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Banana peel as an inexpensive carbon source for microbial polyhydroxyalkanoate (PHA) production

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Abstract

Polluted soil and water samples were used to isolate fourteen different bacterial isolates out of which four bacteria showed the ability to produce Polyhydroxyalkanoates. Banana peel was added to the media in different ratios as a sole carbon source. Polymer producers determined using 16s rRNA studies were found to be Staphylococcus aureus JH1, Geobacillus stearothermophilus R- 35646, Bacillus subtilis JCM 1465 and Bacillus siamensis PD- A10. Cupriavidus necator a reference bacterium for PHA production served as a positive control and the results obtained were 79.73%, 75.94% and 74.69% with C: N ratio of 3:1, 1:1 and 4:1 respectively upto 96hours of incubation. Geobacillus stearothermophilus R- 35646 produced 84.63%, 71.58% and 62.68% of PHA with 4:1, 3:1 and 2:1 C:N ratios respectively upto 96hours of incubation. Bacillus subtilis JCM 1465 accumulated 71.78% and 62.23% of PHA with 4:1 and 3:1 C:N ratios respectively at 24hours of incubation. Bacillus siamensis PD- A10 accumulated 77.55% at 24hours, 69.70% at 72hours and 65.75% at 72hours of incubation with 3:1, 3:1 and 4:1 C:N ratios respectively. While Staphylococcus aureus JH1 showed PHA accumulation of about 70.02% at 24hours and 52.74% at 48hours of incubation with 2:1 and 4:1 C:N ratios respectively.

Keywords: Biopolymers, biodegradable, inexpensive carbon sources, polyhydroxyalkanoates, microbial polysaccharides, zero wastage.

Introduction

Since the introduction of the synthetic plastics in 1950s, they are extensively used material in our daily life, due to which the plastic waste has become a serious environmental threat[1]. Due to the exponential growth of the world population, the demand for the plastic production has crossed100 million tons every year. These plastic products remain unharmed in the environment for longer period of time even in harsh conditions and also remain inert to attacks of microorganisms and chemicals[2]. Thus now-a-days there has been an increase in demand for the research on the degradable plastics which belong to the class of polyesters, known as Poly-(β-hydroxyalkanoate) (PHA)[2-4]. Under unbalanced growth conditions, many microorganisms are known to produce PHAs as storage food reserves[5]. They have similar mechanical properties as that of the synthetic plastics like polyethylene, polypropylene, etc. They also have an added advantage of getting completely mineralized into carbon dioxide and water through the action of various microorganisms. PHAs are known to be produced intracellularly by microorganisms like *Ralstonia eutropha*, *Bacillus megaterium*, *Cupriavidus necator*, *Azotobacter spp*., *Rhizobium spp*., *Pseudomonas spp*., etc under limiting nitrogen substrate and presence of an abundant carbon source[6]. The industries produce Polyhydroxyalkanoates using expensive carbon source, thus resulting in a product that is hardly economically competitive as compared to that of the petroleum derived polymers. Thus the carbon source and downstream processing plays a major role in production costs which represents around 30% (approximately) of final product cost. Hence new alternative strategies are now being studied to lower the final product cost of PHAs. This can be achieved to some extent by incorporating inexpensive carbon sources, usually agro-industrial wastes[7].

Materials and methods

Isolation of bacteria: Collection of samples: Soil samples from the Landfill area of Kalyan (Maharashtra, India) and polluted water samples from Effluent Water Treatment Plant of REVA University campus, Kattigenahalli, Bangalore (Karnataka, India) were used to isolate different bacterial cultures for this study.

Selection of bacteria: Selection of bacterial isolates was done using serial dilution method followed by the spread plate technique. 1ml sample each from three higher dilutions was inoculated onto petri plates containing pre-sterilized nutrient agar and was evenly spread using spread plate technique. The plates were incubated at 37^0C for 24hours. After incubation, bacterial colonies were picked based on the colony morphology using Bergey's Manual of Systematic Bacteriology. Nutrient agar slants were used for maintaining the selected bacterial isolates for further studies.

