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Laccase from *Aspergillus niger*: A novel tool to graft multifunctional materials of interests and their characterization

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Biological polymers; Composite materials; Laccase; *Aspergillus niger*

**Abstract** In the present study, we propose a green route to prepare poly(3-hydroxybutyrate) [(P(3HB)] grafted ethyl cellulose (EC) based green composites with novel characteristics through laccase-assisted grafting. P(3HB) was used as a side chain whereas, EC as a backbone material under ambient processing conditions. A novel laccase obtained from *Aspergillus niger* through its heterologous expression in *Saccharomyces cerevisiae* was used as a green catalyst for grafting purposes without the use of additional initiator and/or cross-linking agents. Subsequently, the resulting P(3HB)-g-EC composites were characterized using a range of analytical and imaging techniques. Fourier transform infrared spectroscopy (FT-IR) spectra showed an increase in the hydrogen-bonding type interactions between the side chains of P(3HB) and backbone material of EC. Evidently, X-ray diffraction (XRD) analysis revealed a decrease in the crystallinity of the P(3HB)-g-EC composites as compared to the pristine individual polymers. A homogeneous P(3HB) distribution was also achieved in case of the graft composite prepared in the presence of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a mediator along with laccase as compared to the composite prepared using pure laccase alone. A substantial improvement in the thermal and mechanical characteristics was observed for grafted composites up to the different extent as compared to the pristine counterparts. The hydrophobic/hydrophilic properties of the grafted composites were better than those of the pristine counterparts.

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1. Introduction

From the last few years, bio-based bio-composite biomaterials are being engineered for their prospective applications in different sectors of the modern world that includes bio- and non-bio subdivisions, with an aim, to address a global
dependence on petroleum-based sources (Zhijiang et al., 2011; Iqbal et al., 2014a, 2015). To develop a polymeric composite material is an original concept/solution to expand and/or impart ideal characteristics (specific strength, thermal properties, surface properties, biocompatibility, and biodegradability) that native/pristine homogeneous materials fail to demonstrate on their own. In recent years, with an ever increasing scientific knowledge, eco-friendly realization and demands of legislative authorities, wasteful protection or de-protection removal of synthetically prepared petroleum-based materials and the development of structured composite are being considered more critically (Datta and Włoch, 2014; Iqbal et al., 2014b).

Green chemistry has many challenges for effective execution of state-of-the-art technologies to reduce or eliminate the use and/or generation of detrimental constituents during the entire engineering/manufacturing processes. The use of enzyme in grafting of biopolymers is a recent practice, where, enzyme offers eco-friendly and safer reaction environment to the current practices, as a green catalyst (Iqbal et al., 2014c, 2015), without the use of additional initiators and/or cross-linking agents. To acquire the anticipated functionality in the final product it is very indispensable to graft two or more polymers with some other suitable moieties (Datta and Włoch, 2014), where individual polymer lacks those functionalities to withstand on its own. From the last few years, among many others, P(3HB) is of particular interest to prepare composites that can be synthesized by combing with other suitable homo-, or co-polymers. This polymer is the focus of the work presented here, to produce composites with EC, either to impart or improve thermal and mechanical characteristics, decrease brittleness, and/or increase tensile strength of the pristine P(3HB).

Oxidoreductases like laccase (EC 1.10.3.2) have tremendous capability to react with a variety of suitable substrates. There is now a new wave of interest in laccase mediated surface activation with a view to grafting materials of interest. There are several advantages in the laccase-assisted grafting methods that include: (i) high added-value; (ii) eco-friendly processing; (iii) convenient preparation methods; (iv) minimum use of water and energy, and finally (v) no or little use of harsh chemicals. This study focuses on the development of green composites with novel characteristics. A pure laccase from Aspergillus niger was used to develop composites under mild and eco-friendly processing conditions.

2. Materials and methods

2.1. Chemicals

A novel laccase obtained from A. niger through its heterologous expression in Saccharomyces cerevisiae was used as a green catalyst for grafting purposes. A unit laccase activity (U) was defined as a μmol of ABTS oxidized per min at pH 4.5 and 30 °C (εmax 36000 M⁻¹ cm⁻¹). All other chemicals used were of analytical laboratory grade and used as-received from Sigma–Aldrich Company Ltd., UK, VWR Chemicals, UK, and DIFCO, UK.

