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Poly-β-hydroxybutyrate biodegradation by mixed culture population vis-à-vis single culture population under varying environmental conditions: A new approach

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Mostly, studies on biodegradation of poly-β-hydroxybutyrate (PHB) employs only single or dual culture of degrading microorganisms and often in single environmental condition. In this study, as a new and sustainable approach, we used natural communities (mixed culture population) for biodegradation of PHB under variable environmental conditions. *Alcaligenes* sp. RZS 4 yielded 5.60 g/L of PHB by submerged fermentation. The absence of polysaccharides, cell component in PHB extract and thin layer chromatography reassert the purity of PHB extract. Eighteen bacterial and 7 fungal cultures were used for assessment of biodegradation PHB. The study using single culture population suggests that the metabolism of fungal and bacterial isolates varies from species to species**,** in solid environment; maximum degradation lasts up to 15 days of incubation while in liquid environment it lasts up to 10 days. The biodegradation of PHB in liquid environment by a mixed population exhibit a much higher biodegradation rate than single culture population and also reduces biodegradation time by 2 days. The times profile suggested that the rate of PHB biodegradation depended on the incubation period and amount of PHB. This study, thus documents the use of natural communities (mixed culture population) in defined media stimulated environmental conditions for efficient biodegradation of PHB.

Keywords: Bioremediation, Liquid environment, Mixed culture, PHB biodegradation, Single culture, Solid environment, Submerged fermentation

Poly-β-hydroxybutyrate (PHB) accumulated intracellularly into bacteria and used as a source of carbon and energy reserve, it is important for the metabolism (enzyme protein, storage compounds), the genetic information (nucleic acid) and the structure (cell wall constituents, protein) of cells. Among biodegradable polymers, the best known is PHB, which is produced on a large scale through bacterial fermentation[1,2]. The accumulated PHB is used by certain bacterial species to achieve a balanced growth even in dynamic substrate conditions, which allows them to utilize the external substrate more efficiently[3] and ultimately outcompete other species within the system[3-6].

Microbial degradation of PHB can take place through the action of enzymes or by products (lipids, peroxides) secreted by microorganisms (bacteria, yeast and fungi)[1]. PHB has been shown to be degraded by bacteria into water and carbon dioxide (aerobically) and into methane (anaerobically) in natural environments including water, soil and compost. The fungal biomass in soil generally exceed the bacterial biomass and thus it is likely that the fungi may play a considerable role in degrading PHB, as they decompose organic matter in the soil ecosystem[3]. Although biodegradation tests with mixed cultures or natural microflora are practical oriented, depend on naturally occurring conditions and are tedious to measure degradation of PHB. Therefore, development of appropriate biodegradation methods to determine the degradation of polymers under defined conditions and within a reasonable time period is important[7].

Several reports are available on PHB degradation by using PHB films[8]. However, in this paper, we report new approach for biodegradation of powder state PHB by single microbial population as well as natural microflora in different environmental conditions such as the solid and liquid environment. The study shows that the fungal as well bacterial isolate has potential biotechnological application in PHB degradation systems.

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Materials and Methods

PHB production and extraction

PHB production was carried out by *Alcaligenes* sp. RZS 4 (Gene bank accession No. JN374993) using two-step cultivation process. In the first step, it was grown (1% inoculum) in 100 mL carbon-rich medium (CRM) containing (g/L) glucose, 20; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 13.3; $MgSO_47H_2O$, 12.; citric acid, 1.7; trace element solution, 1.0 ml/L containing (g/L) $FeSO_4$ $7H_2O$; 10, $ZnSO_47H_2O$, 2.25; $CuSO_45H_2O$, 1.57; $MnSO_45H_2O$, 0.5, $CaCl_22H_2O$, 2.0; $Na_2B_4O_710H_2O$, 0.23; $(NH_4)6Mo7O_{24}$, 0.1; pH was set to 7.2 at 30°C for 48 h at 120 rpm followed by centrifugation at 10000 rpm for 10 min to get the biomass[9]. In the second step, the biomass obtained from CRM was washed with sterile distilled water and used to grow in nitrogen deficient minimal medium (NDMM) consisting of (g/L) Na_2HPO_4, 3.8; KH_2PO_4, 2.65; NH_4Cl, 2; $MgSO_4$, 0.2; fructose, 2 and trace minerals 1.0 ml/L, which contained (g/L) EDTA, 5.0; $ZnSO_47H_2O$, 2.2; $CaCl_2$, 5.45; $MnCl_26H_2O$, 5.06; H_3BO_3, 0.05; $FeSO_47H_2O$, 4.79; NH_4Mo, 24.4; $CoCl_2.6H_2O$, 1.6; $CuSO_45H_2O$, 1.57 at 30°C for 48 h at 120 rpm, followed by centrifugation at 10000 rpm for 10 min[6]. PHB was extracted from biomass by using a mixture of acetone–alcohol (1:1v/v)[10]. This PHB extract was used for biodegradation studies.

Determination of purity of PHB extract

The purity of PHB extract was determined by the Molish's and Iodine tests and also by Thin Layer Chromatography (TLC). The Molish's test was done by acidification of the PHB sample with dilute HCl and change in the colour of the sample was observed. Iodine test was carried out by adding two drops of iodine followed by observing the change in colour.

