UNIVERSIDAD DE VALLADOLID

ESCUELA DE INGENIERIAS INDUSTRIALES

Máster en Ingeniería Química

PRODUCCIÓN DE ENZIMAS DE HONGOS MEDICINALES

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TUTOR:	Full Prof. Dr. Maja Leitgeb, univ. dipl. inž. kem. tehnol.

Resumen

El propósito de esta tesis fue determinar la capacidad de ciertos organismos del reino Fungi para producir una serie de enzimas en su forma activa. En este estudio se utilizaron Pleurotus ostreatus y Ganoderma lucidum.

Para el hongo P. ostreatus, se observó que la mayor concentración de proteína total se obtuvo con 8 minutos de homogeneización (0,8607 mg/mL, y agua destilada). Utilizando el procedimiento de agitación, las actividades enzimáticas más altas se alcanzaron para la α-amilasa (24 h, 8,0413 U/mL, y tampón de citrato de sodio) y la proteasa (3 h, 0,0040 U/mL, y tampón de citrato de sodio).

Para G. lucidum, se observó que la mayor concentración de proteína total se obtuvo con 4 min de homogeneización (0,0338 mg/mL). Además, utilizando el proceso de homogeneización, se obtuvieron las mayores actividades para la α-amilasa (4 min, 16,3459 U/mL) y la SOD (4 min, 9,2615 U/mL).

Palabras clave: hongos medicinales, *Pleurotus ostreatus*, *Ganoderma lucidum*, proteínas totales, actividades enzimáticas.

Abstract

The purpose of this thesis was to determine the ability of certain organisms of the Fungi kingdom to produce a series of enzymes in their active form. *Pleurotus ostreatus* and *Ganoderma lucidum* were used in this study.

For the *P. ostreatus* fungus, it was observed that the highest concentration of total protein was obtained with 8 minutes of homogenization (0.8607 mg/mL, and distilled water). Using the shaking procedure, the highest enzyme activities were achieved for α-amylase (24 h, 8.0413 U/mL, and sodium citrate buffer) and protease (3 h, 0.0040 U/mL, and sodium citrate buffer).

For *G. lucidum*, it was observed that the highest total protein concentration was obtained with 4 min of homogenization (0.0338 mg/mL). In addition, using the homogenization process, the highest activities for α-amylase (4 min, 16.3459 U/mL) and SOD (4 min, 9.2615 U/mL) were obtained.

Keywords: medicinal mushrooms, *Pleurotus ostreatus*, *Ganoderma lucidum*, total proteins, enzymes activities.

Univerza v Mariboru

Fakulteta za kemijo
in kemijsko tehnologijo

Gonzalo Herranz Gómez

Production of enzymes from medicinal mushrooms

Master's thesis

Maribor, June 2022

Univerza v Mariboru

Fakulteta za kemijo
in kemijsko tehnologijo

Production of enzymes from medicinal mushrooms

Master's thesis

Student:	Gonzalo Herranz Gómez
Study program:	Master's study program II. degree in Chemical Engineering
Intended professional title:	Master of Chemical Engineering
Mentor:	Full Prof. Dr. Maja Leitgeb, univ. dipl. inž. kem. tehnol.
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Maribor, 2022

University of Maribor

Faculty of Chemistry and Chemical Engineering

Place and date: MARIBOR, 13.06.2022

On the basis of Article 330 of the Statute of the University of Maribor (Official Gazette of the Republic of Slovenia, No. 41/2021-UPB13), I issue the following

DECISION ON FINAL WORK

GONZALO HERRANZ GÓMEZ, student of 2nd cycle MS study programme Izmenjava MAG, has satisfied all requirements and is allowed to compose the final work.

The topic of the final work is predominantly from the field of the chair/department/institute: KATEDRA ZA KEMIJSKO TEHNOLOGIJO.

Supervisors:

red. prof. dr. MAJA LEITGEB, univ. dipl. inž. kem. tehnol., supervisor
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Title of final work:
Proizvodnja encimov iz zdravilnih gob

Title of final work in English:
Production of enzymes from medicinal mushrooms

The deadline for submission of the final work is 13.06.2023. Final work shall be prepared in line with requirements of the guidelines: *Predloge z navodili za pisanje zaključnega dela* and submitted at the Student Affairs Office. Number of copies: 0. At the same time, a statement from the supervisor is submitted (and possible co-supervisor) on final work adequacy .

Legal precept: an appeal against this decision is possible to the Senate of the faculty within 10 business days from date of receipt of the decision.

UNIVERZA V MARIBORU FAKULTETA ZA KEMIJO IN KEMIJSKO TEHNOLOGIJO 3

red. prof. dr. Zdravko Kravanja, Dean

To be notified:
- candidate,
- supervisor,
- co-supervisor,
- archive.

Univerza v Mariboru

Fakulteta za kemijo in kemijsko tehnologijo

Kraj in datum: MARIBOR, 13.06.2022

Na osnovi 330. člena Statuta Univerze v Mariboru (Uradni list RS, št. 41/2021-UPB13) izdajam

SKLEP O ZAKLJUČNEM DELU

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Tema zaključnega dela je pretežno s področja katedre/oddelka/inštituta: KATEDRA ZA KEMIJSKO TEHNOLOGIJO.

Mentorji:

red. prof. dr. MAJA LEITGEB, univ. dipl. inž. kem. tehnol., mentor/-ica
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Naslov zaključnega dela:
Proizvodnja encimov iz zdravilnih gob

Naslov zaključnega dela v angleškem jeziku:
Production of enzymes from medicinal mushrooms

Rok za izdelavo in oddajo zaključnega dela je 13.06.2023. Zaključno delo je potrebno izdelati skladno z navodili: *Predloge z navodili za pisanje zaključnega dela*, in ga oddati v pristojnem referatu članice. Število izvodov: 0. Hkrati se odda tudi izjava mentorja/-ice (in morebitne/-ga somentorja/-ice) o ustreznosti zaključnega dela.

Pravni pouk: Zoper ta sklep je možna pritožba na Senat članice v roku 10 delovnih dni od dneva prejema sklepa.

UNIVERZA V MARIBORU FAKULTETA ZA KEMIJO IN KEMIJSKO TEHNOLOGIJO 3

red. prof. dr. Zdravko Kravanja, dekan

Obvestiti:
- kandidata/-ko,
- mentorja/-ico,
- somentorja/-ico,
- arhiv.

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Declaration

I declare that I have written this thesis by myself. Any contributions made by others are indicated separately. I have reviewed the literature in the field of my thesis using the following keywords:

Source: Scopus (https://www.scopus.com)

Keywords:	**Number of references**
Pleurotus ostreatus	4284
Pleurotus ostreatus AND enzymes	1235
Ganoderma lucidum	4221
Ganoderma lucidum AND enzymes	905

Source: ScienceDirect (https://www.sciencedirect.com)

Keywords:	**Number of references**
Pleurotus ostreatus	5585
Ganoderma lucidum	4744
Fungal enzyme extraction	49145

Total number of reviewed articles: 53
Total number of books reviewed: 7

Maribor, June 2022 Gonzalo Herranz Gómez

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And, of course, thanks to Maribor and Slovenia. This Erasmus will always represent one of the most important experiences of my life, and I will always be grateful to all the people I have met here.

Production of enzymes from medicinal mushrooms

Abstract

The purpose of this master's thesis was to determine the ability of certain organisms of the kingdom Fungi to produce a series of enzymes in their active form by solid-state fermentation. In this study, two types of fungi were used, *Pleurotus ostreatus* and *Ganoderma lucidum*. For this purpose, different growth media, cultivation times (8 and 10 days), extraction procedures (shaking and homogenization) and extraction medium (distilled water, sodium citrate buffer and sodium phosphate buffer) were used.

First, for *P. ostreatus* mushroom, the optimization of the extraction procedure and time for isolation of enzymes in their active form (α-amylase, glucoamylase, cellulase, laccase, and protease) was studied. It was observed that the highest total protein concentration in mycelium extract was obtained by 8 min of homogenization (0.8607 mg/mL, and distilled water). Using the shaking procedure, the highest enzyme activities were achieved for α-amylase (24 h, 8.0413 U/mL, and sodium citrate buffer) and protease (3 h, 0.0040 U/mL, and sodium citrate buffer). With the homogenization process, the highest activities were achieved for the enzymes glucoamylase (10 min, 6.7113 U/mL, and sodium citrate buffer) and laccase (8 min, 12.2500 U/mL, and sodium citate buffer).

For the mushroom *G. lucidum*, the growth medium and the extraction procedure were optimized, using the same extraction medium (sodium citrate buffer). In this case, α-amylase, glucoamylase, cellulase, laccase, protease, catalase, peroxidase, superoxidase dismutase (SOD), and lipase were studied. It was observed that the highest total protein concentration was obtained with 4 min of homogenization (0.0338 mg/mL). Furthermore, using the homogenization process, the highest activities were achieved for α-amylase (4 min, 16.3459 U/mL) and SOD (4 min, 9.2615 U/mL). With the shaking procedure, the highest activities were achieved for cellulase (3 h, 1.6332 U/mL), lipase (3 h, 16.924 U/mL), glucoamylase (3 h, 14.6737 U/mL), peroxidase (3 h, 0.0156 U/mL), protease (3 h, 0.0080 U/mL) and laccase (24 h, 20.7083 U/mL).

Keywords: medicinal mushrooms, *Pleurotus ostreatus*, *Ganoderma lucidum*, total proteins, enzymes activities.

UDK: 604.4:577.15(043.2)

Proizvodnja encimov iz zdravilnih gob

Povzetek

Namen magistrske naloge je bil ugotoviti sposobnost določenih organizmov iz kraljestva glive, da lahko s fermentacijo v trdnem stanju proizvajajo vrsto encimov v aktivni obliki. V tej študiji sta bili uporabljeni dve vrsti gliv, *Pleurotus ostreatus* in *Ganoderma lucidum*. V ta namen so bili uporabljeni različni rastni mediji, časi gojenja (8 in 10 dni), postopki ekstrakcije (stresanje in homogenizacija) in ekstrakcijski medij (destilirana voda, natrijev citratni pufer in natrijev fosfatni pufer).

Najprej smo za gobo *P. ostreatus* optimizirali postopek ekstrakcije in časa za izolacijo encimov v njihovi aktivni obliki (α-amilaza, glukoamilaza, celulaza, lakaza in proteaza). Ugotovljeno je bilo, da je bila najvišja skupna koncentracija proteinov v ekstraktu micelija dosežena z 8-minutno homogenizacijo (0,8607 mg/mL in destilirana voda). S postopkom stresanja so bile najvišje encimske aktivnosti dosežene za α-amilazo (24 h, 8,0413 U/mL in natrijev citratni pufer) in proteazo (3 h, 0,0040 U/mL in natrijev citratni pufer). S postopkom homogenizacije so bile največje aktivnosti dosežene za encim glukoamilaza (10 min, 6,7113 U/mL in natrijev citratni pufer) in lakaza (8 min, 12,2500 U/mL in natrijev citatni pufer).

Za gobo *G. lucidum* smo rastni medij in postopek ekstrakcije optimizirali z uporabo istega ekstrakcijskega medija (natrijev citratni pufer). V tem primeru smo preučevali α-amilazo, glukoamilazo, celulazo, lakazo, proteazo, katalazo, peroksidazo, superoksid dismutazo (SOD) in lipazo. Ugotovljeno je bilo, da je bila najvišja skupna koncentracija proteinov dosežena s 4 min homogenizacije (0,0338 mg/mL). Poleg tega so bile s postopkom homogenizacije najvišje aktivnosti dosežene za α-amilazo (4 min, 16,3459 U/mL) in SOD (4 min, 9,2615 U/mL). S postopkom stresanja so bile največje aktivnosti dosežene za celulazo (3 h, 1,6332 U/mL), lipazo (3 h, 16,924 U/mL), glukoamilazo (3 h, 14,6737 U/mL), peroksidazo (3 h, 0,0156 U/mL), proteazo (3 h, 0,0080 U/mL) in lakazo (24 h, 20,7083 U/mL).

Ključne besede: zdravilne gobe, *Pleurotus ostreatus*, *Ganoderma lucidum*, skupne beljakovine, encimske aktivnosti

UDK: 604.4:577.15(043.2)

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Symbols and Abbreviations

Symbols

A	absorbance of the sample measured at a given wavelength (/)
ΔA	change in absorbance in the sample between start and end time (/).
c	protein concentration in the sample (Bradford) (mg/mL)
c_g	maltose released concentration (mg/mL).
c_m	glucose released concentration (mg/mL).
df	dilution factor (/)
f	conversion factor (/)
gds	gram of dry substrate
k	slope from the calibration curve (mL/mg)
K	millimolar extinction coefficient (/).
M	molar mass (g/mol)
t	reaction time (min)
Tucas	proteolytic activity (μL)
U	enzyme activity (U/mL)
V	reaction volume (mL)
V_r	reaction volume of the mixture (mL)
V_v	Sample volume (mL)

Greek symbols

ε	extinction coefficient (/)
μ	micro

Abbreviations

ADP	adenosine diphosphate
ºC	Celsius degrees
CAGR	compound annual growth rate
h	hours
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
JCBN	Joint Commission on Biochemical Nomenclature
L	liters
min	minutes
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
PDA	potato dextrose agar
Pi	pyruvate kinase
pH	potential of hydrogen

rpm	revolutions per minute
SMC	spent mushroom compost
SOD	superoxidase dismutase
SSF	solid state fermentation
T	temperature

Chemical compounds

4-APP	4-aminoantipyrine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
DNS	3,5-dinitrosalicylic acid
EDTA	ethylenediaminetetraacetic acid
PBS	potassium phosphate buffer
PNPB	p-nitrophenyl butyrate
TCA	trichloroacetic acid
$CaCl_2$	calcium chloride
$C_6H_8O_7$	citric acid
$FeSO_4 \cdot 6H_2O$	ferrohexahydrite
HCl	hydrochloric acid
KH_2PO_4	potassium dihydrogen phosphate
K_2PO_4	dipotassium hydrogen phosphate
NaOH	sodium hydroxide
Na_2HPO_4	disodium hydrogen phosphate
$NaH_2PO_4 \cdot H_2O$	sodium phosphate monobasic monohydrate
$Na_2HPO_4 \cdot 7H_2O$	sodium phosphate dibasic heptahydrate
$MgSO_4 \cdot 7\ H_2O$	magnesium sulphate heptahydrate
$MnSO_4 \cdot H_2O$	manganese sulphate monohydrate

1 Introduction

1.1 Problem definition

Enzymes are a group of complex proteinoid organic compounds which play an important role in many biological reactions as they accelerate them by decreasing their activation energy. Compared to conventional chemical methods, enzymatic processes have several advantages, such as high selectivity, specificity, and the reduction of by-products and waste during the reaction, which contributes to the terms of green engineering and sustainable development (de Souza et al., 2020). Enzymes can be found in virtually all forms of living organisms, such as animals, plants, or microbial sources. Plant and animal sources are insufficient to meet the large demand, so the focus has turned to microbial sources (El-Gendi et al., 2021). Among these sources, fungi stand out and represent an interesting source of industrial enzymes due to their high production potential at large-scale, easy cultivation, separation and purification, and simultaneous biomass production (Bala and Singh, 2019). Currently, fungal enzymes represent more than 50% of the total enzyme market (Kango et al., 2019). Consequently, the present situation favours the need for the large-scale production of various fungal enzymes.

