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Ph.D. Thesis Summary

Study of the biotechnological conditions for polypyrrole synthesis, by *in situ* polymerization with selected microorganisms

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Table of contents

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Introduction

Conducting polymers (CPs) are an important class of materials that exhibit properties of conventional polymers such as low density, flexibility, processability whilst their inherent nature is electrically conducting. CPs represented a scientific challenge that begun with the Nobel Prize awarding to Heeger [1], MacDiarmid [2] and Shikarawa [3] and was tackled by researchers in chemistry, physics and material science. They are particular in that they consist of single and double bonded organic chains, referred to as conjugated structures. The significance of their identification is related to the opportunity of replacing metallic conductive materials with organic ones that possess similar electrical properties yet higher flexibility in synthesis and processability. The main aim in CPs research and development has been the understanding of their electrochemical properties as derived from their chemical structure and, in turn the multitude of possibilities for modification. The reader is referred to the two volumes of the Handbook of Conducting Polymers [4] for fundamental data on CP synthesis and characterization. Polypyrrole (Ppy) can be considered representative to the field due to its good electrical properties, manageable features and extensive involvement in biological applications, proving stability and biocompatibility.

The method of synthesis employed in preparation of CPs is fundamental as it tailors the final product for the desired applications. In fact, the materials in neutral state are dielectrics and upon oxidation or reduction, they gain electrical charge. Thus, the polymerization does not provide a neutral polymer, in insulating state rather it is endowing it with conductivity and charge according to the synthesis parameters. Several techniques are already established such as electrochemical and chemical methods, which use anodic potential and chemical oxidants for monomer oxidation, leading to formation of radical cations with unpaired electron density that react with their counterparts resulting in formation of polymer chains. Whilst chemically and electrochemically prepared CPs are of great applicability in physicochemical fields such as electrochromic displays, capacitors, batteries, photovoltaics and anti-corrosive materials, bioanalytical fields of research require biocompatible materials with high processability which are preferably, environmentally benign and economically efficient.

Thus, 'green' methods for CP synthesis began being explored and they encompass a wide variety of methods that make use of natural bio-oxidants and/or biocatalytic quantities of oxidants in environmentally friendly conditions such as physiological temperature and pH, without production of toxic byproducts. One of the major approaches is enzymatic catalysis, since enzymes are remarkable biocatalysts that can replace large quantities of chemical catalysts, granting thermodynamically efficient reactions whilst maintaining their biological functions and integrity [5]. Several oxidoreductases have been employed such as peroxidase, laccase and glucose oxidase. Yet, enzyme purification represents a costly tedious process, with liabilities such as enzymatic activity loss and/or denaturation. Therefore, the current study introduces Ppy synthesis within oxidoreductase-yielding microorganism cultures. Although, polymerization of biocompatible plastics using microorganisms has been available, microbe-mediated synthesis of CPs has been only recently attempted, by treatment of microbial components with chemical oxidants able to induce the polymerization process.

Therefore, the PhD thesis termed **'Study of the biotechnological conditions for polypyrrole synthesis, by *in situ* polymerization with selected microorganisms'** provides a new concept for Ppy synthesis between pure enzymatic catalysis and chemical functionalization of microorganisms. The biotechnological pathway proposed is based on the ability of the selected microorganisms to biosynthesize enzymes, with direct or indirect involvement in pyrrole polymerization, in submerge cultivation conditions and encourages the use of cell cultures as 'polymerization bulk solutions'. Thus, the study accomplishes a two-fold purpose: (i) the biocatalytic formation of Ppy through native enzymes synthesized by the cells ; (ii) biochemical process initiated by cell cultures *in vitro* without need for chemical treatment of cellular components. For this purpose,

several types of microorganisms were tested: filamentous bacteria strains belonging to the *Streptomyces* spp. genus and fungi strains ranging from white-rot fungi (*Trametes* spp., *Irpex lacteus*) to common mold fungi (*Aspergillus niger, Rhizoctonia* spp.). The performance of the tested microorganisms was evaluated considering Ppy formation within cell cultures, which was investigated through several microscopy techniques. Validation of the selection study was provided by infrared spectroscopy. The feasibility of Ppy bioproduction within the selected cell cultures was assessed by spectroelectrochemical monitoring of the polymerization process and characterization of the optoelectrical properties of the final product, by comparison with literature data on enzymatic Ppy formation. Ultimately, the benefits of the 'green' route proposed for synthesizing Ppy are relevant particularly in the biomedical and bioanalytical fields of research. Thus, the applicability of Ppy within bio-electrochemical applications was tested by development of several types of amperometric biosensors based on Ppy formed either in cell cultures or culture filtrates of the most performant microorganism strains.

The research followed several main objectives:

- The characterization and selection of microorganism strains capable of initiating pyrrole (Py) oligomerization.
- The analysis of culture mediums used as 'polymerization bulks' concerning protein and enzyme biosynthesis both at intra- or extracellular level.
- The spectro-electrochemical monitoring and evaluation of the oligomerization process and the optoelectrical characterization of the formed Ppy.
- Evaluation of the influence of Ppy formation within cell cultures and culture filtrates by development of amperometric biosensors based on Ppy-modified cell mycelia or crude enzyme extract.

The PhD study is structured in three major sections, as following:

I. Literature review aims to provide a state-of-the-art summary on biocatalytic methods of synthesis for CPs and their extensive employment in bio-application, whilst focusing on Ppy. It is structured in four minor subsections dedicated to: (1) general overview on CPs and their significance in scientific research, highlighting Ppy as illustrative model of analysis; (2) essential information on established methods of synthesis for CPs; details on innovative and environmentally friendly routes of Ppy preparation with brief description of biocatalysts involved in terms of origin and catalytic functions; (3) fundamental models applied in the spectro-electrochemical characterization of Ppy and their interpretation for bioproduced Ppy; (4) brief review of miscellaneous bio-applications which highlight Ppy as a suitable biomaterial, highlighting its purpose within biosensor and biofuel cell technology.

II. Experimental setup entails the detailed research methodology and it is divided in four subsections: (5) cultivation conditions of the tested microorganism strains and procedural details of pyrrole addition *in situ*; (6) description of analytical parameters for protein and enzyme assays; (7) specifications on the spectro-electrochemical methods of characterization including equipment and operational parameters; (8) construction of biosensors, biolayer design and principle of measurements.

III. Results and discussion illustrates the results of the proposed experimental study by contrast with literature data available on the specific topics. It is structured in four subsections, each with its own scientific objective and partial conclusions. Subsection 9 termed **'Selection and characterization of microorganism strains able to induce pyrrole (Py) oligomerization'** aims to illustrate the selection of microorganism strains that possess the ability to induce Ppy formation based

on microscopy techniques (optical microscopy, scanning electron microscopy, focused ion beam) and infrared spectroscopy. Within this study, twenty-five filamentous bacteria strains belonging to the *Streptomyces* spp. genus, five white-rot fungi strains (*Trametes versicolor*, *Trametes pubescens*, *Trametes hirsuta*, *Trametes gibbosa*, *Irpex lacteus*) and six common mold fungi strains (*Aspergillus niger* MIUG 34, *Aspergillus niger* MIUG 35, *Rhizoctonia* spp. coded KM 53 and KM 55, KM 56 and KM 57) were tested in submerge cultivation conditions and the most performant strains were chosen for the further study. Subsection 10 denominated **'Spectroelectrochemical monitoring and characterization of polypyrrole (Ppy) formation within fungi cultures'** is devoted to monitoring and characterization of Py oligomerization process through spectroelectrochemical techniques. During this stage, the best performance in initiating pyrrole polymerization was noticed for two white-rot fungi strains (*Trametes versicolor*, *Trametes pubescens*) and two common fungi strains (*Aspergillus niger* MIUG 34, *Aspergillus niger* MIUG 35), thus Ppy formation was analyzed exclusively in these four cultures. In addition, analysis of the fungi cultures as they constitute 'polymerization bulk solutions' was perfomed in terms of protein and enzymatic yield. Ppy formation pathways according to the above-mentioned enzymatic investigations are included. Subsection 11 named **'Bio-electrochemical applications for polypyrrole (Ppy) formed within fungi cultures'** is focused on testing the applicability of the Ppy obtained through *in situ* biocatalysis within the selected fungi cultures by development of several types of amperometric biosensors. Two different concepts were implemented, one was based on Ppy formation within cell cultures of *Aspergillus niger* for microbial glucose biosensors and one reliant on Ppy formation within culture filtrate of *Trametes pubescens* for enzymatic catechol biosensors.

Subsection 12 represents **'General conclusions, innovations and perspectives'** derived from the experimental investigations, underlining the core importance of the research within the scientific field and offering several perspectives of study follow-up.

The PhD thesis consists of 167 pages including 92 figures and 25 tables. The literature review represents 15% and the experimental study 85%.

The validation of results was performed by publication of four papers in international scientific journals (*Polymer IF 3.68*, *Bulgarian Chemical Communications IF 0.23*, *Bioelectrochemistry IF 3.34*) and international database journals (*Innovative Romanian Food Biotechnology*) and 7 participations at national and international conferences relevant within the biotechnology field.

The research was conducted within several modern research facilities: **The center of research, expertise and technological transfer 'Bioaliment'** *(www.bioaliment.ugal.ro)* of Faculty of Food Science and Engineering; **The Department of Chemistry, Physics and Environment** of Faculty of Science and Environment, 'Dunarea de Jos' Univerisity, Galati; **Center of Nanotechnology and Material Science** and **Department of Physical Chemistry** of Vilnius University, Lithuania; **Department of Polymer Research** of Akdeniz Univeristy, Turkey. The study benefited from scientific and financial support due to author's involvement in Traineeship mobilities (**Erasmus+)** and short-time scientific mission (**SSTM**) funded by **COST Action MP1407**.

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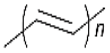
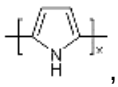
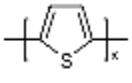
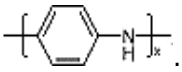
I. Literature review

This review aims to provide data on biocatalytic methods of synthesis for conducting polymers (CPs) and their employment in bio-applications. The manuscript starts by introducing the different types of CPs and their significance in scientific research focusing on polypyrrole (Ppy) as model of analysis. The established synthesis techniques are overviewed, and details are given on 'green' approaches for polymerization. The enzymatic biocatalysts involved are briefly described in terms of origin and catalytic function. Further on, the optoelectrical properties of Ppy are described in accordance with spectroelectrochemical analysis. Ultimately, the extended involvement of Ppy in applications in the biomedical field, biosensors and biofuel cells construction, drug-delivery systems (DDSs) and tissue engineering is specifically discussed.

1. Overview on conducting polymers (CPs)

Some of the most commonly known CPs are polyacetylene (PA)  , polypyrrole (Ppy)  , polythiophene (PT)  , poly(3,4-ethylenedioxythiophene) (PEDOT)  and polyaniline (PANI)  .

1.1 Polypyrrole (Ppy)

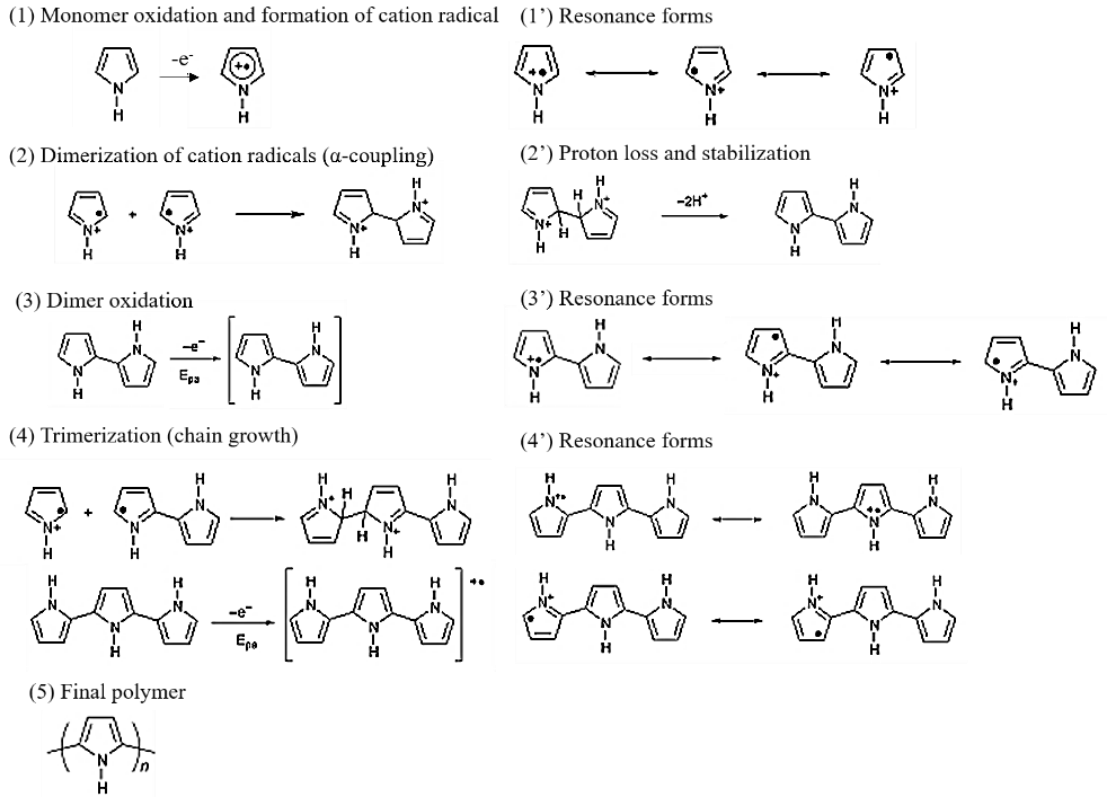


Figure 1.1. The oxidative mechanism for pyrrole polymerization proposed by Diaz et al.[29] (adapted from [24, 30]).

2. Synthesis of conducting polymers (CPs)

The method of synthesis employed in preparation of CPs is fundamental as it tailors the final product for the desired applications. The purpose of this section is the study of CP synthesis methods concerning the suitability for bio-applications.

2.1 Electrochemical synthesis

The electrochemical process consists in using an anodic potential for oxidation of the chosen monomer. Upon oxidation, the monomer moieties become radical cation molecules which due to unpaired electron density are fast to react with their counterparts resulting in dimerization followed by deprotonation leading to formation of polymer chains (Figure 1.1) [8]. The drawback of this method is related to the impediment of large-scale production, because the polymer can only be deposited on conductive and geometrically limited surfaces, thus a limited polymer amount is attained.

2.2 Chemical synthesis

The chemical polymerization offers several advantages over the electrochemical process in that it allows some degree of flexibility of synthesis and polymer processability through covalent modifications [39]. The main hindrance of this type of synthesis is the large amount of oxidant required for monomer oxidation [40].

Whilst chemically and electrochemically prepared CPs are of great applicability in physicochemical fields such as electrochromic displays [45-47], capacitors [48], batteries [49], photovoltaics [50] and anti-corrosive materials [51], bioanalytical fields of research require biocompatible materials with high processability which are preferably, environmentally benign and economically efficient [52]. Thus, 'green' methods of CP synthesis have been explored and will be discussed further.

2.3 Biocatalytic synthesis of conducting polymers (CPs): polypyrrole (Ppy)

Biocatalytic methods for preparation of CPs encompass a wide variety of methods which use natural bio-oxidants and/or biocatalytic quantities of oxidants in environmentally friendly conditions such as physiological temperature and pH without toxic byproducts [53]. For synthesis of π-functional materials, the required enzymes are oxidoreductases, which catalyze the oxidation of monomer molecules into polymer chains [62].

2.3.1 Peroxidase-catalyzed synthesis

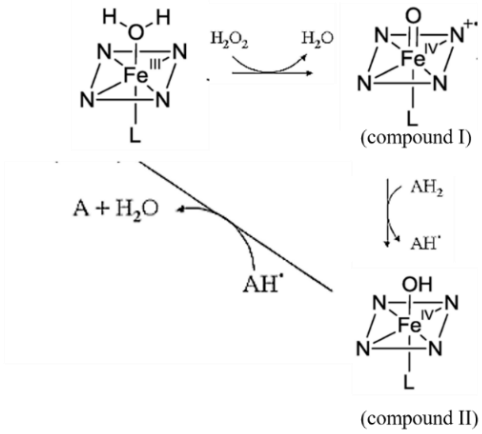


Figure 2.1. Catalytic mechanism of heme-peroxidases (adapted from [62, 74]).

2.3.2 Laccase-catalyzed synthesis

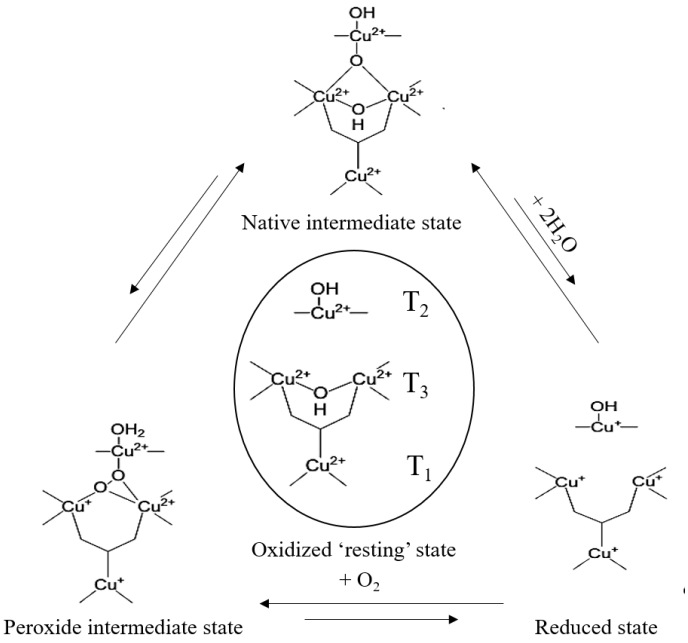


Figure 2.2. Catalytic mechanism of laccases (adapted from [62]).

Laccase catalysis differs to that of peroxidases in that the final electron receiver of the former is molecular oxygen, whilst for the latter hydrogen peroxide is necessary [109]. This is advantageous in CPs synthesis because additional addition of chemical oxidants is not necessary. In addition, laccase exhibits higher stability under acidic conditions [110, 111].

2.3.3 Biosynthesis of conducting polymers (CPs) using other enzymes

The mechanism for CPs polymerization catalyzed by GOx has been introduced relatively recently [27, 151, 152] and it is ingenious in that instead of requiring progressive addition of H_2O_2 as enzyme substrate as it is the case for peroxidases, it uses molecular oxygen and glucose, thus producing H_2O_2 (Figure 2.3).

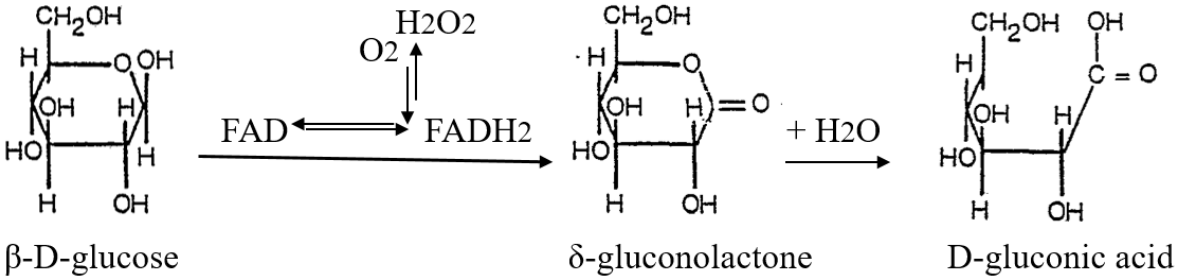


Figure 2.3. Catalytic mechanism of glucose oxidase (adapted from [138]).

2.3.4 Microbial-assisted synthesis of polypyrrole (Ppy)

Polymerization of biocompatible plastics using microorganisms has been available for quite some time [144], however microbe-mediated synthesis of CPs has been only recently attempted. Following aforementioned research that proved that crude enzyme extracts and even iron-proteins in the absence of enzymatic activity can be efficient in polymer synthesis, our research group attempted biosynthesis of Ppy in living cell cultures. *Streptomyces* spp. bacterial cells known to produce phenoloxidases (PPOs) [145] such as laccase were able to induce pyrrole polymerization *in situ* [146]. Further on, *Aspergillus niger* fungi, known GOx producers were similarly employed leading to Ppy-coating of fungal cells though biocatalytic polymerization [147]. The cell-induced Ppy polymerization can also be reliant on oxidative enzymes found within the cell membrane of *Saccharomyces cerevisiae* cells, which due to functionalization with a redox mediator ($Fe(CN)_6$), are able to induce Ppy-yeast cell coating enhancing their applicability in bio-fields as reported by Ramanavicius and colleagues [148, 149]. Another account is given by Zhang et al. [164] which achieved functionalization of *Shewanella oneidensis* cells with Fe^{3+}, that in turn rendered Ppy-coated living bacterial cells, providing enhanced electron transfer in microbial biofuel cells (MBFCs).

3. Characterization and properties of conducting polymers (CPs): polypyrrole (Ppy)

Since the representative feature of CPs is their conductive intrinsic nature, this section will focus on structural and optoelectronic features of Ppy considering the choice in method of synthesis. Particular attention will be given to the spectro-electrochemical properties of Ppy prepared through biocatalysis.

3.1 Fundamental models in the spectro-electrochemical characterization of polypyrrole (Ppy)

The fundamental theoretical model of Ppy characterization was established by Bredas and co-workers [154, 155] and states that the oxidation leads to lengthening of double bonds and shortening of single bonds within the conjugated structure along with localized lattice distortions due to charge carriers. They represent quasiparticles called radical cations or polarons and radical dications or bipolarons. Polarons are formed at low levels of oxidation and possess an electromagnetic spin of ½ and they pair up in double charged spinless bipolarons as the oxidation level increases. Therefore, the electrical conductivity of CPs is a direct result of movement (hopping) of charge carriers (i.e. polarons or bipolarons) along the conjugated chains [10].

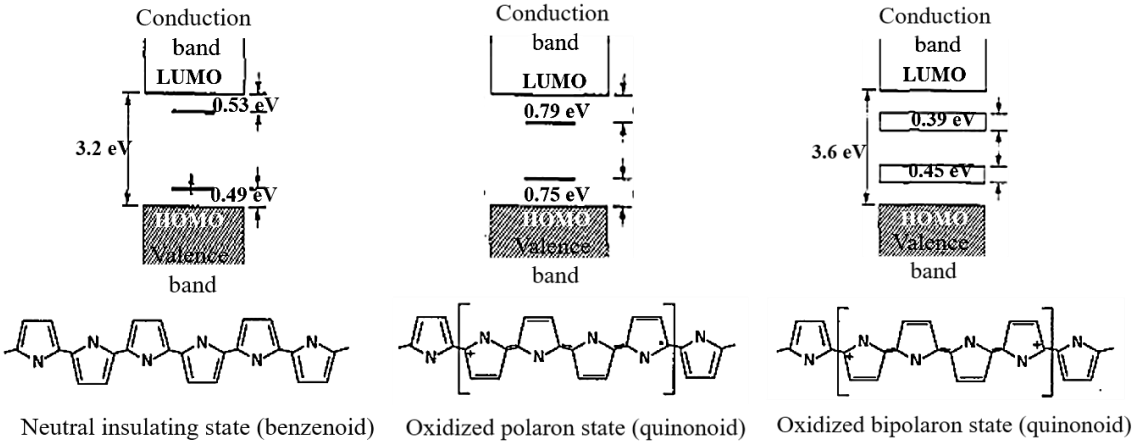


Figure 3.1. Band structure and chemical structure evolution for Ppy at progressive doping stages (adapted from [10, 156]).

The optical characterization correlated to the electrical features of CPs has been further certified by electrochemical [164-168] and structural studies [169, 170]. From an electrochemical perspective, the switching mechanism between conducting and insulating state for Ppy is of paramount importance. The shift in electric features is a result of electron transfer to or from Ppy simultaneously with insertion or repulsion of counterion from the polymer matrix. The reaction can be induced by variation of potential and/or pH and results in mass and volume changes, which are very relevant in bio-fields such as drug-delivery and bioactuators.

3.2 Spectroelectrochemical features of polypyrrole (Ppy) prepared through biocatalysis

Based on the developed models of characterization for electrochemically or chemically prepared Ppy, the interpretation of optoelectrical features of Ppy produced through biocatalysis is available. The spectroelectrochemical characteristics of biosynthesized Ppy prove the mild oxidative effect that the enzymatic biocatalysts possess. The physiological conditions such as aqueous media and neutral pH are seldom suitable for the formation of extensive length and ordered polymeric chains. In most cases, enzymatic catalysis promotes the oligomerization and precipitation of Py moieties due to the nucleophilic effect of aqueous media on the polymer chain. Defects within the conjugated structure are equivalent to loss of electrical conductivity. However, the process can be highly improved by addition of templates and/or redox mediators achieving a final product with adequate conductivity through an environmentally friendly approach. Moreover, the entrapment of enzyme within the polymer structure is a promising feature for bio-applications.

4. Bio-applications for conducting polymers (CPs): polypyrrole (Ppy)

Although CP applications originate in physical chemistry, their biocompatibility was identified and explored for bio-fields from electrical stimulation, growth and adhesion of cells and tissues to drug-delivery, biosensorics and bioactuators [181-186]. Their advantages are low cost, facility and versatility of synthesis as well as ability for tailoring according to specific application requirements. Ppy is representative within bio-applications due to its biocompatibility and electrical properties, namely conductivity and redox switching mechanism. Biosensors and biofuel cells benefit from its conductive nature, interfaced with biomolecules, providing enhanced electron exchange, thus better transduction mechanism, whereas DDSs and tissue engineering applications use the change in mass and volume of Ppy together with its redox shift for entrapment and controlled expulsion of biological molecules and for controlling and monitoring cell activity, respectively. The current section will attempt a review of miscellaneous bio-applications, which highlight Ppy as a suitable biomaterial. In particular, the focus will be aimed at Ppy within an electroanalytic context.

