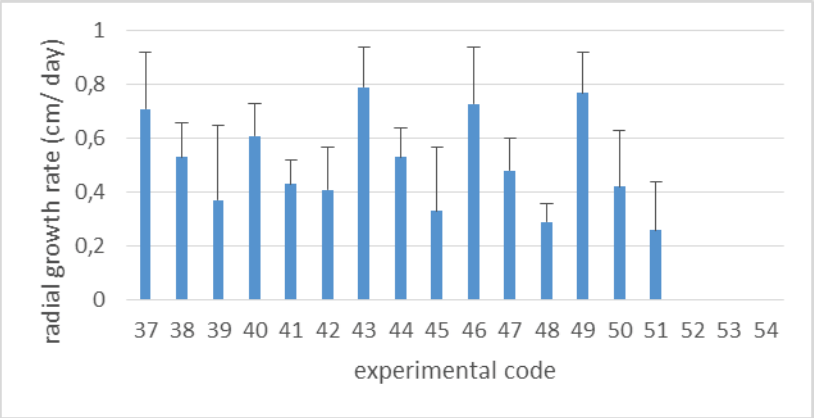


SOURCE: the author (2014).

The strain AbSC51 also maintained its activity, using solely perlite or vermiculite as carrier materials, without needing the addition of glycerol. Growth rates of the reactivated strains varied between 0.25 and 0.83 cm/day (GRAPHIC 4).

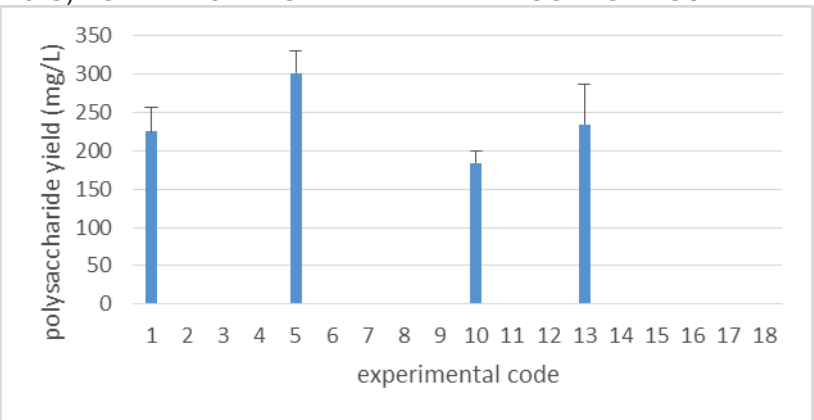
Most of the evaluated techniques for preservation of mycelia at -80ºC were efficient. However, this experiment clearly shows the necessity of at least a carrier material or glycerol, for maintaining the viability of mycelia at this condition. Growth rates of the reactivated strains varied between 0.20 and 0.86 cm/day (GRAPHIC 5).

GRAPHIC 6 - RADIAL GROWTH RATE OF *A. SUBRUFESCENS* MYCELIA, STORED AT LIQUID NITROGEN (-195ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 21.



SOURCE: the author (2014).

GRAPHIC 7 - POLYSACCHARIDE YIELD OF *A. SUBRUFESCENS* MYCELIA, STORED AT FREEZER (-20ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 19.

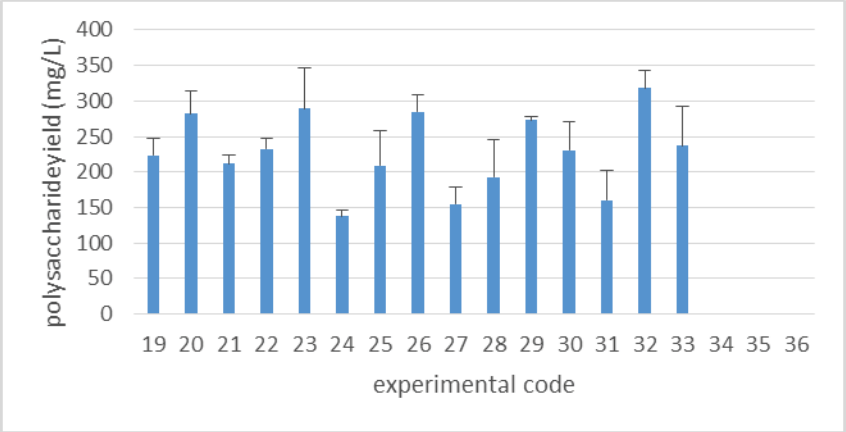


SOURCE: the author (2014).

Most of the evaluated techniques for preserving mycelia were efficient at -195ºC. Results shown in graphic 6 reinforce the importance of using carrier materials or glycerol, for maintaining the viability of cryopreserved mycelia. Growth rates of the reactivated strains varied between 0.26 and 0.91 cm/day, slightly faster than mycelia stored at -80ºC, suggesting that mycelia are even better preserved at -195ºC.

Strains that maintained viable after being conserved at -20ºC for 12 months, also kept their ability of producing exopolysaccharides. The obtained yields were in the range between 180 and 325 mg/L (GRAPHIC 7).

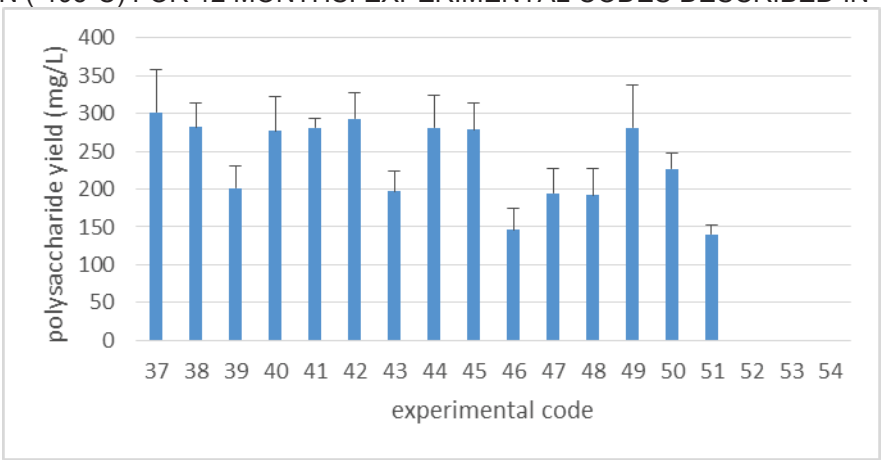
GRAPHIC 8 - POLYSACCHARIDE YIELD OF *A. SUBRUFESCENS* MYCELIA, STORED AT ULTRA FREEZER (-80ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 20.



SOURCE: the author (2014).

All reactivated strains, either conserved at -80ºC or -195ºC, maintained polysaccharide production yield in the range between 140 and 360 mg/L (GRAPHICS 8 and 9). These results suggest that none of the evaluated preservation techniques cause genetic damage to the recovered strains, at least concerning genes related to mycelial growth and exopolysaccharide production.

GRAPHIC 9 - POLYSACCHARIDE YIELD OF *A. SUBRUFESCENS* MYCELIA, STORED AT LIQUID NITROGEN (-195ºC) FOR 12 MONTHS. EXPERIMENTAL CODES DESCRIBED IN TABLE 21.



SOURCE: the author (2014).

Briefly, results show that a carrier material is necessary for mycelium survival to rapid freeze and thaw procedures. Both perlite and vermiculite showed good results

as carrier materials. The addition of any of these materials to the cryoprotection medium was sufficient to maintain the viability of the tested mycelia for twelve months at -80ºC or -195ºC. Common freezer temperature was more harmful to the mycelia (22% recovery) than ultra-freezer or liquid nitrogen temperatures (83% recovery).

These results were obtained, probably because water tends to vitrify at very low temperatures, within cryoprotectants. Due to the fast solidification rates at very low temperatures, water molecules do not have the time to arrange in an organized manner. Opposedly, common freezer temperatures and the absence of cryoprotectants favour water crystallization. The formation of water crystals is the main cause of cell damage during low temperature storage.

After the second subculture, all reactivated strains recovered the original radial growth rate (0.55±0.35 cm/day) and polysaccharide production yield (247±102.5 mg/L) under submerged fermentation, approximately. The described methods are practical, relatively affordable and proven efficient for the tested period of storage.

In comparison to the traditional subculturing method, that allow only nearly two months intervals of storage, the proposed techniques can be performed in at least twelve months intervals. Besides being efficient, vermiculite is also relatively cheap and readily available. It provides a sharp contrast with mycelium color, which facilitates mycelium growth monitoring during incubation (it is sometimes difficult to distinguish mycelium from the substrate when using perlite as a support).

Breaking the pre-incubation in two phases allows both: lower contamination and better aeration during the plate phase and reduced volume and higher resistance during storage in tubes. The protocols here presented do not require highly controlled cooling or thawing processes and reactivation does not require glycerol removal.

5.4 MACROFUNGI MYCELIA AND CARPOPHORES CULTIVATION USING PEJIBAYE PALM (*BACTRIS GASIPAES* KUNTH) SHEATH RESIDUES AS SUBSTRATE

5.4.1 Screening of macromycete strains for adaptation to pejibaye palm (*Bactris gasipaes* Kunth) sheath residues utilization as substrate

Highest radial growth rates were obtained with *Pleurotus ostreatus* (1,38±0,16 cm/day) and *Pleurotus djamor* (1,38±0,1 cm/day) strains using the internal fraction of the pejibaye palm sheath (pH 6,2±0,3) as substrate. The other results were: *Coprinus*

comatus (0,91±0,06 cm/day), *Pleurotus eryngii* (0,72±0,15 cm/day) and *Agaricus subrufescens* (0,49±0,15 cm/day). The assayed *Ganoderma lucidum* strain did not develop over pejibaye residues. None of the evaluated macrofungi mycelia could grow adequately using the external fraction of the pejibaye sheath as substrate, due to the excessively low pH of this material (pH 3,21±0,15) (TABLE 38).

TABLE 38 – RADIAL GROWTH RATE (cm/ DAY) FOR DISTINCT MUSHROOMS SPECIES, USING THE INTERNAL AND EXTERNAL FRACTIONS OF PEJIBAYE PALM SHEATH AS SUBSTRATES *

SPECIES	RADIAL GROWTH (cm/day)	
	INTERNAL FRACTION	EXTERNAL FRACTION
Pleurotus ostreatus	1,38±0,16[a]	0
Pleurotus djamor	1,38±0,1[a]	0
Coprinus comatus	0,91±0,06[b]	0
Pleurotus eryngii	0,72±0,15[bc]	0
Agaricus subrufescens	0,49±0,15[c]	0
Ganoderma lucidum	0[d]	0

* Means followed by the same letter do not differ statistically, as analyzed by ANOVA/ Tukey ($p<0,05$). Values of triplicates means and standard deviations are presented. SOURCE: the author (2014).

Because of these radial growth rate results, further mycelial cultivation experiments were performed exclusively using *Pleurotus* genus strains and the internal fraction of the pejibaye palm sheath as substrate.

5.4.2 Effect of inoculation rate and perforations over *P. ostreatus* yield

Inoculation rate did not influence myceliation time-span. Fewer holes resulted in fewer mushrooms. Time before primordia formation was shortened by a greater number of holes. This was probably due to a better air exchange, lowering CO_2 levels inside the plastic bags, which is recognized as a signal for primordia formation by the mycelium. Number of fructified holes varied randomly (TABLE 39).

Fruiting bodies' dimensions did not vary significantly neither by inoculation rate nor by number of holes variation. However, some interaction between these two variables is observed, in the sense that slightly bigger mushrooms are generated either by the combination of low inoculation rate and lesser holes or the combination of high inoculation rates and more holes, other combinations generated smaller mushrooms.

TABLE 39 – EXPERIMENTAL RESPONSES TO INOCULATION RATE AND NUMBER OF HOLES VARIATIONS, RELATED TO THE BIOLOGICAL PROFILE OF *P. OSTREATUS* CULTIVATED USING PEJIBAYE PALM RESIDUES – 1st PRODUCTION FLUX.

ASSAY	MR	PFI	FRUTIF.	FRUTIFIED HOLES	MUSHROOM DIMENSIONS			PILEUS DIMENSIONS
					WIDTH	HEIGHT	LENGTH	
	(days)	(days)	(units)	(units)	(cm)			
1	33	5-6	3	2-3	9,8	10,3	6,3	4,6
2	33	6-8	2	1-2	12,2	10	8,8	5,8
3	33	4-6	2-4	1-2	10,6	13,4	7,1	5,0
4	33	5-8	2	1-2	9,6	9,8	7,7	3
5	33	5-7	2-3	1-3	10,7	9,0	6,4	4,7
6	33	4-5	2-3	2-3	12	11,8	7,1	4,8
7	33	4-6	2-4	1-3	12,5	10,7	4,2	6,1

MR: mycelial run; PFI: primordia formation initiation; Frutif: frutification. Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). SOURCE: the author (2014).

