FINAL REPORT ON THE WORK DONE ON
UGC MINOR RESEARCH PROJECT

SUSTAINABLE AND ECONOMIC PRODUCTION OF BIOPOLYMER POLYHYDROXYBUTYRATE BY BACTERIAL ISOLATES USING WATER HYACINTH

REPORT NO. FINAL

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Project report

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Introduction

With the turn of the decade, the world - especially plastics - is entering a new era. The demand for plastics in India alone was expected to grow from 7.5 million tons to 15 million tons by 2015. These non-degradable plastics accumulate in the environment at a rate of millions of tons per year. They affect the aesthetic quality of cities, water bodies and natural areas (Full *et al*., 2006). In response to the increasing global focus, the world is gradually turning away from the petrochemical derived plastic materials to alternate source such as biopolymers or the so-called 'green' polymers, having appropriate properties and processbility, in contrast to the conventional synthetic polymers (Bonartsev *et al.*, 2007). The most widely produced microbial biopolymers or bioplastics are polyhydroxyalkanoates (PHAs) and their derivatives. Microbial synthesis of PHB seems to have an inexhaustible potential for the market to grow in the future due to its special characteristics and broad biotechnological applications. Looking ahead to 2018, world bioplastics demand is forecast to reach nearly 2 million tons, with a market value of over US$5 billion (http://www.plastemart.com/Plastic-Technical-Article.asp?LiteratureID=1454&Paper=bioplastics-consumption-2-million-tons-grow-by-2018).

However, despite the numerous advantages and demand of biodegradable plastics, the commercialization of PHB has been met with limited success. The high cost of polymer production, together with high recovery cost, low yield, the lack of high-end market are the major bottlenecks in the commercialization of biodegradable plastics. Wider use of PHB requires a less expensive product; hence, cheaper substrates, improved fermentation strategies and easier downstream recovery methods. 40 to 48% of the total production costs are ascribed to the raw materials where the carbon source could account for 70 to 80% of the total expense (Du *et al.*, 2012).

Finding a less expensive substrate is, therefore, a major need for a wide commercialization of PHB. Among various lignocellulosics, water hyacinth (*Eichhornia crassipes*) has received great attention because of its obstinacy and high productivity especially when grown in domestic sewage lagoons. Thus, water hyacinth can serve as a low-cost carbon source for the production of ecofriendly bioplastic materials thereby reducing

overall production cost. The advantage with using water hyacinth as raw material is that it is available free of cost throughout the year.

Hence, the aim of this study was to optimize the fermentation process by using abundantly available cheap substrate such as water hyacinth for maximizing production of biopolymer polybetahydroxybutyrate using bacterial isolate obtained from soil.

Methodology

Isolation of polyhydroxybutyrate (PHB) producers

Soil samples were collected from car wash area, garden soil, mangrove area, botanical garden soil, petrol pump, garage, dumping ground using standard practices. The organisms were isolated from soil by serial dilution-spread plate technique (dilution 10^{-6}to10^{-9}) on minimal medium containing 2% of glucose. Morphologically distinct colonies were obtained after incubation at 37^{o}C for 24 h. All the isolates were qualitatively screened for PHB production using Sudan Black B dye, followed by secondary screening using Nile blue sulfate staining.

Selection of the most efficient isolate

The most efficient PHB producer was selected using following criteria: PHB production in mineral medium containing 2% glucose after 72 h of incubation; hydrolytic enzyme activities like protease, amylase and lipase to explore their ability to hydrolyze waste materials; antibiotic resistance and heavy metal resistance. The isolate showing highest PHB production with resistance to survive under unfavourable environment by virtue of its resistance to heavy metals and antibiotics were chosen for further work. The isolate was taxonomically identified by 16s rRNA analysis.

Evaluation of different PHB extraction procedures for maximum extraction of PHB

Different methods of recovery such as sodium hypochlorite, acid and alkaline extraction, detergent (SDS), surfactant with chelating agent, chloroform etc. were evaluated for maximum extraction of PHB from the selected isolate.

Monophasic cultivation of *Bacillus aryabhattai* using acid hydrolysate of water hyacinth

Water hyacinth after drying and grinding to a uniform powder was analyzed for its total carbohydrates (by Phenol sulphuric acid method), cellulose (Acetic-Nitric acid method), hemicellulose (difference between total carbohydrates and cellulose content), lignin, ash and moisture content. Acid hydrolysis (2% H_2SO_4 at 15 lbs for 30 min) of water hyacinth at solid to liquid ratio of 1:15 was carried out to obtain fermentable sugars. This hydrolysate was used as a sole source of carbon.

Experimental designs and statistical analysis for determination of the critical medium components for PHB production

Biphasic production of PHB was carried out, where 18h old isolate grown in inoculum medium for 24 h, centrifuged for 10-15 min and the pellet was transferred to the production medium. PHB produced by the isolates were quantified gravimetrically after 45 h of incubation.

Plackett-Burman design, a rapid screening multifactor design was applied to screen the important variables that significantly influence PHB production. It allows the investigator to use N-1 variables with N experiments and assume that there are no interactions between different media components. In this study, a 12-run Plackett-Burman design was applied to evaluate ten factors. Each Plackett - Burman Design can be easily constructed using a "generating vector", in the form of (+ + + - + - -). The elements, + (high level) and - (low level) represent the two different levels of the independent variables examined. One dummy variable was used to estimate experimental errors in data analysis (Table 1). Each variable was examined at two levels: –1 for the low level and +1 for the high level (Table 1). All trials were performed in duplicate and the averages of PHB production were treated as responses.

