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Investigation on Polypyrrole-Induced Modification of Aspergillus flavus: Evaluation of Biocompatibility and Electrochemical Properties

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ABSTRACT

In the present study, development of a conductive polymer, Polypyrrole (PPy), within the cell wall and periplasm of microorganism was examined. PPy was effectively produced by the two different Aspergillus flavus fungi strains (AF1 and AF2). In order to initiate the manufacture of PPy from the enzyme reaction's byproducts, the Aspergillus flavus strain was used because to its ability to synthesize glucose oxidase. The formation of polypyrrole was confirmed by UV-Vis and FTIR analyses, which also yielded information regarding the chain length and oxidative state of the compound. The absorption spectra, cyclic voltammetry (CV), and Fourier transform infrared spectra of developed PPy were used to study its structural features that have changed over time. By utilizing absorption spectroscopy to determine the polaron charges in PPy-modified Aspergillus flavus culture, it was possible to ascertain that the culture functions as a feasible biocatalyst for the synthesis of PPy. While cyclic voltammetry confirmed that PPy exhibited redox activity in cultures, the stability of this activity was higher in the PPy-AF 2 culture.

1. INTRODUCTION

The conjugated chemical structure of conducting polymers (CPs) results in inherent electrical conductivity, allowing for low-energy charge transfers across the polymer backbone and charge delocalization throughout the polymer chain. Because of this, they are able to achieve redox reversibility and electrical conductivity [1]. Environmentally friendly, thermally stable, biocompatible, and electrically responsive, polypyrrole (PPy) is a major conducting polymer. Hence, it is well-suited for a range of bio-applications, such as biosensors, bio-actuators, and drug delivery systems. Various techniques can be used to synthesize PPy, each contributing unique and advanced properties to the final product. The oxidation of pyrrole using chemical compounds or electrochemical techniques is the most common process[2]. When it comes to bio-applications including the immobilization of biomolecules or cells, electrochemical PPy production isn't an option because it can only be used on conducting substrates. On the other hand, the chemical technique requires significant concentrations of oxidative chemicals. Environmentally friendly techniques of synthesis have been the subject of research due to concerns about the purity and influence of PPy on the environment [3].

Enzymes' selectivity and specificity in bioconversion substrate conversion is their principal advantage. Furthermore, they can provide environmentally beneficial catalysts that are 'green' under mild reaction conditions [4]. Oxidoreductases can start polymerization events because of their redox capabilities and ability to produce chemicals that can trigger polymerization reactions. Enzymatic generation of CPs allows for control of kinetic factors using redox enzymes such glucose oxidase, peroxidase, laccase, or lactate oxidase, as demonstrated in successful efforts. Immobilizing biological components into conducting materials is another important aspect of biosensorics. This allows structures to maintain their biological integrity and function for a long time. We are making use of the benefits of biocatalysis in polymer synthesis. Our research focused on generating Polypyrrole (PPy) through cell culture due to the utilization of whole cells as biorecognition elements in biosensors as well as different bioelectronic products. Reason being, enzymes already present in the cell structure can do their jobs without further processing to ensure their purity and stability. A benefit of this approach is that modifying the cells using PPy, which has been utilized to enhance the functionality of biosensors and biofuel cells, just requires a single step.

The current study utilized *Aspergillus flavus* (AF) fungal strain, which are recognized for their ability to manufacture glucose oxidase (GOx) [5], [6]. Hydrogen peroxide is produced when oxygen and glucose are combined by glucose oxidase (GOx). Hydrogen peroxide, when combined with pyrrole monomer, can facilitate the synthesis of polypyrrole (PPy). *Aspergillus flavus*(AF1)and *Aspergillus flavus* Y(AF2), two distinct strains, were cultivated in an aerobic environment that encourages the production of glucose oxidase (GOx) when pyrrole monomer is present. We used spectro-electrochemical methods to measure the biosynthesis rates.

2. EXPERIMENTAL METHODOLOGY

2.1. Pyrrole oxidation and fungi growth

Two strains of *Aspergillus flavus*, named '*Aspergillus flavus* AF1' and '*Aspergillus flavus* AF2', are stored in the Research Centre (Bioaliment) of the Department of Biotechnology, Karpagam Academy of Higher Education, India. The stock cultures were stored using cryopreservation in 25% at -75°C glycerol. The cells were moved from Czapek Dox Agar to 150 mL flasks filled with Czapek broth at 5.5 pH after subculturing. The flasks were incubated at 27ºC with a shaking speed of 200 rpm using Biotechnical Services, Inc.'s SI-300 53 Litre Shaking Incubators[7].