The combination of low inoculation rate and fewer holes resulted in premature second flushes in relation to other conditions tested. Lower inoculation rates resulted in more frutifications in the second flush. Greater number of holes resulted in longer total cultivation time-spans. Number of fructified holes varied randomly.

TABLE 40 – EXPERIMENTAL RESPONSES TO INOCULATION RATE AND NUMBER OF HOLES VARIATIONS, RELATED TO THE BIOLOGICAL PROFILE OF *PLEUROTUS OSTREATUS* CULTIVATED USING PEJIBAYE PALM SHEATH RESIDUES, 2nd FLUX.

ASSAY	PFI	FRUTIF.	TCT	FRUTIFIED HOLES	MUSHROOM DIMENSIONS			PILEUS DIMENSIONS
					WIDTH	HEIGHT	LENGHT	
	(days)	(units)	(days)	(units)	(cm)			
1	9-12	3-4	52-58	1-2	14,4	9,2	10,7	13,7
2	5-8	3-4	50-54	1	13,7	11,3	8,9	9,5
3	7-12	1	54-56	1	12,3	10,2	7,9	8,4
4	9-12	1	53-55	1	14,5	9,4	7,8	6,8
5	9	2-3	53-56	2-3	10,3	6,2	6,1	5,4
6	7-9	3	50-51	3	9,8	8,2	8	7,9
7	9-11	2	53-56	1	11,8	7,7	8,5	8,6

PFI: primordia formation initiation; Frutif: frutification. TCT: total cultivation time. Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). SOURCE: the author (2014).

All treatments produced bigger fruiting bodies in the second flush than in the first one. This happened possibly due to a premature induction of fructification. The excess of remaining nutrients prevented fungi metabolism from switching to

fructification behavior. The combination of lower inoculation rates with a greater number of holes resulted in the largest pileus dimensions in the second flush (TABLE 40). Which confirms the hypothesis of remaining nutritive potential of the substrate. The spawn grains are more nutritive than the production substrates, thus the mycelial phase is priviledged for longer periods in treatments with highest inoculation rates.

Anyway, the yield was lower in the second flush for all tested conditions, as expressed by biological efficiency and productivity results (TABLE 41). Probably, after fructification induction conditions are imposed, the energy accumulated at myceliation phase is released in the form of fructification and not recovered after the first cycle. Results should be different if a second mycelial incubation phase was provided (low aeration, 25°C and light absence) between cycles. Central point condition resulted in the highest yield for the first flush, suggesting that there should be an optimized range, with a middle value, for both assayed parameters. Differently, the best combination of factors for the second flush was 40% inoculation rate and 4 holes.

TABLE 41 – EXPERIMENTAL DATA AND RESPONSE VALUES OBSERVED FOR DISTINCT COMBINATIONS OF INOCULATION RATE AND NUMBER OF HOLES PER BAG. BIOLOGICAL EFFICIENCY (BE) AND PRODUCTIVITY (P) EVALUATED IN THE FIRST AND SECOND FRUTIFICATION CYCLES OF *P. OSTREATUS*, USING PEJIBAYE PALM RESIDUES AS SUBSTRATE.

ASSAY	INDEPENDENT VARIABLES		DEPENDENT VARIABLES			
	X_1	X_2	1st Frut.		2nd Frut.	
			BE*	P*	BE*	P*
1	10 (-1)	4 (+1)	46,75 (±6,01)	12,72 (±3,64)	36,867 (±3,84)	15,979 (±1,15)
2	10 (-1)	2 (-1)	54,8 (±1,56)	14,645 (±3,02)	34,605 (±0,68)	10,382 (±0,20)
3	40 (+1)	4 (+1)	50,414 (±1,15)	16,59 (±2,61)	37,229 (±3,93)	10,185 (±1,18)
4	40 (+1)	2 (-1)	52,877 (±5,72)	15,863 (±1,68)	21,179 (±3,98)	6,353 (±0,29)
5	25 (0)	3 (0)	53,263 (±3,08)	15,979 (±1,68)	31,627 (±0,68)	9,488 (±0,27)
6	25 (0)	3 (0)	53,022 (±3,10)	15,907 (±0,92)	31,473 (±0,58)	9,442 (±0,20
7	25 (0)	3 (0)	53,079 (±3,11)	15,04 (±0,21)	31,99 (±0,76)	11,4 (±0,39)

Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 and 7 (25% inoculation rate and 3 holes). X1 – Inoculation rate (% in dry weight), X2 – Number of holes (units). Standard deviation values are presented in parentheses. * (%). SOURCE: the author (2014).

The 2nd order mathematical model adjusted to the productivity variable, but not to the biological efficiency variable, as shown by lack of fit numbers in TABLE 42.

TABLE 42 – ANOVA, PURE ERROR, LACK OF FIT AND REGRESSION COEFICIENT FOR 2nd ORDER EQUATIONS DESCRIBING BIOLOGICAL EFFICIENCY (BE) AND PRODUCTIVITY (P) IN RELATION TO INOCULATION RATE AND NUMBER OF HOLES PER BAG IN THE CULTIVATION OF *PLEUROTUS OSTREATUS* USING PEJIBAYE RESIDUES FIRST AND SECOND FRUCTIFICATION CYCLES. **

REGRESSION COEFFICIENT		1ST FRUCTIFICATION*		2ND FRUCTIFICATION*	
		BE	P	BE	P
Linear intersection	β_0	39,3357	7,6190	32,3329	23,9628
	β_1	0,4546	0,1525	0,3842	-0,2454
	β_2	2,7629	2,3770	2,4786	-3,1909
Interaction	β_{12}	-0,0931	-0,0442	-0,2298	0,0294
	R^2	0.8586	0.8637	0.9933	0.9374
	Pure error	0.0325	0.2729	0.0705	1,2486
	Lack of fit	0.0055	0.2271	0.0624	0.5461

Assays: 1 (10% inoculation rate + 4 holes); 2 (10% inoculation rate + 2 holes); 3 (40% inoculation rate + 4 holes); 4 (40% inoculation rate + 2 holes); 5, 6 and 7 (25% inoculation rate + 3 holes).
* (%); ** 5% significance level. SOURCE: the author (2014).

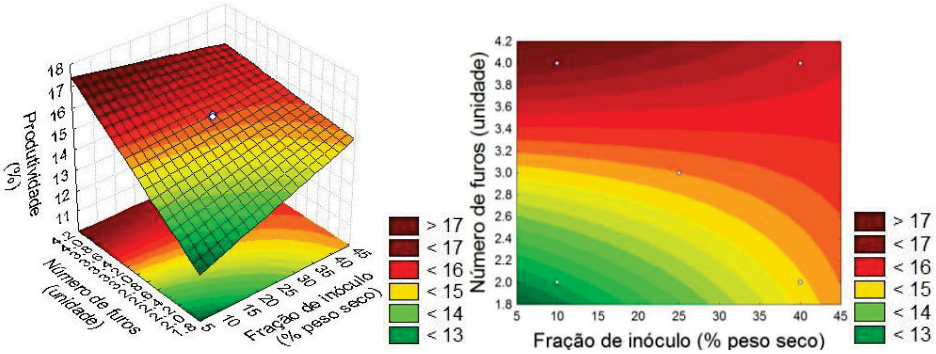
TABLE 43 shows the experimental results of biological efficiency and productivity, for each of the assayed conditions, in the first and second fructification flushes. This table also shows the results predicted by the second order mathematical models adjusted to the experimental points by surface regression.

TABLE 43 – OBSERVED BIOLOGICAL EFFICIENCY (BE) AND PRODUCTIVITY (P) FOR DIFFERENT COMBINATIONS OF INOCULATION RATE AND NUMBER OF HOLES AND RESPONSE VALUES PREDICTED BY THE MATHEMATICAL MODEL, FOR THE 1ST AND 2ND FRUCTIFICATION CYCLES OF *PLEUROTUS OSTREATUS* CULTIVATED USING PEJIBAYE RESIDUES AS SUBSTRATE.

ASSAY	IR	NH	1ST FRUCTIFICATION				2ND FRUCTIFICATION			
	(%)	(units)	BE		P		BE		P	
			Obs.	Pred.	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
1	10	4	46,75	47,54	12,72	13,33	36,87	36,54	15,98	15,72
2	10	2	54,80	55,59	14,64	15,25	34,61	34,27	10,38	10,12
3	40	4	50,41	51,21	14,88	15,48	37,23	36,90	10,19	9,92
4	40	2	52,88	53,67	15,86	16,47	21,18	20,85	6,35	6,09
5	25	3	53,26	52,00	15,98	15,13	31,63	32,14	9,49	10,46
6	25	3	53,02	52,00	15,91	15,13	31,47	32,14	9,44	10,46
7	25	3	52,91	52,00	15,93	15,13	31,99	32,14	11,40	10,46

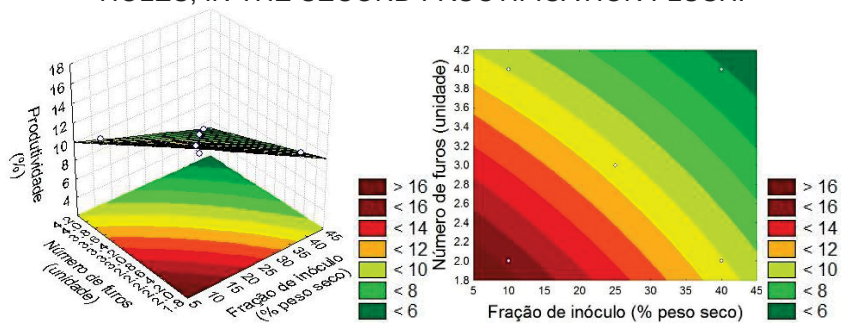
IR: inoculation rate (%); NH: number of holes. Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). SOURCE: the author (2014).

GRAPHIC 10 - SURFACE RESPONSE (LEFT) AND CONTOUR PLOT (RIGHT) FOR PRODUCTIVITY (%) VARIATION IN FUNCTION OF INOCULATION RATE AND NUMBER OF HOLES, IN THE FIRST FRUCTIFICATION FLUSH.



SOURCE: the author (2014).

GRAPHIC 11 - SURFACE RESPONSE (LEFT) AND CONTOUR PLOT (RIGHT) FOR PRODUCTIVITY (%) VARIATION IN FUNCTION OF INOCULATION RATE AND NUMBER OF HOLES, IN THE SECOND FRUCTIFICATION FLUSH.



SOURCE: the author (2014).

The number of holes was the factor that influenced most the productivity in the first flush. The inoculation rate also affected the results, but more pronunciatedly for a smaller number of holes. Bags with more holes were more productive in the first flush, almost independently of inoculation rates. However, for the bags with fewer holes, higher inoculation rates resulted in slightly higher productivity (GRAPHIC 10). For the second flush, both factors affected productivity almost equally. Lower inoculation rates and smaller number of holes clearly resulted in higher productivity (GRAPHIC 11).

The factors (inoculation rate and number of holes) did not affect significantly ($p<0,05$) the carpophores composition in terms of humidity, ashes and lipids, in the assayed levels (TABLE 44). These values are similar to those presented by Duprat (DUPRAT, 2012) for *Pleurotus ostreatus* basidiomata cultivated using pejibaye palm sheath residues as substrate. Ragunathan and Swaminathan (RAGUNATHAN; SWAMINATHAN, 2003) and Urben (URBEN, 2004a) also reported similar values.

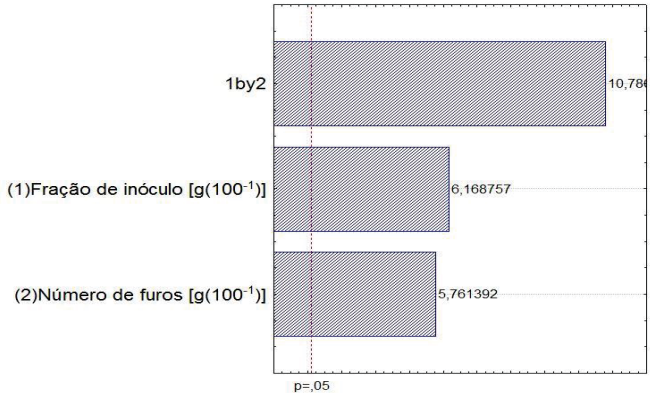
TABLE 44 – PHYSICAL-CHEMICAL COMPOSITION OF *PLEUROTUS OSTREATUS*, USING PEJIBAYE PALM SHEATH RESIDUES AS SUBSTRATE (PRESENTED AS PERCENTAGE IN DRY BASE) *

HUMIDITY	DRY MATTER	ASHES	LIPIDS
90,02 (±1,80)	9,98 (±1,80)	6,52 (±0,65)	3,10 (±0,31)

Standard deviation values are in parentheses. * [g(100^{-1} g)]. SOURCE: the author (2014).

However, significant variations were observed in proteins and carbohydrates contents in function of the assayed variables. Results were significative for the influence of both analysed factors over protein content (GRAPHIC 12).

GRAPHIC 12 - PARETO'S CHART FOR INOCULATION RATE AND NUMBER OF HOLES EFFECTS OVER CARPOPHORES' PROTEIN CONTENT.



