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Biodegradability of *Acinetobacter junii* CNI PHB Copolymerized with PHV

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Abstract: ***Acinetobacter junii CN1 PHB, copolymerized with commercial (Sigma) PHV (1:1 ratio) when subjected to soil burial assay exhibited complete degradation on 60th day. The structural changes occurred in PHBV were then analyzed with AFM, IR spectral readings and TEM as well. A total of 6 PHBV degrading bacterial strains (S1- S6) were isolated not only from the soil in which the PHBV was buried but also from the surface of the degraded PHBV membrane. The isolates were then identified based on their morphological and biochemical characteristics. Further, on screening the six bacterial isolates of the present study, for their PHBV degrading potential, the maximum clear zone was reported with A. faecalis. It is believed that the extracellular PHB depolymerases of the bacterial isolate would serve as the principal enzyme in degradation of PHB / oligomers into dimmers or monomers. These low molecular weight fragments would be further utilized by the microbes as sole carbon and energy sources.***

Keywords: *Acinetobacter junii* CN1 PHB, copolymerization, PHBV, biodegradability

1. Introduction

PHB, a fascinating homopolymer, is existing as a highly crystalline and stiff material. It has also been characterized with poor elastic properties [10]. It can be made better with improved physical and mechanical properties by blending / copolymerization with 5 % valerate [2]. In general, copolymerization enables the polymers to become more flexible and tougher than PHB. Further, they do facilitate easier degradation when discharged into natural environment [28]. Besides, the spectrum of possible applications also expands with the modified physical properties of the polymer blends. It is worth mentioning that the microbial PHB which could copolymerize with 5, 8 and 12 % HV contents are proved to be suitable for tissue engineering [5] , [25].

In general PHB, PHBV and other PHAs are being utilized by the microorganisms as energy sources and degraded in microbial active environments [14], [22], [23]**.** The first report on the function of PHB has been explained in 1958 which demonstrate the rapid biodegradability of PHB produced by *B.megaterium* and *B.cereus* [12]. Biodegradation of PHB under aerobic conditions results in CO_2 and H_2O. Whereas, in anaerobic conditons, the degradative products include CO_2 and CH_4.

The biodegradability of the biopolymer may depend upon the polymer characteristics, organism type and nature of pre-treatment [27]. The pre-treatment of polyethylene is very significant for its biodegradation. Physical rupturing of the polyethylene and chemical washing by ethanol might have added value to its degradability. Supportingly, the improved biodegradation rate of the polymer has been reported with fungal cultures containing polyethylene added with ethanol [29]. However, the fate of these organic polymers in the environment and the time required for their complete mineralization into CO_2 needs to be fully understood [26].

As the microbial degradation of PHAs can take place in natural environments especially in soil, [9] where it is being dumped largely at landfills, the present investigation attempts to degrade the *Acinetobacter junii* CN1 PHB copolymerized with PHV, by subjecting to soil burial assay.

2. Materials and Methods

2.1. Copolymerization

The PHB extracted from *Acinetobacter junii* CN1 [24] was copolymerized with commercial poly -hydroxy valeric acid (Sigma) (1:1 ratio) by dissolving in 5 ml chloroform and fabricated into a film by casting in clean, dry, glass Petri dish in a fume hood [31]. The film was further dried for 24 hrs. The dry copolymerized PHBV film was then removed from the Petri dish using a razor blade and subjected to physical and molecular characterizations.

2.2. Biodegradation of Copolymerized PHBV in Garden Soil

Biodegradability of the copolymerized PHBV was determined by subjecting the sample for soil burial assay [3]. Accordingly, the copolymerized PHBV film was buried in garden soil collected in a large plastic tray maintained at room temperature for a period of 2 months. The structural changes occurred in the polymer were then analyzed with AFM (XE-70, Park System, Korea), IR (Schimadzu - IR Tracer 100, Japan) spectral readings and TEM (Leica SP2, Japan) analysis as well.

2.3. Isolation of Copolymerized PHBV Degrading Microorganisms

The soil sample used in biodegradation assay was serially diluted. From 10^{-6} dilution, 0.1 ml was taken and inoculated in a sterilized plate with mineral salt medium containing PHBV which was fabricated with the extracted PHB obtained on sonication (Sonication for 2 hrs at 90 duty cycles) and commercial PHV (glucose substitute) in 1: 1 ratio. The plates were then incubated at 37 °C for 72 hrs. The isolates which produced the maximum zone of clearance [3] were then subjected not only tomorphological and

biochemical characterizations but also to molecular characterization by 16S rRNA sequencing.