The optimization of CP properties for bio-applications commonly consists of incorporation of bioactive molecules within the polymer structure, achievable through a variety of techniques [187, 188]. Non-covalent methods include physical adsorption and entrapment, whilst a more permanent functionalization can be obtained by covalent modification either pre- or post-polymerization (Figure 4.1).

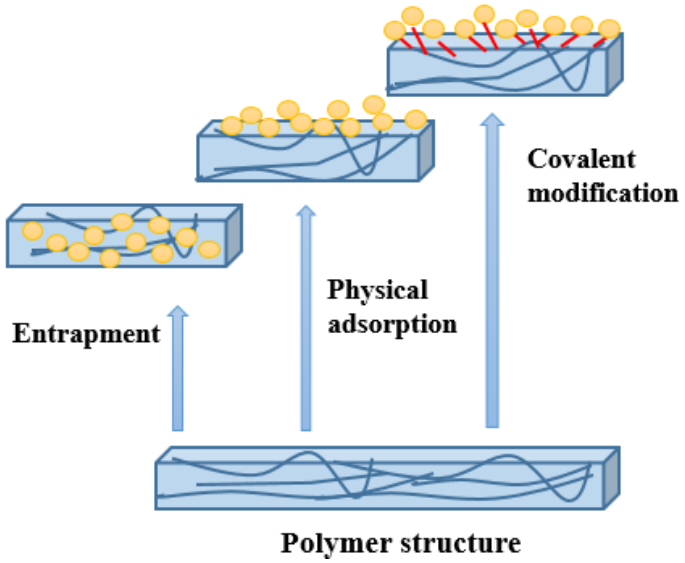


Figure 4.1. Examples for functionalization of CPs (adapted from [189]).

4.1 Biosensors and biofuel cells

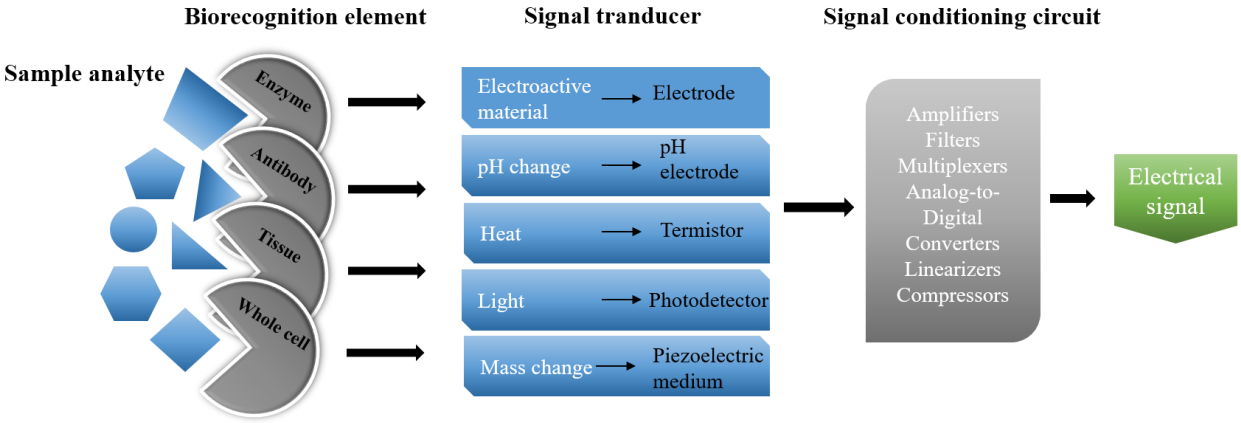


Figure 4.2. Biosensor operating concept (adapted from [233, 235]).

The purpose is to achieve an electrical signal equivalent to the concentration of the specific analyte(s). Commonly, the biomolecule incorporated (e.g. enzyme) exhibits a high level of selectivity, yet it is liable to denaturation because of external factors such as: pH, temperature etc. [236]. Moreover, the bio-specificity can be prejudiced by the electrode selectivity. CPs are suitable for mediating this intimate contact between the biorecognition molecules and the transducer, improving analyte detection [237-239]. However, the hydrophobicity, porosity and surface area of the immobilization matrix must be adjusted accurately.

Overall, Ppy has been widely used in the fabrication of biosensors in numerous fields from medical diagnosis, DNA and immunosensors, to environmental monitoring and food analysis. Even more, the electrical properties of Ppy are employed in biofuel cell development. Biofuel cells are based on the conversion of energy from organic sources to electric current *via* electron transfer [348-350]. The electron transfer can be considerably improved by anode modification with CPs [351], whether in microbial biofuel cells (MBFCs) [352-354] or in enzymatic biofuel cells (EBFCs) [355-357].

Conclusions

The review aimed to highlight the versatility in both synthesis and applicability of CPs. The focus of this study was on the developments made within the requirements of 'green' chemistry and on the advances in biomedical and bioanalytical fields of research. This establishes the prerequisites for *in situ* polymerization of Ppy using biological components such as enzymes, tissues, whole cells etc. together with the applicability of such developed biocompatible Ppy.

II. Experimental setup

5. Biological methods for the preparation of polypyrrole (Ppy)

The microorganism strains tested were provided by the Collection of microorganisms (acronym MIUG) of Bioaliment Research Center, Faculty of Food Science and Engineering, *Dunarea de Jos* University of Galati, Romania. The white-rot fungi strains were acquired from the Culture Collection of Faculty of Biology, *Alexandru Ioan Cuza* University of Iasi. The stock cultures are maintained in 20% glycerol at -70°C. For the proposed study, the following microorganisms were used:

- Bacteria: genus *Streptomyces* spp.
- White-rot fungi: *Trametes* spp.; *Irpex* spp.
- Molds: *Aspergillus spp*. (*A. niger*); *Rhizoctonia* spp.;

Chemicals of the highest analytical grade (Sigma Aldrich) and distilled and ultrapure water were used (Smart-N Water Purification System, 18.2 MΩ.cm), unless otherwise stated. The pH adjustments were done with 1M NaOH or 1M HCl for all experiments. The culture mediums prepared were sterilized before use in autoclave at 121ºC for 20 minutes.

5.1 Cultivation of bacterial strains

All the bacterial strains tested belonged to *Streptomyces* spp. and were cultivated for reactivation in solid state using Gause-agar medium (GMA). The growth media adjusted at pH 7.2-7.4, consisted of agar (2.5 %), potato flakes/starch (2%), K_2HPO_4 (0.05 %), $MgSO_4x7H_2O$ (0.05 %), KNO_3 (0.1%), NaCl (0.05 %), $FeSO_4x7H_2O$ (0.001 %). For enzyme biosynthesis, bacterial strains were further cultivated under submerge cultivation conditions for 14 days at 150 rpm and 25ºC (SI-300 53 Litre Shaker Incubator, Biotechnical Services, Inc.).

5.2 Cultivation of fungal strains

From stock cultures, fungi strains were cultivated in Petri dished on modified Czapek Dox Agar medium consisting of sucrose (6 %), KH_2PO_4 (0.1 %), $MgSO_4$ x $7H_2O$ (0.05 %), $NaNO_3$ (0.6 %), KCl (0.05 %), $FeSO_4$ x $7H_2O$ (0.001 %) adjusted at pH 5.0. For the biosensor experiments, when fungi cultures were used for the preparation of biolayers, the cells were activated in Sabouraud Dextrose Agar (SDA) media. Submerge aerobe cultivation conditions were 27ºC and 180 rpm on shaker incubator (Heidolph® Unimax 1010 Orbital Shaker).

5.3 Pyrrole (Py) addition

Py monomer addition was done in the 6^{th} day of submerge cultivation (30 mM pyrrole/100 mL culture). The chemical polymerization of Py was performed for comparison purposes proceeded by addition of 30 mM monomer and 5 mM hydrogen peroxide (H_2O_2) in 100 mL PBS 0.1 M pH 2.0, method adapted from [17]. The electrochemically synthesized Ppy analyzed with infrared spectroscopy analysis was kindly prepared and provided by the Department of Physical Chemistry of Vilnius University, Lithuania.

6. Culture media/polymerization bulk analysis

6.1 Qualitative test for hydrogen peroxide (H_2O_2)

The test for H_2O_2 was done by taking 1 mL from each cell culture in Eppendorf tubes and subsequent drop-wise addition of $K_2Cr_2O_7$ [367] to observe the reduction of dichromate (VI) to chromate (III) ions by H_2O_2, which results in formation of a deep blue-green color ring. The results were negative for all culture samples.

6.2 Protein content estimation

The protein concentration was determined with Bradford method. The assay provides information about the microgram quantities of protein using the protein-dye binding principle. The Bradford reagent was prepared according to literature protocols [368, 369]. The protein content was deducted from the calibration curve slope (Figure 6.1).

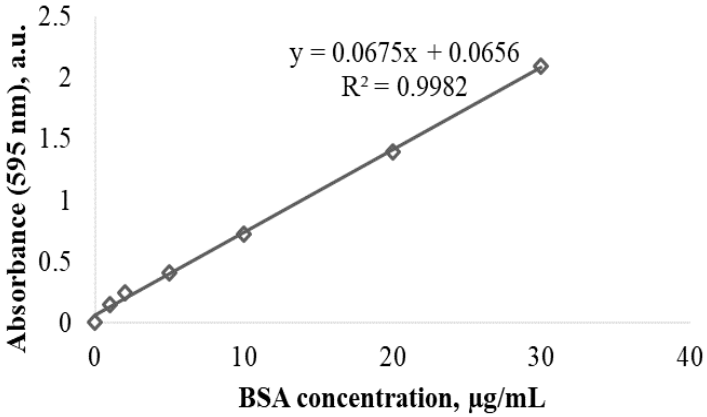


Figure 6.1. Calibration curve for Bradford protocol.

6.3 Phenoloxidase activity assays

All assays were conducted in plastic cuvettes (2 mL; 1 cm path) using the UV-VIS T90$^+$ spectrophotometer (PG Instruments, Lutterworth, UK). The enzyme assay using syrigaldazine as substrate was adapted from Sigma-Aldrich protocols for laccase activity [380]. The total reaction volume (3 mL) consisted of 2.2 mL PBS, 0.3 mL syringaldazine and 0.5 mL enzyme extract. The reaction volume for all other assays except syringaldazine was as 1.5 mL total containing 0.1 M buffer solution, enzyme extract and substrate in equal amounts (0.5 mL/each). The particularities of each buffer solution and the wavelength of each determination are detailed in Table 6.2.

Table 6.2. *Analytical conditions for phenoloxidase activity assays [371-378].*

Substrate	Substrate Concentration, mM	Buffer solution 0.1 M	Wavelength, nm	References (adapted from)
ABTS	1	Acetate pH 5.0	420	[372,373]
Guaiacol	2	Acetate pH 5.0	470	[373,374]
Guaiacol + H_2O_2	2	Acetate pH 5.0	470	[373,374]
Chlorogenic acid	10	Phosphate pH 6.5	400	[374,375]
L-DOPA	10	Phosphate pH 6.5	475	[376]
***p*-cresol**	10	Phosphate pH 6.5	400	[377]
Ferrulic acid	10	Phosphate pH 6.5	410	[378]

Caffeic acid	10	Phosphate pH 6.5	420	[373]
Pyrogallol	10	Phosphate pH 6.5	430	[375]
Pyrocatechol	10	Phosphate pH 6.5	410	[373]
Catechin	10	Phosphate pH 6.5	387	[373]

6.4 Glucose oxidase (GOx) activity assay

The GOx assay was adapted from Sigma-Aldrich protocols [371] and it entailed a mixture of 0.15 mL of 0.21 mM *o*-dianisidine solution, 0.1 mL of 10% D-glucose solution, 0.15 mL peroxidase enzyme solution (60 U/mL) and 0.3 mL enzyme extract or Na acetate buffer solution (50 mM, pH 5.1) for the control sample.

For all the enzymatic assays, the absorbance increase relates linearly to the enzyme content. One unit of enzyme activity was considered the optimal quantity necessary for producing an absorbance change of 0.001 per minute, at specific temperature and wavelength.

6.5 Separation of biomass and intracellular enzyme assays

Intracellular protein estimation and phenoloxidase and GOx enzyme assays were performed for mold (*A. niger*) and white-rot biomass (*Trametes versicolor* and *Trametes pubescens*). In all experiments, wet biomass (1g) was separated from the culture by centrifugation at 1000 rpm for 10 minutes (Hettich® Universal 320R, Sigma-Aldrich), washed with saline solution, subjected to mechanic disruption by grinding with a pestle and 1g silica sand in a mortar and further subjected to ultrasonic homogenization for 10 minutes at 27ºC ((Professional Ultrasonic, GT SONIC). The final enzymatic extract was obtained by filtration of the centrifuged liquid phase [379].

7. Polypyrrole (Ppy) characterization through phisyco-chemical methods and microscopy

7.1 Absorption spectroscopy

The primary analysis was performed in plastic cuvettes (2 mL; 1cm path) using optical spectrophotometer Lambda 25 from Perkin Elmer (Waltham, US).The optical data recorded in the 4th oligomerization day in samples from cell cultures and cell-free culture filtrates (diluted 1:10 with ultrapure water) was considered for the strain selection. Subsequently, samples were collected each day (24h) for 8 days for process monitoring, diluted with ultrapure water (1:20) and pipetted in quartz cuvettes (2 mL; 1 cm path). Spectra was recorded with the UV-VIS T90^{+} spectrophotometer (PG Instruments, Lutterworth, UK). The oligomerization initiated by H_2O_2 was monitored for 6 days.

7.2 Electrochemistry

7.2.1 Cyclic voltammetry (CV) and open circuit voltage (OCV) measurements

The Bio-logic potentiostat SP -150 (Claix, France) featuring a 20 mL electrochemical cell with Pt wire – auxiliary electrode; SCE – reference electrode and GCE - working electrode was used for CV and OCV measurements. The BASi® polishing kit was used for polishing of electrodes, which were also rinsed with ethanol/water before each experiment. The voltammetric measurements were performed at room temperature within the potential range of E = $\pm$ 1V *vs* SCE, at different scan rates from 1-100 mV/s.

Samples for analysis were: (i) 10 mL liquid phase/ culture filtrate separated by centrifugation (1:10 dilution) before and after Py addition; (ii) Ppy-cell culture precipitates suspended in PBS 0.1 M pH 6.0 (iii) Ppy-cell culture precipitates suspended in ultrapure water.

7.2.2 pH measurements

pH measurements were performed using the Multiparameter Consort C862 pH-meter (Turnhout, Belgium) every 24h for 14 days.

7.3 Microscopy techniques

A digital webcam recorder (USB Digital Microscope 500X) was used for imagistic of topographical characteristics of the fungi pellets used in the biosensor construction.

7.3.1 Optical microscopy (OM)

For optical imaging, 1 mL of Ppy-culture precipitate was placed on a glass slide and dried in incubator or at room temperature. Optical data was recorded with Brightfield microscope BX51 from Olympus (Japan) at 40x magnification.

7.3.2 Scanning electron microscopy (SEM)

SEM investigations of the Ppy particles were performed on the same Ppy-culture sediment deposited on an iron slide/covered in aluminium foil (SEM) or on a gold slide (SEM-FIB). For SEM-FIB, additional coating with Pt foil. JEOL electron microscope from JEOL (Boston, US) and FEI Helios Nanolab™ DualBeam™ (Thermo Fisher Scientific) with Ga^+ ion beam were used.

7.4 Fourier-Transform Infrared spectroscopy (FTIR)

FTIR spectroscopy for Ppy formed in bacterial cultures was performed by two methods: (i) KBr pellet method; (ii) Attenuated total reflection (ATR) method. KBr pellet spectra were recorded with 'BRUKER VERTEX 70' FTIR (USA). ATR spectra were recorded with BRUKER ALPHA' FTIR (USA) spectrophotometer. FTIR spectra on Ppy formed in fungi cultures were recorded using the Nicolet iS50 Analytical FTIR Spectrometer and the Software from Thermo Scientific (Waltham, Massachusetts, USA). The collection range was 4000–400 cm^{-1} at the resolution of 4 cm^{-1}.

8. Biosensor construction and principle of measurements

8.1 Amperometric and scanning electrochemical microscopy (SCEM) measurements for *Aspergillus niger* MIUG 35 cells

8.1.1 Treatment of the working electrode and biolayer preparation

The graphite rods (GR) were modified with 0.5 mg wet biomass immobilized with 4.5 µL 1,10-phenanthroline-5,6-dione (PD) and secured with a carbonate membrane (3 µm pores) by a silicone tube to avoid cells from detaching into the solution.

8.1.2 Principle of measurements

Electrochemical investigations were conducted using the PGSTAT 30/Autolab potentiostat (EcoChemie, Utrecht, The Netherlands) and GPES 4.9 software. The three-electrode system was based on a modified graphite electrode, as working electrode, a Pt electrode - counter electrode, and an $Ag/AgCl_{(3M\ KCl)}$ Metrohm reference electrode (Herisau, Switzerland). The experiments were conducted at ambient temperature in PBS 1M, pH 6.0, with 0.1 M KCl and 0.01 M hexacyanoferrate as redox mediator. Electrochemical detection was performed at progressive additions of glucose within the range of 5-300 mM.

8.1.3 Cell immobilization for scanning electrochemical microscopy (SECM) measurements

The wet biomass (1.5 mg) was immobilized with a droplet of poly-L-lysine (0.5 µL) and further cross-linked with glutaraldehyde vapors. Biosensor design is depicted in Figure 11.1.

8.1.4 Evaluation of biomass activity with scanning electrochemical microscopy (SECM)

The electrochemical system included a potentiostat PGSTAT 30 Autolab from EcoChemie, (Utrecht, The Netherlands), SECM from Sensolytics (Bochum, Germany) and a three-electrode based electrochemical cell. A Pt ultramicro electrode (UME) with a diameter of 20 µm was the working electrode, Ag/AgCl - a reference electrode and Pt wire - auxiliary electrode. The SECM analysis was based on collection-generation method by moving the UME in a vertical z axis at 10 µm s^{-1} velocity at +0.4 V potential. From the approaching curve, information such as electrochemical activity and maximal current values were derived.

8.2 Amperometric measurements for *Aspergillus niger* MIUG 34 cells

8.2.1 Biolayer preparation

For simple microbial biosensor, fungi mycelia (wet biomass) after 48 h of cultivation and separation (subsection 6.5) was used. Py monomer (30 mM/100 mL) was added in the *A. niger* MIUG 34 culture at this point and the resulting Ppy-wet biomass mixture was collected after another 72h for construction of the Ppy-microbial biosensor. Biosensor design is depicted in Figure 11.7.

8.2.2 Principle of measurements

The amperometric measurements were performed under continuous constant magnetic stirring (120 rpm) and the oxygen consumption was monitored at negative potential in the range -0.55 - -0.7 V *vs* Ag/AgCl. When hexacyanoferrate ($K_3[Fe(CN)_6]$) was used, the measurements were recorded at +0.4 V *vs* Ag/AgCl. The experiments were conducted at ambient temperature in PBS 0.1 M. Experiments were conducted using Gamry potentiostat and Gamry Echem Analyst software for data analysis. A conventional electrochemical system using a reference electrode (Ag/AgCl), auxiliary electrode (Pt wire) and a working electrode (GCE) was used. The BASi® polishing kit was used for polishing of electrodes, which were also rinsed with ethanol/water prior to each experiment.

8.3 Amperometric measurements for *Trametes pubescens* (*TP*) enzyme extract

8.3.1 Biolayer preparation

For the first biosensor (*TP* biosensor), the crude enzyme (72 h of submerge cultivation) was used. Py monomer (30 mM/100 mL) was added in the culture at this point. The resulting Ppy was collected after 96h for for development of a second type of biosensor (BioPpy biosensor). The third type of biosensor was based on an equal mixture (1:1) of bioproduced Ppy and enzyme extract (BioPpy-*TP* biosensor). The fourth type of biosensor entailed an equal mixture (1:1) of chemically produced Ppy (ChemPpy) and enzyme extract (ChemPpy-*TP* biosensor). Biosensor design is depicted in Figure 11.16.

8.3.2 Principle of measurements

The measurements were based on oxygen consumption at negative potential within -0.55 and -0.8 V *vs* Ag/AgCl. Catechol was selected as the model phenolic compound. Progressive concentrations of catechol were added in steady-state conditions under continuous constant magnetic stirring (120 rpm) and the oxygen consumption was monitored amperometrically. Experiments were performed using Gamry potentiostat and Gamry Echem Analyst software for data

analysis. A conventional 3-electrode electrochemical system using Ag/AgCl reference electrode, Pt wire as counter electrode and GCE as working electrode was used for amperometric measurements The BASi® polishing kit was used for polishing of electrodes, which were also rinsed with ethanol/water prior to each experiment.

8.3.3 Real sample investigations

For analysis in real system, three fruit wines: blueberry, blackberry and pomegranate (Kutman Winery brand) were selected. To compare the performance of the *TP*-based biosensors, Folin-Ciocalteau spectrophotometric assay for phenol detection was employed [382]. For spectrophotometric wine analysis, the gallic acid was replaced with 1:10 diluted wine sample (20 µL). The results were calculated by interpolating with the absorbance values for the calibration samples.

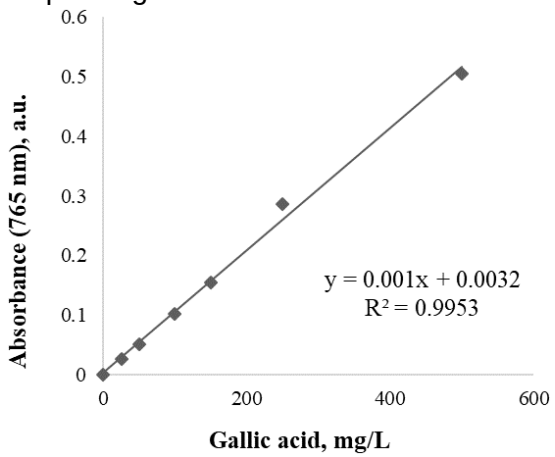


Figure 8.4. Folin-Ciocalteau standard calibration curve for total phenol content.

8.4 Statistic analysis and interpretation of results

All the experiments were performed in triplicate and the media between minimum three separate determinations was used. Microsoft Excel was used as software for determination of mean values, standard deviation and variance. Hyperbolic dependency between amperometric signals and analyte concentration was observed and the calculation of kinetics parameters such as the maximal current (I_{max}) and the apparent Michaelis-Menten constant (Km) were determined from the analysis of the reciprocal curve to the calibration plot (amperometric current (I) *vs* substrate concentration (C)), i.e. the Lineweaver–Burk slope (1/I *vs* 1/C). Biosensor sensitivity was calculated as follows: Sensitivity (µA/mMcm^{-1}) = Slope/Electrode Area (cm^{-1}). The limit of detection (LOD) was calculated based on the response to minimum ten separate additions of 0.01 mM analyte using the formula: 3x Standard Deviation of Low Concentration/ Slope [381].

III. Results and discussion

9. Selection and characterization of microorganism strains involved in the bioproduction of polypyrrole (Ppy)

The primary objective of this research was to test different types of microorganisms in order to select the ones able to initiate pyrrole (Py) bioconversion into polypyrrole (Ppy). The performance of the tested microorganism cultures was based on Ppy formation, which was assessed by a number of techniques. Firstly, microscopy imaging including optical microscopy (OM), scanning electron microscopy (SEM) and focused ion beam microscopy (FIB) were employed for direct visualization of Ppy. Additionally, infrared spectroscopy (FTIR) analysis was performed for chemical identification of

Ppy through detection of chemical bonds. The selection of suitable cultures and the primary characterization of the Ppy formed within them will be the focus of the present chapter.

9.1 Polypyrrole (Ppy) bioproduction within filamentous bacteria cultures

9.1.2 Formation of polypyrrole (Ppy) within *Streptomyces* spp. cultures

A qualitative indicator of strain suitability was the visible darkening of the cultures after Py monomer addition. Out of 25 strains, 11 strains displayed visible darkening within 24h, indicator of Py bioconversion into 'dark' Ppy. The suitable strains are listed in Table 9.2.

Table 9.2. *Ability of Streptomyces spp. cultures to initiate Ppy bioproduction.*

***Streptomyces* spp. code**	**Ppy bioproduction**	***Streptomyces* spp. code**	**Ppy bioproduction**
MIUG 12p	☑	RA	☒
MIUG 4.88	☑	RD	☑
MIUG 4.89	☑	RE	☑
LP1	☑	1b4	☒
LP2	☑	b2	☒
B	☑	41	☑
3Fi	☒	46	☒
3Fs	☒	72	☑
3Fsc	☒	107	☒
S1	☑	LH	☒
S3	☒	8p	☒
MIUG 4.38	☒	SNA	☒
V3C	☒		

9.1.3 Identification of polypyrrole (Ppy) formed in *Streptomyces* spp. cultures by optical microscopy (OM)

The formation of dark round Ppy microparticles around bacterial colonies was observed within 48h. Two Ppy-modified cultures samples belonging to *Streptomyces* spp. coded MIUG 12p and MIUG 4.88 were taken as model for analysis.