The third day marked the commencement of cell proliferation. In the sixth day of cell development, while the cells were in the exponential phase, the culture was supplemented with 35 mM/100 mL of pyrrole monomer. The cells were kept in the same culture medium for an extra eight days after that. The addition of monomer caused the culture media to darken with time, which in turn produced dark PPy. The pH of 2.5 phosphate buffer solution (PBS) was used in an additional experiment. Hydrogen peroxide was present in the solution at concentrations as low as 6 mM and pyrrole monomer as high as 35 mM. Next, we evaluated the pyrrole chemical polymerization. Our prior research on PPy production in bacterial cultures involved raising the pyrrole concentration required for effective biocatalytic polymerization [8], [9].

All other compounds used were of the highest possible purity and were purchased from commercial sources such as Sigma-Aldrich.

2.2. PPy formation classification

2.2.1. UV-Visible spectrum

Cell cultures that had been treated with pyrrole were taken out of the flasks every 24 h for 8 days, beginning 24 h after the monomer was added. The samples were subsequently spun in a Sigma-Aldrich Hettich® Universal 320R centrifuge at 8,000 x g for a duration of 15 minutes. The liquid phase was observed with a UV-Vis T90+ spectroscopy through PG Tools. The quartz test tubes having a volume of 2 mL and an optical path length of 1 cm were used for this purpose[10], [11]. The first five days were devoted to studying the polymerization of pyrrole caused by hydrogen peroxide. To prepare the samples for spectrum analysis, they had to be diluted with ultrapure water at a ratio of 1:10.

2.2.2. CV and open circuit potential (OCP) for Electro-chemical analysis

For a duration of 8 days, flasks containing culture samples that had been treated with pyrrole were sampled every 48-h following the addition of the monomer. One option was to assess the culture samples after dilution with ultrapure water (1:10), while another was to use centrifugation to separate the solution precipitate, wash it thoroughly, and then immerse it in ultrapure water (An 18.2 micrometer Smart-N water purification system)[12].

The apparatus made use of a 20 mL electrochemical cell with three electrodes: a glassy carbon working electrode, a saturated calomel (SCE) reference electrode, and a Pt wire counter electrode. One component of the setup was a Bio-logic potentiostat/galvanostat SP-150. Alumina and diamond slurries were used in the BASi® polishing kit to polish the working electrode. The object was washed with ultrapure water and ethanol afterward every electro-chemical testing. The evaluations were conducted at room temperature, at potential (E) ± 1 V compared to saturated calomel. The scanning speeds varied between 1 and 150 mV s^{-1}.

2.2.3. FTIR analysis

Infrared spectroscopy was used to examine the identical pyrrole-modified culture samples following centrifugation. The Thermo Scientific (Waltham, Massachusetts, USA). In order to get FTIR spectra, the Nicolet iS50 Analytical FTIR Spectrometer was utilized. This instrument has a built-in ATR accessory, DTGS detector, and KBr beam splitter. The range of 4000-400 $cm^{-1}$was covered by 32 scans that were combined at a resolution of 4 cm^{-1}[13]. In order to eliminate background noise, the air spectrum was used as a reference before each sample spectrum was registered. Following spectral registration, an ethanol solution was used to clean the ATR plate.

2.2.4. Measurement of pH

A pH-meter manufactured by Turnhout, Multiparameter (Consort C862, India) was used for the measurement.

3. RESULTS AND DISCUSSIONS

3.1. Core idea of polymerization for biologically induced polymers

Enzymatic activity evaluation, namely glucose oxidase production, informed the selection of *Aspergillus flavus* strains AF 1 and AF2. In order to assess the kinetics of polymerization, it was found that *Aspergillus flavus*, AF2 produced more enzymes than *Aspergillus flavus*AF 1 (data not provided). The growth media for microorganisms had a pH of 5.0, was supplemented with carbon and nitrogen sources, and had trace levels of iron and magnesium. As shown in Scheme 1, the process begins with the conversion of glucose to gluconic acid and the production of hydrogen peroxide by glucose oxidase (GOx) in the presence of molecular oxygen[14]. So, this polymerization process is biocatalytic, since it relies on the enzymatic generation of an oxidant. Enzymatic gluconic acid generation, as stated before [15], leads to a localized lowering of pH, which improves the polymerization process. The culture media had a low salt concentration. To ensure that pyrrole does not polymerize in the culture media prior to fungal cultivation, control studies were conducted.