Screening of PHA producers: Sudan Black B staining method was done to study the ability of the selected bacterial isolates to produce PHA. 0.3% of stain in 70% ethanol was used to perform the staining procedure. All the bacterial cultures were inoculated onto sterile nutrient agar containing 1% glucose. After incubation at 37^0C for 24hours, the petriplates were flooded with Sudan Black B stain and kept undisturbed for 30mins. Later pure ethanol solution was poured onto the colonies to remove the leftover excess stain. The colonies which showed bluish black coloration were considered as PHA producers. Out of 14 bacterial strains, 4 bacterial strains showed positive results. The same staining procedure was repeated using minimal agar media and the recurring results confirmed the PHA producers[8].

Biochemical testing: Biochemical testing of the selected PHA producers was performed[9,10]. All the studies performed here after were done on the four selected PHA producers.

Catalase test: Agar slants were prepared and the selected isolates were inoculated into each tube and incubated for 24hours at 37^0C. After incubation, all the slants were flooded with hydrogen peroxide and checked for the effervescence which indicated catalase positives.

Indole test: Tryptophan broth was prepared and the selected isolates were inoculated into each tube and incubated for 24hours at 37^0C. After incubation, 5drops of 1% Kovac's reagent was added. The tubes showing the formation of pink color were positives.

Simmon's Citrate Agar test: Simmon's Citrate Agar slats were prepared and selected isolates were inoculated onto each slant and incubated at 37^0C. The results were checked after 24 hours and 48 hours. The change of color of the slant indicated positive result for citrate production.

Triple Sugar Iron agar test: Triple Sugar Iron agar media slants were prepared and the selected isolates were inoculated and incubated for 24 hours at 37^0C. After incubation the slants were observed for the change in the color of the media. The type of sugar used by bacteria for fermentation was indicated, presence of black precipitate indicated H_2S production and presence of gas bubbles indicated hydrogen gas production.

Methyl Red Voges Proskauer test: Sterile MRVP broth was prepared and the isolates were inoculated and incubated for 24hours at 37^0C. For Methyl Red test was performed by adding 5drops of methyl red to all the test tubes and the broth was observed for the color change. For Voges Proskauer test, 0.6ml of Baritt's reagent A and 0.2ml of Baritt's reagent B was added to all tubes and were allowed to stand for 30mins and observations for the color change were made.

Growth Optimization: Effect of different parameters on the growth of bacterial isolates was investigated using nutrient broth media. UV visible spectrophotometer was used for determining the turbidity at 600nm[11-13].

Effect of incubation time: The growth pattern of all the selected isolates were studied at 24hours, 48hours, 72hours and 96hours keeping rest of the parameters constant like the incubation temperature; 37^0C, pH; 7, carbon source; glucose and nitrogen source; yeast extract.

Effect of Incubation temperature: The growth pattern of all the selected isolates were studied at 25^0C, 35^0C, 45^0C and 55^0C keeping rest of the parameters constant like the incubation time; 24hours, pH; 7, carbon source; glucose and nitrogen source; yeast extract.

Effect of pH: The growth pattern of all the selected isolates were studied at pH 5, 6, 7, 8 and 9 keeping rest of the parameters constant like the incubation temperature; 37^0C, incubation time; 24hours, carbon source; glucose and nitrogen source; yeast extract.

Effect of different carbon sources: The growth pattern of all the selected isolates was studied using glucose, lactose, sucrose and maltose keeping rest of the parameters constant like the pH; 7, incubation temperature; 37^0C, incubation time; 24hours and nitrogen source; yeast extract.

Effect of different nitrogen sources: The growth pattern of all the selected isolates was studied using ammonium chloride, potassium nitrate; peptone and yeast extract keeping rest of the parameters constant like the pH; 7, incubation temperature; 37^0C, incubation time; 24hours and carbon source; glucose.

Carbohydrate estimation of inexpensive raw materials: The inexpensive raw material used as a carbon source was banana peel. Total carbohydrate estimation of selected raw material was done using Phenol sulphuric acid method[14,15].

C:N ratios: Banana peel was used as carbon source and a substitute for glucose and yeast extract was used as nitrogen supplement. Different ratios of carbon and nitrogen were used (1:1, 2:1, 3:1 and 4:1) at different incubation periods (24hours, 48hours, 72hours and 96 hours). Results are summarized in Table-5[16].

Preparation of media for PHA production: Media was prepared containing all components of nutrient broth except for the carbon source. Banana peel was added in said ratios against the yeast extract. 100ml of media for each ratio was prepared with desired pH for the particular bacterial isolate. Inoculum was prepared by taking 10ml media in separate sterile test tubes and inoculating them with the bacterial isolates to obtain the active culture. After incubation the media containing active bacterial cells was poured into the corresponding flask and further incubated for 4days and samples were withdrawn for PHA production every 24hours.