Table 1: Levels of the variables tested in Plackett-Burman design

Code	Variable	Range		
		-1	0	+1
A	Hydrolysate of water hyacinth (g/L)	20.0	30.0	40.0
B	Beef extract (g/L)	4.0	5.5	7.0
C	Trace element(g/L)	0.5	0.1	1.5
D	$MgSO_4$. $7H_2O$(g/L)	0.2	0.5	0.8
E	$FeSO_4$. $7H_2O$(g/L)	0	0.01	0.02
F	$CaCl_2$(g/L)	0.02	0.06	0.10
G	Sodium acetate(g/L)	0	0.5	1.0
H	Sodium citrate(g/L)	0	0.5	1.0
I	Na2HPO4(g/L)	3.0	4.5	6.0
J	KH_2PO_4(g/L)	1.5	2.5	3.5
K	Dummy	0	0	0

Locating the region of optimum response by the Path of Steepest Ascent (PSA)

The factors that were screened using the PBD were further optimized using the PSA to move toward the vicinity of the optimum results. To improve PHB production, concentrations of variables were increased or decreased using stepwise units according to the sign of the main effects. The zero level of PBD was identified as the base point of PSA and, for every point in the PSA, an experimental run was performed. The step along the path was determined by practical experience. Experiments were performed along the steepest ascent path until the response showed no further increase. This point would be near the optimal point and could be used as the center point of CCD.

Response surface methodology by using Central composite Design (CCD)

Response Surface Methodology (RSM) was employed in order to determine the optimum values of the most effective factors and to obtain an empirical model of the process to improve phenol degradation. Independent factors obtained from Plackett - Burman Design analysis were applied into CCD to study the interactions between the significant factors and also to determine their optimal levels of factors. The selected variables were coded in five levels which will be –α, -1, 0 and +1, + α. The factors were coded according to the following equation:

$$X_i = \frac{(X_i - X_o)}{\Delta X}, i = 1, 2, \ldots, k$$

Where, Xi is the coded independent factor, Xi is the real independent factor; X_0 is the value of Xi at the center point; ΔX is the step change value.All experimental designs were randomized to exclude any bias. The data obtained from the RSM with regards to PHB production were subjected to analysis of variance (ANOVA) to check for errors and the significance of each parameter. The general form of the second-order polynomial equation is:The second-order model used to fit the response to the independent variables is shown in Eq. (2):$Y = \beta 0 + \Sigma \beta i\ xi + \Sigma \beta ii\ xi2 + \Sigma \beta ijxixj$, i= 1, 2, 3, . . . , k,

Where Y, was the predicted response, β_0 was the intercept, xi and xj were the coded independent factors, βi was the linear coefficient, βii was the quadratic coefficient and βij was the interaction coefficient.

Characterization of PHB

Characterization of PHB was done by Determination of melting temperature, crystalline content, and lamellar thickness distribution of the PHB film using Differential Scanning Colorimetry (DSC), Determination of the structure and purity of PHB films by Fourier Transform Infrared Spectroscopy (FTIR) analysis, Determination of molecular

weight distribution of PHB using Nuclear Magnetic Resonance Spectroscopy (NMR) and determination of components of PHB by Gas Chromatography Mass Spectrometry (GC-MS). Biodegradability testing was also done to determine the fate of PHB in the soil.

Results and discussion

Isolation and screening of PHB producers

294 representative bacteria were isolated, purified and maintained as pure cultures from 12 samples collected from different sources. Based on primary screening for PHB production using Sudan black B and secondary screening using Nile blue sulphate, as many as 119 were found to be positive for PHB production (40.5%).

Selection of the most efficient isolate

All of the 119 putative PHB producers were evaluated for PHB production in minimal medium using 2% glucose to select the most efficient isolate (Table 2).

Table 2: PHB production by isolates

Isolates	Dry weight g/L	PHB g/L	% PHB
1	0.117±0.026	0.024±0.0005	20.17
2	0.234±0.005	0.018±0.0004	7.71
3	0.277±0.006	0.012±0.0003	4.33
4	0.636±0.014	0.016±0.0003	2.44
5	0.189±0.004	0.016±0.0004	8.73
6	0.177±0.004	0.056±0.0013	31.73
7	0.533±0.012	0.007±0.0002	1.31
8	1.457±0.033	0.186±0.0042	12.81
9	0.878±0.020	0.115±0.0026	13.13
10	1.284±0.029	0.129±0.0029	10.05
11	0.732±0.016	0.018±0.0004	2.39
12	2.010±0.045	0.007±0.0001	0.32
13	1.314±0.030	0.017±0.0004	1.27
14	1.166±0.026	0.020±0.0004	1.67
15	1.182±0.027	0.015±0.0003	1.27
16	0.935±0.021	0.024±0.0006	2.62
17	0.500±0.011	0.012±0.0003	2.41
18	0.703±0.016	0.061±0.0014	8.68

19	0.779±0.018	0.051±0.0011	6.48
20	0.697±0.016	0.011±0.0002	1.58
21	0.902±0.020	0.032±0.0007	3.57
22	0.034±0.001	0.001±0.0000	3.43
23	0.561±0.013	0.031±0.0007	5.57
24	1.238±0.028	0.022±0.0005	1.79
25	0.635±0.014	0.005±0.0001	0.77
26	0.622±0.014	0.005±0.0001	0.79
27	0.897±0.020	0.025±0.0006	2.84
28	1.207±0.027	0.023±0.0005	1.86
29	1.266±0.028	0.033±0.0007	2.61
30	1.284±0.029	0.018±0.0004	1.44
31	1.308±0.029	0.020±0.0004	1.49
32	1.083±0.024	0.021±0.0005	1.99
33	0.863±0.019	0.014±0.0003	1.62
34	2.065±0.046	0.024±0.0005	1.15
35	1.745±0.039	0.024±0.0006	1.40
36	1.470±0.033	0.024±0.0005	1.65
37	1.600±0.036	0.063±0.0014	3.96
38	1.450±0.033	0.075±0.0017	5.15
39	1.790±0.040	0.058±0.0013	3.26
40	2.010±0.045	0.083±0.0019	4.15
41	1.568±0.035	0.076±0.0017	4.85
42	1.910±0.043	0.081±0.0018	4.25
43	3.018±0.068	0.083±0.0019	2.76
44	3.182±0.072	0.077±0.0017	2.40
45	4.083±0.092	0.042±0.0009	1.02
46	3.368±0.076	0.075±0.0017	2.23
47	0.640±0.014	0.047±0.0011	7.29
48	0.624±0.014	0.070±0.0016	11.20
49	0.700±0.016	0.044±0.0010	6.26
50	1.254±0.028	0.065±0.0015	5.22

