Enzymatic Depolymerization of Lignin Enhanced by Electrochemistry

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ABSTRACT

Lignin is an important component of biomass that has recently become a subject of interest to the scientific community as a highly attractive renewable source of chemicals, materials and fuels. However, lignin has complex structure with high stability bonds, making lignin depolymerization a challenging mission.

The main goal of this thesis was to depolymerize lignin combining three distinct approaches: enzymatic, chemical and electrochemical depolymerization.

Therefore, laccase from *Trametes versicolor*, was tested with the mediator, ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) to identify the optimal conditions, in order to obtain the higher enzymatic activity possible.

There are several studies about laccase behavior in the presence of ILs to degrade lignin, but since these solvents are expensive, the goal was to try other solvents with similar physical-chemical properties, like sodium xylene sulfonate (SXS) and a deep eutectic solvent (DES).

The reaction system was composed by the enzyme, solvent and lignin. In order to understand the behavior of the system components in the presence of electrochemistry, two different potentials, 0.05 and 0.25 V, were applied to a stirred reactor, to be subsequently compared with the system without electropotential.

Results showed that the ideal conditions for the laccase production involved *Pichia* Trace Metals (PTM_1) medium supplemented with kanamycin, at pH 5. Laccase activity decreases with the solvents concentration, especially with DES. In the further experiments, there were used 10 w/v % of SXS and 80 v/v % of DES. Enzymatic activity was improved applying a certain potential for all solvents, reaching higher values with the higher potential studied.

Finally, lignin depolymerization products were analyzed by two different analytical techniques, GPC (to get product molecular weight information) and GC-MS (for product characterization and quantification).

KEYWORDS: Lignin; Laccase; Electrochemistry, Deep Eutectic Solvent (DES), Sodium Xylene Sulfonate (SXS)

1. Introduction

Since the second half of the 20th century, the interest of biomass as a potential substitute of liquid fuels has increase. Lignin valorization has an important role in the modern biorefinery scheme, and its structure and composition offer several useful chemicals [1].

For many years, lignin has been considered a low-quality and low-added-value material, having no relevant industrial application. Instead, lignin is burned as a low-value fuel, to be used for internal heat and power generation [2]. Not using this highly complex chemical system appears a waste of potential. This is mainly because of its complex structure and the several types of linkages is the lignin molecule, making it difficult to depolymerize lignin into single organic compounds [3].

Lignin organic composition, structure and availability as a natural resource, suggest that such a resistant and stable biopolymer may become an important key to the renewable energy industry, having the potential to be a sustainable alternative to fossil fuels [4].

Several different lignin depolymerization strategies to harvest value-added phenolic compounds such as ionic-liquid catalyzed, oxidative, enzymatic and electrochemical depolymerization, can be found in the literature [1][3].

This thesis aimed to evaluate the feasibility of an integrated biological and chemical strategy for lignin depolymerization.

In order to depolymerize lignin through an enzymatic method, a laccase variant [5] from *Trametes versicolor* was expressed in *Pichia pastoris* and used, since this fungal laccase is an oxidoreductase, working as a biocatalyst in the depolymerization reaction. The only resultant by-product is water, making it non-pollutant process [6].

There are several studies about laccase in the presence of ionic liquids (ILs) to degrade lignin, however ILs consumption is not the most affordable method. Therefore, two other types of solvents with similar characteristics were preferred. Sodium Xylene Sulfonate (SXS) solvent was one of the options, since in this solvent lignin solubility is independent from the pH values, allowing enzymatic processes. Deep Eutectic Solvent (DES) [7] ChCl:EtGly, a mixture of choline chloride with ethylene glycol, is a nonaqueous solvent also chosen to depolymerize lignin that is currently investigated with this objective.

The third approach used in this work to depolymerize lignin was electrochemistry. By applying a certain potential to an electroactive lignin solution is possible to degrade lignin structure into smaller molecules. Electrochemical depolymerization is a controllable and affordable method that does not require oxidizing agents to treat lignin.