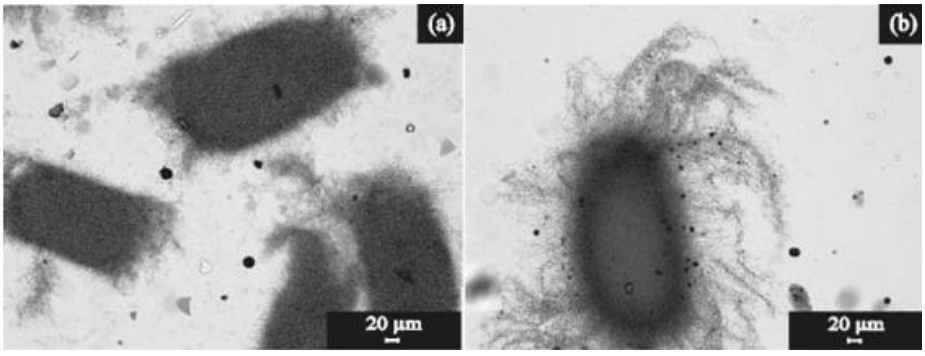


Figure 9.3 Ppy identification within *Streptomyces* ssp. cultures: (a) MIUG 12p; (b) MIUG 4.88.

Further on, optical evaluation of Ppy formation in all the suitable *Streptomyces* ssp. cultures (Table 9.2) was conducted and the identification of the Ppy formed is displayed in Figure 9.4.

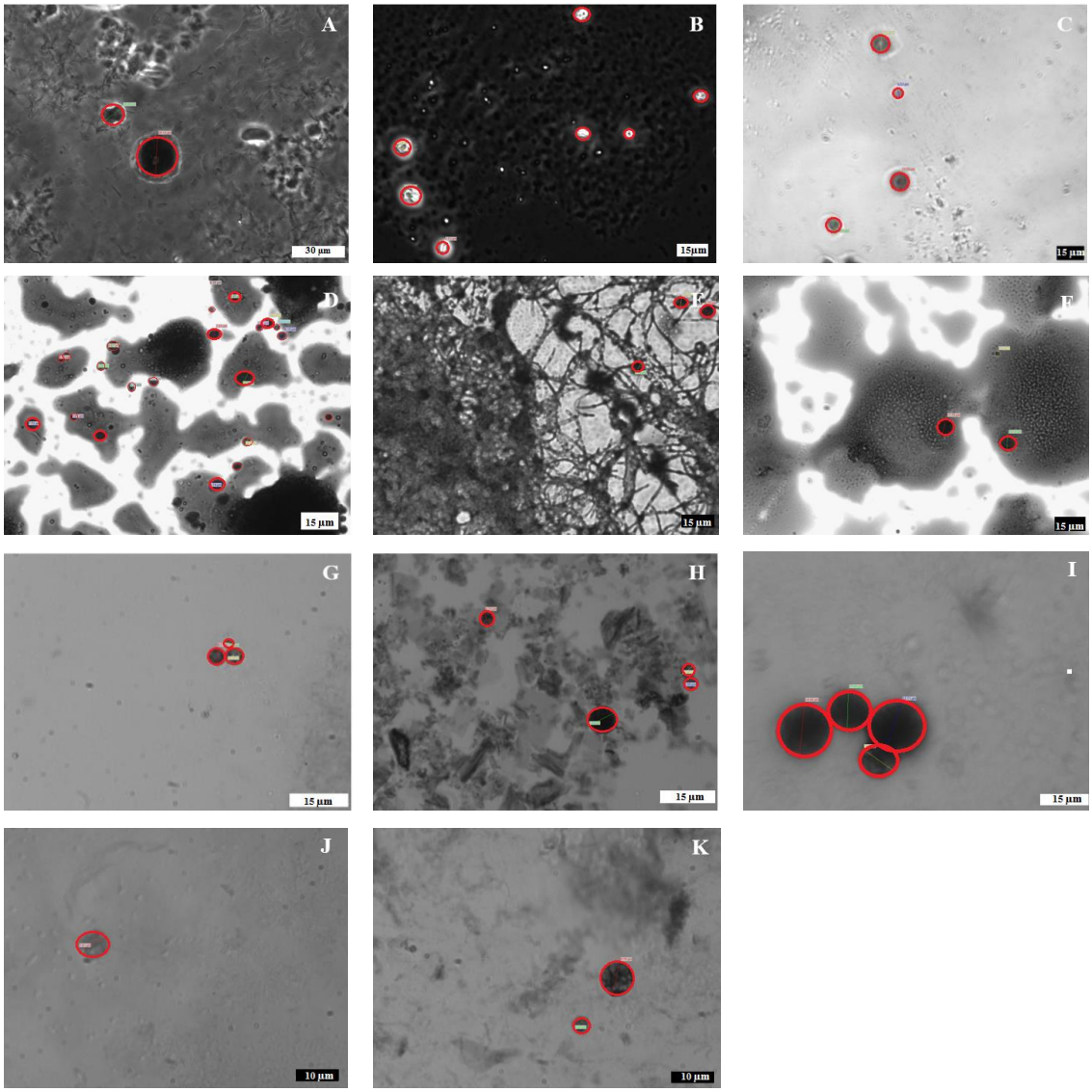


Figure 9.4. Ppy identification within *Streptomyces* ssp. cultures coded (from left to right): (A) MİUG 12p; (B) MİUG 4.88; (C) MİUG 4.89; (D) LP1; (E) LP2; (F) B; (G) S1; (H) RD; (I) RE; (J) 41; (K) 72.

The differences in size and frequency noticed by identification of Ppy through OM (Figure 9.4 (A-K)), can be attributed to the different oxidative efficiencies of the *Streptomyces* spp. cultures, catalyzing the Py bioconversion at different rates. On such note, it was observed that a low concentration of Ppy particles was associated with a high particle size as is the case of Ppy-MIUG 12p and a higher frequency of Ppy particles was associated with smaller Ppy particles as described for Ppy-MIUG 4.88. Presumably, a fast oligomerization process in some cultures, such as *Streptomyces* ssp. MIUG 12p, leads to conglomeration of particles, explaining the high diameter and low particle frequency.

9.1.4 Identification of polypyrrole (Ppy) formed within *Streptomyces* spp. cultures by scanning electron microscopy (SEM) and focused ion beam (FIB)

Additional morphological and topographical investigation on the Ppy structures was provided by SEM and SEM-FIB.

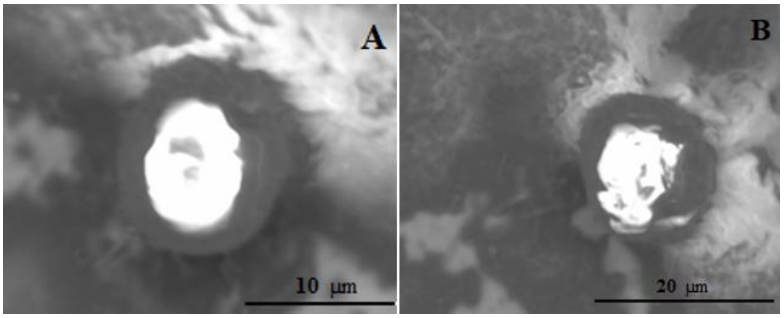


Figure 9.5. Ppy identification within *Streptomyces* ssp. cultures by SEM: (A) MIUG 12p; (B) MIUG 4.88.

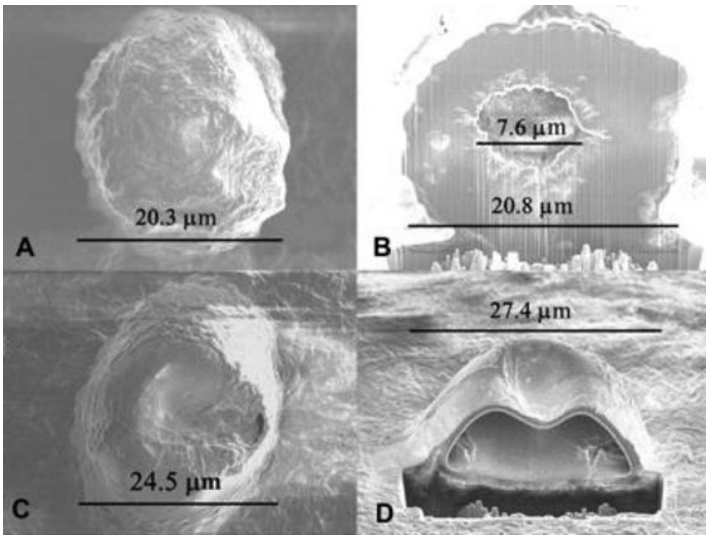


Figure 9.6. FIB-SEM images of: (A, B) Ppy - MIUG 12p; (C, D) Ppy - MIUG 4.88; A, C - surface view; B, D - interior section.

The cross-section perspective introduced by FIB technology confirmed the hollow interior of the Ppy particles, which is an interesting feature that can be useful for applications that require immobilization of desired components and controlled release such as drug-delivery systems (DDSs) [183, 215].

As observed previously from OM, Ppy-MIUG 12p particles tend to have higher dimensions and lower frequency than Ppy-MIUG 4.88 ones. Thus, the compactness of the Ppy-MIUG 12p structure depicted above can be related to the conglomeration process that was previously discussed. It is feasible that the fast polymerization rate allows for additional polymer growth with agglomeration of smaller particles into more compact structures.

9.1.5 Structural identification and characterization of polypyrrole (Ppy) formed within *Streptomyces* spp. cultures by infrared spectroscopy (FTIR)

FTIR represents an infrared spectroscopy technique that is able to identify chemical compounds through detection of chemical bonds. The present study is based on the premise that Py addition in oxidoreductase-yielding cultures leads to Ppy formation. The validation of this assumption was ultimately given by infrared analysis of the formed microparticles.

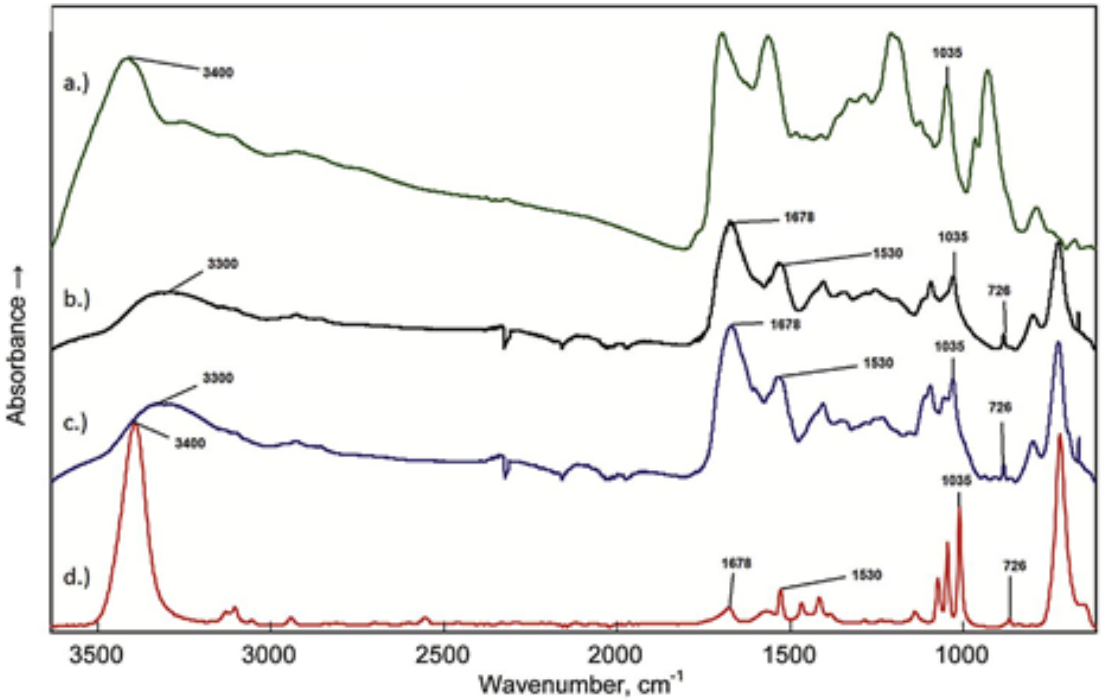


Figure 9.7. FTIR spectra of: (a) Electro-Ppy; (b) Ppy- MIUG 12p; (c) Ppy-MIUG 4.88; (d) Py.

The characteristic Ppy peaks are present, as follows: 1530 cm^{-1}, indicative of stretching of the pyrrole ring (C-C/C=C); 1450-1300 cm^{-1} ,C-C and C-N conjugated stretching; 1236 cm^{-1}, C-N in-plane deformation; 1035 cm^{-1} and 726 cm^{-1}, descriptive of N-H wagging vibrations. The extensive band recorded between 3300-3400 cm^{-1} in the studied samples is attributed to the stretch vibrations of the pyrrole ring N-H and is commonly associated with pristine Ppy [386, 387]. The absorption band at 1678 cm^{-1} is known to correspond to the stretching vibration of C=O group, due to the aqueous polymerization environment and its effect on the polymer structure.

9.2 Polypyrrole (Ppy) bioproduction within fungi cultures

Upon analysis of Ppy formation within bacterial cultures, the process was further explored. The observations on bacterial culture-assisted catalysis such as the preparation facility, environmentally friendly and economical aspects inspired further exploring of microbial mediated pathways for Ppy formation.

9.2.2 Formation of polypyrrole (Ppy) within fungi cultures

Following the visual observation of culture darkening as indicator for the ability of microorganism cultures to initiate Ppy bioproduction, two out of five white-rot cultures were highlighted, listed in Table 9.5. Two out of five common mold cultures were able to initiate Ppy bioproduction, listed in Table 9.6.

Table 9.5. *Ability of white-rot fungi cultures to initiate Ppy bioproduction.*

Strain classification	Ppy bioproduction
Trametes versicolor	☑
Trametes pubescens	☑
Trametes hirsuta	☒
Trametes gibosa	☒
Irpex lacteus	☒

Table 9.6. *Ability of common mold fungi cultures to initiate Ppy bioproduction.*

Strain isolation code	Ppy bioproduction
MIUG 34	☑
MIUG 35	☑
KM 53	☒
KM 56	☒
KM 55	☒
KM 57	☒

9.2.3 Identification of polypyrrole (Ppy) formed in fungi cultures by microscopy techniques

The microscopy evaluation of Ppy particles will be presented onward for the two different types of fungi tested.

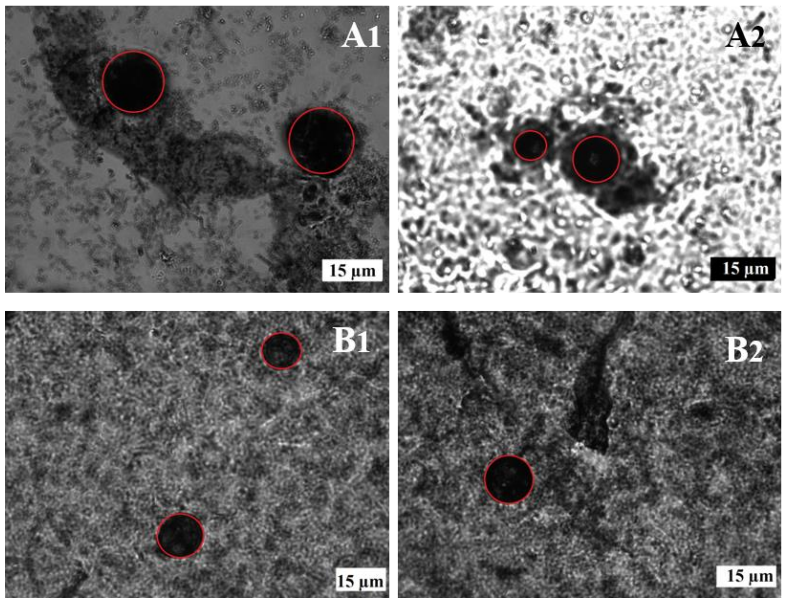


Figure 9.12. Ppy identification within white-rot fungi cultures: (A_1, A_2) *TV*, (B_1, B_2) *TP*.

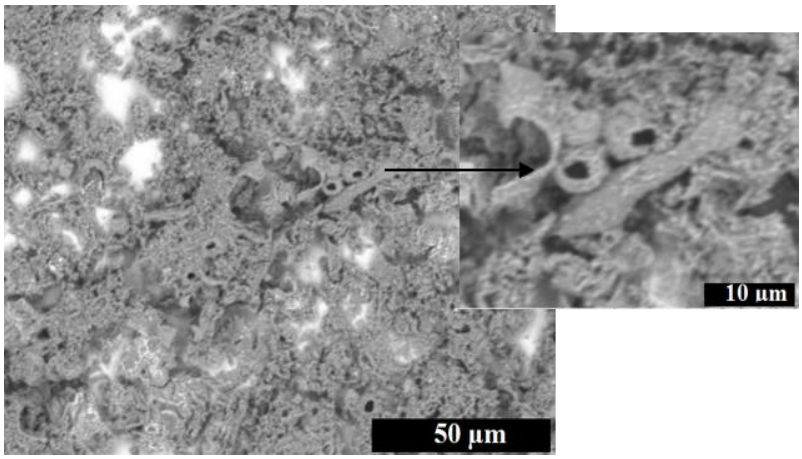


Figure 9.13. Ppy identification within *TV* culture by SEM.

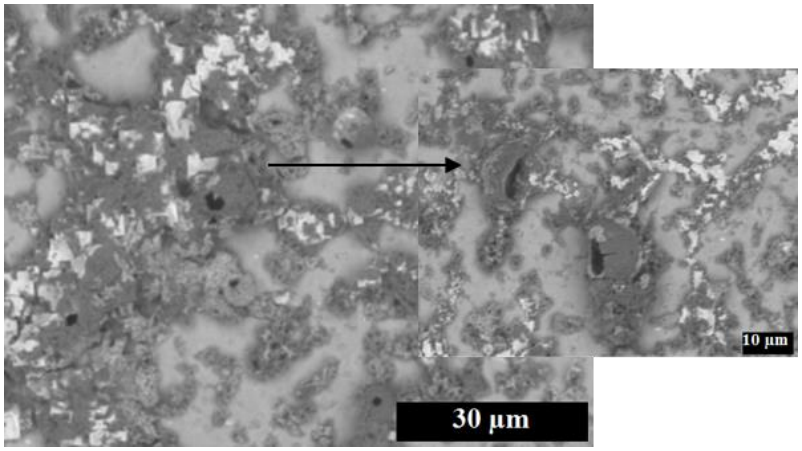


Figure 9.14. Ppy identification within *TP* culture by SEM.

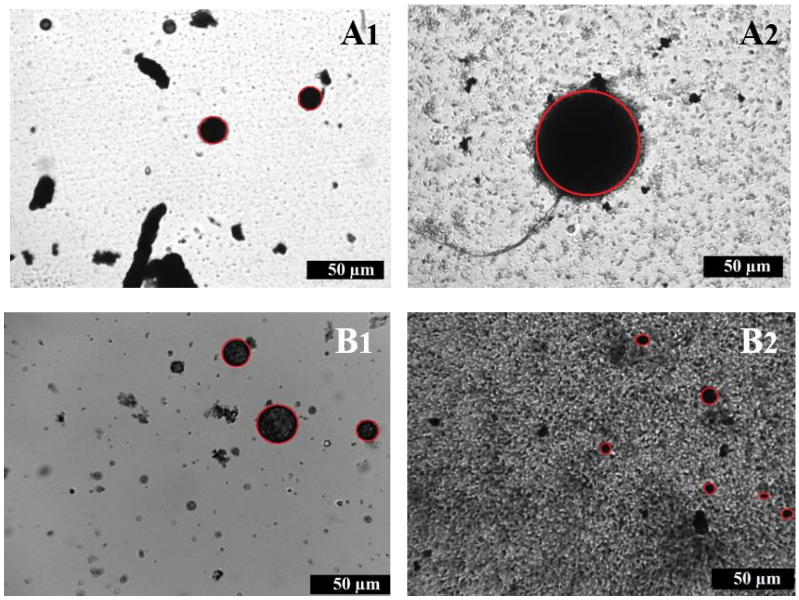


Figure 9.15. Ppy identification within common mold fungi cultures: (A_1, A_2) *A. niger* MIUG 34; (B_1, B_2) *A. niger* MIUG 35.

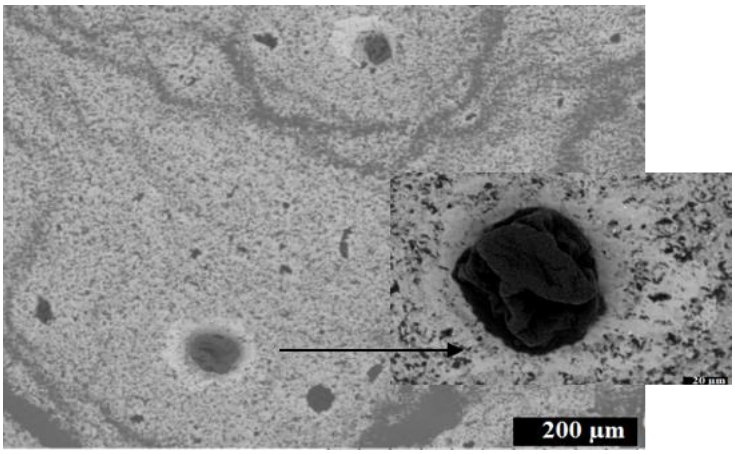


Figure 9.16. Ppy identification within *A. niger* MIUG 34 culture by SEM.

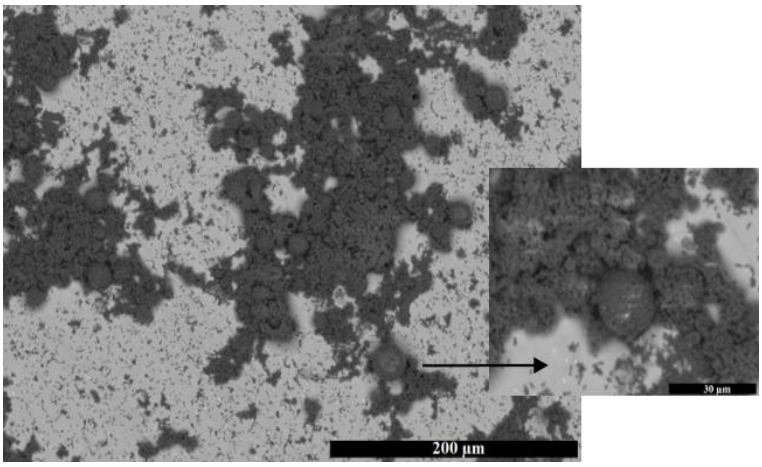


Figure 9.17. Ppy identification within *A. niger* MIUG 35 culture by SEM.

From the optical evaluation conducted, it can be assessed that the frequency of Ppy particles is lower in white-rot cultures than was observed in *Streptomyces* spp. cultures whilst the particle size is similarly in the diameter range of 10-20 µm.

Investigating the Ppy formed with common fungi during submerge cultivation, a few conclusions can be drawn: (1) the frequency of Ppy-MIUG 35 particles is higher than Ppy-MIUG 34; (2) the particle size is considerably higher for Ppy-MIUG 34 particles. The diameter of the Ppy particle illustrated in Figure 9.16 (Ppy-MIUG 34) is approx. 75 µm while the ones in Figure 9.17 (Ppy-MIUG 35) vary in diameter between 10-30 µm.

SEM imagistic showed that the Ppy particles formed in common fungi cultures have similar structural characteristics as the ones identified in white-rot fungi and bacterial cultures such as low density and tendency for mechanical distortion under action of external factors. It was observed that Ppy bioproduction is particularly favoured in fungi cultures rather than bacterial ones due to shorter timeframe for formation of Ppy particles. This can be attributed to higher yield of oxidoreductases and/or more suitable pH of synthesis.

9.2.4 Structural identification and characterization of polypyrrole (Ppy) formed in fungi cultures by infrared spectroscopy (FTIR)

FTIR spectra provides valuable insight into the structural features of charged species such as CPs due to ability to visualize the increased level of conjugation, i.e. stretching of double bonds and shrinking of single bonds, equivalent to conductivity increase [177, 393-395]. The tentative assignments of the main infrared bands according to literature data [387, 396, 397] are listed in Tables 9.7 and 9.8 (details on FTIR protocols are given in Section II, subsection 7.4).