SOURCE: the author (2014).

The high levels of both inoculum rate and number of holes produced significative increases in the protein content of the carpophores. This graphic also shows that the interaction of these factors was significative and synergistic. This increase in fruiting bodies protein content can be attributed both to the higher protein concentration in spawn grains in relation to the production substrate and a possible mechanism of air nitrogen fixation enhancing at higher aeration levels. Protein content found in mushrooms cultivated using pejibaye palm sheath residues as substrate varied between 14,22 [g($100g^{-1}$)] and 18,84 [g($100g^{-1}$)] (table 45). These results are in accordance with the values reported by Duprat (2012), 19,32[g($100g^{-1}$)].

TABLE 45 – TOTAL PROTEIN AND CARBOHYDRATES CONTENT IN *P. OSTREATUS* BASIDIOMATA, CULTIVATED USING PEJIBAYE PALM RESIDUES AS SUBSTRATE, WITH VARIED INOCULATION RATES AND NUMBER OF HOLES PER BAG.

ASSAY	INDEPENDENT VARIABLES		DEPENDENT VARIABLES	
	X_1	X_2	Proteins [g(100g^{-1})]	Total carbohydrates [g(100g^{-1})]
1	10 (-1)	4 (+1)	15,56	15,59
			(±0,86)	(±2,34)
2	10 (-1)	2 (-1)	14,32	19,02
			(±0,26)	(±0,12)
3	40 (+1)	4 (-1)	14,22	14,25
			(±0,13)	(±0,79)
4	40 (+1)	2 (-1)	18,76	18,79
			(±0,62)	(±0,60)
5	25 (0)	3 (0)	16,84	16,91
			(±0,37)	(±0,55)
6	25 (0)	3 (0)	17,21	16,91
			(±0,36)	(±0,33)
7	25 (0)	3 (0)	16,84	16,91
			(±0,37)	(±0,58)

Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). X1 – Inoculation rate (% in dry weight), X2 – Number of holes (units). Standard deviation values are in parentheses. * (%). SOURCE: the author (2014).

Nevertheless, the calculated second order mathematical models fitted well the relation between inoculum rates/ number of holes and protein content. In the other hand, the calculated model for the relation between the assayed variables and carbohydrates content showed a significative lack of fit (TABLE 46).

These results can also be observed in TABLE 47. Predicted results are better fitted to the experimental data for proteins than for carbohydrates content.

These preliminary results show that interesting variations in the nutritional content of fruiting bodies can be obtained by manipulating simple process parameters, such as inoculation rate and number of holes.

GRAPHIC 13 clearly shows the significative synergistic interaction between inoculation rate and number of holes. The simultaneous high levels of both factors resulted in carpophores with much higher protein concentrations than those obtained with any other tested combination of levels of these factors.

TABLE 46 – ANALYSIS OF VARIANCE (ANOVA), PURE ERROR, LACK OF FIT AND REGRESSION COEFICIENT FOR SECOND ORDER POLYNOMIAL EQUATIONS DESCRIBING TOTAL PROTEINS AND CARBOHYDRATES CONTENT IN RELATION TO INOCULATION RATE AND NUMBER OF HOLES PER BAG IN THE CULTIVATION OF *PLEUROTUS OSTREATUS* USING PEJIBAYE PALM SHEATH RESIDUES AS SUBSTRATE. **

REGRESSION COEFFICIENT		PROTEINS [G(100G^{-1})]*	TOTAL CARBOHYDRATES [G(100G^{-1})]*
Linear intersection	β_0	19,77738	78,5524
	β_1	-0,23386	-0,2299
	β_2	-1,63595	-0,4890
Interaction	β_{12}	0,09633	0,0290
	R^2	0,7798	0,7362
	Pure error	0,2212	0,9896
	Lack of fit	0,2156	0,0391

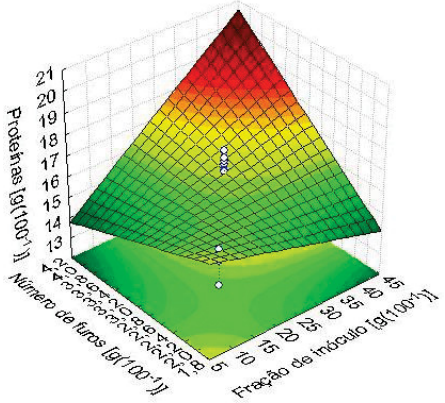
Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). * (%) ** 5% significance level. SOURCE: the author (2014).

TABLE 47 – OBSERVED EXPERIMENTAL TOTAL PROTEINS AND CARBOHYDRATES CONTENT FOR DIFFERENT COMBINATIONS OF INOCULATION RATE AND NUMBER OF HOLES AND RESPONSE VALUES PREDICTED BY THE PROPOSED MATHEMATICAL MODEL, FOR *PLEUROTUS OSTREATUS* CARPOPHORES CULTIVATED USING PEJIBAYE PALM SHEATH RESIDUES AS SUBSTRATE.

Assay	**IR**	**NH**	**Proteins [g(100g^{-1})]**		**Total carbohydrates [g(100g^{-1})]**	
			Obs.	Pred.	Obs.	Pred.
1	10	4	15,56	16,09	5,20	1,73
2	10	2	14,32	14,86	6,34	2,11
3	40	4	14,22	14,75	4,75	1,58
4	40	2	18,76	19,29	6,26	2,09
5	25	3	16,84	16,25	5,64	1,88
6	25	3	17,21	16,25	5,64	1,88
7	25	3	16,84	16,25	5,64	1,88

IR: inoculation rate (%); NH: number of holes. Assays: 1 (10% inoculation rate and 4 holes); 2 (10% inoculation rate and 2 holes); 3 (40% inoculation rate and 4 holes); 4 (40% inoculation rate and 2 holes); 5, 6 e 7 (25% inoculation rate and 3 holes). SOURCE: the author (2014).

GRAPHIC 13 - SURFACE RESPONSE OF CARPOPHORES' PROTEIN CONTENT IN RELATION TO INOCULATION RATE AND NUMBER OF HOLES FACTORS.



SOURCE: the author (2014).

5.5 MUSHROOM SPENT SUBSTRATE AS SOIL FERTILIZER FOR LETTUCE (*LACTUCA SATIVA* VAR. VERÔNICA) CULTIVATION

TABLE 48 – ROUTINE ANALYSIS FOR THE EVALUATION OF SOIL FERTILITY, WITH P_R (PEJIBAYE PALM SHEATH RESIDUES) AND M_R (MUSHROOM CULTIVATION RESIDUES), AFTER 40 COMPOSTING DAYS PRIOR TO LETTUCE CULTIVATION. EXCHANGEABLE ACIDITY (Al^{+3}) AND TITRABLE ($H^+ + Al^{+3}$), POTASSIUM (K^+), SECONDARY MACRONUTRIENTS (Ca^{+2} AND Mg^{+2}) AND BASES SUM (Ca^{+2}, Mg^+2 AND K^+).

Treatment	pH	Al^{+3}	$H^+ + Al^{+3}$	Ca^{+2}	Mg^{+2}	K^+	BS
	($CaCl_2$)	($cmol_c/dm^3$)					
T1	6,30	0,00	3,00	5,30	2,70	0,98	8,98
T2	6,20	0,00	3,00	2,80	2,80	0,93	6,53
T3	6,30	0,00	2,70	4,00	2,60	1,15	7,75
T4	6,30	0,00	2,50	4,00	2,60	1,18	7,78
T5	6,00	0,00	4,40	4,60	2,90	1,21	8,71
T6	6,00	0,00	3,40	4,50	3,10	1,50	9,10
T7	6,10	0,00	2,50	4,10	2,90	1,33	8,33

Treatments: T1 (Soil), T2 (Soil + 5% P_R), T3 (Soil + 10% P_R), T4 (Soil + 15% P_R), T5 (Soil + 5% M_R), T6 (Soil + 10% M_R) and T7 (Soil + 15% M_R). SOURCE: the author (2014).

Results summarized in TABLE 48 show that:

Soil pH values between 6 and 6,3, as those found for T1-T7 treatments, are adequate for lettuce cultivation, according to Olenik (2004).

Analysed samples did not show significant amounts of Al^{+3}. Consequently, this type of soil can be classified as very low potencial acidity.

Calcium (Ca^{+2}) concentrations were quantified as medium (between 2,41 and 4,80 $cmol_c/dm^3$) or high (over 4,80 $cmol_c/dm^3$), classified as good and very good for plants' physiology, according to Ribeiro *et al.* (1999).

Magnesium (Mg^{+2}) presented values higher than 0,90 $cmol_c/dm^3$ (classified as soil with high magnesium content), adequate for photosynthesis processes and carbohydrates metabolism (RIBEIRO *et al.*, 1999).

Potassium (K^+) presented values higher than 0,3 $cmol_c/dm^3$ for all treatments (classified as very high), allowing sugars processing during respiration, through protein and starch synthesis and oxidative and photosynthetic phosphorylation (REIS, 1997).

Bases sum (BS), which consists in the sum of $Ca^{+2} + Mg^{+2} + K^+$ showed results higher than 3,6; classified as good/ very good for all treatments (RIBEIRO *et al.*, 1999).

TABLE 49 - ROUTINE ANALYSIS FOR SOIL FERTILITY EVALUATION, INCLUDING CATIONIC EXCHANGE CAPACITY (E), PHOSPHORE (P), CARBON (C), ORGANIC MATTER (OM), BASE SATURATION PERCENTAGE (V), ALUMMINIUM (m) AND Ca/Mg IN THE SOIL, WITH THE ADDITION OF P_R AND M_R, AFTER 40 DAYS OF COMPOSTING.

TREATMENT	E	P	C	OM	V	m	Ca/Mg
	(mg/dm^3)		(g/dm^3)		(%)		
T1	11,98	210,3	18,2	31,38	75	0	1,96
T2	9,53	65	18,2	31,38	69	0	1,00
T3	10,45	56,7	17,2	29,66	74	0	1,54
T4	10,28	47,4	17,2	29,66	76	0	1,54
T5	12,11	51,1	16,2	27,93	72	0	1,59
T6	12,5	52,8	18,2	31,68	73	0	1,45
T7	10,83	49,5	19,2	33,10	77	0	1,41

Treatments: T1 (Soil), T2 (Soil + 5% P_R), T3 (Soil + 10% P_R), T4 (Soil + 15% P_R), T5 (Soil + 5% M_R), T6 (Soil + 10% M_R) and T7 (Soil + 15% M_R). SOURCE: the author (2014).

The routine analyses for soil fertility evaluation are shown in TABLE 49.

Cationic exchange capacity (E) was classified as good for all treatments, allowing greater storage potential of cationic nutrients in exchangeable form (COSTA, 2008). Phosphore (P) concentrations were classified as high, for all treatments, being available for carbohydrates metabolism and lettuce respiration (OLENIK, 2004). According to Ribeiro *et al.* (1999), carbon was converted in organic matter (OM) to a good extent, for all treatments.

Bases saturation percentages (V) were higher than 50%. Consequently, these soils were classified as eutrophic, or fertile for all treatments, with high cations amount, allowing good productivity (50~80% range) (RONQUIM, 2010).

Aluminum saturation percentage (m) was null for all treatments, pointing a productive, nutrient rich soil, needing no acidity correction and allowing radicular growt of plants (COSTA, 2008).

After this initial soil analysis, all treatments were considered fertile and were used as lettuce culture substrates for 63 days. After harvesting, the soil was re-analyzed, as shown in TABLES 50 and 51.

After 63 days of lettuce cultivation, soil samples characteristics remained very similar to those pre-cultivation, with a slight increasing in carbon and organic matter, and a slight decreasing in phosphorus amounts.

GRAPHICS 14 and 15 show the average lettuce growth after 23 and 63 days of cultivation, respectively, in height, width and roots length (for day 63).

TABLE 50 – ROUTINE ANALYSIS FOR EVALUATION OF SOIL ADDED WITH P_R AND M_R FERTILITY, AFTER 63 DAYS OF LETTUCE CULTIVATION. EXCHANGEABLE ACIDITY (Al^{+3}) AND TITRABLE ($H^+ + Al^{+3}$), POTASSIUM (K^+), SECONDARY MACRONUTRIENTS (Ca^{+2} and Mg^{+2}) AND BASES SUM (Ca^{+2}, $Mg^{+}2$ and K^+).