In complementing research preceding this work, the purpose of this thesis is to connect the three distinct strategies described before to depolymerize lignin, in order to obtain high-value organic chemicals and identify them.

2. Materials and Methods

2.1. Yeast strain, enzyme and culture medium

The strain *Pichia pastoris* SMD1168H was used in the protein expression, from the *P. pastoris* glycerol stock (500 µL 50% glycerol and 500 µL M3 culture). The enzyme used was laccase lcc2 variant M3 from *Trametes versicolor*. Media components including yeast extract, yeast nitrogen base – 10x YNB (57.10 g yeast nitrogen base without amino acids and 7.40 g ammonium sulfate), peptone and glucose were purchased from AppliChem and Sigma-Aldrich.

In this work, two different expression media were studied, Hartwell's complete medium - $HC_{glucose}$ (100 mL 10x YNB, 100 mL 10x HC dropout solution; 100 mL 10 w/v % glucose; 100 mL ddH_2O and 2 mL 0.25 M $CuSO_4$)and *Pichia* Trace Metals solution - PTM_1 (6 g $CuSO_4$; 0.08 g NaI; 3 g $MnSO_4.H_2O$; 0.2 g $Na_2MoO_4.2H_2O$; 0.5 g $CoCl_2$; 20 g 4ZnCl2; 65 g FeSO4; 0.02 g H3BO4; 0.2 g biotin; 19.20 mL H_2SO_4 and fill up to 1 L with ddH_2O) with basal salt medium (50 mL glycerol; 1.18 g $CaSO_4.2H_2O$; 18.2 g K_2SO_4; 5.7 g $MgSO_4$; 9 g $(NH_4)2SO_4$ and fill up to 1 L with ddH_2O) (4.35 mL PTM_1 per 1 L basal salt medium)

2.2. Antibiotics

Working with concentrations (µg/mL) of Ampicillin (50.0), Kanamycin (50.0) and Zeocin (12.5) were used from 100 mg/mL stock solutions.

2.3. Expression of lcc2

The lcc2 cDNA gene isolated from *T. versicolor* was cloned and expressed in *P. pastoris*. *P. pastoris* cells were streaked on yeast extract-peptone-dextrose (YPD) agar plate, and incubated at 30ºC for two days. Then, a *P. pastoris*/lcc2 colony was transferred from the agar plate into a 250 mL Erlenmeyer flask containing 25 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose). Incubation was performed under 250 rpm shaking at 30ºC until OD_{600} reached 2-6. Cell growth in this preculture was monitored daily

through OD_{600} readings. Samples were diluted in ddH_2O (1:10) [6]. Then, a second preculture was performed, by transferring 25 mL of the first preculture into a 1000 mL Erlenmeyer flask containing 200 mL YPD, incubated overnight at 30ºC, 250 rpm. The expression was performed for 5 days, in 1000 mL of the expression medium, in a 2000 mL Erlenmeyer flask. The Erlenmeyer was incubated at 20ºC under 250 rpm shaking, with the start OD_{600} of the culture of 0.4. The expression was performed in several conditions, using two different media ($HC_{glucose}$ and PTM_1) supplemented with different antibiotics (1 mL/1 L expression medium).

The supernatant was recovered by centrifugation at 4ºC, 1519 RCF, 10 minutes and stored at 4ºC. The enzyme was concentrated by using Amicon Ultra-15 Centrifugal Filter Devices (30 kDa), centrifuging at 4000 rpm, for 10 minutes at 4ºC. Then, 300 µL of culture were pipetted to an Eppendorf tube, always in a sterile environment, and then centrifuged at 3100 RCF for 10 min. The supernatant was transfered to another Eppendorf tube and the cell pellet is discarded. The supernatant obtained was used for the activity determination.

2.4. Laccase Activity Screening Assays

The laccase activity was studied by the oxidation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The non-phenolic dye is oxidized by the enzyme and forms a stable radical accountable for the green color. The concentration of this green cation radical can be correlated to the enzyme activity, through the Lambert Beer law [8].