51	0.890±0.020	0.033±0.0007	3.69
52	0.767±0.017	0.041±0.0009	5.39
53	0.652±0.015	0.027±0.0006	4.09
54	0.480±0.011	0.040±0.0009	8.33
55	1.376±0.031	0.056±0.0013	4.06
56	1.272±0.029	0.071±0.0016	5.60
57	2.850±0.064	0.057±0.0013	2.00
58	0.247±0.006	0.024±0.0005	9.87
59	2.258±0.051	0.064±0.0014	2.84
60	0.978±0.022	0.067±0.0015	6.85
61	0.557±0.013	0.035±0.0008	6.29
62	1.410±0.032	0.046±0.0010	3.26
63	0.788±0.018	0.018±0.0004	2.33
64	0.454±0.010	0.057±0.0013	12.56
65	2.08±0.0468	0.066±0.0015	3.18
66	0.99±0.0222	0.065±0.0015	6.59
67	1.63±0.0366	0.185±0.0042	11.38
68	1.62±0.0364	0.215±0.0048	13.30
69	1.03±0.0232	0.120±0.0027	11.65
70	0.89±0.0199	0.080±0.0018	9.03
71	0.86±0.0192	0.035±0.0008	4.09
72	1.35±0.0303	0.025±0.0006	1.86
73	1.73±0.0389	0.025±0.0006	1.45
74	1.04±0.0235	0.030±0.0007	2.87
75	1.15±0.0258	0.010±0.0002	0.87
76	0.25±0.0056	0.025±0.0006	10.02
77	2.28±0.0514	0.065±0.0015	2.84
78	1.29±0.0290	0.050±0.0011	3.88
79	1.51±0.0340	0.090±0.0020	5.96
80	3.30±0.0743	0.015±0.0003	0.45
81	3.14±0.0707	0.315±0.0071	10.03
82	1.79±0.0404	0.055±0.0012	3.06

83	2.13±0.0479	0.195±0.0044	9.15
84	0.45±0.0102	0.020±0.0005	4.40
85	2.33±0.0526	0.020±0.0005	0.86
86	2.96±0.0666	0.075±0.0017	2.53
87	1.18±0.0266	0.020±0.0005	1.69
88	1.72±0.0388	0.060±0.0014	3.48
89	0.82±0.0186	0.065±0.0015	7.88
90	0.66±0.0150	0.055±0.0012	8.27
91	1.43±0.0322	0.030±0.0007	2.10
92	1.57±0.0352	0.055±0.0012	3.51
93	1.44±0.0323	0.025±0.0006	1.74
94	1.12±0.0252	0.010±0.0002	0.89
95	2.71±0.0610	0.140±0.0032	5.17
96	2.86±0.0644	0.050±0.0011	1.75
97	2.33±0.0523	0.010±0.0002	0.43
98	2.74±0.0617	0.020±0.0005	0.73
99	2.10±0.0473	0.035±0.0008	1.67
100	2.37±0.0533	0.030±0.0007	1.27
101	2.31±0.0519	0.030±0.0007	1.30
102	1.11±0.0250	0.020±0.0005	1.80
103	1.54±0.0346	0.015±0.0003	0.98
104	2.15±0.0484	0.010±0.0002	0.47
105	3.07±0.0691	0.015±0.0003	0.49
106	1.29±0.0290	0.025±0.0006	1.94
107	1.59±0.0357	0.015±0.0003	0.95
108	2.01±0.0452	0.010±0.0002	0.50
109	2.04±0.0458	0.040±0.0009	1.97
110	2.44±0.0549	0.140±0.0032	5.74
111	1.96±0.0441	0.015±0.0003	0.77
112	2.25±0.0506	0.075±0.0017	3.33
113	2.28±0.0512	0.165±0.0037	7.25
114	1.74±0.0392	0.015±0.0003	0.86

115	3.49±0.0784	0.010±0.0002	0.29
116	0.89±0.0200	0.015±0.0003	1.69
117	2.16±0.0486	0.010±0.0002	0.46
118	2.89±0.0649	0.025±0.0006	0.87
119	2.05±0.0460	0.010±0.0002	0.49

Four isolates namely, 8, 9, 10 and 81were shortlisted based on the PHB production (g/L). Isolates were identified as *Bacillus flexus, Ensifer adhaerens, Paenibacillus sp.* and *Bacillus aryabhattai* respectively based on 16s rRNA analysis. They were further evaluated on the basis of their ability to produce various enzymes, metal resistance and antibiotic resistance.

Table 3: Characterization of the selected PHB producers

Name of the Organism	Enzymes produced	Resistance to metal (µg/mL)	Resistance to antibiotic
Bacillus flexus	Cellulase, Amylase and Protease	Chromium (50), Mercury (200), Lead (100), Zinc (100)	Cephalothin, Ampicillin
Ensifer adhaerens	Nil	Chromium (100), Mercury (200), Cobalt (200), Lead (500), Zinc (50), Cadmium (50)	Nalidixic acid, Nitrofurantoin, Cephalothin, Ampicillin, Co-trimoxazole, Norfloxacin
Paenibacillus sp.	Nil	Cobalt (25), Lead (200), Zinc (25)	Nalidixic acid, Nitrofurantoin, Cephalothin, Ampicillin, Co-trimoxazole, Norfloxacin
Bacillus aryabhattai	Cellulase, Amylase and Protease	Chromium (100), Cobalt (50), Lead (500)	Ampicillin

The *Bacillus* sp. showed ability to produce cellulase, amylase, and protease which would enable them to utilize various types of wastes thereby considerably lowering the overall cost of production. All the isolates showed resistance to various heavy metals and antibiotics indicating their ability to survive under unfavourable environment. This ability can be explored to use waste substrates like industrial effluents or waste waters (Table 3). *Bacillus aryabhattai* was chosen for further work based on higher PHB production (0.315±0.0071 g/), its hydrolytic potential and resistance towards metals.

