ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2019; 5(10): 335-343
www.allresearchjournal.com
Received: 22-08-2019
Accepted: 26-09-2019

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Isolation, screening and selection of efficient Polyhydroxybutyrate synthesizing bacteria and its production using cheap sources

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Abstract
Bioplastics are biomass based biodegradable plastics which can be derived from corn starch, pea starch, vegetable fats and oils as well as microorganism like bacteria, algae etc. They may be used for packaging purposes and catering items like bowls, pots, straws, cutlery etc., for making bottles for soft drinks, bags, trays etc. plastics is one of the major pollutant at present time in the world, which is used for daily use like packaging materials, carry bags, well as to reduce the increasing environmental pollution an alternative must developed. This need of plastic can be fulfilled by the use of bioplastics. Polyhydroxyalkanoate are produced by bacteria among which Polyhydroxybutyrate (PHB) is one major group. The property of PHB is similar to synthetic plastics. So, it can be used as a suitable alternative to the present day conventional practices for sustainability. Several bacterial species like *Actinobacillus*, *Azotobacter*, *Agrobacterium*, *Rhodobacter* and *Sphaerotilius* have been under focus for their ability of converting organic waste to bacterial PHA. For industrial production of PHB, with some bacterial species like *Bacillus* spp, *Pseudomonas* spp, *Aeromonas* spp, *Cupriavidus* spp have been extensively used for their potential to produce PHB. Since the production of bioplastic is expensive many techniques adopted for large scale production. But, to obtain PHB in larger amount the selection of proper strain of bacteria, capable of producing or accumulating PHB in less costly way has become an absolute necessity. So in this study, isolating, selecting and screening of an efficient Polyhydroxybutyrate producing microorganism has been done with comparison of the production of PHB (Bio-plastic) in agro industrial waste to find out which one has the potency to produce more PHB.

Keywords: Bioplastic, PHB, bacteria, agro industrial waste

1. Introduction
One of the serious threats to our world is plastic pollution. Plastic constitutes approximately 90% of all trash floating in the ocean's surface, with 46,000 pieces of plastic per square mile. Marine over 100,000 mammals and one million seabirds die each year from ingesting or become entangled in plastic. The plastic will degrade in 500-1000 years. The dominant raw material for plastic production is petroleum which is non-renewable and also the main source of energy. Due to non-biodegradability of conventional plastics have thus driven various entities to look for more sustainable alternatives such as polyhydroxyalkanoates (PHAs), polylactic acids (PLA), aliphatic polyesters, polysaccharides, blends of starch and polypropylene and other copolymers to replace petroleum derived plastics. (Lee, 1996) [14]. Biological transformations by mixed cultures offer a wide potential for chemical or energy production. These types of plastics are biodegradable and better for the environment. Investment is just starting to tap into bioplastics are they are becoming more sophisticated in performance, more efficient in biomass yields and more cost effective in process ability (Chen *et al*., 2012) [8]. Among all these biodegradable plastics, PHA is of major interest because it possesses similar characteristic to conventional plastics and is completely biodegradable in the environment. PHA is synthesized from renewable carbon sources, based on agricultural or industrial wastes, allowing a sustainable production (Braunegg *et al*., 1998; Sudesh and Iwata, 2008) [6, 12]. Microorganisms are able to incorporate up to 60 different monomers into their storage polymers and series of PHAs with different monomeric composition (Doi *et al*., 2000) [19].

The polymer form within the bacterial cells is discrete granules, which generally have diameters between 100 and 800nm. However, the number and size of the granules and the molecular weight of the polymer, vary depending on the type of bacteria, the growth conditions and the method of extraction. A challenging combination of biomedical and biodegradable properties of PHB is a perspective tool in design of novel medical devices and tissue engineering. They have also been used to manufacture disposable utensils and razors, well as in the packaging industry, to make shampoo bottles and food containers.

2. Polyhydroxybutyrate

2.1 General Characteristics of PHB

Poly (3-hydroxybutytrate) [P (3HB)] and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P (3HB-co-3HV] are the most studied polyesters in the PHA family. These polymers share the physical and mechanical properties similar to petroleum derived thermoplastics polypropylene (PP) and polyethylene (PET). The polymer typically constitute up to 30% of the dry mass of the cell, but under controlled condition, involving excess carbon and a limited supply of nutrients like nitrogen, the yield can be increased to as high as 80% of dry weight. It is insoluble in water, but poor resistance to acids and bases and dissolves in chlorinated solvents. The unfavourable characteristic of P (3HB) such as high crystalline, stiffness, brittleness and low extension to break limit its range of application (Freier, 2006) [10]. Another challenge it possesses it is difficult to process PHB in a molten state because it starts to degrade at temperature not much higher than melting temperature of 175 °C. To overcome some of these problems, the bacterial production process can be modified to produce PHB that is copolymerised with PHV. Copolymerisation reduces the melting temperature as low as 75 °C depending upon the composition. The degradation time depends on the composition, as well as the environment. It can be as little as few months where there are lot of bacteria or it may take few years where there are fewer bacteria. Much attention has been spent on optimizing the PHA production process, recovery and blending with other polymers to reduce the PHA production cost.