Table 9.7. *Peak assignments for FTIR spectra of Ppy produced within white-rot fungi cultures: Ppy-TV and Ppy-TP.*

Assignment	Ppy-*TV*	Ppy-*TP*
C-H / N-H in-plane deformation	1035 cm^{-1}	1035 cm^{-1}
C-N stretching	1396 cm^{-1}	1396 cm^{-1}
C–C / C=C backbone stretching	1532 cm^{-1}	1532 cm^{-1}
C=O stretching vibrations	1624 cm^{-1}	1624 cm^{-1}
CH_2 asymmetric stretching and symmetric vibrations	2920 cm^{-1}	2920 cm^{-1}
N-H / C-H stretching vibrations	3270 cm^{-1}	3270 cm^{-1}

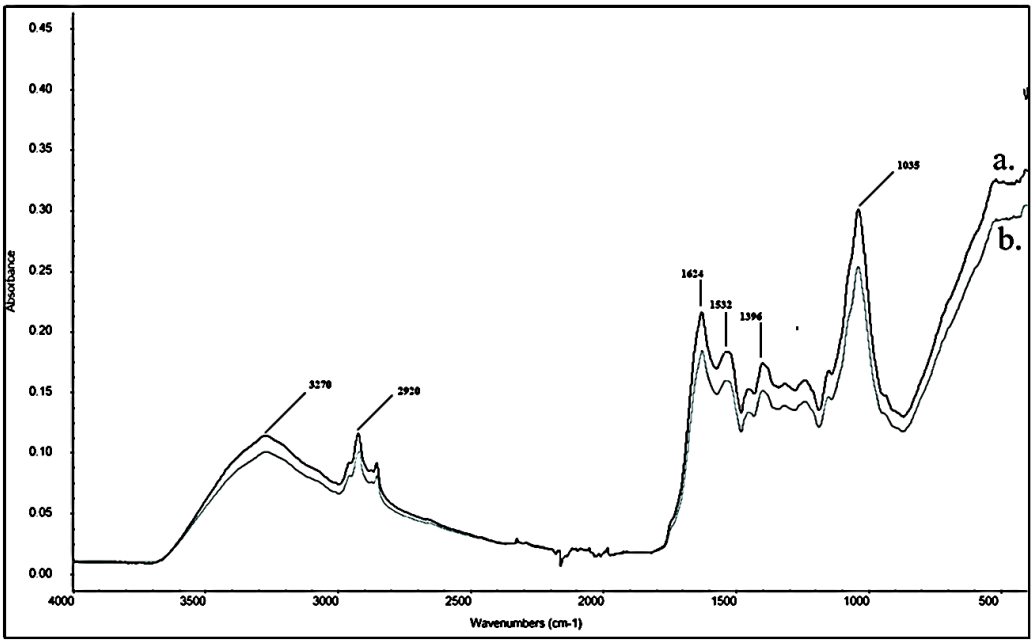


Figure 9.18. FTIR spectra of Ppy produced within white-rot fungi cultures: (a.) Ppy-*TV*; (b.) Ppy-*TP.*

Table 9.8. *Peak assignments for FTIR spectra of Ppy produced within common fungi cultures: Ppy - MIUG 34 and Ppy - MIUG 35.*

Assignment	Ppy-MIUG 34	Ppy-MIUG 35
C-H / N-H out-of-plane deformation	1027 cm^{-1}	1027 cm^{-1}
ring breathing vibration	1148 cm^{-1}	1148 cm^{-1}
C-H / C-N in-plane deformation	1247 cm^{-1}	1247 cm^{-1}
C-N conjugated stretching	1374 cm^{-1}	1374 cm^{-1}
C–C / C=C backbone stretching	1548 cm^{-1}	1548 cm^{-1}
C=O stretching vibrations	1744 cm^{-1} - 1640 cm^{-1}	1744 cm^{-1} - 1640 cm^{-1}
CH_2 asymmetric stretching and symmetric vibrations	2923 cm^{-1} - 2853 cm^{-1}	2923 cm^{-1} - 2853 cm^{-1}
N-H / C-H stretching vibrations	3276 cm^{-1}	3273 cm^{-1}

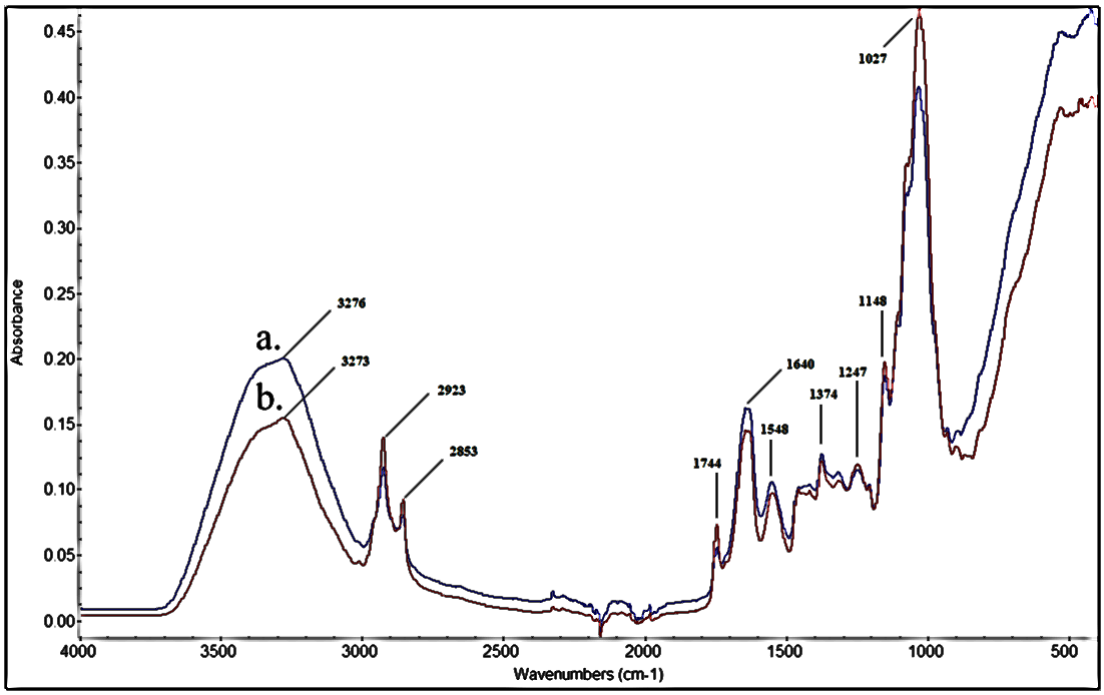


Figure 9.19. FTIR spectra Ppy produced within common fungi cultures: (a.) Ppy-MIUG 34; (b.) Ppy-MIUG 35.

The spectrums presented in both figures proves that the microparticles formed in fungi cultures have the chemical bonds descriptive of Ppy.

Conclusions

The leading aim of this research was the selection of microorganism strains able to induce Py bioconversion into Ppy in an environmentally friendly, economical and efficient manner. For this purpose, twenty-five filamentous bacteria strains belonging to *Streptomyces* spp. genus, five white-rot fungi strains belonging to *Trametes* spp and *Irpex* spp. genres and six common fungi strains belonging to *Aspergillus niger* and *Rhizoctonia* spp. genres were tested through cultivation followed by Py addition. *Streptomyces* ssp. strains were firstly tested and Ppy particles were observed in eleven out of twenty-five sampled cultures. From the fungi strains tested, Ppy particles formed only in four culture samples, two white-rot fungi belonging to *Trametes* spp. and two common fungi strains belonging to *Aspergillus niger* sp..

The observation of Ppy particles was performed through different microscopy techniques. OM showed the Ppy structures formed as spherical micro-dimensioned particles in all samples. The culture samples had different efficiencies in initiating Ppy bioproduction, thus Ppy particles size and frequency proved to be dependent on the microorganism culture used as 'polymerization bulk'. Subsequently, SEM and FIB microscopy were employed for three-dimensional and cross-section perspectives of the polymer particles formed.

FTIR analysis identified the particles as composed of Ppy through detection of chemical bonds. Moreover, this technique is able to provide information on the structure of CPs. The prevalent vibrational bands, specific of Ppy were similar in the three different type of cultures (bacterial, white-rot and common fungi). Apart from the main vibration bands owing to chemical structure of Ppy, the additional peaks were indicative of low oxidation level of the Ppy formed and of effect of acetate group within polymer backbone due to the aqueous media.

Although microbial cultures represent highly complex environments that cannot allow Ppy yield quantification, based on the performance observed in this step of the study, only the four fungi strains (*TV*, *TP*, *A. niger* MIUG 34, *A. niger* MIUG 35) were selected to be further employed for the research. The proposed research is in its preliminary stages, promoting the novelty in Ppy bioproduction, using sustainable resources.

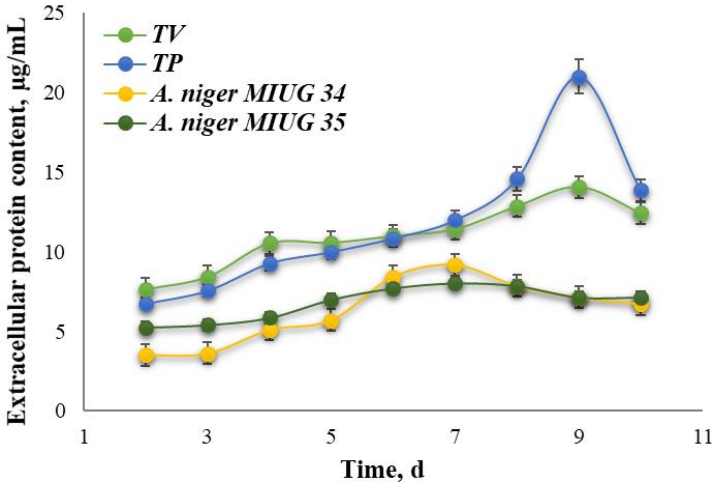
10. Spectroelectrochemical monitoring and characterization of polypyrrole (Ppy) formation within fungi cultures

This chapter is devoted to monitoring and characterization of the process leading to Ppy bioproduction. Since the proposed concept is based on the hypothesis that the selected microorganism strains yield oxidoreductases in submerge cultivation conditions, the primary objective was the analysis of the four fungi cultures used as 'polymerization bulk solutions'. Protein yield estimations and enzyme assays for phenoloxidases (PPOs) and glucose oxidase (GOx) were performed for each culture media. Ppy formation pathways according to the enzymatic investigations are proposed.

Insight into the evolution of the oligomerization process and on the optolelectric properties of the formed Ppy was acquired through absorbance spectroscopy and electrochemical measurements (i.e. cyclic voltammetry, open circuit potential, pH).

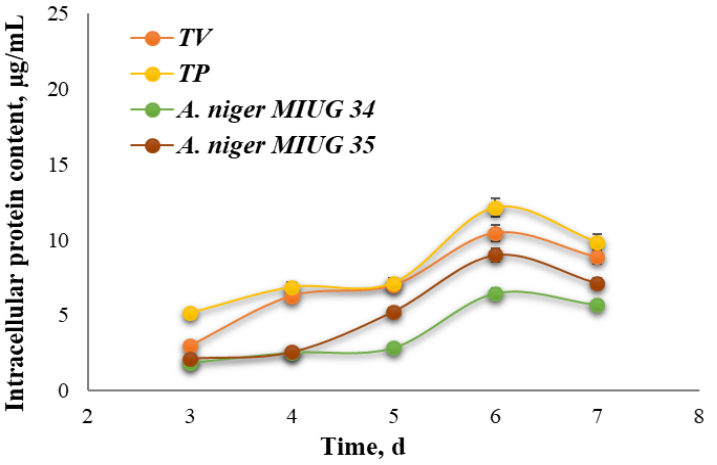
10.1 Characterization of the fungi cultures as polymerization bulk solutions

10.1.2 Protein biosynthesis within fungi cultures



The results describe higher protein yield in the white-rot fungi cultures (*TV*, *TP*) than in the common fungi cultures (*A. niger* MIUG 34, *A. niger* MIUG 35) both at extracellular and intracellular level. Yet, the difference between culture samples is emphasized mostly extracellularly. The importance of assessing protein biosynthesis within the fungi cultures derives from the fact that protein secretion is a key process in fungal growth [400].

Figure 10.1. The estimation of extracellular protein content within fungi cultures.

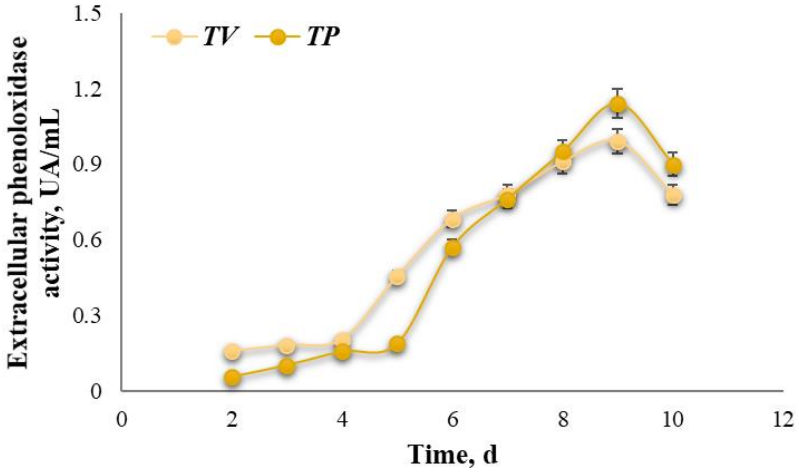


Since the proposed concept is based on the yield of oxidative components by the fungi strains, able to initiate Py oligomerization, the moment of Py addition was decided according to the evolution of fungal growth.

Figure 10.2. The estimation of intracellular protein content within fungi cultures.

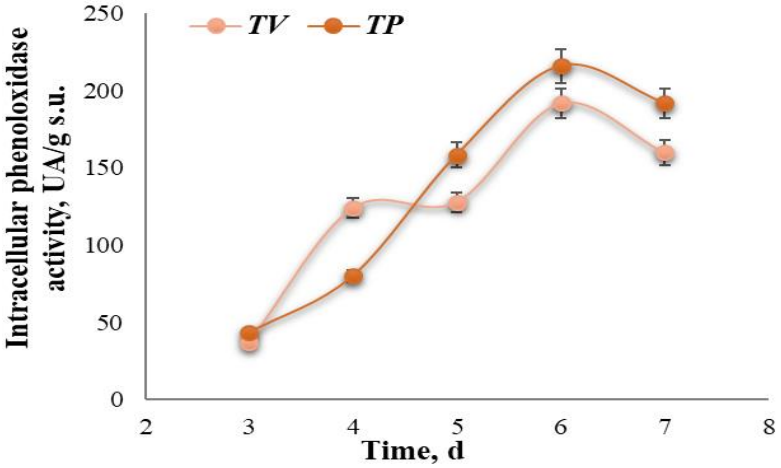
10.1.3 Polyphenoloxidase (PPO) biosynthesis within fungi cultures

Some of the most common biocatalysts employed for this purpose are reviewed in Section I, subsection 2.3 and include peroxidases, laccase and glucose oxidase (GOx). The research ruled out peroxidase because no H_2O_2 was added during oligomerization and the qualitative test for endogenous H_2O_2 was negative (subsection 10.1.1). Thus, the enzymatic assays focused on laccase as part of the polyphenoloxidase (PPO) group and GOx.



The extracellular assay shows increasing PPO activity until 9th day of cultivation for both culture samples (Figure 10.3).

Figure 10.3. Extracellular phenoloxidase activity within white-rot fungi cultures.



Intracellular determinations depict increasing activity until 6th day of cultivation (Figure 10.4).

TP culture reveals slightly higher enzyme yield in comparison with *TV*, as anticipated from protein content estimations. The results of enzymatic assays are in agreement with literature data on PPO biosynthesis by white-rot fungi strains [412-414].

Figure 10.4. Intracellular phenoloxidase activity within white-rot fungi cultures.

The enzyme yield indicates that the white-rot fungi cultures are suitable as polymerization environments whether the process occurs in the presence or absence of living cells since enzyme biosynthesis occurs both at extra- and intracellular level.

The difficulty in differentiating between laccase and other types of phenol oxidases derives from assay sensitivity, which is highly dependent on substrate efficiency [416]. Thus, to obtain additional insight on the type of PPOs biosynthesized within the selected white-rot fungi cultures, the extracellular enzyme extract was tested against 11 different substrates. This should provide an understanding of the catalytic origin and efficiency of these two cultures and their role in Py oxidation.

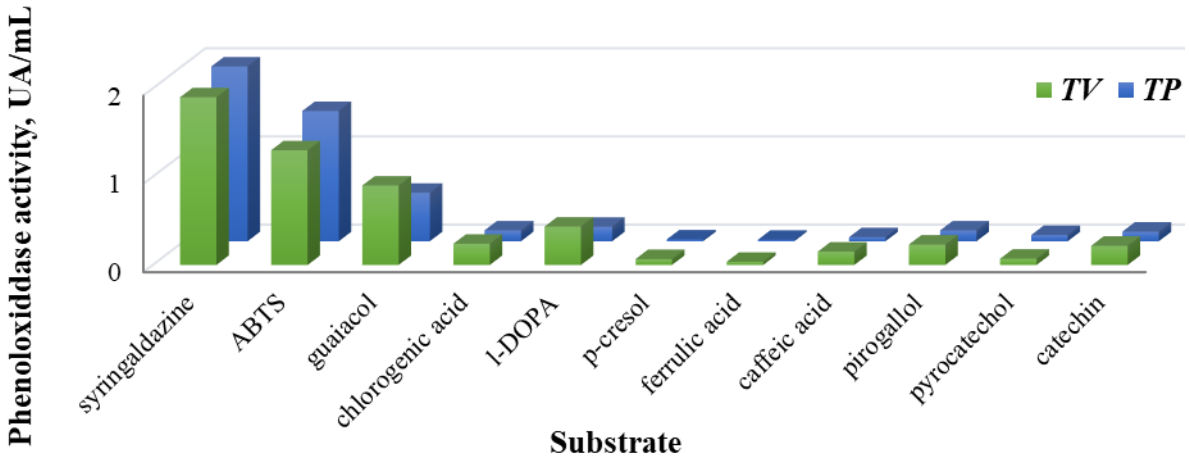


Figure 10.5. Extracellular PPO activity towards different substrates.

Highest enzyme activity was recorded for common laccase substrates such as ABTS and syringaldazine, thus suggesting that the prevalent enzyme produced within the two fungi cultures behaves like a laccase [97, 105, 417].

The mechanism of laccase-based Ppy production was identified in literature data and it entails the redox reaction between Cu(II) and Cu(I) occurring at the active center of the enzyme, resulting in the oxidation of four Py moieties by the four copper ions, followed by dimerization and polymerization. Accordingly, the proposed mechanism for laccase-catalyzed Ppy formation is depicted in Figure 10.6.

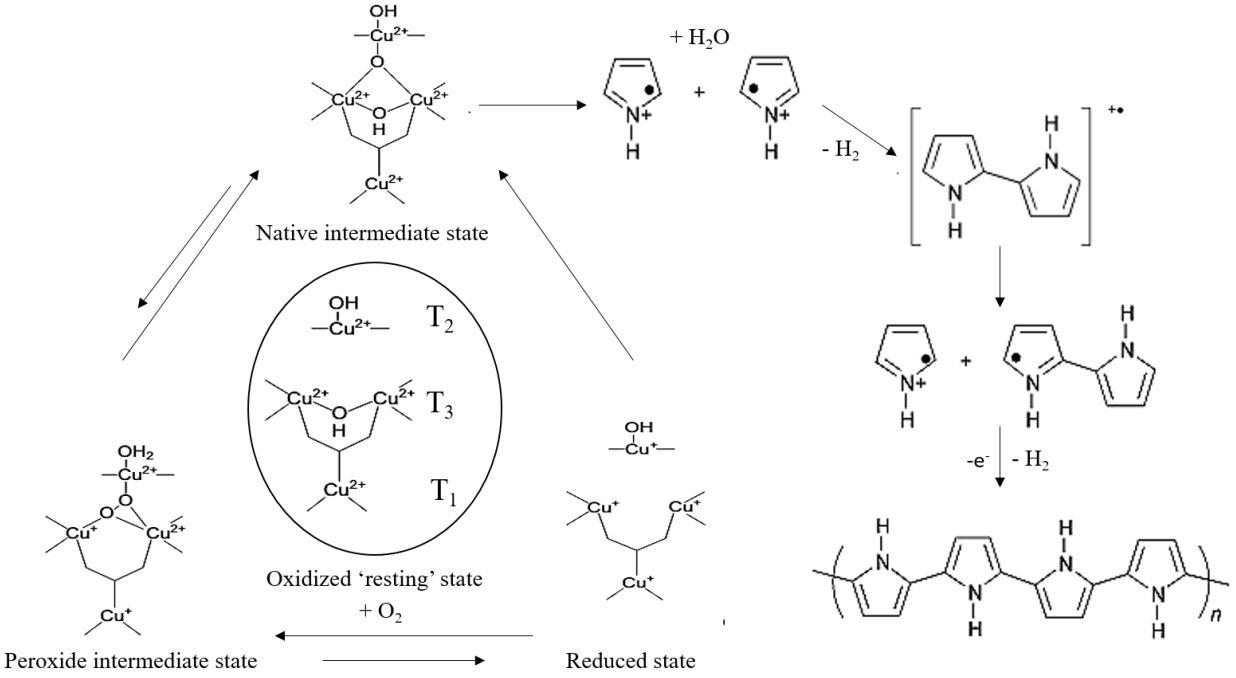
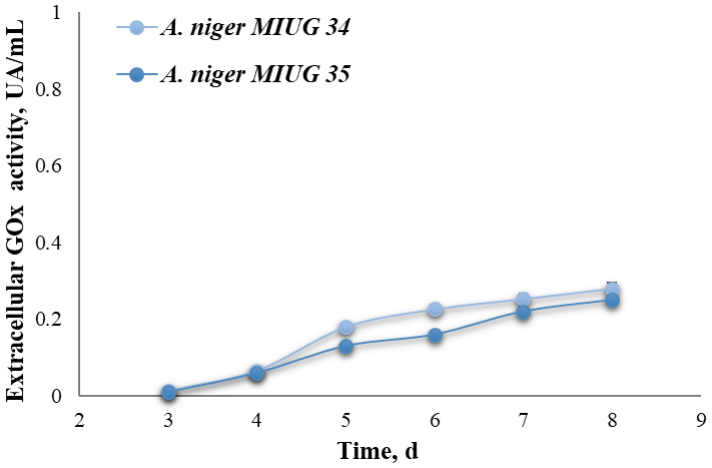


Figure 10.6. The proposed route for Ppy production through the biocatalytic action of laccase (inspired from [62, 117])

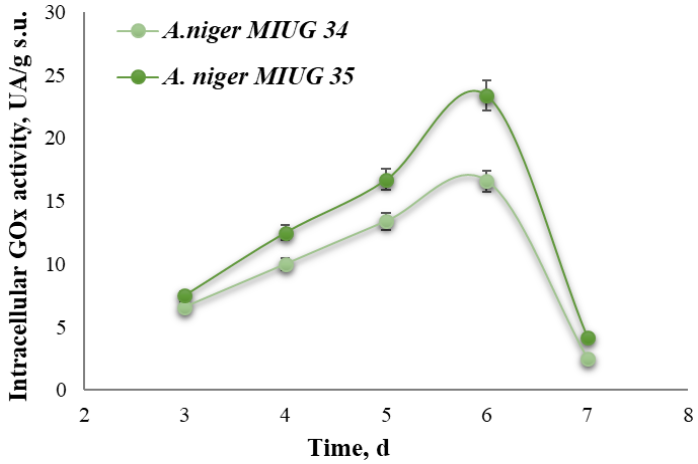
10.1.4 Glucose oxidase (GOx) biosynthesis within fungi cultures

The GOx assay results were negative for the white-rot fungi culture samples (*TV*, *TP*), which were previously established as PPO producers. The enzyme activity assayed in *A. niger* MIUG 34 and *A. niger* MIUG 35 culture samples both extra- and intracellularly is depicted in Figures 10.7 and 10.8.



Extracellularly, GOx activity is similar in both samples, while intracellularly, *A. niger* MIUG 35 showed higher enzyme yield.

Figure 10.7. Extracellular glucose oxidase activity within common fungi cultures.



Lower GOx yield is observed at extracellular level than intracellularly for the two common mold fungi cultures, which is in accord with literature reports on GOx localization within the mycelia for *A. niger* strains [133, 134].

Figure 10.8. Intracellular glucose oxidase activity within common fungi cultures.

While the process in the selected white-rot fungi cultures (*TV*, *TP*) cannot be attributed to a singular enzyme since multiple copper-proteins are available in the media, in the common mold fungi cultures, it is safe to assume that the catalytic action of GOx is dominant in the catalysis of Py oligomerization. Even though other enzymatic factors might be present, H_2O_2 represents a strong oxidant, that is certainly prevalent in initiating Py oxidation.

The mechanism through which GOx catalyzes Ppy bioproduction is as follows: GOx converts D-glucose into gluconic acid in the presence of oxygen, generating H_2O_2, which in turn initiates the oxidation of Py. The biocatalytic mechanism, as inspired from literature data [135, 136, 343] is illustrated in Figure 10.9.

The predilection for intracellular production of GOx within common mold fungi indicates that Py oligomerization is endorsed in the presence of living cells rather than in culture supernatant due

to limited enzyme secretion outside of the cell membrane. This enforces the bioproduction of Ppy using living cell cultures and offers predisposition towards bio-applications that use microbial components such as microbial biosensors or microbial fuel cells.

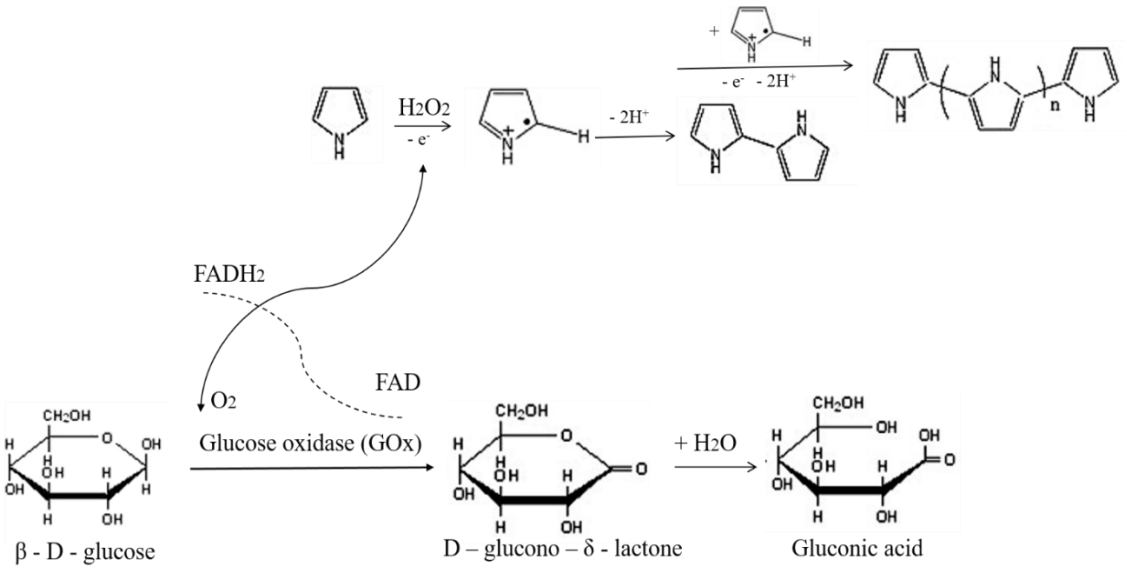


Figure 10.9. The proposed route for Ppy production through glucose oxidase catalysis (inspired from [27, 28]).

