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Extraction and Immobilization of the Urease enzyme in Polyvinylpyrrolidone films: Applications in Biosensors

Extração e Imobilização da enzima Urease em filmes de Polivinilpirrolidona: Aplicações em Biossensores

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ABSTRACT

Enzymes play a fundamental role in the manufacture of biosensors, acting as biological recognition elements due to their sensitivity and selectivity in the reactions they catalyze. Among them, urease stands out as an efficient enzyme found in nature, whose function involves the hydrolysis of urea. This enzyme has been used when extracted from legume seeds, as well as various bacteria and fungi. The objective of this study was the extraction and immobilization of the urease enzyme in polyvinylpyrrolidone (PVP) films. Crude jack bean extracts were prepared at concentrations of 15, 25 and 35% in PBS/PVP, centrifuged and refrigerated. Subsequently, aliquots of each solution were applied to plates to form films, being characterized by enzymatic activity, pH, FTIR and UV-vis. The affinity with urea was evidenced by enzymatic activity, pH and UV-vis, compatible with the Michaelis-Menten model. In FTIR, the polypeptide bands characteristic of the amino acids present in the structure of the enzymes, such as those of amides I, II and III, were confirmed.

Keywords: Urease; immobilization; biosensors; jack bean.

INTRODUCTION

Brazil has a wide variety of plant tissues rich in enzymes that can be used in the construction of biosensors. They have specific analytical properties for their use, in addition to being low cost and easy to extract. The lifespan of these tissues that contain enzymes is much longer than the lifespan of purified enzymes, improving the cost-benefit ratio. (Soares, 2011; Souza, 2023).

Jack bean (*Canavalia ensiformis* (Jack bean)) is a legume grown in tropical and equatorial regions. The main value of this species lies in its remarkable hardiness and adaptation to low-fertility soils with the property of immediately enriching them. Phytochemical studies show that jack bean contains a series of compounds, such as saponins, terpenoids, alkaloids and tannins. In addition, extracts from various regions of the plant have shown potential herbicidal and fungicidal activity (Soares, 2011; Mutia *et al.*, 2024).

Several studies use this plant tissue as a source of the urease enzyme with applications in several areas, including the construction of biosensors for urea (Bezerra *et al.*, 2017; Paiva *et al.*, 2021; Kumar *et al.*, 2024; Sharma *et al.*, 2024).

Biosensors are analytical devices formed by the combination of a biological element and a transducer, used in the most diverse applications, this is due to their advantageous characteristics compared to conventional laboratory methods (Metkar e Girigoswami, 2019; Haleem *et al.*, 2021).

In enzymatic biosensors, enzymes immobilized on solid supports are used, being an important tool for enzyme stabilization, as it allows reuse and reduces inactivation due to the influence of temperature and solvents, which can be attractive for the industrial sector (Bezerra *et al.*, 2017; Paiva *et al.*, 2021; Carlos, 2023; Diniz, 2023).

The enzyme immobilization process can induce two factors: first, the amount of immobilized enzyme, which is related to the surface area/volume of the matrix, and second, the porosity of the matrix, which provides rapid diffusion of the solution to the immobilized enzyme and directly affects the performance of enzymatic biosensors (Malhotra e Turner, 2003; Bollella e Katz, 2020; Pandey, 2020; Leguillier *et al.*, 2024).

The most commonly used methods for enzyme immobilization are physical or chemical adsorption; encapsulation; gel occlusion; covalent bonding and cross-covalent bonding (Liu *et al.*, 2018; Maghraby *et al.*, 2023; Han e Sari, 2024).

Several materials are used to immobilize biological elements, however, polymeric matrices such as gelatin, polyvinylpyrrolidone, polyacrylamide, collagen, cellulose triacetate and alginate are the most used in the development of enzymatic biosensors, due to their physical-chemical, biological properties and availability (Ismail e Baek, 2020; Paiva *et al.*, 2021).

