Parthenium hysterophorus: low cost substrate for the production of polyhydroxyalkanoates

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Parthenium hysterophorus **is considered as one of the most devastating and hazardous weed; abundantly available in several parts of the world, it is utilized as a substrate for the production of polyhydroxyalkanoates (PHA). Bacterial strain *Bacillus aerophilus,* isolated from oil contaminated soil, was studied for its potential to accumulate PHA. Utilizing this cheap substrate, the highest yield of PHA content obtained was 5.4 g/l PHA with 11.92 g/l cell biomass. PHA produced was extracted using sodium hypochlorite method and the polymer synthesized was characterized as polyhydroxybutyrate (P3 (HB)) by Fourier transform infrared spectroscopy and nuclear magnetic resonance analysis.**

Keywords: Biopolymer, *Bacillus aerophilus*, biomass, *Parthenium hysterophorus*, P3 (HB).

PARTHENIUM HYSTEROPHORUS is an aggressive annual herbaceous plant native to tropical America[1]. This weed of global significance, responsible for major biodiversity problem, was first recorded in India by Roxburgh in 1814 (refs 2, 3). The weeds infestation has caused 40% loss in the yield of several crops and reduced forage production by 90%. In 2005, it was estimated that over 5 million acres of land in India was infested by this weed[4]. In the past few decades, production of plastic materials from non-renewable resources increased to a greater extent due to their myriad applications in our day-to-day life, but in turn, they have also created massive environmental problems relying on their non-degradable nature. There are two main factors for which researchers are trying to find an effective solution for the problems posed by conventional plastics – first, the global environmental concern created by the recalcitrance of thermoplastics and secondly, the diminution of non-renewable natural resources for their production.

Polyhydroxyalkanoates (PHAs) are biopolymers exhibiting similar material properties with that of conventional plastics, thus making them an effective alternative for utilization[5,6]. They represent a complex class of storage polyesters that are synthesized and deposited as insoluble cytoplasmic inclusions by a wide range of prokaryotes and some archae[7]. PHAs are accumulated as discrete granules and are believed to play a crucial role as sink for carbon and reducing equivalents in microbes[8]. Few bacterial species accumulate PHA under unbalanced growth conditions with a high level of carbon and a limitation of other nutrients such as nitrogen, sulphur, phosphorus or oxygen[8–10]. The economics of PHA production is a major limitation in commercializing them, as the feedstock alone contributes to more than 50% of the total cost[11]. Hence, various cheaper substrates were explored to cut down the cost to bring about a feasible economical strategy.

In the present study, P3HB was synthesized utilizing *P. hysterophorus* as feedstock. By supplementing simple media components and optimizing fermentation conditions, a high yield of PHB was obtained along with successful management of weed. Spectroscopic analysis provided valuable information about the type of polymer produced, as the functional groups present in them have a great influence on the physical and chemical properties of the polymer thus aiding their implementation in diverse fields of application[12].

For sampling and isolation of microorganism, soil samples were collected from oil contaminated sites like diesel loco sheds and petrol bunks (Erode, Tamil Nadu, India) and serially diluted for isolation of PHA producing bacteria. The isolated pure bacterial colonies were preserved on nutrient agar slants and glycerol stocks were maintained at 4°C, until further use.

The weed (*P. hysterophorus*) was collected locally from Bharathiar University Campus, Coimbatore. All parts of the weed (excluding the root) were chopped into small pieces and dried in hot air oven at 60°C for 2–3 days. After complete drying, they were ground to make a fine powdery substrate.

Hydrolysate of the substrate was prepared by the modified method of Pumiput *et al.*[13]. The substrate (40 g/l) was exposed to steam at 121°C for 20 min, water was added to the wet pretreated material and boiled for 30 min at 80°C, and the final volume was made to 1 litre with distilled water. Using filter paper the hydrolysate was filtered. Concentrated HCl (1% V/V) was added to the filtrate and autoclaved for 30 min at 121°C. The pH of the acid post hydrolysate was adjusted to 6.8 ± 0.2 and it was again filtered[14].