In this context, the possibility of extracting certain types of enzymes from two mushroom species, *P. ostreatus* and *G. Lucidum*, has been studied by solid state fermentation (SSF). Among the wide variety of enzymes studied, some of the most important are, for example, the superoxidase dismutase (SOD), which is one of the most potent endogenous antioxidants in the human body, and the laccase, which has the potential to polymerizate natural phenols to form compounds useful for textile decolorization, treatment of dye effluents from the textile industry, and to remove a broad range of antibiotics effectively (Manavalan et al., 2013).

1.2 Purpose, hypotheses, and goals

The purpose of this research was to isolate different enzymes in their active form from different types of fungi – medicinal mushrooms *P. ostreatus* and *G. lucidum*. Optimization of the extraction technique was performed to isolate the enzymes in their active form. The influence of different extraction techniques, the time of extraction, and the choice of extraction medium on the production of proteins and enzymes was studied. The high content of isolated proteins and enzymes in their active form could positively contribute to their use in various industries, such as food, pharmaceutical, and cosmetic industries.

2 Theoretical part

2.1 Medicinal mushrooms

Certain edible fungi can produce enzyme complexes that degrade cellulose, lignocellulose, and starch, which are applied in numerous food, textile, paper, and biofuel industries. The cultivation of these fungi can be transformed into an ecological bioconversion process by using agro-industrial waste as a substrate. In addition, the revaluation of these wastes can reduce their impact on the environment (Colunga et al., 2021).

2.1.1 *Pleurotus ostreatus*

P. ostreatus is a saprophytic lignicolous fungus, commonly known as oyster mushroom (Figure 2-1). It belongs to the class *Basidiomycetes*, order *Agaricales*, and family *Agaricacea* (Furci, 2007). *P. ostreatus* possesses specific enzymes capable of degrading lignin, phenols, and polyphenols up to 60% of the original content, making it one of the most widely used species in research on residues suitable for cultivation (Varnero et al., 2009).

Figure 2-1. *P. ostreatus.* Source:(Sarmiento, 2022).

Several authors have demonstrated the ability of this group of fungi to develop on a wide variety of lignocellulosic residues by solid-phase fermentation, characterizing the stages of mycelial growth and carpophore production. In a study Lim et al., efficiently performed extraction of lignocellulolytic enzymes amylase, cellulase, laccase, and xylanase from spent mushroom compost (SMC) of *P. ostreatus*, *P. eryngii*, and *P. cornucopiae*. They achieved an optimal enzyme recovery with 50 mM sodium citrate (pH 4.5) buffer at 4 ºC for 2 h extraction procedure. Each sample was filtered through miracloth (pore size: 22~25 μm) and then centrifuged at 10,000×g at 4 ºC for 15 min. The supernatant, which constituted the crude enzyme extract, was assayed. In this way, amylase, cellulase, and xylanase activity values of 2.97 U/g, 1.67 U/g, and 91.56 U/g were obtained for *P.ostreatus*, respectively (Lim et al., 2013). Melanouri et al. performed *P. ostreatus* enzyme's extraction from 2 g of the lyophilized fermented substrate of each sample with 20 mL phosphate buffer (0.05 M, pH 5.0) in 100 mL Erlenmeyer flasks, with an agitation of 120 ± 5 rpm for 1 h at 20-22 ºC. The absorbances were measured spectrophotometrically at 525 nm, obtaining 1987.25 U/g of laccase (Melanouri et al., 2022). In another research, the activities of different enzymes from *P. ostreatus* were studied using SSF and wheat bran as solid phase. The extraction was performed with distilled

water (20 mL) with 15 min of agitation followed by centrifugation at 6000 rpm for 20 min to obtain the supernatant. Using specific enzymatic assays, laccase and peroxidase activity values reached 9210 and 20 U/L, respectively (Elisashvili et al., 2003; Kumla et al., 2020).

2.1.2 *Ganoderma lucidum*

G. lucidum (Figure 2-2) is a macromycete fungus recognized for its medicinal properties and its content of bioactive compounds, including polysaccharides, triterpenoids, and immunomodulatory proteins, among others, which has generated a remarkable increase in its production (Montoya et al., 2018). It belongs to the class *Agaricomycetes*, order *Polyporales*, and family *Ganodermataceae* (Loyd et al., 2018).

Figure 2-2. *G. lucidum*. Source: (Kamado, 2021)

This genus has a great potential to produce widely used enzymes in the pharmaceutical and food industries. Yuliana et al., studied the potential of *G. lucidum* to produce enzyme laccase using substrate comprising corncob and rice straw under submerged fermentation conditions by incubation in the dark for 14 days at 30 ºC in an incubator shaker at 100 rpm. Extraction was performed by protein precipitation with saturated ammonium sulphate (80%), cooling at 4 ºC for 2 h, followed by centrifugation at 10,000×g for 15 min, obtaining the highest activity of 68.75 U/mL (Yuliana et al., 2020). Sharma et al. conducted a study in which production of laccase under SSF was performed in 250 ml Erlenmeyer flasks containing dry wheat bran (5.0 g) moistened with mineral salt solution and pH 5.4. The inoculum consisted of 10% v/w of 9-day-old crushed fungal of *G. lucidum* and incubated at 30 ºC. Extraction was carried out with 50 mL of citrate phosphate buffer (100 mM, pH 5.4) and centrifugated at 10,000 rpm at 4 ºC for 10 min, achieving a maximum laccase activity of 2989 U/g (Sharma et al., 2019).

2.1.3 Spent mushroom compost (SMC)

In 2019, the ten Member Countries of the European Mushroom Growers' Group (EMGG) accounted for 90% of the overall European production of mushrooms (Figure 2-3), which amounted to 1,095,000 tons (EMGG, 2019). SMC is a kind of waste material produced by mushroom production farms after the mushroom harvesting period. It is a mixture of mushroom mycelium, rubber sawdust, rice husk, and calcium carbonate (Kamarudzaman et al., 2022). In general, for every kilogram of mushrooms, 5 kg of SMC are produced (Williams et al., 2001), implying that mushroom farms dispose of approximately 24 tons of compost each month on average (Singh et al., 2003), resulting in approximately 5,475,000 tons of SMC in

Europe in 2019. In addition, the current processing cost of SMC in Europe is between 10 and 50 EUR/ton, creating an enormous financial burden of up to 15 billion EUR/year for the mushroom industry (Beckers et al., 2019).

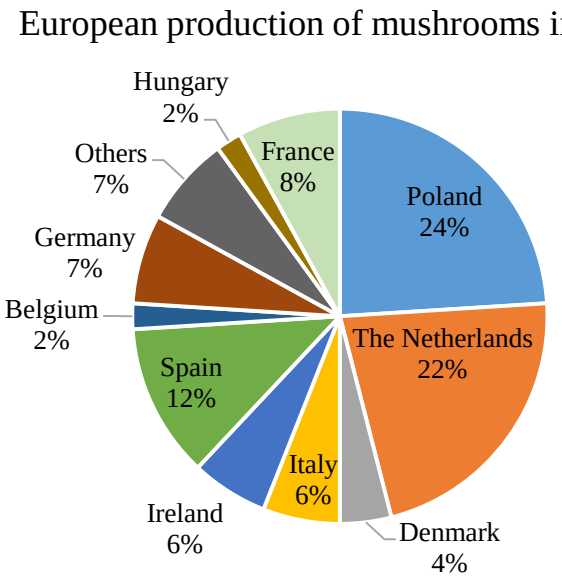

Figure 2-3. Mushrooms production of the member countries of the EMGG. Adapted from: (EMGG, 2019)

For both mushroom farmers and the environment, disposal of unused mushroom compost is a significant problem, therefore one approach is to increase demand by researching new alternatives for recycling and re-utilization. SMC can be either discarded or used for the production of value-added products and different purposes, such as biogas production (Pérez-Chávez et al., 2019), bioconversion to organic fertilizers (Jasińska, 2018), biosorbent of heavy metals, pesticides and dyes (Álvarez-Martín et al., 2017), as animal feed supplement (Chang et al., 2016), pentachlorophenol degradation (Singh et al., 2003) and bulk enzyme production (Corrêa et al., 2016; Phan and Sabaratnam, 2012), especially cellulases, hemicellulases, and lignin-degrading enzymes (Mayolo-Deloisa et al., 2009).

2.2 Enzymes

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), in consultation with the IUPAC-IUBMB (International Union of Pure and Applied Chemistry- International Union of Biochemistry and Molecular Biology) and the Joint Commission on Biochemical Nomenclature (JCBN) (NC-IUBMB, 2022), enzymes can be classified into seven groups (EC 1 to 7): oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases (Table 2-1, 2-2, 2-3).

Table 2-1. General classification of enzymes with examples of enzymes produced by fungal organisms and different applications.

Group	Description	Reaction	Enzymes produced by fungi	Applications	Reference
Oxidoreductases (EC 1)	Electron or proton transfer reactions (redox reactions)	$AH_2 + B \rightarrow A + BH_2$	Laccase	Textile industry (degradation of dyes), pulp industry (biopulping), soil bioremediation, degradation of herbicides and pesticides	(Guimarães et al., 2017) (Sharma et al., 2014)
			Peroxidase	Pharmaceutical industry (preparation of ELISA kits), textile industry (degradation of various dyes), and paper industry (degradation of wood components)	(Singh et al., 2019)
Transferases (EC 2)	Transfer of a functional group from one substance to another	$A - X + B \rightarrow A + X - B$	Fructosyltransferase	Catalyzes the transformation of sucrose into fructooligosaccharides	(El-Gendi et al., 2022)

Table 2-2. (Continued) General classification of enzymes with examples of enzymes produced by fungal organisms and different applications.

Group	Description	Reaction	Enzymes produced by fungi	Applications	Reference
Hydrolases (EC 3)	Catalyzes the hydrolysis reaction by using water	$A-B+H_2O \rightarrow A-OH+BH$	Protease	Food industry (meat tenderization and cheese preparation, fortification of fruit juices, and preparation of protein-rich diet), leather processing, wastewater treatment, and manufacturing of laundry detergents	(Adrio and Demain, 2014) (Amore and Faraco, 2015) (Białkowska et al., 2016)
			Lipase	Food industry (flavor enhancer), dairy industry (production of butter, fats, oils, etc.), and biodiesel production	(Amore and Faraco, 2015) (Białkowska et al., 2016)
			Amylase	Food, textile, detergent, paper, and pharmaceutical industries	(Mehta and Satyanarayana, 2016)
			Cellulase	Pulp and paper, detergent, textiles, and laundry, food and agricultural, and bioethanol production	(Abdel-Azeem et al., 2019) (Kour et al., 2019)

Table 2-3. (Continued) General classification of enzymes with examples of enzymes produced by fungal organisms and different applications.

Group	Description	Reaction	Enzymes produced by fungi	Applications	Reference
Lyases (EC 4)	Catalyzes the addition of groups to double bonds and the formation of double bonds by the removal of groups	$A = B \leftrightarrow A - B$	Pectin lyase	Textile industry, paper making, pectic wastewater pre-treatment, juice clarification, and oil extraction	(Yadav et al., 2009)
Isomerases (EC 5)	Catalyzes the transfer of groups within molecules to yield isomeric forms (isomeric reactions)	$A - B - C \leftrightarrow A - C - B$	Glucose/xylose isomerase	Food industry (production of high fructose corn syrup) and pharmaceutical industry (production of sucralfate)	(Nam, 2022)
Ligases (EC 6)	Catalyzes the condensation of two molecules with the expense of ATP hydrolysis	$A + B + ATP \leftrightarrow A - B + ADP + Pi$	tRNA ligase	Repair the RNA breaks in fungal cells	(El-Gendi et al., 2022)
Translocases (EC 7)	Catalyzes the movement of ions or molecules across membranes or their separation within membranes	/	ATP synthase	Anticancer activity, immunomodulation, wound healing, drug carriers, vaccine adjuvants, or innate defense regulator	(Ahmad et al., 2013) (Yeung et al., 2011)

ATP Adenosine triphosphate; Pi Pyruvate kinase

2.2.1 Enzyme production by fermentation

Currently, enzymes are mainly produced by submerged fermentation (Christopher et al., 2005). However, in recent years, the use of SSF to produce enzymes using residual cultures has gained much importance (Singhania et al., 2009). SSF is the technique that takes place in a solid matrix with practically no free water present. The main advantages of the SSF compared to submerged fermentation include higher product yields (Sharma et al., 2019), cost-effectiveness (Osma et al., 2011), and the use of undemanding and simple instruments (Szendefy et al., 2006), and moreover, the production of enzymes by SSF is mainly when the fermented substrate can be used directly for bioprocessing. Additionally, SSF with fungi for enzyme production is particularly advantageous as it mimics their habitat, thus giving higher product yields (Steudler and Bley, 2015). The following tables (Tables 2-4, 2-5) show different enzymes produced from fungal organisms from different substrates using SSF, as well as the activity of each enzyme and the optimal growth conditions.