Polyvinylpyrrolidone (PVP) is a homopolymer of N-vinyl-2-pyrrolidone, an amorphous, synthetic polymer of the vinyl family, obtained through radical polymerization in water or isopropyl alcohol, compatible with most resins and solvents due to its hydrophilic (pyrrolidone) and hydrophobic (alkyl group) components. It has inherent properties such as thin film formation, adhesiveness, biocompatibility, non-toxicity, stability at various pH levels and mechanical resistance, demonstrating it to be an efficient biomaterial (Sizílio *et al.*, 2018; Mishra *et al.*, 2019; Franco e De Marco, 2020; Kurakula *et al.*, 2020).

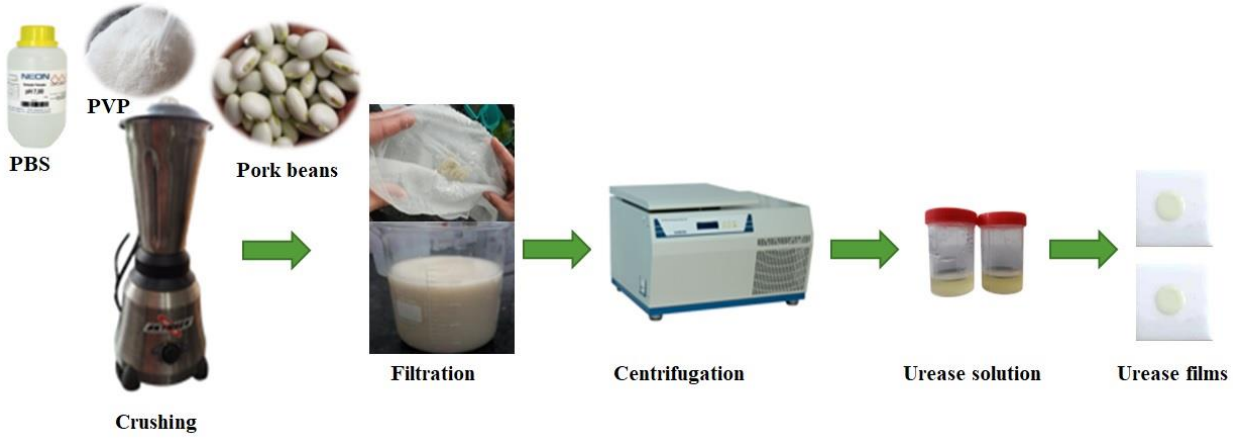
The aim of this study was the extraction and immobilization of the urease enzyme in polyvinylpyrrolidone (PVP) films, aiming at its potential application in enzymatic biosensors.

MATERIALS AND METHODS

As sementes do feijão de porco foram obtidas por meio da BRSEEDS. As soluções salinas tampão fosfato (PBS) e ureia foram adquiridas da Sigma Aldrich. O Polivilpirrolidona (PVP) foi adquirido da ACS Científica.

PVP films with immobilized urease enzyme were obtained from jack bean seeds using a methodology adapted from the work of Fatibello. First, a PBS solution with a concentration of 0.1 mol/L and pH 7 was prepared, to which the protective agent PVP was added at a concentration of 2.5% m/v. Then, 15, 25 and 35 grams of jack bean were ground separately for 3 minutes in a blender, together with 100 mL of the PBS/PVP solution, until a paste was formed. Using an organza cloth, the liquid extract was separated from the pasty mass. The liquids obtained were centrifuged at 15,000 rpm, at 4 °C, for 20 minutes, to remove the remaining solid part. The solutions were transferred to collection bottles and stored in the refrigerator at approximately 8 °C. Aliquots of 1 mL were placed on Teflon plates and allowed to dry at room temperature (25 °C) to form films. The procedure is illustrated in Figure 1.

Figure 1 – Process of obtaining and immobilizing the urease enzyme in PVP films.