TREATMENT	pH	Al^{+3}	$H^+ + Al^{+3}$	Ca^{+2}	Mg^{+2}	K^+	SB
	($CaCl_2$)	($cmol_c/dm^3$)					
T1	6,10	0,00	3,20	5,10	3,30	0,52	8,92
T2	6,20	0,00	3,20	5,20	3,10	0,85	9,15
T3	6,40	0,00	2,70	5,30	3,10	1,27	9,67
T4	6,40	0,00	2,50	4,50	2,80	1,07	8,37
T5	6,20	0,00	3,20	5,00	3,00	1,03	9,03
T6	6,40	0,00	3,00	5,00	2,90	1,14	9,04
T7	6,50	0,00	2,70	4,50	2,80	1,29	8,59

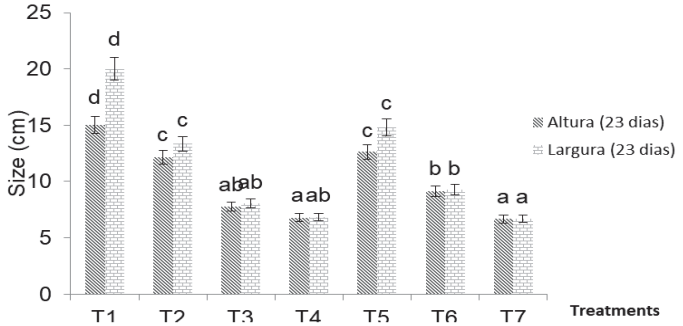
Treatments: T1 (Soil), T2 (Soil + 5% P_R), T3 (Soil + 10% P_R), T4 (Soil + 15% P_R), T5 (Soil + 5% M_R), T6 (Soil + 10% M_R) and T7 (Soil + 15% M_R). SOURCE: the author (2014).

TABLE 51 – ROUTINE ANALYSIS FOR EVALUATION OF SOIL ADDED WITH P_R AND M_R FERTILITY, AFTER 63 DAYS OF LETTUCE CULTIVATION. CATIONIC EXCHANGE CAPACITY VALUES (E), PHOSPHORE (P), CARBON (C), ORGANIC MATTER (OM), BASES SATURATION PERCENTAGE (V), ALUMINIUM (m) and CALCIUM/ MAGNESIUM (Ca/Mg).

Treatment	E	P	C	OM	V	m	Ca/Mg
	(mg/dm^3)		(g/dm^3)		(%)		
T1	12,12	40,80	23,20	40,00	74	0	1,55
T2	12,35	34,00	26,40	45,51	74	0	1,68
T3	12,37	46,00	23,20	40,00	78	0	1,71
T4	10,87	32,60	23,20	40,00	77	0	1,61
T5	12,23	39,10	19,20	33,10	74	0	1,67
T6	12,04	32,20	17,20	29,65	75	0	1,72
T7	11,29	33,10	21,20	36,55	76	0	1,61

Treatments: T1 (Soil), T2 (Soil + 5% P_R), T3 (Soil + 10% P_R), T4 (Soil + 15% P_R), T5 (Soil + 5% M_R), T6 (Soil + 10% M_R) and T7 (Soil + 15% M_R). SOURCE: the author (2014).

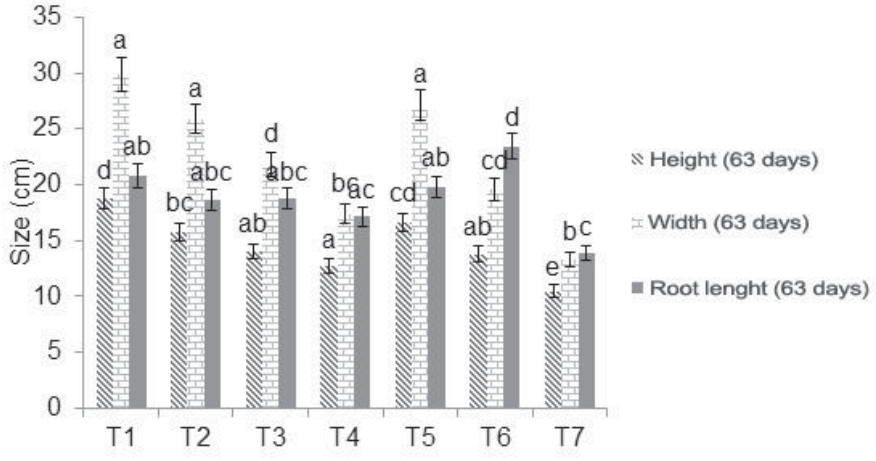
GRAPHIC 14- LETTUCE GROWTH (cm) IN HEIGHT AND WIDTH, AFTER 23 DAYS OF CULTIVATION, FOR EACH TREATMENT. VALUES IDENTIFIED WITH THE SAME LETTER DO NOT DIFFER STATISTICALLY, AS ANALYSED BY ANOVA/ TUKEY (P<0,05), BASED IN 20 REPETITIONS. TREATMENTS: T1 (SOIL), T2 (SOIL + 5% P_R), T3 (SOIL + 10% P_R), T4 (SOIL + 15% P_R), T5 (SOIL + 5% M_R), T6 (SOIL + 10% M_R) AND T7 (SOIL + 15% M_R).



SOURCE: the author (2014).

At the end of the cultivation period, lettuces were harvested and it was verified that treatments with 5% P_R or M_R (T2 and T5, respectively) resulted in similar plant growth. However, only the treatment T5 (5% M_R) had no statistical difference to the control (T1) group in height. Treatment T2 showed slightly lower results for this parameter. As pejibaye palm residues are fibrous, tough and show low nitrogen content, they difficult the composting process (FDR, 2014; TAVARES *et al.*, 2013).

GRAPHIC 15 – LETTUCE GROWTH (cm) IN LEAVES HEIGHT AND WIDTH AND ROOTS LENGTH, AFTER 63 CULTIVATION DAYS. VALUES IDENTIFIED WITH THE SAME LETTER DO NOT DIFFER STATISTICALLY, AS ANALYSED BY ANOVA/ TUKEY (P<0,05), BASED IN 20 REPETITIONS. TREATMENTS: T1 (SOIL), T2 (SOIL + 5% P_R), T3 (SOIL + 10% P_R), T4 (SOIL + 15% P_R), T5 (SOIL + 5% M_R), T6 (SOIL + 10% M_R) AND T7 (SOIL + 15% M_R).



SOURCE: the author (2014).

The presented results suggest that mycelial action is significatively efficient for the degradation of pejibaye fibers, converting its biomass and releasing nutrients for other composting processes, making nutrients more rapidly available for plants.

It is remarkable that treatments with the addition of 15% M_R showed considerably smaller growth of lettuces than those with the addition of 15% P_R. This suggests that the best proportion of M_R addition should be carefully investigated.

It is interesting to note a significative increase in root length in treatment T6 (10% M_R) in relation to T3 (10% P_R) and the control T1. However, as shown in TABLE 52, the mycelium containing treatments (M_R) did not present more nitrogen than those only containing crude pejibaye sheath residues (P_R).

TABLE 52 – NITROGEN CONTENTS OF SAMPLES FROM TREATMENTS T1-T7, AFTER 63 DAYS OF LETTUCE CULTIVATION.

TREATMENT	N_2 (g/Kg)	EQUIVALENCE (%)
T1	2,19	-
T2	2,16	-1,36
T3	2,44	+10,24
T4	2,62	+16,41
T5	2,58	+15,12
T6	2,59	+15,44
T7	2,52	+13,10

Treatments: T1 (Soil), T2 (Soil + 5% P_R), T3 (Soil + 10% P_R), T4 (Soil + 15% P_R), T5 (Soil + 5% M_R), T6 (Soil + 10% M_R) and T7 (Soil + 15% M_R). SOURCE: the author (2014).

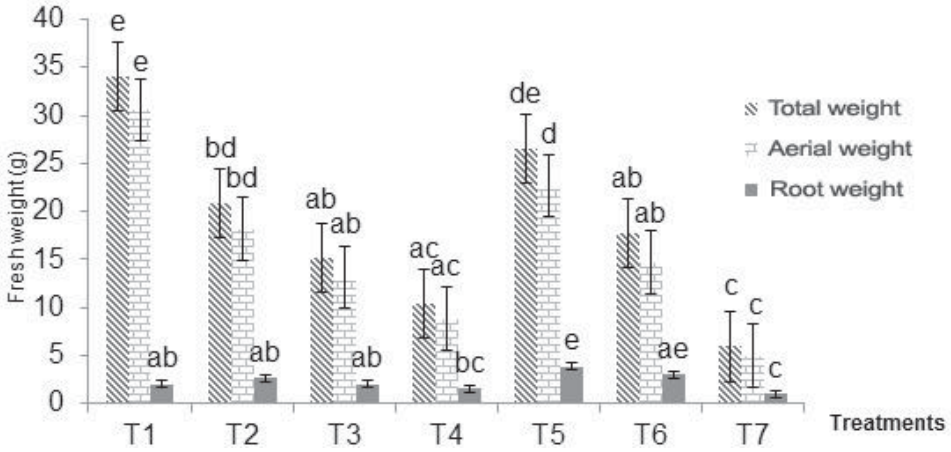
FIGURE 32 shows the effect of each of the assayed substrate compositions. T5 (5% M_R) lettuces showed similar leaves and roots growth as the control group (T1). An evident inverse correlation is observed between residues addition and lettuces development. That is, when more residues are added, lower is the growth.

FIGURE 32 - LETTUCES' LEAVES AND ROOTS DEVELOPMENT FOR TREATMENTS T1-T7.

SOURCE: the author (2014).

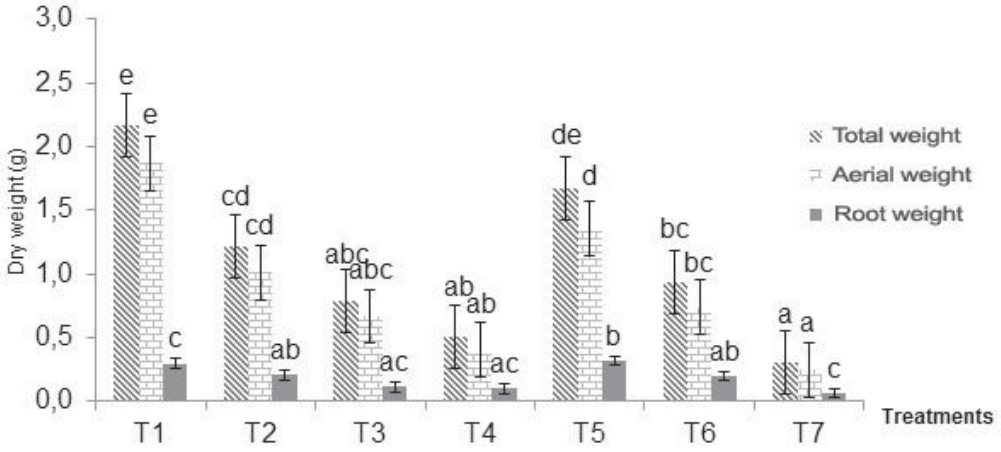
The same tendency is observed in GRAPHICS 16 and 17. The performance of soils with M_R addition was better than those with P_R (nearly 40% increase in yield), but the best substrate stills the control T1. More experiments are needed in order to determine whether the mycelium cultivation time influences the process.

GRAPHIC 16 – TOTAL FRESH WEIGHT, AERIAL PARTS FRESH WEIGHT AND ROOTS FRESH WEIGHT FOR T1-T7 TREATMENTS, AFTER 63 CULTIVATION DAYS. TREATMENTS: T1 (SOIL), T2 (SOIL + 5% P_R), T3 (SOIL + 10% P_R), T4 (SOIL + 15% P_R), T5 (SOIL + 5% M_R), T6 (SOIL + 10% M_R) AND T7 (SOIL + 15% M_R).



SOURCE: the author (2014).

GRAPHIC 17 – TOTAL DRY WEIGHT, AERIAL PARTS DRY WEIGHT AND ROOTS DRY WEIGHT FOR T1-T7 TREATMENTS, AFTER 63 CULTIVATION DAYS. TREATMENTS: T1 (SOIL), T2 (SOIL + 5% P_R), T3 (SOIL + 10% P_R), T4 (SOIL + 15% P_R), T5 (SOIL + 5% M_R), T6 (SOIL + 10% M_R) AND T7 (SOIL + 15% M_R).



SOURCE: the author (2014).

5.6 ANTIOXIDANTS PRODUCTION BY SUBMERGED CULTIVATION OF MYCELIA, USING AGRO-INDUSTRIAL RESIDUES AS SUBSTRATE

5.6.1 Preliminar Plackett-Burman experimental design for evaluation of significative factors affecting the process

TABLE 53 shows the three analysed response values for each of the experimental runs, in the cultivation of *P. djamor*, using Pejibaye palm liquid residues

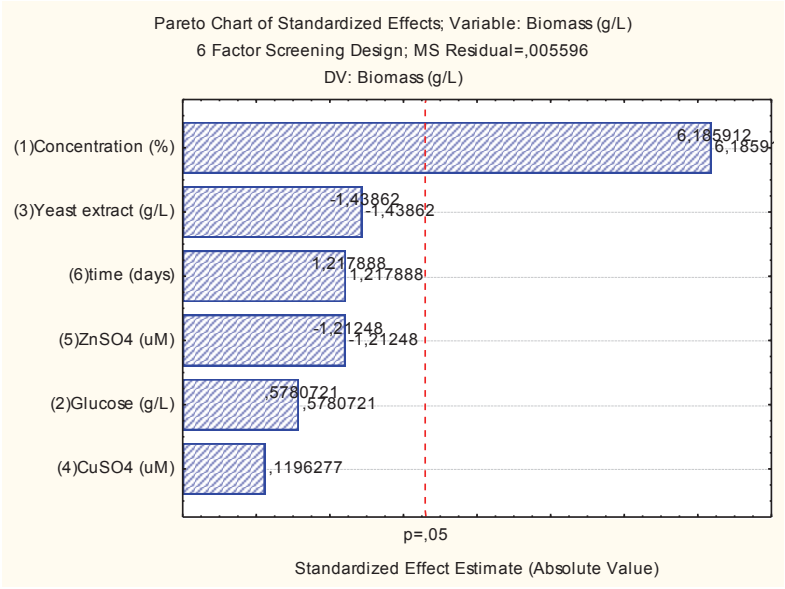
as substrate. The assayed factors were: substrate concentration (liquid residue was diluted with water) and the addition of glucose (Gluc.), yeast extract (YE) and the salts $CuSO_4$ and $ZnSO_4$. Pareto's charts allow a better visualization of the most significative factors affecting the response variables and whether the effect is positive or negative.