Extraction of PHA: Chloroform extraction procedure was done for the extraction of PHA. The pellet was obtained by centrifugation of bacterial cells at 5000*(g)* for 10mins. Further the pellet was dried and Dry Cell Weight (g/L) was obtained. Sodium hypochlorite was added to the pellet and was allowed to stand for 2hours at 37^0C in an incubator, during which, there was a complete digestion of cellular components except PHA. Later the PHA granules were collected by centrifugation of the mixture and discarding the supernatant. Further, the mixture was washed twice with 10ml distilled water and centrifuged. Resultant PHA granules were further washed with acetone: methanol: diethyl ether (1:1:1) twice. Later, boiling chloroform was added to the PHA granules and evaporated by air drying until completely dried powder of PHA was obtained which was then weighed to obtain extracted PHA (g/L).[17-23].

Quantification of PHA: Percentage PHA accumulation was calculated by applying the following formula[22,24].

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA } \left(\frac{g}{L}\right)}{\text{Dry Cell weight (DCW)} \left(\frac{g}{L}\right)} x100$$

Residual Biomass (g/L) = Dry Cell weight (g/L) – Dry weight of extracted PHA (g/L) (DCW)

Bacterial Identification and PCR studies: Selected bacterial isolates were further subjected to identification using 16s rRNA technique which revealed that the bacteria used in the studies were *Bacillus subtilis* JCM1465, *Geobacillus stearothermophilus* R- 35646, *Bacillus siamensis* PD- A10, *Staphylococcus aureus* JH1.

Isolation of genomic DNA from the culture was done using Norgen DNA isolation kit Cat No: 17900. Using the ~1.5kb consensus primer and Taq DNA polymerase, 16srRNA gene was amplified and later the PCR product was sequenced by using 100ng of forward and reverse primer each. Closest homologous microbes were obtained by aligning and analyzing the sequence data.

PCR conditions

94^0C	94^0C	56^0C	72^0C	72^0C
5mins	30secs	30secs	1.30secs 35cycles	10mins

Using Blast N programme, the sequence of DNA was compared with that of the NCBI database sequences. The best found matches for all the bacterial isolates are given below in the table (Table-1).

Table-1: Bacterial Identification.

Sample	Homology	Identification results
Bacterial isolate 1	*Bacillus subtilis* NBRC 13719	*Bacillus subtilis* JCM 1465
Bacterial isolate 2	*Geobacillus stearothermophilus* strain NBRC- 12550	*Geobacillus stearothermophilus* R- 35646
Bacterial isolate 3	*Amyloliquefaciens plantrum* FZB42	*Bacillus siamensis* PD- A10
Bacterial isolate 4	*Staphylococcus aureus strain NBRC 100910*	*Staphylococcus aureus* JH 1

Results and discussion

Selection and screening of bacteria: Fourteen different bacterial isolates were selected using spread plate technique and later screened for their ability to produce PHA using above described methods. Out of 14 isolates, 4 bacterial isolates showed positive for the Sudan Black B staining.

Biochemical testing: The selected isolates were tested for the Indole test (indicating their ability to convert tryptophan to indole) (Table-2), catalase enzyme activity (indicating their ability to release oxygen) (Table-2), Methyl Red and Voges-Proskauer test (indicating mixed acid fermentation or 2, 3-butanediol fermentation pathway) (Table-2), Simmon's Citrate test (indicating ability to use citrate as sole source of carbon) (Table-3) and Triple Sugar Iron test (indicating utilization of sugars) (Table-4).

Growth optimization: The growth pattern of selected isolates were studied using different incubation time, incubation temperature, pH, carbon sources and nitrogen sources and the optimum growth conditions obtained for each isolate was found out (Table-5).

Carbohydrate estimation of the inexpensive raw material: The total carbon content of the banana peel was analyzed in order to provide exact amount of carbon content to be provided to the bacterial isolates as per the C: N ratio (Table-6).

Table-2: Catalase, Indole and MRVP tests.