2.2 PHB pathway

Microbial biosynthesis of PHB starts with the consideration of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA by acetoacetyl-CoA reductase. Then with the help of PHB synthase the PHB is synthesized.

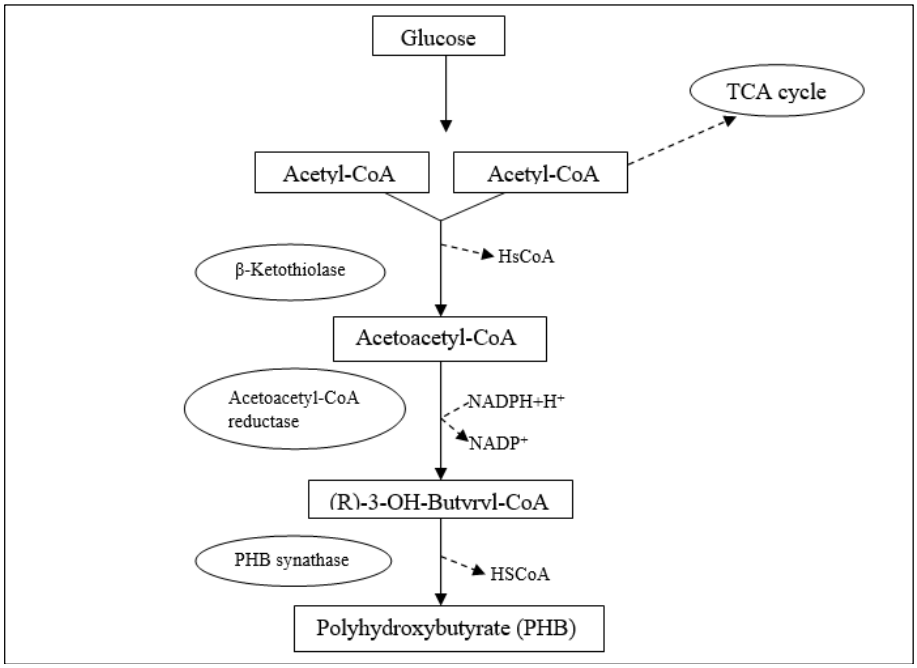

Fig 1: PHB Pathway

Table 1: Polyhydroxybutyrate production from Bio wastes by diverse microorganism.

Microorganism	Substrate	Culture mode	Time(h)	PHB		Reference
				Yield (%)	Conc (g/l)	
Arachaea						
Haloferaxmediteranei	Extruded rice bran	Fed batch	120	55.6	77.8	Huang *et al*., 2006 [20]
	Extruded corn starch			38.7	24.2	
Firmicutes						
Bacillus megatirium	Date syrup/ beet molasses	Batch	48	52	1.76	Omar *et al*., 2001 [15]
α-Proteobacteria						
Methylobacterium extorquens	Methanol	Fed batch	186	40	114	Bourque *et al*., 1995 [5]
M. rhodesianum	Glycerol and casein hydrosylates	Batch	45	50	11	Bormann and Roth, 1999 [4]
β-proteobacteria						
A. eutropha DSM545	Potato processing wastes	Batch	120	77	5	Rusendi and Sheppard, 1995 [18]
A. latus DSM1124	Malt waste	Fed batch	69	32.4	18.4	Yu *et al*., 1999 [23]

	Soya waste			22.7	6.0	
Ralstonia eutropha	Tapioca hydrosylate	Fed batch	59	58	61	Kim and Chang, 1995 [2]
R.eutropha	Food scraps	Batch	80	72.6	11.3	Du and Yu, 2002 [11]
γ-Proteobacteria						
A. vinelandiiiUWD	Molasses	Fed batch	36	66	22	Page and Cornish, 1993 [22]
A. chroococcum	Starch	Fed batch	70	46	25	Kim, 2000 [1]
		Batch	58	73.9	0.864	

3. Materials and Methods

3.1 Cleaning of glasswares

The glassware to be used were subjected to cleaning by first washing it with chromic acid after which it was washed with tap water, then was cleaned with soap solution, at last a final rinse was given with distilled water.

3.2 Sample collection

Sewage and soil samples were collected from 16 different sewage places across the Chennai city. Sterile plastic containers were used to collect the samples and to store it. Gloves and masks were worn while collecting the sample.

3.3 Sample processing

The thus collected were subjected to serial dilution from 10^{-1} to 10^{-7}. Pour plate technique were used to isolate the microorganisms. Based on the colony morphology**,** positive isolates were obtained and maintained separately in a nutrient agar slant at 4 °C.

3.4 Screening for PHB production

Screening for PHB production was done by using reagents such as Sudan black and Safranine.

3.4.1 Preparation of Sudan Black

Sudan black	0.3g
Ethanol (70%)	100ml

3.4.2 Preparation of Safranine

Safranine	0.5g
Distilled water	100ml

3.4.1.1 Staining for PHB (Byrom *et al*., 1991) [7].

- Flame the loop and allow it to cool. Remove the cap from the culture bottle, flame the neck, take a loop full of broth, flame the neck again and close the bottle.
- Spread the culture on a clean glass slide, using the loop. Fix the smear by holding the slide with forceps and passing it horizontally through a small Bunsen flame 2-3 times.
- Place a few drops of Sudan black solution on the fixed preparation. Immerse the slide in xylene until it is completely decolorized.
- Flood the slide with counterstain, safranine solution. Then rinse the slide with running water and allow it to dry. The PHB can be seen as very dark granules inside pink cells.