TABLE 53 - EXPERIMENTAL PLANNING AND RESPONSES TABLE.

CP	Conc. (%)	Gluc. (g/L)	YE (g/L)	CuSO4 (uM)	ZnSO4 (uM)	days						Biom. (g/L)	Prot. (mg/mL)	TEAC (uM TROLOX / % extract)	Fr
1	80,0	0,00	4,00	0,0	0,0	14	1	1	-1	1	-1	1,7755	2,245	1,98141254	5
1	80,0	10,00	0,00	100,0	0,0	7	1	1	1	-1	-1	1,7660	2,956	1,01133183	2
1	20,0	10,00	4,00	0,0	100,0	7	-1	1	1	1	-1	1,3169	2,419	4,09701173	0
1	80,0	0,00	4,00	100,0	0,0	7	-1	-1	1	1	1	1,5813	3,626	2,36248877	5
1	80,0	10,00	0,00	100,0	100,0	14	-1	-1	-1	1	-1	1,7135	2,136	-1,93258775	3
1	80,0	10,00	4,00	0,0	100,0	7	1	-1	-1	-1	1	1,6551	3,682	2,09804147	3
1	20,0	10,00	4,00	100,0	0,0	14	-1	1	-1	-1	1	1,4653	0,975	5,19774468	0
1	20,0	0,00	4,00	100,0	100,0	14	1	-1	1	-1	-1	1,3774	0,782	4,76353254	0
1	20,0	0,00	0,00	100,0	100,0	7	1	1	-1	1	1	1,4699	0,489	-1,64528049	0
1	80,0	0,00	0,00	0,0	100,0	14	-1	1	1	-1	1	1,6680	2,262	-0,823570135	4
1	20,0	10,00	0,00	0,0	0,0	14	1	-1	1	1	1	1,5160	0,834	-2,82916108	1
1	20,0	0,00	0,00	0,0	0,0	7	-1	-1	-1	-1	-1	1,4109	0,605	-2,93989826	0
0	50,0	5,00	2,00	50,0	50,0	11	0	0	0	0	0	1,4356	1,537	-1,06337458	3
0	50,0	5,00	2,00	50,0	50,0	11	0	0	0	0	0	1,4858	2,540	-1,12134487	3
0	50,0	5,00	2,00	50,0	50,0	11	0	0	0	0	0	1,4832	1,765	-1,06441608	2

Conc. = substrate concentration (the Pejibaye palm sheath residues juice was diluted with water); Gluc. = glucose; YE = yeast extract; Biom. = biomass concentration; Prot. = protein concentration; TEAC = trolox equivalent antioxidant capacity; Fr. = fructification level. SOURCE: the author (2014).

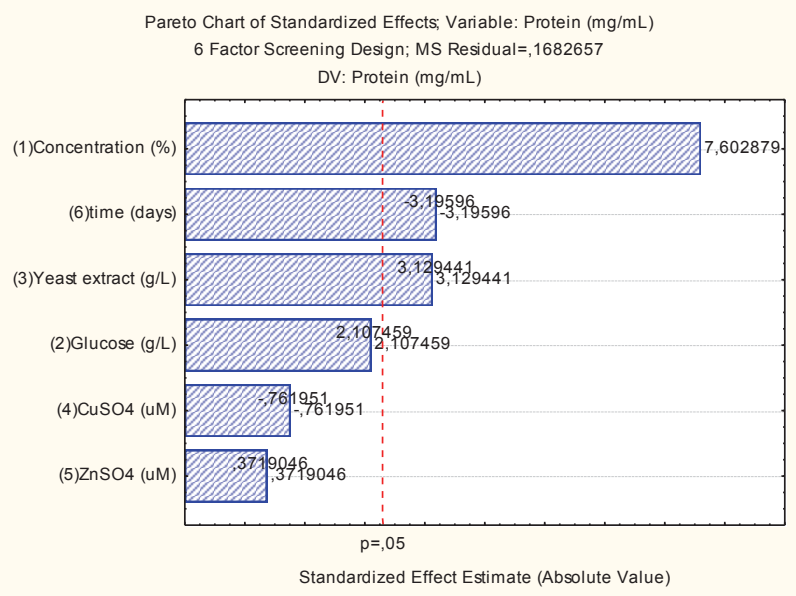
GRAPHIC 18 - MAIN EFFECT OF THE 6 FACTORS OVER THE BIOMASS PRODUCTION.



SOURCE: the author (2014).

Biomass yield was affected only by the variation in the concentration of the substrate. The higher concentration of pejibaye palm liquid residue resulted in higher biomass production (GRAPHIC 18). None of the other factors were significative.

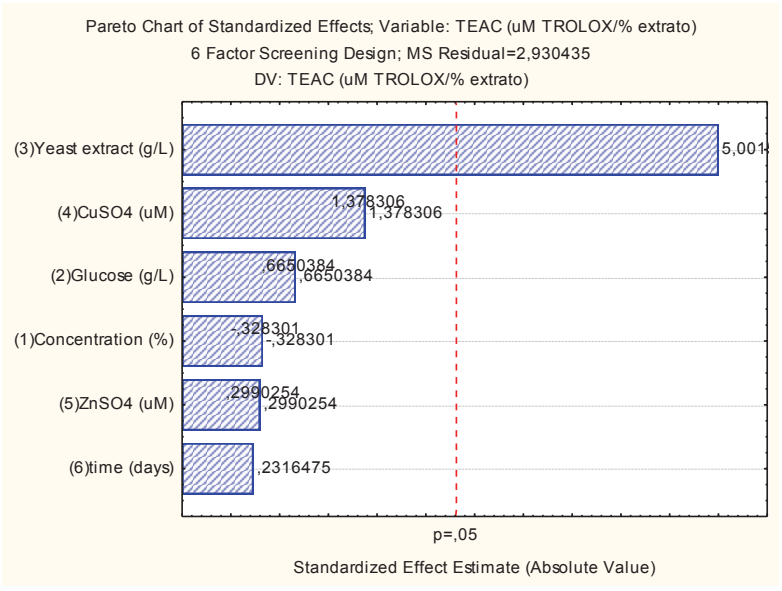
GRAPHIC 19 - MAIN EFFECT OF THE 6 FACTORS OVER THE PROTEIN CONTENT.



SOURCE: the author (2014).

The higher concentration of pejibaye palm liquid residue and yeast extract addition resulted in higher final protein content in the fermented broth. Protein content decreased with time, between day 7 and day 14 (GRAPHIC 19). None of the other assayed factors affected significatively the final protein content.

GRAPHIC 20 - MAIN EFFECT OF THE 6 FACTORS OVER THE ANTIOXIDANT ACTIVITY (TEAC).

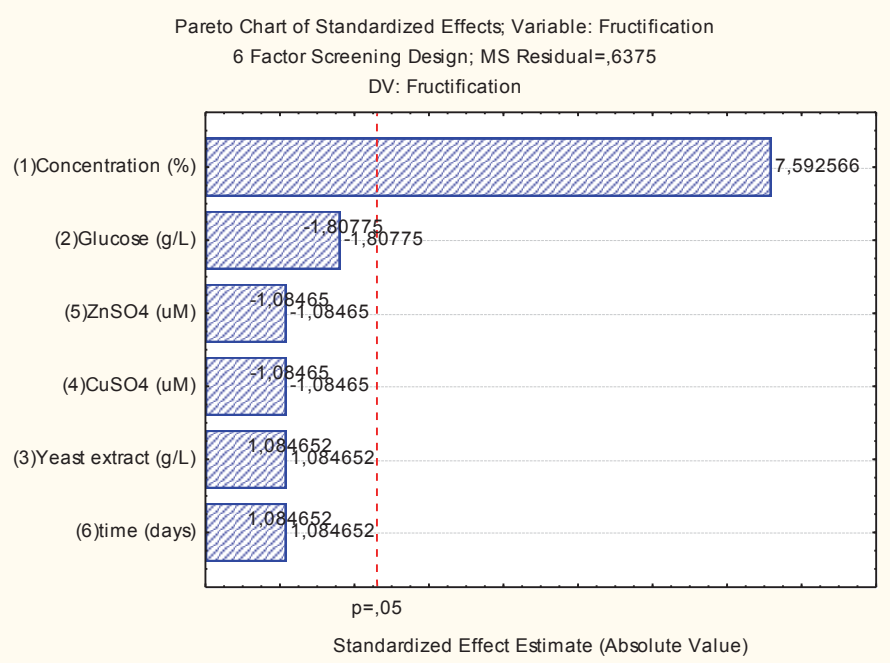


SOURCE: the author (2014).

The only factor that affected the antioxidant activity of the final product significatively was yeast extract addition. Higher concentrations of yeast extract in the culture medium resulted in higher final total antioxidant activity (GRAPHIC 20). Although in a subtle amount, the addition of $CuSO_4$ improved the antioxidant activity of the product and should be assayed more carefully in further experiments. The addition of $ZnSO_4$ should also be better studied, because it affected the process even less, but also in a positive manner in relation to the antioxidant activity.

This antioxidant activity improvement within yeast extract addition can be attributed to different mechanisms. Macromycetes mycelia are known to be producers of oxidative enzymes. Some authors observed lower oxidative enzymes activities when mycelia were cultivated using nitrogen richer media. Thus, the observed improvement in antioxidant activity within yeast extract addition could be in fact the result of lower oxidative enzymes activities. The subtle contribution of salts to the improvement of antioxidant activity can be attributed to their roles as antioxidant enzymes cofactors.

GRAPHIC 21 - MAIN EFFECT OF THE 6 FACTORS OVER FRUCTIFICATION INDUCTION.



SOURCE: the author (2014).

The only significative factor affecting fructification induction was the pejibaye residue concentration in the substrate. Higher concentrations resulted in higher fructification rates (GRAPHIC 21).

The concentration of substrate was a significative factor for three of the response variables analyzed. The higher concentration of substrate resulted in:

- higher final biomass production;
- higher final protein concentration and
- higher induction of fructification level.

The main factor that affected the antioxidant activity of the final product was yeast extract addition. It also resulted in higher final protein content.

The other factors affected the response variables only slightly, and cannot be considered significative. However, absolute numbers show that:

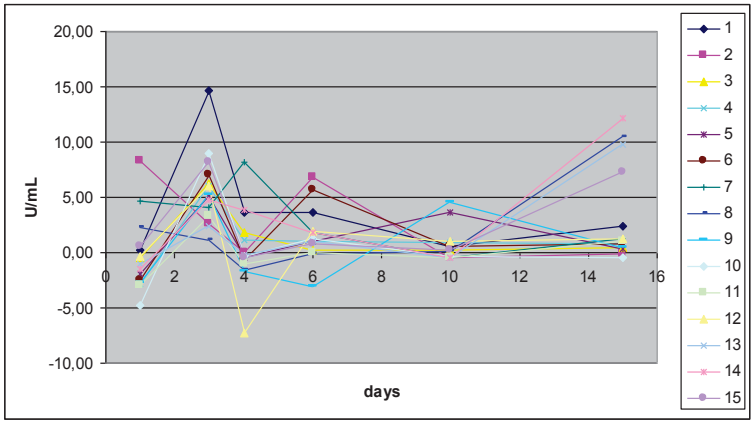
Biomass concentration increased very little after the 7th day of fermentation, so as the antioxidant activity and fructification induction level.

Glucose addition resulted in a slight increasing in final biomass production, protein concentration and antioxidant activity and subtly decreased fructification induction level. The addition of yeast extract reduced the biomass production a little, as it increased fructification rates also in a small amount.

The addition of $CuSO_4$ decreased the biomass production, but increased the final protein concentration. Inversely, the addition of $ZnSO_4$ caused an increase in biomass production and a decrease in protein concentration. Both salts caused a slight decrease in fructification levels.

5.6.2 Plackett-Burman experimental design for evaluation of inducers of SOD and CAT activities

GRAPHIC 22 - CAT ACTIVITY KINETICS FOR THE PLACKETT-BURMAN DESIGN EXPERIMENT.