10.2. Monitoring and evaluation of pyrrole (Py) oligomerization by absorption spectroscopy

UV-Vis spectroscopy is a suitable technique for observation of the Py oligomerization process since optical absorption peaks are indicative of Ppy chain formation and its intrinsic electrical properties (reviewed in Section I, subsection 3). The electrical characteristics of the conducting polymer are direct results of the gap length between the valence band (VB) and the conduction band (CB), equivalent to the energy levels of the molecular orbitals. Formation and oxidation of the polymer chains have as direct result the formation of localized electronic states within the gap which are translated as energy shifts to higher wavelengths [10, 152, 155, 159]. The degree of oxidation of the polymer leads to formation of cationic charge carriers or polarons and/or dicationic charge carriers or bipolarons, each with assigned specific energy levels (wavelengths).

The current study attempted the monitoring of the Py oligomerization through UV-Vis spectroscopy due to the innovative aspect of Ppy formation through *in situ* biocatalysis. This allowed for observation of oxidative effects of the selected fungi cultures by estimation of Py oligomers length and conductivity.

10.2.2 Evaluation of pyrrole (Py) oligomerization within fungi cultures

The monitoring of the Py oligomerization for each selected strain in (i) cell cultures and (ii) culture filtrates was conducted (procedural details in Section II, subsection 7.1). This analysis confirmed the connection between the enzymatic activity estimated in the previous section and the formation of Ppy.

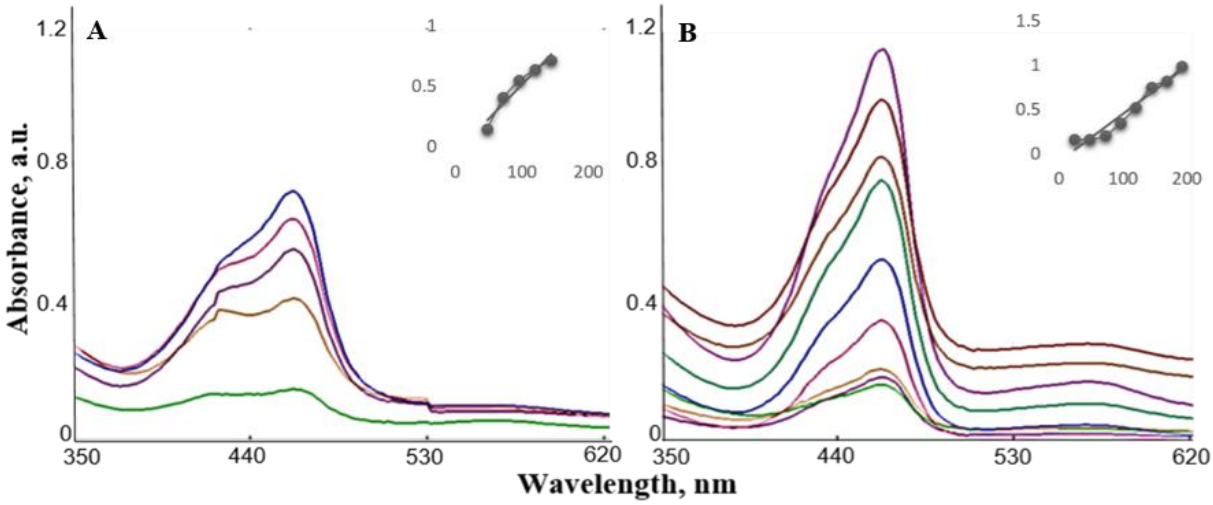


Figure 10.12. Absorbance spectroscopy monitoring of Ppy production in *TV*: A. culture filtrate; B. cell culture; The absorbance increase at 460 nm over time (h) is inserted.

Following the optical absorbance intensity, it can be observed that the oligomerization evolution is faster in cell culture (Figure 10.12B) rather than in culture filtrate (Figure 10.12A). The discrepancy is presumably due to the progressive PPO yield in the presence of living cells, which sustains the process longer.

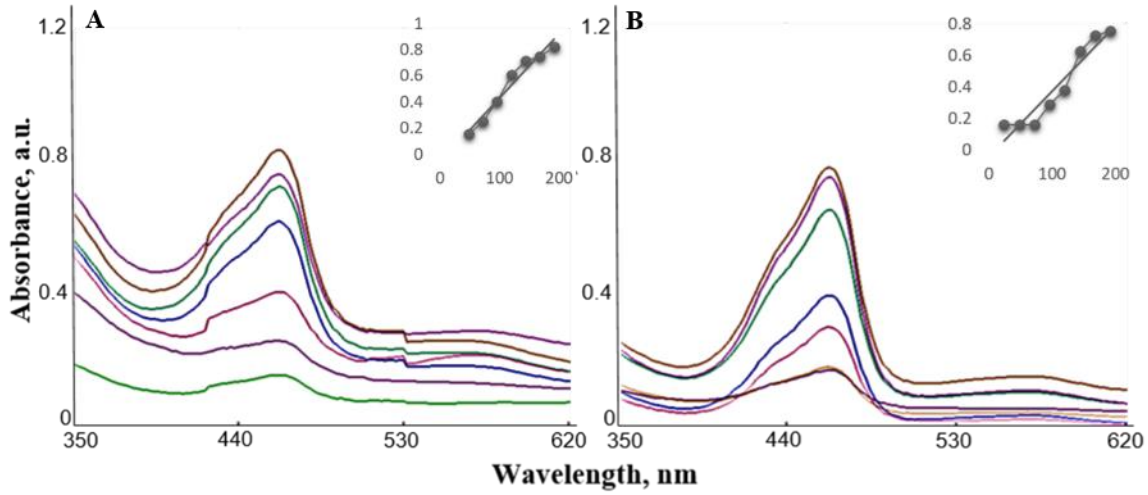


Figure 10.13. Absorbance spectroscopy monitoring of Ppy production in *TP*: A. culture filtrate; B. cell culture; The absorbance increase at 460 nm over time (h) is inserted.

Ppy formation in culture filtrate (A) and cell culture (B) of *TP* are depicted in Figure 10.13. Ppy chain evolution appears similar in both cases based on the absorption intensity. Resemblance to the oligomerization process recorded in *TV* is observed, in that culture filtrate appears to endorse the formation of shorter oligomer chains, which further connect and form an extended chains whilst cell culture promotes a direct formation of extensive Ppy chains.

The optical monitoring of Ppy formation through biocatalysis by common mold fungi strains is depicted in Figure 10.14.

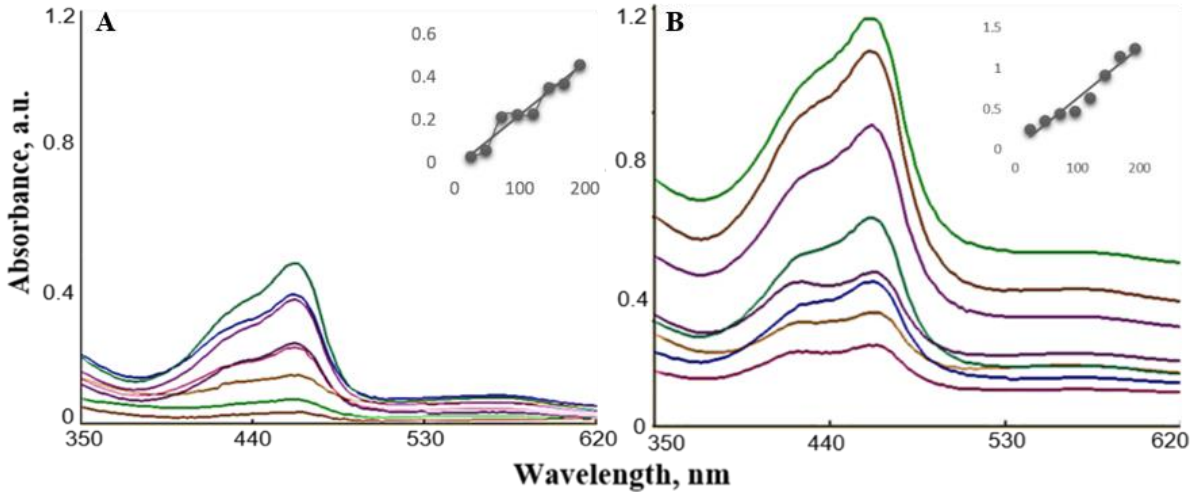


Figure 10.14. Absorbance spectroscopy monitoring of Ppy production in *A. niger* MIUG 34: A. culture filtrate; B. cell culture; The absorbance increase at 460 nm over time (h) is inserted.

As observed from Figure 10.14, the difference between Ppy formation in culture filtrate (Figure 10.14A) and cell culture (Figure 10.14B) of *A. niger* MIUG 34 is considerable. The oligomerization proceeds in a quasi-linear manner in both cases: formation of a shorter oligomeric chain, indicated by absorption at 420 nm wavelength (Ppy II) and a longer one (λ_{max} = 460 nm - Ppy I). However, the absorption intensity is almost three times higher in cell culture than in culture filtrate, which is descriptive of the ability or lack thereof to sustain Py bioconversion in the absence of living cells when using common fungi strains. It appears that the different Py oligomer chains increase in length separately due to repetitive oligomerization reactions, thus short chains are consecutively formed and merge into two major Ppy chains. Eventually, one of the chains gains extensive length (λ_{max} = 460 nm). Peaks at longer wavelengths were not observed meaning that the extended Ppy chain formed with *A. niger* MIUG 34 did not assume electrical charge.

The discrepancies between Ppy formation in culture filtrate (Figure 10.15A) and cell culture (Figure 10.15B) of *A. niger* MIUG 35 are not as remarkable. Although absorption intensity is almost double in the cell culture media, the oligomerization evolves similarly in both cases. As such, the formation of a short oligomeric chain is negligible and the formation of an extensive Ppy chain (λ_{max} = 460 nm) with high absorbance intensity is prevalent. In addition, a peak at 570 nm wavelength, consistent with charge gain within the Ppy backbone was observed especially in cell culture media.

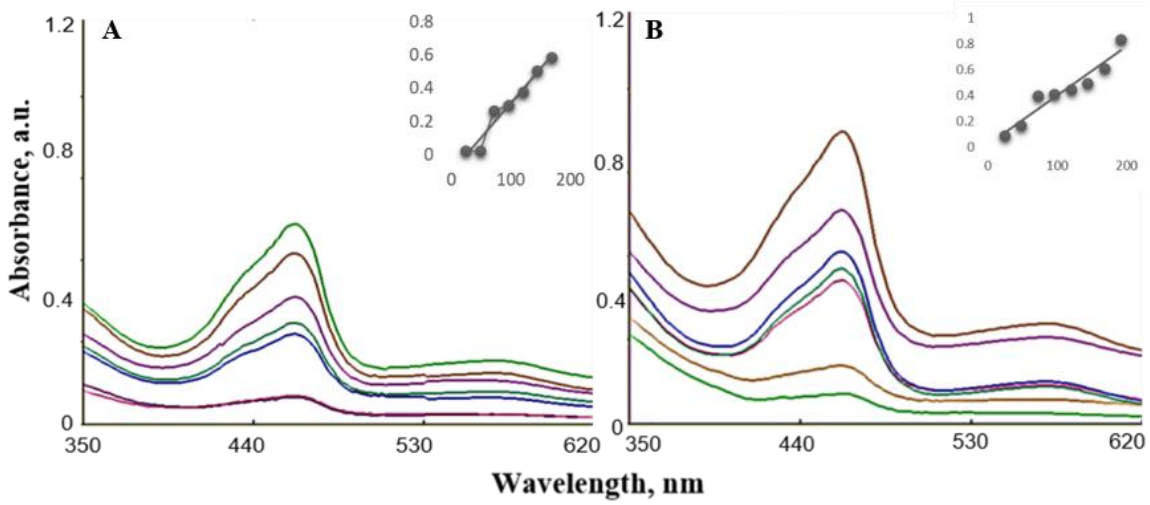


Figure 10.15. Absorbance spectroscopy monitoring of Ppy production in *A. niger* MIUG 35: A. culture filtrate; B. cell culture; The absorbance increase at 460 nm over time (h) is inserted.

The monitoring of Py oligomerization catalyzed by common fungi cultures revealed some kinetic differences to the process recorded in white-rot fungi cultures. The discrepancy consists in the process evolution in the absence *vs* in the presence of living cells. If similar oligomerization occurred in *TV* (Figure 10.12) and *TP* (Figure 10.13) whether cell culture or culture filtrate was employed, when using the common mold fungi (*A. niger* MIUG 34 and *A. niger* MIUG 35), the performance of cell culture was clearly distinguished. This relates to enzyme biosynthesis and localization.

10.3. Monitoring and characterization of pyrrole (Py) oligomerization by electrochemical techniques

Optical absorption measurements were useful in assessing the evolution of Py oligomerization into Ppy chains, yet for a more comprehensive understanding of the intrinsic electrical properties of the formed Ppy, electrochemical techniques were used including cyclic voltammetry (CV), open circuit voltage (OCV) and pH measurements (methodology details in Section II, subsection 7.2).

10.3.1 Characterization of pyrrole (Py) oligomerization within fungi cultures by cyclic voltammetry (CV)

At the low scan speed of 1 mV/s, monomer oxidation is supported according to literature data [172], so the process was recorded in the 2nd and 8th day of oligomerization, offering the possibility to compare and contrast monomer depletion in each sample. Figures 10.21 (*TV*, *TP*) and 10.22 (*A.niger* MIUG 34, *A.niger* MIUG 35) depict the initial potential cycles, since Py oxidation is of interest at this point and following cycles would not have been suitable for interpretation.

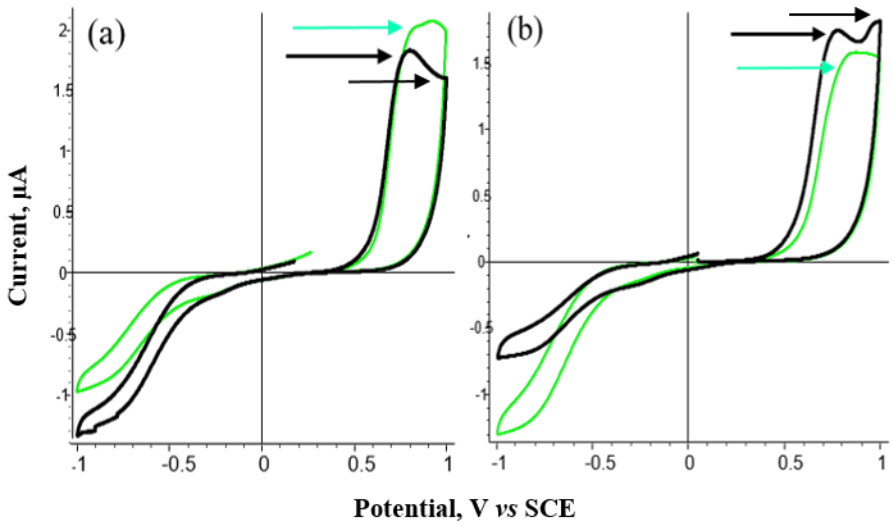


Figure 10.21. Cyclic voltammograms of Py oligomerization in cell cultures of: (a) *TV*; (b) *TP* within 2 d. (**-**), 8 d. (**-**); Potential scan speed of 1 mV/s.

Py monomer depletion progressed slightly different between the two white-rot culture samples. In cell culture of *TV*, increment in current values with time was observed, indicative of ongoing Py oxidation. The reverse evolution took place in cell culture of *TP*, signifying that the formed Ppy is unavailable to further electrochemical oxidation. Disregarding these distinctions, the anodic potential for Py oxidation is very similar in both culture samples, indicating analogy between processes.

Higher current intensity was observed for Ppy formation in common fungi cultures (Figure 10.22) than in white-rot cultures (Figure 10.21). In *A. niger* MIUG 34 culture (Figure 10.22(a)), Py oxidation evolves towards higher current with time, as it was the case in cell culture of *TV*, although additional oxidation peaks are negligible. For Py oligomerization in cell culture of *A. niger* MIUG 35, current intensity maximum was recorded in the first two days, then the current decreased considerably.

This indicates that the oligomerization occurs fast in cell culture of *A.niger* MIUG 35 and Ppy-MIUG 35 is not available for further electrochemical oxidation upon process completion, much like Ppy-*TP*. The electrochemical features for the formation of Ppy-MIUG 34 within eight days (Figure 10.22 (a)-green line) roughly resemble the evolution of Ppy-MIUG 35 within two days (Figure 10.22 (b)-black line), thus these two common fungi cultures display similarity in conducting Py bioconversion, although at different rates.

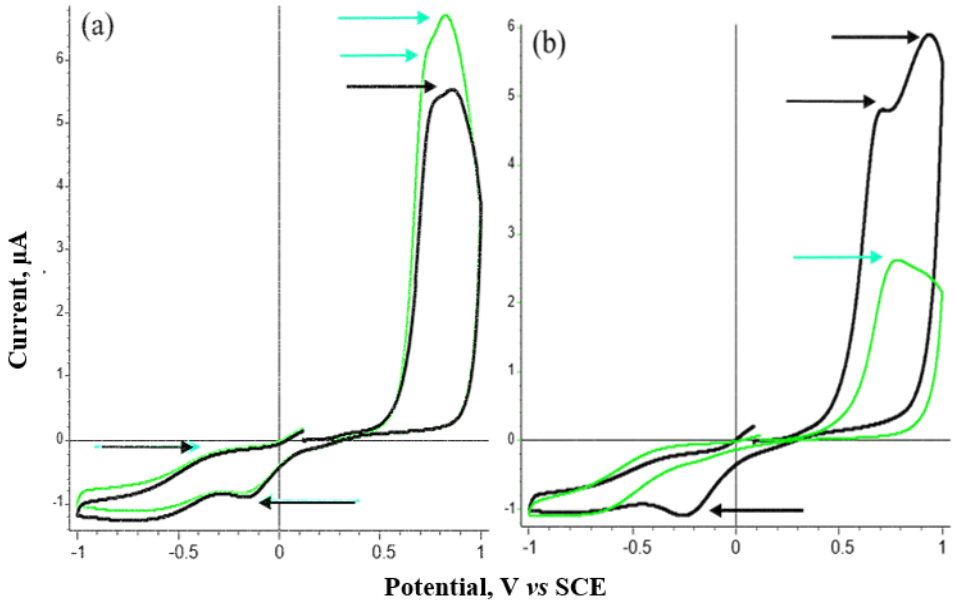


Figure 10.22. Cyclic voltammograms of Py oligomerization in cell cultures of: (a) *A. niger* MIUG 34, (b) *A. niger* MIUG 35 within 2 days (-), 8 days (-); Potential scan speed of 1 mV/s.

Further on, potential sweep rate was increased to 100 mV/s. These current-potential diagrams are descriptive of the redox exchange during Py oligomerization process. The analysis of the electrochemical parameters (Tables 10.7 and 10.8) reveals the reversibility exhibited by Ppy during oligomerization. The electron exchange is given by the value of ΔE_p, which ideally is 0.059 for a fast single electron transfer at 298 K [431] and the redox reversibility is indicated by current peak ratio (I_{pa}/I_{pc}), which is equal or close to unity for reversible and quasi-reversible and less than unity for irreversible processes [432]. Figures 10.27 (*TV*, *TP*) and 10.28 (*A. niger* MIUG 34, *A. niger* MIUG 35) depict secondary cycles of CVs registered at 100 mV/s.

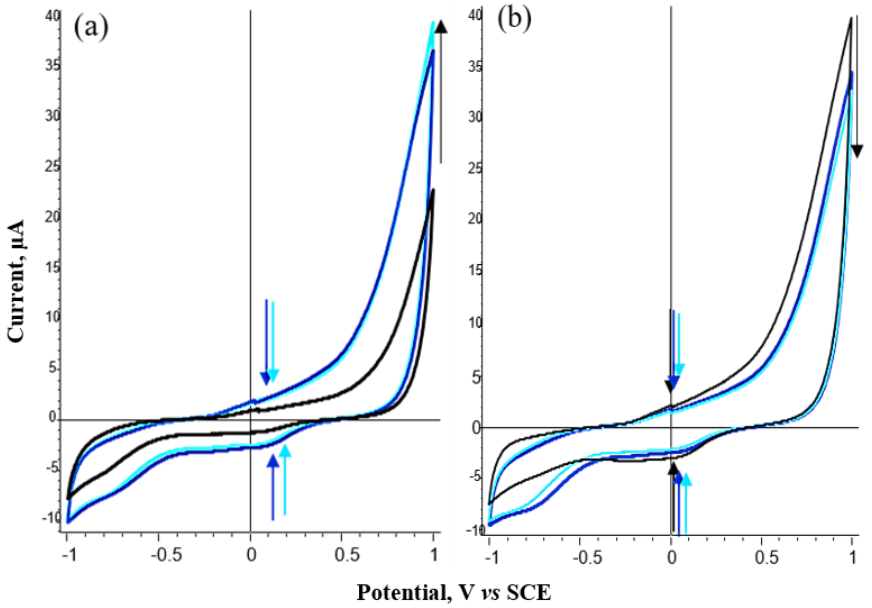


Figure 10.27. Cyclic voltammograms of Py oligomerization in cell cultures of: (a) *TV*, (b) *TP* within 2 d. (-), 4 d. (-), 6 d. (-) and 8 d. (-); Lateral arrows indicate scan evolution; Potential scan speed of 100 mV/s.

Table 10.7. *Electrochemical parameters on Py oligomerization evolution within white-rot fungi cultures as registered at potential scan speed of 100 mV/s.*

Scan speed: 100 mV/s		**E_{pa}, V**	**I_{pa}, µA**	**E_{pc}, V**	**I_{pc}, µA**	**ΔE_p**	**I_{pa}/I_{pc}**
Ppy-*TV*	2nd d.	0.143	1.156	0.083	- 1.138	0.060	1.01
	4th d.	0.099	2.173	0.086	- 2.367	0.013	0.91
	6th d.	0.071	2.246	0.124	- 2.385	0.053	0.94
Ppy-*TP*	2nd d.	0.024	2.051	0.076	- 2.785	0.052	0.73
	4th d.	0.022	1.628	0.081	- 2.269	0.059	0.71
	6th d.	0.021	1.441	0.086	- 1.928	0.065	0.74

A single reversible electron exchange is noticed for Ppy-*TV* within the first two days of reaction. Later on, the reversibility decreases making the electron exchange unfeasible. Ppy-*TP* evolves towards a quasi-reversible electron exchange within six oligomerization days. The CV graphs obtained on the 8th oligomerization day superimpose the ones from the 6th day, indicative of no further evolution i.e. steady-state phase.

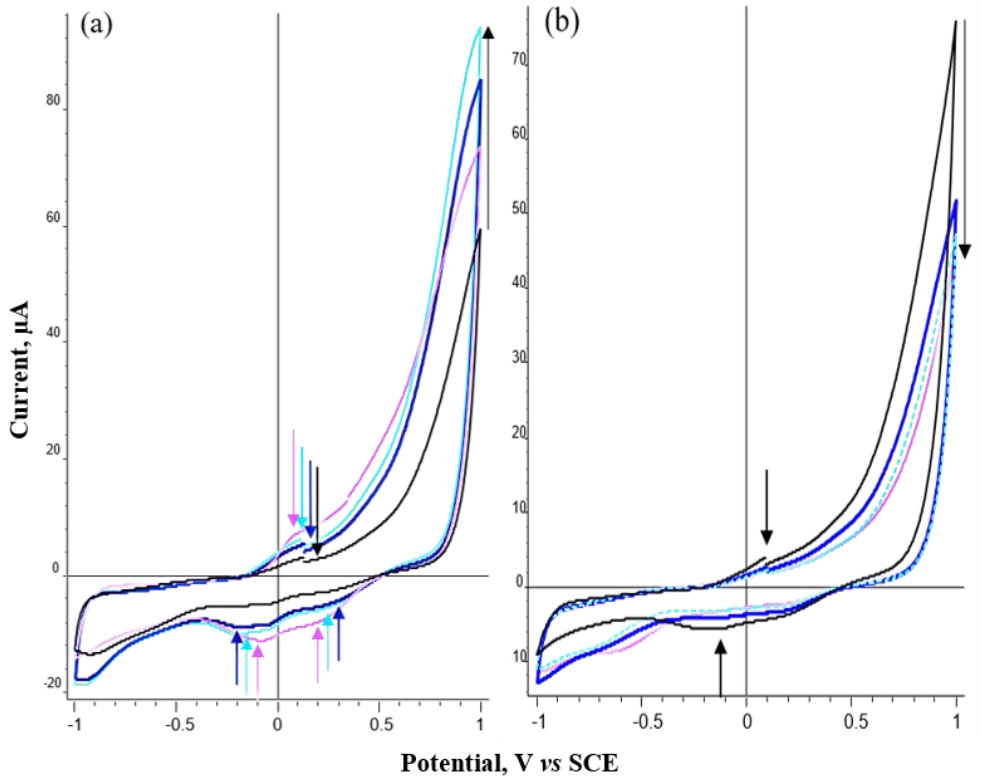


Figure 10.28. Cyclic voltammograms of Py oligomerization in cell cultures of: (a) *A. niger* MIUG 34; (b) *A. niger* MIUG 35 within 2 d. (**-**), 4 d. (-), 6 d. (-) and 8 d. (-); Lateral arrows indicate scan evolution; Potential scan speed of 100 mV/s.

