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Production of Polyhydroxybutyrate (Bioplastic) and its Biodegradation by *Pseudomonas Lemoignei* and *Aspergillus Niger*

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Abstract: The biodegradation of polyhydroxybutyrate (PHB) and its copolymer polyhydroxy butyrate-co-hydroxyvalerate (PHB-co-HV) was studied. Bacterial as well as fungal isolates were isolated from the local industrial ecosystem. All these isolates were tested for the degradation of the above polymers in assay agar medium as well in liquid cultures. The culture biomass and the clear zone around the colonies were measured to evaluate the activity of these isolates. In all, the fungal isolates were found to degrade these polymers more rapidly when compared to bacteria, due to their versatile depolymerase activities.

Keyword: Polyhydroxy butyrate Poly-3-hydroxybutyrate, Polyhydroxyvalerate, Polyhydroxy alkanoate, Depolymerase activity.

Introduction

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-biodegradable residues is affecting the potential survival of many species.

For this reason, many countries have promoted special programmes directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere and have designed novel strategies aimed at facilitating the transformation of contaminants. Biomaterials are natural products that are synthesized and catabolised by different organisms and that have found broad biotechnological applications. They can be assimilated by many species (biodegradable) and do not cause toxic effects in the host (biocompatible)[1-4], conferring upon them a considerable advantage with respect to other conventional synthetic products. Bioplastics are a special type of biomaterial.

They are polyesters, produced by a range of microbes, cultured under different nutrient and environmental conditions[5]. These polymers, which are usually lipid in nature, are accumulated as storage materials (in the form of mobile, amorphous, liquid granules), allowing microbial survival under stress conditions[6,7] The number and size of the granules, the monomer composition, macromolecular structure and physicochemical properties vary, depending on the producer organism[8-11]. They can be observed intracellularly as light refracting granules or as electron lucent bodies that, in overproducing mutants, cause a striking alteration of the bacterial shape.

Atomic force microscopy and confocal Raman spectroscopy are techniques currently used for poly(3-hydroxyalkanoate) (PHA)-granule analysis. Bioplastics can be isolated by centrifugation (cell-free extracts) or by solvent extraction (dried intact bacteria) with chloroform, trifluoroethanol, dichloroethane, propylene carbonate, methylene chloride or dichloroacetic acid[12–15]. Their molecular weights (ranging from 50,000 to 1,000,000 Da) have been established by light scattering, gel permeation chromatography, sedimentation analysis and intrinsic viscosity measurements[16,17] and their monomer compositions have been determined by gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) analyses[18]. Other physical properties, such as crystal structure, polydispersity, melting temperature, enthalpy of fusion, glass transition temperature and mechanical properties were established using different procedures[19].

Most known bioplastics contain, as monomers, different *b*-oxidation intermediates [(R)-3-hydroxyacyl–CoAs], which are enzymatically polymerised by the condensation of the carboxy function, present in a monomeric–CoA thioester with the 3-hydroxy group (or the thiol group) of the next one[7]. Other bioplastics, containing unusual monomers (*e.g*. 4-, 5- or 6 hydroxyalkanoic acids or glutamic acid), are synthesized through different pathways[18-23] suggesting that biosynthetic enzymes are widely distributed and that the production of a particular type of polyester is a strain specific event.

Currently, the main limitations for the bulk production of bioplastics are its high production and recovery costs[24]. However, genetic and metabolic engineering has allowed their biosynthesis in several recombinant organisms (other bacteria, yeasts or transgenic plants)[25-28], by improving the yields of production and reducing overall costs[29,30].

Lemoigne described a bioplastic-poly(3-hydroxybutyrate) (PHB) in *Bacillus megaterium*[31]. This initial observation was almost forgotten until the mid-1970s when, because of the petroleum crisis, a scientific movement aimed at discovering alternative sources of fossil fuel reserves was undertaken. However, the structure, biosynthetic pathways and applications of many bioplastics have now been established.

Microbes belonging to more than 90 genera-including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters[18]. The occurrence of bioplastics has been discussed elsewhere[32]. The most widely produced microbial bioplastics are PHB, PHA and their derivatives[19].