3. Results and Discussion

3.1. Copolymerized PHBV

The copolymerized PHBV of the present study (Plate 1), was characterized with Atomic Force Microscope. It is a powerful tool to observe the

Plate 1: Copolymerized PHBV

thickness of the membrane. While screening the topography of PHBV under AFM, it revealed 2-5µm thickness and 79.219 nm roughness (Plate 2 & 3; Table 1). It is noteworthy, that the roughness seemed to be increased with the degradation of the biopolymer [31].

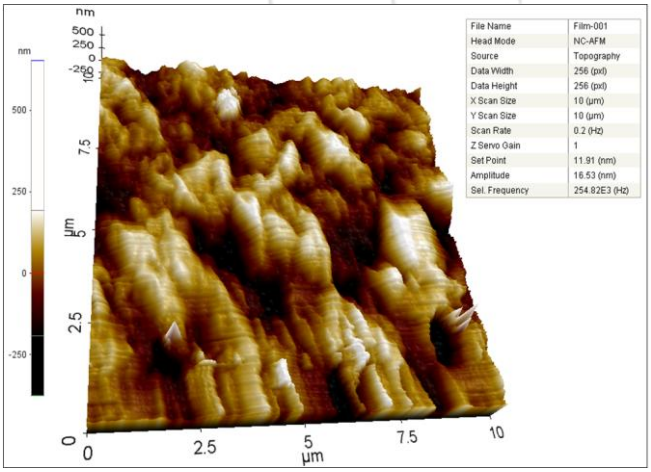

Plate 2: AFM image of copolymerized PHBV measuring thickness

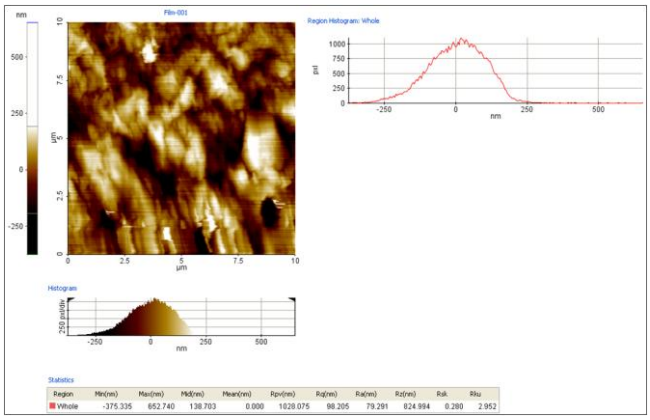

Plate 3: AFM image of copolymerized PHBV measuring roughness

Table 1: Physical characterization of PHBV with AFM

Physical Properties	*Measurement*
Thickness	2-5 µm
Roughness	79.291 nm

3.2. Biodegraded Copolymerized PHBV in Garden Soil

Initially, on visual observation, the PHBV film degraded in soil by a fortnight, revealed tiny holes, tear or thinning out of the film and some cracks in certain areas. Subsequently, the similar trend has also been reported [32]. The wide visible changes observed on polymer degradation, includes roughness of the surface, formation of holes or cracks, defragmentation, changes in colour and /or formation of biofilms on the surface [8]. These visual changes can be used as a first indication of microbial attack.

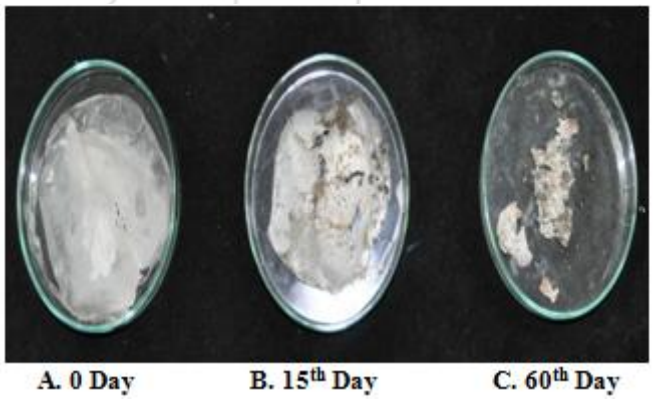

Plate 4: PHBV subjected to soil burial assay

Further, the chemical changes due to the microbial activity in the course of biodegradation studies of polymer can also be assessed with FTIR techniques [16]. On soil burial assay, the remarkable changes were reported on 60th day and the IR spectrum revealed the peaks at 3424.73, 2928.04 , 1722.49, 1632.80, 1383.97, 1280.78, 1099.46 and 1035.81 cm^{-1}(Plate 4 - 6; Figure 1 & 2).