Bacterial isolate	Catalase test	Indole test	Methyl red	Voges-Proskauer
Bacillus subtilis JCM 1465	++++	Negative	Negative	Negative
Geobacillus stearothermophillus R35646	+++	Negative	Negative	Negative
Bacillus siamensis PD- A10	++++	Negative	Negative	Negative
Staphylococcus aureus JH 1	++++	Negative	Negative	Negative

Table-3: Simmon's Citrate Test.

Bacterial strain	Hours of incubation	Media color change		Result
		From	To	
Bacillus subtilis JCM 1465	24	Green	Blue	Positive
	48	Blue	Blue	Positive
Geobacillus stearothermophillus R 35646	24	Green	Blue	Positive
	48	Blue	Blue	Positive
Bacillus siamensis PD- A10	24	Green	Blue	Positive
	48	Blue	Blue	Positive
Staphylococcus aureus JH 1	24	Blue	Blue	Positive
	48	Blue	Blue	Positive

Table-4: Triple Sugar Iron Test.

Bacterial strain	Slant color	Butt color	Sugar fermented	Black precipitate	Gas formation
Bacillus subtilis JCM 1465	Red	Yellow	Glucose	+	-
Geobacillus	Red	Yellow	Glucose	-	-
Stearothermophillus R35646					
Bacillus siamensis PD- A10	Red	Yellow	Glucose	-	-
Staphylococcus aureus JH 1	Yellow	Yellow	Lactose/ Glucose	++	++

Table-5: Optimization of growth of the selected PHB producers.

Bacterial strain	Incubation time	Incubation temperature	pH	Carbon source	Nitrogen source
Bacillus subtilis JCM 1465	96 hours	25^0C	8	Glucose	Yeast extract
Geobacillus stearothermophillus R35646	48 hours	25^0C	9	Glucose	Yeast extract
Bacillus siamensis PD- A10	72hours	35^0C	7	Glucose	Yeast extract
Staphylococcus aureus JH 1	48 hours	35^0C	8	Sucrose	Yeast extract

Table-6: Estimation of total carbohydrate content.

Name of the sample	Component	Concentration	Results
Orange peel	Total carbon (as carbohydrate)	Percentage	20%

Extraction and Quantification of PHA: During the extraction procedure, the bacterial isolates were provided with the optimal conditions suitable for their growth, as per the optimization of the growth pattern except for the carbon source, which was supplied in the form of banana peel. The PHA production in each of the different C:N ratio flask was monitored every 24hours for 4days. The PHA accumulation and DCW obtained helped us to quantify the percentage of PHA produced by each bacterial isolate. Results obtained are summarized (Figures-1, 2, 3 and 4). The detailed report of the polymer extracted and quantified is given in the tables below (Table-7, 8, 9 and 10).

Extraction and quantification of PHA by Cupriavidus necator: The reference strain (positive control) *Cupriavidus necator* was procured from MTCC Chandigarh. The strain was inoculated in the media containing Banana peel using four different C: N ratios and PHA production was analyzed every 24hours for 4days (Figure-5). The exact amount of PHA obtained by Cupriavidus necator is given below (Table-11).

Table-7: PHA production obtained by *Bacillus subtilis* JCM 1465.

Hours of incubation	C:N Ratio			
	1:1	2:1	3:1	4:1
24hours	16.17	52.47	62.23	71.78
48hours	11.55	9.31	20.62	9.15
72hours	49.64	23.79	31.95	22.99
96hours	18.01	8.36	14.72	7.15

Table-8: PHA production obtained by *Geobacillus stearothermophillus* R35646.

Hours of incubation	C:N Ratio			
	1:1	2:1	3:1	4:1
24hours	7.16	12.16	18.97	25.19
48hours	17.19	12.77	41.26	20.33
72hours	7.98	32.17	34.15	50.01
96hours	14.19	62.68	71.58	84.63

Table-9: PHA production obtained by *Bacillus siamensis* PD-A10.