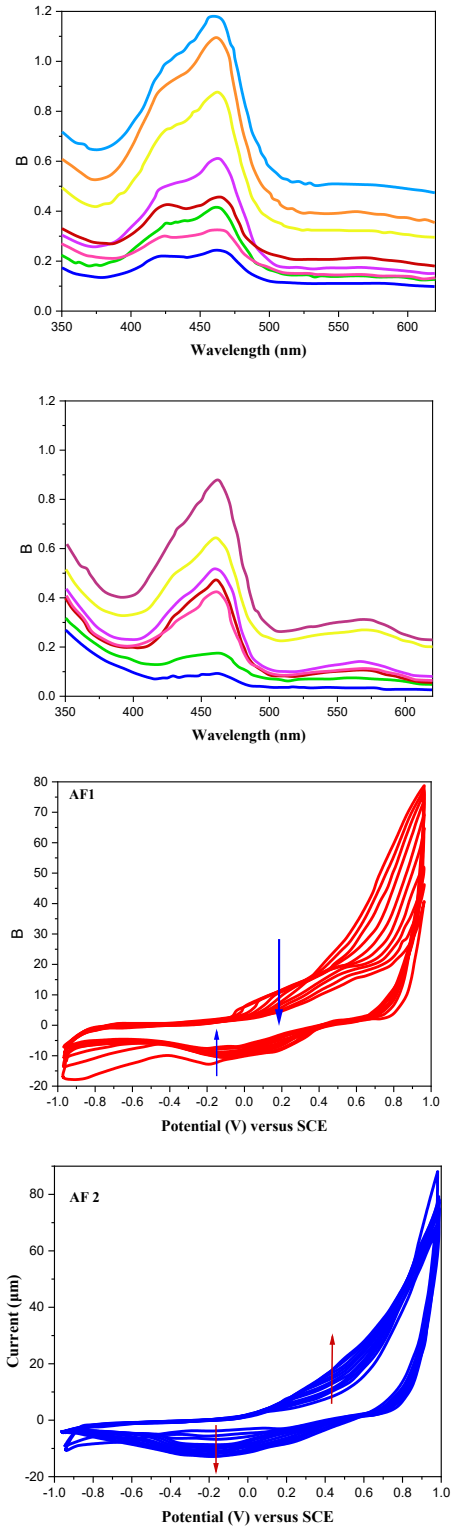

Figure 1. Process of PPy creation by the enzymatic activity of glucose oxidase

Researchers examined the biocatalytic function of microbes in a series of studies that ran in parallel. The pyrrole was introduced to cell suspensions in a phosphate buffer, culture filtrates, and whole cell cultures in these investigations. There was an 8-day duration to the trial in every type. According to the results, PPy synthesis is more likely to occur in the living cells of the entire culture rather than in the culture filtrate. The optical absorbance spectra and the fact that the polymerization environment became darker provided empirical evidence of this.

These specific *Aspergillus flavus* strains have an increase in extracellular GO x synthesis. Because there weren't enough nutrients in the PBS cell solution, the fungus cells autolyzed, preventing the matrix polymerization phase. Throughout the experiment, it was seen that the fluid included pyrrole monomer droplets. When kept in nutrient-rich conditions that encouraged optimal cell growth and enzyme synthesis, including GOx, catalysis in cell culture was most efficient.

The process of chemical polymerization was once compared to hydrogen peroxide, an acidic compound, in a published experiment [16]. Our previous investigation already had the pyrrole monomer concentration set at 30 mM. The presence of polypyrrole in the fungal culture was noted within 48 hours of monomer application, and the cells remained viable for a maximum of 8 d during the interaction.

3.2. UV-Visible spectroscopy analysis

The intrinsic electrical characteristics of conducting polymers can be determined using absorption spectroscopy, a well-established approach. One can determine the electrical resistance of a substance by measuring its conduction band width and/or the gap width among its valence band, which are associated with the energy stages of the most working orbital molecules and lessvacant molecule orbitals [17], [18]. P-doping induces oxidation of the polymer chains, leading to the formation of a localized electronic state within the band gap. This causes interband changes to shift towards lower energy levels or longer wavelengths. The presence of polaron or bipolaron species, as per the polaron/bipolaron structural concept, reflects the degree of oxidation. In optical absorption research, these species can be discovered by means of electronic transitions.