Thin layer chromatography of PHB extract and PHB standard (Sigma Aldrich, USA) was performed on a silica gel sheet (20 X 20 cm, Merck) with solvent system toluene:ethyl acetate:acetic acid (60:30:5). The detection of PHB was carried out by exposing the silica plate in the iodine chamber[11]. The retention value [Rf] was calculated and compared with that of the standard PHB.

Biodegradation of PHB

Source of biodegrading cultures

Fungal cultures like *Cercospora arachichola*, *Aspergillus flavus* NCIM 650, *Metarhizium anisopliae* NCIM 1311, *Verticillium lecanii* NCIM 1312, *Fusarium oxysporum* NCIM 1281, *Aspergillus niger* NCIM 1025 and bacterial strains like *Alcaligenes faecalis* NCIM 2949, *A. faecalis* NCIM 2262, *Pseudomonas* sp. NCIM 2866, *Pseudomonas* sp. NCIM 2208 were procured from the National Collection of Industrial Microorganism [NCIM], NCL, Pune, India. *Alternaria alternata* IARI 715 was procured from the Indian Agricultural Research Institute (IARI), New Delhi, India while other strains were isolated from local winery effluent as mentioned below.

Isolation and identification of cultures

Bacterial strains like *Alcaligenes* sp RZS 4, *Alcaligenes* sp. RZS 2, *A. faecalis* sp. JAS1, *A. faecalis* PAOAC 171, *Pseudomonas* sp RZS 1, *P. aeruginosa* strain 67, *P. putida*, *P. aeruginosa* RZS 3, *Acinetobacter* sp. K2 and *Acinetobacter* sp. 2109 were previously isolated and identified by GC-FAME (Gas Chromatography-Fatty Acid Methyl Esters) analysis, phenotypic fingerprinting and 16s RNA sequencing[1].

Several *Streptomycetes* sp. were isolated from garden soil of PSGVPM's Art, Science, Commerce College, Shahada, Maharashtra, India by plating on glycerol asparagine agar (ISP medium)[4] containing aspargine, 1 g/L; glycerol, 10 ml/L; K_2HPO_4, 1 g/L; agar, 20 g/L; trace salt solution (1 ml/L) containing $FeSO_4$, 0.1 g/100 mL; $MnCl_2$, 0.1 g/100 mL; $ZnSO_4$, 0.1 g/100 mL[12] and the plates were incubated at 30°C for 7-10 days. Isolates were identified by both macroscopic and microscopic examination and maintained on glucose asparagine agar. The bacterial isolates were maintained on nutrient agar (NA) and fungal cultures were maintained on potato dextrose agar (PDA). All the cultures were preserved at 4°C.

Screening of PHB degrading organism

In order to study the ability of various isolates and improvements in biodegradation of PHB, 7 fungal and eighteen bacterial isolates were screened for their ability to degrade PHB. For this purpose, each fungal and bacterial strain was separately grown in minimal salt agar (MSA) medium containing (g/L) K_2HPO_4, 0.5; KH_2PO_4, 0.04; NaCl, 0.1; $CaCl_2$, 0.002; $(NH_4)_2SO_4$, 0.2; $MgSO_4$, 0.02; $FeSO_4$, 0.001; agar, 20 supplemented with 0.1 % PHB as a sole source of carbon. The growth of each PHB degrading organism was observed and utilization of PHB from the media

was determined. Isolates capable of efficiently utilizing PHB as carbon source were taken for further degradation studies.

PHB biodegradation by single culture population

Biodegradation in solid environment

The PHB degrading ability of fungal and bacterial strains was determined in the MSA by over layer plate assay. A thin basal layer of the MSA was poured and solidified at low temperature and the same agar medium supplemented with 0.2% (w/v) PHB powder was then over layered on the basal layer and allowed to solidify quickly. The overlayered plates were streaked individually with a loopful of fresh inoculum of individual bacterial and fungal isolate followed with their incubation at 30°C. The PHB degrading ability and the extent of degradation of each isolate was determined by measuring zone of PHB hydrolysis surrounding the growth[9].

PHB biodegradation profile

The degradability of PHB extract was determined by tube assay; the MSA medium tubes supplemented with varying concentrations of PHB (0.1, 0.2, 0.3 and 0.4 %) were inoculated with fungal spores (50 µL) and bacterial suspension (50 µL) at the surface and incubated at 30°C for several days. The PHB degrading ability and the extent of degradation of the individual isolates was determined by the formation of zone of PHB hydrolysis beneath the surface growth and measuring the depth of the zone[13].

Biodegradation in liquid environment

PHB degradation in liquid medium was determined by growing the efficient PHB degrader in liquid MSM containing PHB (0.2% i.e. 2 mg/mL) as sole carbon source. The MSM medium was inoculated with different bacterial and fungal cultures and incubated for several days at 100 rpm at 30°C. Biodegradation of PHB was determined by measuring the extent of growth as biomass (mg/mL), optical density (OD) at 660 nm, the amount of residual PHB by gravimetric analysis (mg/mL) and crotonic acid concentration (µg/mg)[9].