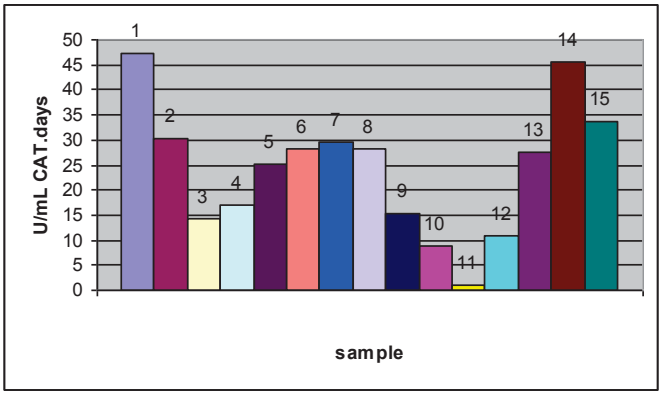


SOURCE: the author (2014).

The kinetics of CAT activity showed an apparently random oscillation in enzymatic activity during the cultivation period (GRAPHIC 22).

For a better comparison between treatments, enzymatic activities at each experimental condition were integrated (GRAPHIC 23). However, additional experiments should be performed in order to better understand these oscillations.

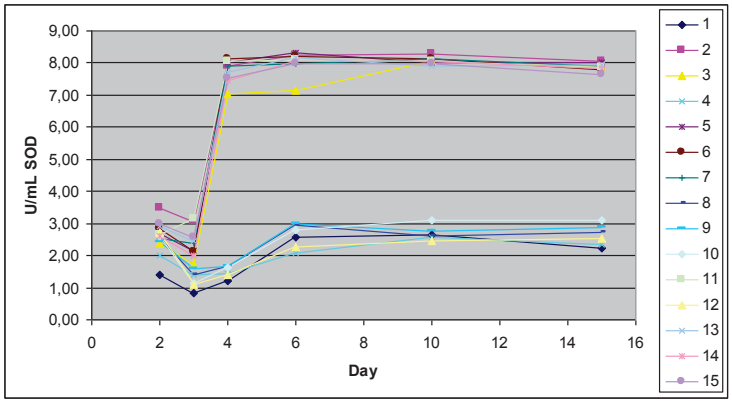
GRAPHIC 23 - AREAS UNDER CURVES FOR CAT ACTIVITY OF EACH RUN.



SOURCE: the author (2014).

Differently, kinetic curves for SOD activity clearly show a drastic increase in SOD activity in all treatments that included the addition of lignin sulfonate to the cultivation media (GRAPHIC 24). SOD activity increased sharply after the third day of cultivation and kept high until the 15th day.

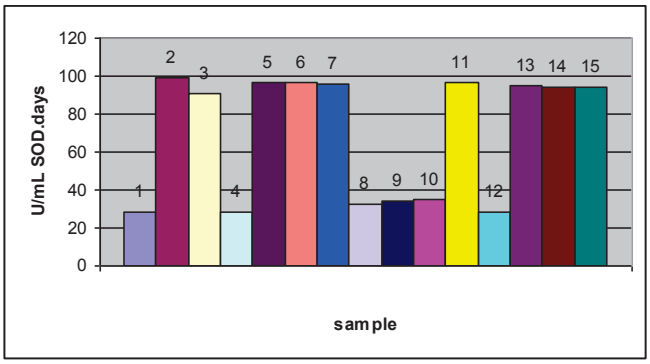
GRAPHIC 24 - SOD ACTIVITY KINETICS FOR THE PLACKETT-BURMAN DESIGN EXPERIMENT.



SOURCE: the author (2014).

The integration of SOD activity results at each experimental condition, reinforce the previous discussion. These data suggest that lignin sulfonate is a strong inducer of SOD activity in the submerged cultivation of *P. ostreatus* mycelium (GRAPHIC 25).

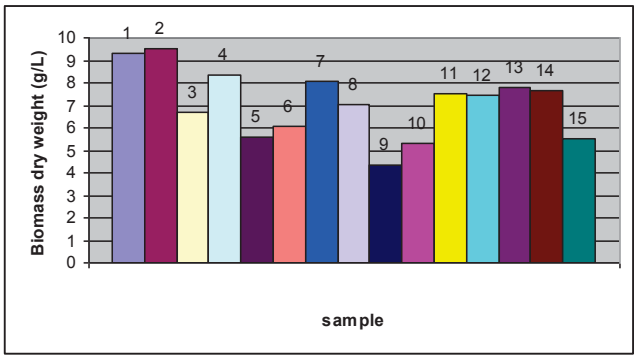
GRAPHIC 25 - AREAS UNDER CURVES FOR SOD ACTIVITY OF EACH RUN.



SOURCE: the author (2014).

Biomass production was not followed during the cultivation period. Instead, at the end of the experiment, biomass of each experimental condition was recovered by filtration, dried and weighted. The obtained results are expressed in GRAPHIC 26. Condition 2 resulted in the highest biomass yield and condition 9, in the lowest.

GRAPHIC 26 - BIOMASS DRY WEIGHT FOR EACH RUN.



SOURCE: the author (2014).

The integrated values for SOD and CAT activities, and dry biomass yields, for each experimental run, were used for the Plackett-Burmann analyses (TABLE 54).

As intended with this experimental design, it is possible to point the most effective inducers for each of the enzymatic activities, in the assayed experimental levels and whether these factors affect biomass growth. Pareto's charts help the visualization of these results (GRAPHICS 27, 28 and 29).

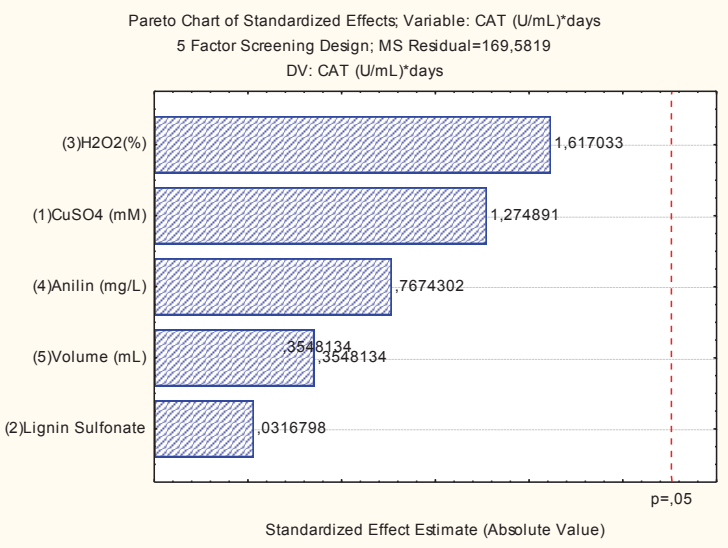
For the production of CAT, the most effective inducer was H_2O_2, as logical, because the main role of CAT is to process H_2O_2. The second most effective inducer was $CuSO_4$. All of the other tested substances also had positive effects over the production of CAT, although smaller (GRAPHIC 27).

TABLE 54 - THE THREE RESPONSES OBTAINED FOR EACH EXPERIMENTAL RUN.

RUN	CUSO4 (mM)	LIGNIN SULFONATE (g/L)	H_2O_2 (%)	ANILINE (mg/L)	VOLUME (mL)	CAT (U/mL)*DAYS	SOD (U/mL)*DAYS	DRY BIOMASS (g/L)
1	0,5	0	0,05	0	125	47,13	28,57	9,28
2	0,5	15	0	80	125	30,26	98,84	9,52
3	0	15	0,05	0	62,5	14,31	90,89	6,72
4	0,5	0	0,05	80	125	16,95	28,09	8,32
5	0,5	15	0	80	62,5	25,16	96,54	5,60
6	0,5	15	0,05	0	62,5	28,34	96,31	6,08
7	0	15	0,05	80	125	29,70	95,87	8,08
8	0	0	0,05	80	62,5	28,09	32,63	7,04
9	0	0	0	80	62,5	15,19	33,85	4,32
10	0,5	0	0	0	62,5	8,95	34,91	5,28
11	0	15	0	0	125	0,98	96,79	7,52
12	0	0	0	0	125	11,02	28,68	7,44
13 (C)	0,25	7,5	0,025	40	93,75	27,66	95,32	7,79
14 (C)	0,25	7,5	0,025	40	93,75	45,64	94,50	7,68
15 (C)	0,25	7,5	0,025	40	93,75	33,80	94,28	5,55

SOURCE: the author (2014).

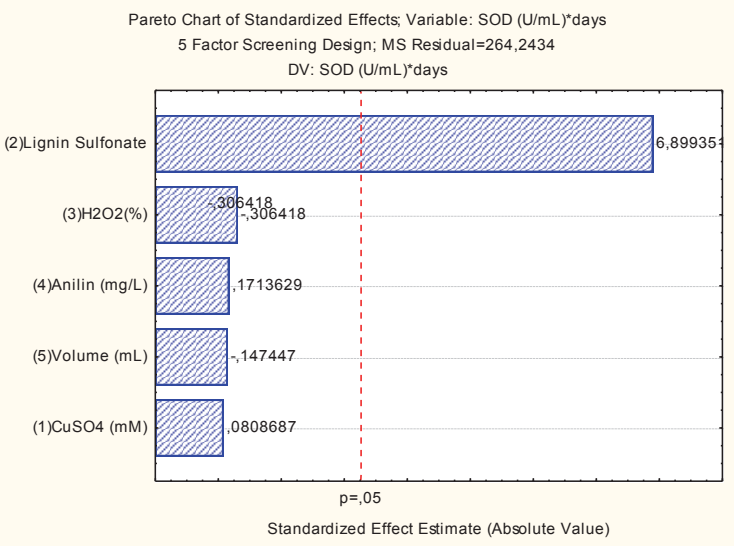
GRAPHIC 27- MAIN EFFECT OF THE 5 FACTORS OVER CAT ACTIVITY.



SOURCE: the author (2014).

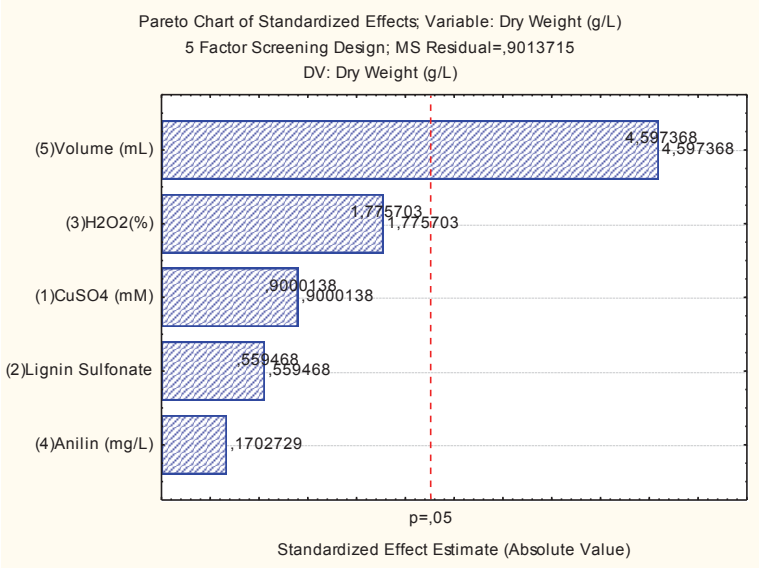
For the production of SOD, the most effective inducer was, by far, lignin sulfonate. Other observed effects were: a little inhibition by H_2O_2 (that would be explained as an inhibition by product, because hydrogen peroxide is the product of SOD enzymatic activity); and a little induction by aniline, which is a generator of superoxide radicals, the substrate of SOD (GRAPHIC 28).

GRAPHIC 28 - MAIN EFFECT OF THE 5 FACTORS OVER SOD ACTIVITY.



SOURCE: the author (2014).

GRAPHIC 29 - MAIN EFFECT OF THE 5 FACTORS OVER BIOMASS PRODUCTION.



SOURCE: the author (2014).

Cultivations with higher volume/ surface ratio produced higher quantities of biomass. All of the added substances had positive impact on biomass production, demonstrating a low toxicity of these substances for the mycelium in the tested levels. Also, it suggests that higher concentrations should be tested, in order to further induce enzymatic activities and stimulate biomass production (GRAPHIC 29).