Figure 2a shows that polypyrrole (abbreviated PPy-AF 1), which is produced by *Aspergillus flavus* grown at AF 1, exhibits an absorption at 470 nm peak and a nearby peak at 420 nm. The formation of polypyrrole oligomers is analogous to the interband transition, to which this term alludes. However, the fact that there are no peaks at longer wavelengths rules out the presence of bipolaronic and polaronic species. An absorption spectrum with a peak at about 460 nm and no shoulder at shorter wavelengths was displayed by PPy-AF2, an example of a cultured polypyrrole produced by Aspergillus flavus. In addition, figure 2b shows an extra peak at around 580 nm. The 460 nm peak was believed to be caused by the π-π * transition, whilst the 580 nm peak could be linked to the creation of polarons or the change from bonding to antibonding energy levels [19].

The size of the conjugated π-electron system can be determined in absorption spectroscopy by examining the λ_{max} values, which decrease as the conjugated polymer's wavenumber and degree of polymerization increase. For PPy-AF1, the absorption peaks at 400 and 470 nm were nearly identical up to day 5. After that, the absorbance at 460 nm starts to dominate. This points to the formation of initially identical-length conjugated oligomer chains that, after extensive oxidation, fused into a single, substantially longer chain.

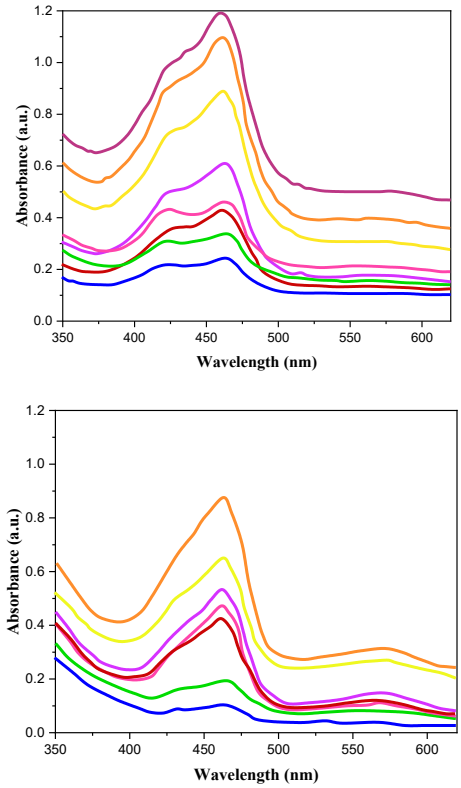

Figure 2. Polypyrrole production in fungal culture specimens: a UV-Vis study: *Aspergillus flavus*a) AF 1; b) AF 2. Measurements were taken daily for 8 days

Oligomerization produced cationic charge carriers in the instance of PPy-AF2, which was an extended conjugated chain[20].

The coupling process allows charged oligomers to create intermediate states, which, as chain length increases, become more stable. Conjugated oligomers take up charges and expand into polymer chains when protonation is removed. Despite using the same biocatalytic production method, the two different *Aspergillus flavus* cultures exhibit interestingly different oxidation rates. In PPy-AF1, a lengthy conjugated oligomer stage was caused by intermediates created through continuous oxidation of the pyrrole group. In contrast, a shorter chain was engaged in charge production during oxidation in PPy-AF 2. This will explain why the PPy-AF 1 spectra have a higher absorbance intensity than the PPy-AF 2 spectra. The sluggish polymerization rate probably allows for the observation of intermediate species with short lifetimes.

The procedure produces a somewhat oxidized version of polypyrrole, as is visible upon closer inspection. Here the results are reported in line with earlier studies on biocatalytic PPy synthesis that did not use redox mediators or dopant templates [21], [22]. To learn how biocatalytic and chemical oxidation-generated PPy differ, PPy was prepared in a very acidic pH environment with H_2O_2. Its optical data is displayed in Figure 3.