Quantification of PHB degradation

After incubation, the zone of PHB hydrolysis appeared in circular and cylindrical forms in Petri plates and test tubes respectively. PHB biodegradation was quantitatively expressed as deprivation of polymer in term of weight loss (WL_{PHB}) with time and expressed as mg/mm^2 using equation (I)[14].

$$WL_{PHB} = A_Z \, X \, W_{PHB}/A_T \quad \text{..... (I)}$$

whereas,

WL_{PHB} = Deprivation of polymer in term of weight loss, A_Z = Area of zone of PHB degradation in Petri dish/Test tube (mm), W_{PHB} = Weight of PHB seeded in medium (mg), and A_T = Total area of Petri dish/Test tube (mm^2)

The A_Z is the area of the zone of degradation appears in Petri plate and a test tube while A_T is the total area of Petri plate and test tube. Therefore, the area of the zone of degradation (A_Z), total area (A_T) of Petri plate and Test tube was measured by using equation (II) and (III), respectively. It was expressed as mm.

$$A_Z \text{ or } A_T = n\, r^2 \quad \text{..... (II)}$$

$$A_Z \text{ or } A_T = 2\, n\, r\, h \quad \text{..... (III)}$$

whereas,

π = 3.14 (constant), r = Radius of zone of degradation (d/2), and h = Height of test tube up to media filled.

However, the resulting area of the zone of degradation (A_Z) and total area (A_T), was used in an equation (I) calculate biodegradation of PHB.

Biodegradation by mixed culture population

To find the role and activity of ecological microflora in PHB degradation, various samples from natural environment were used for the study. They were labeled and collected as SW (Water solution of campus roadside soil), LWW (Waste water from laboratory), TRW (TAPI river water collected at Piloda Tal, Shirpur, Maharashtra), NTWW (Waste water collected from textile industries at Nandurbar, Maharashtra), GTWW (Wastewater collected from textile industries at Surat, Gujrat), SWW (Waste water collected from sugar factory at Shahada, Maharashtra) and DWW (Domestic waste water from a local area). These water samples belonging to natural locations are known to harbour wide variety of microflora, and hence used as mixed culture populations of microorganisms for PHB biodegradation. These water samples (500 µL) were inoculated in MSM seeded with 0.2% PHB, incubated for several days at 100 rpm at 30°C and biodegradation was recorded as described previously.

Results and Discussion

PHB production and extraction

Growth of *Alcaligenes* sp. RZS 4 in NDMM yielded 5.60 g/L of PHB with 46.1% rate of PHB

accumulation. These yields are much higher than the earlier reported yields[12,15,16]. Kumar and Prabakaran[17] reported 0.8 g/L PHB in *A. eutrophus* MTCC 1285 and Mohamed *et al*.[18] have also reported similar PHB yields from *Bacillus* sp.

Determination of purity of PHB extract

The impurities like polysaccharides or any microbial cell component left over during the extraction process are commonly found in PHB extract. Purity of PHB sample confirmed by detection of such impurities in the PHB extract sample. All PHB samples showed negative Molish's and iodine test indicating the absence of polysaccharides and microbial cell component.

After evaluating PHB extract by TLC, greenish black colored bands appeared on plate. The Rf value calculated as 0.714, which matched with the Rf value of standard PHB. Kumar and Prabakaran[17] also reported 0.71 as the Rf value of PHB. The absence of polysaccharides, any cell component and Rf values confirmed the purity of PHB extracted from *Alcaligenes* sp. RZS 4.

Biodegradation of PHB

Source of biodegrading cultures

Ten different bacterial strains belonging to *Alcaligenes* sp. (04 strains), *Pseudomonas* sp. (04 strains) and *Acinetobacter* sp (02 strains) were isolated and identified as mentioned in our previous study[2]. Other 7 fungal and 4 bacterial strains were procured from collection centers as mentioned above. All the fungal strains belonged to plant pathogenic genera, were tested for possible use of PHB in agricultural application. All the bacterial strains (PHB producer)[2] were tested for their potential in self biodegradation of PHB. Four *Streptomycetes* sp. Were isolated from the local garden soil. The *Streptomycetes* sp. formed irregular, wrinkled, grayish-white and reddish-white colonies having branched aerial with vegetative mycelia; these aerial mycelia produced sporophores of different morphological forms bearing spores in the chain. They were Gram positive, non-motile, oxidase, catalase positive and showing differences in colonial morphology and diffusible pigment[19,20]. These *Streptomycetes* sp. were labeled as NSG1, NSG2, NSG3 and NSG4. These strains are predominantly found in soil and decaying vegetation, and hence were used for PHB degradation studies.

Screening of PHB degrading organism

The morphological appearances of isolates indicate the diversity among them[1]. The fungal and bacterial strains during screening (based on their comparative performance) for selection of efficient PHB degrading strains revealed utilization and degradation of PHB extracted from *Alcaligenes* sp. RZS 4 on their comparative performance.

Out of different fungal strains used, *A. niger* NCIM 1025, *C. arachichola*, *M. anisopliae* NCIM 1311, *A. flavus* NCIM 650, *F. oxysporum* NCIM 1281 and *A. alternata* IARI 715 showed growth on minimal medium containing PHB as sole carbon source except *V. lecanii* NCIM 1312. However, among the different bacterial strains used *Alcaligenes* sp. RZS 4, *Pseudomonas* sp. RZS 1, *A. faecalis* NCIM 2949, *Pseudomonas* sp. NCIM 2866, *P. aerugenosa* RZS 3, *Streptomycetes* sp. NSG 2 and *Streptomycetes* sp. NSG 3 showed growth on minimal medium containing PHB extract of *Alcaligenes* sp. RZS 4. The growth on minimal media suggests utilization of PHB indicating the biodegradation of PHB and evokes them as PHB degrading organisms. Mergaert *et al*.[19] have also isolated and screened 295 microorganisms capable of degrading PHB from the soil, according to growth on minimal media containing PHB as sole carbon source.