5.6.3 Central composite, non-factorial, surface response experimental design for optimization of inducers of SOD and CAT activities

5.6.3.1 Phase I – “cube”

The following results were generated by the analysis of experimental data, aiming the approximation of a first order model, with the R software:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	23.05667	0.24414	94.4401	2.617e-06 ***
x1	1.09750	0.29901	3.6704	0.03499 *
x2	0.17750	0.29901	0.5936	0.59455

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Analysis of Variance Table
Response: SOD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
FO(x1, x2)	2	4.9441	2.47203	6.9123	0.07529
Residuals	3	1.0729	0.35763		
Lack of fit	2	0.9216	0.46082	3.0467	0.37547
Pure error	1	0.1512	0.15125		

Direction of steepest ascent (at radius 1):

x1	x2
0.9871726	0.1596566

Corresponding increment in original units:

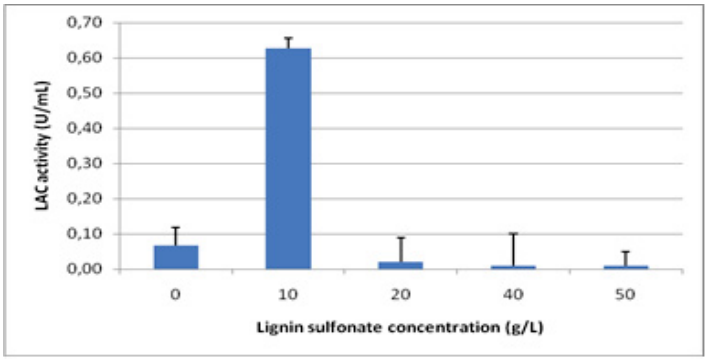
lignin.s	H2O2
9.871726	3.193132

Briefly, results confirmed the induction of SOD by lignin sulfonate addition. But, for this experiment, H_2O_2 also worked as an inducer of SOD. Both CAT and LAC showed very low activities within this experimental setting, probably due to mass transferrence problems, affecting aeration, nutrient diffusion and mycelial growth, caused by the small dimension of the cultivation recipient.

Nevertheless, it was possible to determine that the assayed concentrations of inducers were distant from the optimum. This experiment rendered inclined response surfaces, without significative curvatures. The first order model adjusted adequately to experimental data (“lack of fit” showed little significancy). This suggested paths of steepest ascent for increasing SOD activities in further experiments.

5.6.3.2 Phase II – induction curves

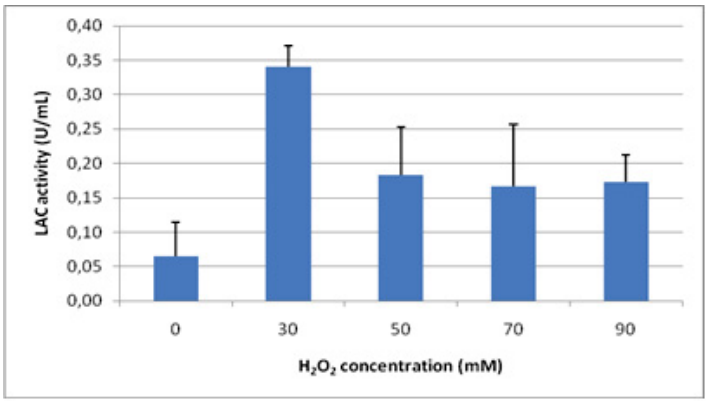
GRAPHIC 30 - LAC INDUCTION WITH LIGNIN SULFONATE.



SOURCE: the author (2014).

Interestingly, both lignin sulfonate and H_2O_2 induced LAC activity. The best concentration of lignin sulfonate for LAC induction was 10g/L (this condition resulted in almost tenfold units of LAC production in relation to the control) (GRAPHIC 30).

GRAPHIC 31 - LAC INDUCTION WITH H_2O_2.

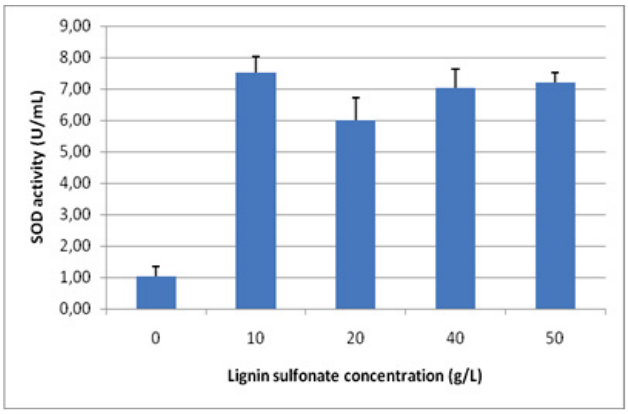


SOURCE: the author (2014).

The optimal H_2O_2 concentration for LAC induction was 50mM (nearly six fold increasing of LAC activity, compared to the control) (GRAPHIC 31).

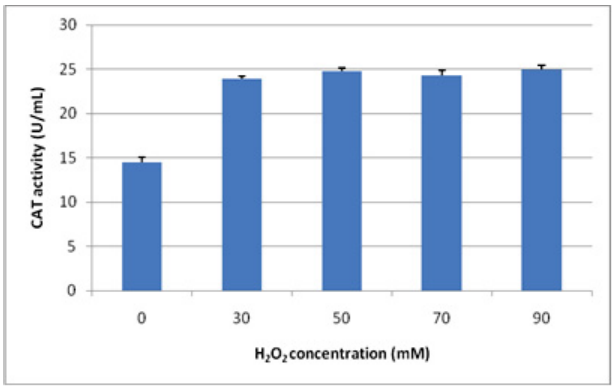
This experiment suggested that the best concentration of lignin sulfonate for inducing the production of SOD, by *Pleurotus ostreatus*, in the described conditions, is around 10 g/L (GRAPHIC 32). SOD activity was increased more than sevenfold with the addition of lignin sulfonate to the cultivation medium.

GRAPHIC 32 - SOD INDUCTION WITH LIGNIN SULFONATE.



SOURCE: the author (2014).

GRAPHIC 33 - CAT INDUCTION WITH H_2O_2.



SOURCE: the author (2014).

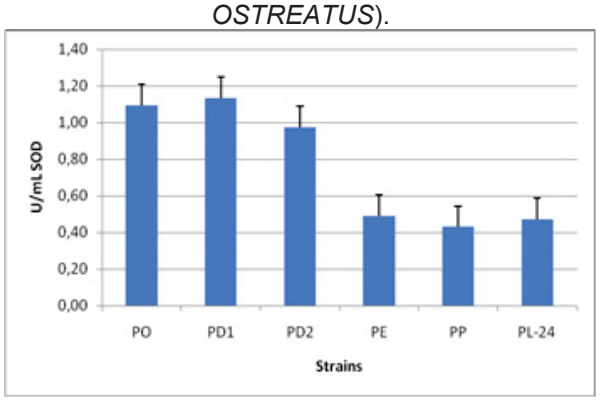
Results also suggest that the best concentration of H_2O_2 for the induction of CAT is above 30mM. The sensitivity of the analytic method was extrapolated, requiring an additional experiment to determine the optimal value more precisely (GRAPHIC 33). CAT activities increased up to 46%.

5.6.4 Characterization of antioxidant enzymes produced by four different species of the *Pleurotus* genus

5.6.4.1 Activity assays

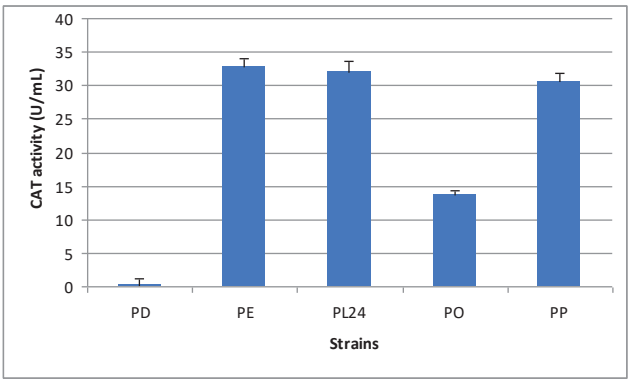
Strains with greatest SOD activity are PO, PD1 e PD2. The least active ones are: PE, PP and PL-24. *P. djamor* and *P. ostreatus* PO presented more than double SOD activity than the other strains (GRAPHIC 34).

GRAPHIC 34 - SOD ACTIVITY OF FERMENTED BROTHS. STRAINS: PO (*P. OSTREATUS*), PD1 (*P. DJAMOR*), PD2 (*P. DJAMOR*), PE (*P. ERYNGII*), PP (*P. PULMONARIUS*), PL-24 (*P. OSTREATUS*).



SOURCE: the author (2014).

GRAPHIC 35 - CAT ACTIVITY OF FERMENTED BROTHS. STRAINS: PO (*P. OSTREATUS*), PD1 (*P. DJAMOR*), PD2 (*P. DJAMOR*), PE (*P. ERYNGII*), PP (*P. PULMONARIUS*), PL-24 (*P. OSTREATUS*).



SOURCE: the author (2014).

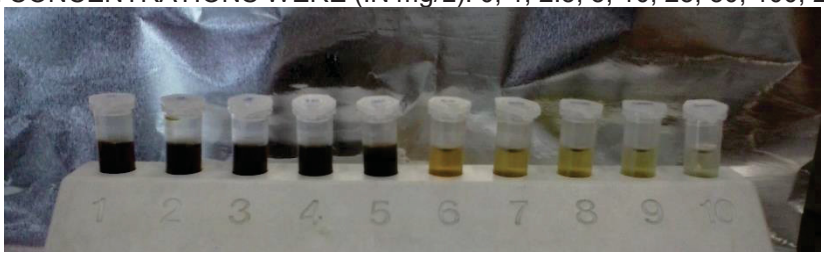
The strains which showed the highest CAT activity were: PE, PL24 and PP. and PO, moderate activity. PE, PL24 and PP activities were about 59% higher than that of PO. PD showed virtually no measurable CAT activity (GRAPHIC 35).

5.6.4.2 Lignin sulfonate precipitation curve

This experiment evidenced a CaOH concentration threshold at 25mM, above which there was a significative precipitation of lignin sulfonate (FIGURE 33).

Enzymatic activity assays showed that the resulting supernatant still contains SOD after this processing. However, SOD is not stable in the final pH, at 4ºC, as confirmed by activity assays performed 24 hours after the precipitation procedure.

FIGURE 33 - PRECIPITATION CURVE OF LIGNIN SULFONATE, WITH THE ADDITION OF CaOH. CaOH FINAL CONCENTRATIONS WERE (IN mg/L): 0, 1, 2.5, 5, 10, 25, 50, 100, 200 AND 300.

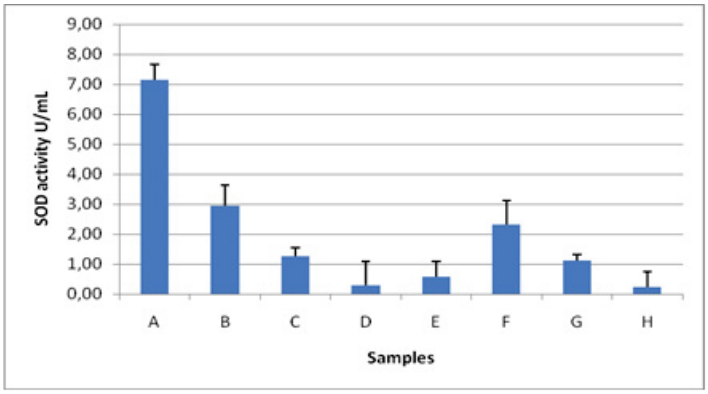


SOURCE: the author (2014).

5.6.4.3 SOD activities after samples dialysis

The lignin sulfonate removal was efficient with a single step of CaOH precipitation, however, at the cost of some enzymatic activity. The enzyme was not stable at the resulting pH. The obtained product lost almost all the activity in less than 24 hours.

GRAPHIC 36 - SOD ACTIVITY OF SAMPLES IN EACH PURIFICATION STEP.



SOURCE: the author (2014).

A dialysis step was added at the end of the process, aiming pH neutralization and SOD concentration. The best combination of lignin sulfonate removal efficiency and SOD activity retaining was achieved by treatment F. Notwithstanding this method was performed in microscale (2mL). For scaling up to nearly ten times larger volumes (treatment G), the activity of the final product fallen to almost half of the product obtained by the microscale method (GRAPHIC 36).

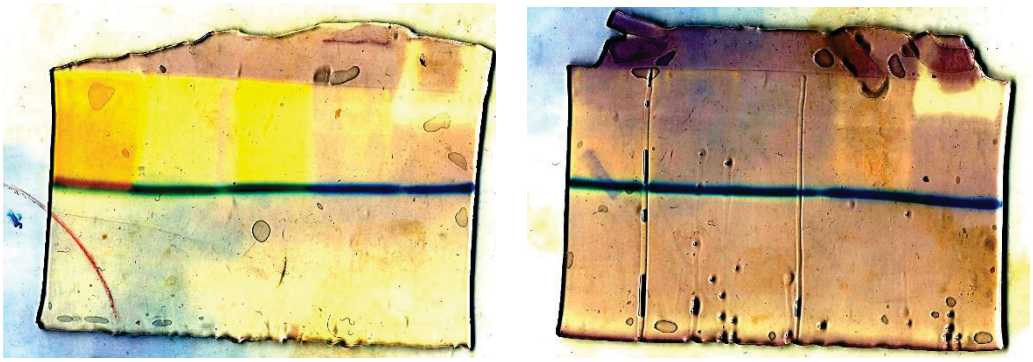
This way, it was shown that larger scale processes can be more economic, but small scale processes are advisable when small volumes of a more active product are desired. As only minute amounts of enzymatic solution were needed in order to perform the zymograms, treatment F was chosen for further experiments. It consisted

of the following steps: 1- centrifugation at 10.000 rpm for 15 minutes, in order to decant biomass; 2- precipitation of lignin sulfonate by adding CaOH; 3- centrifugation at 10.000 rpm, for 15 minutes in order to decant the precipitated lignin sulfonate; 4- dialyzing of the supernatant with 10kD membranes, using 50mM potassium phosphate buffer, ph7.5, to adjust pH, concentrate the desired proteins and remove impurities, such as salts and small molecules present in lignin sulfonate.