Evaluation of different PHB extraction procedures for maximum extraction of PHB

Various extraction processes were carried out to maximize the yield of PHB produced by *Bacillus aryabhattai* as shown in Table 4. Use of 4% NaOCl at 37° for 135 min yielded the highest amount of PHB and hence was chosen throughout the study.

Table 4: Evaluation of various extraction procedures

Method	PHB (g/L)	% PHB
1% SDS	0.004	2.0
10% SDS	0.007	3.5
TRITON X 100+ EDTA	0.004	2.0
H_2O_2 80° 5% 60 min	0.003	1.5
H_2O_2 80° 5% 120 min	0.005	2.5
H_2O_2 80° 20% 60 min	0.005	2.5
H_2O_2 80° 20% 120 min	0.007	3.5
2N HCl sonication	0.001	0.5
0.2N NaOH 30° 300 min +sonication	0.002	1.0
Overnight chloroform 24h	0.02	10.0
4% NaOCl 45° 60 min	0.0144	7.2
4% NaOCl 37° 60 min	0.0174	8.7
4% NaOCl 37° 75 min	0.0182	9.1
4% NaOCl 37° 90 min	0.0196	9.8
4% NaOCl 37° 105 min	0.0214	10.7
4% NaOCl 37° 120 min	0.0226	11.3
4% NaOCl 37° 135 min	0.024	12.0
4% NaOCl 37° 150 min	0.0212	10.6
4% NaOCl 37° 180 min	0.016	8.0

Monophasic cultivation of *Bacillus aryabhattai* using acid hydrolysate of water hyacinth

Water hyacinth was found to contain 73.53% carbohydrates, 32.47 % cellulose, 41.06 % hemicellulose, 7.5% lignin, 6.35% extractives, 4.25% ash and 8.4% crude protein. Hydrolysate was prepared using acid hydrolysis with 2% H_2SO_4 at solid to liquid ratio of 1:15. The mixture was autoclaved at 12 lb for 30 min. Hydrolysis yielded a total carbohydrate content of 55.5g%. The hydrolysate was diluted to obtain sugar content of 2% and used as a sole source of carbon in MSM medium using monophasic cultivation. PHB production was studied over a period of 30 to 75 h. Maximum biomass and PHB were obtained after 50 h.

Table 5: Monophasic cultivation over a period of 75 h

Time (h)	Dry weight (g/L)	PHB (g/L)	% PHB
30	3.22	0.14	4.34
40	4.2	0.2	4.76
45	3.72	0.3	8.06
50	4.1	0.66	16.09
55	3.4	0.58	17.05
65	3.46	0.5	14.45
70	3.5	0.44	12.57
75	3.6	0.32	8.88

In order to increase the PHB production, biphasic cultivation was carried out. Initially, the organisms were grown in nutrient rich medium to obtain large amount of biomass and then the cells were transferred to nutrient limiting medium to induce PHB production (Table 6).

Table 6: Biphasic cultivation over a period of 75 h

Time (h)	Dry weight (g/L)	PHB (g/L)	% PHB
30	2.86	0.58	20.28
40	5.22	0.64	12.26
45	6.9	0.94	13.62
50	6.18	0.78	12.62
55	5.88	0.66	11.22
65	5.76	0.58	10.07
70	4.62	0.54	11.69
75	4.18	0.42	10.05

Biphasic cultivation proved to be beneficial not only in increasing the yield of PHB, but also in decreasing the total time from 50 h to 45 h.

Screening of parameters using PBD

In order to study the effect of various parameters on PHB production, a 12-run PBD along with three runs at zero level (in duplicate) was used in the present study to screen the important variables that significantly influenced PHB production. Ten variables, viz. water hyacinth hydrolysate, beef extract, trace element solution, $MgSO_4$. $7H_2O$, $FeSO_4$. $7H_2O$, $CaCl_2$, sodium acetate, sodium citrate, Na_2HPO_4 and KH_2PO_4 were chosen as the independent input variables and the PHB production was used as a dependent response variable. The data listed in Table 7 indicate a wide variation in the amount of PHB produced, ranging from 0.41 to 2.39 g/L, in the 12 trials run in duplicate.

Regression analysis was performed on the results and the first-order polynomial equation was derived by representing the amount of PHB produced as a function of the independent variables:

PHB g/L = -0.504 + 0.00738 Water hyacinth hydrolysate + 0.2651 beef extract + 0.2531 trace element + 0.214 $MgSO_4$ + 27.18 $FeSO_4$ - 1.11 $CaCl_2$ + 0.4500 sodium acetate - 0.2412 sodium citrate - 0.0578 Na_2HPO_4 - 0.1774 KH_2PO_4 (Eq.1)

Analysis of the regression coefficients and the *t* values of ten factors (Table 8) showed that water hyacinth hydrolysate, beef extract, trace element solution, $MgSO_4$, $FeSO_4$ and sodium acetate had positive effect on PHB production, whereas $CaCl_2$, sodium citrate, Na_2HPO_4 and KH_2PO_4 had a negative effect on PHB production. The corresponding probability values (*P* values) indicate the significance of each of the coefficients, which in turn govern the patterns of interactions between the variables. The smaller the value of *P*, the more significant is the corresponding coefficient. The model was significant ($P< 0.05$) and R^2 = 0.9802 indicated that 98.02 % of the total variability in the response could be explained using this model.