The fluorescence plate assay serves as an efficient screening method for identification of PHA producers[15]. Nile blue A dissolved in dimethyl sulphoxide (DMSO) was added to the agar medium (final concentration of 0.5 μg/ml). Test cultures grown for 16 h in nutrient agar slants were patched onto Nile blue agar plates and incubated at 37°C for 3–4 days. Bacterial colonies on the plate were examined under UV illumination to detect the accumulation of PHA. Genomic DNA was then extracted from bacterial strains exhibiting high PHA productivity and amplified by PCR using 16S rRNA universal primers (forward: GAGTTTGATCGTGGCTCAG; reverse: AGGGCTACCTTAGACTT)[16]. The amplified PCR products

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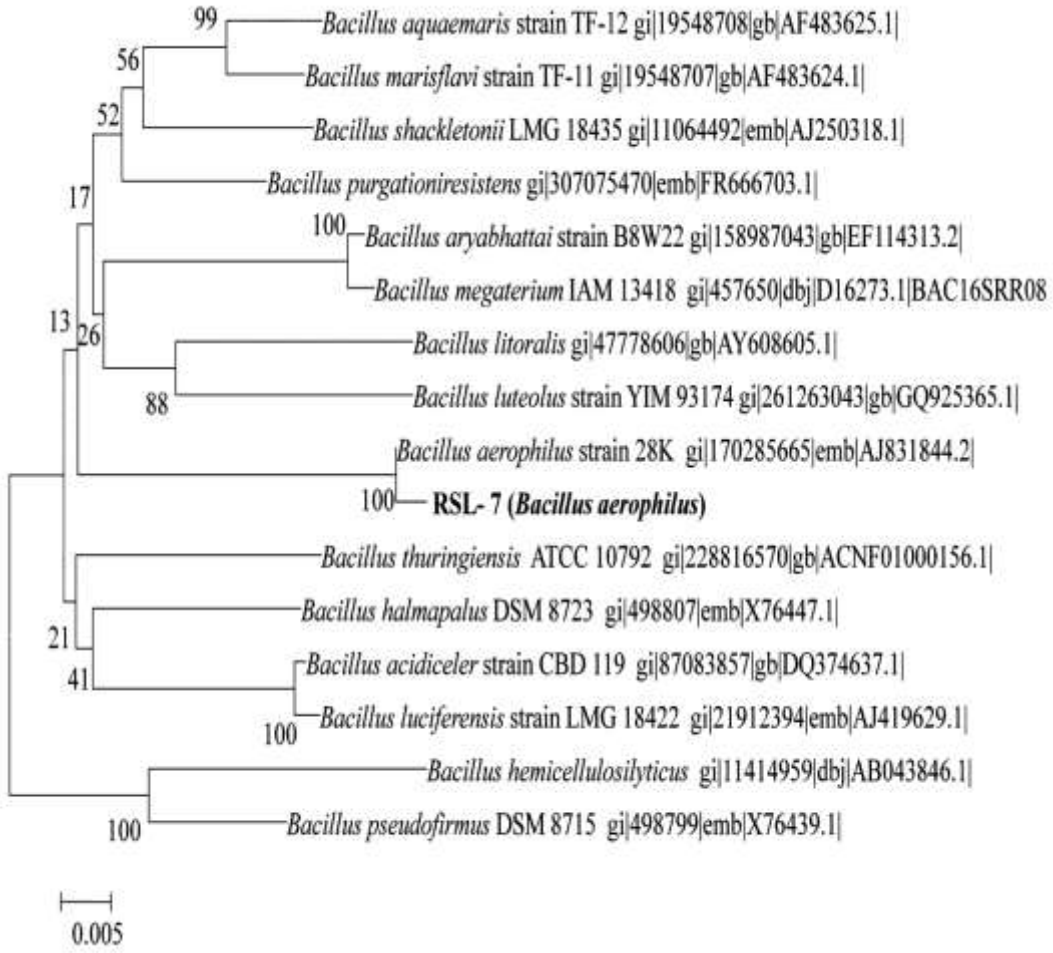

Figure 1. Phylogenetic tree for the isolate RSL-7 by neighbor-joining method[30,31].

were sequenced (SciGenom, Kochi, Kerala) and their phylogenetic analysis was done.