Table 2-4. Fungal enzymes from different organisms, substrates, activity, and optimal growth conditions.

Fungal enzymes	**Organisms**	**Substrate**	**Enzyme activity**	**Optimized conditions**	**Reference**
Amylase	*Thermomyces lanuginosus*	Wheat bran, molasses bran, rice bran, and crushed wheat	534 U/mL	pH: 6.0 Incubation period: 120 h T:50 °C Inoculum: 10%	(Kunamneni et al., 2005)
	Aspergillus fumigates NTCC1222	Pomegranate peel, wheat bran	164.1 U/mL	pH: 6.0 Incubation period: 60 min T: 55 °C Inoculum:10%	(Singh et al., 2014)
Lipase	*Trichoderma harzianum*	Wheat bran supplemented with 2% olive oil	14.3 U/mL	pH: 8.03 Incubation period: 120 h T: 40 °C Inoculum:10%	(Toscano et al., 2013)
Protease	*Aspergillus tamarii*	Wheat bran	401.42 U/mL	pH: 8 Incubation period: 72 h T: 40 °C Inoculum: 10%	(da Silva et al., 2016)
	Aspergillus brasiliens BCW2	Wheat bran and 10% orange peel as carbon source	1734 U/mL	pH: 9.0 Incubation period: 72 h T: 30 °C Inoculum: 10% Moisture content: 60%	(Chimbekujwo et al., 2020)

Table 2-5. (Continue) Fungal enzymes from different organisms, substrates, activity, and optimal growth conditions.

Fungal enzymes	Organisms	Substrate	Enzyme activity	Optimized conditions	Reference
Cellulase	*Trichoderma reesei*	Fermentation medium containing municipal solid waste (wheat bran, wheat gluten, soybean meal)	20 U/mL	pH: 4.8 T: 30 °C Incubation period: 168 h Moisture content: 60% Inoculum: 10%	(Darabzadeh et al., 2019)
Laccase	*Ganoderma sp.*	Wheat bran	2400 U/gds	pH: 3 Incubation period: 7 days Temperature: 25 °C Inoculum: 10% Moisture content: 70%	(Revankar et al., 2007)
	Coriolopsis caperata RCK2011	Wheat bran induced with 0.5 mM xylidine	1623.55 U/gds	pH: 5.0 Incubation period: 5 days Temperature: 30 °C Inoculum: 10%	(Nandal et al., 2013)

U/gds: units per gram of dry substrate

In addition to the value-added products that can be generated by the degradation of lignocellulosic biomass, lignocellulosic enzymes as such are of great utility for different applications. Therefore, microbial enzyme production is becoming an essential part of the interests of the biotechnology industry worldwide due to its important uses in various industry sectors such as pharmaceutical, energy, and food, among others (Singh et al., 2016). The global market for the enzyme production industry is growing at tremendous speeds. It was estimated at $8.6 billion in 2019 and is projected to reach $14.5 billion in 2027 at a compound annual growth rate (CAGR) of 6.5% between 2020 and 2027 (Figure 2-4).

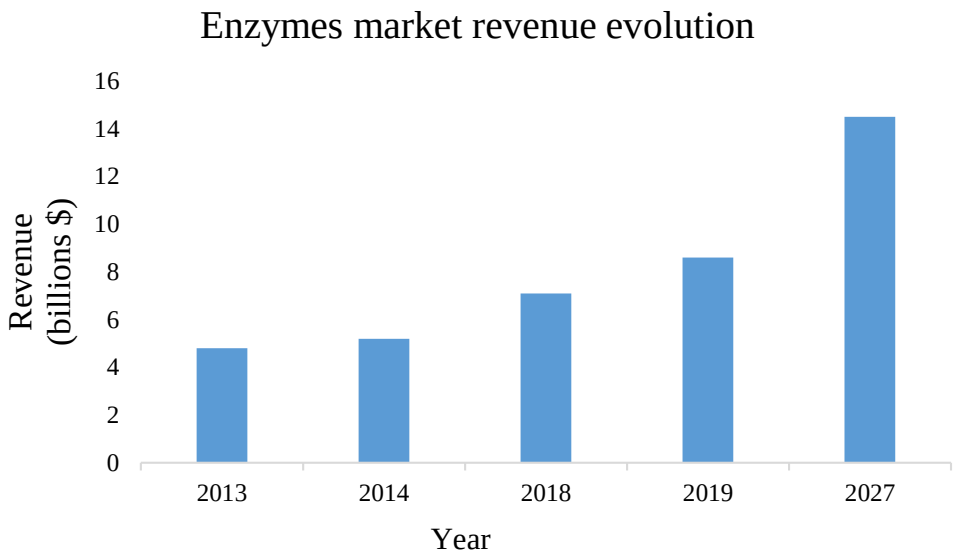

Figure 2-4. Enzymes market revenue evolution and projection. Source: (Snehal Manjrekar, Trupti Wadekar, 2021).

Therefore, the use of fungal organisms for enzyme production represents a promising alternative for the future due to their safety and economic viability.

3 Materials and methods

3.1 Materials and reagents

The laboratory material used was:

- Aluminum foil.
- Beaker.
- Cork borer.
- Cotton wool.
- Disposable pipettes.
- Erlenmeyer flasks.
- Falcon tubes.
- Graduated cylinder.
- Magnetic stir bar.
- Microcentrifuge tubes.
- Micropipettes.
- Petri dishes.
- Potato dextrose (glucose) agar (PDA).
- Reagent bottles.
- Safety glasses.
- Spatula.
- Test tubes.
- Thermometer.
- Tweezers.
- Volumetric flasks.
- Wash bottle.
- Watch glass.
- Wheat bran.

The reagents used were:

- 4-APP
- ABTS
- $C_6H_8O_7$
- $CaCl_2$
- Distilled water.
- DNS
- EDTA
- $FeSO_4 \cdot 6H_2O$
- HCl
- K_2PO_4
- KH_2PO_4
- $MgSO_4 \cdot 7H_2O$
- Milli-Q water
- $MnSO_4 \cdot H_2O$
- Na_2HPO_4
- $Na_2HPO_4 \cdot 7H_2O$
- $NaH_2PO_4 \cdot H_2O$
- NaOH
- PBS

- PNPB
- TCA

3.2 Laboratory equipment

The laboratory equipment used was:

- Autoclave (NÜVE).
- Bunsen burner.
- Centrifuge (Eppendorf 5804 R).
- Excella E24 Incubator Shaker Series (New Brunswick Scientific).
- Homogenizator (IKA® T 10 basic ULTRA-TURRAX®).
- Laminar chamber (Telstar AH-100).
- Spectrophotometer (Varian Cary 50 Probe).
- Stirring hot plate (Rotamix 550 MMH).

3.3 Inoculation procedure of mycelium

First, we prepared agar plates for the mycelium inoculation procedure. 19.5 g of PDA were dissolved in 500 mL of distilled water and sterilized in an autoclave (Figure 3-1) using moist heat sterilization. The necessary laboratory equipment, namely a cork borer, spatula, and tweezers, were sterilized using dry heat sterilization.

Figure 3-1. Autoclave for moist heat and dry heat sterilization.

Using pre-sterilized laboratory equipment, three holes were made in the prepared sterile PDA agar plates, into which three cuttings of mycelium from previous mycelial cultures were inoculated. This was performed in accordance with the aseptic technique, which means that

all the work was performed in the laminar chamber near the flame of a Bunsen burner. After inoculation, these agar plates were sealed and incubated for 9 days at 27 °C. After 9 days, the mycelium grew over the entire surface of the PDA agar, as shown in the Figure 3-2. Until later use, agar plates with fully grown mycelium were stored in the refrigerator at 4 °C.

Figure 3-2. Fully grown mycelium on the surface of PDA agar after 9 days of incubation at 27 °C.

3.4 Preparation of culture and growth medium

The production of various enzymes under SSF was performed in 250 mL Erlenmeyer flasks, containing 5.0 g of dry medium-size wheat bran (0.8 mm) moistened with 20 mL of different growth media.

The composition of the growth medium used for *P. ostreatus* can be seen in Table 3-1.

Table 3-1. Growth medium for *P. ostreatus*.

Growth medium	**Compound**	**Concentration (g/L)**
1-*P. ostreatus* medium	Yeast extract	2
	Pepton	5
	$MnSO_4 \cdot H_2O$	0.075
	KH_2PO_4	1
	$MgSO_4 \cdot 7\ H_2O$	0.5

In addition to the above-mentioned growth medium, three additional ones were used for *G. lucidum* (Table 3-2) in order to optimize mushroom growth to obtain the maximum amount of total proteins and enzymes in their active form.

Table 3-2. Different composition of each growth medium for the *G. lucidum* mycelium growth optimization.

Growth medium	Compound	Concentration (g/L)	References
2-Sucrose medium	Sucrose	35	(Fang and Zhong, 2002)
	Peptone	5	
	Yeast extract	5	
	K_2PO_4	0.8831	
	$MgSO_4 \cdot 7H_2O$	0.5	
3-Glucose medium	Glucose	49.2	(Hsu et al., 2017)
	Yeast extract	4.9	
	KH_2PO_4	0.88	
	$MgSO_4 \cdot 7H_2O$	0.5	
4-Yeast extract medium	Yeast extract	2.5	(Manavalan et al., 2013)
	KH_2PO_4	1	
	$MgSO_4 \cdot 7H_2O$	1.024	
	Na_2HPO_4	0.05	
	$CaCl_2$	0.01	
	$FeSO_4 \cdot 6H_2O$	0.0171	

Erlenmeyer flasks with wheat bran and growth medium were sealed with cotton wool and aluminum foil and then sterilized in an autoclave with moist heat sterilization (Figure 3-3).

Figure 3-3. Sterilized Erlenmeyer flasks with wheat bran and growth medium.

After that, the cultivated mycelium on agar plates was transferred to the Erlenmeyer flasks using a pre-sterilized cork borer, spatula, and tweezers. This step was performed in a laminar chamber near a Bunsen burner to achieve a sterile environment. Once the mycelial transfer was completed, the flasks were resealed with their corresponding cotton wool and aluminum foil (Figure 3-4). In addition, these flasks were also weighed after the end of incubation, namely after 8 or 10 days at 27 ºC, in order to check the biomass production.

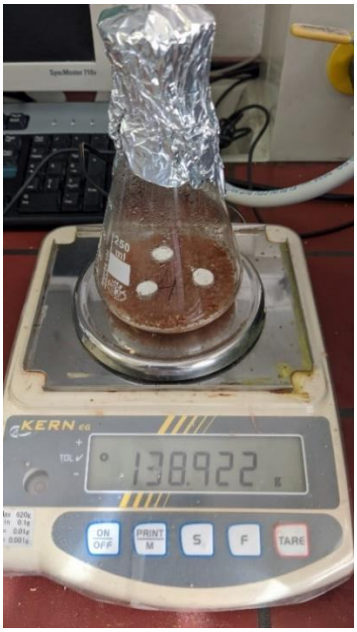

Figure 3-4. Weighing after mycelia inoculation.

Figure 3-5 shows Erlenmeyer flasks with grown mushroom mycelium after incubation at 27 °C.

Figure 3-5. Incubation process after inoculation of mycelia.

3.5 Extraction media and procedure

The extraction of proteins and enzymes in their active form from *P. ostreatus* mycelium was performed under the use of three different extraction media, containing 20 mL of distilled water or previously prepared 0.05 M sodium citrate buffer or 0.05 M sodium phosphate buffer, at 23 °C, with different procedures and times of extraction, such as 1, 1.5, 3 and 24 h of shaking at 200 rpm and 4-, 8-, and 10-min of homogenization at 8000 rpm. The extraction of proteins and enzymes in their active form from *G. lucidum* mycelium was performed using only 0.05 M sodium citrate buffer, as it has proven to be the best extraction medium in optimization for mushroom *P. ostreatus*. The extraction was carried out at 23 °C using the shaking procedure at 200 rpm for 3 and 24 h and using the homogenization process for 4 and 10 min, which were the best conditions for optimizing the extraction process of *P. ostreatus* mycelium.

0.05 M sodium citrate buffer was prepared by adding 4.803 g of citric acid ($C_6H_8O_7$) into a beaker with Milli-Q water and calibrating the pH of the solution to 4.8 with 0.1 M sodium hydroxide (NaOH). The solution was then poured into a 500 mL flask, adding Milli-Q water to the mark. 0.05 M sodium phosphate buffer was prepared by adding 3.872 g of $Na_2HPO_4 \cdot 7H_2O$ and 1.457 g of $NaH_2PO_4 \cdot H_2O$ into a beaker with Milli-Q water and calibrating the pH of the solution to 7.0 with NaOH or HCl. The solution was then poured into a 500 mL flask, adding Milli-Q water to the mark

For all experiments, after the shaking or homogenization procedure (Figure 3-6), the slurry was broken up in the laminar chamber with a spatula and transferred into a cotton cloth (Figure 3-7).

Figure 3-6. Samples after shaking or homogenization procedure.

Figure 3-7. Obtaining enzyme extracts from mycelium.

Subsequently, all the enzyme extract was squeezed out from the slurry, the extracts were transferred to Falcon tubes (50 mL) (Figure 3-8) and centrifugated at 5000 rpm for 20 min at 4 °C (Figure 3-9). The obtained pellet (solid phase) was discarded, and the supernatant was filtered, diluted, and stored in the freezer at -20 °C until further use.

Figure 3-8. Obtained sample before centrifugation.

Figure 3-9. Obtained sample after centrifugation.

For each sample, we prepared a boiled and unboiled sample to determine the total content of proteins (Bradford method) and the activity of each type of enzyme (α-amylase, glucoamylase, laccase, cellulase, and protease for the *P. ostreatus*, and α-amylase, glucoamylase, laccase, cellulase, catalase, peroxidase, SOD, and lipase for the *G. lucidum*) by means of light absorption measured in spectrophotometry equipment (Varian Cary® 50 UV-Vis Spectrophotometer, Figure 3-10).