2.2. Bacillus subtilis and maintenance

A Gram⁺ bacterial strain B. subtilis NCTC 3610 was collected from the University of Westminster’s bacterial culture collection unit and used as a potential candidate to produce P(3HB) polymer. The collected strain was streaked in its pure form on the sterilized nutrient agar plates and subsequently used for inoculum development. In order to obtain homogeneous bacterial spore suspension, a pure colony was grown overnight in 50 mL sterile nutrient broth at 30 °C and 120 rpm. The initial bacterial concentration was maintained approximately at 10⁶ CFU/mL in the freshly prepared spore suspension by comparing OD₅₆₀ using McFarland standard method.

2.3. P(3HB) production and extraction

P(3HB) polymer was produced from B. subtilis NCTC 3610 under some previously optimized fermentation using as-reported modified G medium (MG) (Akaraonye et al., 2010). After the stipulated fermentation time (72 h), as-reported chloroform–hypochlorite dispersion method (Rai et al., 2011) was adopted to extract P(3HB) polymer from the fermented culture. Following that the freshly extracted polymer was placed in the desiccated jar to avoid free moisture absorption by the P(3HB) and used further in subsequent graft synthesis experiments.

2.4. Grafting tool

A fungal laccase from A. niger, through its heterologous expression in S. cerevisiae, was produced. Briefly, a Bioflo3000 bioreactor was used to ferment 0.5 L culture and the fermentation conditions were: 20 g/L glucose, pH 5.5, aeration of 0.6 VVM (volume of air/volume of culture medium/min), 10⁶ spore/mL inoculum and 120 mg/L syringaldazline as substrate. A simple chromatographic purification step (anionic exchanger, 50% final yield) allowed us to recover 2.3 g of lac state with a specific activity of 50 U/mg and a satisfactory level of homogeneity as controlled by UV/VIS spectroscopy and denaturing gel electrophoresis (Mekmouché et al., 2014).

2.5. Grafting of P(3HB) onto the EC

A pure laccase from A. niger was used with and without ABTS as a laccase-mediator to develop composites between P(3HB) and EC using P(3HB): EC (50: 50) under an ambient environment. Briefly, P(3HB) obtained from B. subtilis NCTC 3610, and EC were treated with laccase from A. niger at 25 °C for 30 min. The laccase-assisted reaction mixture comprising of P(3HB) and EC was poured into the labeled sterile petri plates. This was then followed by incubation at 50 °C for 24 h to cast the composites. Finally, the composites were designated as P(3HB)-g-EC⁻⁴ (prepared using laccase supplemented with ABTS as a mediator) and P(3HB)-g-EC⁻⁸ (prepared using pure laccase alone). The resulting enzymatically grafted composites were then characterized using various analytical techniques as described below.
2.6. Characterization of P(3HB)-g-EC composites

FT-IR spectra were recorded from the wavelength region of 4000–500 cm\(^{-1}\) using a Perkin Elmer System 2000 FT-IR spectrophotometer. Morphological characterization was performed in ultra-high vacuum mode using scanning electron microscope (Philips, XL-30, FEG SEM; EFI, Netherlands). To investigate the thermal and mechanical characteristics a Pyris Diamond Differential scanning calorimeter (Perkin–Elmer Instruments, USA) and a Perkin–Elmer dynamic mechanical analyzer (DMA) Q800 were used respectively. A wide-angle X-ray diffraction with a thin film attachment using a Brüker D-8 Advance X-ray diffractometer equipped with Ni filtered Cu–K radiation was used to investigate crystalline structures. A pendant drop method was adopted to measure hydrophobic and hydrophilic characteristics using a KSV Cam 200 optical contact angle analyzer (KSV instruments Ltd., Finland).