The oxidation was carried out at different pH values in the presence of different solvents in microtiter plates for 4 hours at room temperature and monitored at 420 nm using Tecan Sunrise™ microtiter plate reader. All of the assays were performed in duplicate.

To determine the optimal pH value to obtain the higher enzymatic activity possible, the Britton-Robinson (BR) buffer (pH 3-7) (0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH) was used. The media composition for screening was: 30 µL BR (pH 3-7); 30 µL supernatant; 60 µL 5 mM ABTS and 80 µL ddH_2O.

To study the laccase activity in the presence of different concentrations of the solvents, SXS and DES, the pH was set to 5.0. For the assay buffer 48.50 mL 0.1 M citric acid and 51.50 mL 0.2 M Na_2HPO_4 were mixed. ABTS assays with 40 w/v % SXS and DES were also performed.

2.5. Cyclic Voltammetry

Cyclic voltammetry is a fast technique usually used for obtaining qualitative information about the electrochemical behavior of electrolytes which allows to establish the location of redox potentials of the electroactive solution. The current is measured continuously during the sweep against the applied potential (current vs potential). Using NOVA 2.0 software is possible to control the potentiostat/galvanostat (Metrohm Autolab). This allows to choose the upper and lower potential, scan rate and the number of stop crossings that defines the number that the scan should cross the "stop potential", in order to stop the measurement. In the set-up used, a stirred reactor of 100 mL was connected to the potentiostat by graphite electrodes, one anode and one cathode, where the electrochemical cleavage of lignin was carried out, using $Hg/HgSO_4$ reference electrode, since this electrode and the solutions prepared have similar ions. The graphite electrodes were chosen because graphite is electrochemically stable in acid environment.

Cyclic Voltammetry was performed to the different solvents used in this thesis: buffer (48.50 mL 0.1 M Citric Acid - 51.50 mL 0.2 M Na_2HPO_4), 10 w/v % SXS and 80 v/v % DES, all of them at pH 5. Using NOVA 2.0 software, the CV stair case (voltage range) defined was -1.5 V and 1.5 V, to the lower and upper vertex potential, respectively.

The number of stop crossing that was chosen was 8, meaning that the CV is going to make 4 cycles, but the first one is ignored. Three different scan rates were operated by this technique, 0.050, 0.025 and 0.010 V/s.

This experiment was designed in order to understand the electrochemical behavior of the different components of the system, when combined. Five different cyclic voltammograms were performed: only solvent; solvent combined with ABTS; solvent and lignin; solvent, ABTS and enzyme; and solvent, ABTS, enzyme and lignin.

When only one solvent was used, the reactor was filled with 80 mL of the solvent in study. The lignin solution was prepared solubilizing the Kraft lignin in 40 w/v % SXS or pure DES (5 g lignin/L).

2.6. Chronoamperometry Analysis

Another electrochemical technique is the chronoamperometric approach. This method is operated at a defined and constant potential value, which is chosen based on the cyclic voltammetry results, considered as reference value, and the resultant current is monitored as a function of time (current vs time) [9].

By using the chroamperometry analysis it is possible to study the behavior of the system components over time. The NOVA 2.0 software is also used in CA and the experiment setup was the same as the one described in section 2.3. There were applied distinct set potentials, 0.05 and 0.25 V, for 8 hours, to understand better the laccase activity using the electrochemical approach to depolymerize lignin, in order to compare the enzymatic activity with and in the absence of potential. This analysis also has the intended to study the influence of electrochemistry in the system by itself, i.e., preparing buffer, 10 w/v % SXS and 80 v/v % DES solutions (pH 5) with and without the enzyme.

During the experiment, at each hour a sample (200 µL) was collected from the reaction system and its absorbance was measured using a Tecan Sunrise™ microtiter plate reader (ABTS assay). This technique does not allow to possible to analyze the system in the presence of lignin, since it is impossible to measure its absorbance at 420 nm.