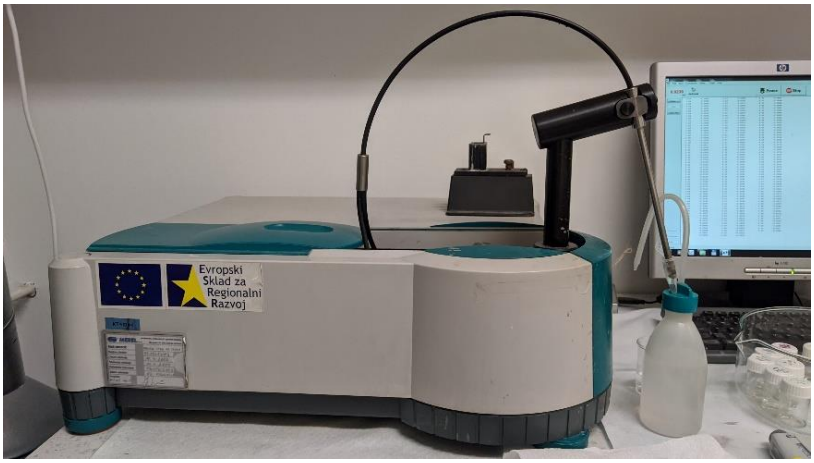

Figure 3-10. Spectrophotometer used to measure the absorbances of the samples for each enzymatic assay.

3.6 Determination of total protein concentration

3.6.1 Bradford method

The determination of total protein concentration was performed by the Bradford method. Prior to performing the assay, the calibration curve was obtained using albumin protein with different concentration ranges (0 to 10 mg/mL), diluting with Milli-Q water. 1 mL of the Bradford reagent was pipetted into 1.5 mL microcentrifuge tubes along with 20 μL of the sample. The mixture was vortexed and incubated for 15 min at room temperature to finally measure the absorbance at 595 nm. For the blank, 20 μL of Milli-Q water was used instead of the sample.

Using the calibration curve (Annex 1, Figure 7-1), the total protein concentration was calculated (equation 3-1):

$$c = \frac{A}{k} \qquad 3\text{-}1$$

Where:

- A absorbance of the sample measured at a wavelength of 595 nm (/).
- c protein concentration in the sample (mg/mL).
- k slope from the calibration curve (mL/mg).

3.7 Enzymatic assays

3.7.1 α-Amylase

0.02 M sodium phosphate buffer with 0.006 M NaCl was prepared to dissolve starch from wheat and obtain 1% starch solution. Also, 3,5-dinitrosalicylic acid (DNS) and maltose solution were prepared in advance. The calibration curve was obtained from the maltose solution in different concentrations diluted with Milli-Q water. The enzymatic activity of α-amylase was performed by pipetting 0.5 mL of sample or boiled sample into large tubes and by adding 0.5 mL of the 1% starch solution at time intervals. The mix was incubated 3 min at room temperature and stirred. At time intervals, 1 mL of DNS was added to stop the reaction and simmered for 5 min (gentle boiling). After cooling to room temperature, 10 mL of Milli-Q water were added (Figure 3-11) and later vortexed well to measure the absorbance at 540 nm. For the blank, instead of the sample, 0.5 mL of Milli-Q water was added to calibrate the spectrophotometer.

Figure 3-11. Samples and a blank sample (yellow color) just before measurement.

From the calibration curve (Annex 2, Figure 7-2), the concentration of released maltose was calculated by the equation 3-2:

$$c_m = \frac{(A_{unboiled} - A_{boiled})_{540\,nm}}{k} \qquad 3\text{-}2$$

Then, the enzymatic activity of α-amylase was calculated by the equation 3-3:

$$\frac{U}{mL} = \frac{(c_m \cdot V_1 \cdot 10^3 \cdot df)}{M \cdot t \cdot V_2} \qquad 3\text{-}3$$

Where:

- U enzymatic activity (U/mL).
- c_m maltose released concentration (mg/mL).
- V_1 reaction volume (substrate volume + sample volume = 1 mL).
- M molar mass of maltose (g/mol).
- t reaction time (min).
- V_2 sample volume (mL).
- df dilution factor (/).

3.7.2 Glucoamylase

First, 0.02 M sodium acetate buffer (0.005 M, pH 4.5 at 55 ºC) was prepared to dissolve starch from potato and obtain 1% starch solution. Also, DNS and glucose solution were prepared in advance. The calibration curve was obtained from the glucose solution in different concentrations diluted with Milli-Q water. For glucoamylase, 0.5 mL of starch solution were added into large tubes, incubating them for 10 min at 55 ºC (water bath). At time intervals, 0.5 mL of sample or boiled sample were added. After 3 min of incubation at 55 ºC, 1 mL of

DNS was added and simmered for 5 min (gentle boiling). 10 mL of Milli-Q water were added when the mix was at room temperature and later vortexed well to measure the absorbance at 540 nm. For the blank, instead of the sample, 0.5 mL of Milli-Q water was added to calibrate the spectrophotometer.

From the calibration curve (Annex 3, Figure 7-3), the concentration of released glucose was calculated by the equation 3-4:

$$c_m = \frac{(A_{unboiled} - A_{boiled})_{540\,nm}}{k} \qquad 3\text{-}4$$

Then, the enzymatic activity of glucoamylase was calculated by the equation 3-5:

$$\frac{U}{mL} = \frac{\left(c_g \cdot V_1 \cdot 10^3 \cdot df\right)}{M \cdot t \cdot V_2} \qquad 3\text{-}5$$

Where:

- c_g glucose released concentration (mg/mL).

3.7.3 Cellulase

For cellulase, different reagents were prepared in advance, such as a sodium acetate buffer (0.05 M, pH 5), which was used to dissolve Sigmacell cellulose to obtain a 5% Sigmacell solution. For the assay, 4 mL of the Sigmacell solution were pipetted into centrifuge tubes and incubated for 3 min at 37 ºC. Then, 1 mL of sample, boiled sample, or Milli-Q water (for the blank) was added. The centrifuge tubes were incubated on a shaker for 2 h at 37 ºC. After 2 h, the tubes were transferred to an ice bath to cool and sediment. Therefore, samples were centrifuged for 2 min at 11000 rpm, and the supernatant was carefully transferred into microcentrifuge tubes. 750 µL of glucose reagent were pipetted into fresh 1.5 mL microcentrifuge tubes. Just before measuring the kinetics at a wavelength of 340 nm (Enzyme Kinetics mode), 25 µL of sample supernatant or blank sample were added, mixed rapidly and immediately began measuring the absorbance for 5 min.

Cellulase enzyme activity is calculated by the equation 3-6:

$$\frac{U}{mL} = \frac{(\Delta A_{340\,nm\,(sample)} - \Delta A_{340\,nm\,(blank)}) \cdot V_1 \cdot V_2}{K \cdot f \cdot V_3 \cdot V_4} \qquad 3\text{-}6$$

Where:

- ΔA change in absorbance in the sample between start and end time (/).
- K millimolar extinction coefficient β – NADH at 340 nm (/).
- f conversion factor from 2h to 1h as given in the unit definition (/).

3.7.4 Laccase

Before starting the assay, two reagents were prepared: ABTS (0.001 M) and sodium acetate buffer (0.1 M, pH 5). Laccase assay was performed by adding 600 µL of sodium acetate buffer and 400 µL of the sample or boiled sample into a 1.5 mL microcentrifuge tubes and then adding 200 µL of ABTS, vortexed for 1 min and immediately measured the absorbance at a wavelength of 420 nm (Figure 3-12). For the blank, 1 mL of sodium acetate buffer and 200 µL of ABTS were used.

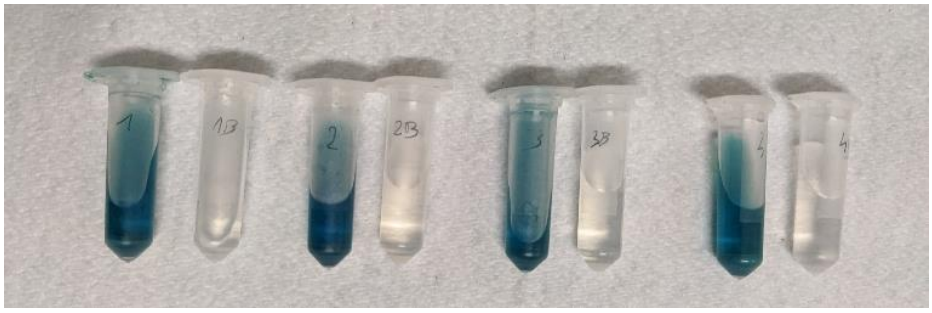

Figure 3-12. Example of a laccase enzymatic assay result. The green-colored microcentrifuge tubes represent samples with laccase in their active form, and the transparent ones represent boiled samples, where no enzyme is present.

Laccase enzyme activity is calculated by the equation 3-7:

$$\frac{U}{mL} = \frac{(\frac{A_{420\,nm}}{t}) \cdot V_r \cdot df}{\varepsilon \cdot V_v} \qquad 3\text{-}7$$

Where:

- V_r reaction volume of the mixture (mL).
- ε extinction coefficient (/).
- V_v sample volume (mL).

3.7.5 Protease

A phosphate buffer was prepared beforehand to subsequently dissolve casein (Hammarsten bovine) to obtain a 1% casein solution. In addition, TCA was dissolved to get a 5% TCA solution. Protease assay was performed by pipetting 1 mL of casein solution into the centrifuge tubes and incubating them for 3 min at 35 ºC. Then, 500 µL of sample or boiled sample and phosphate buffer were added and incubated at 35 ºC for 20 min. After incubation, 3 mL of 5% TCA solution was added and left for 30 min at room temperature. Therefore, samples were centrifuged for 20 min at 6000 rpm. The supernatant was carefully transferred into microcentrifuge tubes and the absorbance was measured at a wavelength of 280 nm. The spectrophotometer was calibrated with a blank, substituting the sample with 500 µL of Milli-Q water.

To calculate the proteolytic activity in the sample, the equation 3-8 was used:

$$1\ Tucas\ (\mu L) = \frac{V_v \cdot f}{\Delta A_{280\ nm}} \quad 3\text{-}8$$

Then, the total proteolytic activity was calculated by the equation 3-9:

$$x = \frac{V_v}{1\ Tucas \quad (\mu L)} \quad 3\text{-}9$$

Where:

- 1 Tucas: proteolytic activity (µL).

3.7.6 Catalase

Prior to the enzymatic assay, sodium phosphate buffer (50 mM) and 0.036% hydrogen peroxide (H_2O_2) solution were prepared. 2.9 mL of H_2O_2 were pipetted into tubes. For the blank, 0.1 mL of the boiled sample was used to calibrate the spectrophotometer. Just before measuring the kinetics, 0.1 mL of the sample was added to 2.9 mL of the 0.036% H_2O_2 solution. Immediately after that, kinetics was measured for 3 min at a wavelength of 240 nm.

The enzymatic activity of catalase was calculated by the equation 3-10:

$$\frac{U}{mL} = \frac{f \cdot df}{t \cdot V} \quad 3\text{-}10$$

Where:

f conversion factor corresponding to a degradation of 3.45 µmol of H_2O_2 in 3 mL of the reaction mixture, resulting in a decrease in absorbance of 0.45 to 0.40.

t time during which the absorbance decreases from 0.45 to 0.40.

3.7.7 Peroxidase

Before starting with the assay, three reagents were prepared namely PBS buffer (0.2 M, pH 7), hydrogen peroxide (0.0017 M), and 4-APP (0.0025 M). For the peroxidase assay, 1.4 mL of 4-APP, 1.5 mL of 0.0017 M H_2O_2, and 0.1 mL of PBS buffer were pipetted into small tubes. Just before the measurement, 0.5 mL of sample or boiled sample were added, and measured the kinetics for 4 min at a wavelength of 510 nm. For the blank, 0.5 mL of Milli-Q water was added to calibrate the spectrophotometer instead of the sample.

The enzymatic activity of peroxidase was calculated by the equation 3-11 and 3-12:

$$A_{510\ nm} = \frac{A_{510\ nm,\ f} - A_{510\ nm,\ i}}{4} \quad 3\text{-}11$$

$$\frac{U}{mL} = \frac{\Delta A_{510\,nm}}{\varepsilon \cdot V \cdot c} \qquad 3\text{-}12$$

3.7.8 Superoxidase dismutase

To perform this assay, two solutions were prepared in advance:

- Solution A: 0.1 mol/L Tris-HCl buffer solution with 1 mmol/L EDTA at pH 8.20.
- Solution B: 4.5 mmol/L pyrogallol solution in HCl.

SOD assay was performed by mixing 2.35 mL of solution A (0.1 mol/L Tris-HCl buffer solution with 1 mmol/L EDTA at pH 8.20) with 1.80 mL of Milli-Q water in a small tube. Then, 200 µL of the sample was added, 0.15 mL of solution B (4.5 mmol/L pyrogallol solution in HCl) and vortexed immediately. At a wavelength of 325 nm, the absorbance was measured in kinetics mode for 1 min. To calibrate the spectrophotometer, 2.35 mL of solution A and 2.15 mL of Milli-Q water were mixed in the small tubes.

The enzymatic activity of SOD was calculated by the equation 3-13:

$$\frac{U}{mL} = \frac{\frac{\Delta A_{325\,nm,\ boiled} - \Delta A_{325\,nm,\ sample}}{\Delta A_{325\,nm,\ boiled\ sample}} \cdot 100\%}{50\%} \cdot 4.5 \cdot \frac{1}{V} \cdot df \qquad 3\text{-}13$$

3.7.9 Lipase

Beforehand, sodium phosphate buffer (100 mM, pH 7.2, at 37 ºC with 150 mM sodium chloride and 0.5% triton) and PNPB (prepared in acetonitrile) were prepared. For lipase assay, 0.90 mL of sodium phosphate buffer and 0.1 mL of sample or boiled sample were pipetted into 1.5 mL microcentrifuge tubes, vortexed, and incubated at 37 ºC for 3 min. For the calibration of the spectrophotometer, 0.01 mL of Milli-Q water was added to the blank sample immediately before measurement. 0.01 mL of PNPB was added to the samples immediately before measurement, vortexed, and immediately began measuring kinetics for 5 min at 400 nm.