For producing PHA, a simple medium containing 1% glucose (W/V), 1% peptone (W/V), 0.5% yeast extract (W/V) and 1% sodium chloride (W/V) along with the hydrolysate of *Parthenium* was used. PHA producing organisms were inoculated into 250 ml Erlenmeyer flask containing 50 ml of sterile medium (autoclaved at 121°C for 15 min). The cell cultures were kept in a shaking incubator (KEMI, India) for incubation at 150 rpm at 37 ± 2°C for 72 h. After incubation the broth was centrifuged at 10,000 rpm for 10 min. The pellet was collected and dried until constant weight was obtained for calculation of dry cell weight. PHA was extracted by treating the cells in sodium hypochlorite (4% v/v) for 1–2 h (37°C) enabling complete digestion of non-PHA biomass. PHA was then recovered by centrifugation (8,000 rpm at 4°C for l0 min) followed by washing with distilled water, acetone : diethyl ether (1 : 1)[17–19]. The resultant polymer granules were dissolved in boiling chloroform and quantified gravimetrically after complete drying of the obtained PHA[20]. The experiments were performed in triplicates. PHA yield was calculated by the following formula

$$\text{PHA accumulation \%} = (\text{PDW}/\text{DCW}) \times 100,$$

where PDW is PHA dry weight and DCW is dry cell weight.

Optimum incubation time for maximum PHA production was determined by incubating the inoculated media for a total of 72 h. The samples were analysed at a regular interval of 24 h for calculating PHA content and DCW. To optimize C : N ratio, the culture medium was supplemented with different percentages (0, 1, 2 and 3) of glucose and peptone. The ability of the organism to produce PHA by utilizing the weed as sole carbon and nitrogen source was also studied by preparing a medium devoid of both glucose and peptone in the hydrolysate. Different concentrations of acid treated substrate (2, 4, 6 and 8%) were used to understand the capability of the organism to uptake nutrients from the substrate. To analyse and confirm functional groups present in the biopolymer, a

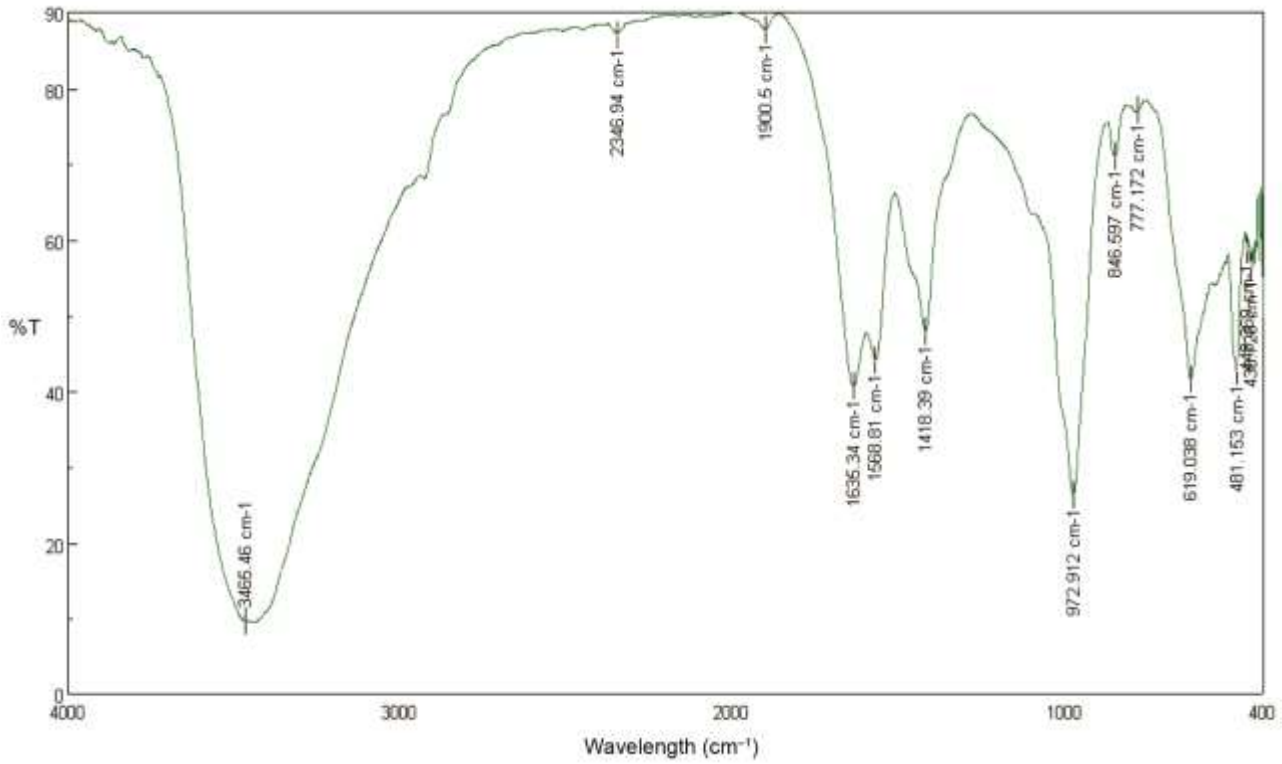

Figure 2. FTIR spectra of PHA produced from *Parthenium* (substrate) by RSL-7.