Table 10.8. *Electrochemical parameters on Py oligomerization evolution within common fungi cultures as registered at potential scan speed of 100 mV/s.*

Scan speed: 100 mV/s		E_{pa}, V	I_{pa}, µA	E_{pc}, V	I_{pc}, µA	ΔE_p	I_{pa}/I_{pc}
Ppy-MIUG 34	2nd d.	0.124	3.058	- 0.026/0.280	-4.81/- 2.57	0.098/0.156	0.63/1.18
	4th d.	0.117	5.332	- 0.060/0.270	-8.54/- 4.61	0.057/0.153	0.62/1.15
	6th d.	0.110	5.981	- 0.080/0.260	- 9.71/- 5.88	0.030/0.150	0.61/1.01
	8th d.	0.091	7.154	- 0.090/0.230	-11.08/- 6.91	0.001/0.139	0.64/1.03
Ppy-MIUG 35	2nd d.	0.096	3.90	- 0.160/0.170	-5.75/- 4.16	0.064/0.074	0.67/0.93
	4th d.	0.098	3.26	0.140	- 3.90	0.042	0.83
	6th d.	0.105	2.69	0.120	- 3.79	0.012	0.70
	8th d.	0.171	2.20	0.100	- 3.04	0.071	0.72

Ppy-MIUG 34 exhibits one anodic and two cathodic peaks with increasing current intensity over time. Double reversible electron transfer is noticed between the pair of redox peaks, while the secondary peak observed during cathodic sweep is not reversible indicating only expulsion of ions from the Ppy structure. Electrochemical activity is maintained during the eight days of oligomerization. The electron exchange for Ppy-MIUG 35 is negligible and quasi-reversible with loss of current intensity over time.

Upon electrochemical analysis of Py oligomerization a few conclusions can be drawn: (i) weak electron exchange is observed for Ppy formed in white-rot fungi cultures, thus moderate oxidative efficiency; (ii) good redox behaviour for Ppy formed in common fungi cultures, especially for Ppy-MIUG 34, indicative of substantial oxidative performance; (iii) faster oxidation might lead to loss of electroactivity/oxidation susceptibility over time for Ppy-*TP* and Ppy-MIUG 35, due to lack of reactive components in the culture mediums; (iv) lower oligomerization rate results in ongoing reactivity towards electrochemical oxidation for Ppy-*TV* and Ppy- MIUG 34, presumably due to leftover unreacted monomer and/or oligomers.

10.3.2 Electrochemical features of polypyrrole (Ppy) in phosphate buffer solution (PBS)

Upon analysis of redox behaviour for Ppy within the polymerization cultures, Ppy precipitate was collected. Although, the culture did not exhibit electro-character, it is safe to assume that previous outcomes were very much dependent on the interaction at the Ppy/cell culture interface. Since the capacity of a material is a function of both the conductivity and concentration of the electrolyte solution [434, 435], the collected Ppy was suspended in PBS and analyzed regarding its redox behaviour. The Ppy suspended in PBS (pH=6.0, 0.1M, procedural details in Section II, subsection 7.2) was analyzed at 100 mV/s to observe the phosphate ion migration in and out of the polymer matrix and the electrochemical sustainability.

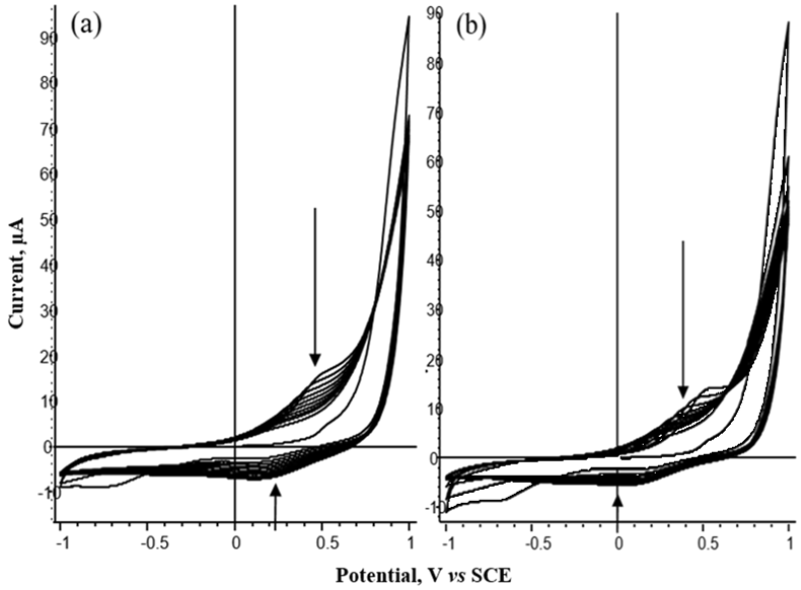


Figure 10.31. CV of the redox behaviour of Ppy produced in: (a) *TV*; (b) *TP* within 8 d. and suspended in PBS; Arrows indicate scan evolution; Potential scan speed of 100 mV/s.

The redox exchange described in Figure 10.31 accounts for decent electroactivity for Ppy-*TV* and Ppy-*TP*. The oxido-reductive peaks are most visible for Ppy-*TV*, especially during anodic sweep, equivalent to tendency of ion incorporation within Ppy structure.

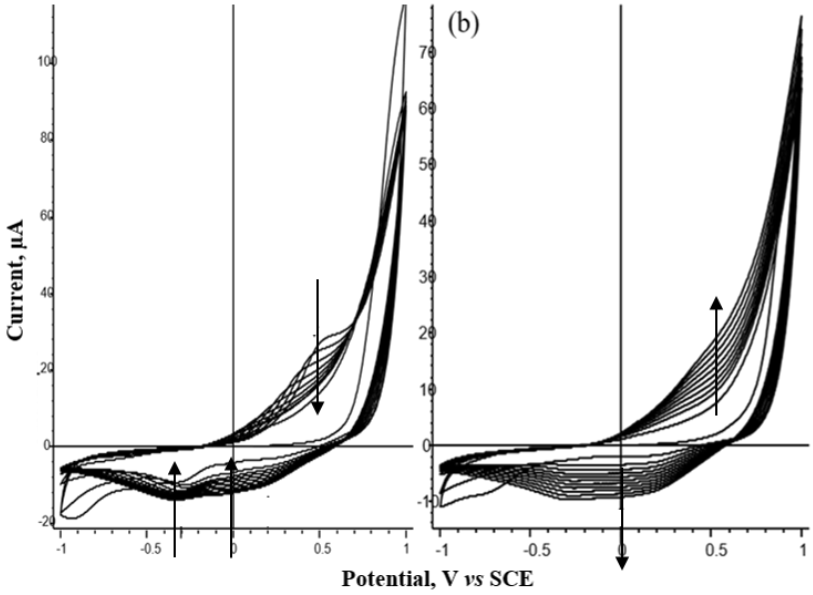


Figure 10.32. CV of the redox behaviour of Ppy produced in: (a) *A. niger* MIUG 34; (b) *A. niger* MIUG 35 within 8 d. and suspended in PBS; Arrows indicate scan evolution; Potential scan speed of 100 mV/s.

Ppy-MIUG 34 exhibits one anodic and two cathodic peaks, as previously observed in cell culture, proving that it performs step-by-step exclusion of ions from its structure. Yet, the intensity of the electro-exchange is diminished when compared with cycling within cell culture. The best redox activity is noticed for Ppy-MIUG 35, confirming its predilection for ion expulsion rather than insertion, probably due to a higher oxidative state achieved during oligomerization. This also explains the

minimal interaction with the culture media illustrated previously and the lack of leftover monomer/oligomers available in the media for electrochemical oxidation.

The relevance of analyzing the redox behaviour of the prepared Ppy by CV in buffer solution is derived from Ppy suitability for ion-exchange/purification membranes, based on the ability of the material to extract ions from blood or physiological fluids [436, 437].

10.3.3 Electrochemical features of polypyrrole (Ppy) in ultrapure water

The characterization of capacitive behaviour for Ppy was done by suspending the polymer collected from each culture in ultrapure water. In this manner, both anion and cation movements can be disregarded. The capacitive sustainability is recorded during 10 consecutive CV cycles at 100 mV/s scan rate (Figures 10.33 (*TV*, *TP*) and 10.34 (*A. niger* MIUG 34, *A. niger* MIUG 35)).

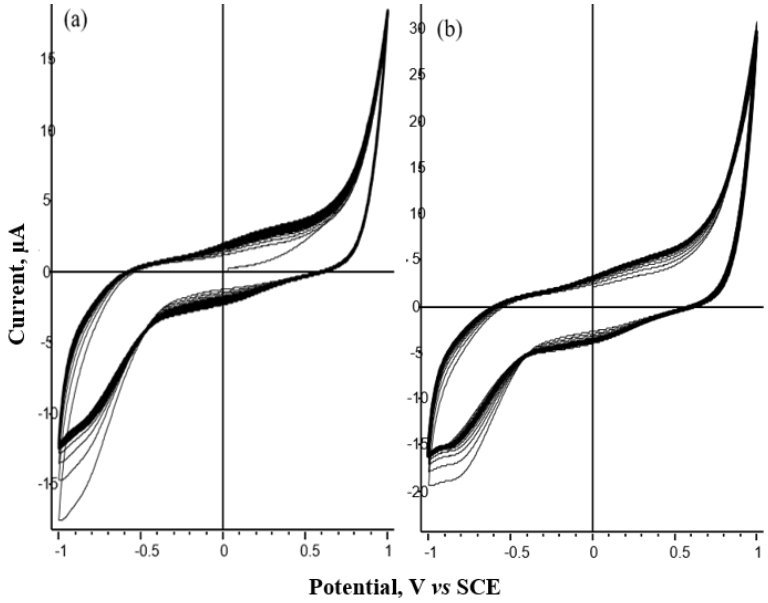


Figure 10.33. CV of the redox behaviour of Ppy produced in: (a) *TV*; (b) *TP* within 8 d. and suspended in ultrapure water; Potential scan speed of 100 mV/s.

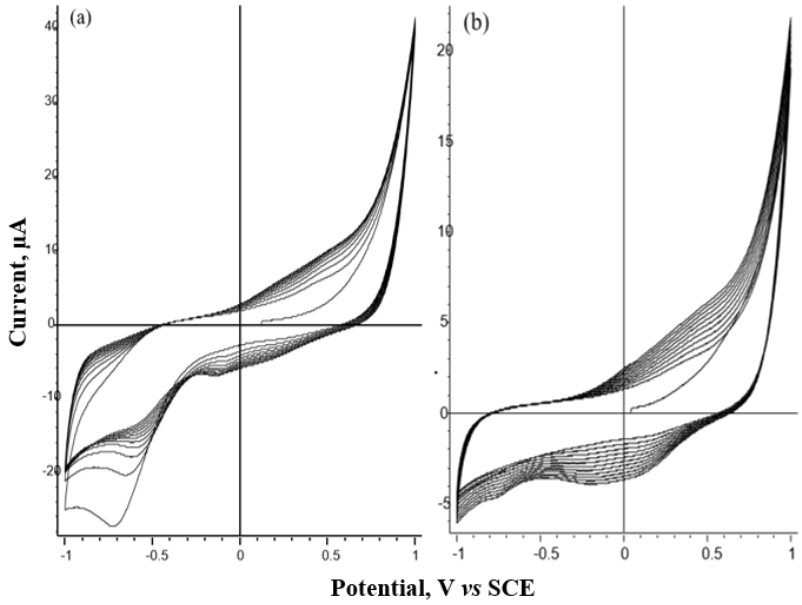


Figure 10.34. CV of the redox behaviour of Ppy produced in: (a) *A. niger* MIUG 34; (b) *A. niger* MIUG 35 within 8 d. and suspended in ultrapure water; Potential scan speed of 100 mV/s.

The capacitive behaviour of the formed Ppy is important in applications that employ conductive material composites for electrochemical capacitors. The features of the Ppy formed within fungi cultures are suitable, considering the roughly rectangular shapes recorded by CV and current-potential symmetry recommended by literature data [173, 430, 438, 439].

10.3.5 Open circuit voltage (OCV) evolution during pyrrole (Py) oligomerization within fungi cultures

OCV technique was employed as additional to CV due to its ability to reveal the oxidative state of the formed Ppy. It allows observation of redox susceptibility in the absence of a constantly applied potential. OCV evolution during Py oligomerization in each culture media is depicted in Figures 10.36 (*TV*, *TP*) and 10.37 (*A.niger* MIUG 34, *A.niger* MIUG 35).

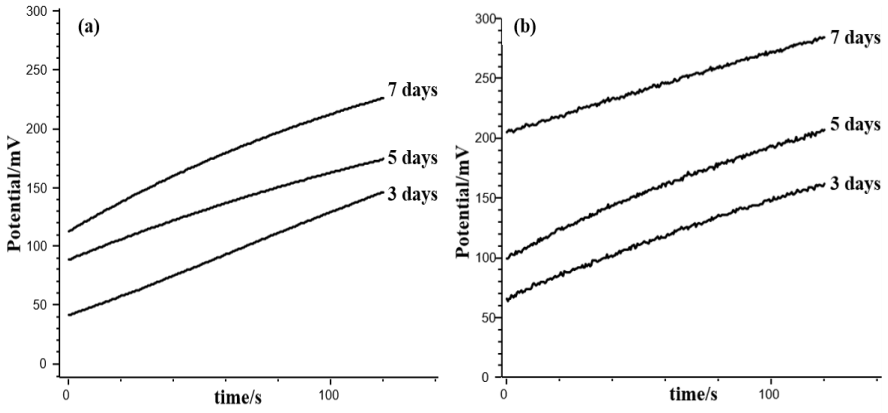


Figure 10.36. Open circuit voltage evolution during Py oligomerization in white-rot fungi cultures: (a) *TV*; (b) *TP*.

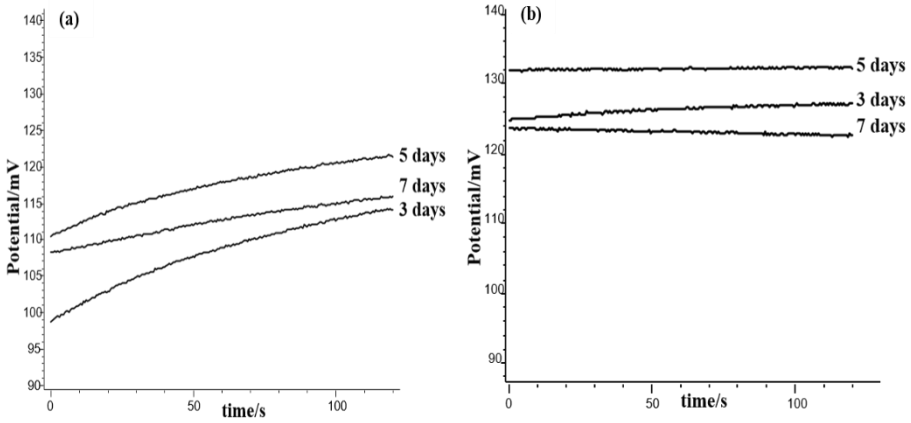
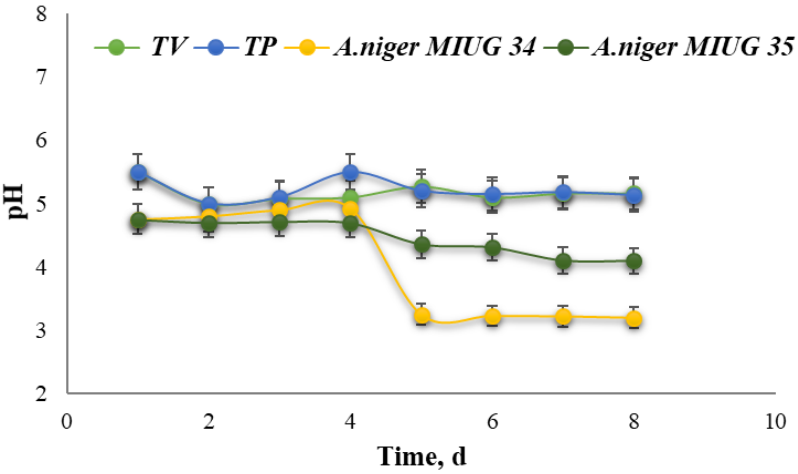


Figure 10.37. Open circuit voltage evolution during Py oligomerization in common fungi cultures: (a) *A. niger* MIUG 34; (b) *A. niger* MIUG 35.

The OCV evolution during Py oligomerization in white-rot fungi cultures is ascending, indicative of susceptibility to oxidation. The OCV values recorded in common fungi cultures are slightly increasing in cell culture of *A. niger* MIUG 34 and relatively steady, even decreasing in cell culture of *A. niger* MIUG 35. Ppy-MIUG 34 evolves into a more oxidized state over time, whilst Ppy-MIUG 35 does not present any susceptibility to oxidation (i.e. incorporation of ions), even more, reveals tendency towards reduction (i.e. expulsion of ions).

10.3.6 pH evolution during pyrrole (Py) oligomerization within fungi cultures

pH monitoring allows observations on the evolution of each fungi culture during Py oligomerization (Figure 10.38).



pH evolution in white-rot fungi cultures begins at 5.5 and is relatively stable with slow decrease until pH 5.1. The pH in common fungi cultures was adjusted at 5.0 before inoculation and it decreases steadily during Py oligomerization due to synthesis of both H_2O_2 and gluconic acid through GOx catalysis.

Figure 10.38. pH evolution during Py oligomerization within fungi cultures

Since the best electrical properties were identified for Ppy formed in the common fungi cultures, rather than in the white-rot cultures, it is reasonable to assume that it is contingent on the pH of the polymerization media. Reports on chemical synthesis of Ppy using H_2O_2 entailed lower pH values such as 2.0 [17], as attempted herein for comparison purposes or 3.1 [73]. For peroxidase catalysis, buffers with pH values of 3.5 [92] up to 4.0 [91] were recommended, due to liability for enzyme inactivation at pH = 2.0 [89]. Concerning Ppy formation by laccase catalysis, pH values employed were higher such as 3.5 [119] up to 4.0 [117] or 5.0 [118]. Therefore, the pH employed in this study is within limits that allow Py oligomerization without inhibition of biocatalyst.

Conclusions

The aim of this subsection was the in-depth analysis of Py oligomerization parameters. Previously, the selection of the most suitable microorganism strains was conducted and four fungi strains exhibited the best performance: *TV*, *TP*, *A. niger* MIUG 34 and *A. niger* MIUG 35. The analysis of each of these fungi cultures regarding protein and enzyme biosynthesis was accomplished. The white-rot cultures proved to be PPO producers both extra- and intracellularly with similar enzymatic activity, elevated for *TP*. The most prominent enzyme appeared to be laccase and the pathway for Py bioconversion into Ppy based on its catalytic mechanism was envisioned. GOx production was determined for the common mold fungi cultures mostly at intracellular level with best values for *A. niger* MIUG 35. Since its catalytic mechanism is based on the production of H_2O_2, and several reports on Ppy formation using pure GOx are available, the mechanism for Py oxidation was accordingly described.

Monitoring and evaluation of the Py oligomerization process was achieved through spectroelectrochemical methods. It was observed that the oligomerization proceeded similarly within the white-rot fungi cultures, good performance being achieved whether in the presence or absence of living cells. This relates to similar enzymatic yield both extra- and intracellularly. Within the common fungi cultures, the oligomerization was superior from both timeframe and electrical features standpoints. This is presumably due to stronger oxidative effect of these type cultures due to H_2O_2 production by GOx. The process was favored in the presence of biomass, as anticipated from elevated enzyme biosynthesis intracellularly.

Electrochemical investigations such as CV, OCV and pH measurements offered insight into the redox characteristics of Ppy. Suitable redox switching and good capacitive behaviour were observed for Ppy formed in each culture, which was visible by potential cycling in PBS and ultrapure water, with best electrochemical behaviour proven by Ppy-MIUG 35.

Overall, the difference between fungi culture efficiency in Py bioconversion is related to several factors such as: (i) the enzymatic catalyst; (ii) the polymerization pathway; (iii) the optimum pH. The process was validated and characterized through spectroelectrochemical techniques and perspectives for improvement include the addition of templates and/or redox mediators in the polymerization bulk solution, in order to prepare a highly ordered and conducting Ppy through a biocatalytic, economic and environmentally friendly pathway.

11. Bio-electrochemical applications for polypyrrole (Ppy) formed within fungi cultures

The objective of this subsection imposes the final aim of the study, i.e. testing the applicability of the Ppy obtained through *in situ* biocatalysis within fungi cultures. Considering the extensive research dedicated to microbial electrochemical applications [310, 313, 314, 440, 441] and the reported benefits of using Ppy in the design of biosensors [135, 267, 268, 329, 347, 442, 443] (reviewed in Section I, subsection 4), several types of amperometric biosensor configurations were devised.

The polymer formed within cell cultures of common fungi (Ppy-coated cells) was assessed from the perspective of preservation of function and integrity for the fungi cells along with ability to improve the analytical characteristics of a microbial biosensor, conferring double purpose for the GOx-yielding microbial components: (i) ability to catalyze Py oligomerization; (ii) preservation of enzymatic function enough for sensitive detection.

In the case of polyphenoloxidase (PPO)-producers (i.e. white-rot fungi), Ppy formed within culture filtrates was employed, as Ppy-PPO extract mixtures. This was considered an advantageous alternative to the use of pure enzyme in biosensing, without further need for purification along with one-step bioproduction of Ppy.

The outcomes reflect: (i) the ability of Ppy to improve detection in electrochemical devices; (ii) the simplicity of the proposed concept; (iii) the biocompatible features of the bioproduced Ppy (BioPpy).

11.1 Microbial glucose biosensors based on polypyrrole (Ppy) formed within common mold fungi cultures

11.1.1 The influence of polypyrrole (Ppy) production within *Aspergillus niger* MIUG 35 culture in glucose detection

Amperometric analysis and scanning electrochemical microscopy (SCEM) techniques were applied for observation of electrochemical response towards glucose by simple and Ppy-modified *A. niger* MIUG 35 biomass. In this configuration, a double-mediator system was used involving a lipophilic mediator (1,10-phenanthroline-5,6-dione (PD) or 9,10-phenanthrenequinone (PQ)), able to enhance the permeability of the cellular membrane and a hydrophilic mediator (potassium ferricyanide), responsible for enhancement of the catalytic reaction in solution (Figure 11.1).

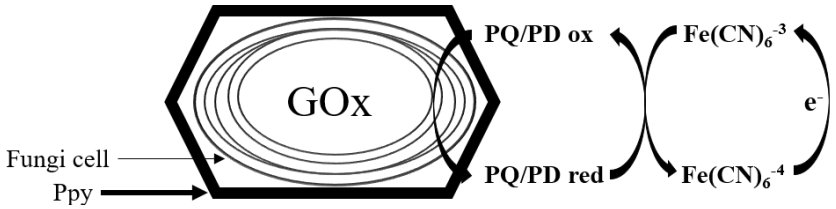


Figure 11.1. Biolayer design for investigation of current response to glucose for simple and Ppy-modified *A. niger* MIUG 35 biomass; Two redox mediators: $Fe(CN)_6^{3-}/Fe(CN)_6^{4}$, PQ/PD (9,10-phenanthrenequinone/ 1,10-phenanthroline-5,6-dione).

The visual aspect of *A. niger* MIUG 35 cell culture observed prior to Py addition and within four days of Ppy formation was digitally recorded and is depicted in Figure 11.2.

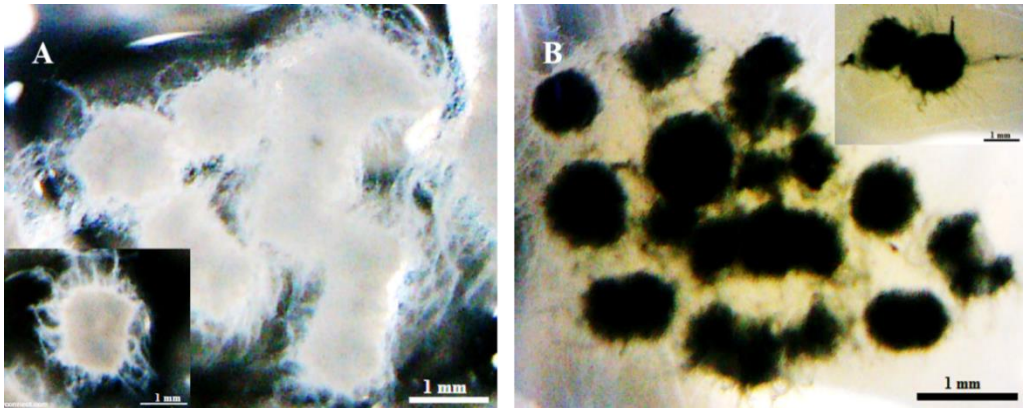


Figure 11.2. Aspect of *A. niger* MIUG 35 culture: a) prior to Py addition and b) within 4 d. of Ppy production.