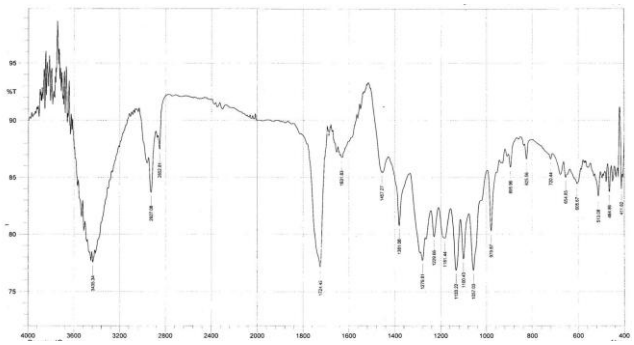

Figure 1: IR spectrum of PHBV

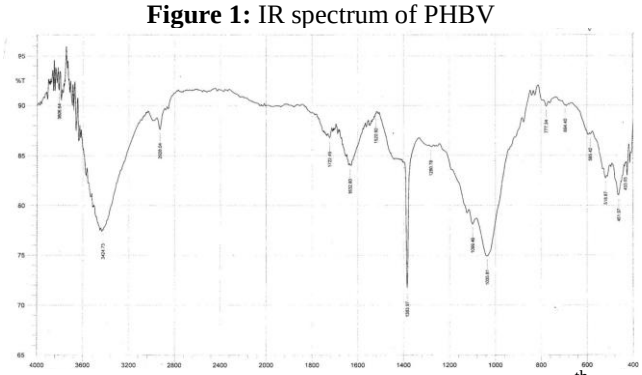

Figure 2: IR spectrum of soil degraded PHBV on 60th day

Further, the results of the TEM analysis of the PHBV subjected to soil burial assay (Plate 7) had revealed a rapid reduction in thickness from 2 µm to 1 µm on 15 th day, 1 µm – 0.5 µm on 30 th day and 0.5 – 0.2 µm on 60 th day. On an observation under TEM for the morphological changes occurred in the thin PHB film on enzymatic degradation, both the surface (4-5 nm) and lamellar crystal (8 -10 nm) were reported to exhibit reduction in thickness. This would suggest the possibility of preferential hydrolyses of the crystals by PHB depolymerase. The biodegradation of different commercial biopolymers at moderate temperature in different soil types with 30% weight loss after 3 months period has also been demonstrated [17].

Consequently, several authors [20], [6], [7] , [30] have also observed the complete degradation of PHBV on 6, 7 and 350 weeks in sewage, soil and sea water respectively.

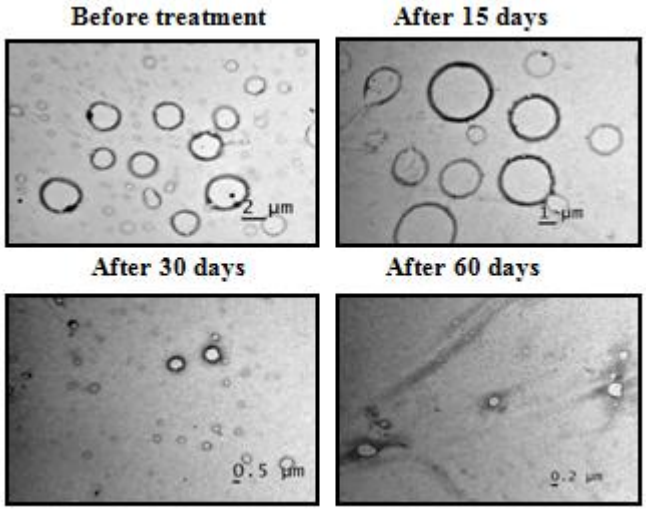

Plate 5: TEM image of PHBV degraded in soil revealing the thickness

3.3. PHBV Degrading Microorganisms

In the present study, a total of 6 PHBV degrading bacterial strains (S1- S6) were isolated not only from the soil in which the PHBV was buried but also from the surface of the degraded PHBV membrane. The isolates were then identified based on their morphological and biochemical characteristics (Table 2).

With the results of the morphological and biochemical characterizations, the PHBV degrading bacterial strains S1, S2, S3, S4, S5 and S6 of the present investigation were identified as *Bacillus*, *Bifidobacterium*1 , *Bifidobacterium* 2, *Corneybacterium*, *Bifidobacterium* 3 and *Alcaligenes faecalis* respectively (Table 2).