However, other polyesters can also be produced by microorganisms. Most of them either require similar biosynthetic enzymes or lack current industrial applications and hence we shall only describe the genes and enzymes involved in the production of PHBs and PHAs. In this review, we discuss the occurrence, biosynthesis, catabolism and biotechnological applications of poly 3-hydroxyalkanoates.

PHB is water insoluble and relatively resistant to hydrolytic degradation. PHB shows good oxygen permeability. PHB has good UV-resistant but has poor resistance to acids and bases.

PHB is soluble in chloroform and other chlorinated hydrocarbons. PHB is suitable for medical applications like bone plates, nails, screws[33] and in the treatment of osteomyelitis[34]. PHB has its melting point at 175 °C and glass transition temperature of 15 °C. PHB is non-toxic. PHB sinks in H_2O, while polypropylene floats. But sinking of PHB facilitates its anaerobic biodegradation in sediments.

PHB is used in the following productions; Shampoo bottles; Food containers; Suitable dishwasher use due to the good impact and temperature resistance of the material; Disposable razors; Disposable utensils; Fishing nets; Composite-glial growth factors; Drug carriers; PHB coated papers; Golf tees, *etc*.

Experimental

For production, the bio-industrial effluent such as sugarcane effluent waste was collected in sterile bottle and they were preceded for PHB production. The sugarcane effluent was collected from Trichirappalli, Tamilnadu, India. For degradation, the soil samples were collected from natural habitats from the garden land.

Effluent was collected from paper and sugarcane industry in clean bottles. Mother culture was prepared with 99 mL of distilled water added to 1 mL of paper and sugarcane industry effluent to make up the volume to 100 mL (10^{-2}) and then mixed well. Five sterile test tubes were taken and 9 mL of sterile distilled water was added to it. Then 1 mL of sample from 10^{-2} dilution was pipetted into the test tube containing 9 mL of sterile distilled water using a sterile pipette, representing 10^{-3} dilution. This procedure was repeated using sterile pipettes for each dilution up to 10^{-7} dilutions. Specific medium were prepared and autoclaved at 121 °C for 5 min. After sterilization the medium was cooled and poured 15 to 20 mL on plates and allowed for solidifying. After solidification, 0.1 mL of sample from each dilution tube was taken and allowed to spread on the surface of agar plates using L-rod. All the plates were incubated at 37 °C for 24-48 h. Separate plates were prepared for paper and sugarcane effluent. After incubation, plates were observed.

50 mL of specific broth for *Bacillus megaterium* (Glucose monohydrate-33 g, ammonium sulfate-5 g, potassium dihydrogen phosphate-2.2 g, magnesium sulfate-0.3 g, yeast extract - 0.5 g, trace elements-2 g such as $MnCl_2$, CaC_{l2}, $FeSo_4.7H_2O$, CoC_{l2}) and 50 mL of specific broth for *Alcaligenes eutrophus* (Disodium hydrogen orthophosphate-3.8 g, potassium dihydrogen phosphate-2.7 g, ammonium chloride-2 g, glucose-20 g, magnesium chloride-0.2 g, trace minerals-1 g such as FeCl, CaC_{l2}, $CuSO_4$, CoC_{l2}) were prepared and sterilized. One loop full of culture was inoculated onto the paper and sugarcane substrates. Flasks were placed in the shaker at 110 rpm for 24 h. It was incubated for one day. The occurrence of turbidity was overseen frequently. Slowly the quantity was raised and the mass production was carried on.

After 48 h incubation at 37 °C, 10 mL of culture was taken and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 10 mL sodium hypochlorite and the mixture was incubated at 30 °C for 2 h. After incubation, the mixture was centrifuged at 5000 rpm for 15 min and then washed with distilled water, acetone and methanol respectively for washing and extraction. After washing, the pellet was dissolved in 5 mL of boiling chloroform and was evaporated on sterile glass tray and kept at 4 °C. After evaporation, the powder was collected for further analysis.

The sample (0.5 mL) was treated with 4.5 mL concentrated sulfuric acid and placed in boiling water bath for 10 minutes. On cooling, absorbance was noted at 235 nm in UV spectrophotometer.