The SCEM technique allowed current measurements with the ultramicroelectrode (UME) tip at specific distances from the microbial samples. Electrochemical surface reactivity was compared between the two different samples in the presence of 10 µm glucose at several distances.

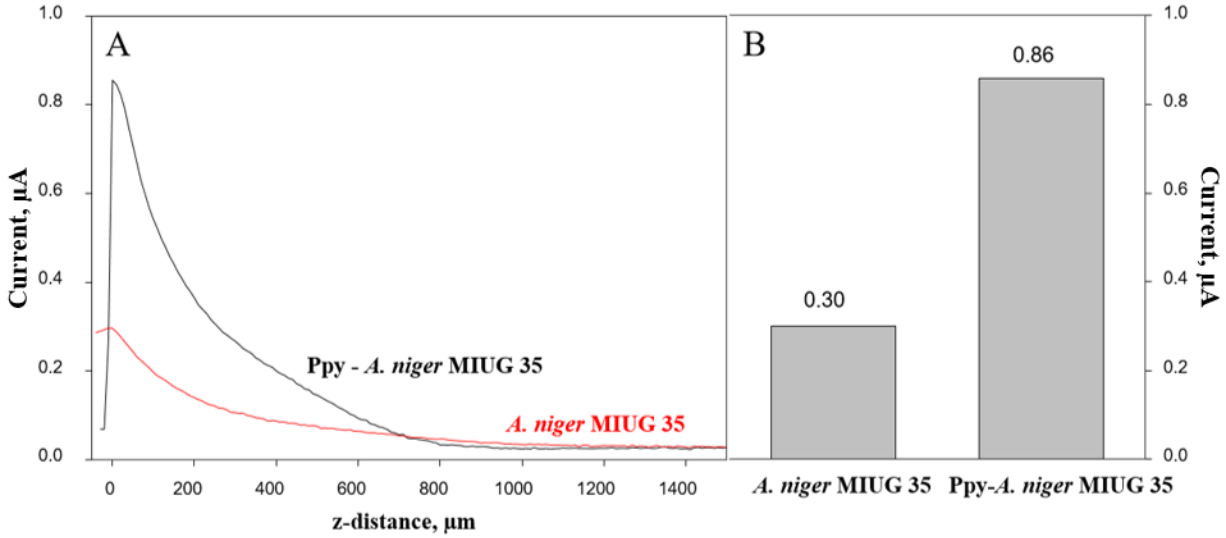


Figure 11.3. A. The approaching curve at 0 µm distance to the immobilized microbial samples registered by SECM generation-collection mode; B. Electrochemical activity for simple and Ppy-modified *A. niger* MIUG 35 biomass at 0 µm with 10 µm glucose; Operational potential: 0.4 V *vs* Ag/AgCl, scanning speed of 10 µm/s.

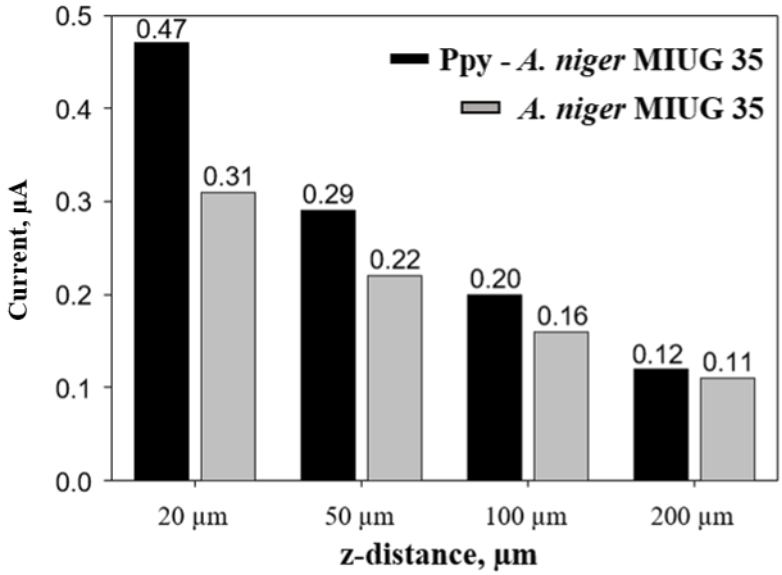


Figure 11.5. Maximum current differences registered at varying heights during SCEM horizontal scans for simple and Ppy- modified *A. niger* MIUG 35 biomass.

If at 0 µm scan distance, the current value registered for the Ppy-microbial sample was three times higher than for the simple microbial sample, at 20 µm, the difference was around 1.5 times and at 200 µm the current values were similar. This is due to concentration gradient from spherical diffusion. Through this technique, it has been ascertained that the fungi biomass preserves its metabolic activity after modification with Ppy produced *in situ* and even more, analyte detection is facilitated through the conductive nature of Ppy. This is the premise of this electroanalytical study, therefore, a microbial amperometric biosensor for glucose detection was developed using both type of samples (simple and Ppy-modified).

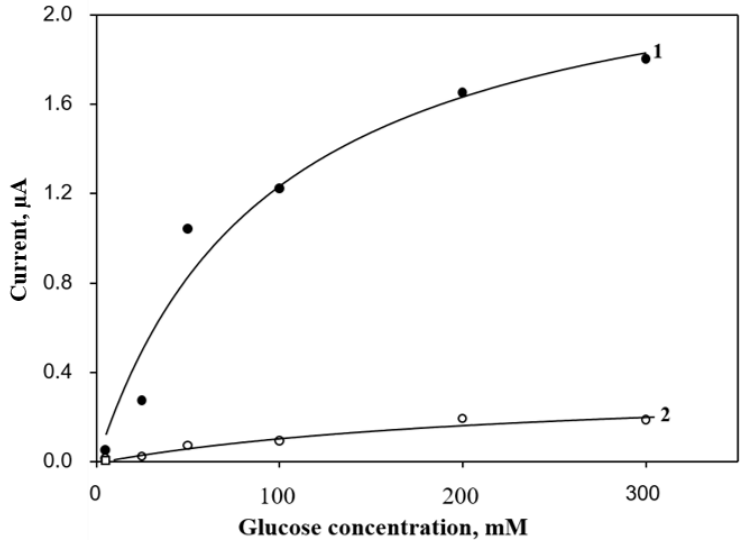


Figure 11.6. Amperometric response to glucose for the microbial biosensors based on: simple (○, curve 2) and Ppy-coated (●, curve 1) fungi cells; PBS pH 6.0, 10 mM $K_3[Fe(CN)]_6$; 0.3 V *vs* Ag/AgCl/3 M KCl.

The amperometric analysis was performed within a wide concentration range for an overview on glucose detection ability. An elevated current response was observed with the Ppy-microbial biosensor, six times higher than with the simple microbial biosensor within 0-300 mM glucose concentration range.

The suitable results obtained using *A. niger* MIUG 35 biomass with Ppy inspired further investigations on the bioelectrochemical potential of Ppy-coated cells. It appears that Ppy, through its conductive nature, improves the communication between biomolecule and transducer within biosensor configurations. Further on, *A. niger* MIUG 34 biomass was tested for glucose detection.

11.1.2 Microbial glucose biosensors based on polypyrrole (Ppy) formed within *Aspergillus niger* MIUG 34 culture

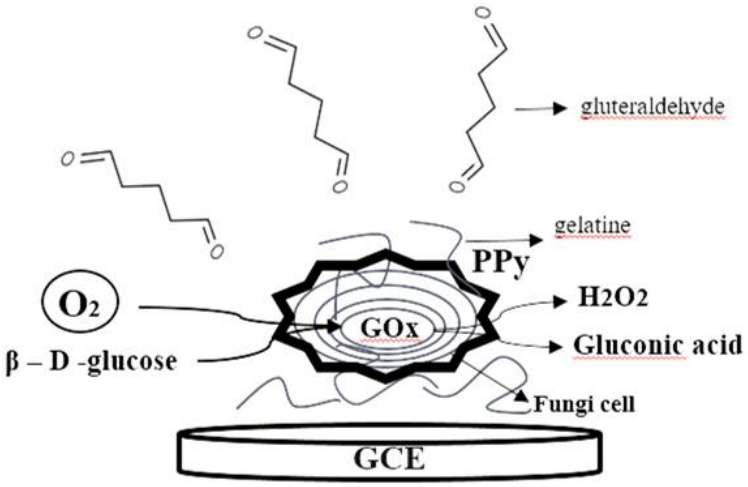


Figure 11.7. Biolayer design for the glucose biosensors based on simple and Ppy- modified *A. niger* MIUG 34 biomass immobilized on top of a glassy carbon electrode (GCE).

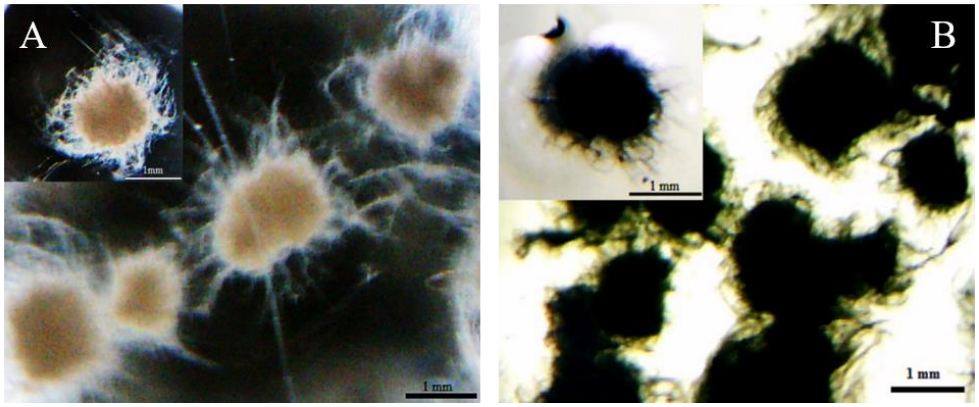
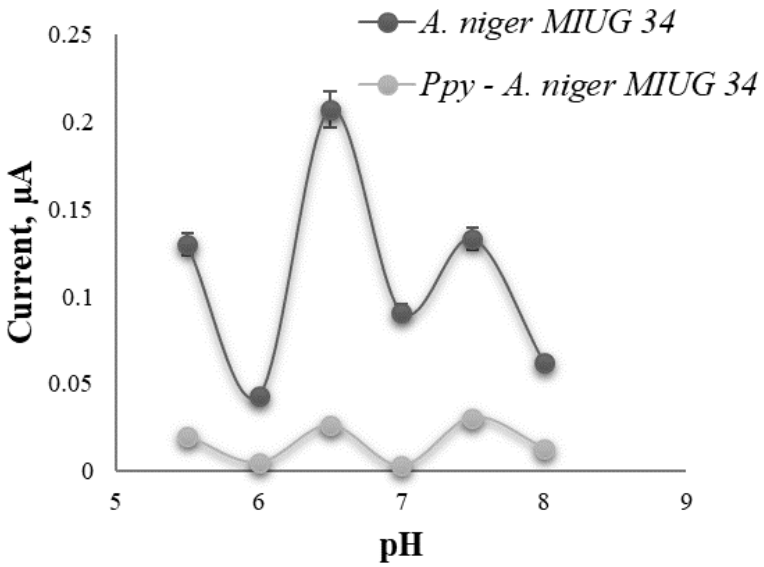


Figure 11.8. Aspect of *A. niger* MIUG 34 culture: a) prior to Py addition and b) within 4 d. of Ppy production.

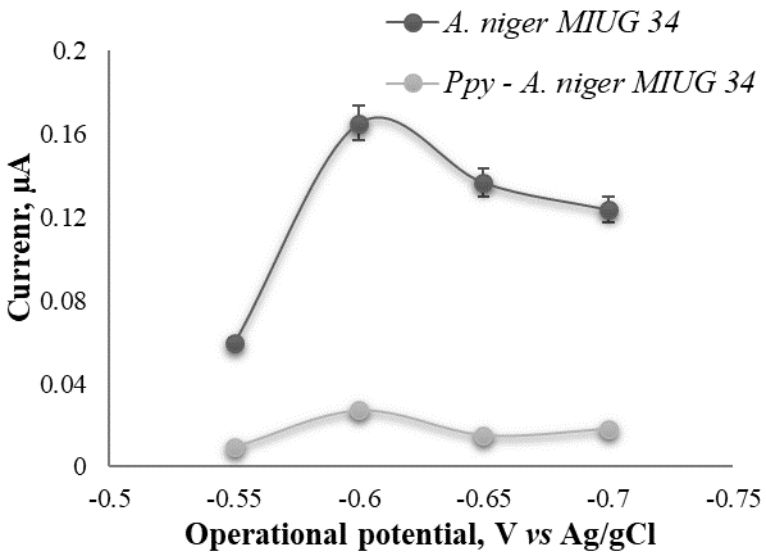
The effect of pH on both types of microbial biosensors was examined in the range 5.5 – 8.0 in PBS 0.1M and optimum behaviour was noticed at pH 6.5 and 7.5 for simple and Ppy-microbial biosensors, respectively (Figure 11.9).

Progressive glucose additions started at the concentration of 0.01 mM and each following addition determined a current increase that upon stabilization was considered the new steady-state value. Subsequently, the difference between two consecutive steady state current values was calculated as the biosensor response to the correspondent glucose concentration and the current response evolution was plotted against the substrate concentration resulting in the calibration curve of the microbial biosensors. Glucose addition stopped when the calibration curve started to deviate from linearity, indicative of enzyme saturation.



Both pH and operational potential optimum values were similar by comparison between simple and Ppy-microbial biosensors, which indicates that Ppy does not have a drastic effect upon the conditions of the enzymatic reaction that leads to analyte detection.

Figure 11.9. Optimum pH for glucose detection for simple and Ppy-microbial biosensors based on *A.niger* MIUG 34 biomass; 0.1M PBS, V=-0.6 V *vs* Ag/AgCl; additions of 0.5 mM glucose.



Moreover, the values obtained as suitable in these configurations are in accordance with optimum values for pure GOx biosensors [243, 444], which suggests that Ppy coating did not alter the pH profile of the enzyme.

Figure 11.10. Optimum operational potential for simple and Ppy-microbial biosensors based on *A.niger* MIUG 34 biomass; 0.1 M PBS, pH = 6.5/7.5; additions of 0.5 mM glucose.

The calibration curves for the two types of biosensors are depicted in Figures 11.12 and 11.13. The response to glucose was divided in two sections: over an extended concentration range (a) and at low concentrations (b), to achieve a proper comparison of the two biosensor systems.

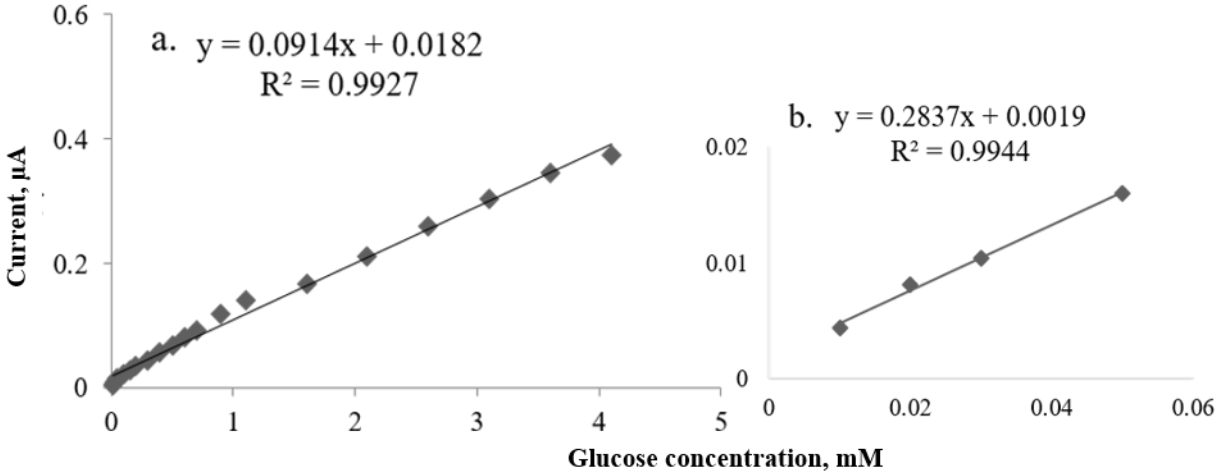


Figure 11.12. Calibration curve for glucose obtained with *A. niger* MIUG 34 biosensor within: a. an extended concentration range (0.01 – 4.1 mM) and b. a low concentration range (0.01 – 0.05 mM).

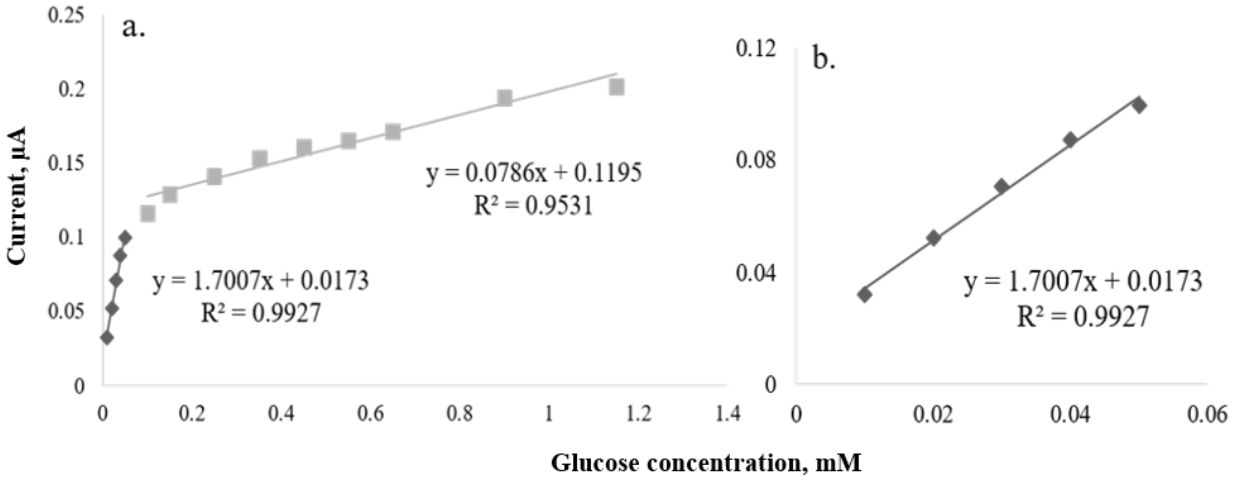


Figure 11.13. Calibration curve for glucose obtained with Ppy-*A. niger* MIUG 34 biosensor within: a. an extended concentration range (0.01 – 1.15 mM) and b. a low concentration range (0.01 – 0.05 mM).

While the microbial biosensor proved linearity both over an extended glucose concentration range of 0.01-4.1 mM (Figure 11.12a) and at low concentration between 0.01 – 0.05 mM (Figure 11.12b), the linearity of the Ppy microbial biosensor was limited to lower concentrations of 0.01 – 0.05 mM glucose (Figure 11.13b). However, the sensitivity of the Ppy microbial biosensor was 13 times higher than that of the simple microbial biosensor considering the extended linear range. Certainly, the widening of linear range towards higher substrate concentrations is interrelated to the decrease of sensitivity for the simple microbial biosensor. Yet, the amperometric response was still over five times higher for the Ppy biosensor when the exact same linear ranges were compared (Figure 11.12b *vs* Figure 11.13b). The benefits of Ppy modification are most noticeable at low analyte concentrations, the standard curve deviates promptly from linearity when glucose concentration exceeds 0.05 mM. Presumably, this accounts for an endorsed enzymatic reaction in the presence of Ppy, leading to very sensitive analyte detection but also to faster enzyme saturation.

Accordingly, the limit of detection (LOD) determined for the Ppy microbial biosensor is superior, up to seven times lower than the LOD obtained with the simple microbial biosensor considering the exact same linear range (0.01 - 0.05 mM). The analytical parameters derived from the calibration curves are presented in Table 11.2.

Table 11.2. *Analytical characteristics of the microbial biosensors.*

Type	Linear range, mM	Sensitivity*, µA/mMcm^{-1}	LOD, mM	I_{max}, µA	Km, mM
A. niger MIUG 34 biosensor	0.01 – 4.1	1.97±0.45	0.114	0.068	0.148
	0.01 – 0.05	5.03 ±1.05	0.036		
Ppy- *A. niger* MIUG 34 biosensor	0.01 - 0.05	**27.25±1.84**	**0.005**	0.186	0.048

*Result of triplicate measurements

Research on glucose microbial biosensors based on GOx-producing fungi [445, 446] reveals similar sensitivity values with the ones provided herein by the simple microbial biosensor, improved usually by the use of redox compounds that increase the enzymatic reaction rate. Another alternative

for improvement is the use of conducting polymers (CPs). Literature data reports several immobilizations of GOx within electropolymerized Ppy which has led to extensive linear ranges but moderate sensitivities [254, 265, 447-449]. This can be attributed to loss and/or denaturation of enzyme during electropolymerization. The widening of the linear range commonly leads to lowering of the sensitivity, yet the activation energy necessary for enzyme catalysis appears to be lower when Ppy is involved [262]. Advanced methods of immobilization have been devised in order to take advantage of the conductive properties of electrochemically prepared Ppy without loss of enzymatic activity, providing both an extensive linear range and good sensitivity [264, 266, 275].

In the current study, due to *in situ* biocatalysis, Ppy becomes a conductive coating for the GOx-yielding cells. This confers multiple advantages: (i) increase in cellular membrane permeability and conductivity; (ii) increase in contact between intracellular enzyme and transducer; (iii) less liability for enzyme loss/denaturation. Ppy not only improves the sensitivity at minimal glucose concentrations, but also facilitates the enzymatic reaction.

The applicability of the biosensors developed was evaluated by detection of glucose in beverages. Cappy sour cherry and mix fruit juice were chosen as real samples and the reference glucose concentrations were determined by the HPLC Laboratory of Akdeniz University, Antalya. The measurements were performed in triplicate to compare the detected amount of glucose by biosensor *vs* the known glucose amount and the recovery rates are displayed in Table 11.4.

***Table 11.4**. Glucose detection in fruit juices with the microbial biosensors*.

Fruit juice	**Glucose concentration (g/dL)**	**Determined glucose concentration (g/dL) (n=3)**		**Recovery (%) (n=3)**	
		Microbial biosensor	PPy - microbial biosensor	Microbial biosensor	PPy - microbial biosensor
Sour cherry	4.35	4.56 ± 0.33	4.24 ± 0.22	104.82	97.47
Mix fruit	3.23	3.27 ± 0.3	3.29 ± 0.14	101.23	101.85

Recovery rates for sour cherry juice are higher than the recovery rated for mix fruit juice, attributable to higher amount of interfering compounds in the former. Moreover, the slightly lower recovery rate noticeable for the Ppy microbial biosensor in this case is probably related to the minimization of such interference. Similar recovery rates were reported for enzymatic glucose biosensors: based on GOx [451,452] or pyranose oxidase [453]. Since microbial biosensors usually possess less specificity, the recovery results obtained are suitable indicating that the proposed systems are an accurate method for sensitive detection of glucose due to involvement of Ppy.

11.2 Catechol biosensors based on polypyrrole (Ppy) formed within *Trametes pubescens* culture filtrate

A secondary approach is that of pure enzyme incorporation within conductive matrices during electrodeposition. This is challenging because of liabilities such as enzyme leaching and/or denaturation and the current study provides an alternative. Since the formation of Ppy is also available in white-rot fungi cultures (i.e. PPO producers) both at extra and intracellular level, the Ppy formed extracellularly (in culture filtrates) represents an adequate 'green' alternative for enzymatic biosensor systems.

Simple *TP* culture was used as crude extract, resulting in the first type of biosensor (*TP* biosensor). Upon formation of Ppy within *TP* culture, biocatalytic Ppy was used for the second type of biosensor (BioPpy biosensor). For comparison of enzymatic activity preservation upon Ppy formation within *TP* culture, a third type biosensors was developed, based on the biocatalytic Ppy collected previously mixed (1:1 ratio) with simple *TP* extract (BioPpy-*TP* biosensor) to observe if its sensitivity is superior to the BioPpy biosensor. Lastly, a chemical alternative for Ppy formation was employed (described in Section II, subsection 5.3) resulting in chemically synthesized Ppy (ChemPpy). The ChemPpy was mixed with the *TP* extract (1:1 ratio), resulting in the fourth type of biosensor (ChemPpy-*TP*), providing an adequate comparison for the bio-electrochemical performance of the biocatalytic Ppy formed within *TP* culture whether simple or mixed with additional *TP* enzymatic extract.