Table 7: Plackett–Burman design of variables (in coded levels) with experimental and predicted values of PHB produced (g/L) as response

	Coded values										PHB (g/L)	
Run no.	A	B	C	D	E	F	G	H	J	K	Estimated	Predicted
1	1	-1	1	1	-1	1	-1	-1	-1	1	0.41	0.44
2	-1	1	1	-1	1	-1	-1	-1	1	1	1.39	1.42
3	-1	1	1	1	-1	1	1	-1	1	-1	1.86	1.72

4	1	-1	-1	-1	1	1	1	-1	1	1	1.02	0.88
5	-1	1	-1	-1	-1	1	1	1	-1	1	0.89	0.91
6	1	1	-1	1	1	-1	1	-1	-1	-1	2.39	2.42
7	1	1	1	-1	1	1	-1	1	-1	-1	1.91	1.76
8	1	1	-1	1	-1	-1	-1	1	1	1	0.80	0.65
9	-1	-1	-1	1	1	1	-1	1	1	-1	0.49	0.52
10	1	-1	1	-1	-1	-1	1	1	1	-1	0.76	0.79
11	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.50	0.35
12	-1	-1	1	1	1	-1	1	1	-1	1	1.28	1.13
13	0	0	0	0	0	0	0	0	0	0	1.10	1.08
14	0	0	0	0	0	0	0	0	0	0	1.11	1.08
15	0	0	0	0	0	0	0	0	0	0	1.13	1.08

R^2= 0.9802

Table 8: Analysis of the regression coefficients

Variables	Effect	Coefficient	Standard error	T-value	P-value
constant		1.1413	0.0495	23.06	0
Water hyacinth hydrolysate	0.1475	0.0738	0.0495	1.49	0.233
Beef extract	0.7953	0.3976	0.0495	8.03	0.004
Trace element	0.2531	0.1266	0.0495	2.56	0.083
$MgSO_4$	0.1283	0.0642	0.0495	1.3	0.286
$FeSO_4$	0.5436	0.2718	0.0495	5.49	0.012
$CaCl_2$	-0.0889	-0.0445	0.0495	-0.9	0.435
Sodium acetate	0.45	0.225	0.0495	4.54	0.02
Sodium citrate	-0.2412	-0.1206	0.0495	-2.44	0.093
Na_2HPO_4	-0.1733	-0.0867	0.0495	-1.75	0.178
KH_2PO_4	-0.3548	-0.1774	0.0495	-3.58	0.037
Ct Pt		-0.292	0.111	-2.64	0.078

The maximum production of PHB was obtained at high nitrogen concentration (beef extract) i.e. 1.5389 g/L, while at lower beef extract concentration the grand mean of PHB production dropped down to 0.7436 g/L. Under normal conditions, bacteria synthesize their body materials like proteins and grow. But, during nutrient limiting conditions, bacteria may

shift their protein synthesis to PHB synthesis for survival. In absence of nitrogen, PHB synthesis generally increases. The reasons may be during nitrogen starved conditions, reduced amino acid synthesis may be accompanied by increase in Acetyl CoA and the activity of Phosphoacetyltransferase (β- Ketothiolase). This in turn activates PHB synthase enzyme (Asada *et al*., 1999).Wang and Lee (1997) have shown that nitrogen limited condition along with continuous feeding of water hyacinth hydrolysate increases the production of PHB. As for the optimum PHB production the C:N ratio should be maintained. It was observed that when the amount of beef extract in medium was 7 g/L, the C:N was 19.22: 1. These results agree with the results obtained by Chandrashekharaiah, 2005.

It was seen that there was increase in production of PHB at higher $FeS0_4$ concentration showing a positive effect on the system. The mean increased from to 0.869 g/L to 1.4131 g/L with an increase in $FeS0_4$ concentration. As Fe^+ ions are required for the maximum production of PHB it can be concluded that higher amount of Fe^+ ions will maximize the PHB yield. Trace elements and $MgSO_4$ also had a positive effect on PHB production.

It was seen that increasing the sodium acetate concentration in the system increased the PHB production, i.e., it had a positive effect on the system. The mean increased from 0.9163 to 1.3663 g/L when the sodium acetate was increased to +1 level. As acetate is the intermediate in the PHB production pathway, its addition in the medium will increase the PHB yield.

KH_2PO_4 showed a negative effect on the production of PHB, decreasing the PHB production from 1.318g/L to 0.9639 g/L. hence this showed that KH_2PO_4 was significant at -1 level and have profound effect on PHB production. It can be concluded that remaining other factor were not significant and were kept constant for further experimentation.

The highest production of PHB 2.39 g/L after running Plackett–Burman experiments was obtained under the following conditions: water hyacinth hydrolysate 40 g/L; beef extract 7g/L; trace element solution 0.5g/L; $MgSO_4$ 0.8g/L; $FeSO_4$ 0.02 g/L; $CaCL_2$ 0.02 g/L; sodium acetate 1 g/L; sodium citrate 0 g/L; Na_2HPO_4 3 g/L; and KH_2PO_4 1.5 g/L.

Locating the region of optimum response by the PSA

In the current investigation, PSA was employed to move from the current operating conditions to the optimum region in the most efficient way by using the minimum number of experiments. PSA was based on the zero level of the PBD and moved along the direction in which the beef extract, $FeSO_4$ and sodium acetate concentration increased and KH_2PO4 concentration decreased. The non significant factors, viz. water hyacinth hydrolysate, trace

element, $MgSO_4$ was used in all trials at its +1 level (40 g/L, 1.5 mL/L and 0.8 g/L respectively) for its positive contribution, while $CaCl_2$, sodium citrate and Na_2HPO_4 was kept at its -1 level (0.02 g/L, 0 g/L and 3 g/L respectively) for its negative contribution. The experimental design and results are shown in Table 9. The highest production was found to be PHB yield of 2.403 g/L with beef extract 7 g/L, $FeSO_4$ 0.0168 g/L, sodium acetate 0.7828 g/L and KH_2PO_4 2.054g/L. This point was concluded to be near the optimal point and was chosen for optimization by RSM using CCD.