The isolates thus showing positive for PHB accumulation were individually inoculated into 50ml of production medium and incubated for 48hrs at 37°C in 120rpm for polyhydroxybutyrate production. After the incubation period the different isolates were PHB was extracted by alkaline digestion method to be determined by Law Splepecky method.

3.5 Production

3.5.1 Preparation of Luria-Bertani medium

Peptone	1g
Yeast extract	1g
$Nacl_2$	1g
Distilled water	100ml

The chemicals were all dissolved in 100ml of distilled water and sterilised at 121 °C at 15lbs for 15 minutes.

3.5.2 Preparation of mineral Salt medium (Whittenbury *et al*., 1970) [21].

Fructose	2g
NH_4Cl	0.05g
KH_2PO_4	0.23g
Na_2HPO_4	0.23g
$MgSO_4$	0.05g
$NaHCO_3$	0.05g
$CaCl_2$	0.001g

The chemicals were dissolved in 100ml of distilled water and sterilised at 121 °C at 15lbs for 15 minutes.

3.6 Estimation and determination of PHB

The estimation and determination of PHB was done by alkaline digestion method and crotonic acid assay.

After 24 hour incubation at 37 °C, 10ml of culture was taken and centrifuged at 10,000 rpm for 15minutes. The supernatant was discarded and the pellet was treated with 10ml of sodium hypochlorite and the mixture was incubated at 30 °C for 2 hours. The residue was collected by centrifugation at 8000 rpm for 20 min and performed a series of washing step using distilled water, acetone and finally ethanol. The polymer was dissolved in chloroform and kept for complete evaporation (Nish *et al*., 2009) [16]. Then 5ml concentrated H_2SO_4 was added and heated for 40minutes at 100 °C in a water bath. The resultant crotonic acid was measured at 235nm against H_2SO_4 as blank in UV-VIS spectrophotometer (Beckman DU 40). The concentration of PHB was determined by comparing the OD value with a standard graph prepared using P3HB (sigma Aldrich) (Law and Splepecky 1961) [13].

3.7 Optimization of various parameters for production of PHB

Effect of time, temperature and pH on the production of PHB from the bacteria isolate was observed using LB medium (Nish *et al*., 2009) [16].

3.7.1 Effect of incubation time on PHB production

Around 500ml of sterile production medium is prepared and 1% inoculum was added aseptically. The inoculated medium was incubated 37 °C temperature in a shaker flask at 120rpm. Around 10ml culture was aseptically collected periodically at 12 hours intervals up to 120 hours. The PHB

production was determined using alkaline digestion method.

3.7.2 Effect of temperature on PHB production

100ml of sterile production medium was prepared in different conical flask and inoculated with 1% inoculum. Each flask was incubated at different temperatures such as 27 °C, 32 °C, 37 °C, 42 °C and 45 °C for 48 hours. The PHB production was determined using alkaline digestion method.

3.7.3 Effect of pH on PHB production

100ml of sterile production medium was prepared in different conical flask and each flask was adjusted to different pH such as 6, 6.5, 7, 7.5 and 8 using 0.1N NaOH and 0.1N HCl. After sterilisation flasks were inoculated with 1% inoculum and incubated at 37 °C for 48 hours. The production was determined using alkaline digestion method.

3.8 Genomic DNA Isolation

- Transfer the 1.5ml of overnight bacterial culture (grown in LB medium) to a 1.5ml Eppendorf tube and centrifuged at 10,000 for 1min to pellet the cells.
- Discard the supernatant, re suspend the cell pellet in 600µl lyses buffer and vortex to completely re suspend the cell pellet. Incubate for 1hour at 37 °C.
- Add equal volume of phenol/chloroform and mix well by inverting the tube until the phases are completely mixed. Do not vertex the tube as it can shear the DNA.
- Spin at maximum speed for 5 min at room temperature. Carefully transfer the upper aqueous phase to a new tube by using 1ml pipette. The steps are repeated until the white protein layer disappears.
- To remove the phenol, add an equal volume of chloroform to the aqueous layer. Again mix well by inverting the tube. Spin at 10,000rpm for 5 min. remove the aqueous layer to the new tube.
- To precipitate the DNA, add 2.5 or 3ml of cold ethanol (ethanol is stored at -20 °C freezer) and mix gently (DNA precipitation can be visible). Incubate the tube at -20 °C for 30 min or more.
- Spin at 10,000rpm for 15min at 4 °C. Discard the supernatant and rinse the pellet with 1ml of 70% ethanol.
- Spin at 7,500rpm for 2 min. carefully discard the supernatant and air-dry the DNA pellet. Re suspend the DNA in TE buffer.