PHB biodegradation by single culture population

Biodegradation in solid environment

In plate assay degradation of PHB began from 2nd day of incubation and reached to maximum level after 8 days of incubation. The visualization of growth and formation of zone of PHB hydrolysis around the colonies indicated degradation of PHB. Degradation of PHB into water-soluble products under influence of PHB depolymerase has been reported by Nishida and Tokiwa[22]. The extent of PHB degradation by all isolates ranged between 0.39- 32 mg/mm^2. The plate containing over layered medium provides a solid environment having more surface area that help for higher growth and therefore higher biodegradation. After 8 days of incubation, maximum biodegradation was obtained with *Streptomycetes* sp. NSG 2 (32 mg/mm^2) and *C. arachichola* (28.54 mg/mm2) whereas *A. alternata* IARI 715 gave minimum (1.20 mg/mm2) degradation. Among the various cultures, *Streptomycetes* sp. NSG 2 came out as potent PHB degraders (Fig. 1). This potent biodegrdation by *Streptomycetes* may be due to its diversity of using wide range of substrate and ability

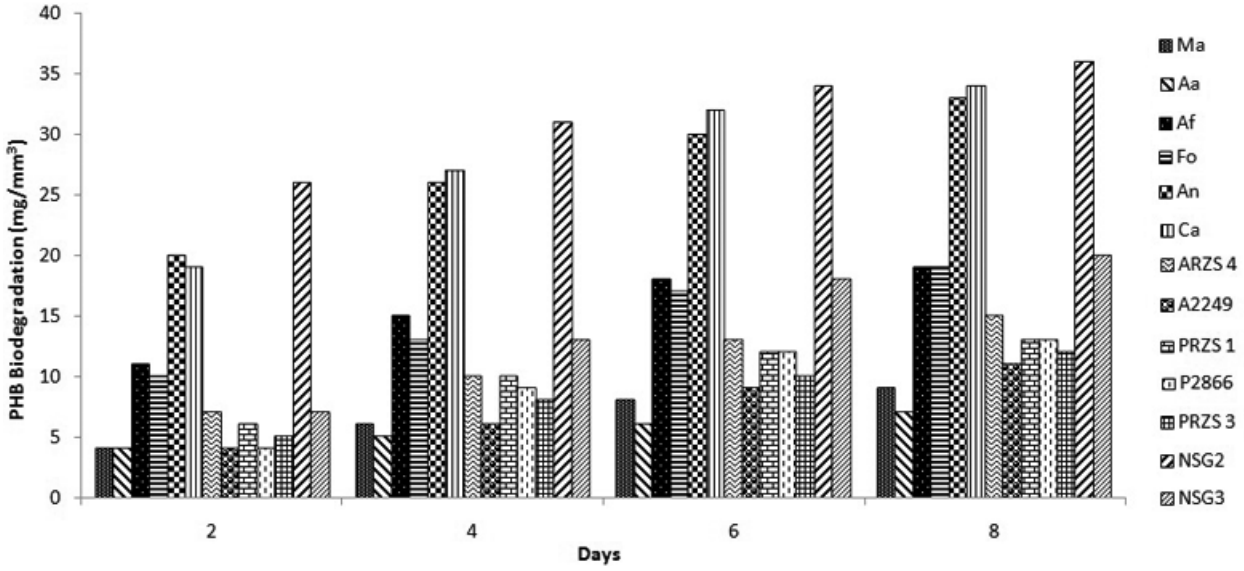

Fig 1—PHB biodegradation in solid environment by plate assay.

to secrete more than one depolymerase enzyme[22]. Mergaert and Swings[23] have reported the versatility of *Streptomycetes* sp. to degrade P(3HB), P(3HB-co-3HV) and P(3HB-co-3HD). Biodegradation of PHB by bacterial culture, *Alcaligenes* sp. RZS 4 was less (5.55 mg/mm^2). This strain has been reported to produce PHB[2]. This dual ability (production and degradation of PHB) of *Alcaligenes* sp. RZS 4 is due to secretion of PHB synthase and PHB depolymerase as well. Shirakuara *et al.*[24] reported D-3-hydroxybutyrate oligomer hydrolases and extracellular PHB depolymerase from *A. faecalis* T1.

PHB biodegradation profile

The profile (with respect to time and PHB level) of PHB biodegradation by fungal and bacterial isolates indicated a gradual increase in the biodegradation with an increase in the incubation period as well as PHB concentration. The biodegradation by fungal and bacterial strains started from 3rd to 6th day of incubation and lasts up to the 15th day of incubation (Table 1). Delay in the beginning of PHB biodegradation in tube assay might be due to the small surface area available that restricted the microbial growth, and hence PHB degradation. Moreover, slow microbial growth lengthened the incubation period up to 15 days, which turned down PHB degradation by delaying the expression of enzymatic activity of PHB degrading strains because the concentration of PHB regulates PHB depolymerase gene expression[11]. Increase in substrate concentration remarkably decreased the depth of the clear zone formed, but increased the PHB biodegradation (Table 1).