Table 9: Experimental design and response value of path of steepest ascent

Sr. No.	Items	Beef extract	$FeSO_4$	Sodium acetate	KH_2PO_4	PHB yield (g/L)
1	Base point	5.5	0.01	0.5	2.5	
2	Origin step unit	1.5	0.01	0.5	1	
3	Slope	0.3976	0.2718	0.225	0.1774	
4	Corresponding range	0.5964	0.002718	0.1125	0.1774	
5	New step unit	0.75	0.003418	0.141474	0.223089	
6	New step unit with decimal	0.75	0.0034	0.1414	0.223	
	Experiment No. 1	5.50	0.01	0.5	2.5	1.01
	Experiment No.. 2	6.25	0.0134	0.6414	2.277	1.49
	Experiment No. 3	7.00	0.0168	0.7828	2.054	2.40
	Experiment No. 4	7.75	0.0202	0.9242	1.831	1.44
	Experiment No. 5	8.50	0.0236	1.0656	1.608	2.05
	Experiment No. 6	9.25	0.027	1.2070	1.385	1.49
	Experiment No. 7	10.00	0.0304	1.3484	1.162	1.97
	Experiment No. 8	10.75	0.0338	1.4898	0.939	0.76
	Experiment No. 9	11.50	0.0372	1.6312	0.716	1.44
	Experiment No.10	12.25	0.0406	1.7726	0.493	1.28

Optimization of significant variables using CCD

CCD was employed at the specified combinations of four independent significant factors (Beef extract, $FeSO_4$, Sodium acetate, KH_2PO_4,) at five levels ($-\alpha$, -1, 0, +1, $+\alpha$) to study the interactions between them and to determine their optimum levels (Table 10). The

levels of Water hyacinth hydrolysate, trace element, MgS04, CaCL2, Sodium citrate, Na2HPO4, were kept similar to the trial runs in PSA.

Table 10: Experimental ranges and levels of the independent process variables in the central composite design

Factor	Variable	Range and level				
		-2	-1	0	1	2
B	Beef extract g/L	6.25	6.625	7	7.375	7.75
E	$FeSO_4$ g/L	0.015025	0.0159	0.016775	0.01765	0.018525
G	Sodium acetate g/L	0.6414	0.7121	0.7828	0.8535	0.9242
K	KH_2PO_4 g/L	1.831	1.9425	2.054	2.1655	2.277

The design matrix of tested variables in 31 experimental runs along with the experimental results and the results of theoretically predicted responses (using the model equation) are shown in Table 11. The PHB production increased to 3.55 g/L after running the response surface design using the following conditions: Beef extract 7.375 g/L, FeSO4 0.0159 g/L, Sodium acetate 0.721 1.9425 g/L and KH_2PO_4 1.9425 g/L. Multiple regression analysis was used to analyze the data to obtain an empirical model for the best response and thus a second-order polynomial equation (Eq. 2) was derived as follows:

Table 11: Central composite design matrix with experimental and predicted values

Run order	Coded values				PHB g/L	
	Beef extract	$FeSO_4$	Sodium acetate	KH_2PO_4	Estimated	Predicted
1	1	1	-1	-1	2.08	1.99
2	2	0	0	0	2.87	2.81
3	-1	1	1	-1	2.00	2.00
4	0	0	2	0	2.18	2.04
5	-1	1	-1	1	2.06	1.97
6	0	0	0	2	1.48	1.23
7	0	0	0	-2	1.34	1.59
8	-1	-1	-1	-1	2.21	2.35
9	1	-1	1	-1	1.71	2.01
10	0	0	0	0	2.57	2.49
11	1	-1	1	1	1.81	1.80

12	0	0	-2	0	2.64	2.78
13	1	-1	-1	-1	3.55	3.29
14	0	0	0	0	2.65	2.49
15	1	1	-1	1	1.74	1.80
16	-1	1	-1	-1	2.30	2.11
17	0	0	0	0	2.55	2.49
18	0	-2	0	0	1.76	1.59
19	-1	-1	1	1	0.50	0.78
20	1	1	1	-1	2.14	2.00
21	-1	-1	-1	1	1.58	1.53
22	0	0	0	0	2.57	2.49
23	0	0	0	0	2.48	2.49
24	-1	1	1	1	2.46	2.52
25	0	0	0	0	2.38	2.49
26	1	1	1	1	2.42	2.47
27	1	-1	-1	1	2.22	2.42
28	-2	0	0	0	1.87	1.92
29	0	2	0	0	1.85	2.02
30	0	0	0	0	2.24	2.49
31	-1	-1	1	-1	1.20	0.94

R^2= 0.9190

Y= -97.8 + 16.90 Beef extract + 5631 $FeSO_4$ – 135.9 sodium acetate + 44.6 KH_2PO_4 – 0.215 beef extract*beef extract – 224303 $FeSO_4$*$FeSO_4$ – 3.95 sodium acetate*sodium acetate – 21.74 KH_2PO_4*KH_2PO_4 – 811 beef extract*$FeSO_4$ + 1.16 beef extract*sodium acetate – 0.29 beef extract*KH_2PO_4 + 5235 $FeSO_4$*sodium acetate + 1751 $FeSO_4$*KH_2P0_4 + 21.19 sodium acetate*KH_2PO_4 (Eq. 2)

The mathematical expression of the relationships between the independent variables and dependent response is given in terms of uncoded factors. Apart from the linear effect of the parameter for PHB production, the RSM also gives an insight into the quadratic and interaction effect of the parameters. These analyses are done by means of Fisher's F test and Student's t test. Student's t test is used to determine the significance of the regression coefficients of the parameters. In general, the larger the magnitude of t and smaller the value

of P, the more significant is the corresponding coefficient term. The regression coefficient and the F and P values for all the linear, quadratic, and interaction effects of the parameters are given in Table 7. From very small P values, it was observed that the coefficients for the linear, quadratic and interaction effects of the factors were highly significant except the quadratic effect for KH_2PO_4 (P = 0.068), interaction effects for beef extract and sodium acetate (P = 0.587), and beef extract and KH_2PO_4 (P = 0.829). These measures indicated that the accuracy and general ability of the polynomial model were good and that analysis of the response trends using the model was reasonable.