3.8.1 Primers

Forward: 5'-GGAAGTAAAAGTCGTAACAAGG-3'
Backward: 5'-TCCTCCGCTTATTGATATGC-3'
The amplified fragment includes ITS1, 5.8S and the ITS2 of rDNA. Amplification was performed in 50µl reaction mixture containing the following ingredients.

3.8.2 16S rRNA Sequence Analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well defined queries and alignments. BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm. nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments, regions of similarity may provide important clues to the function of uncharacterized nucleotides and proteins.

3.9 Cheap source processing

The agro industrial wastes used were starch water extract and sapota peel extract. It was first washed with tap water and then rinsed thrice with distilled water. Later it was processed by drying it under the sun for 48 hours. After which it was powdered and stored in sterile vials. 1g of the powder was mixed with 100ml of distilled water and was kept in a shaker for 48 hours. The processed wastes were mixed with mineral salts medium in different concentrations from 10% till 100% in a 250ml conical flask. An overnight culture was inoculated into each test tube and was incubated at 30 °C for 48 hours. The growth in each medium was then determined by extracting the PHB by alkaline digestion method and subjecting it to crotonic acid assay.

3.10 Partial purification of PHB (Reddy *et al.*, 2009)

The isolate was grown in 250ml Erlenmeyer flasks containing 50ml of mineral medium with different carbon sources. These flasks were incubated at 28 °C for 48 hours on scientific environmental shaker at 1d50rpm. Cell suspension 10ml was centrifuged at 6,000rpm for 10 minutes. The cell pellet was washed once with 10ml saline and was centrifuged to get the pellet. The cell pellet was then suspended in 5ml sodium hypochlorite (4% active chlorine) and incubated at 37 °C for 10 minutes with stirring. This extract was centrifuged at 8,000rpm for 20 minutes and the pellet of PHB was washed with 10ml cold diethyl ether. The pellet was again centrifuged at 8,000rpm to get purified PHB.

3.11 Nuclear magnetic resonance (NMR)

The polymer was suspended in spectro chem. Grade deuterochloroform (CDC13). The ^{1H} NMR spectra of sample was obtained at the range of 0-13 MHz while that of 13C NMR spectra in the range of 0-200MHz using a brucker AV III NMR spectrometer (Bruker Biospin AG, Switzerland) from Sophisticated Analytical Instrument Facility, IIT, Madras. The spectra thus obtained were compared with the standard NMR spectra to confirm the presence of C atoms.

4. Result and Discussion

Investigations were conducted to isolate bacteria from various environmental samples and screened them for accumulation if polyhydroxybutyrate (PHB) within their cells. The efficient isolates were selected and their culture parameters for maximum PHB production were optimized. Different agro industrial wastes were tested as cheaper substrates for PHB production by the promising isolates.

4.1 Isolation of bacteria

As many as 15 different sewage samples (Fig 1) were collected from sources listed in table 2. Bacteria from these samples were isolated on Nutrient agar by pour plate technique. From the samples studied, 13 bacterial strains were isolated (Fig 2) based on their morphology and was screened for PHB production.

Table 2: Origin and description of the samples used for the isolation of PHB producing bacteria.

S No	Sample type	Place of sampling	No. OF bacteria isolated	No. of sudan black B positive strains	Name of the isolates
1	Sewage samples	MRC Nagar	3	1	SE 1, SE2, SE3
		Mylapore	1	0	SE4
		ICF clolony	0	0	-
		Choolaimedu	0	0	-
		Chintharipet	2	0	SE5, SE6
		Anna nagar	3	1	SE7, SE8, SE9
		R.A. Puram	0	0	-
		Anakapathur	0	0	-
		Kundrathur	1	0	SE10
		Saidapet	0	0	-
		kottupuram	1	0	SE11
2	Soil	Adyar	0	0	-
		Koyembedu	1	1	S01
		sriperambathur	0	0	-
3	Sugar industry waste		1	1	S1W1

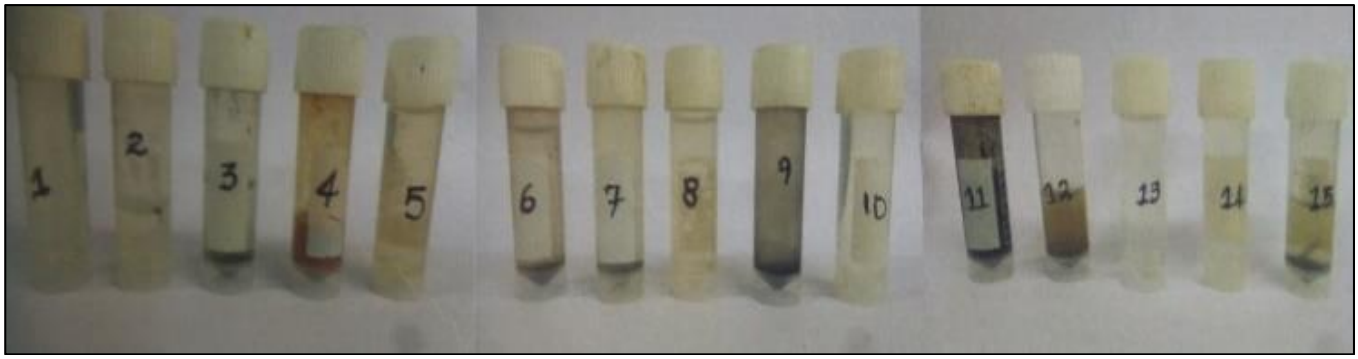

Fig 2: Fifteen different sewage samples collected in sterilized vials

Fig 3: Bacterial strains isolated by quadrant streak method

4.2 Screening of the isolates for PHB production

The 13 isolates were subjected for PHB production using Sudan black B staining method (Fig 3). It was observed that out of 13 isolates studied 4 were found to accumulate PHB (Table 3). Three of which were isolated from sewage effluents (SE1, SE7, SE11) while one was isolated from the sugar industry waste water (S1W1)