Table 1. Analysis of PHA production using *Parthenium* without supplementation of glucose and peptone

Acid treated substrate without glucose and peptone			
Incubation time (h)	DCW (g/l)	PDW (g/l)	%PHA (w/w)
24	1.25 ± 0.23	0.65 ± 0.02	52
48	9.5 ± 0.12	0.7 ± 0.01	7.3
72	7.25 ± 0.26	0.47 ± 0.03	6.5

Data represents the mean of 3 different readings ± standard deviation.

Fourier transform infrared (FTIR) analysis was performed between 400 and 4000 cm^{-1} wave number using a Perkin Elmer FTIR spectrophotometer (Jasco FTIR-6100, Japan) with KBr disc[21].

To understand and authenticate the monomeric units of PHB, ^{13}C and ^{1}H-NMR were performed following the methods described by Bhuwal *et al.*[22]. The polymer was dissolved in deuterochloroform ($CDCl_3$) at a concentration of 10 mg/ml and analysed on a Bruker Avance III 400 MHz spectrometer at 25°C, with a 13.85-ms pulse width (30° pulse angle), 2-s pulse repetition and 10,330-Hz spectral width.

The bacterial strain RSL-7 was found to be Gram-positive, rod-shaped, exhibiting high level of fluorescence in Nile blue agar plates (see Supplementary Information online), thus indicating the presence of PHA[23,24]. From the 16s rRNA sequencing, the organism was identified as *Bacillus aerophilus*, acc. no. SP KT364633 (Figure 1).

From early reports it was evident that the carbohydrate (164.0 ± 3.46 mg/g) content of weed was higher than total protein (4.17 ± 0.15 mg/g) and amino acids (0.88 ± 0.07 mg/g)[25,26]. As the C : N ratio plays an intricate role in PHA accumulation, *Parthenium* was successfully utilized as a substrate for its production. The organism exhibited capability to utilize complex substrate, as the highest amount of PHB accumulation of 52% was noted with a DCW of 1.25 g/l and PDW of 0.65 g/l (Table 1). In order to enhance the yield of PHB, the hydrolysate was supplemented with synthetic sources of carbon and nitrogen at different concentrations which had a stimulatory effect on both PHB yield and biomass production. Supplementation of 1% glucose and peptone also exhibited a positive impact on the yield (2.05 g PHB/l; DCW-8.75 g/l).

Increase of glucose levels showed an increase in biomass with simultaneous decrease in the productivity level and PHB content (1.20 g PHB/l in 2% glucose and 1.35 g PHB/l in 3% glucose at 24 h) and it was shown that 24 h incubation time was optimal for high yield of PHB (Table 2). This might be due to the fact that higher concentration of nutrients in media affects bacterial metabolism and inoculum morphology, which resulted in the decrease of PHA productivity.

It has been reported that PHA production was enhanced under limiting conditions of nitrogen, phosphorus and sulphur[27]. It was interesting to note that the high yield of PHB was because of the additional source of carbon and