Hours of incubation	C:N Ratio			
	1:1	2:1	3:1	4:1
24hours	17.63	50.55	77.55	45.22
48hours	10.54	12.49	12.51	15.32
72hours	29.68	40.88	69.07	65.75
96hours	9.29	11.67	9.11	18.73

Table-10: PHA production obtained by *Staphylococcus aureus* JH1

Hours of incubation	C:N Ratio			
	1:1	2:1	3:1	4:1
24hours	39.19	70.02	32.42	31.22
48hours	13.76	8.57	14.82	52.74
72hours	18.93	10.66	16.61	30.43
96hours	11.59	11.05	36.22	21.66

Table-11: PHA production obtained by *Cupriavidus necator.*

Hours of incubation	C:N Ratio			
	1:1	2:1	3:1	4:1
24hours	28.69	16.96	33.05	22.00
48hours	7.88	6.12	11.67	34.06
72hours	6.82	16.01	16.85	26.05
96hours	75.94	48.34	79.73	74.69

Discussion: All the PHA producers were isolated from the polluted environments like landfill and the effluent water treatment plant, so it was necessary to compare their PHA yield with that of the extensively studied strain and a well known producer *Cupriavidus necator*. Best values of PHA production obtained by *Cupriavidus necator* using banana peel were 79.73% at 96hours, 75.94% at 96hours and 74.69% at 96hours with C:N ratios of 3:1, 1:1 and 4:1 respectively. PHA production more than that of *Cupriavidus necator* was obtained by *Geobacillus stearothermophilus* R- 35646 (84.63% at 96hours with C:N ratio 4:1). Same bacterium produced about 71.58% of PHA at 96hours with C: N ratio 3:1. *Bacillus subtilis* JCM 14645 produced 71.78% of PHA at 24hours with C: N ratio 4:1 which is equal to PHA produced by the reference strain under similar conditions. Same bacterium produced 62.23% of PHA at 24hours with C:N ratio 3:1. *Bacillus siamensis* PD- A10 produced 77.55% at 24hours with C: N ratio 3:1, 69.70% at 72hours with C: N ratio 3:1 and 65.75% of PHA at 72hours with C: N ratio 4:1 while *Staphylococcus aureus* JH1 produced 70.02% of PHA at 24hours with C: N ratio 2:1 and 52.74% of PHA at 48hours with C: N ratio 4:1. In other studies, *Bacillus thuringiensis* have shown to accumulate about 72.5% with starch (10g/L) as carbon source and *Bacillus megaterium* showed 20% of PHB production with glucose as carbon source[25,26]. In other comparative studies different *Bacillus* strains were used to study the amount of PHA production and maximum amount obtained was 66.6% by *Bacillus cereus* EUG3[27]. In a similar study, *Bacillus megaterium* was used as a

reference strain and some *Bacillus* species were isolated from soil samples. 5% glucose was added to the PHA producing media in which the reference bacterium produced 83.78% of PHB while other *Bacillus* species showed the accumulation of around 75.22% and 79.59% of PHA[28]. The work done by other researchers proved that all the strains studied so far produced more or less equal amounts of PHA as compared to that of the reference strain. However, our investigation established that we can improve upon PHA production by isolating bacteria from polluted environments rather than lab maintained strains as the new isolates adapt themselves to biowastes provided in a better manner. To further improve upon PHA production, recombinant DNA technologies and genetic engineering can be performed.