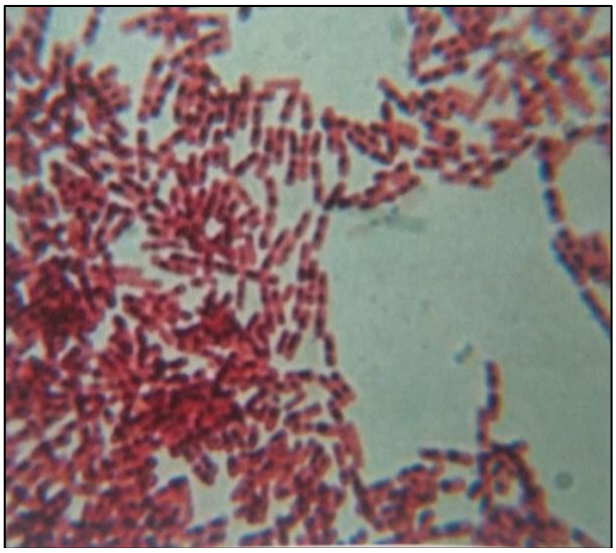

Fig 4: Bacterial isolate stained by Sudan black B

4.3 Quantitative screening of the isolates for PHB production

Quantification screening of the four isolate were done. The organism were inoculated in LB broth supplemented with 2% glucose and were incubated for 48 hours at room temperature in orbital shaker. After the incubation time samples were collected and quantified. From the four isolates studied maximum production was observed (Table 3) for SE1 isolate with a yield of 0.064g/100ml. Other isolates such as SE7, SE11, S1, W1 gave 0.015, 0.043 and 0.051g/100ml yields respectively.

Table 3: PHB production by Sudan black positive isolates

S No	Name of the isolates	PHB production (g/100ml)
1	SE1	0.033
2	SE7	0.015
3	SE11	0.024
4	S1W1	0.019

4.4 Selection of promising bacterial isolate

Based on the PHB yields, (Table 2) the most promising isolate, SE1 (0.064g/100ml) was selected and stored for further study.

4.5 Optimization studies

Various parameters such as incubation time, temperature and pH were optimized for the PHB production by SE1 isolate.

4.5.1 Effect of time for PHB production

The SE1 isolate was inoculated in a 500ml flask containing 250ml of LB broth and at an interval of every 6 hours the culture was taken and studied for its production of PHB for a total of 72 hours (Table 4).

Table 4: Yield of PHB recovered at different incubation time

S. No	Incubation time(in hours)	PHB production(g/100ml)
1	6	0.002
2	12	0.010
3	18	0.012
4	24	0.015
5	30	0.024
6	36	0.026
7	42	0.030
8	48	0.032
9	54	0.031
10	60	0.030
11	66	0.028
12	72	0.026

The maximum yield of the SE1 isolate was obtained after 48 hours of growth (0.032g/100ml). From the 54th hour a gradual decrease in the growth of the bacteria was noted till 72nd hour.

4.5.2 Effect of temperature for PHB production

The SE1 isolate was incubated in LB broth at different temperatures for 48 hours (Table 5).

Table 5: Yield of PHB recovered at different temperatures

S No	Temperature (°C)	PHB production (g/100ml)
1	27	0.031
2	34	0.030
3	37	0.033
4	42	0.028
5	45	0.026

The maximum PHB production was observed at 37 °C with a yield of 0.033g/100ml. The minimum production was observed at the maximum temperature of 45 °C (0.026g/100ml).

4.5.3 Effect of pH for production

The SE1 isolate was inoculated in LB broth of different pH for 48 hours at 37 °C (Table 6)

Table 6: Yield of PHB recovered at different pH

S No	pH	PHB production (g/100ml)
1	6	0.025
2	6.5	0.030
3	7	0.033
4	7.5	0.036
5	8	0.028

The maximum yield of 0.036g/100ml was obtained at a pH of 7.5. The minimum yield of 0.025g/100ml was obtained at a pH of 6.

The optimization of the PHB production resulted in maximum yield at an incubation time of 48 hours (0.032g/100ml) in a temperature of 37 ^{0}C (0.033g/100ml) with a pH of 7.5 (0.036g/100ml).

4.6 Effect of different cheap sources on PHB yield

Different cheap sources (Fig 4) in different ratios along with mineral salt medium were inoculated with SE1 isolate. The growth in each test tube was extracted by alkaline digestion method and the PHB production was estimated by crotonic acid assay (Fig 5) obtaining the OD values and computing it with a standard graph to get the yield (Table 7).