The enzymatic activity of lipase was calculated by the equation 3-14 and 3-15:

$$\Delta A_{400\,nm} = \frac{A_{400\,nm,\,f} - A_{400\,nm,\,i}}{5} \qquad 3\text{-}14$$

$$\frac{U}{mL} = \frac{\Delta A_{400\,nm} \cdot V_{suspension} \cdot df}{f \cdot V_{sample}} \qquad 3\text{-}15$$

4 Results and discussion

4.1 Biomass production

The growth of mycelium of two medicinal mushrooms, *P. ostreatus* and G. *lucidum* was monitored. Erlenmeyer flasks with wheat bran and growth medium, where the mycelium of individual mushrooms was grown, were weighed on the first and eighth or tenth day, respectively. Therefore, we monitored mycelial growth and biomass production over time.

For both mushrooms, *P. ostreatus* and *G. lucidum*, an increase in biomass could be visually observed due to the growth and development of mycelium inside the Erlenmeyer flasks (Figure 4-1).

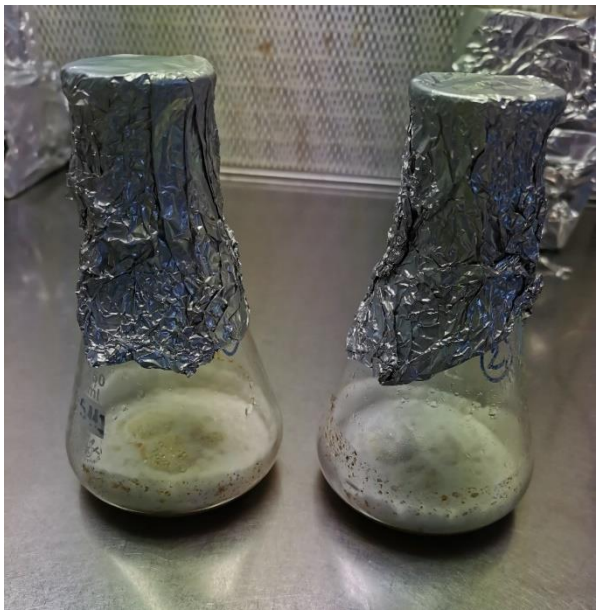

Figure 4-1. Mycelium after 8 days of cultivation.

In terms of numerical results, a decrease in mass was obtained during the growth period in each Erlenmeyer flask. This was due to the vaporization of the growth medium, and the mycelium also consumed part of the nutrients for its growth. Figure 4-2 shows the biomass production of *P. ostreatus* and Figure 4-3 for *G. lucidum*. In Annex 4 (Tables 7-1 to 7-7), all masses before and after each incubation period are presented.

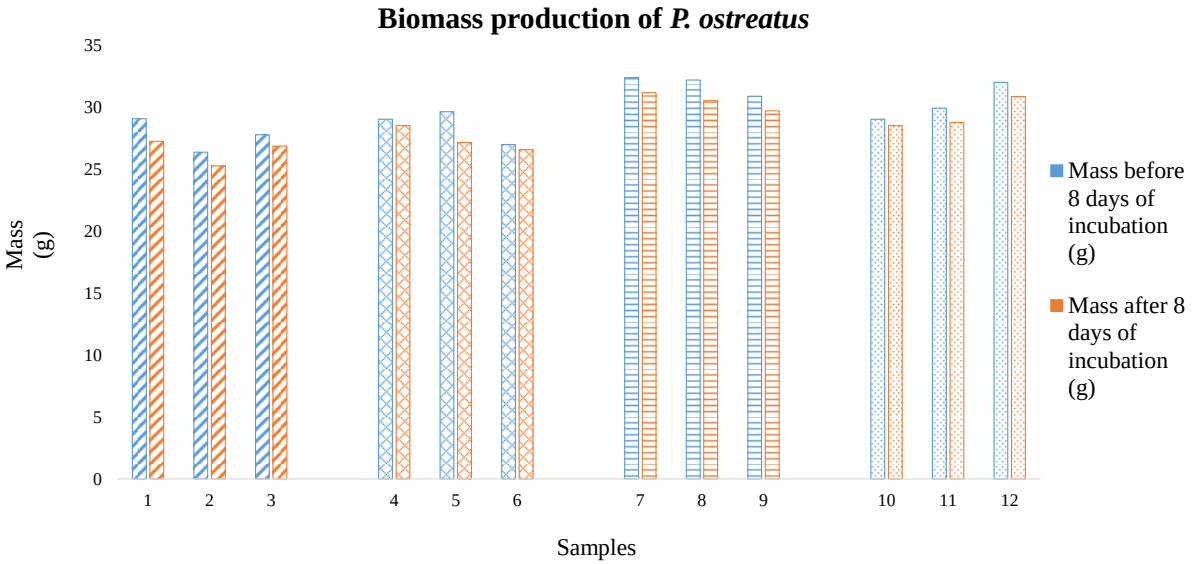

Figure 4-2. Biomass production of *P. ostreatus* after 8 days of cultivation with growth medium 1-*P. ostreatus* medium.

Legend: **1**: Distilled water, 1.5 h shaking; **2**: Sodium citrate buffer, 1.5 h shaking; **3**: Sodium phosphate buffer, 1.5 h shaking; **4**: Sodium citrate buffer, 8 min homogenization; **5**: Sodium phosphate buffer, 8 min homogenization; **6**: Distilled water, 8 min homogenization; **7**: Sodium citrate buffer, 1 h shaking; **8**: Sodium citrate buffer, 3 h shaking; **9**: Sodium citrate buffer, 24 h shaking; **10**: Sodium citrate buffer, 8 min homogenization; **11**: Sodium citrate buffer, 4 min homogenization; and **12**: Sodium citrate buffer, 10 min homogenization.

Figure 4-2 shows that the largest difference between the masses before and after 8 days of incubation was in sample 5, with a difference of 2.4942 g, and the smallest difference in sample 4, with a difference of 0.5179 g. Biomass production for *G. lucidum* is presented in Figure 4-3.

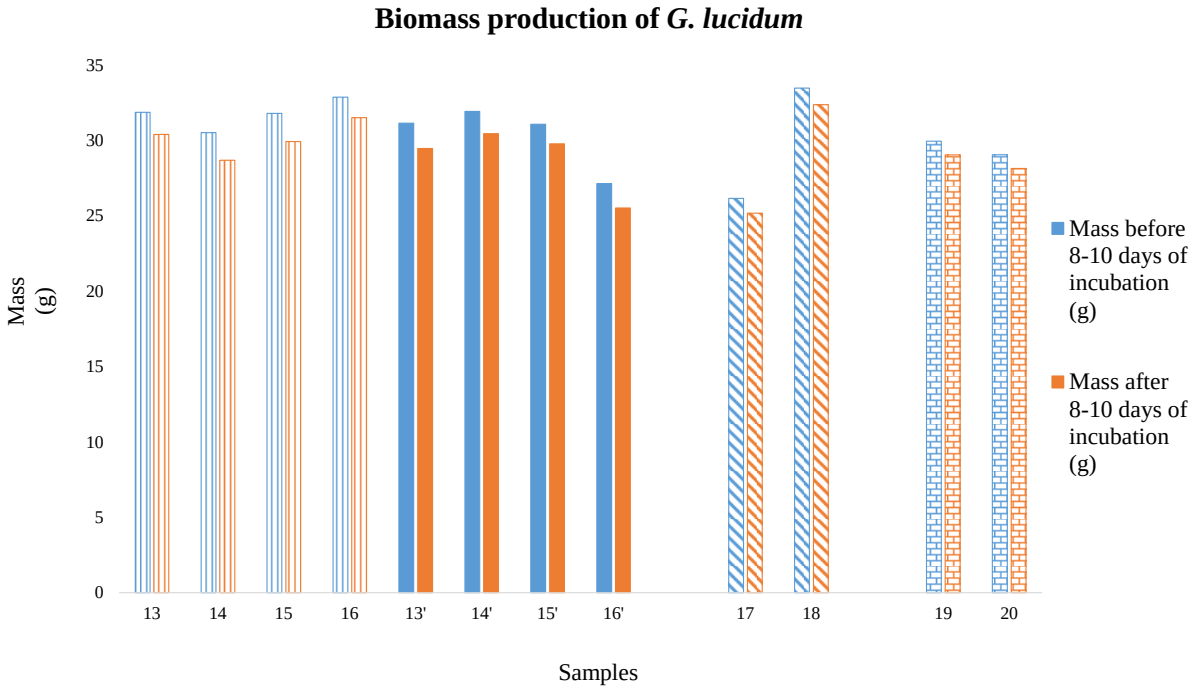

Figure 4-3. Biomass production of *G. lucidum* after 8 or 10 days of cultivation with four different growth media (*P. ostreatus*, sucrose, glucose, and yeast extract medium) (Table 3-1, 3-2). In all cases, sodium citrate buffer was used as an extraction medium.

Legend: **13**: 8 days of cultivation, 1 - *P. ostreatus* medium, 1.5 h shaking; **14**: 8 days of cultivation, 2 - sucrose medium, 1.5 h shaking; **15**: 8 days of cultivation, 3 - glucose medium, 1.5 h shaking; **16**: 8 days of cultivation, 4 - yeast extract medium, 1.5 h shaking; 13': 10 days of cultivation, 1 - *P. ostreatus* medium, 1.5 h shaking; **14'**: 10 days of cultivation, 2 - sucrose medium, 1.5 h shaking; **15'**: 10 days of cultivation, 3 - glucose medium, 1.5 h shaking; **16'**: 10 days of cultivation, 4 - yeast extract medium, 1.5 h shaking; **17**: 8 days of cultivation, 3 - glucose medium, 3 h shaking; **18**: 8 days of cultivation, 3 - glucose medium, 24 h shaking; **19**: 8 days of cultivation, 3 - glucose medium, 4 min homogenization; and **20**: 8 days of cultivation, 3 - glucose medium, 10 min homogenization.

It can be seen from Figure 4-3 that the highest difference between the masses before and after incubation was achieved in sample 15, with a difference of 1.8650 g, and the smallest difference was in sample 20, with a difference of 0.9030 g.

4.2 *Pleurotus ostreatus*

4.2.1 Optimization of the extraction medium

Several experiments were carried out with samples obtained from *P. ostreatus* mycelium to determine the optimal extraction medium. For this purpose, three different extraction media were used: distilled water, sodium citrate buffer, and sodium phosphate buffer.

First, the shaking procedure of 1.5 h was chosen, and the results of the total protein content in the obtained samples are shown in Figure 4-4. The highest total protein concentration (0.4826 mg/mL) was obtained with sodium citrate buffer as an extraction medium. This concentration was 46.9 and 276.7% higher than when using distilled water and sodium phosphate buffer, respectively.

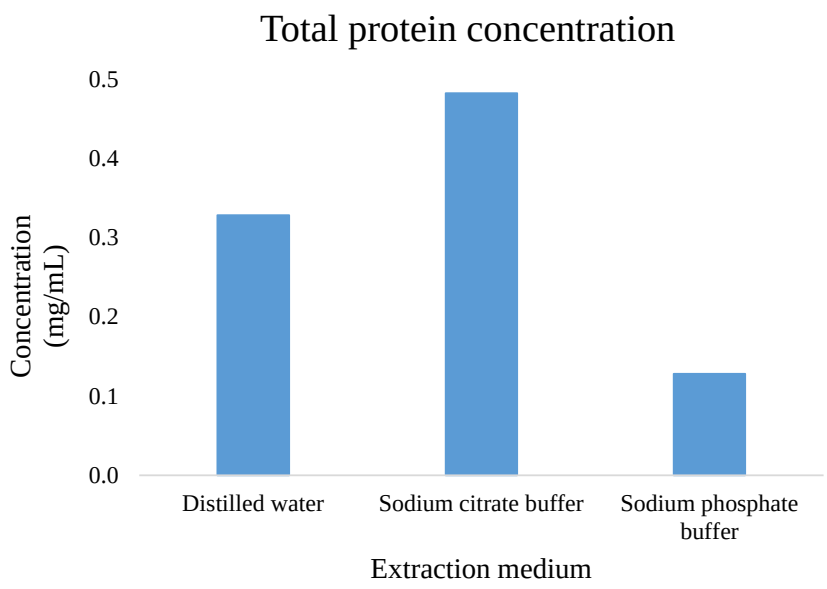

Figure 4-4. Total protein concentration in extracts from *P. ostreatus* mycelium using 3 different extraction media (distilled water, sodium citrate buffer, and sodium phosphate buffer), with 1.5 h of shaking.

Regarding enzyme activities in obtained mycelium extracts, the results are shown in Table 4-1. It is worth noting that with distilled water as an extraction medium, better results were obtained for α-amylase and glucoamylase, with activity values of 3.3712 and 6.0844 U/mL, respectively. While for laccase and protease, better results were achieved using sodium citrate buffer. For enzyme laccase, when the extraction procedure was carried out using sodium citrate buffer, the activity value was 7.1754 U/mL, which is 188.3 and 71.1% higher than using distilled water and sodium phosphate buffer, respectively. For protease, the value using sodium citrate buffer was 0.0099 U/mL. In contrast, the highest activity of enzyme cellulase was obtained with sodium phosphate buffer, with 1.0747 U/mL.

Table 4-1. Enzymes activities in extracts from *P. ostreatus* mycelium using 3 different extraction media, with 1.5 h of shaking.

		U/mL				
Sample	**EM**	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**
1	Distilled water	3.3712	6.0844	0.3959	2.4886	/
2	Sodium citrate buffer	2.4381	5.3936	0.7665	7.1754	0.0099
3	Sodium phosphate buffer	1.3085	4.7564	1.0747	4.1943	0.0085

Extraction media (EM); α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.).

Furthermore, the same extraction media were used with the second extraction procedure, i.e., homogenization for 8 min. The results of the total protein content in the obtained samples using homogenization are shown in Figure 4-5. It can be seen that the highest protein concentration (0.8607 mg/mL) was obtained using distilled water as an extraction medium.

