Biodegradation of microbial polyesters P(3HB) and P(3HB-co-3HV) under the tropical climate environment

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Abstract

The biodegradation of poly(3-hydroxybutyrate), P(3HB), and its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV) produced by a locally isolated bacteria identified as \textit{Erwinia} sp. USMI-20 were carried out by using soil burial test and immersion test method at various places under the tropical environment in West Sumatra, Indonesia. The isolation of P(3HA)-degrading microorganisms was done by the in vitro rapid plate test method and was further characterized by using biochemical reactions. Our results showed that P(3HB) biodegraded at a rate of 3.6\% per week in activated sludge, 1.9\% per week in soil, 1.5\% per week in lake water and 0.8\% per week in Indian Ocean sea water. The degradation rates for P(3HB-co-3HV) were 17.8\% per week in activated sludge, 6.7\% per week in soil, 3.2\% per week in lake water and 2.7\% per week in Indian Ocean sea water. The biodegradation of both polymers were highest after burial into activated sludge with a half-life (T\textsubscript{1/2}) of 14 weeks and the time for 100\% degradation (T\textsubscript{100\%}) at 28 weeks for P(3HB), and a T\textsubscript{1/2} of 3 weeks and T\textsubscript{100\%} at 6 weeks for P(3HB-co-3HV). In this study, 10 bacteria which were responsible for the biodegradation of P(3HB) and P(3HB-co-3HV) film were isolated and identified from the various places studied under the tropical environment. They were \textit{Bacillus} sp. FAAC-2202, \textit{Enterobacter} sp. FAAC-2207, \textit{Bacillus} sp. FAAC-2209 and \textit{Proteus} sp. FAAC-2203 obtained from activated sludge, \textit{Bacillus} sp. FAAC-2201 and \textit{Alcaligenes} sp. FAAC-2210 from soil, \textit{Alcaligenes} sp. FAAC-2205, \textit{Micrococcus} sp. FAAC-2206 and \textit{Pseudomonas} sp. FAAC-2208 from lake water and \textit{Proteus} sp. FAAC-2204 from Indian Ocean sea water.

Keywords: Biodegradation; Microbial polyesters; Tropical climate; Poly(3-hydroxybutyrate); Poly(3-hydroxybutyrate-co-valerate)

1. Introduction

A variety of bacterial poly(3-hydroxyalkanoate), P(3HA), has been reported and associated as an intracellular storage polymer of carbon and energy under various nutritional and environmental condition [1,2]. The polyester is accumulated as distinct granules in the cells and can be isolated from these cells by means of solvent extraction method [3]. P(3HA) are biodegradable and biocompatible thermoplastics [4]. Among the P(3HAs) which have been widely reported are poly(3-hydroxybutyrate), P(3HB), and its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV) [5]. These polymers have been produced and marketed by Monsanto of USA, by using fed-batch cultivation of \textit{Ralstonia eutropha} [6]. By varying the production strains, substrates and co-substrates, a number of polyesters can be synthesized which differ in the monomer composition [7]. Thus, P(3HAs) with tailored interesting physical features can be produced by employing biotechnological processes.

A soil microorganism, \textit{Erwinia} sp. USMI-20, has been reported to produce P(3HB) and P(3HB-co-3HV) from a mixture of palm oil and various second carbon sources [8,9]. The bacteria can achieve 60 wt.\% of P(3HB) and a dry cell weight of 3.6 g/l from a batch fermentation in a 10 l fermentor from initial concentration of 4.6 g/l of palm oil. Also, in the production of the copolymer P(3HB-co-3HV), the highest mole fraction of 3-hydroxyvalerate, 3HV, units can be as high as 47 mol\% from a single feeding of valeric acid upon initial growth on palm oil. The mechanical and physical properties of these polyesters have been characterized and reported [10].
Recently, we reported the degradation kinetics of P(3HB) produced by Erwinia sp. USM1-20 under nonaqueous condition of chloroform–methanol mixture in the presence of either one of the two following catalysts, 4-toluenesulphonic acid and imidazole and in aqueous condition of increasing pH [11]. From this study, a random chain scission of P(3HB) occurred in the nonaqueous condition while the degradation of P(3HB) in the presence of water occurred through surface hydrolysis with no change in molecular weight. For the surface degradation of the polymer, the rate was increased with higher pH value.

In this paper, the biodegradation profile of two microbial polyester films consisting of P(3HB) and P(3HB-co-3HV) under the tropical climate was studied. The degradation processes were characterised by monitoring the time dependent change in weight loss (erosion) of these polymer films. The microorganisms responsible for the degradation of these films were isolated and characterized at the genus level. Until now a number of aerobic and anaerobic P(3HAs)-degrading bacteria and fungi have been isolated from various environments, which include Acidovorax facilis [12], Aspergillus fumigatus [13], Pseudomonas lemoignei [14] from soil, Alcaligenes faecalis [15,16] from activated sludge, Comamonas testosteroni [17] from sea water, and Pseudomonas stutzeri from lake water [18]. These microorganisms have been demonstrated to excrete extra-cellular P(3HA)-depolymerases to degrade P(3HAs) into the water soluble monomers and oligomers, and utilised these degraded products as a carbon source. In addition, the extra-cellular P(3HA)-polymerases have been isolated and characterized from A. faecalis [15,16], C. testosteroni [17], P. fluorescens and P. lemoignei [19].