Among the fungal cultures, *A. flavus* NCIM 650 and *A. niger* NCIM 1025, and among bacterial cultures, *Streptomycetes* NSG 2 exhibited maximum PHB degradation (Table 1). Biodegradation potential of fungal cultures was comparatively higher than that of bacterial cultures. These results are in accordance with the findingss of Kim and Rhee[3]. For effective biodegradation of PHB, 14:0.4 was found to be the best time concentration ratio for *C. arachicola*, *F. oxysporum* NCIM 1281, *Alternata*, *Pseudomonas* sp. NCIM 2866, *P. auriginosa* RZS 3 and *Streptomycetes* sp. NSG 2; 12:0.4 for *A. flavus* NCIM 650 and *A. niger* NCIM 1025; 13:0.4 for *Alcaligenes* sp. RZS 4 and *M. anisopliae* NCIM 1311; 14:0.3 for *A. faecalis* NCIM 2949 and *Streptomycetes* sp. NSG 3; 11:0.4 for *Pseudomonas* sp. RZS 1 (Table 1).

Biodegradation of PHB in tube assay was higher than the plate assay by all microorganisms, whereas only *Streptomycetes* sp. showed comparatively lesser degradation which might be due to superficial growth of *Streptomycetes* sp.

The depth and clarity of clear zones in the tube varied with different organisms, suggesting differences in metabolic states of the degrading isolates, diffusion rates of different enzymes in the medium and the amounts and activities of the enzyme[24].

Table 1—PHB biodegradation profile

	PHB (%)	Period in Days														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	0.1	-	+	1.17	2.35	3.52	5.88	8.23	10.58	12.94	15.29	15.29	16.47	17.64	17.64	17.64
Af	0.2	-	+	2.35	4.70	11.76	16.47	21.17	25.88	30.58	35.29	40.00	42.35	42.35	42.35	42.35
	0.3	+	+	3.52	10.58	14.11	21.17	28.23	35.29	42.35	49.41	52.94	52.94	56.47	60.00	60.00
	0.4	-	-	4.70	9.41	18.82	23.52	32.94	42.35	51.76	61.11	70.58	80.00	80.00	80.00	80.00
	0.1	-	+	+	1.17	2.35	4.70	5.88	7.05	9.41	10.58	12.94	14.11	15.29	15.29	15.29
Ma	0.2	-	+	2.35	4.70	7.05	11.76	16.47	18.82	23.52	28.23	30.58	35.29	35.29	35.29	35.29
	0.3	-	+	+	3.52	7.05	14.11	21.17	28.23	31.76	38.82	45.88	45.88	49.51	49.51	49.51
	0.4	-	+	4.70	9.41	14.11	18.82	28.23	32.94	42.35	51.76	51.76	61.17	65.88	65.88	65.88
	0.1	-	-	1.17	2.35	4.70	5.88	7.05	9.41	10.58	11.76	12.94	14.11	15.29	15.29	15.29
Ca	0.2	-	2.35	7.05	9.41	11.76	16.47	21.17	23.52	25.88	30.58	32.94	32.94	35.29	35.29	35.29
	0.3	-	-	3.52	10.58	14.11	21.17	24.70	31.76	35.29	42.35	45.88	45.88	49.41	49.41	49.41
	0.4	-	+	4.70	9.41	18.82	28.23	32.94	42.35	47.05	51.76	61.17	61.17	61.17	65.88	65.88
	0.1	-	+	1.17	2.35	3.52	4.70	5.88	7.05	8.23	9.41	10.58	12.94	14.11	14.11	14.11
Fo	0.2	-	+	2.35	4.70	7.05	9.41	11.76	14.11	16.47	21.17	25.88	30.58	32.94	32.94	32.94
	0.3	-	+	3.52	7.05	10.58	10.58	17.64	21.17	24.70	31.76	38.82	42.35	45.88	45.88	45.88
	0.4	-	+	+	4.70	9.41	14.11	18.82	23.52	28.23	32.94	42.35	51.76	56.47	61.17	61.17
	0.1	-	+	1.17	2.35	4.70	7.05	8.23	9.41	11.76	14.11	16.47	18.82	18.82	18.82	18.82
An	0.2	+	2.35	4.70	9.41	14.11	18.82	23.52	28.23	30.58	35.29	40.00	42.35	42.35	42.35	42.35
	0.3	-	+	3.52	10.58	17.64	24.70	28.23	31.76	38.82	45.88	52.94	60.00	60.00	60.00	60.00
	0.4	-	+	4.70	14.11	23.52	28.23	37.64	47.05	56.47	65.88	75.29	80.00	80.00	80.00	80.00
	0.1	-	-	+	1.17	2.35	3.52	4.70	5.88	8.23	9.41	12.94	15.29	16.47	16.47	17.64
Aa	0.2	-	-	+	2.35	2.35	4.70	7.05	11.76	18.82	21.17	30.58	37.64	37.64	40.00	40.00
	0.3	-	-	-	+	3.52	7.05	10.58	14.11	21.17	24.70	31.76	38.82	42.35	42.35	45.88
	0.4	-	-	-	+	4.70	9.41	14.11	14.11	18.82	23.52	32.94	42.35	47.05	51.76	51.76
	0.1	-	-	-	-	+	++	1.17	1.17	2.35	2.35	3.52	4.70	4.70	5.88	5.88
ARZS4	0.2	-	-	-	+	++	2.35	2.35	4.70	4.70	7.05	9.41	9.41	11.76	14.11	14.11
	0.3	-	-	-	-	+	++	3.52	7.05	10.58	14.11	14.11	17.64	17.64	21.17	21.17
	0.4	-	-	-	-	+	++	4.70	4.70	9.41	9.41	14.11	14.11	18.82	18.82	18.82
	0.1	-	-	-	-	+	++	++	1.17	2.35	2.35	3.52	3.52	4.70	4.70	4.70
A2949	0.2	-	-	-	-	+	++	2.35	4.70	7.05	7.05	9.41	9.41	9.41	11.76	11.76
	0.3	-	-	-	-	+	++	3.52	7.05	10.58	10.58	14.11	14.11	14.11	17.64	17.64
	0.4	-	-	-	-	+	++	++	4.70	4.70	9.41	9.41	14.11	14.11	14.11	18.82
	0.1	-	-	-	-	+	++	1.17	1.17	2.35	2.35	3.52	3.52	3.52	4.70	4.70
P2866	0.2	-	-	-	+	++	2.35	2.35	4.70	7.05	7.05	7.05	9.41	11.76	11.76	11.76
	0.3	-	-	-	-	+	++	3.52	7.05	10.58	10.58	14.11	14.11	17.64	17.64	17.64
	0.4	-	-	-	-	+	++	4.70	4.70	4.70	9.41	9.41	14.11	14.11	18.82	18.82
	0 .1	-	-	-	-	+	++	1.17	2.35	2.35	3.52	3.52	4.70	4.70	5.88	5.88
PRZS3	0.2	-	-	-	+	++	2.35	4.70	4.70	7.05	9.41	9.41	11.76	14.11	14.11	14.11
	0.3	-	-	-	+	++	3.52	7.05	7.05	7.05	10.58	14.11	14.11	17.64	17.64	17.64
	0.4	-	-	-	-	+	++	4.70	4.70	9.41	9.41	14.11	14.11	14.11	18.82	18.82
	0.1	-	-	-	+	++	1.17	2.35	2.35	2.35	3.52	3.52	4.70	4.70	4.70	5.88
PRZS 1	0.2	-	-	+	+	2.35	4.70	7.05	7.05	9.41	9.41	11.76	11.76	11.76	14.11	14.11
	0.3	-	-	+	++	3.52	7.05	10.58	10.58	14.11	14.11	17.64	17.64	17.64	17.64	21.17
	0.4	-	-	+	++	++	4.70	9.41	9.41	14.11	14.11	18.82	18.82	18.82	18.82	18.82
	0.1	-	-	+	++	1.17	2.35	3.52	4.70	5.88	8.23	10.58	11.76	12.94	15.29	15.29
NSG 2	0.2	-	-	+	++	2.35	4.70	7.05	11.76	16.47	18.82	23.52	30.58	37.64	40.00	40.00
	0.3	-	-	+	++	3.52	7.05	10.58	14.11	21.17	21.17	28.23	31.76	38.82	42.35	42.35
	0.4	-	-	+	++	++	4.70	9.41	14.11	18.82	23.52	32.94	42.35	47.05	51.76	51.76
	0.1	-	-	+	++	1.17	2.35	3.52	4.70	7.05	9.41	10.58	14.11	14.11	15.29	15.29
NSG 3	0.2	-	-	+	++	2.35	4.70	7.05	9.41	11.76	16.47	21.17	25.88	30.58	32.94	32.94
	0.3	-	-	+	++	3.52	3.52	7.05	10.58	10.58	21.17	24.70	31.76	42.35	45.88	45.88
	0.4	-	-	+	++	4.70	4.70	9.41	9.41	14.11	18.82	23.52	28.23	37.64	37.64	37.64