Fig 5: Sapotta peel waste and its extract

Table 7: PHB production at different concentration of cheap sources

S No	Fruit waste extract concentration in production medium	PHB (g/100ml)	
		Sapota peel waste extract	Starch water from rice
1	10%	0.01	0.012
2	20%	0.017	0.014
3	30%	0.019	0.016
4	40%	0.021	0.018
5	50%	0.023	0.020
6	60%	0.020	0.021
7	70%	0.018	0.019
8	80%	0.016	0.017
9	90%	0.014	0.015
10	100%	0.012	0.013

As it is seen from Table 7, there is a gradual increase in the production of PHB at the increasing concentration of sapota peel extract and also starch water extract in the production medium. The growth of the SE1 isolate was observed maximum (0.023g/100ml) in sapota peel waste extract at a ratio of 5:5 with the production medium and in starch water extract from rice (0.021g/100ml) at 6:4 ratio with the production medium. Further the production starts to decrease when it crosses 60% amendment in the final production medium.

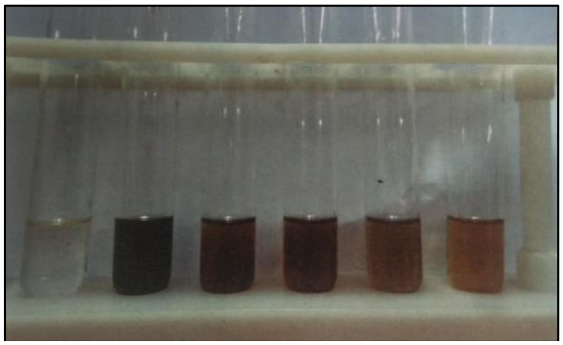

Fig 6: Crotonic acid assay of SE1 isolate in starch water

4.7 Identification of the isolate (Molecular Studies)

4.7.1 Isolation of genomic DNA

Genomic DNA was isolated using 24 hours old bacterial culture, followed by visualisation under UV light after agarose gel separation for determination of molecular weight. Polymerase chain reaction was performed in thermocycler (PTC-100 TM Programmable Thermal Controller, USA) to produce multi-copies of the specified DNA. The PCR reaction was allowed for 30 cycles for amplification of 16S rRNA gene and the product was run on 2% agarose gel electrophoresis along with 100bp DNA ladder mix and visualised under UV light.

The nucleotide sequence of PCR products of both forward and reverse sequences was that of the Bacillus subtilis 16S rRNA gene.

4.7.2 Molecular identification of *Bacillus Subtilis*

The potential PHB producing bacteria, *Bacillus subtilis* was ascertained its systematic position based on 16S rRNA sequence analysis and with aid of computational programme, BLAST homology analysis was also carried out to compare with other 16S rRNA sequences available in the Genebank of NCBI. It revealed that the sequence of SE1 isolate was *Bacillus subtilis.*

4.8 NMR analysis

NMR analysis was used to determine quality of PHB structural composition. The 1H and 13C NMR spectra obtained from PHB samples produced using starch water extracts waste was shown in (Fig 7) and (Fig 8), respectively compared with the commercial PHB (Fulka, Sigma-Aldrich chemicals, USA). Both spectra were found

to match perfectly with each other. The peak observed in the spectra coincide, corresponding to the different types of carbon atoms presented in the PHB structure, [-O-CH-$(CH)_3$-$(CH)_2$-(C=O)-]n. The chemical shift signals of 13C NMR spectrum obtained in the present work in the commercial PHB were agreed with those obtained by (Fabiane *et al*., 2007) [9] in table 8.

Table 8: The chemical shift signals obtained the 13C NMR spectra for PHB sample and commercial PHB, compared to the results by Fabiane *et al*. (2007) [9]

Chemical shift (ppm)		
C atom	**PHB produced by *Bacillus subtilis***	**PHB (Fabiane *et al*., 2007) [9]**
CH_3	19.76	19.65
CH_2	40.80	40.66
CH	67.62	67.48
C=O	169.16	169.03