2. Experimental

2.1. Materials

Polymers were extracted from the lyophilized cells of Erwinia sp. USM1-20 after 48 h following a single batch cultivation in a 10 l fermentor containing palm oil as a single carbon source for producing P(3HB) or a mixture of palm oil and valeric acid for producing P(3HB-co-3HV) [8]. Extraction processes were carried out in a Soxhlet apparatus with hot chloroform and these polymers were purified by re-precipitation with methanol [10]. The films of P(3HB) (Mw 800 kDa) and P(3HB-co-40 mol% 3HV) (Mw 850 kD) samples were prepared by a solvent-casting technique from chloroformic solutions of the polyester being poured into glass Petri dishes and being left to completely dry for 3 days. The solution cast films were aged for 2 weeks to reach equilibrium crystallinity prior to analysis [20].

2.2. Degradation in tropical environment

Degradation studies of polyester films were conducted under various conditions: activated sludge, soil and lake water in Sungai Buluh village, District Agam, West Sumatra, Indonesia and Indian Ocean sea water at Purus Beach, Padang, West Sumatra, Indonesia. The degradation test in activated sludge and soil was carried out by using the soil burial test method while the immersion test method was used for lake and sea water [21]. Polyester films measuring 20 mm×20 mm×0.2 mm thick with an average weight of 80 mg were either buried into activated sludge or soil (10 cm depth) or immersed into lake or sea water (100 cm underwater). Samples were periodically removed, washed with distilled water and dried to constant weight in vacuo before further analysis by gravimetric measurement.

2.3. Screening and identification of P(3HA)-degrading microorganisms

The screening for P(3HA)-degrading microorganisms was done by the in vitro rapid plate test method [22,23]. These microorganisms were isolated from various study sites by inoculating onto suspended P(3HA) in agar growth media. Upon cultivation, those microorganisms showing a clear zone around their colonies were further isolated. These microorganisms were then purified and characterized microscopically and macroscopically [24,25]. Identification of the isolated microorganisms was done by using a commercially available identification kit (API 20-E kit) obtained from bioMerieux, France. This identification system utilised 21 biochemical tests which include a number of biochemical reactions with the various enzymes within the cells. A positive result would indicate that the strain under investigation exhibited these enzymes within their metabolic system. Otherwise, a negative reaction indicates the absence of such enzymes. In addition, the growth of the bacterium on some common carbon sources was also tested which include glucose, mannitol and sucrose. For this purpose, the identification of the microorganisms was done at the genus level only.

3. Results and discussion

Figs. 1 and 2 show the weight loss of P(3HB) and P(3HB-co-3HV) films after burial in activated sludge and soil as well as after immersion into lake water and sea water as a function of the degradation time. It was observed that the highest degradation rate occurred after burial in activated sludge followed by soil, lake water and in Indian Ocean sea water. Fig. 3 shows the appearance of P(3HB-co-3HV) film samples after burial in soil. Since the weight loss from these films was noted
to follow a zero-order pattern as shown in our previous study [11], a linear regression plot of the weight loss from polyester films was used to characterize the degradation profile of these films. By assuming this, surface erosion is the main degradation process with the degradation taking place through a constant surface area such that the degradation from the sides of the films were not significant as compared to the degradation from main planar surface. In addition, polyester biodegradation normally proceeds via surface attack by bacteria [26–28] with both the weight and thickness of film decreasing with time. It has been found that the extra-cellular P(3HB) depolymerase hydrolyzes polyester chains in the surface layer of the film. These bacteria can
excrete extra-cellular enzymes to solubilize the P(3HA) surface on which they are growing [29]. The soluble degradation products are then absorbed through the cell wall and metabolized. The rate constant values, as derived from the gradient of the slope for P(3HB) and P(3HB-co-3HV) films at the various places are shown in Table 1. P(3HB) biodegraded at a degradation rate of 3.6% per week in activated sludge, 1.9% per week in soil, 1.5% per week in lake water and 0.8% per week in Indian Ocean sea water. The degradation rate for P(3HB-co-3HV) were 17.8% per week in activated sludge, 6.7% per week in soil, 3.2% per week in lake water and 2.7% per week in Indian Ocean sea water. The biodegradation of both polymers were highest after burial in activated sludge with a half-life ($T_{1/2}$) of 14 weeks and the time for 100% degradation ($T_{\sim 100\%}$) of 28 weeks for P(3HB), and a $T_{1/2}$ of 3 weeks and $T_{\sim 100\%}$ at 6 weeks for P(3HB-co-3HV). By comparison, the biodegradation rate of copolymer P(3HB-co-3HV) was about five times faster than homopolymer P(3HB). Therefore our results indicate that the degradation rate of these two polyesters is dependent on the composition of second monomer in the polyester.