Table 12: Estimated regression coefficients and corresponding t and P values of the central composite design

Term	Effect	Coefficient	Standard error	T-value	P-value
Beef extract	0.8899	0.4449	0.0905	4.92	0
$FeSO_4$	0.4322	0.2161	0.0905	2.39	0.03
Sodium acetate	-0.7352	-0.3676	0.0905	-4.06	0.001
KH_2PO_4	-0.3544	-0.1772	0.0905	-1.96	0.475
Beef extract*beef extract	-0.242	-0.121	0.166	-0.73	0.001
$FeSO_4$*$FeSO_4$	-1.374	-0.687	0.166	-4.14	0.64
Sodium acetate*sodium acetate	-0.158	-0.079	0.166	-0.48	0
KH_2PO_4*KH_2PO_4	-2.162	-1.081	0.166	-6.52	0
Beef extract*$FeSO_4$	-2.129	-1.064	0.222	-4.8	0
Beef extract*sodium acetate	0.246	0.123	0.222	0.55	0.587
Beef extract*KH_2PO_4	-0.097	-0.049	0.222	-0.22	0.829
$FeSO_4$*sodium acetate	2.591	1.295	0.222	5.85	0
$FeSO_4$*KH_2PO_4	1.367	0.683	0.683	3.08	0.007
Sodium acetate*KH_2PO_4	1.337	0.668	0.668	3.02	0.008

The statistical significance of the ratio of the mean square variation due to the regression and mean square residual error was also tested using analysis of variance (ANOVA) as shown in Table 13. The ANOVA of the quadratic regression model demonstrated that the model was highly significant, as was evident from the low P value of the Fisher's F test. The model was found to be adequate for prediction within the range of variables employed. The coefficient of determination $R^2 = 0.9190$ implied a good agreement between the experimental and predicted values of PHB yield, which can be attributed to the

given independent variables. It is thus envisaged that Eq. (2) can capture 91.90% of the variation in the measured values of PHB yield as function of the four independent conditions within the ranges considered in the present study. The ANOVA thus indicated that the second order polynomial model for Eq. 2 was highly significant and adequate to represent the actual relationship between the response (PHB yield g/L) and variables, with P<0.000 and a high value of the coefficient of determination (91.90 %).

Table 13: ANOVA for response surface quadratic model

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	14	8.90719	0.63623	12.96	0
Linear	4	2.46717	0.61679	12.56	0
Beef extract	1	1.18783	1.18783	24.2	0
$FeSO_4$	1	0.28014	0.28014	5.71	0.03
Sodium acetate	1	0.8108	0.8108	16.52	0.001
KH_2PO_4	1	0.1884	0.1884	3.84	0.068
square	4	0.67447	0.67447	13.74	0
Beef extract*beef extract	1	0.02626	0.67447	0.53	0.475
$FeSo_4$*$FeSO_4$	1	0.84334	0.67447	17.18	0.001
Sodium acetate*sodium acetate	1	0.01113	0.67447	0.23	0.64
KH_2PO_4*KH_2PO_4	1	2.08837	0.67447	42.54	0
2-way interaction	6	0.62369	0.67447	12.7	0
Beef extract*$FeSO_4$	1	1.13305	0.67447	23.08	0
Beef extract*sodium acetate	1	0.01511	0.67447	0.31	0.587
Beef extract*KH_2PO_4	1	0.00236	0.67447	0.05	0.829
$FeSO_4$*sodium acetate	1	1.6779	0.67447	34.18	0
$FeSO_4$*KH_2PO_4	1	0.46716	0.67447	9.52	0.007
Sodium acetate*KH_2PO_4	1	0.44657	0.67447	9.1	0.008
Error	16	0.04909	0.67447		
Lack-of-fit	10	0.06668	0.67447	3.37	0.075
Pure error	6	0.11869	0.67447		
Total	30	9.69269			

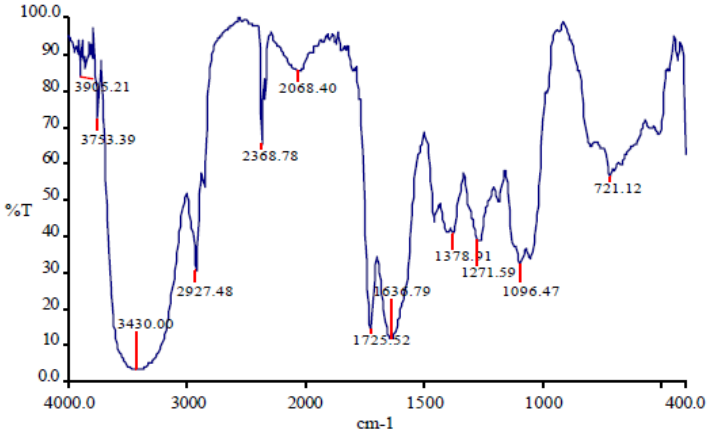
Spectrophotometric estimation of PHB

The PHB estimated by gravimetric method was assessed for its purity by performing crotonic acid assay. The extracted PHB may sometimes contain proteins or residual biomass as contaminants. PHB in presence of sulphuric acid is converted to crotonic acid showing maximum absorption at 235 nm. The purity of the PHB obtained was found to be 92.92 ± 0.2091%.