Source: The authors (2024)

CHARACTERIZATIONS

Since the enzyme was obtained from a crude extract of jack bean, the enzyme activity and kinetics test was performed to confirm its catalytic function and affinity for the urea substrate. This affinity can also be monitored by analyzing the change in reaction pH, since the solution becomes basic as ammonia is formed. For the characterization of enzyme activity and hydrogen potential (pH), it was decided to use the crude extract of the urease solution with a concentration of 25% m/v.

To perform the enzymatic activity and hydrogen potential (pH) measurements, urea solutions with concentrations ranging from 0, 2, 4, 8, 20, 60, 80 and 140 mmol/L were prepared in phosphate buffer (PBS). To each solution, 1 mL of the crude urease extract was added, waiting for a reaction time of 10 minutes. Subsequently, the pH readings were performed using a benchtop digital pH meter, model T-100 TEKNA. Then, the enzymatic activity measurements were performed using equation 1, following an adapted procedure proposed by Sigma-Aldrich. This method is based on a titrimetric approach.

$$\frac{Unidades}{mL_{enzima}} = \frac{0{,}1\ mol/L\ V_{HCl}*(1000)*(df)}{10min.*(1mL)enzima} \quad (1)$$

In order to confirm the immobilization of the urease enzyme in the PVP films, the Fourier Transform Infrared Spectroscopy (FTIR) and UV-vis Spectrophotometry tests were performed.

The films formed were removed from the Teflon plates and the FTIR test by Attenuated Total Reflectance (ATR) was performed at room temperature (25 °C), covering scanning ranges from 4000 to 400 cm^{-1}, using the Spectrum 400 equipment from Perkin Elmer. Each sample was coded specifically, as detailed in Table 1.

Table 1 - Código de preparação das amostras.

Film Samples	Solution PVP/PBS (m/v)	Pork beans (m/v)
PVP	2,5 %	-
Urease 15	2,5 %	15 %
Urease 25	2,5 %	25 %
Urease 35	2,5 %	35 %

Source: The authors (2024)

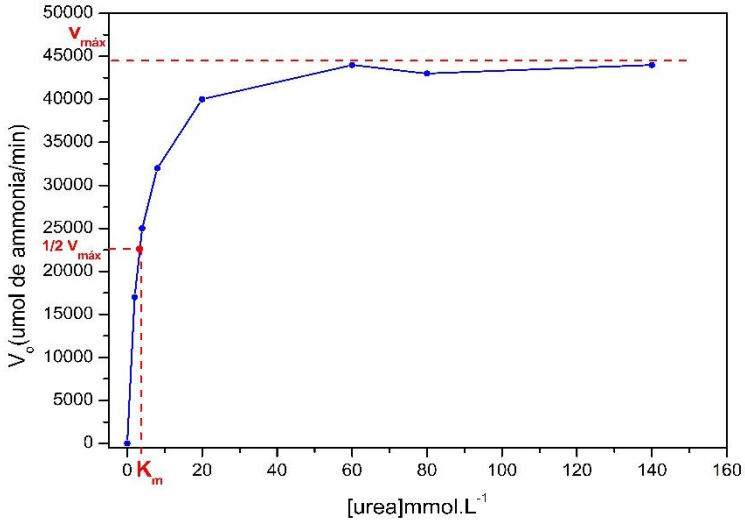
For UV-vis spectrophotometry analysis, four urea solutions were prepared at concentrations of 6 mg/dL, 30 mg/dL, 60 mg/dL and a blank solution. Teflon plates containing the immobilized enzyme were placed at the bottom of beakers and the prepared urea solutions were added. After 2 minutes of reaction, 0.2 mL of Nessler reagent was added and the reading was performed with a Perkim Elmer Lambda 35 UV-Vis spectrophotometer in order to detect dimercury ammonium iodide, a product of the reaction between ammonia (obtained by the catalytic reaction of the urease enzyme and urea) and the Nessler reagent, being identified in a wavelength range between 500 and 350 nm.