[(-), No growth; (+), Growth; (++), More growth Af, *A. flavus* NCIM 650; Ma, *M. anisopliae* NCIM 1311; Ca, *C. arachicola*; Fo, *F. oxysporum* NCIM 1281; An, *A. niger* NCIM 1025; Aa, *A alternate* ARZS4, *Alcaligenes* sp. RZS 4; A2949, *A. faecalis* NCIM 2949; P2866, *Pseudomonas* sp. NCIM 2866; PRZS3, *P. auriginosa* RZS 3; PRZS 1, *Pseudomonas* sp. RZS 1; NSG 2, *Streptomycetes* sp. NSG 2; and NSG 3, *Streptomycetes* sp. NSG 3. Values are of PHB Biodegradation (mg/mm2)]

The colour of MSA changed from transparent to yellow due to the excretion of metabolites like acetic, butyric, and valeric acids from 3-hydroxybutyric acid and 3-hydroxyvaleric acid[25]. Such metabolites and other monomers are oxidized to D-β-hydroxybutyrate by NAD dependent D-β-hydroxybutyrate dehydrogenase enzyme which leads to the formation of acetoacetate and acetoacetyl-CoA. The activated acetoacetyl CoA is then hydrolyzed into two molecules of acetyl-CoA, which can then enter the TCA cycle[26-28].

Biodegradation in liquid environment

Biodegradation of PHB in liquid environment started from 2nd day and reached to maximum on 10th day. Fungal cultures such as *A. niger* NCIM 1025, *M. anisopoliae* NCIM 1311 and *Alternata* IARI 715 showed the highest biomass (54, 35.5 and 33 mg/mL, respectively) at 6th day of incubation while *F. oxysporum* NCIM 1281 and *C. arachicola* showed the highest biomass (31.5 and 23 mg/mL, respectively) at 4th day of incubation. However, *A. flavus* NCIM 650 also showed the highest biomass (35.5 mg/mL) at 8th day (Fig. 2). *Pseudomonas* sp. NCIM 2866 and *A. faecalis* NCIM 2949 at the 8th day of incubation showed the highest biomass (40.50 and 31 mg/mL, respectively), while *Streptomycetes* sp. NSG 2 and *Streptomycetes* sp. NSG 3 showed higher biomass (43 and 33.5 mg/mL, respectively) at 4th day of incubation period (Fig. 2B).