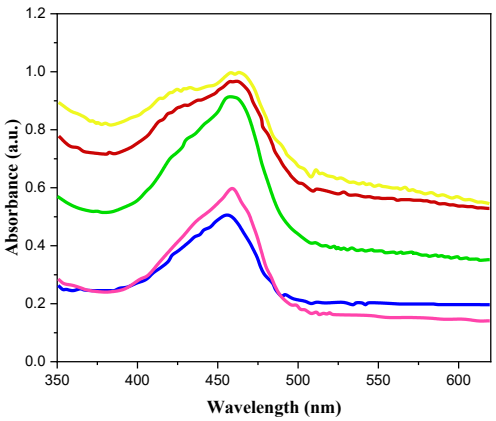

Figure 3. UV-Vis analysis of polypyrrole synthesis in phosphate-buffered saline solution with hydrogen peroxide at pH 2. Measurements were taken every 24 hours following the administration of pyrrole for the initial 5 days

A primary peak around 470 nm and a secondary peak around 430 nm are visible in the absorption spectra of chemically produced PPy. Thus, it is reasonable to assume that this will produce a polymerization kinetic similar to that observed in AF1 culture. On the fourth day of polymerization, the second peak emerged, indicating that the process was almost finished. A peak absorption at 470 nm in the spectra shows that the main PPy oligomeric chain that developed during chemical polymerization did not advance further in the absence of hydrogen peroxide. At 430 nm, a shoulder appeared, indicating the start of another short-conjugated chain. These results show that even when cells are present, the proposed biocatalytic polymerization still follows kinetics that are comparable to chemical oxidation. Although the oxidant operates faster, depletes faster, and achieves a steady-state phase sooner in chemically treated media, the process takes longer in culture media because of diminished oxidative effects.

3.3. Electrochemical analysis of microorganism cultures modified with polypyrrole (PPy)

The redox systems PPy/PPy^{+} or PPy/PPy^{2+} in polypyrrole, as well as potential cycling in general, include electron transfer. The introduction of positive charges during an oxidation sweep promotes the intercalation of counterions into the polymer matrix, while the elimination of positive charges during a reduction sweep releases the counterions into solution. Redox switching ability, the process of capturing and releasing molecules in the PPy structure, is vital for materials that are π conjugated[23].

Two samples were tested for their impact on the polymer using cyclic voltammetry (CV): one contained cells from a full cell culture, and the other was culture liquid that had no cells in it before the monomer was added. Almost identical results were obtained from both PPy samples; however, redox exchange was slightly better in the PPy-cell sample. This was likely because of its greater polymer output and/or improved electrochemical characteristics. When tested using cyclic voltammetry, control samples derived from unaltered cells did not exhibit any redox

activity. Following the same procedures as before, PPy-modified cell samples were examined. Scanning at speeds between 1 and 100 mV/s allowed us to examine the process kinetics. Scan rates greater than 50 mV/s enable the examination of the redox process involving the polymerization medium and pyrrole monomer/polymer, but scan rates lower than 20 mV/s can result in the oxidation of remaining monomers. The catalytically approved monomer oxidation process was continually monitored during the polymerization time at a scan rate of 1 mV/s. We chose to compare the data from the second and eighth days so we could see how the process changed in each culture. These circumstances were used to obtain the initial voltammetric cycles, which are shown in Figure 4. The first oxidation rendered subsequent cycles invalid.

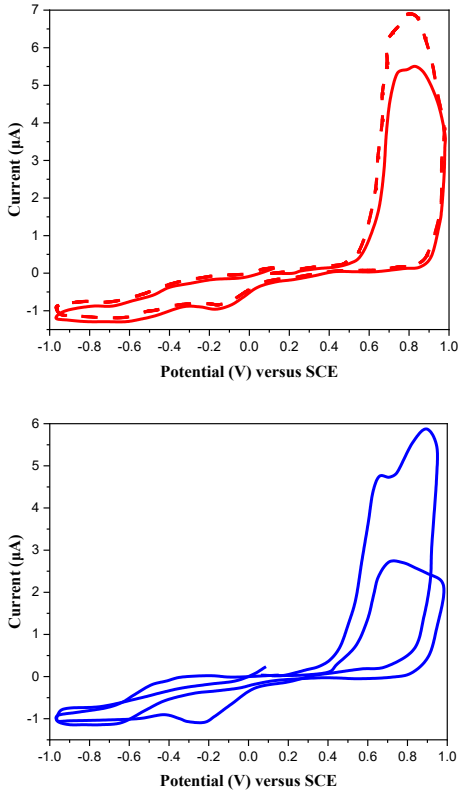

Figure 4. Assessment of Polypyrrole production in fungal cell cultures using cyclic voltammetry. *Aspergillus flavus* (a) AF 1; (b) AF 2; Day 2 to Day 8 of the reaction; The potential sweep rate was 1 mV/s

As the polymerization process progresses, cyclic voltammetry examination of PPy synthesis in *Aspergillus flavus* AF 1 culture reveals a steady current increase and a continuous electron exchange. The remaining monomers undergo oxidation, leading to the observation of an additional oxidation peak. It is possible that the hastened polymerization process caused the highest current, clearest redox peaks, and extra oxidation shoulder to be observed in AF 2 produced on the second day of the experiment. During the extended polymericprogression, reduced current levels and limited electron exchange were noted. The second scenario's increased response rate suggests that the *Aspergillus flavus* AF 2 culture is undergoing more effective biocatalysis[24].