RESULTS AND DISCUSSIONS

Enzyme Activity

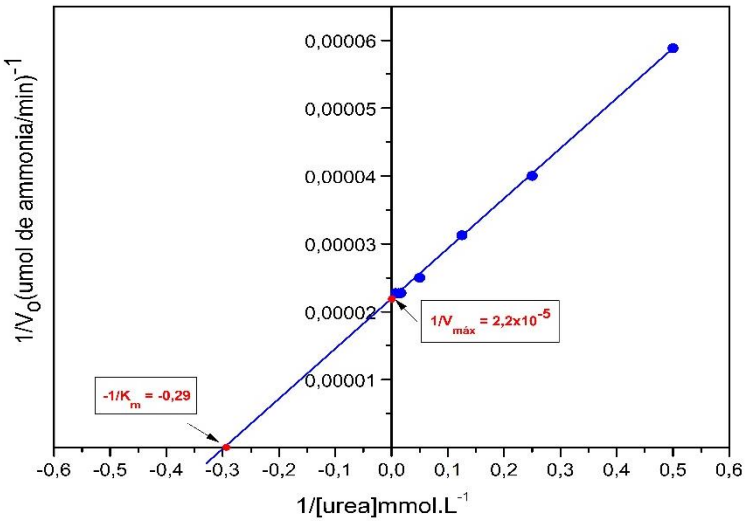
Several concentrations of urea were prepared and their rate kinetics and parameters (Km and Vmax) were determined. Figure 2 illustrates the rate of the enzymatic reaction in μmol of ammonia min-1 as a function of the urea concentration in $mmol.L^{-1}$, according to the Michaelis-Menten model. Figure 3 represents the linear fit of the graph obtained by using the inverse of the initial rate as a function of the inverse of the urea concentration, according to the model described by Lineweaver–Burk. Both models were used to determine the maximum rate and the Michaelis-Menten constant.

Figure 2 - Enzymatic reaction rate as a function of urea concentration



Source: The authors (2024)

Figure 3 - Linear adjustment of the graph obtained by the inverse of the initial velocity vs the inverse of the urea concentration to determine the maximum velocity and the Michaelis-Menten constant



Source: The authors (2024)

In Figure 2, it is possible to notice that the rate of the enzymatic reaction increases in line with the increase in the urea concentration, reaching a maximum rate value ($V_{máx}$) around 45,000 µmol of ammonia.min^{-1}. Subsequently, the increase in the urea concentration no longer impacts the rate of the reaction, indicating that the Michaelis constant (K_m) is the concentration of the substrate when the rate reaches half of the maximum value. In this context, the value obtained for Km was 3.5 mmol.L^{-1}.

The analysis of the kinetic parameters (K_m and $V_{máx}$) was also performed using the Lineweaver-Burk method (Figure 3), where linearization involved creating a graph of $1/V_0$ in relation to 1/[urea]. This resulted in a straight line with an angular coefficient

corresponding to K_m/V_{max}, with the intersection of $1/V_{máx}$ on the $1/V_0$ axis and $-1/K_m$ on the 1/[urea] axis. The values found were 3.44 $mmol.L^{-1}$ for Km and 45,400 μmol of $ammonia.min^{-1}$ for $V_{máx}$ in the urease in crude extract form.

The K_m parameter reflects the affinity of the enzyme for the substrate, with lower values indicating a higher enzyme/substrate affinity. This aspect is relevant for the sensitivity of the biosensor (Krajewska, 2009; Pacheco e Colla, 2019).