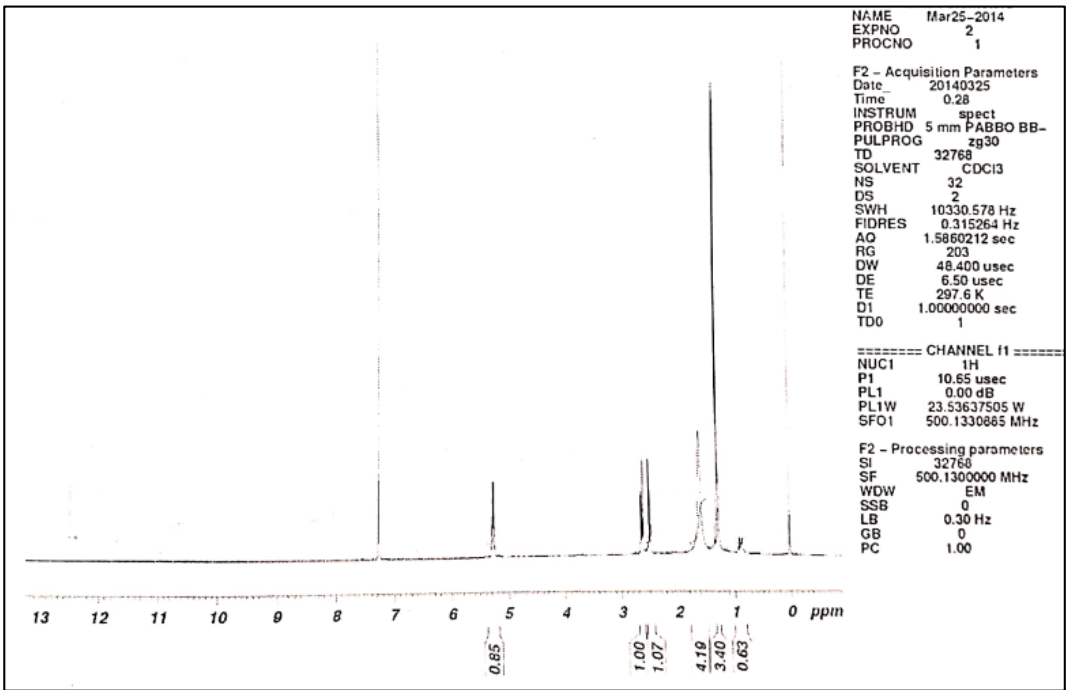

Fig 7: 1H NMR spectra of PHB produced from *Bacillus subtilis*

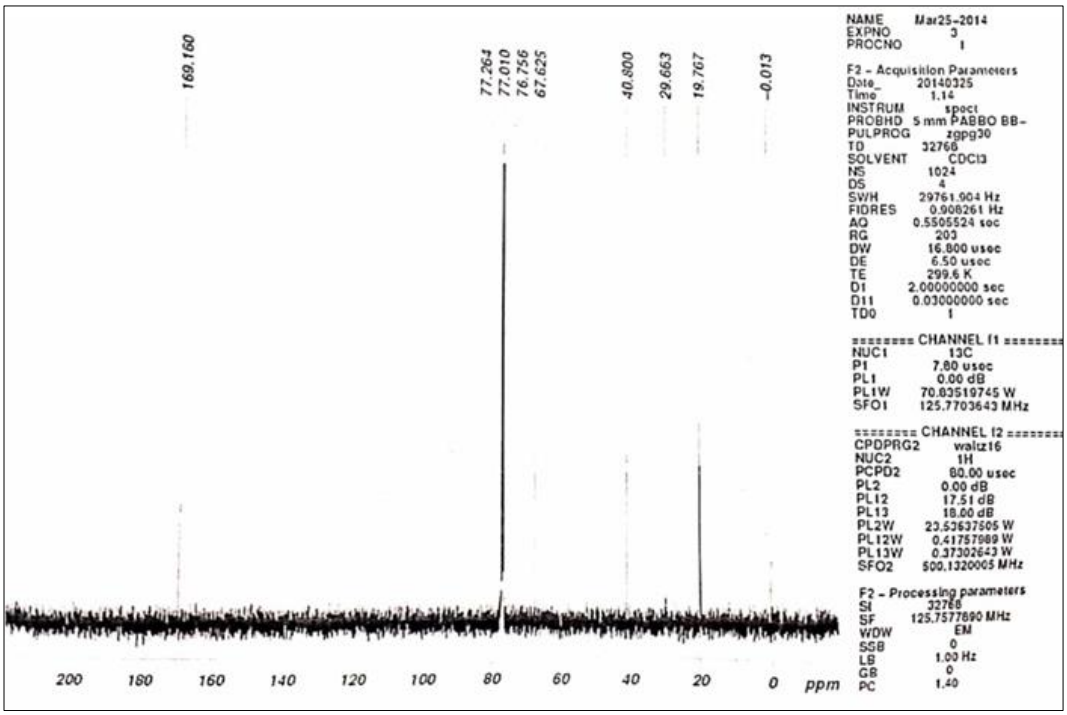

Fig 8: 13C NMR spectra of PHB produced from *Bacillus subtilis*

All the carbon atom peak obtained by PHB produced from *Bacillus subtilis* were in accordance with the standard peak. The CH_3 atom was at 19.76 in or sample while it was 19.65 in the standard, similarity the CH_2,CH and C=O atom's peak of the sample was obtained with a minute difference at 40.80, 67.62, 169.16 while that of the standard was obtained with a minute difference at 40.66, 67.48, 169.03 respectively.

Furthermore, 1H NMR spectrum of PHB produced is seen as Fig 6. The figure showed a number of characteristics of PHB peaks at δ=4.19, 3.40 and 1.07 which corresponds to a -CH doublet, -CH_2 multiplet and –CH_3 doublet respectively (Bonartsev AP *et al*., 2006) [3]. Two additional peaks at δ= 0.63 and δ=1.00 were found may be due to impurities present.