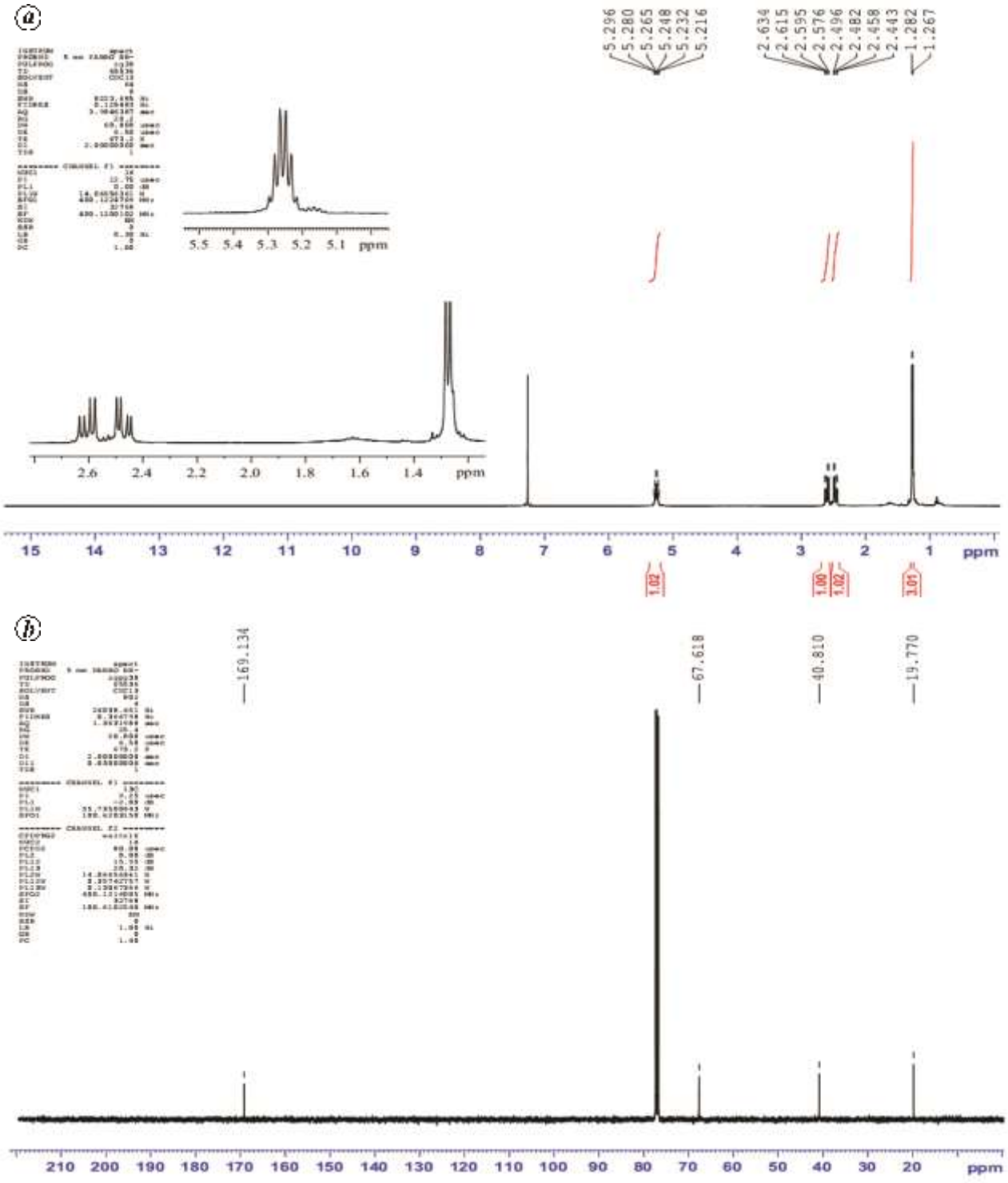

Figure 3. ***a***, ^{1}H NMR spectrum of extracted PHB. ***b***, ^{13}C NMR spectrum of PHB produced by *Bacillus aerophilus.*

nitrogen than the complex nutrients already available in hydrolysate, and by increasing the level of peptone (3%), a drastic decrease in PHB content (0.15 g/l, 23% accumulation) was observed. Hence, lesser amount of peptone yielded higher amount of PHA, indicating that the organism relied on nitrogen limitation for higher PHB productivity (Table 2). Despite increase in the supplementation of synthetic nutrients to improve PHB yield, change in the substrate concentration had remarkable effect on the yield of PHB content. With 6% substrate concentration,

Table 2. Optimization of incubation time and percentage of carbon and nitrogen source using *Parthenium*

Parthenium media with different percentages of glucose												
	0%			1%			2%			3%		
Incubation time (h)	DCW (g/l)	PDW (g/l)	% PHA (w/w)	DCW (g/l)	PDW (g/l)	% PHA (w/w)	DCW (g/l)	PDW (g/l)	% PHA (w/w)	DCW (g/l)	PDW (g/l)	% PHA (w/w)
24	4.5 ± 0.12	1.72 ± 0.02	38.22	8.75 ± 0.09	2.05 ± 0.06	23.42	6.01 ± 0.22	1.20 ± 0.05	19.96	3.25 ± 0.22	1.35 ± 0.05	41.53
48	6.5 ± 0.10	1.30 ± 0.07	20.0	10.5 ± 0.17	0.92 ± 0.01	8.76	8.25 ± 0.31	0.75 ± 0.01	9.09	6.75 ± 0.13	0.92 ± 0.02	13.62
72	11.34 ± 0.05	1.02 ± 0.03	8.99	13.2 ± 0.22	0.47 ± 0.09	3.50	9.75 ± 0.33	0.25 ± 0.02	2.56	7.06 ± 0.30	0.59 ± 0.03	8.35
Parthenium media with different percentages of peptone												
24	14.25 ± 0.21	0.77 ± 0.01	5.4	3.5 ± 0.15	0.77 ± 0.02	22.14	6.0 ± 0.12	0.37 ± 0.02	6.16	1.25 ± 0.21	0.05 ± 0.04	4.0
48	9.25 ± 0.23	1.02 ± 0.03	11.08	8.75 ± 0.19	1.8 ± 0.01	20.57	10.25 ± 36	0.95 ± 0.06	9.26	3.75 ± 0.11	0.25 ± 0.02	6.6
72	7.5 ± 0.15	1.97 ± 0.04	25.33	12 ± 0.17	0.97 ± 0.02	8.12	9.5 ± 0.44	0.65 ± 0.04	6.84	6.45 ± 0.14s	0.15 ± 0.07	2.3