2.7. Lignin Depolymerization

With the aim of depolymerizing lignin, the experimental setup described in section 2.3. was used, applying the same set potentials that were used in the chronoamperometry analysis. The experiment was running for 32 hours and six samples of 2 mL were collected, at 0, 1, 2, 8, 24 and 32 h, to a 50 mL falcon tube. All of the depolymerization reaction systems analyzed contain the solvents, ABTS, lignin and the enzyme.

2.7.1. Lignin Precipitation

After collecting every sample, with 2 mL of lignin solution (5 g/L), 6 mL of H_2SO_4 (0.01 M) were added to the falcon tubes containing 5 g lignin/L buffer and 80 v/v % DES solutions (dilution 1:4) and 18 mL of H_2SO_4 (0.01 M) to the tubes belonging to 5 g lignin/L 10 w/v % SXS (dilution 1:10). All of the falcon tubes were subsequently centrifuged at 12400 RFC per 10 minutes, at room temperature. In the next step the supernatant and the pellet (solid lignin) were completely separated and the liquid phase was transferred into 50 mL falcon tubes. The solid phase was washed again with 5 mL of H_2SO_4 (0.01 M), and resuspended using a vortex. The solution was centrifuged again at 8000 rpm, at room temperature for 10 min. The liquid was separated again from the solid phase and the washed H_2SO_4 were added to the supernatant falcon tube. The supernatant (approximately 11 mL for buffer and 80 v/v % DES solutions and 23 mL for the 10 w/v % SXS solution) was stored at 4ºC and the solid lignin was frozen at -20ºC, overnight.

The solid phase was freeze dried (Alpha 1-4 LSC plus, *CHRIST*) for two days, and resuspended in 1 M NaOH (2 mL), using the Vortex. 1 mL of the suspension was transferred into HPLC vials, to be then analyzed by GPC.

2.8. Lignin and Products Liquid-Liquid Extraction

The liquid phase stored from the lignin precipitation was used in two different ways:

4 mL of the supernatant were mixed with 4 mL of MIBK (4-methyl-2-pentanone) in a 15 mL falcon tube, followed by a liquid-liquid extraction (two phase system). The tubes were stirred for 5 hours, and then the solution was suspended by Vortex (1-2 min). The separation was carried out using the centrifuge ate 2000 rpm for 3 min, at room temperature. The resultant lower density liquid, containing the MIBK, was separated and transferred into a HPLC vial (1 mL), to be analyzed by GC. This extraction is for lignin products.

4 mL of the supernatant were mixed with 4 mL of ethyl acetate in a 15 mL falcon tube. The tubes were stirred the tubes for 5 hours, and suspended the solution by Vortex after that. The liquid-liquid extraction was carried out using the centrifuge at 2000 rpm for 3 min, at room temperature. 3 mL of the lower density liquid (ethyl acetate) were placed in new 15 mL falcon tubes and dried overnight under the fume hood. When observed that all the liquid is dried, 0.5 mL of NaOH were added, solubilizing the solid in the bottom of the tube by Vortex (2-3 min). 0.5 mL of the NaOH is transferred to a HPLC vial for analysis by GPC. This extraction is for soluble lignin.

2.9. Gel Permeation Chromatography (GPC)

The GPC, is used after lignin depolymerization to determine the molecular weight of the chemical mixtures produced during the process. The samples were prepared as described in the sections 2.7. and 2.8. The average molecular weight (M_w) of each sample was evaluated by this method. Measurements were performed using an Agilent 1200 system equipped with a refractive index detector at a wavelength of 280 nm. The eluent solution was done with water (HPLC grade, Carl Roth), adding 0.1 mol/L of sodium hydroxide (NaOH, 99 %) and 0.01 wt % sodium azide (NaN3, extra pure). The internal standard was a glucose monohydratate solution (12.5 mg/mL).

The analytical GPC procedure employs a variety of detectors to scan the samples. A variable wavelength detector and a refractive index detector are responsible to receive the signals of interest, to be further analyzed by the WinGPC Unichrom software (by PSS).