The rate of biodegradation is also influenced by the microbial population in a given environment, the temperature, pH, humidity and the properties of the plastic material to be degraded [26,27,30]. From our study, the highest degradation rate of values of 3.6 and 17.8% per week for P(3HB) and P(3HB-co-3HV) films, respectively was achieved in activated sludge, while the lowest degradation rates of 0.8 and 2.7% per week for P(3HB) and P(3HB-co-3HV) films, respectively, was after immersion in Indian Ocean sea water. Due to these factors, the population of microorganisms at various places was determined by using an agar-plate count as shown in Table 2. The microbial population in the order of increasing number follows the sequence: Indian Ocean sea water, lake water, soil, activated sludge.

Table 1
The comparative data of biodegradation rate of P(3HB) dan P(3HB-co-3HV) at various places under the tropical environment

<table>
<thead>
<tr>
<th>Polymer sample and degradation parameter</th>
<th>Places</th>
<th>Activated sludge</th>
<th>Soil</th>
<th>Lake water</th>
<th>Indian Ocean sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB) film</td>
<td>$k$ (% per week)</td>
<td>3.6</td>
<td>1.9</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>$T_{1/2}$ (Week)</td>
<td>14</td>
<td>26</td>
<td>34</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>$T_{\sim 100%}$ (Week)</td>
<td>28</td>
<td>52</td>
<td>68</td>
<td>136</td>
</tr>
<tr>
<td>P(3HB-co-3HV) film</td>
<td>$k$ (% per week)</td>
<td>17.8</td>
<td>6.7</td>
<td>3.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>$T_{1/2}$ (Week)</td>
<td>3</td>
<td>8</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>$T_{\sim 100%}$ (Week)</td>
<td>6</td>
<td>15</td>
<td>31</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 2
The microbial population in various places under the tropical environment

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Activated sludge (cells g$^{-1}$) x 10$^{-6}$</th>
<th>Soil (cells g$^{-1}$) x 10$^{-6}$</th>
<th>Lake water (cells ml$^{-1}$) x 10$^{-6}$</th>
<th>Indian Ocean sea water (cells ml$^{-1}$) x 10$^{-6}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>186</td>
<td>142</td>
<td>122</td>
<td>104</td>
</tr>
<tr>
<td>Fungi</td>
<td>153</td>
<td>116</td>
<td>124</td>
<td>101</td>
</tr>
</tbody>
</table>
Because of this, the degradation rate was found to be the highest in activated sludge. The other parameters noted during this study were as follows: average temperature of 27 °C, humidity index of 80% and the pH of activated sludge, soil, lake water and Indian Ocean sea water of 6.6, 6.1, 6.4 and 7.3, respectively.

The isolation of P(3HAs)-degrading microorganisms was done by an in vitro rapid plate test method and were further characterized by a number of biochemical reactions. Ten bacteria strains which were responsible for the biodegradation of P(3HB) and P(3HB-co-3HV) film were isolated. Further identification of these strains showed that these microorganisms were as follows (see also Table 3): Bacillus sp. FAAC-2202, Enterobacter sp. FAAC-2207, Bacillus sp. FAAC-2209 and Proteus sp. FAAC-2203 obtained from activated sludge, Bacillus sp. FAAC-2201 and Alcaligenes sp. FAAC-2210 obtained from soil, Alcaligenes sp. FAAC-2205, Micrococcus sp. FAAC-2206 and Pseudomonas sp. FAAC-2208 obtained from lake water and Proteus sp. FAAC-2204 obtained from Indian Ocean sea water. Further investigation is now being carried out to observe the P(3HA) extra-cellular enzymes produced by this bacteria.

4. Conclusion

Our studies showed that P(3HB) and P(3HB-co-3HV) films were degraded in the tropical environment. P(3HB) was biodegraded at a degradation rate of 3.6% per week in activated sludge, 1.9% per week in soil, 1.5% per week in lake water and 0.8% per week in sea water. The degradation rates for P(3HB-co-3HV) were 17.8% per week in activated sludge, 6.7% per week in soil, 3.2% per week in lake water and 2.7% per week in sea water, respectively. Ten bacteria were found to bio-degrade P(3HB) and P(3HB-co-3HV) films which were identified as Bacillus sp. FAAC-2202, Enterobacter sp. FAAC-2207, Bacillus sp. FAAC-2209 and Proteus sp. FAAC-2203 (activated sludge), Bacillus sp. FAAC-2201 and Alcaligenes sp. FAAC-2210 (soil), Alcaligenes sp. FAAC-2205, Micrococcus sp. FAAC-2206 and Pseudomonas sp. FAAC-2208 (lake water) and Proteus sp. FAAC-2204 (Indian Ocean sea water).

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