5. Conclusion

Organic wastes are rich in various nutrients as well as they provide many environmental stress conditions to their inhabitants which are the ultimate resources for PHB producers. In this regard, the current study revealed the presence of PHB producers in such an environment studied which can be used for the production of bioplastics in both laboratory as well as industrial scale. The characterization of PHB by various analytical techniques showed the production of pure PHB by the selected isolates which can be studied further by various blending technique to get user friendly, economical goods. The most potent among the isolates were identified to be *Bacillus subtilis* (SE1). Bacillus sp. are ubiquitous in nature and have been reported to possess the capability of the stress condition by various mechanisms. Though starch from rice have been reported to be PHB producers, less study have been conducted so far in this regard. When the PHB production capability was compared between the isolates from organic wastes like sapota peel extract and starch water from rice, the bacteria from both organic wastes showed reasonable amount of production. Hence, the continuous search from the various environmental conditions may provide some more suitable isolates and their genetic modification, for efficient PHB production for commercial use.

6. Reference

1. Beom Soo Kim. Production of poly (3-hydroxybutyrate) from inexpensive substrates. Enzyme and Microbial Technology. 2000; 27(10):774-777.
2. Beom Soo Kim, Ho Nam Chang. Control of glucose feeding using exit gas data and its application to the production of PHB from tapioca hydrolysate by *Alcaligenes eutrophic.* Biotechnology Techniques. 1995; 9(5):311-314.
3. Bonartsev AP, Bonartseva GA, Makhina TK, Mashkina VL, Luchinina ES, Livshits VA *et al.* New poly-(3-hydroxybutyrate) based systems for controlled release of dipyridamole and indomethacin. Prikl Biokhim Mikrobiol. 2006; 42(6):710-5.
4. Bormann EJ, Roth M. The production of polyhydroxybutyrate by *Methylobacterium rhodesianum* and *Ralstonia eutropha*in media containing glycerol and casein hydrolysates. Biotechnology Letters. 1999; 21(12):1059-1063.
5. Bourque D, Pomerleau Y, Groleau D. High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens:* production of high-molecular-mass PHB. Applied Microbiology and Biotechnology. 1995; 44(3-4):367-376.
6. Braunegg G, Lefebvre G, Genser KF. Polyalkanoates, Biopolyester from renewable resources: Physiological and Enginneering aspects. Journal of biotechnology. 1998; 65:127-161.
7. Byrom J, Byrom TK. 'Biopol-Nature's plastic' NCBE Newsletter Summer. 1991; 9-11.
8. Chen G, Patel M. Plastics derived from biological sources: present and future: technical and environmental review. Chemical Reviews. 2012; 112(4):2082-2099.
9. Fabiane CO, Marcos LD, Leda RC, Denise MGF. Characterization of poly (3-hydroxybutyrate) produced by *Cupriavidus necator* in solid state fermentation. Bioresource Technology. 2007; 98:633-638.
10. Freier T. Biopolyesters in tissue engineering applications. Adv. polym. Sci. 2006; 203:1-61.
11. Guocheng Du, Jian Yu. Green Technology for Conversion of Food Scraps to Biodegradable Thermoplastic Polyhydroxyalkanoates. Environ. Sci. Technol. 2002; 36(24):5511-5516.
12. Kumar Sudesh, Tadahisa Iwata. Sustainability of Biobased and Biodegradable plastics. CLEAN- Soil, Air, Water. 2008; 36(5-6):433-442.
13. Law JH, Slepecky RA. Assay of poly-hydroxybutyric acid. J. Bacteriol. 1961; 82:33-36.
14. Lee SY. Review Bacterial Polyhydroxyalkanoates. Biotechnology & Bioengineering. 1996; 49:1-14.
15. Mona K Gouda, Azza E Swellam, Sanaa H Omar. Production of PHB by a *Bacillus megaterium*strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiological Research. 2001; 156(3):201-2.
16. Nisha V, Ramadas, Sudheer Kumar Singh, Carlos Ricardo Soccol, Ashok Pandey. Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. 2009; 52(1):17-23.
17. Reddy CSK, Ghai R, Rashmi, Kalia VC. Polyhydroxyalkanoates: an overview. Bioresource Technology. 2003; 87:137-146.
18. Rusendi D, John D Sheppard. Hydrolysis of potato processing waste for the production of poly-β-hydroxybutyrate. Bioresource Technology. 1995; 54(2):191-196.
19. Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog. Polym. Sci. 2000; 25:1503-1555.
20. Ting Yen Huang, Kow Jen Duan, Shih Yow Huang, C Will Chen. Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferax mediterranei*. Journal of Industrial Microbiology and Biotechnology. 2006; 33(8):701-706.
21. Whittenbury R, Philip KC, Wilinkson JE. Enrichment, isolation and some properties of methane utilizing bacteria. J Gen Microbial. 1970; 61:205.
22. William J Page, Anthony Cornish. Growth of *Azotobacter vinelandii* UWD in Fish Peptone Medium and Simplified Extraction of Poly-β-Hydroxybutyrate. Appl Environ Microbiol. 1993; 59(12):4236-44.
23. Yu PH, Chua H, Huang AL, Lo W, Chen GQ. Conversion of food industrial wastes into bioplastics. Applied Biochemistry and Biotechnology. 1998; 70(1):603-614.