2.10. Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS allowed the detection and quantification of the monomers and oligomers produced during the lignin electrochemical depolymerization and consequent extraction. The lignin target compounds in this work are: alpha-methylstyrene; guaiacol;.p-cumenol; phenylacetic; syringol; 4-hydroxybenzaldehyd; eugenol; vanillin; 4-hydroxyacetophenon; acetovanilone; di-butylhydroxytoluol; syringaldehyd; acetosyringone and bisphenol-A (BPA).

The samples were prepared as described in the subchapter 2.6. For this analytical method an Agilent 5975 Inert Mass Selective Detector equipped with a J&W 122-0132 DB-1MS capillary column, measuring 30 m x 0.25 mm, with a film thickness of 0.25 µm was used. The carrier gas used was helium with an initial flow of 0.8 mL/min.

The phenol total amount was given by the GC-MS data and the original amount of lignin can be calculated.

3. Results and Discussion

3.1. Laccase optimal conditions

To find the optimal conditions for the expression of lcc2 variant M3 in *P. pastoris* and to obtain the higher enzymatic activity possible, two different mediums, three antibiotics and five pH values were tested. The first step was to evaluate two media, Hartwell's complete medium and *Pichia* Trace Metals solution, and then combine them with different antibiotics, ampicillin, zeocin

and kanamycin (Kan), in order to achieve the best enzymatic activity.

The results showed that laccase presents the highest activity in the presence of the trace salt PTM_1 combined with kanamycin at pH 5.

3.2. Laccase activity in the presence of solvents

Laccases are selective enzymes, which have the ability to degrade lignin, but since it is a polymer with a huge and complex structure, it is not possible for the laccase to oxidize it directly. To overcome this problem, a mediator is required to depolymerize the compound. There are several studies about laccase behavior in the presence of ILs to degrade lignin [5].

A hydrotrophic solution and a deep eutectic solvent were chosen, sodium xylene sulfonate (SXS) and ChCl:EtGly (1:2 M), respectively, and ABTS assays were performed to check the enzymatic activity of the laccase in the presence of these solvents during four hours.

One of the main reasons to use the SXS solution is due to the fact that this solvent does not change the lignin solubility when changing its pH, allowing to reach suitable pH values for enzymatic activity.

Different concentrations of the hydrotrophic solution were evaluated (0 to 30 w/v % SXS) in order to determine the best condition for laccase activity. In the presence of SXS solution, the enzyme presents its highest activity with the concentration of 10 w/v % SXS. This concentration was used in further experiments.

It is already known that DES is a good solvent for wood delignification and many studies are being carried out about its interaction with lignin, and if it is possible to depolymerize this wood compound. Since DES is a nonaqueous solvent, combining it with the laccase could be a problem, since this enzyme usually only acts in aqueous systems.

According to the experiment the higher enzymatic activity is reached at 5 v/v % of DES, which was expected, since this enzyme typically does not work in nonaqueous systems. Nonetheles, 80 v/v % of DES was used in the further experiments, since this concentration of solvent enables lignin solubilization. The enzymatic activity is very low in the presence of 80 v/v % of this solvent in comparison with lower concentrations, although some activity was retained.

This first stage of the experimental work allowed to establish the concentration of each solvent, as 10 w/v % of SXS and 80 v/v % of DES, to be used in further experiments.

3.3. Cyclic Voltammetry Results

Since the enzyme activity is too slow to be possible to observe in the CV, only the results of the scan rate of 0.010 V/s are presented to the electrochemical activity study of the different components of the system [10].

3.3.1. Buffer Cyclic Voltammograms

In the buffer CV, it was only possible to check the oxygen evolution. No hydrogen evolution can be observed, since it is very low. Adding the laccase to the system it was just possible to see a little shift, the behavior is similar in both conditions.