Characterization of PHB produced by *B. aryabhattai*

FTIR analysis

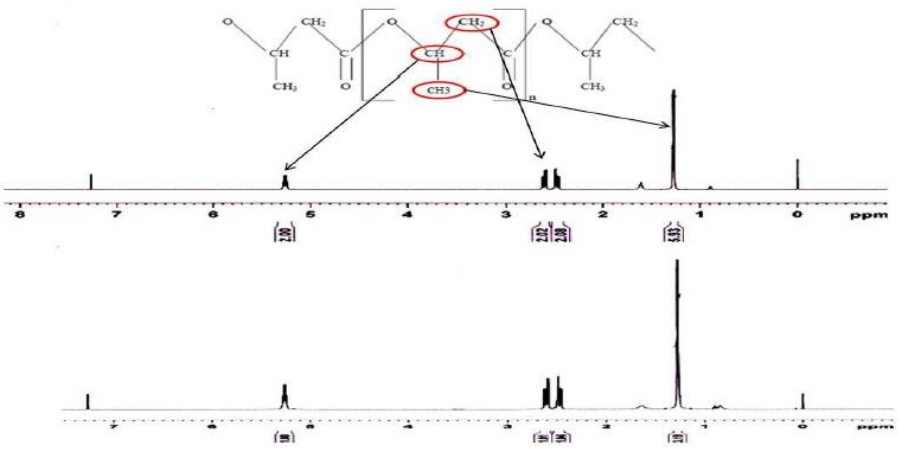
Fig. 1: FTIR spectra of PHB produced by *B. aryabhattai*



FTIR analysis of PHA produced by *Bacillus aryabhattai* showed bands characteristic of PHB (Fig.1.). The band found at 1725.52 cm-1 corresponds to ester carbonyl group (C=O). The band found at 1378.91 cm-1 is the equivalent for CH3 groups and the band at 1271.59 cm-1 corresponds to the –CH group. Also bands of minor relevance, such as those found at 3430 cm-1, originated due to water adsorption onto the sample, are found in all spectra.

1H NMR analysis

Fig. 2: 1H NMR spectra of PHB produced by *B. aryabhattai*



The 1H NMR spectra obtained from extracted PHB from *Bacillus aryabhattai* was compared with the commercial PHB (Sigma-Aldrich Chemicals, USA). Both spectra were found to match perfectly with each other (Fig. 2). The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms in the PHB structure. The spectrum shows a doublet at 1.29 ppm which is attributed to the methyl group coupled to one proton. The doublet of quadruplet at 2.57ppm is attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom. The multiplet at 5.27ppm is characteristic of methylene group. Two other signals are observed, a broad one at 1.56 ppm which is due towater and another one at 7.25ppm attributed to the solvent used i.e. chloroform.

Thermogravimetric analysis

The thermal degradation of extracted PHB proceeds by a one-step process with a maximum decomposition temperature at 291^{o}C. This thermal degradation at maximum decomposition temperature of approximately 300^{o}C is mainly associated with the ester cleavage of PHB component by β-elimination reaction. However, the thermal decomposition patterns of blends followed a considerably different pattern from the single-step reaction of the PHB. Maximum decomposition temperature also increased from 291 ^{o}C to 500 ^{o}C. The temperature of 291^{o}C was found to be the maximum decomposition temperature for biofilm made with extracted PHB and it was almost same for standard PHB from Sigma (302^{o}C). The decomposition temperature for all the blends made in this experiment was beyond 300^{o}C.

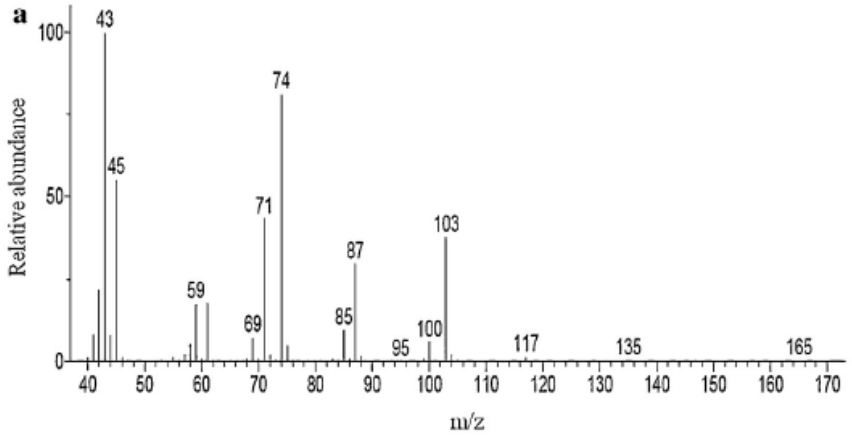
Differential scanning calorimetry

Non – isothermal DSC studies of PHB were carried out in order to have an understanding of the effect on crystallinity of PHB. The PHB extracted, PHB Sigma and PHBTS showed two endothermal peaks in between 140 and 200 °C (Fig. 6). The peak at the higher temperature is attributed to the melting of the crystalline film. Another endothermal peak appearing at a lower temperature is also clearly shown which is probably due to the melting of the imperfect crystals formed during the sample preparation. The melting enthalpy (_Hf) was obtained from the area of the two endothermal peaks. The crystallinity degree (Xc) was calculated based on the melting enthalpy of 146 J/g of 100% crystalline PHB. Intensified cold crystallization of the blend samples at about 65 °C may be the results from the inability of all the crystallizable chains to crystallize completely during the cooling cycles. When the PHB content was lowered and the TS and PLA content increased, the PHB microcrystal's or ordered chains could be more easily removed to pack into a denser or perfect crystalline structure as PHB is still a highly crystalline polymer with low crystallization rate. The melting temperature (Tm) for standard PHB, the extracted PHB, PHB-TS blends were almost

same and for PHB – PLA blend is slightly higher. The enthalpy of melting (_Hf) is 36.5 J/g for standard PHB and for extracted one is 29.09 J/g.

GC analysis

Fig. 3:



GC-MS analysis carried out to determine the constituents present in the PHB revealed the major peak, resembling to methyl 3-hydroxybutyrate with retention time of 2.6 min.

Biodegradability tests

PHB film was buried for a period of four weeks in composted soil. It showed 80% decrease in weight indicating biodegradability.

Conclusion

Use of inexpensive and renewable carbon substrates such as agro industrial wastes and by-products as feedstock can contribute to as much as 40-50% reduction in the overall production cost. Finding a less expensive substrate is, therefore, a major need for a wide commercialization of PHB. Considering all the economic, environmental and social issues, the ultimate goal is to obtain an economically viable PHBs production based on clean and safe processes, such that the final commercial products can be environmentally compatible. In this study, water hyacinth, a troublesome waste was utilized to produce PHB, which has a high commercial value. This study also succeeded in reducing the waste management problem.

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