The structural changes to PPy that take place in each microbial culture can be uncovered by analyzing the current-potential diagrams acquired at a scan rate of 100 mV/s. Cyclic voltammograms were taken every 48 hours for 8 days, as shown in Figure 5, to account for the relaxation of the polymer matrix in the first cycle.

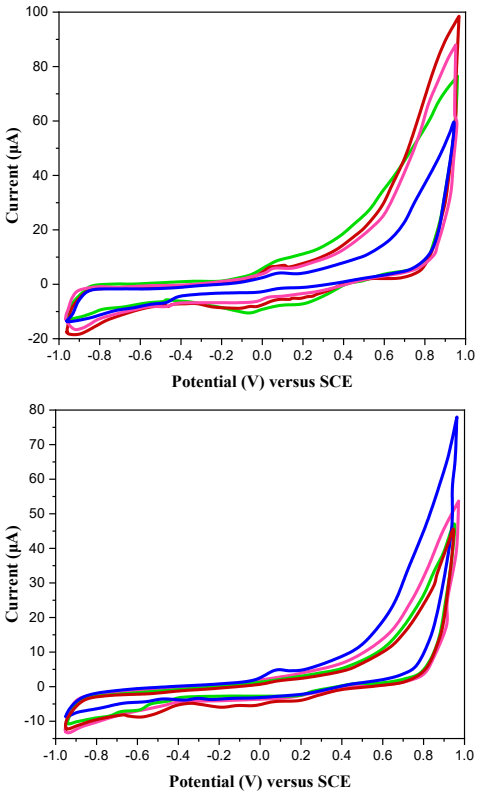

Figure 5. The formation of polypyrrole in fungal cell cultures of *Aspergillus flavus* AF 1 and *Aspergillus flavus* AF 2 was evaluated using cyclic voltammetry on the second, fourth, sixth, and eighth day of the reaction. At 100 mV/s, the sweep rate was implemented

In cultures of *Aspergillus flavus* AF 1 and AF 2, the peak current intensity occurred in the first two days after PPy formation and six days after PPy development, respectively. An advanced electron exchange process was seen in the PPy-AF 1 culture, characterized by an anode peak at 80mV and two cathode peaks (Epc_1, Epc_2 at -50mV, 20mV respectively). Alternatively, the electrochemical activity of the PPy-AF 2 culture dropped dramatically during the first 48 hours of polymerization, when it exhibited the maximum redox exchange. Past studies have demonstrated that larger polymeric chains of PPy are produced by *Aspergillus flavus* AF 2 culture, as opposed to shorter oligomer forms, by *Aspergillus flavus* AF 1 culture. The presence of multiple PPy chains with varying degrees of π conjugation causes two reduction peaks to be seen in the PPy-AF 1 instance. With every decline peak, there is a correlation with the loss of counterions from charged oligomeric chains. Further contact with the culture media cannot be observed in the *Aspergillus flavus* AF 2 culture due to the quick pyrrole polymerization, which depletes the monomer[25].

3.3.1. Characterization ofPolymer

In order to evaluate the polymer's electrochemical stability, the polypyrrole that was generated in fungal cultures was extracted and mixed with ultrapure water. In complex polymerization settings, previous investigations had to account for both cation and anion migration. It is now possible to comprehend the additionand/or removal of ions from the polymer matrix by subsequent measurements. Ten separate cyclic voltammetry cycles were obtained from the polypyrrole precipitate in every fungal culture, and they are shown in Fig. 6.