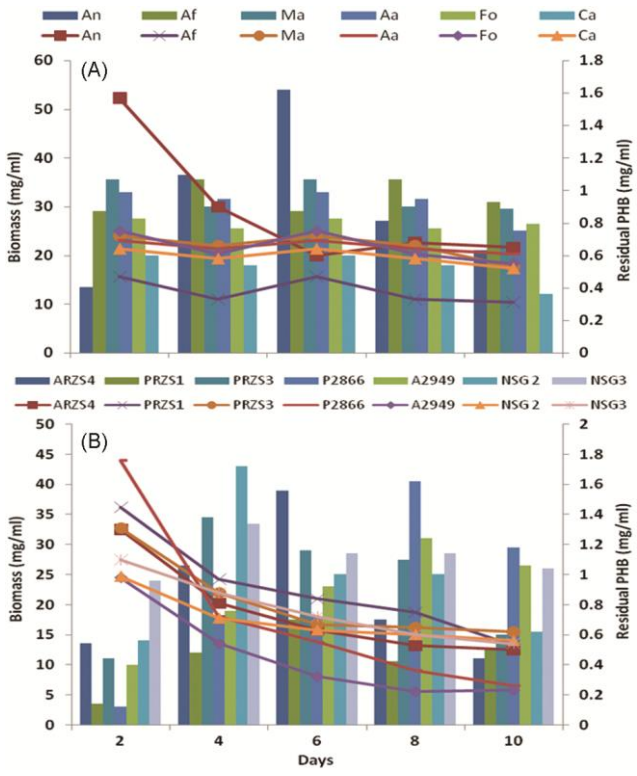

Fig 2—Biodegradation of PHB in liquid environment by (A) fungal culture; and (B) bacterial cultures.

Among the fungal cultures, *A. flavus* NCIM 650 degraded maximum PHB and remained 0.31 mg/mL PHB behind in broth (Fig. 2A) whereas *A. faecalis* NCIM 2949 remained 0.23 mg/mL up to 10 days of incubation (Fig. 2B). The concentration of residual PHB was continuously decreased as the incubation period as well as biomass was increased. It is found that the concentration of residual PHB has been declining on increase in growth of microorganisms. The rate of PHB biodegradation in broth is directly proportional to the growth of microorganisms; this indicates biodegradation of PHB is growth associated process (Fig. 2 A and B). Bacterial cultures are highly efficient in PHB biodegradation under liquid environment due to their capacity to accumulate PHB, as we reported in our previous study[2] and the activity of bacteria is closely connected to the presence of water. The supply of nutrients to the microorganism and the transportation of excreted enzymes and metabolic products take place by diffusion in an aqueous environment is natural one for all metabolic reactions. The estimation of crotonic acid also indicates a continuous decline in a concentration of PHB in broth. OD showed the growth status of each microorganism and its relation in biodegradation of the PHB (Table 2).

The variation observed in degradation of PHB extract among the fungal as well as bacterial cultures may be due to the powdery nature of PHB. The PHB degradation in an amorphous state has been reported to be 20 times higher than in the film sheet[29]. The powdery nature of PHB permits mobility of water within the polymer and makes it more accessible to PHB depolymerases.

Biodegradation by mixed culture population

Sometimes PHB degradation by single microbial culture may not give an accurate understanding of the pattern of PHB degradation in multicomponent ecosystems under varying environmental conditions. Biodegradation of PHB by natural microbial community started from 2nd day and lasts up to 8th day in liquid environment (Fig. 3). Microbial communities present in all the environmental samples showed the presence of PHB degraders. This is predictable, in view of the biodiversity of PHB producing microorganisms in these environments and the consequent availability

Table 2—Biodegradation of PHB extract by single population and mixed population

Strain/ Sample	2 days		4 days		6 days		8 days		10 days	
	Crotonic acid (µg/ml)	OD	Crotonic acid (µg/ml)	OD	Crotonic acid (µg/ml)	OD	Crotonic acid (µg/ml)	OD	Crotonic acid (µg/ml)	OD
An	726	Nd	421	Nd	282	Nd	276	Nd	186	Nd
Af	453	Nd	432	Nd	453	Nd	432	Nd	188	Nd
Ma	501	Nd	403	Nd	501	Nd	403	Nd	356	Nd
Aa	625	Nd	573	Nd	625	Nd	573	Nd	555	Nd
Fo	544	Nd	465	Nd	544	Nd	465	Nd	315	Nd
Ca	618	Nd	593	Nd	618	Nd	593	Nd	528	Nd
ARZS4	519	0.017	437	0.041	364	0.040	303	0.034	248	0.026
PRZS1	852	0.021	687	0.96	645	0.009	605	0.889	576	0.037
PRZS3	762	0.015	418	0.038	336	0.073	334	0.068	370	0.032
P2866	860	0.017	537	0.035	267	0.069	186	0.065	158	0.034
A2949	645	0.002	373	0.020	298	0.020	208	0.011	179	0.006
NSG 2	675	Nd	410	Nd	401	Nd	372	Nd	342	Nd
NSG 3	812	Nd	684	Nd	569	Nd	397	Nd	365	Nd
SW	821	0.014	773	0.016	671	0.021	653	0.015	-	-
LWW	879	0.008	859	0.012	848	0.009	842	0.010	-	-
TRW	874	0.006	880	0.007	832	0.015	791	0.015	-	-
NTWW	892	0.010	821	0.015	802	0.017	800	0.014	-	-
GTWW	824	0.013	762	0.016	694	0.018	625	0.018	-	-
KWW	811	0.021	704	0.028	587	0.036	579	0.037	-	-