3. Results and discussion

3.1. FT-IR spectroscopy

The IR absorption found at 3450 cm\(^{-1}\) for P(3HB) was shifted to 3358 cm\(^{-1}\) for P(3HB)-g-EC\(^{A}\) (Fig. 1). The peak assigned to the O–H stretching vibration at 3358 cm\(^{-1}\) intensified in the P(3HB)-g-EC\(^{A}\) composite because of the contributions from the –OH group of EC. Furthermore, IR characteristic bands at 2980 and 2867 cm\(^{-1}\) belonging to stretching vibration of CH\(_3\) and aliphatic C–H bond from alkyl groups of P(3HB). A sharp band observed at 1721 cm\(^{-1}\) is assigned to the C\(^{\prime}\)O stretching vibration. The bands with characteristic peaks at 1260, 1353, 1456, 1721, and 2926 cm\(^{-1}\) were particularly attributed to the bending modes of C–H, stretching vibration of C\(\cdots\)O, and symmetric and asymmetric stretching vibration of CH\(_3\), respectively (Liu et al., 2009). Previously, FT-IR spectroscopy has been used to prove the existence of hydrogen bonding interactions in the different composites based on cellulose/PHBV blends at different compositions (Hameed et al., 2011). A broad peak between 3376 and 3500 cm\(^{-1}\) indicating cellulose O–H vibration, while the peak at 1050–1100 cm\(^{-1}\) region is due to the stretching of the C–O–C linkage of ethyl cellulose molecules (Mohammed-Ziegler et al., 2008; Iqbal et al., 2014a).

3.2. X-ray diffraction (XRD)

XRD profile for pristine P(3HB) showed distinct peaks at 2\(\theta\) values of 28\(^{\circ}\), 32\(^{\circ}\), 34\(^{\circ}\), 39\(^{\circ}\), 46\(^{\circ}\), 57\(^{\circ}\), 64\(^{\circ}\), 78\(^{\circ}\), and 84\(^{\circ}\). The results obtained through XRD analysis evidently support that the extent of P(3HB) crystallinity was greatly reduced due to the homogeneous incorporation of semi crystalline material i.e., EC, in this study, into the polymeric matrix (Fig. 2). Ethyl cellulose, as a semi-crystalline material, can suppress and break the crystalline domains of the crystalline polymers (Davidovich-Pinhas et al., 2014), thus the crystallization of P(3HB) in P(3HB)-g-EC composites would be restricted as evidenced by XRD analyses. For the EC, a main scattering intensity peak can be identified at 2-Theta value of 21.2\(^{\circ}\) as shown in the Fig. 2, which is assigned to the reflexion planes of cellulose I.

3.3. Scanning electron microscopy (SEM)

Fig. 3 confirms the morphological changes via surface topographies of the pristine polymers and grafted composites i.e., P(3HB)-g-EC\(^{A}\) and P(3HB)-g-EC\(^{B}\) observed using SEM micrographs. As compared to the P(3HB)-g-EC\(^{B}\), P(3HB)-g-EC\(^{A}\) showed homogenous dispersion of P(3HB) within the backbone polymer. Based on the data reported in an earlier literature graft copolymerization can modify the physiochemical and morphological characteristics of the

![Figure 1](image-url)

**Figure 1** Typical FT-IR spectra of individual polymers i.e., P(3HB) and EC and their grafted composites i.e., P(3HB)-g-EC\(^{A}\) and P(3HB)-g-EC\(^{B}\).
grafted materials, which in turn affect their bio-compatibility and biodegradability (Iqbal et al., 2014b). This change in surface morphology supported the successful occurrence of graft polymerization (Yu et al., 2012).

### 3.4. Differential scanning calorimetry (DSC)

The results obtained after DSC analyses are summarized in Table 1. In comparison to the two forms of laccase (with and without ABTS) used two different profiles were recorded. In comparison to the untreated P(3HB) an increase in the $T_g$ and $T_m$ values was observed in case of the P(3HB)-g-EC composite prepared using laccase as a catalyst. One possible explanation of this increase in the $T_g$ and $T_m$ values in case of P(3HB)-g-EC is a re-joining of short segments would cause an increase in the $T_g$ and $T_m$ due to the better impregnation of thermally stable cellulosic component as reinforcement into the P(3HB) matrix. Herein, the parameter $T_c$ is used to measure the overall rate of crystallization, and the smaller the $T_c$ the greater the rate of crystallization (Liu et al., 2009; Yu et al., 2012).