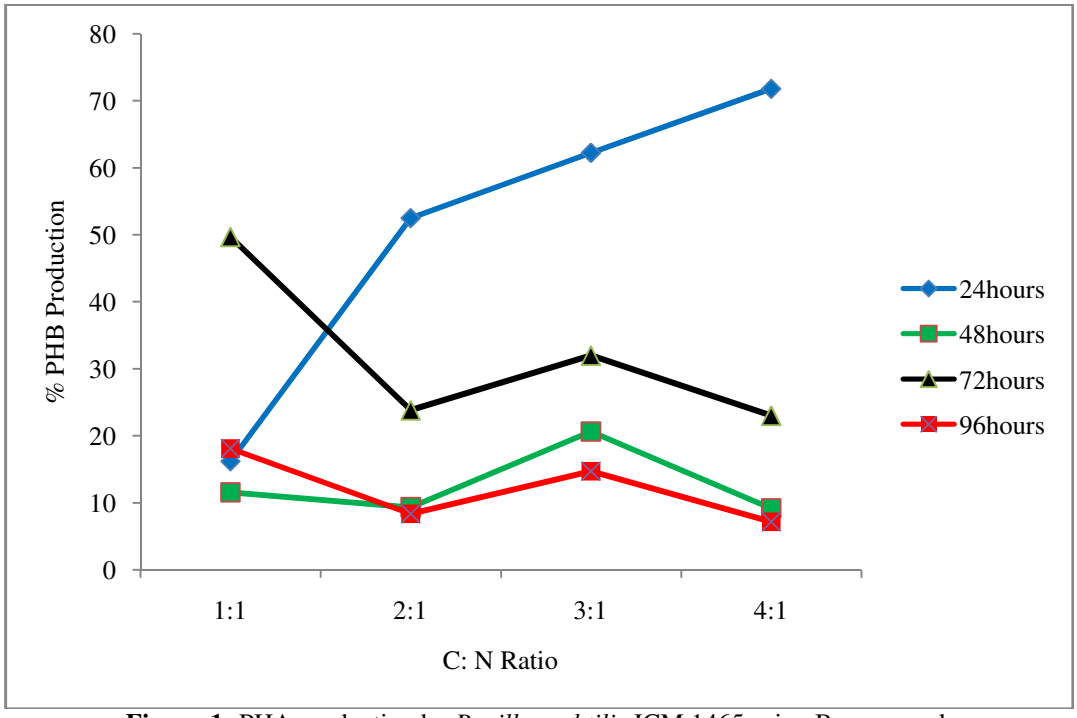

Figure-1: PHA production by *Bacillus subtilis* JCM 1465 using Banana peel.

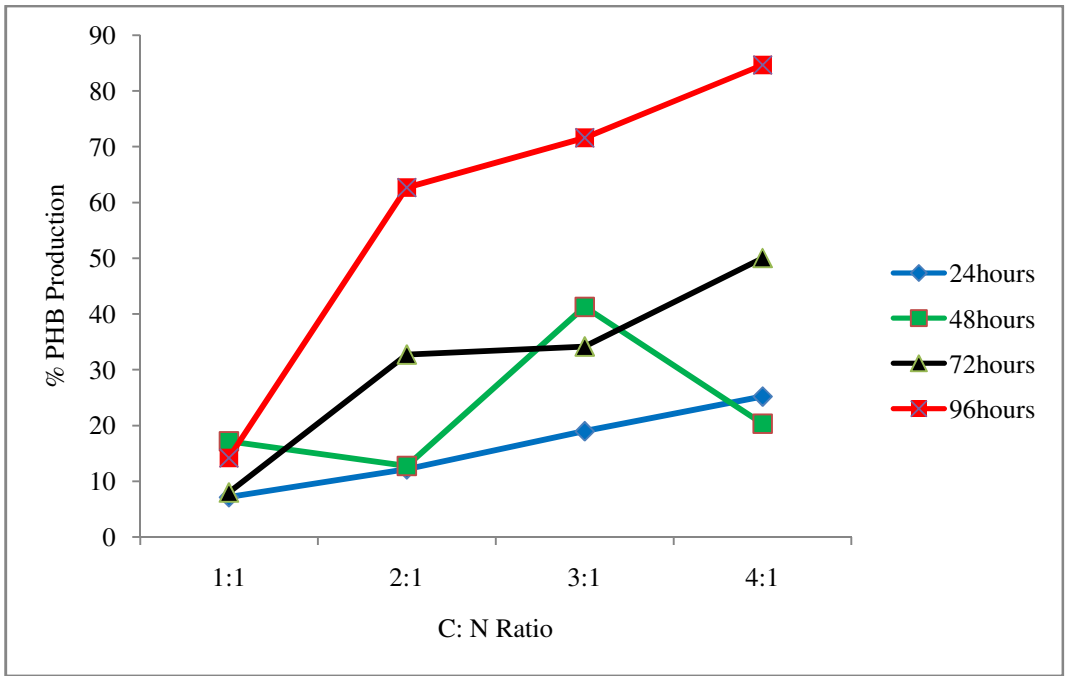

Figure-2: PHA production by *Geobacillus stearothermophillus* R 35646 using Banana peel.