In Fig. 1, two anodic peaks are present with potential at 0.3 and 0.8 V, corresponding to the oxidation of the ABTS to its cation radical ($ABTS^{\bullet+}$), and then to its dication ($ABTS^{2+}$). It is also possible to observe a single cathodic peak, at -0.2 V, resulting from the reduction of the ABTS cation radical [11]. In the same voltammogram, when adding the laccase, the behavior is quite the same as in the graph that just contain the buffer combined with the substrate.

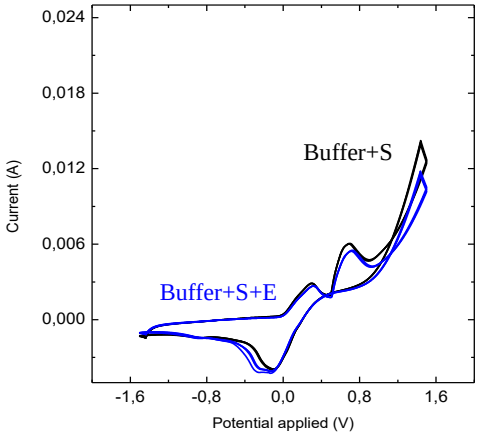

Figure 3.1 – Cyclic voltammogram of the buffer combined with the ABTS substrate (S) and buffer mixed with the substrate and enzyme (E)

Analyzing the results, when looking at the cyclic voltammograms with the substrate, it can be concluded that electropotential by itself is converting the substrate, since the results are similar with and without the enzyme. These results can mean that in both experiments the reactions can be just the result of ABTS conversion by electrochemistry, without enzyme influence.

3.3.2. 40 w/v % SXS Cyclic Voltammograms

After studying the electrochemical behavior of the buffer, the 10 w/v % SXS (Fig. 2) and 80 v/v % DES (Fig. 3)activity was investigated, which was the main focus of this procedure, to check if it was possible to see any interaction between the solvents, enzyme and lignin.

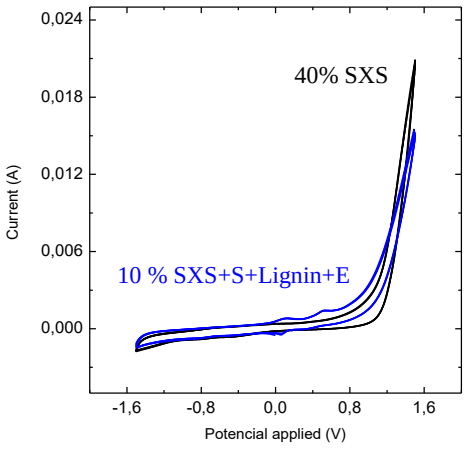

Figure 2 – Cyclic voltammogram of 40% SXS and 10% SXS mixed with the ABTS, lignin and enzyme

By mixing the solvent with the substrate and lignin, is possible to observe two anodic peaks at 0.1 V and 0.7 V, and one cathodic peak at 0.0 V which characterize the substrate activity under electrochemical processes.

3.3.3. DES Cyclic Voltammograms

Attending at the Fig. 3, the DES CV shows a prominent cathodic peak, at 0.1 V, that could be originated by the reaction between the solvent with the graphite of the electrodes. It is also possible to observe an oxidation peak in the oxidative side of the CV that may correspond to the degradation of the solvent. Since it pure DES was used, it was not possible to observe the oxygen evolution, but other gases could have been developed during the reaction. Preparing a solution with the DES and lignin to study its electrochemical activity, in comparison with the CV that just contains the solvent, the cathodic peak became smaller, and the oxygen evolution presented a slight shift.