### 3.5. Dynamic mechanical analyzer (DMA)

Stress–strain curves were used to calculate the mechanical characteristics of the tested materials which showed simultaneous improvement in the tensile strength and Young’s modulus as compared to the individual P(3HB) counterpart because the high strength of EC allowed the mechanical properties of the composite to improve. The results obtained after DMA measurements are summarized in Table 2. A gradual increase in the tensile strength and Young’s modulus values was observed in case of the P(3HB) containing P(3HB)-g-EC composites as compared to the pristine P(3HB) polymer (Table 2). A persistent reinforcement or low filler loaded network bargains incredible enhancement in the mechanical assets, in particular, the tensile strength and Young’s modulus (Iqbal et al., 2014b).
3.6. Water contact angle (WCA)

The surface energy of the solid materials can be estimated by means of contact angle measurement together with a theory of intermolecular forces. Therefore, in this experiment, the drop contour analysis was used for determining the surface tension and the water contact angle. The characteristic WCA features of the resulting composites are shown in Fig. 4. A dramatic reduction from 68° to 35° was observed in the contact angle of P(3HB) as compared to the composite prepared using laccase from *A. niger*. However, an increase in the surface tension properties from 20.5 mN/m to 72.5 mN/m was recorded in the P(3HB)-g-EC\(^\text{A}\). The WCA profile of P(3HB)-g-EC\(^\text{A}\) and P(3HB)-g-EC\(^\text{B}\) indicates that the hydrophilic property of the composite is much better than pure P(3HB). According to the literature, such a result is probably accounted because of the high hydrophilic nature of EC and P(3HB) interaction involving the carbonyl group with the hydroxyl group from EC. It has also been reported in the literature that the grafted polymers with more hydroxyl groups of short chains exhibited more hydrophilic characteristics than other copolymers (Yu et al., 2012).

### Table 1 Thermal characteristics of individual polymers *i.e.*, P(3HB) and EC and their grafted composites *i.e.*, P(3HB)-g-EC\(^\text{A}\) and P(3HB)-g-EC\(^\text{B}\).

<table>
<thead>
<tr>
<th>Samples</th>
<th>(T_g) (°C)</th>
<th>(T_m) (°C)</th>
<th>(T_c) (°C)</th>
<th>(\Delta H_m) (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB)</td>
<td>9 ± 0.05</td>
<td>174 ± 0.45</td>
<td>135 ± 0.18</td>
<td>64 ± 0.02</td>
</tr>
<tr>
<td>EC</td>
<td>ND</td>
<td>188 ± 0.09</td>
<td>48 ± 0.05</td>
<td>86 ± 0.69</td>
</tr>
<tr>
<td>P(3HB)-g-EC(^\text{A})</td>
<td>28 ± 0.33</td>
<td>192 ± 1.21</td>
<td>72 ± 1.25</td>
<td>62 ± 0.93</td>
</tr>
<tr>
<td>P(3HB)-g-EC(^\text{B})</td>
<td>13 ± 0.96</td>
<td>175 ± 2.32</td>
<td>99 ± 1.34</td>
<td>81 ± 1.15</td>
</tr>
</tbody>
</table>

Where, ND: not detected.

### Table 2 Mechanical characteristics of individual polymers *i.e.*, P(3HB) and EC and their grafted composites *i.e.*, P(3HB)-g-EC\(^\text{A}\) and P(3HB)-g-EC\(^\text{B}\).

<table>
<thead>
<tr>
<th>Samples</th>
<th>TS (MPa)</th>
<th>YM (GPa)</th>
<th>EB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EC</td>
<td>122 ± 9.85</td>
<td>3.38 ± 0.85</td>
<td>8.2 ± 1.25</td>
</tr>
<tr>
<td>P(3HB)-g-EC(^\text{A})</td>
<td>78 ± 3.35</td>
<td>0.91 ± 2.15</td>
<td>19.2 ± 2.65</td>
</tr>
<tr>
<td>P(3HB)-g-EC(^\text{B})</td>
<td>46 ± 1.33</td>
<td>1.10 ± 2.36</td>
<td>10.4 ± 1.42</td>
</tr>
</tbody>
</table>

Where, ND: not detected; TS: tensile strength; YM: Young’s modulus and EB: elongation at break.

4. Conclusions

In summary, laccase-assisted grafting is quite a new and attractive technique from green chemistry technologies. Therefore, the composite materials developed using natural biopolymers in combination with enzymatic catalysis would prove to be green in their preparation and performance. The above-discussed newly synthesized composites are expected to find potential applications in various sectors, where environmentally friendly polymer composites with novel characteristics are of utmost importance. The newly grafted composites under an enzymatic environment exhibited some unique functionalities and characteristics such as good tensile strength, and mechanical strength due to its unique graft structure.

![Figure 4](image-url) WCA profile of individual polymers *i.e.*, P(3HB) and EC and their grafted composites *i.e.*, P(3HB)-g-EC\(^\text{A}\) and P(3HB)-g-EC\(^\text{B}\).
Acknowledgements

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