Cell dry weight was measured in sugarcane effluent. Soil samples were collected from natural habitats from the area of garden soil for the study of polymer degradation capabilities of fungi and bacteria. The fungi were isolated on saboraud dextrose agar medium (SDA). The media was purchased from Hi-Media. Samples were suspended by vortexing in sterile distilled water and allowed to stand for several minutes. The supernatants were then serial diluted. A 0.1 mL portion from each dilution was plated onto the SDA plates and incubated at room temperature for 2-7 days. The isolated fungi were maintained on SDA slants.

Serial dilutions were made in sterile distilled water and the samples were directly poured and plated onto Pseudomonas isolation agar plates. The media was purchased from Hi-Media. The plates were then incubated at 37 ^{0}C for 2-3 days. Colonies with different morphology, color, pigmentations, *etc*., were isolated in pure form and maintained on slants. The polymer degrading ability of the isolated bacteria and fungi was determined by different techniques. For the fungal isolates, the basal medium was used which was supplemented with 0.01 g yeast extract, containing about 0.02 g of dry polymer (PHB) as a sole carbon source and solidified with 1% agar at pH 6.0. The plates were incubated at room temperature for 1-3 weeks. The bacterial isolates were screened by using minimal media containing 0.03 g of the polymer (PHB) as a sole carbon source, which was solidified with 2 g agar at pH 7.0. The isolates were transferred onto plates with this minimal media and were incubated at 37 ^{0}C ± 2 ^{0}C for 7-12 days. In case of degradation of the polymers by fungal isolates, it was detected by the formation of a clear zone surrounding the growth and the extent of degradation was measured from the width of the clear zone formation. The bacterial isolates were seen as clear colonies on media with the polymer as a sole carbon source.

The degradation of the polymers in the liquid media was determined by growing the bacterial and fungal isolates in the assay medium supplemented with 0.02% of the polymer. After inoculation, the samples were incubated on rotator shaker for 7 to 10 days at 110 rpm at 37 °C. The leftover residual amount of the polymer after incubation was determined using Law and Slepecky method (1961)[37], after extraction with chloroform and evaporation to dry. The amount of the dry biomass was determined to calculate the growth of the isolates.

Results and Discussion

In this study, two different microorganisms were isolated for the production and degradation of PHB respectively. They were characterized based on their characteristics according to Bergey's manual of systematic bacteriology[38]. They were then identified to be *Bacillus megaterium* and *Alcaligenes eutrophus* (for production of PHB) and *Pseudomonas lemoignei* and *Aspergillus niger* (for degradation of PHB). According to Williamson & Wilkinson[35], their lipid inclusions were observed. In *Alcaligenes eutrophus*, the inclusion is found to be larger in size when compared with *Bacillus megaterium.* In sugarcane effluent, the dry weight of PHB produced of *Alcaligenes eutrophus* is found to be more when compared with *Bacillus megaterium.* The property of PHB production was observed by naked eye visualization by forming a layer.

Table 1. Cell dry weights in sugarcane effluent with different medium

S. No.	Organism	Cell mass, g	
		Nitrogen deficient medium	Industrial waste medium
1	*Bacillus megaterium*	0.250	0.358
2	*Alcaligenes eutrophus*	0.262	0.375

Alcaligenes eutrophus produced a thin layer in both the medium. *Bacillus megaterium* requires high temperature for the growth. Green black color bands were observed in TLC for

all the cases and from R_f values the *Alcaligenes eutrophus* found to be produced more PHB that was followed by *Bacillus megaterium.* According to, Rawate T and Mavinkruve[39], the R_f values were measured and PHB was read by Spectrophotometry *Alcaligenes* produced more PHB both in paper and sugarcane effluent.

Table 2. R_f value for the production of PHB from sugarcane effluent in industrial waste medium

S. No.	Organism	Solvent distance, cm	Solute distance, cm	R_f Value
1	*Bacillus megaterium*	16.8	3.5	0.21
2	*Alcaligenes eutrophus*	16.5	4.0	0.24

In the present study, *Alcaligenes eutrophus* was found to produce more PHB in sugarcane effluent rather than *Bacillus megaterium.* The degradation properties of polymer (PHB) were studies with the isolated microorganisms from garden soil. They were then characterized. Degradation of PHB was affected significantly when the PHB containing media was supplemented with consumable carbon sources. Result of supplementation studies have indicated that the degradation of PHB was significantly retarded by the entire carbon source supplemented in the degrading medium.