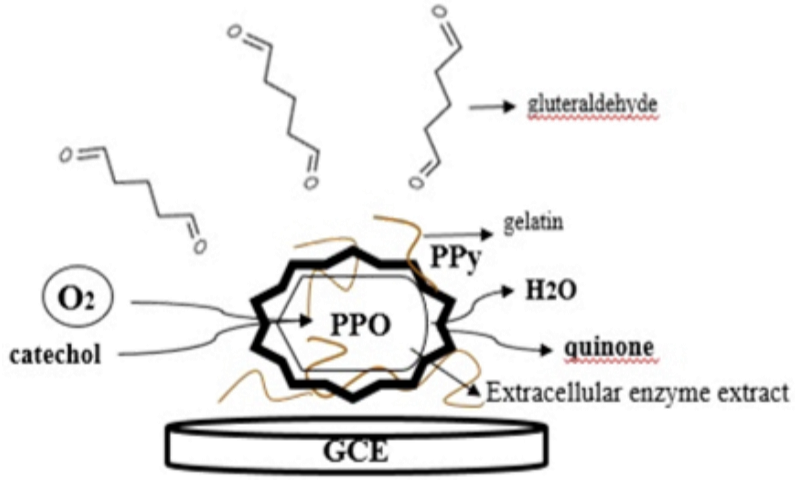
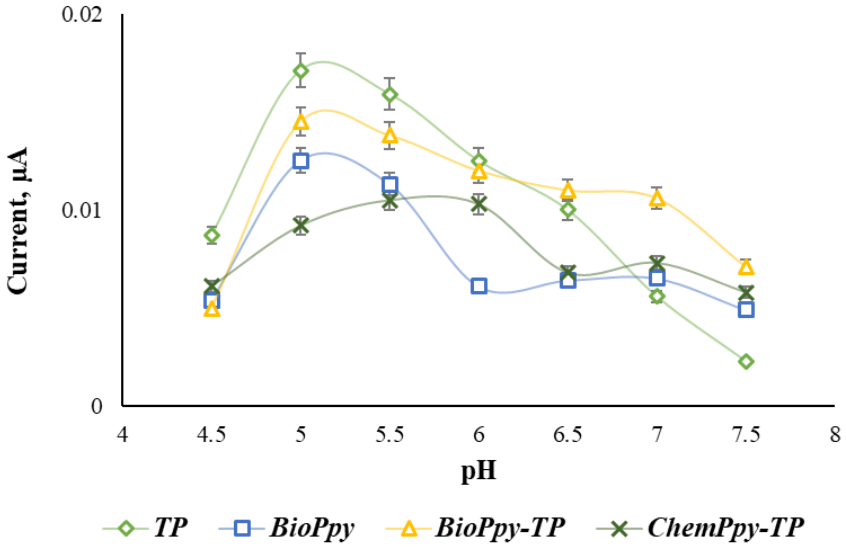


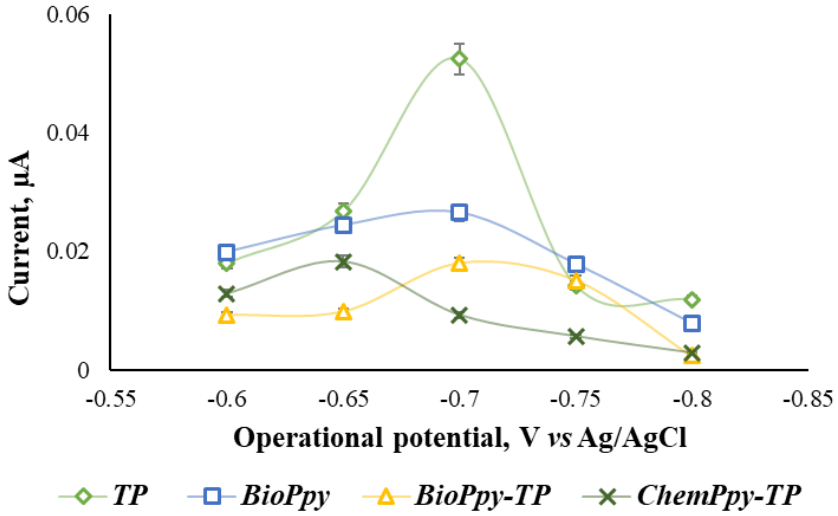
Figure 11.16. Biolayer design for the catechol biosensors based on: *TP* enzyme extract either simple or modified with Ppy and immobilized on top of a glassy carbon electrode (GCE).

For each type of biosensor developed, pH and operational potential optimizations were performed.



The most suitable pH value for catechol detection was 5.0 for three out of four biosensors (*TP*, BioPpy, BioPpy-*TP*). The fourth type responded best to pH 5.5, presumably due to slight modification of enzymatic reaction parameters by ChemPpy.

Figure 11.17. Optimum pH for catechol detection with the *TP* - based biosensors; 0.1 M PBS, V=-0.7/-0.65 V *vs* Ag/AgCl; additions of 0.1 mM catechol.



This is confirmed by operational potential optimization, in which the three systems based on *TP* extract and BioPpy responded best at -0.7 V *vs* Ag/AgCl and ChemPpy-*TP* biosensor had an optimum result at -0.65 V *vs* Ag/AgCl.

Figure 11.18. Optimum operational potential for catechol detection with the *TP* - based biosensors; 0.1 M PBS, pH 5/5.5; additions of 0.1 mM catechol.

Interesting remarks considering the optimum pH for catechol detection using *TP* extract-based biosensors are: (i) pH values in the range 5.0-5.5 are commonly suitable for laccase catalysis proving the predominant enzyme behaves as a laccase as anticipated from analysis of PPOs biosynthesis within white-rot fungi cultures (Section III, subsection 10.1.3); (ii) several catechol biosensors using pure laccase report similar operational pH values [454-456], thus BioPpy did not alter the usual enzymatic reaction conditions; (iii) a secondary peak around pH = 7.0 can be observed and it is correspondent to optimum pH for tyrosinase catalysis (as reported in tyrosinase-based catechol detection [287, 445, 457]), concluding that the *TP* extract used in this study is a multi-enzymatic system based on both laccase and tyrosinase, although laccase predominance can be attested.

Catechol detection was based on oxygen consumption during PPO-catalyzed bioconversion of phenols. The amperometric response was examined in optimized conditions of potential and pH after a steady state current value was reached. Progressive catechol additions started at concentrations of 1.0 µM and each addition led to amperometric current increase, which was considered proportional with the catechol concentration added. Thus, standard plots of amperometric current *vs* catechol concentration were determined.

The calibration curve of the *TP* biosensor is illustrated in Figure 11.21 and the comparison between the calibration curves of the three Ppy-containing biosensors is given in Figure 11.22.

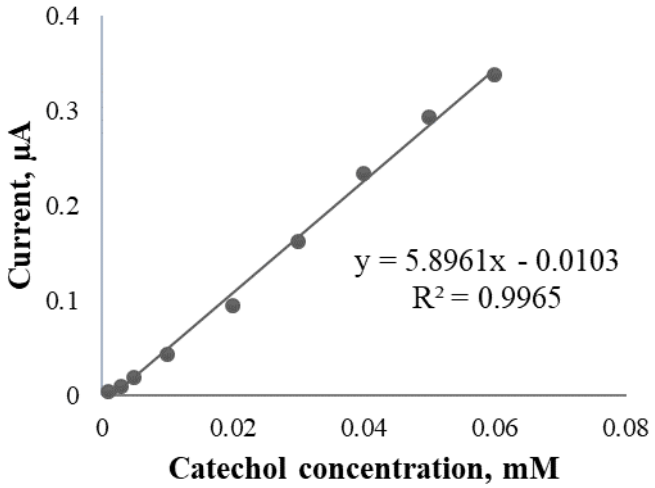


Figure 11.21. Calibration curve for catechol obtained with the *TP* biosensor (0.001-0.06 mM).

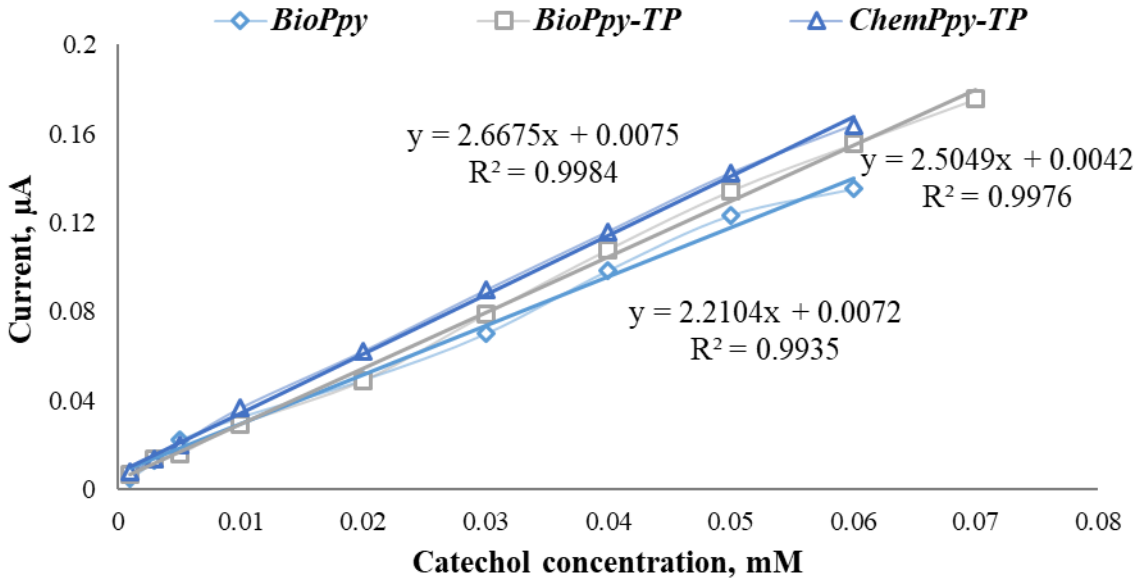


Figure 11.22. Calibration curves for catechol obtained with the: BioPpy biosensor (0.001-0.06 mM); BioPpy-*TP* biosensor (0.001-0.07 mM); ChemPpy-*TP* biosensor (0.001-0.06 mM).

The linearity for catechol detection was similar for all the prepared biosensors. However, detection sensitivity using the simple *TP* biosensor was considerably higher in comparison with the three Ppy-containing biosensors. Illustration of the analytical parameters of the developed biosensors as derived from the calibration curves are given in Table 11.5.

Table 11.5. *Analytical characteristics of the TP-based biosensors.*

Type	Linear range, mM	Sensitivity*, μA/mMcm^{-1}	LOD, mM	I_{max}, μA	Km, mM
***TP* biosensor**	0.001 – 0.06	83.39±1.96	0.0018	10.84	0.7
BioPpy biosensor	0.001 - 0.06	30.40±0.45	0.005	0.134	0.026
BioPpy-*TP* biosensor	0.001 – 0.07	34.61±1.28	0.0021	0.497	0.153
ChemPpy-*TP* biosensor	0.001- 0.06	37.45±0.48	0.0018	0.267	0.057

* Result of triplicate measurements

All the *TP*-based biosensors proved effective for very sensitive detection of catechol. All the linear ranges presented are between 1-60/70 μM with sensitivities from 83.39 μA/mMcm^{-1} for the simple *TP* biosensor to 30.04 μA/mMcm^{-1} for the BioPpy biosensor. Also, adequate LOD values were obtained in the range 1.8-5.0 μM. The highest Km value was 0.7 for the simple *TP* biosensor and is due to high I_{max}, since the two are interrelated. The Ppy-containing biosensors exhibit lower Km values, related to increased affinity for analyte, but also due to lower I_{max} values. Firstly, it is important to note that developing a biosensor based on crude enzyme extract is highly efficient, in addition to overcoming extra tedious steps of enzyme purification. Another aspect to be considered is that all the Ppy-containing biosensors exhibit very similar behaviour, from which some conclusions are derived: (i) the similarity between BioPpy and BioPpy-*TP* biosensors accounts for confirmation of enzyme entrapment within the *in situ* formed Ppy structure, otherwise the BioPpy-*TP* biosensor would have exhibited superior performance; (ii) the biocatalysis of Ppy is valid and comparable with a chemical polymerization since BioPpy and ChemPpy biosensors exhibit similar performance; (iii) Ppy has the same influence within the biosensor systems regardless of its synthesis.

All the four types of biosensors prepared were tested for phenol detection in real samples, fruit wines (blueberry, blackberry and pomegranate wine). For comparison and as a standard method for determining total phenolic content, Folin-Ciocalteau method was employed and the phenolic content was detected in each wine sample (Folin-Ciocalteau protocol detailed in Section II, subsection 8.3.3). Table 11.7 depicts the recovery rates for each biosensor and each type of wine.

Table 11.7*. Real sample analysis for the TP-based biosensors.*

Wine sample	Recovery (%) (n=3)			
	***TP* biosensor**	**BioPpy biosensor**	**BioPpy-*TP* biosensor**	**ChemPpy-*TP* biosensor**
Blackberry	98.66±0.46	97.20±0.55	101.28±0.25	101.71±0.30
Blueberry	101.97±0.87	112.34±0.93	109.4±0.33	102.85±0.49
Pomegranate	94.80±0.18	97.53±0.46	103.7±0.19	97.83±0.14

Recovery rates are similar for all types of biosensors prepared and are adequate. It is noticeable that the simple *TP* biosensor is most effective when detecting phenols in the blueberry

wine sample, which has the lowest phenolic content (around 1299.18 mg/L) while the BioPpy-*TP* and ChemPpy-*TP* biosensors are more suitable for detection of a medium phenol content as encountered in the blackberry wine sample (around 2214.5 mg/L). When higher phenolic content is present as in the pomegranate wine sample (around 3110.2 mg/L), the Ppy-containing biosensors are the most suitable based on recovery rates, although slightly under 100%. These results show that the biosensor based on simple *TP* enzyme extract is very efficient in sensitive detection, whilst the biosensors including Ppy are more appropriate for medium range phenol detection. This is due to high sensitivity of the enzyme extract to small substrate concentrations and rapid enzyme saturation phenomenon when the substrate concentration increases.

Overall, the preparation of biosensors based on white-rot fungi enzyme extract appears to be a very good alternative to pure enzymatic biosensors based on PPOs.

Conclusions

The development of microbial electrodes is reliant on the facility of preparation and economical aspects. Commonly, the sensitivity and selectivity of such systems are amenable to improvement. Since the performance of a biosensor is contingent on the charge transport between the biorecognition element and the transducer, the modification with a conducting polymer is advantageous, as extensively reported in literature data. The benefits of this study are based on Ppy biosynthesis within GOx-yielding fungi cultures. The process consists of one-step biocatalysis rendering Ppy-enveloped cells with the ability for very sensitive glucose detection.

A secondary approach taken in bio-electrochemical analysis of biosynthesized Ppy was that of developing enzymatic biosensors based on simple and Ppy-modified enzymatic extract. Since the white-rot fungi cultures (*TV*, *TP*) showed availability for Ppy formation whether in the presence or absence of living cells due to suitable biosynthesis of PPOs both intra- and extracellularly, it was beneficial to take advantage of the extracellular enzymatic extract of *TP* for development of very sensitive catechol biosensors.

The comparative studies reported here revealed the advantages of production of Ppy *in situ* within fungi cultures that are oxidoreductase yielding. Certainly, the biosensors developed herein represent just model concepts in establishing the suitability of biosynthesized Ppy for bio-electrochemical applications.

12. General conclusions, innovations and perspectives

The PhD thesis termed **'Study of the biotechnological conditions for polypyrrole synthesis, by *in situ* polymerization with selected microorganisms'** was devoted to the investigation of Ppy production through biocatalysis conducted by microorganism cultures *in vitro*. Due to its innovative aspects and complexity, the research was focused on some of the major prospective goals. These were the main research objectives:

(i) The selection of microorganism strains with ability for Ppy bioproduction based on their biochemical characteristics;

(ii) The analysis of the submerge cultures that proved ability for Py bioconversion by protein content and enzymatic assays;

(iii) The evaluation of the Py oligomerization process and the characterization of the formed Ppy through spectroelectrochemical techniques;

(iv) The investigation of bio-electrochemical potential of the obtained Ppy by amperometric analysis.

The general conclusions will be presented accordingly:

(i) Eleven out of twenty-five filamentous bacteria strains (*Streptomyces* spp.), two out of five white-rot fungi strains (*Trametes* spp.) and two out of six common fungi strains (*Aspergillus niger*) proved ability to induce Py oligomerization *in situ* in submerge cultivation conditions. The Ppy was produced mostly in colloidal form as Ppy microparticles and their size and frequency were dependent on the specific microorganism culture used.

Infrared spectroscopy analysis identified the particles as composed of Ppy through detection of chemical bonds. Apart from the main vibration bands owing to chemical structure of Ppy, the additional peaks were indicative of low oxidation level of the Ppy formed and of effect of acetate group within polymer backbone due to the aqueous polymerization media.

The best timeframe for the oligomerization process along with favorable characteristics were observed for Ppy produced in the fungi cultures (*Trametes versicolor*, *Trametes pubescens*, *A. niger* MIUG 34 and *A. niger* MIUG 35 strains), thus further study was exclusively focused on them.

(ii) The highest protein biosynthesis was observed for the white-rot fungi cultures (*Trametes versicolor*, *Trametes pubescens*) both extra- and intracellularly. Enzymatic assays were divided in polyphenoloxidase (PPO) and glucose oxidase (GOx) determinations and the white-rot fungi proved to be PPO producers, whilst the common mold fungi (*A. niger* MIUG 34; *A. niger* MIUG 35) yielded GOx. The common mold fungi cultures had the highest enzyme yield at intracellular level, especially *A. niger* MIUG 35.

(iii) The UV-Vis evaluation of Py oligomerization proved similar evolution for *Trametes versicolor* and *Trametes pubescens*, adequate performance being achieved whether in the presence or absence of living cells. This relates to similar enzymatic yield both extra- and intracellularly. Lengthy Py oligomeric chains were described by UV-Vis data, however little to no electrical charge was noticed within the final product. Within the common fungi cultures, the oligomerization was superior from electrical features standpoints, with best performance in the presence of living cells. Two different tendencies were observed, the oligomerization catalyzed by *A. niger* MIUG 34 consisted in consecutive formation of oligomer intermediates reaching extensive chain lengths and the process induced by *A. niger* MIUG 35 was focused on the formation of a single π-conjugated chain, followed by charge gain with the polymeric backbone. In this respect, the bioconversion product coded Ppy-MIUG 35 shows the best

potential for further use in bio-electrochemical fields. For comparison purposes, the chemical synthesis of Ppy with H_2O_2 was conducted and it revealed similar evolution with the biocatalytic processes, validating the proposed approach.

The electrochemical investigations (CV, OCV and pH) indicated that the most suitable electrochemical behaviour is rendered by Ppy-MIUG 35. Suitable redox switching and good capacitive behaviour were observed for Ppy formed in each culture, when potential cycling was performed in PBS and ultrapure water. OCV evolution confirmed the neutral to moderate oxidized state of Ppy-*TV* and Ppy-*TP* and moderate to highly oxidized state for Ppy-MIUG 34 and Ppy-MIUG 35 bioproducts. The evolution of pH showed steady decrease for the common mold fungi cultures due to production of gluconic acid and H_2O_2 by GOx, which was favorable for Py bioconversion. The pH in white-rot cultures remained within weakly acidic range, which is slightly higher than commonly necessary for Ppy production.

(iv) Two main types of biosensors were constructed: glucose microbial biosensors using simple and Ppy-modified biomass from *A. niger* MIUG 34 and *A. niger* MIUG 35 cultures and catechol enzymatic biosensors based on simple and Ppy-modified crude *Trametes pubescens* enzyme extract.

The Ppy - *A. niger* MIUG 35 biosensor showed increased sensitivity within a wide glucose concentration range (0-300 mM) and the Ppy- *A. niger* MIUG 34 biosensor proved ability for very sensitive glucose detection. The linear range was limited to 10-50 µM and the sensitivity increased up to six times, LOD value reached 5.0 µM and Km decreased to a third of its initial value upon modification with Ppy. Thus, in this context Ppy has a two-fold purpose: that of a redox mediator that aids in permeability of cellular membrane, facilitating the enzymatic reaction and that of a conducting polymer, improving the contact between biomolecule and transducer within a biosensor system. The detection of glucose in fruit juices revealed good recovery rates even comparable with enzymatic biosensors.

Four different biosensor configurations were developed based on *Trametes pubescens* crude enzyme extract, allowing observation of Ppy within the system, produced either chemically or through biocatalysis. The linear range for all the catechol biosensors was limited between 1-60/70 µM and the best sensitivity of 83.04 $\mu A/mMcm^{-1}$ was obtained with the *TP* extract biosensor. The Ppy-containing biosensors revealed diminished sensitivities, albeit adequate LOD and Km values. This is presumably related to some sort of enzyme inhibition as observed in literature data for *in situ* biocatalytic formation of Ppy with pure enzyme. Nevertheless, this proved that the biocatalytic Ppy is comparable with chemically synthesized Ppy, which makes it an economical and environmentally friendly alternative. Real sample analysis of fruit wines (blackberry, blueberry and pomegranate) rendered good recovery rates, comparable with literature data on pure enzyme biosensors.

Overall, the innovative quality of the study is given by the use of cell cultures as 'polymerization bulk' solutions, which represents a creative alternative between using pure enzyme, that requires costly and tedious enzyme purification and using chemically functionalized microorganisms. Considering that there are several types of microorganisms that produce the enzymes that are usually employed in purified form, the proposed concept represents an economical and environmentally friendly pathway for Ppy biosynthesis. Concerning the oligomerization process, the difference between fungi culture efficiency in Py bioconversion is related to several factors such as: (i) the enzymatic catalyst yielded by the culture; (ii) the polymerization pathway derived from the biocatalyst; (iii) the optimum pH for microorganism growth. The process is valid and comparable with literature data on Ppy production through pure enzyme catalysis. Regarding the quality of the end product, it is dependent on the oxidative strength, which in 'green' approaches for Ppy production, is

seldom sufficient. Yet, the addition of templates and/or redox mediators in the polymerization environment aids in producing an ordered and conducting Ppy through a biocatalytic, economic and environmentally friendly pathway. The potential for bio-applications proved by biosensor development is the ultimate advantage of the current study.

This type of investigation have not been reported previously, to the best of our knowledge, which emphasizes the originality of the study along with its preliminary character. Several study parameters can be tuned for achievement of advanced results due to the biochemical diversity and metabolic potential of the microorganisms. The complexity of the research endeavor allows additional study perspectives. For instance, the selection of microorganisms able to induce Py bioconversion can be extended and the bio-preparation of other conducting polymers such as PANI can be attempted. On this note, the accountability of pH values during cultivation is required since PANI, more so than Ppy, requires an acidic polymerization environment. For better understanding of the polymerization process, molecular dynamic simulations can be effectively used to assess the function of biomolecules within this context. Further on, the bio-applications can be diversified from biosensors and biofuel cells to ion exchange membranes or drug-delivery systems. The polymer can be obtained whether in the presence or absence of living cells, thus crude enzymatic extract or cells are available for prospective applications.

Validation of results

List of author's publications summarized in the dissertation:

- **Web of Science**

1. **Roxana-Mihaela Apetrei**, Geta Carac, Gabriela Bahrim, Almira Ramanaviciene, Arunas Ramanavicius. ***Modification of Aspergillus niger by conducting polymer, Polypyrrole, and the evaluation of electrochemical properties of modified cells***, in: Bioelectrochemistry, Volume 121, 6 January 2018, Pages 46 –55 (Impact Factor **3.346**) https://doi.org/10.1016/j.bioelechem.2018.01.001

2. **R.-M. Apetrei**, G.-E Bahrim, G. Carac. ***Spectroelectrochemical characteristics of Polypyrrole synthesized by different methods***, in: Bulgarian Chemical Communications, Volume 49 Special Issue C (pp. 74 – 83) 2017 (Impact Factor **0.238**) http://www.bcc.bas.bg

3. Arunas Stirke, **Roxana-Mihaela Apetrei**, Monika Kirsnyte, Lina Dedelaite, Vladimiras Bondarenka, Vitalija Jasulaitiene, Milda Pucetaite, Algis Selkis, Geta Carac, Gabriela Bahrim, Arunas Ramanavicius. ***Synthesis of polypyrrole microspheres by Streptomyces spp.*,** in: Polymer, Volume 84, 10 February 2016, Pages 99-106 (Impact Factor **3.684**) https://doi.org/10.1016/j.polymer.2015.12.029

- **International Database**

1. **Roxana-Mihaela APETREI**, Geta CARAC, Gabriela-Elena BAHRIM. ***Bioproduction and relevance of conducting polymers: polypyrrole***, in: Innovative Romanian Food Biotechnology, Vol. 17, Issue of November, 2015 www.bioaliment.ugal.ro

List of Conferences, where the results of the dissertation were presented:

- **International**

1. **Roxana-Mihaela Apetrei**, Geta Cârâc, Gabriela Bahrima Pinar Camurlu. ***Catechol biosensor based on Trametes pubescens and polypyrrole***, 3rd World Congress on Material Science & Engineering, Spain, 2017

2. **Roxana-Mihaela Apetrei**, Geta Cârâc, Gabriela Bahrim, Pinar Camurlu. ***Glucose biosensor based on whole cells of Aspergillus niger MIUG 34 coated with polypyrrole***, EUROBIOTECH, Croatia, 2017

3. **Apetrei R.M**., Gabriela Bahrim, Geta Carac. ***Testing of microorganism strains for biosynthesis of polypyrrole***, EUROBIOTECH, Latvia, 2016

- **National**

1. **Roxana-Mihaela Apetrei**, Geta Cârâc, Gabriela Bahrima Pinar Camurlu. ***Microbial biosensor based on Aspergillus niger MIUG 34 for sensitive determination of glucose***, CSSD-UDJG, Galati, 2017

2. **Roxana-Mihaela Apetrei**, Geta Cârâc, Gabriela Bahrim, Arūnas Ramanavicius. ***Spectroelectrochemical characterization of pyrrole polymerization in situ with selected fungi strains***, International Conference of Physical Chemistry ROMPHYSCHEM, Galati, 2016

3. **Roxana-Mihaela Apetrei**, Geta Cârâc, Gabriela Bahrim. ***Screening of fungi strains for polypyrrole biosynthesis in situ***, CSSD-UDJG Galati, 2016

4. **Apetrei R.M**., Gabriela Bahrim, Geta Carac. ***An approach for obtaining polypyrrole microparticles using Streptomyces spp. strains***, CSSD-UDJG Galati, 2015