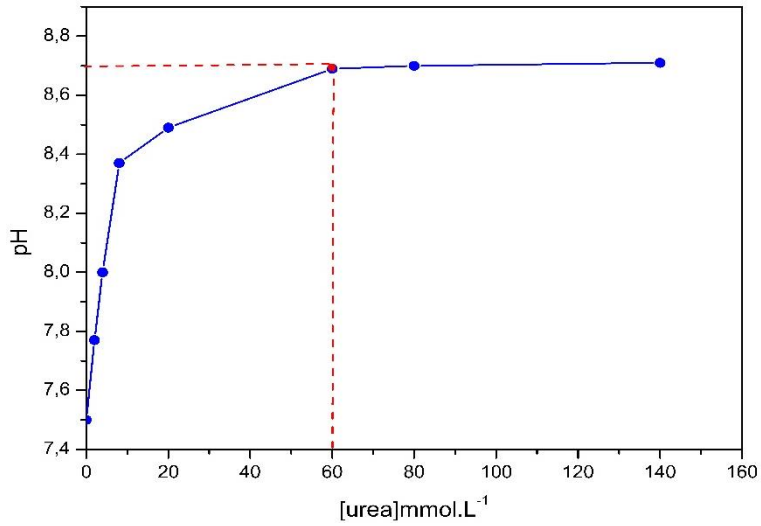
According to the literature, urease extracted from jack bean demonstrates a very simplified Michaelis-Menten behavior. Generally, K_m values are between 2.9 and 3.6 $mmol.L^{-1}$, as evidenced in this study for urease from jack bean (Krajewska, 2009; Pacheco e Colla, 2019).

The results obtained are considered satisfactory, as they confirm that the urease present in the crude extract follows the Michaelis-Menten equation, indicating the enzyme's affinity for urea.

Potential of Hydrogen (pH)

Urea solutions with concentrations of 2, 4, 8, 10, 20, 60, 80 and 140 mmol/L were prepared in phosphate buffer (PBS) and 1 mL of the crude urease extract was added to each solution, a reaction time of 10 minutes was waited and immediately after the pH measurement was performed. A figura 4 representa o pH das soluções de ureia em função de suas concentrações após o período de 10 minutos de reação catalítica com a enzima urease.

Figure 4 – pH as a function of urea concentration in $mmol.L^{-1}$.



Source: The authors (2024)

Fourier Transform Infrared Spectroscopy

FTIR analysis was conducted to evaluate the extraction and immobilization of the urease enzyme on the PVP structure. The FTIR spectra are presented in Figure 5a. The occurrence of interactions between the PVP structure and the urease enzyme is evident, highlighted by the appearance of a band at 1543 cm^{-1} and the reduction in the intensity of the band at 1298 cm^{-1}.