Data represents the mean of 3 different readings ± standard deviation.

Table 3. Study of PHA accumulation using various substrate concentrations

Media supplemented with different percentages of substrate												
	2%			4%			6%			8%		
Incubation time (h)	DCW (g/l)	PDW (g/l)	%PHA (w/w)	DCW (g/l)	PDW (g/l)	%PHA (w/w)	DCW (g/l)	PDW (g/l)	%PHA (w/w)	DCW (g/l)	PDW (g/l)	%PHA (w/w)
24	6.96 ± 0.17	2.24 ± 0.02	32.18	11.34 ± 0.12	1.72 ± 0.04	15.16	11.92 ± 0.23	5.4 ± 0.06	45.30	8.8 ± 0.18	3.72 ± 0.05	42.27

Results shown are mean ± standard deviation.

the highest yield of PHB noted was 5.4 g PHB/l with 11.92 g/l DCW (Table 3). It was important to note that a four-fold increase in PHA productivity resulted when *Parthenium* was used as a feedstock.

The infrared (IR) spectrum of PHA produced from *Parthenium* provided marked peaks when compared with the standard polyhydroxybutyrate[28] (see Supplementary Information online). IR spectrum of PHB exhibited $\nu_{(OH)}$ vibration in the region at 3465.46 cm^{-1}. A band appeared at around 2345.94 cm^{-1} corresponding to a possible $\nu_{(C–H)}$ vibration. In the spectrum of PHA, a band was observed at around 1418 cm^{-1} due to asymmetric stretching vibration of $–CH_3$ and $–CH_2$ groups. In addition, the characteristic absorption band corresponding to the presence of ester carbonyl group $\nu_{(C=O)}$ was also present at 1635 cm^{-1} (Figure 2).

The ^{1}H-NMR spectrum of PHA was recorded in $CDCl_3$ at a field strength of 400 MHz showing all the expected signals. In PHA spectrum, a doublet corresponding to the CH_3 group appeared at δ 1.26–1.28 ppm (J = 6 Hz). Two doublets appeared at δ 2.443–2.496 (J = 5.6, 15.6 Hz) and δ 2.576–2.634 (J = 7.6, 15.6 Hz) ppm, were assigned to the presence of CH_2 group protons of PHA. In addition, a multiplet was observed at δ 5.216–5.296 ppm corresponding to –CH proton of PHA[29] (Figure 3 *a*). In ^{13}C-NMR spectrum also, the PHA was recorded in $CDCl_3$ at field strength of 400 MHz showing all the expected signals. A peak at δ 19.770 ppm was assigned to methyl carbon (CH_3) and a peak at δ 40.810 ppm corresponded to methylene carbon (CH_2). Methine carbon (CH) appeared at δ 67.618 ppm and the peak corresponded to carbonyl carbon (C=O) at δ 169.134 ppm, thus confirming the production of P3 (HB) polymer by fermentation of *Parthenium* with *Bacillus aerophilus* (Figure 3 *b*).

The present study reported detailed information about the ecofriendly production of 'bioplastic P3(HB)' from *P. hysterophorus*. An incubation time of 24 h, with a substrate concentration of 6% and 1% glucose and peptone, was found to be optimal for high yield of PHB content of about 5.4 g PHB/l from 11.92 g/l biomass. From the basic data obtained it was evident that *Bacillus aerophilus* (RSL-7) produced about 52% of PHB, which can be further exploited industrially. Spectroscopic studies revealed the structure of the compound to be P3 (HB). Till date

there have been no previous reports on the production of PHB from *P. hysterophorus* as a substrate, to the best of our knowledge.

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