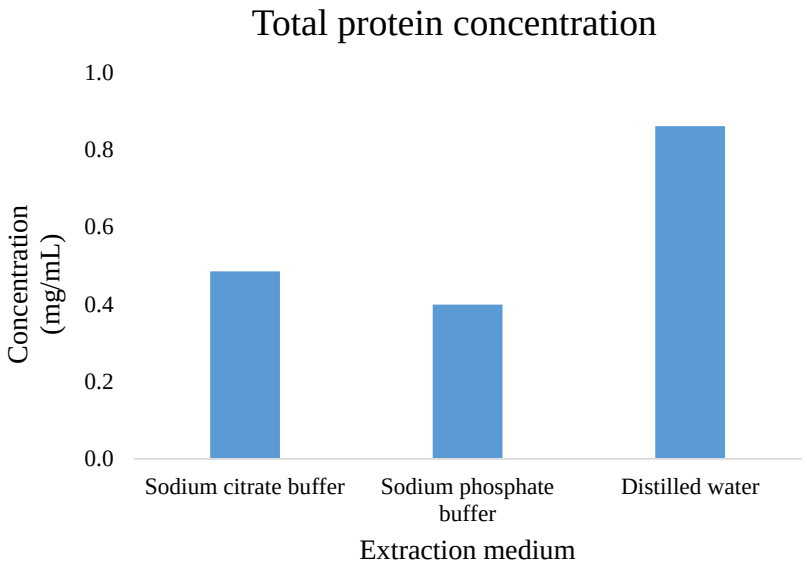

Figure 4-5. Total protein concentration in extracts from *P. ostreatus* mycelium using 3 different extraction media (distilled water, sodium citrate buffer, and sodium phosphate buffer), with 8 min of homogenization.

Different results were obtained for the enzymatic activities. The results are shown in Table 4-2. For α-amylase, the highest activity value (4.0384 U/mL) was observed using sodium phosphate buffer as an extraction medium, for glucoamylase, and cellulase with distilled water (5.7268 and 0.6124 U/mL, respectively), and for laccase and protease with sodium citrate buffer (12.250 and 0.0035 U/mL, respectively).

Table 4-2. Enzyme activities in extracts from *P. ostreatus* mycelium using 3 different extraction media, with 8 min of homogenization.

		U/mL				
Sample	**EM**	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**
6	Water	1.2201	5.7268	0.6124	3.0796	/
4	Sodium citrate buffer	2.2424	4.7194	/	12.2500	0.0035
5	Sodium phosphate buffer	4.0384	/	0.1956	6.2805	/

Extraction media (EM); α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.).

It can be concluded that, in general, the extraction medium where the highest activities of selected enzymes were detected was sodium citrate buffer. Therefore, for further experiments, this was the medium of choice. Regarding the growth medium, medium 1 – *P. ostreatus* (Table 3-1) was used for all experiments for the cultivation of *P. ostreatus* mycelium. Incubation lasted 8 days, as the growth of this mushroom was already optimized (Kodba, 2016), namely after 8 days of cultivation, the activity of enzymes present in the mycelium began to decrease.

4.2.2 Optimization of the extraction procedure

Once the optimal extraction medium was determined, further the procedure and time of extraction of proteins and enzymes were optimized. First, with the shaking procedure, different extraction times were tested, namely shaking for 1, 3, and 24 h, respectively.

Results for the total protein concentration are presented in Figure 4-6. An increasing trend could be observed with increasing time of shaking. The best results were obtained with 24 h of shaking (0.4110 mg/mL).

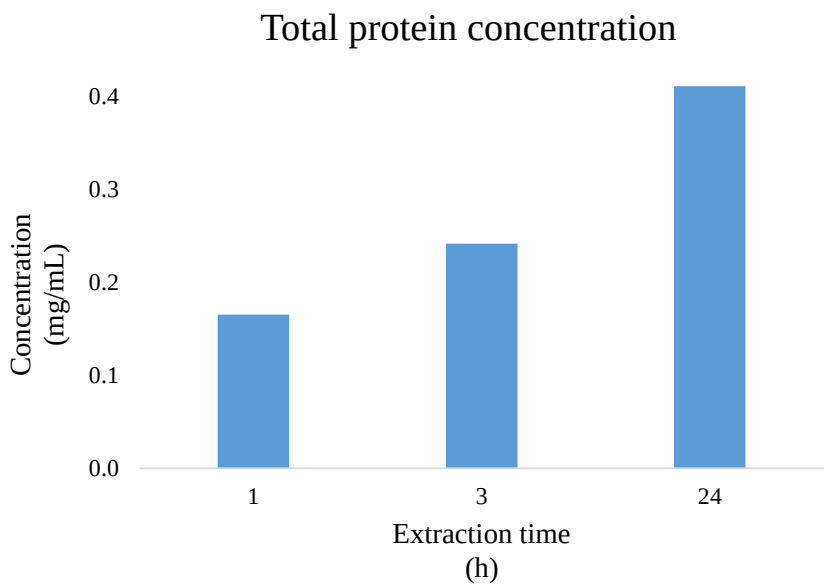

Figure 4-6. Total protein concentration in extracts from *P. ostreatus* mycelium using sodium citrate buffer as extraction medium, with 1, 3, and 24 h shaking.

The results of enzymatic assays are shown in Table 4-3. For the enzymes α-amylase, glucoamylase and cellulase, it was observed that with a prolonged time of shaking, a higher amount of those enzymes in their active form is isolated. The highest enzyme activity values were determined when the extraction was performed using shaking procedure for 24 h, with 8.0413 (α-amylase), 6.2120 (glucoamylase), and 1.0457 U/mL (cellulase), respectively. On the other hand, for laccase and protease, the highest activities were obtained with 3 h of shaking, with activity values of 0.2458 and 0.0040 U/mL, respectively.

Table 4-3. Enzyme activities in extracts from *P. ostreatus* mycelium using sodium citrate buffer as extraction media, with 1, 3 and 24 h shaking.

			U/mL				
Sample	**EM**	**ST (h)**	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**
7	Sodium citrate buffer	1	1.5865	3.2369	0.1380	1.7889	0.0171
8		3	3.8127	4.9243	0.2458	2.2684	0.0040
9		24	8.0413	6.2120	1.0457	1.6629	0.0018

Extraction media (EM); Shaking time (ST); α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.).

Subsequently, the homogenization time was optimized by exposing the samples to homogenization for 4, 8, and 10 min. With the Bradford test, the concentration of total proteins was determined, the results are presented in Figure 4-7. The highest total protein concentration was obtained with 8 min of homogenization, with a value of 0.4854 mg/mL, which was only 4.4% higher than the concentration obtained with 10 min.

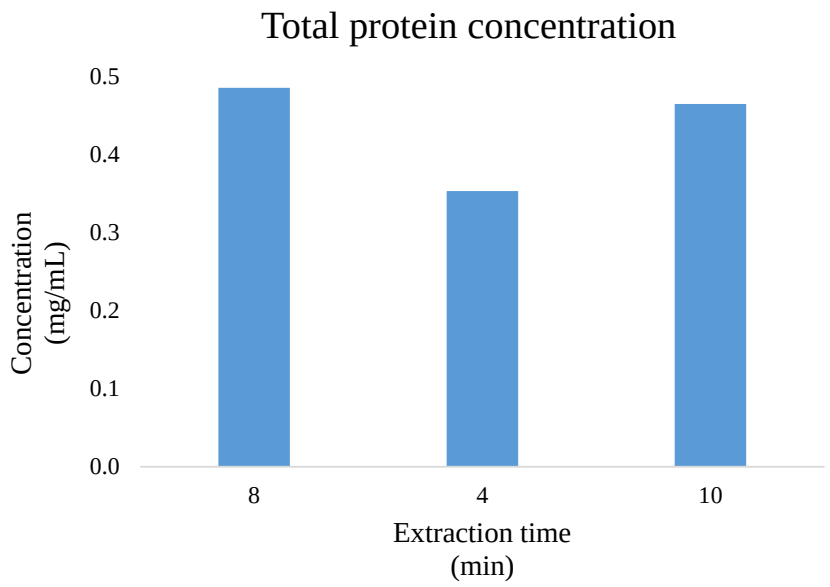

Figure 4-7. Total protein concentration in extracts from *P. ostreatus* mycelium using sodium citrate buffer, with 4, 8 and 10 min of homogenization.

Regarding the enzyme activities, as can be seen from Table 4-4, the highest values for α-amylase (3.5418 U/mL) and glucoamylase (6.7113 U/mL) were observed at 10 min of homogenization process. In contrast, for laccase and protease, higher activities were obtained with exposure to homogenization for 8 min, with values of 12.2500 and 0.0035 U/mL,

respectively. The homogenization process was not suitable for obtaining enzyme cellulase in its active form.

Table 4-4. Enzyme activities in extracts from *P. ostreatus* mycelium using sodium citrate buffer as extraction media, with 4-, 8- and 10-min of homogenization.

			U/mL				
Sample	**EM**	**HT (min)**	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**
11	Sodium citrate buffer	4	0.1658	3.9714	/	2.8166	0.0141
10		8	2.2424	4.7194	/	12.2500	0.0035
12		10	3.5418	6.7113	/	4.6655	0.0026

Extraction media (EM); Homogenization time (HT); α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.).

Comparing both procedures of extraction for isolation of proteins from mycelium, it was observed that the highest total protein concentration was obtained using 8 min of homogenization (0.4854 mg/mL), which is 18.1% higher than that with 24 h of shaking (0.4110 mg/mL). Higher values of enzyme activities were also obtained with the homogenization process for enzymes glucoamylase and laccase. The activity value of the enzyme glucoamylase with 10 min of homogenization was 6.7113 U/mL, which is 8.0% higher than with 24 h of shaking (6.2120 U/mL). For laccase 440.0% higher activity with 8 min of homogenization (12.2500 U/mL) compared to the activity achieved with 3 h of shaking (2.2684 U/mL) was observed. In contrast, for α-amylase and protease, higher activities were obtained with the shaking procedure. For α-amylase, with 24 h of shaking, the enzyme activity (8.0413 U/mL) was 127.0% higher than with 10 min of homogenization (3.5418 U/mL). With 3 h of shaking, the activity value for protease (0.0040 U/mL) was 14.29% higher than with 8 min of homogenization (0.0035 U/mL).

In general, it can be concluded that, with the shaking procedure, higher activities were obtained for enzymes α-amylase, cellulase, and protease. Extracellular enzymes, which catalyze oxidative reactions, are lignin peroxidases, laccases, manganese peroxidases, and hydrogen peroxide-producing enzymes (Palmieri et al., 2001; Wang et al., 2022). Based on these results, we hypothesized that the shaking procedure worked best for the extracellular enzymes α-amylase, cellulase, and protease. On the other hand, the homogenization process was more suitable for achieving higher total protein concentration and higher activities of glucoamylase and laccase due to increased cell rupture. So, in this study, we assume that these two enzymes (glucoamylase and laccase) were found in *P. ostreatus* at a higher intracellular level.

4.3 *Ganoderma lucidum*

4.3.1 Optimization of the growth medium

First, we optimized the growth of *G. lucidum* mycelium in order to determine the optimal cultivation time to obtain the highest possible amount of enzymes in their active form. For the optimization, four different growth media (1 – P. ostreatus, 2 - sucrose, 3 - glucose, and 4 - yeast extract medium) and different incubation times, namely 8 and 10 days were used. The composition of the used growth media is presented in Table 3-1 and Table 3-2

Figure 4-8 presents the results of obtained concentration of total proteins using the Bradford method. The highest protein concentration was obtained with medium 4 -Yeast extract medium when the mycelium was cultivated for 10 days (0.0193 mg/mL, sample 15').

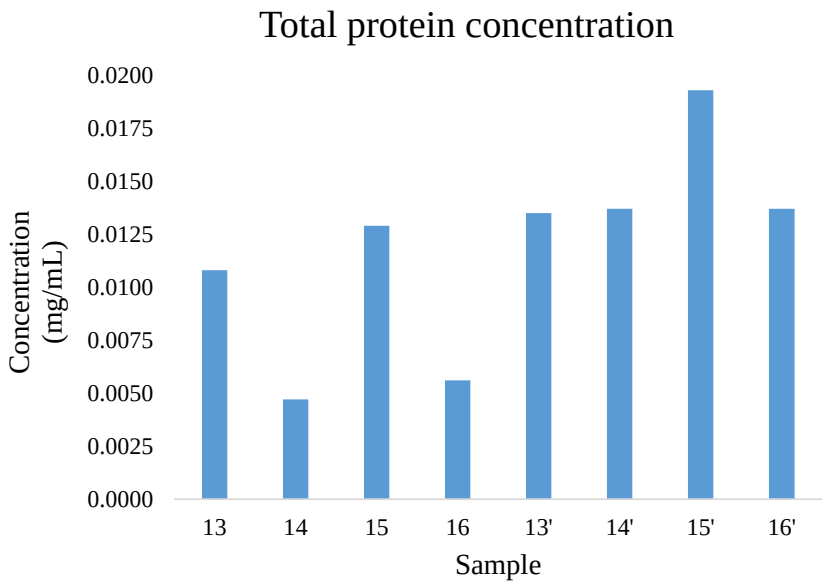

Figure 4-8. Total protein concentration in extracts from *G. lucidum* mycelium using 4 different growth media (1 - *P. ostreatus*, 2 - sucrose, 3 - glucose, and 4 - yeast extract media) with 1.5 h shaking and sodium citrate buffer as extraction media.

Legend: **13**: 8 days of cultivation, *P. ostreatus* medium, 1.5 h shaking; **14**: 8 days of cultivation, sucrose medium, 1.5 h shaking; **15**: 8 days of cultivation, glucose medium, 1.5 h shaking; **16**: 8 days of cultivation, yeast extract medium, 1.5 h shaking; **13'**: 10 days of cultivation, *P. ostreatus* medium, 1.5 h shaking; **14'**: 10 days of cultivation, sucrose *medium*, 1.5 h shaking; **15'**: 10 days of cultivation, glucose *medium*, 1.5 h shaking; and **16'**: 10 days of cultivation, yeast extract *medium*, 1.5 h shaking.