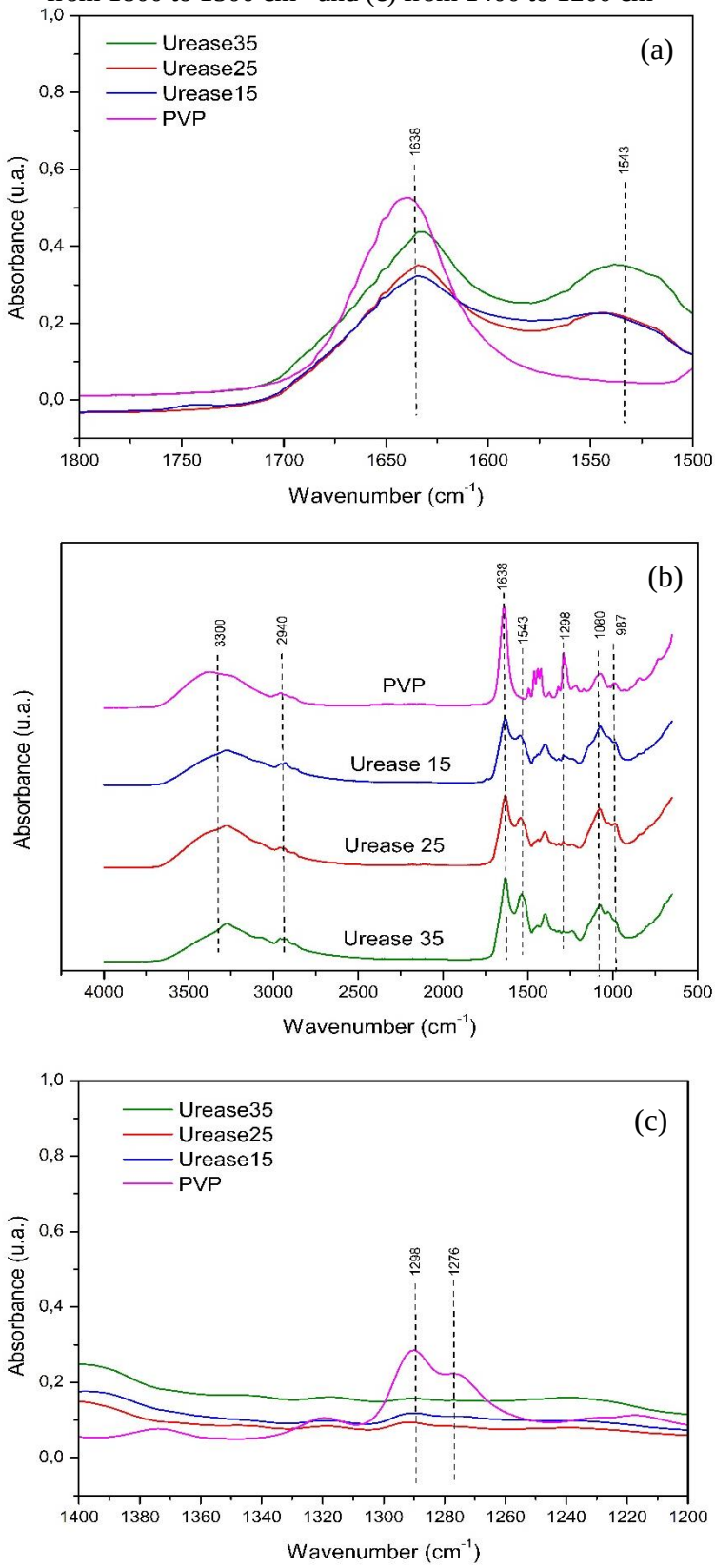
When examining Figure 5a, it is noticeable that the PVP present in the film structure exhibits a broad band around 3300 cm^{-1}, indicative of the stretching vibration of the hydroxyl group (OH) due to the presence of water in the film, since PVP is highly hydrophilic. In addition, an absorption band located at 2956 cm^{-1} corresponds to the asymmetric CH_2 stretching vibration. The vibrational band at approximately 1638 cm^{-1} is associated with the C=O stretching (Mohamed *et al.*, 2016; Safo *et al.*, 2019; Zidan *et al.*, 2019; Consendey *et al.*, 2021).

The presence of the urease enzyme in the PVP structure can be evidenced by analyzing the polypeptide bands and the R groups (CH_3, –CH $(CH_3)_2$, etc.) belonging to the amino acids. Vibrations are evident at 1638, 1543 and 1239 cm^{-1} corresponding to the C=O stretching of amide I, the N-H deformation of amide II and the mixed vibrations involving C-N and N-H of amide III, respectively (Ogura *et al.*, 1998; Soares, 2011).

In details presented in Figure 5b, the emergence of a band at 1543 cm^{-1} can be noted, whose intensity increases proportionally to the amount of enzyme, as observed in the spectrum of urease 35.

In Figure 5c, it is possible to observe a reduction in the intensity of the bands at 1298 cm^{-1} and 1276 cm^{-1}, attributed to the torsional vibrations of CH_2 or to the shaking movement of PVP.