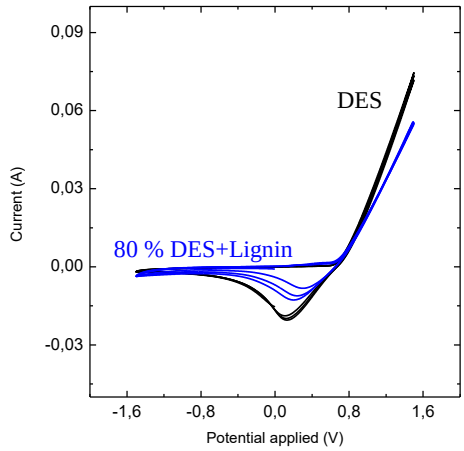

***Figure 3** - Cyclic voltammograms of DES and 80 % DES with lignin*

The CVs belonging to the 80 v/v % DES solution mixed with the different components of the system present a similar behaviour in comparison with the 40 w/v % SXS CVs. Adding the lignin to DES with the substrate, the usual cathodic peak disappears, having no visible reduction of the ABTS to its first radical cation.

3.4. Chronoamperometry Experiments

Fig. 4 is divided in three distinct sections, one for each solvent.

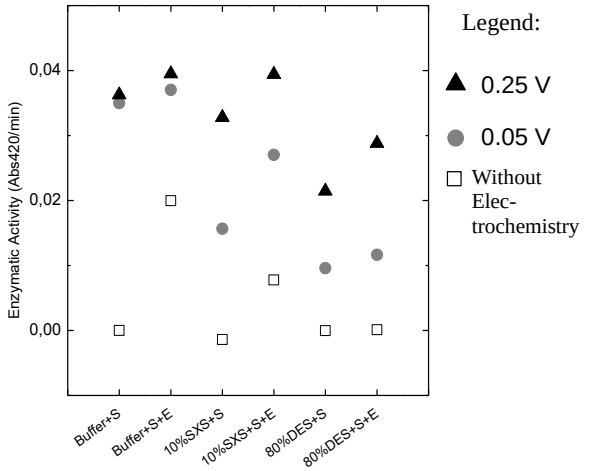

Figure 4 - Chronoamperometric results with two different potentials, 0.05 and 0.25 V, and without electrochemistry, to the buffer, 10 % SXS and 80 % DES, with and without the enzyme, during 4 hours

In every section, without electrochemistry, there is no enzymatic activity when the enzyme is not present in the solution. However, upon enzyme addition activity noticeably increases. Then, with both of the potentials it is possible to notice activity in the presence of laccase or without it, having a slight increase in the activity when the enzyme is mixed in the solution.

With the chronoamperometry analysis, it can be concluded that the activity is always improved with the use of electrochemistry, and combining the enzyme with this approach the results are even more promising. Looking at the results where there is no enzyme present, but where electropotential was applied is possible to check activity in all of the results, which could mean that electrochemistry is converting ABTS by itself, which was already concluded by the cyclic voltammetry results.

3.5. Lignin and Products Chracterization

3.5.1. GPC Results

GPC has been used to obtain information on the molecular weight distributions of polymers of the different lignin fractions [12].

GPC measurement graphs, i.e, chromatograms of lignin with the three different solvents in the presence of laccase applying two potentials, 0.05 and 0.25 V, and without electrochemistry, were performed to study lignin depolymerization during 32 hours.

All of the chromatograms usually have two distinct peaks, the first corresponds to molecules with small structure and the seconds is related with larger analytes. The buffer chromatogram with a 0.05 V potential presented a depolymerization of the small molecules and a polymerization of the larger ones over time. The others buffer chromatograms present a constant behavior.

Without applying potential or with a 0.25 V potential, 10 w/v % SXS chromatograms presented a depolymerization of the small molecules and a polymerization of the larger ones during the experiment. With a 0.05 V potential the opposite was observable, from time zero until 8 hours, small molecules were polymerized and the macromolecules were depolymerized, from 8 to 32 hours the lignin structure returned to its initial state, the small molecules were depolymerized and the others were polymerized.

Without electrochemistry, 80 v/v % DES chromatograms presented a depolymerization of the small molecules and a polymerization of the larger ones during the process time. Applying the lower potential the molecules structure seems to be intact. With a 0.25 V potential, from time zero until 8 hours, all of the molecules appeared to be polymerized.