5.6.4.4 Zymograms

The obtained zymograms show the importance of the previously described sample purification steps. Lignin sulfonate makes this assay impossible. Its multiple molecular weights components make a smear in protein electrophoresis gels (FIGURE 34, left). After processing as described in section 4.6.4.2 (protocol F), all samples exhibited some SOD activity bands in the gel (FIGURE 34, right).

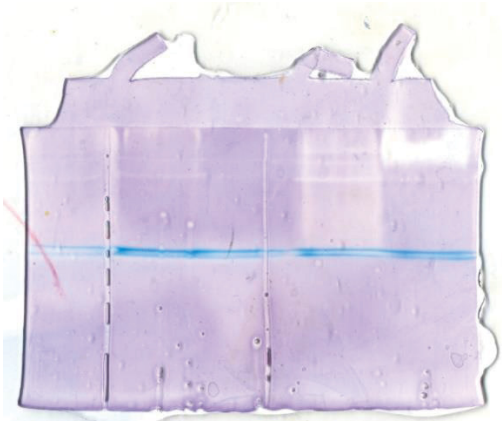
FIGURE 34 – FIRST GEL, SHOWING THE INTERFERENCE OF LIGNIN SULFONATE IN THE ANALYSIS AND SECOND GEL, SHOWING SAMPLES AFTER PRECIPITATION AND CONCENTRATION. SUBTLE ACTIVITY BANDS APPEARED AFTER THE DESCRIBED SAMPLE PROCESSING.



SOURCE: the author (2014).

Three distinct bands can be seen in all samples, but stronger for PO strain (FIGURE 35). These experiments results suggest that the assayed species of the *Pleurotus* genus present three different SOD isoforms, and no polymorphism among their superoxide dismutase enzymatic profiles. The discovery of new isoforms could lead to a path of artificial improvement of mushroom strains expressing more active SOD enzymes, through recombinant DNA methods.

FIGURE 35 - GEL SHOWING BANDS OF SOD ACTIVITY FOR THE FOLLOWING STRAINS: PD, PE, PP AND PO. THE LAST LANE AT RIGHT IS THE SOD STANDARD.



SOURCE: the author (2014).

5.7 AUTOMATION AND CONTROL OF BIOPROCESSES USING FREE HARDWARE AND SOFTWARE

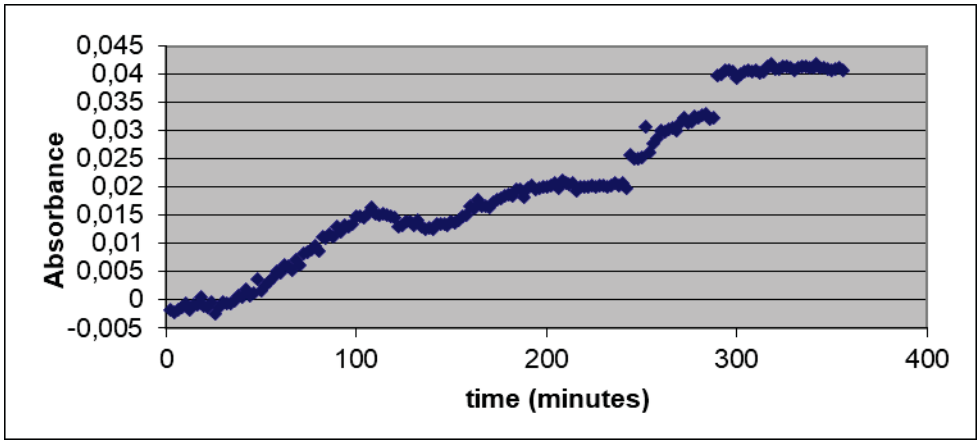
All examples described were successfully implemented and worked reliably for at least ten days, continuously. Countless other systems were built within concepts here outlined. The learning curve both for Pure Data and Arduino is very fast if compared with other microcontrollers and programming languages. This work environment provides extreme flexibility, allowing the development of fully customizable applications in short periods of time and at relatively low costs. The main difficulties were system instability and interface limitations. Although interface limitations do not represent central concerns in prototyping, these can be probably solved with Flash interfaces that communicate with PD through Flashserver. Some stability problems were found to be related with antivirus software's mechanisms and automatic operational system actualizations.

With proper hardware and software configuration, PD programs involving Arduino ran for many days without crashing, dealing with large amounts of data and showing almost unnoticeable delay.

The heterogeneity of the culture broths caused noticeable error in the laser absorption system. For yeast, some of the cells are naturally decanted and some flotate, resulting in underestimation of cell multiplication, especially in static non-aerated reactors. Agitation for sample collection reduces the problem, but does not solve it completely. However, the absorbance readings of the suspended phase are still useful for monitoring and control of the process.

GRAPHIC 37 shows the variation of turbidity read by an optical density measuring system based in laser absorption, during the cultivation of brewer's yeast (*Saccharomyces cerevisiae*).

GRAPHIC 37 – KINETIC OF ABSORBANCE OF YEAST SUSPENSION ALONG CULTIVATION PERIOD.



SOURCE: the author (2014).

For macromycetes mycelia, the approach of analog absorbance reading could not be applied due to the very heterogeneous nature of the broth (opaque pellets surrounded by limpid culture medium). In order to solve this issue, series of readings and mathematic processing should be tested.

For big or opaque bioreactors, as well as for opaque culture broths, it was necessary to shorten the optical path for the absorbance readings in order to adequate the system's sensitivity to the measured object. The inclusion of a separate cell allowed readings to be within the equipment's sensitivity range. Nevertheless, some inherent problems aroused and some design adaptions were necessary. With the first design tried, some yeast cells decanted inside the reading cell and connection pipes and it was impossible to apply the system to macromycetes mycelia, because the pellets were too large to be pumped through the connection pipes. A reading cell washing step was included by allowing samples to flow through the reading cell for some seconds before absorbance measuring. This helped to eliminate some interference caused by the sedimentation of biomass on the sampling pipes and reading cell.

All Pure Data example patches developed for this thesis are freely available at http://puredata.info/Members/chicobiotec.

6 CONCLUSIONS

The main objective of this thesis was accomplished as processes for producing antioxidant substances by the cultivation of macromycetes' mycelia using organic residues as substrate were successfully developed. Secondary objectives were also fully accomplished, as new protocols for cryopreservation and DNA extraction from mycelial cultures, as well as new processes for edible mushrooms and soil fertilizers production resulted from this work; free hardware and software utilization was explored for prototyping and building systems for bioprocesses automation and control. This resulted in the assembling of a prototype system for online absorbance measuring of bioreactors cultivation broths, which, among other applications, can monitor biomass growth and control continuous or fed-batch systems.

Experiments showed that at least a cryoprotectant substance or a carrier material is necessary for mycelial preservation at low temperatures. Vermiculite can be used as an efficient carrier material for the preservation of viable *Agaricus subrufescens* mycelia. The described methods are practical and proven efficient for the tested period of storage. Some of these methods are relatively affordable and can be useful to mycologists, spawn producers and germplasm banks. It is expected that the described techniques can be successfully applied to other fungi species.

Pejibaye palm sheath residues were proven as an efficient substrate for the cultivation of *Pleurotus* spp. carpophores. Preliminary results show that interesting variations in the nutritional content of fruiting bodies can be obtained by manipulating simple process parameters, such as inoculation rate and number of holes.

Mushroom spent substrate showed reasonable results as soil fertilizer. However, further experiments, composting this material before its utilization as fertilizer should be carried in order to make nutrients more available to the plants.

Methodologies were developed to induce the production of two antioxidant enzymes: CAT and SOD, by fungi of the *Pleurotus* genus, in submerged culture. Methods to evaluate enzymatic activity and zymography were adapted.

Several automation and control systems, useful for biotechnological processes were assembled using Pure Data programming language and Arduino hardware. These included a photoperiod timer, a thermostatic device and an online optical density sensor for bioreactors, based in laser absorption. The described techniques should be useful for a wide range of other automation and control applications.

7 PERSPECTIVES

The experimental results here presented point many interesting paths for future investigations. It was possible to draw initial steps in this vast and important research field. It is expected that the obtained results contribute to the development of science and economy, instigating new questions and offering technical bases for future research. Much stills need to be researched about native mushrooms in Brazil.

The cultivation techniques here presented should be adapted for the utilization of other agro-industrial residues as substrate. Substrates should be screened for obtaining the most active antioxidant products possible. In addition, other fungi strains should be screened with the same methodologies, for the development of better enzymes, aiming industrial and commercial applications.

The logical step after the success with the zymograms would be to recover protein bands for sequencing. The obtained sequences can be useful for comparing the proteic structure with one another and with other previously studied enzymes, allowing phylogenetic approaches and technological applications, such as the amplification of the respective genes and transformation of host organisms for the expression of the desired enzymes in great quantities. Products such as food, cosmetics and pharmaceuticals can be developed with these active molecules. In addition, these enzymes can be modified and expressed in other macromycetes, aiming the extension of mushrooms shelf-life.

This is only the beginning of a technological path, which can lead to multiple benefits to human life, including in aspects such as environment, economy and health.

A growing number of researchers is currently involved with the advance of mycotechnology in all regions of our country. Due to the amplitude of the theme, a crescent specialization is being required to cover the diverse related topics. This multidisciplinary and complementary nature will require an integration only possible with the current informatics and communications tools.

A great and unknown molecular universe is being unveiled. There is a great expectative that new substances for curing diseases yet incurable and molecules that contribute to the creation of new processes and products will be discovered.

Data originated from genomics, proteomics and metabolomics will be integrated in accurate models, reasonably approximate to the biochemical phenomena that

constitute the macromycetes physiology, in an area denominated fluxomics. These models will alow the simulation of bioprocesses in a resolution each time more approximate to the real functioning of fungi cells, helping the design of installations, equipments, substrates formulation, strains selection and genetic engineering.

Genetic manipulation techniques will allow the modification of these organisms for obtaining strains with engineered properties, aiming diverse applications. Highly aggressive, fast and productive mushrooms strains will be used as expression vectors for interest genes, allowing the conversion of a wide range of renewable organic materials and even residues, in useful and highly valued substances, such as pharmaceuticals, cosmetics, food and food additives, biomaterials and enzymes (for many applications, such as biopulping, effluent treatment and biofuels production).

Even non-cultivable organisms genes can be cloned and expressed, using metagenomic techniques. Not only these new techniques will allow the expression of proteins originated from any organism in any other, but will also permit the rational modification of these molecules, in order to create improved versions of these molecules, previously inexistent in nature. This will lead to the creation of new and more active pharmaceuticals, more efficient industrial enzymes and biomaterials with designed physical-chemical characteristics, for example; in an area that can be called biomolecules rational design.

Electronics can become as accessible to common people as informatics is nowadays. It depends on technological evolution and information diffusion. With this improved accessibility, general view over automation tools can change. Machines would evoke positive concepts as freedom, creativity, flexibility and customization instead of the classic symbols associated with them: restriction, standardization, repetition, hermetism and impersonality. This thesis can be viewed as a sample of this accessibility improvement process, which is already happening at some level.

One idea that can be extracted from this study is that simplicity and flexibility are as important as power and stability for creative tools. Both Pure Data and Arduino show a good balance of these factors. Programming techniques described in this thesis allow control of Arduino outputs over time and according to sensors data with great flexibility. It was also shown how to use Pure Data to register data collected by sensors and output it graphically.This technology was developed aiming the control of bioreactors for the submerged mycelial cultivation of macromycetes in

order to produce antioxidant substances. Notwithstanding, similar techniques can be applied for other cell types, such as bacteria, yeast or even vegetal and animal cells.

There is no doubt that fungiculture will play a crucial role in systems compromised with sustainability. Their ability to degrade organic matter will be useful for effluents detoxification and residues recycling. Without technological control over these organisms, an accumulation of organic effluents and residues is likely to occur, and consequently a spontaneous proliferation of other organisms, including disease vector insects and pathogenic species of microorganisms, converting this material in undesirable products, such as toxins and pollutants.

More than a strategic field from the economical point of view, the technological study of macromycetes may constitute a technical requirement, needed for the development of a productive model that allow the long-term survival of humanity.

It is hoped that this thesis can inspire interesting future developments in the bioprocesses engineering and biotechnology area.

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