In this context, the bacterial isolates *viz.*, *Pseudomonas* (both fluorescent & non-fluorescent forms), *Bacillus*, *Azospirillum*, *Mycobacterium* and *Streptomyces* have been detected over the degraded PHB films [4]. Besides, it has been observed that the fungal isolates exhibit much higher capabilities than the bacteria in degrading PHAs [11].

Further, on screening the six bacterial isolates of the present study, for their PHBV degrading potential, the maximum clear zone was reported with *A. faecalis* (Plate 6). The similar zones of
clearance have been reported around the colony **[3].** This is mainly due to the hydrolysis of the suspended polyesters by the target organism.

Table 2: Morphological and biochemical characteristics of PHBV degrading bacteria

Characterization	Inference					
	S1	**S2**	**S3**	**S4**	**S5**	**S6**
Gram reaction	+	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Motility	Non motile	Non motile	Non motile	Non motile	Non motile	Non motile
Colony morphology	White irregular colonies	Light white, smooth, flat, irregular	Light cream, smooth, flat, irregular	Dull white small, flat, circular	Light cream, smooth, flat, irregular	Light cream, smooth, flat, irregular
Indole test	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+
Voges- proskauer test	+	-	-	-	-	-
Citrate test	+	-	-	+	-	-
Catalase test	-	-	-	+	-	-
Oxidase test	+	-	-	-	-	-
Triple- sugar iron agar test	+	+	+	+	+	+
Urease test	+	+	+	+	+	+
Gelatin hydrolysis test	+	+	+	+	-	+
Probable identification	*Bacillus*	*Bifido bacterium* 1	*Bifido bacterium* 2	*Corney bacterium*	*Bifido bacterium* 3	*Alcaligenes faecalis*

The clear zone test gives an opaque appearance in the polymer coated medium. The formation of a clear halo around the colony also indicates the first step of degradation and reveals the degradability of the microorganism [21], [1]. Further, the semi quantitative results can also be obtained by analyzing the growth of clear zones [3].

It is believed that the principal enzyme pertaining to the degradation of PHB / oligomers is PHB depolymerase. The extracellular PHB depolymerases are being isolated from different bacteria *viz., A. faecalis, R. rubrum, B. megaterium, A.beijerinckii* and *P. lemoignei*. Besides, polyhydroxyalkanoic acid degrading fungi have also been isolated from various environments such as freshwater, sea water and sludge sample [15]. It has been established that many fungal taxa belonging to *Basidiomycotina*, *Denteromycotina* and *Ascomycotina* are the predominant degraders of PHAs in natural environment [19] .

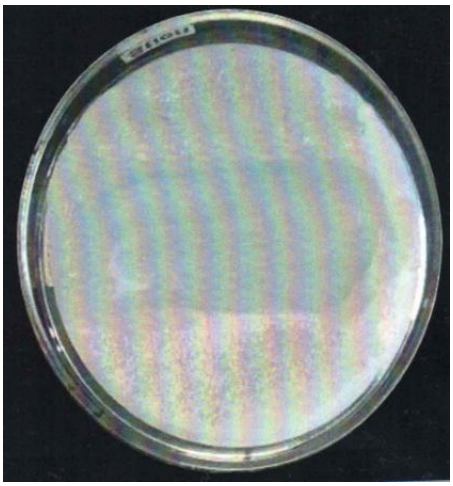

Plate 6: Zone of clearance exhibited by PHBV degrading *A.faecalis*

By 1992, *Aspergillus* has been reported as one of the predominant genera involved in PHB degradation [13]. In addition to *Aspergillus*, a number of other mesophilic fungi belonging to the genera *Penicillium* and *Paecilomyces* are found to be responsible for degrading PHAs in soil and aquatic environments [11].

PHAs degrading organisms, at once when get attached to the surface of the polymer they get started to grow by exploiting them (PHAs) as the carbon source. In the primary degradation, the cleavage of the main chain leads to the formation of dimmers or monomers. These low molecular weight fragments are further utilized by the microbes as sole carbon and energy sources [28].

The resultant breakdown fragments must be completely used by the microorganisms, otherwise the remnants would cause brutal environmental and health hazards [18]. However, the biodegradability of biopolymer depends primarily on its molecular weight, molecular structure and crystallinity as well. Nevertheless, the degradability decreases with the increase in molecular weight.

4. Acknowledgement

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Author Profile

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