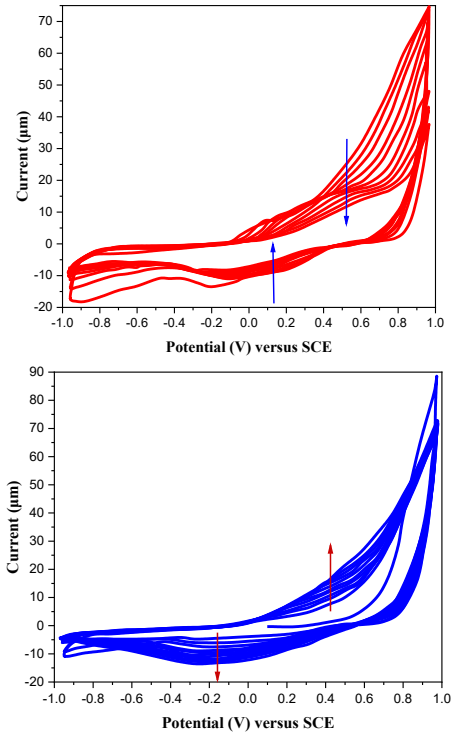

Figure 6. *Aspergillus flavus* AF 1 and AF 2 PPy precipitate redox behavior evaluation by cyclic voltammetry; increasing number of scans denoted by arrows; potential sweeping rate of 100 $mV.s^{-1}$

Cyclic voltammetry investigation of PPy precipitate shows sufficient electrochemical activity, particularly for PPy-AF 2. Using cyclic voltammetry-induced electrochemical processes and the participation of fungal cells are examined in depth in this comparison. AF 2 culture of Aspergillus flavus is likely to undergo a rapid oxidative process induced by GOx, which concludes the polymerization reaction within forty-eight hours. The process can be prolonged beyond 8 days due to the decreased quantity of glucose oxidase generated during the cultivation of Aspergillus flavus AF 1. PPy, which was generated at a rapid synthesis rate using an Aspergillus flavus AF 2 culture, exhibited favorable electrochemical stability and efficient electron exchange, resulting in the most favorable outcomes.

3.4. pH and OCPmeasurements

Experiments using OCP were carried out to evaluate the oxidation potential fluctuation that occurs during cell culture polymerization. The open circuit potential of the polymer undergoes a slight reduction in its oxidized state, whereas neutral forms exhibit a rapid increase. Because of impurities in the electrolyte solution oxidizing the polymer backbone [26]. The *Aspergillus flavus* AF 1 culture generated a polypyrrole that was essentially neutral in nature, as illustrated in Figure 7a. The produced PPy remained susceptible to oxidation seven days after processing, as shown by the cyclic voltammograms in Figures 3 and 4. As the polymerization process progresses, there is a progressive decrease in the open circuit potential change; however, the polymer remains in a state of low oxidation.

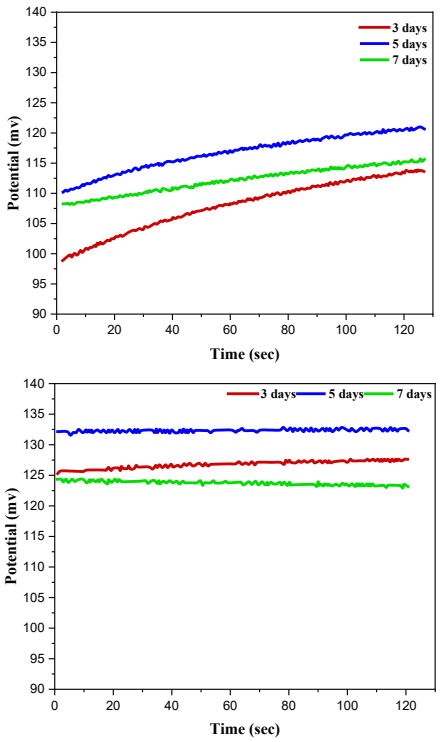

Figure 7. OCP evaluation during py polymerization in the following fungal cultures were performed: (a) AF 1 and (b) AF 2

A procedure is applied to *Aspergillus flavus* AF 2 culture, which produces a polymer that is slightly oxidized. After 3 days, the open circuit potential signifies the susceptibility to oxidation. By the end of the reaction, it has stabilized after 5 days and is gradually decreasing. The findings support previous research on the redox properties of the grown material from this fungus. The marginal variations in open circuit potential render PPy an advantageous coating material for cellular components and living cells, as it facilitates moderate enzymatic oxidative reactions that preserve biological integrity.

Initially, the pH was adjusted to 5.0 in order to promote optimal conditions for microbial proliferation. As a result of the production of hydrogen peroxide and gluconic acid in the culture medium, it decreased. The conditions for polypyrrole production were favorable in *Aspergillus flavus* AF 1 culture, where the pH fell to 3.2, and in *Aspergillus flavus* AF 2 culture, where it rose to 4.0. Although the pH is not optimal for GOx activity, the enzymatic response is primarily enhanced at the cell membrane

in the presence of intact cells, provided that membrane disruption or autolysis does not occur.