Table 4-5 shows the enzyme activities obtained from the performed enzymatic assays. Samples 13 and 13' were incubated with growth medium 1- *P. ostreatus* medium, 14 and 14' with growth medium 2 - sucrose medium, 15 and 15' with growth medium 3 - glucose medium and 16 and 16' with growth medium 4 - yeast extract (Tables 3-1, 3-2). For α-amylase, glucoamylase, cellulase, and lipase, the highest values of enzyme activities were achieved with growth medium 3 - glucose medium when the mycelium was cultivated for 8 days, with

activities of 13.1896, 62.2482, 2.1091, and 23.392 U/mL, respectively. For laccase and peroxidase, the highest activities were determined with medium 4 - yeast extract with 8 days of incubation (35.8583 and 0.0035 U/mL, respectively). The highest protease production was achieved with medium 1 with 8 days of cultivation (0.002 U/mL). In contrast, no catalase or SOD production in *G. lucidum* mycelium was observed. Therefore, the activities of these enzymes in our samples may have been so low that they could not be determined using selected enzymatic assays.

Table 4-5. Enzyme activities in extracts from *G. lucidum* mycelium using 4 different growth media with 1.5 h shaking and sodium citrate as extraction media. (Samples 13-16 were incubated for 8 days and samples 13'-16' were incubated for 10 days).

	U/mL								
Sample	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**	**Cat.**	**Perox.**	**SOD**	**Lip.**
13	7.3578	6.0652	0.7819	18.8000	0.0020	/	0.0015	/	5.0500
13'	4.4230	1.1045	0.3115	15.4500	/	/	/	/	22.6568
14	4.1638	1.7238	0.7373	10.5583	0.0016	/	0.0015	/	23.1117
14'	2.4080	2.1835	0.2131	5.4833	/	/	/	/	17.5158
15	13.1896	62.2482	2.1091	23.4750	0.0010	/	0.0010	/	23.3392
15'	4.5192	2.5218	1.1301	8.7750	/	/	0.0009	/	5.8689
16	2.6630	8.4211	1.8079	35.8583	/	/	0.0035	/	9.5995
16'	0.6982	9.4809	0.5046	17.4250	/	/	0.0019	/	3.2302

α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.); Catalase (Cat.); Peroxidase (Perox.), Superoxidase dismutase (SOD); Lipase (Lip.).

Overall, the best results for total protein content and enzyme activities were achieved with 8 days of incubation, as growth medium 3 - was generally the most optimal for the production of enzymes in their active form.

4.3.2 Optimization of the extraction procedure

In the previous subsection (Subsection 4.3.1) it was concluded that the optimal growth medium to produce proteins and enzymes from *G. lucidum* mycelium was 3 - glucose medium. Therefore, the extraction procedure was optimized using growth medium 3 – glucose medium. For this purpose, different shaking times (3 and 24 h) and homogenization times (4 and

10 min) were used (Table 4-6). In addition, sodium citrate buffer was employed as an extraction medium, whereas optimization of the extraction medium for *P. ostreatus* with this medium has generally yielded the best results. For the same reason, only the two most appropriate times for the shaking and homogenization procedure were selected.

The results of the Bradford method performed for the determination of total protein content are shown in Figure 4-9. The highest total protein concentration was obtained when the *G. lucidum* mycelium was exposed to 4 min of homogenization (0.0338 mg/mL).

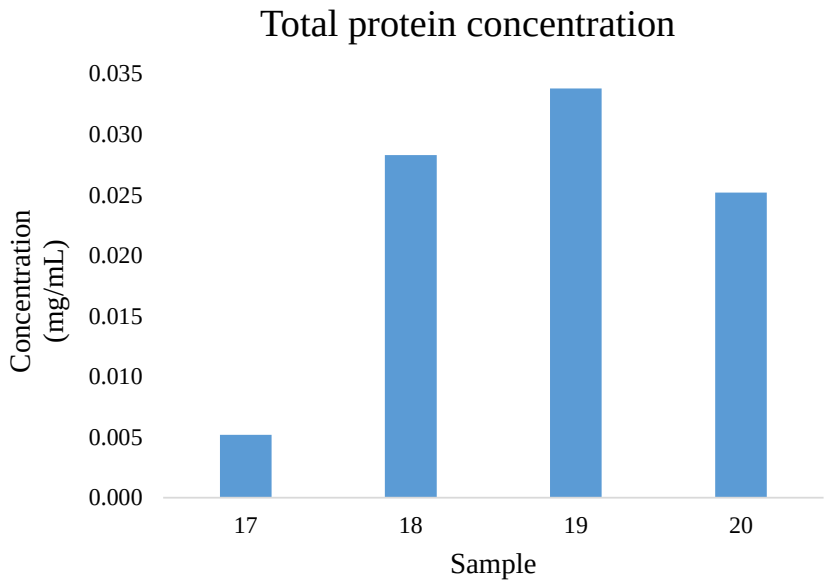

Figure 4-9. Total protein concentration in extracts from *G. lucidum* mycelium. Sodium citrate buffer as extraction medium, using growth medium 3 - glucose medium.

Legend: **17**: 8 days of cultivation, 3 h shaking; **18**: 8 days of cultivation, 24 h shaking; **19**: 8 days of cultivation, 4 min homogenization; and **20**: 8 days of cultivation, 10 min homogenization.

Table 4-6 summarizes the results of the determined enzyme activities. For α-amylase, the highest activity was obtained using the homogenization procedure for 4 min (16.3459 U/mL). Also, under the same extraction conditions, the enzyme SOD in its active form was successfully isolated with an activity value of 9.2615 U/mL. For cellulase and lipase, the highest production yields were determined after 3 h of shaking, with activity values of 1.6332 and 16.924 U/mL, respectively. Glucoamylase in its active form was isolated only using the shaking procedure, with 1426.1% higher value after 3 h than after 24 h of shaking (14.6737 with respect to 0.9615 U/mL). Also, the highest peroxidase activity (0.0156 U/mL) was obtained after 3 h of shaking. These were also the only extraction conditions for isolation of protease with an activity of 0.008 U/mL. For laccase, the highest activity was determined after 24 h of shaking (20.7083 U/mL). Among all analyzed enzymes, laccase was isolated in the largest amount in its active form. The obtained samples again did not contain the enzyme catalase, or its activity in the samples was so low that it was not detected by the enzymatic assay used.

According to the results, it was determined that the most suitable extraction procedure to produce the highest amounts of enzymes in their active form was when the shaking procedure was used for 3 h.

Table 4-6. Enzyme activities in extracts from *G. lucidum* mycelium using growth medium 3 - glucose medium, sodium citrate buffer as extraction medium with 3 and 24 h of shaking (samples 17 and 18, respectively) and 4 and 10 min of homogenization (samples 19 and 20, respectively).

	U/mL								
Sample	**α-Amy.**	**Gluc.**	**Cell.**	**Lacc.**	**Prot.**	**Cat.**	**Perox.**	**SOD**	**Lip.**
17	14.6737	67.8026	1.6332	12.0075	0.0008	/	0.0156	/	16.9243
18	0.9615	120.4742	0.7382	20.7083	/	/	0.0025	/	6.6878
19	16.3459	/	0.7766	17.1283	/	/	0.0013	9.2615	1.3649
20	13.5868	/	0.5367	18.4517	/	/	0.0027	/	14.8770

α-Amylase (α-Amy.); Glucoamylase (Gluc.); Cellulase (Cell.); Laccase (Lacc.); Protease (Prot.); Catalase (Cat.); Peroxidase (Perox.), Superoxidase dismutase (SOD); Lipase (Lip.).

With the comparison between 1.5 (Table 4-5), 3 and 24 h of shaking and 4 and 10 min of homogenization (Table 4-6), it can be observed that α-amylase activity was 11.40% higher after 4 min of homogenization (16.3459 U/mL) with respect to 3 h of shaking (14.6737 U/mL). As for cellulase production, after 1.5 h of shaking (2.1091 U/mL), a 171.58% higher result was obtained compared to 4 min of homogenization (0.7766 U/mL). With laccase, the value achieved after 1.5 h of shaking (23.4750 U/mL) was 27.22% higher than after 10 min of homogenization (18.4517 U/mL). Peroxidase activity was equal, after 10 min of homogenization (0.0027 U/mL) 8% higher than after 24 h of shaking (0.0025 U/mL). Finally, after 1.5 h of shaking (23.3392 U/mL), lipase production was 56.88% higher than after 10 min of homogenization (14.8770 U/mL). In general, it could be concluded that better results were obtained with the shaking procedure for glucoamylase, cellulase, laccase, and protease enzymes. In this case, for *G. lucidum*, we assume that the highest activity of extracellular enzymes glucoamylase, cellulase, laccase, and protease (Melouka et al., 2015) was obtained by the shaking procedure. On the other hand, by homogenization the highest values were obtained for α-amylase, peroxidase, and SOD enzymes. In this case, we assume that the homogenization procedure achieved a better cell disruption resulting in better results for α-amylase, peroxidase, and SOD enzymes, so we assume that these enzymes were found in *G. lucidum* at a higher intracellular level (Gomes et al., 2020).

5 Conclusions

In this study, the total protein content, and activities of different enzymes from *P. ostreatus* and *G. lucidum* mycelium. were determined. For this purpose, different cultures were prepared in Petri dishes with PDA. These already grown mycelia were incubated by SSF in Erlenmeyer flasks with wheat bran and different growth media (medium number 1 – *P. ostreatus* medium for *P. ostreatus* and medium number 1- *P. ostreatus* medium, 2 – sucrose medium, 3 – glucose medium, and 4 – yeast extract medium for *G. lucidum*) (Table 3-1, 3-2). After the incubation, different extraction procedures, such as shaking and homogenization, were tested, in which the time was optimized for each of them. In addition, different extraction media (distilled water, sodium citrate buffer, or sodium phosphate buffer) were tested. The total protein content was determined using the Bradford method for the obtained extracts. With specific enzymatic assays for each individual enzyme, such as α-amylase, glucoamylase, laccase, cellulase, protease, catalase, peroxidase, SOD, and lipase, enzyme activities were examined. All analyses were performed by means of light absorption measured in spectrophotometry equipment. Based on the obtained results, optimal conditions were determined for isolation of enzymes in their active form.

First, the optimization of the extraction medium and extraction procedure was carried out with *P. ostreatus*. The obtained results showed a better performance using sodium citrate buffer regarding the extraction medium. For the extraction procedure, it can be concluded that the highest activity for selected enzymes were obtained at following conditions: for α-amylase (24 h; 8.0413 U/mL, and sodium citrate buffer), cellulase (24 h; 1.0457 U/mL), and protease (1 h; 0.0171 U/mL, and sodium citrate buffer) with the shaking procedure, and for total protein concentration (8 min; 0.8607 mg/mL, and distilled water), glucoamylase (10 min; 6.7113 U/mL, and sodium citrate buffer) and laccase (8 min; 12.2500 U/mL, and sodium citrate buffer) with homogenization process. For *G. lucidum*, the optimal growth period and medium were first investigated, resulting in 8 days of cultivation and medium number 3 – glucose medium (Table 4-5). According to these results, the extraction procedure was subsequently optimized. In general, 1.5 h of shaking was the best of the tested methods and conditions studied to produce the highest number of maximum values for different enzymes (such as glucoamylase, cellulase, laccase, protease, and lipase).

Figure 5-1 shows the highest enzymatic activities obtained from *P. ostreatus* mycelium. For α-amylase and protease (8.0413 and 0.0171 U/mL, respectively) were achieved with sodium citrate buffer as extraction medium and shaking procedure (24 and 1 h, respectively). The highest activity for glucoamylase and laccase (6.7113 and 1.0747 U/mL, respectively) were also achieved with sodium citrate buffer with 10 and 8 min of homogenization, respectively. In contrast, the maximum activity value for cellulase (12.2500 U/mL) was obtained with phosphate buffer and 1.5 h of shaking.

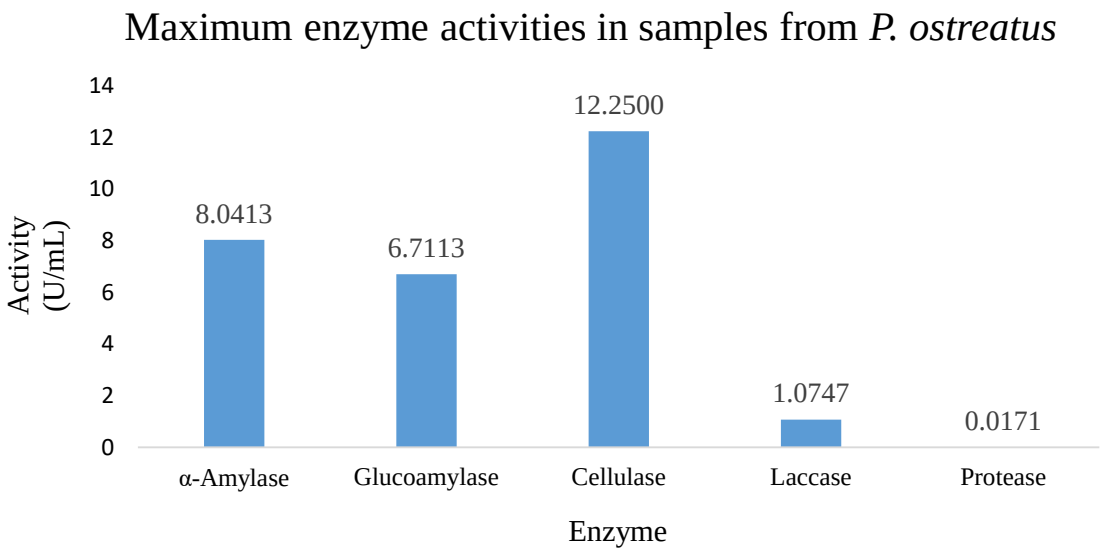

Figure 5-1. Maximum enzymatic activity achieved in samples obtained from *P. ostreatus* mycelium.