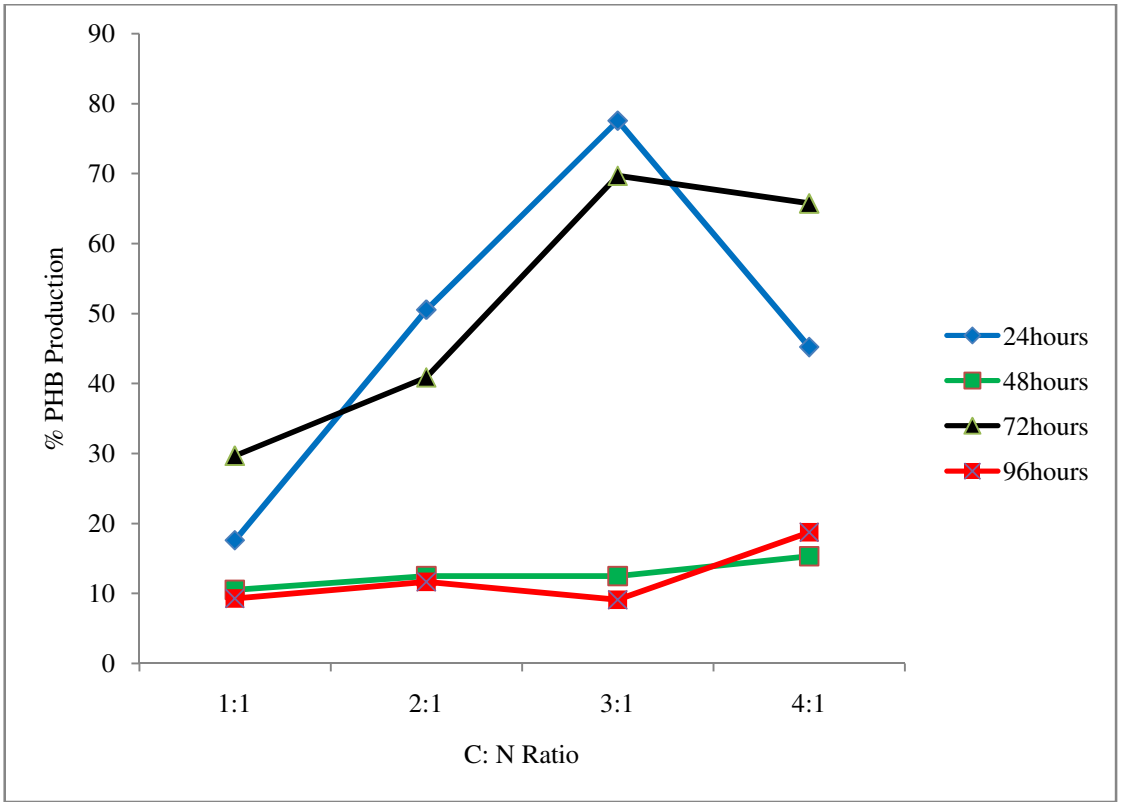

Figure-3: PHA production by *Bacillus siamensis* PD- A10 using Banana peel.