Figure 5 - FTIR spectrum of PVP and Urease concentrations (a) from 4000 to 500 cm^{-1};(b) from 1800 to 1500 cm^{-1} and (c) from 1400 to 1200 cm^{-1}



Source: The authors (2024)

When comparing the intensities of the bands related to amide I (1638 cm^{-1}) and amide II (1543 cm^{-1}) in the spectra of Figure 5b, we noticed a proportional increase in

these intensities as we increased the amount of beans. This phenomenon may be related to a greater presence of amide I and II groups in the structure, which may be crucial for the response speed in the biosensors produced.

UV-vis spectrophotometry

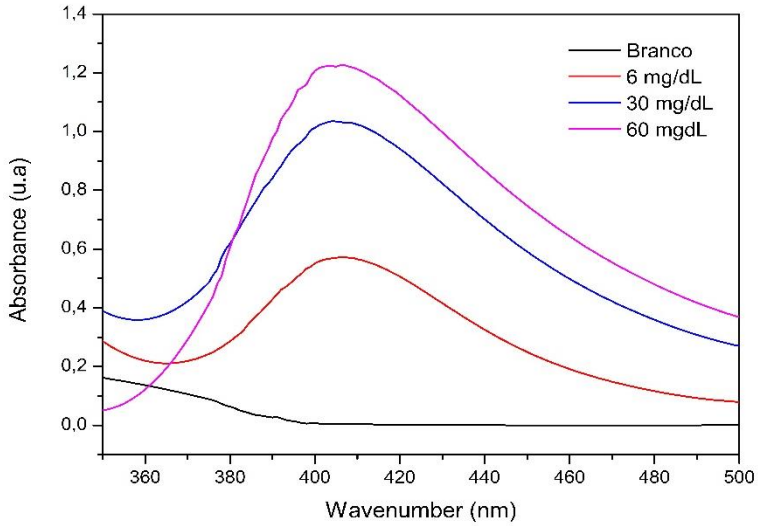
Urease is an enzyme that, in an aqueous medium, catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Nessler's reagent (alkaline potassium iodine-mercurate) is decomposed, in the presence of ammonia, into a compound (dimercury ammonium iodide) with a color ranging from red-orange to brown. Figure 6 represents qualitatively the catalytic reaction of the urease enzyme in the formation of dimercury ammonium iodide.

Figure 6 – Formation of dimercury ammonium iodide from the reaction of urease with urea concentrations of 0; 6; 30 and 60 mg/dL and Nesller.

Source: The authors (2024)

Figure 6 shows the formation of the compound dimercurioammonium iodide, with the color intensity increasing as the concentration of urea in the reaction increases, demonstrating the catalytic action of the enzyme immobilized in polyvinylpyrrolidone (PVP) films. This compound formed was confirmed through UV-Vis spectra (Figure 7) obtained by a spectrophotometer with a wavelength between 500 and 350.

Figure 7 – Uv-vis spectrum of urea solutions at concentrations of 0; 6; 30 and 60 mg/dL.



Source: The authors (2024)

Figure 7 shows that an increase in urea concentration increases the intensity of the absorption band of the compound dimercurioammonium iodide, demonstrating the catalytic action of the urease enzyme immobilized in polyvinylpyrrolidone, which can also be evidenced by the increased color intensity of the compound formed (Figure 6). Levels above 45 mg/dl in adults or 18 mg/dl in children are an early indication of renal failure (Ridley *et al.*, 2018).

CONCLUSIONS

Based on the results obtained, it is concluded that the immobilization of the urease enzyme in polyvinylpyrrolidone (PVP) films using the jack bean extract was successful. The presence of the enzyme in the films was confirmed by the characteristic polypeptide bands of the amino acids, identified by FTIR, corresponding to amides I, II and III. In addition, the affinity of urease for urea was evidenced by enzymatic activity tests, pH measurements and UV-Vis spectrophotometry analyses, indicating that the developed system has potential for application in biosensors, such as glucometers.

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