3.5.2. Products Characterization – GC-MS phenols

By doing this analysis is possible to get information about the products obtained during the electrochemical depolymerization/polymerization and subsequent extraction.

Without electrochemistry the main lignin products obtained during the experiment without applying potential in the presence of the buffer solution were Acetosyringone and BPA, and they

are both degraded during the experiment. Using the 10 w/v % SXS solution, there was no substantial production of none of the target compounds. With the 80 v/v % DES solution, the concentration of guaiacol increases with the time of operation, which is a positive result; in this experiment vanillin was also obtained.

Applying a potential of 0.05 V, using the buffer and the 10 w/v % SXS solutions, there was almost no production of the lignin target compounds. Using 80 v/v % DES, the lignin target compound presented in higher concentration is guaiacol.

Applying a potential of 0.25 V in the presence of the buffer solution, there is almost no production of the lignin target compounds. Using the 10 w/v % SXS solvent, in the beginning of the experiment, acetosyringone and BPA were the compounds obtained with higher concentrations, but after 1 hour up to the end of the experiment these values tend to decline, reaching almost zero. Vanillin is one of the lignin products obtained, using 80 v/v % DES, and its concentration grows during the experiment. Another target compound produced with these conditions was guaiacol, being the lignin product obtained in higher amount in this experiment.

4. Conclusions and Outlook

Lignin is the most abundant aromatic resource on Earth that has profitable uses, representing an important and promising feedstock in terms of renewable production of fuels and chemicals in the future. Nowadays, the larger amount of lignin is produced from the pulp and paper industry, but it is mainly disposed of and burned as an energy source to feed the process, not using its full potential.

Since lignin possesses a huge and complex aromatic structure and because of its large availability as a renewable carbon source, the main goal of this project was to study three different approaches to depolymerize lignin into high value aromatic compounds: enzymatic, chemical and electrochemical depolymerization.

The biologic methodology, using a laccase from *T. versicolor*, enabled the determination of the optimal laccase medium/conditions were PTM_1 combined with kanamycin at pH 5, by ABTS assays.

Furthermore, the enzymatic activity was studied in the presence of SXS and DES, revealing that laccase activity decreases with the solvents concentration, especially with DES. Nevertheless, the chosen concentration of each solvent to use in the next steps of this experimental work were 10 w/v % of SXS and 80 v/v % of DES. Although 80 v/v % DES is not the best condition of the enzyme, it is known that at this percentage is possible to observe lignin solubility.

In the electrochemical approach the electrochemical behavior of the different components of the system was studied. The cyclic voltammetry demonstrated to be not a sensitive method for enzyme redox activity. The chronoamperometry analysis revealed that the enzymatic activity is improved applying a certain potential for all solvents, reaching higher values with the higher potential used (0.25 V). Regarding to solvents combined with electrochemistry, the laccase presents higher activities in the buffer and 10 w/v % SXS systems.

For further insight, two analytical methods were used: GPC, to gain information about the molar mass distribution of lignin solutions and GC-MS, to quantify the different phenolic product molecules.

The GPC demonstrated that lignin was polymerized and depolymerized during the time, in different conditions, which means that the performance on depolymerization of lignin is not fully satisfactory, as full depolimerization is not achieved.

Looking at component identification results, vanillin, guaiacol, acetosyringone and BPA are the most common products, but the total amount of phenols tends to decrease with time, except in the 80 v/v % DES system with a 0.25 V potential. In the experiments using the buffer and 80 v/v % DES

solutions without applying electrochemistry present acetosyringone and BPA at time zero, but after that the products concentration tends to decrease during the tests. Applying a 0.05 V potential, it is just possible to observe some phenols using the 80 v/v % DES solution, having constant concentration values over the time.

For achieving better results, the product characterization should be also performed with different potentials using DES as solvent. For future research, additional kinds of lignin and enzyme should be tested. More analytical methods could be employed to help the identification of products derived from the process, like liquid chromatography-mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC), which have been used by several researchers to analyse lignin solutions.

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