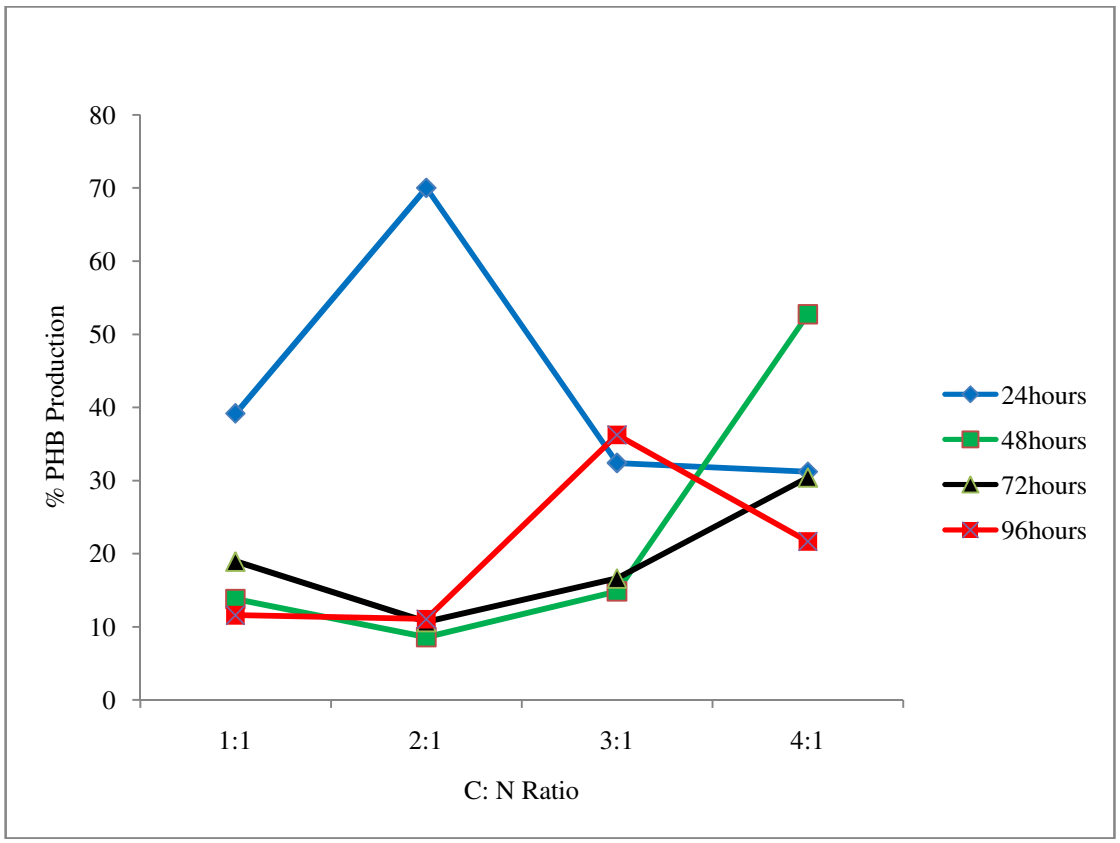

Figure-4: PHA production by *Staphylococcus aureus* JH1 using Banana peel.

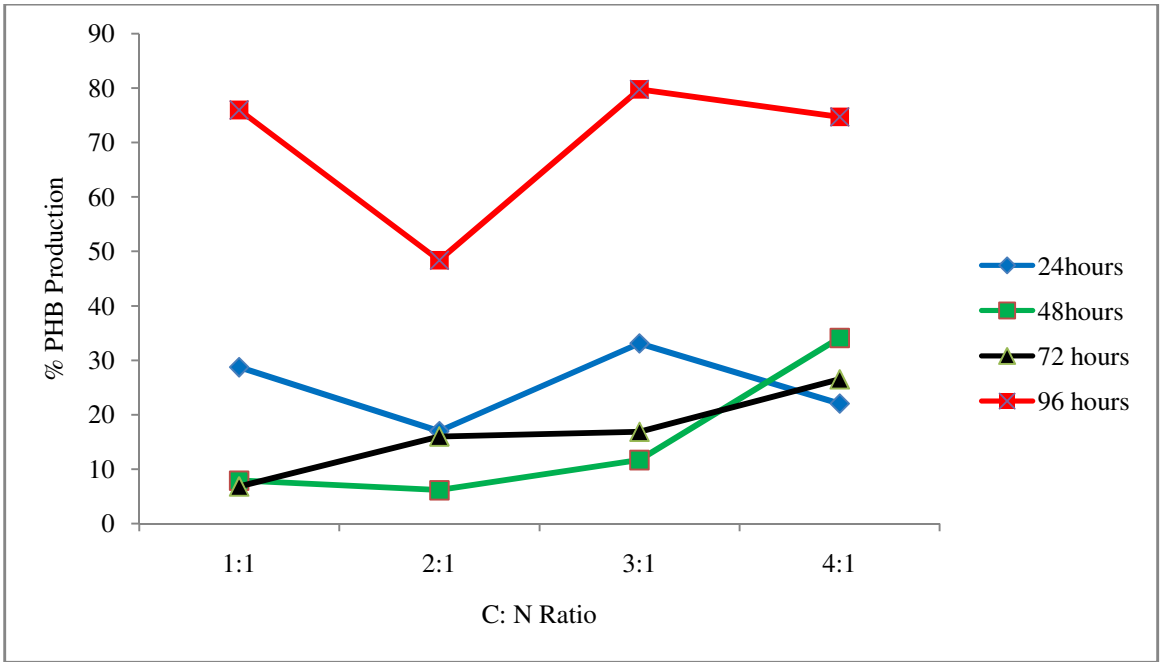

Figure-5: PHA production by *Cupriavidus nectaor* using Banana peel.

Conclusion

The findings in this study indicate that the banana peels can prove to be a good substitute for the carbon source which is available easily at free of cost and still supports PHA production upto considerable amount of levels by almost all selected bacterial isolates. Thus the problem of waste disposal is addressed on one hand and a value added product is being produced on the other.

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