[Nd, Not determine; Af, *A. flavus* NCIM 650; Ma, *M. anisopliae* NCIM 1311; Ca, *C. arachicola*; Fo, *F. oxysporum* NCIM 1281; An, *A. niger* NCIM 1025; Aa, *A alternate*; ARZS4, *Alcaligenes* sp RZS 4; A2949, *A. faecalis* NCIM 2949; P 2866, *Pseudomonas* sp. NCIM 2866; PRZS3, *P. auriginosa RZS3*; P RZS 1, *Pseudomonas* sp. RZS 1; *S* NSG 2, *Streptomycetes* sp. NSG 2; *S* NSG 3, *Streptomycetes* sp. NSG 3; SW, Soil water; LWW, Laboratory waste water; TRW, TAPI river water; NTWW, Textile waste water from Nandurbar; GTWW, textile waste water from Gujarat; KWW, waste water from sugar factory; and DWW, Domestic waste water]

of PHB as a nutrient source[1]. Earlier workers[30-32] have also reported significant degradation through the action of mixed culture.

Among the various natural microbial communities studied for biodegradation of PHB, DWW microflora showed the highest utilization of PHB and produced a biomass of 27.5 mg/mL at the end of the 8th day of incubation. This was followed by KWW, SW, GTWW, LWW and NTWW microflora. Lowest biomass was observed with NTWW microflora (5.5 mg/mL) in liquid medium containing PHB. DWW microflora was found to be involved in maximum PHB degradation up to 1 mg/mL of PHB, at the end of the 8th day of incubation (Fig. 3). Gradual increase in OD and simultaneous reduction in crotonic acid indicates the pattern of growth as well as PHB biodegradation by a mixed culture population in liquid environment (Table 2). Yellow colour began to appear in medium during incubation, suggesting PHB biodegradation due to release of degradation products[16,31,32]. The changes in residual PHB and biomass suggests that the biodegradability of PHB is related to the number of PHB degraders in different ecosystems in different regions and also depends upon the growth conditions required for easy proliferation in the environment.

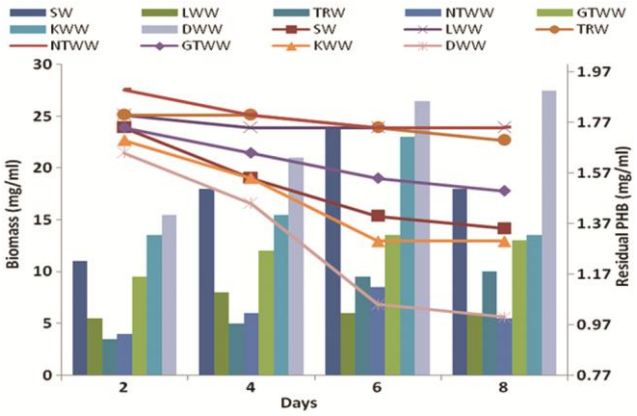

Fig 3—Biodegradation of PHB by mixed population.

The biodegradation of PHB in liquid environment by mixed culture population exhibited a much higher degradation rate than the single culture population and reduced period of degradation. After determining the efficiency of PHB degradation by a mixed culture population in comparison with single culture population, versatile nature of the natural communities (mixed culture population) of PHB biodegradation has been revealed. Such communities have potential to degrade PHB in natural conditions as well as defined liquid environment. Biodegradation

of PHB in the field with different environments have a number of limitations and problems, parameters such as temperature[28,29], water quality can vary during the test period and monitoring of such biodegradation process is tedious. To overcome these difficulties biodegradation of PHB in defined liquid environment under controlled laboratory tests simulates natural environment is best to investigate the PHB biodegradation.

Biodegradability of PHB in liquid environment was considerably higher than in a solid environment. Since PHB degradation process is a hydrolytic reaction of polymers and PHB depolymerase are water soluble. Therefore, the enzymatic degradation was easier to take place in aquatic ecosystem than in the solid environment.

Conclusion

The selection and isolation of microorganism based on PHB utilization minimizes the difficulties occurring in isolation of PHB degraders. Solid media provides more surface area for the growth of microorganisms, but it doesn't promote PHB biodegradation, while liquid medium gives direct access to PHB depolymerase secreted by microorganisms to degrade PHB. The rate of PHB biodegradation in solid environment was lesser and slows due to the less accessibility of PHB to microorganisms in a solid environment. The efficient PHB biodegradation was dependent on the time period, PHB concentration as well as microorganism used.

Liquid environment is highly suitable for PHB biodegradation than the solid environment. Moreover, biodegradation of PHB is well supported in defined liquid media stimulated with environmental conditions.

Natural microflora having mix microbial communities gave maximum PHB degradation in less time[29]. However, assessment of PHB degradation by natural microflora is difficult task, as the proportion of microorganisms varies from location to location, and also each culture may produce variety of depolymerases. Presence of other enzymes that stimulate or inhibit the PHB depolymerase is yet another aspect in PHB biodegradation.

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