The different media, procedures, and times for obtaining the maximum values are shown in the following table (Table 5-1).

Table 5-1. Summary of conditions and parameters of maximum values of enzymatic activities of *P. ostreatus.*

Enzyme	**Growth medium**	**Growth time (d)**	**Extraction medium**	**Extraction procedure**	**Extraction time**
α-Amylase	1 - *P. ostreatus*	8	Sodium citrate buffer	Shaking	24 h
Glucoamylase			Sodium citrate buffer	Shaking	1 h
Cellulase			Sodium citrate buffer	Homogenization	10 min
Laccase			Sodium phosphate buffer	Shaking	1.5 h
Protease			Sodium citrate buffer	Homogenization	8 min

The highest enzyme activities in samples obtained from *G. lucidum* mycelium are shown in Figure 5-2. All maximum productions occurred when the mushroom mycelium was cultivated for 8 days. In general, the highest enzyme activities with growth medium 3 – glucose medium and 1.5 h of shaking were determined such as lipase and cellulase (23.1117, and 2.1091 U/mL, respectively) and for glucoamylase and peroxidase (67.8026 and 0.0156 U/mL, respectively) after 3 h of shaking. Using the same growth medium (3 – glucose medium), the highest activities were achieved after 4 min of homogenization for α-amylase and SOD (16.3459 and 9.2615 U/mL, respectively). In contrast, the highest laccase and protease productions (35.8583 and 0.0020 U/mL, respectively) were obtained after 1.5 h of shaking and with growth medium 4 – yeast extract medium and 1 – *P. ostreatus* medium, respectively.

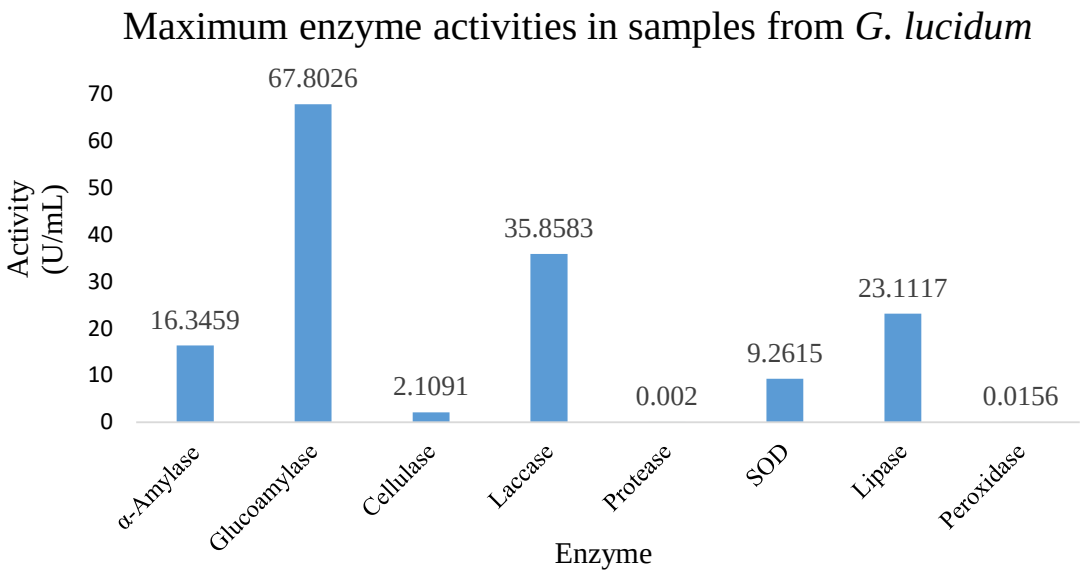

Figure 5-2. Maximum enzymatic activity achieved in samples obtained from *G. lucidum* mycelium.

The different media, procedures, and times for obtaining the maximum values are shown in the following table (Table 5-2).

Table 5-2. Summary of conditions and parameters of maximum values of enzymatic activities of *G. lucidum.*

Enzyme	**Growth medium**	**Growth time (d)**	**Extraction medium**	**Extraction procedure**	**Extraction time**
α-Amylase	3 - Glucose	8	Sodium citrate buffer	Homogenization	4 min
Glucoamylase	3 - Glucose			Shaking	1.5 h
Cellulase	3 - Glucose			Shaking	1.5 h
Laccase	4 - Yeast extract			Shaking	1.5 h
Protease	1 - P. ostreatus			Shaking	1.5 h
SOD	3 - Glucose			Homogenization	4 min
Lipase	3 - Glucose			Shaking	1.5 h
Peroxidase	3 - Glucose			Shaking	3 h

Thus, the feasibility of producing proteins and a series of enzymes in their active form from *P. ostreatus* and *G. lucidum* was demonstrated. From certain growth media, incubation times, and extraction media with different procedures, it was possible to determine the total protein content and the activity of various enzymes, which are of great importance in industrial processes such as food, pharmaceutical, and cosmetics.

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7 Annexes

7.1 Annex 1

The calibration curve used for the Bradford test for determination of total protein content for *P. ostreatus* and *G. lucidum* is as follows:

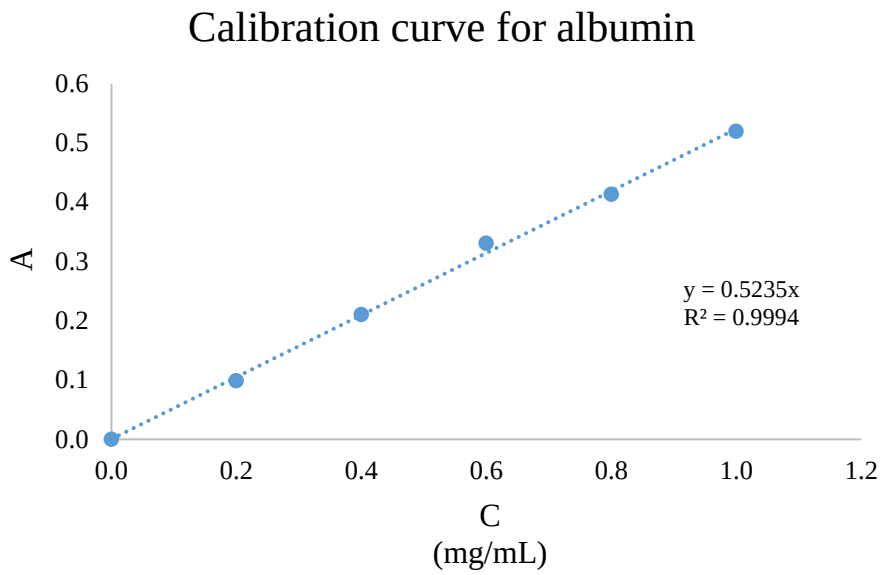

Figure 7-1. Calibration curve for albumin used for the determination of the total protein concentration.

7.2 Annex 2

The calibration curve for maltose used for the α-amylase assays is as follows:

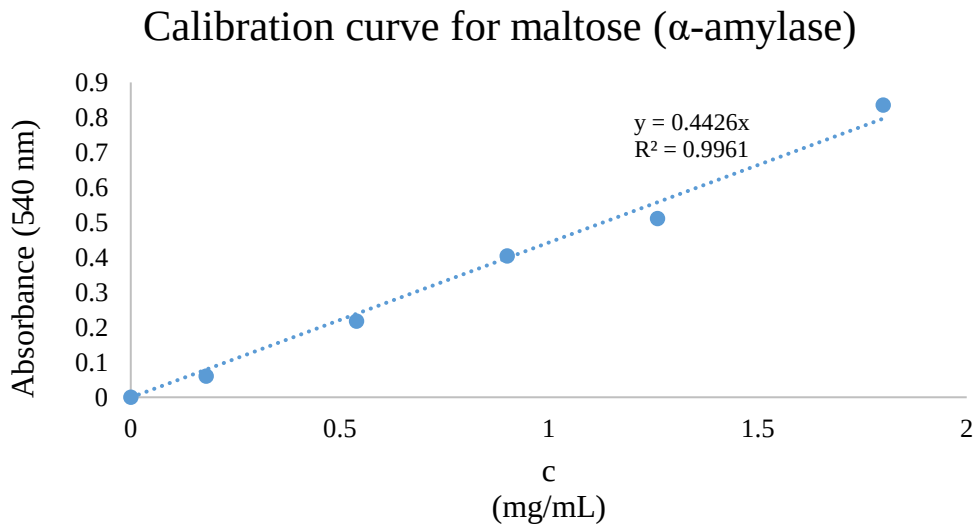

Figure 7-2. Calibration curve for maltose for α-amylase activity calculations.

7.3 Annex 3

The calibration curve for glucose used for the glucoamylase assays is as follows:

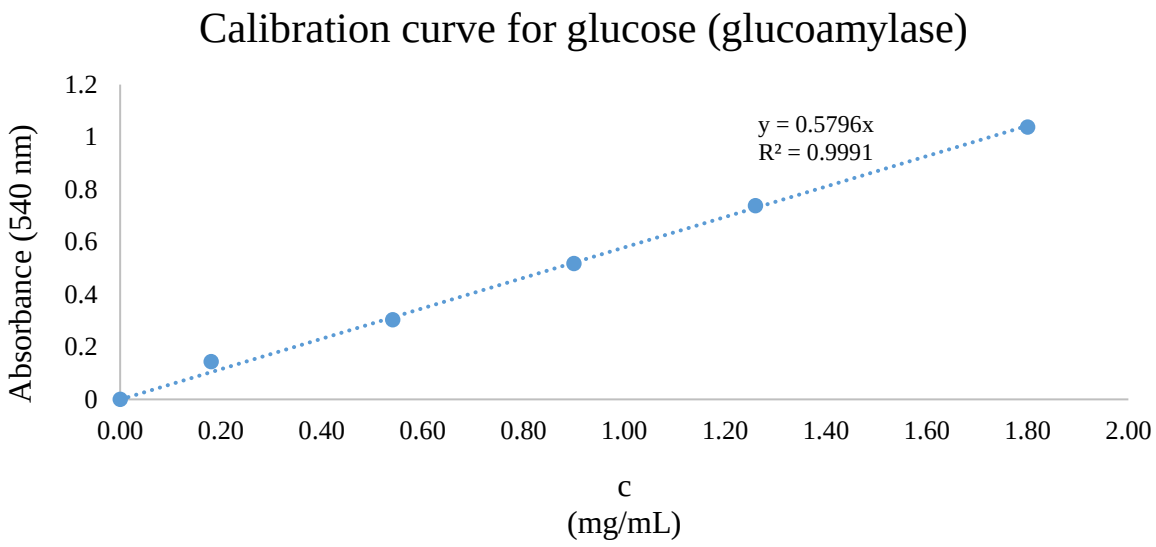

Figure 7-3. Calibration curve for glucose for glucoamylase activity calculations.

7.4 Annex 4

The following tables (Tables 7-1 to 7-7) show all the numerical results corresponding to the masses before and after each incubation period:

Table 7-1. Biomass production in samples of *P. ostreatus* using 3 different extraction media with 1.5 h of shaking.

Sample	Extraction media	Mass before (g)	Mass after 8d (g)	Biomass production (g)
1	Water	29.1013	27.2819	-1.8194
2	Sodium citrate buffer	26.4032	25.2935	-1.1097
3	Sodium phosphate buffer	27.8082	26.8828	-0.9254

Table 7-2. Biomass production in samples of *P. ostreatus* using 3 different extraction media with 8 min of homogenization.

Sample	Extraction media	Mass before (g)	Mass after 8d (g)	Biomass production (g)
6	Distilled water	27.0212	26.6043	-0.4169
4	Sodium citrate buffer	29.0582	28.5403	-0.5179
5	Sodium phosphate buffer	29.6721	27.1779	-2.4942

Table 7-3. Biomass production in samples of *P. ostreatus* using sodium citrate buffer as extraction media with 1, 3 and 24 h of shaking.

Sample	Extraction media	Shaking time (h)	Mass before (g)	Mass after 8d (g)	Biomass production (g)
7	Sodium citrate buffer	1	32.4320	31.2075	-1.2245
8		3	32.2297	30.5586	-1.6711
9		24	30.9186	29.7340	-1.1846

Table 7-4. Biomass production in samples of *P. ostreatus* using sodium citrate as extraction media with 4, 8 and 10 min of homogenization.

Sample	Extraction media	Homogenization Time (min)	Mass before (g)	Mass after 8d (g)	Biomass production (g)
10	Sodium citrate buffer	8	29.0582	28.5403	-0.5179
11		4	29.9504	28.8046	-1.1458
12		10	32.0365	30.8909	-1.1456

Table 7-5. Biomass production in samples of *G. lucidum* using 4 different growth media (1 – *P. ostreatus*, 2 – sucrose, 3 – glucose, and 4 – yeast extract medium) with sodium citrate buffer as extraction media and 1.5 h of shaking.

Sample	Growth media	Growth time (d)	Mass before (g)	Mass after 8d (g)	Biomass production (g)
13	1	8	31.8730	30.4120	-1.4610
14	2		30.5320	28.6920	-1.8400
15	3		31.8030	29.9380	-1.8650
16	4		32.8820	31.5230	-1.3590
13'	1	10	31.1560	29.4750	-1.6810
14'	2		31.9370	30.4570	-1.4800
15'	3		31.0810	29.7790	-1.3020
16'	4		27.1500	25.5310	-1.6190

Table 7-6. Biomass production in samples of *G. lucidum* using growth media 3 – glucose medium with sodium citrate buffer as extraction media and 3 and 24 h of shaking.

Sample	Shaking time (h)	Mass before (g)	Mass after 8d (g)	Biomass production (g)
17	3	26.1690	25.1890	-0.9800
18	24	33.4880	32.3810	-1.1070

Table 7-7. Biomass production in samples of *G. lucidum* using growth media 3 – glucose medium with sodium citrate buffer as extraction media and 4- and 10-min of homogenization.

Sample	Homogenization time (min)	Mass before (g)	Mass after 8d (g)	Biomass production (g)
19	4	29.9560	29.0510	-0.9050
20	10	29.0580	28.1550	-0.9030

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