PHB depolymerase synthesis by the *Aspergillus spp*., therefore appeared to be regulated by the presence of easily consumable carbon source in the degradation medium. The fungal isolates were identified as largely *Aspergillus* strains from their spore morphology. All the samples showed evidence of PHB degraders. This is predictable, in view of the biodiversity of the polymer-producing prokaryotes in these environments[36] and the consequent availability of PHB as a nutrient source. Fungi that degrade PHB and copolymer were mostly isolated from soil compost, garden soil.

Table 3. Assay of PHB by spectrometry for sugarcane effluent

S. No.	Organism	Cell Mass, g		
		Original	Duplicate	Average
1	*Bacillus megaterium*	0.924	0.908	0.916
2	*Alcaligenes eutrophus*	1.274	1.258	1.266

Bacteria are considerable degraders of PHB through their capability of production of intracellular depolymerase enzyme. Hence, their presence in these environmental samples was studied through a basal minimal media containing 0.03% of the polymer as a carbon source. The results indicate that the soil from garden soil exhibited highest number of PHB degrading bacteria.

Table 4. Degradation of polymer in liquid culture against bacteria

S. No.	Organisms	Optical density, 660 nm		
		Day	Control, nm	Medium with polymer, nm
1	*Pseudomonas lemoignei*	1	0.132	0.134
2		2	0.139	0.141
3		3	0.144	0.149
4		4	0.149	0.156
5		5	0.147	0.167
6		6	0.143	0.174
7		7	0.137	0.179

According to law and Slepecky[37] the cultivation of a few selected fungal and bacterial isolates in liquid cultures was carried out to study the amount of polymers utilized by these isolates during their growth. The fungal isolates showed a high capability of utilizing PHB

as growth substrate than bacterial isolates. PHB degrading bacteria secrete specific PHB depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolytic products are taken up by the cells and metabolized. The main objective in this work was to determine the biodegradability of the PHB and co-polymers, by various bacterial and fungal isolates from a local ecosystems.

Table 5. Degradation of polymer in liquid culture against fungi

S. No.	Organisms	Optical density, 660 nm		
		Day	Control, nm	Medium with polymer, nm
1		1	0.136	0.138
2		2	0.144	0.149
3		3	0.151	0.158
4		4	0.162	0.173
5	*Aspergillus niger*	5	0.168	0.182
6		6	0.164	0.185
7		7	0.158	0.197
8		8	0.155	0.213
9		9	0.149	0.227
10		10	0.144	0.234

According to Law and Slepecky (1961)[37] the cultivation of a few selected fungal and bacterial isolates in liquid cultures was carried out to study the amount of polymers utilized by these isolates during their growth. The fungal isolates showed a high capability of utilizing PHB as growth substrate than bacterial isolates. PHB degrading bacteria secrete specific PHB depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolytic products are taken up by the cells and metabolized. The main objective in this work was to determine the biodegradability of the PHB and co-polymers, by various bacterial and fungal isolates from a local ecosystems.

Conclusion

From the present study, we can conclude that we can produce the PHB from an industrial effluent (sugarcane effluent) and the degradation of PHB by microorganisms on soil-garden soil. This effluent discharged from these industries spoils the agricultural practice and aquatic organisms, thereby affecting the ecosystem around them. These industrial effluents utilization in an effective way will be a boom for agriculturalist and industrialists to get rid of the hazardous problems. This study thus revealed that this effluent can be used as substrate for PHB (bio-plastic) production. These can be degraded by microorganisms without any chemicals. In case of using chemicals it may cause environmental pollutions but microbes do not cause any serious problems. So we can conclude bio-plastic, an eco-friendly and very valuable product. Fungi isolates degrade the PHB (bio-plastic) much higher than that of bacteria.

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