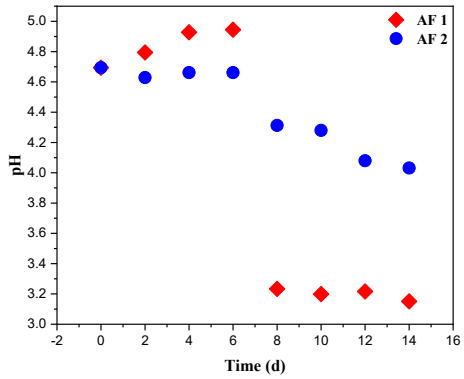

Figure 8. Pyrrole polymerization pH determinations in (a) *Aspergillus flavus*AF 1 and (b) *Aspergillus flavus*AF 2 cultures

3.5. FTIR analysis

The electronic transitions and molecular structure of charged species in conducting polymers can be elucidated with the help of FTIR studies. As the chemical structure changes from benzoid to quinoid, the oxidation process causes the constriction of single bonds and the elongation of double bonds [27]. Our research shows that the main vibrational bands of PPy-AF 1 and PPy-AF 2 are in the same relative positions, suggesting that the charge delocalization of the polymer chains is also similar (Figure 9).

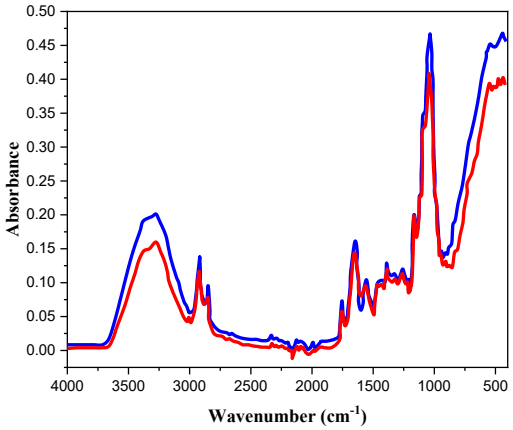

Figure 9. FTIR spectrum for PPy of (a) AF 1 and (b) AF 2

The presence of N-H and C-H stretching vibrations at 3341 cm^{-1} suggests a minimal level of oxidation and confirms the purity of the polymer as polypyrrole. In metallic systems, charged particles are strongly and extensively absorbed, shielding the bands. Similar to what was previously stated, the CH_2 absorption peaks at 2952 cm^{-1} and 2872 cm^{-1} indicate symmetric and asymmetric stretching and vibrations, respectively. Signals at 1752 cm^{-1} and 1651 cm^{-1} suggest that carbonyl groups are affecting the PPy structure. Interference in aqueous media polymerizations occurs due to water's nucleophilic attack on the polymer's structure. The main vibrational bands that the polypyrrole chain is thought to display were at 1556 cm^{-1} (corresponding to C=C/C-C stretching vibrations), 1390 cm^{-1} (indicating C-N conjugated stretching), 1251 cm^{-1} (representing C-H and C-N in-plane deformation modes), 1152 cm^{-1} (indicating ring breathing vibration), and 1027 cm^{-1} (indicating C-H and N-H out-of-plane deformations)[28-31].

4. CONCLUSION

A biosynthesis of polypyrrole was conducted utilizing two strains of *Aspergillus flavus* that produce GOx. Ensuring optimal conditions for cultivation, pyrrole monomer was introduced into the submerged culture during the exponential growth phase of the microorganisms. At varying rates of polymerization, these two cultures generated PPy. Biocatalysis is essential for producing the conducting polymer polypyrrole, with the concentration of glucose oxidase in the cell cultures being a critical factor. Our research revealed that the estimated concentration of the enzyme was greater within cells. As a result, polymerization occurred more rapidly in cultures of living cells in their entirety. So, it would be useful to increase the microbe cultures' ability to produce redox enzymes (like glucose oxidase) that may be used in later polymerization procedures. Furthermore, in contrast to enzymatic synthesis, in situ polymerization yields polypyrrole, which defeats the need for time-consuming and expensive enzyme purification processes, thereby reducing the risk of enzyme inactivation.

The early bioconversion of pyrrole to polypyrrole was effectively documented through the utilization of bulk solutions comprising complete cultures of glucose oxidase-producing fungi. Prospective uses of PPy-encased entire cells in bioelectroch emical devices are promising directions for future research.

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