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Optimization, Purification and Characterization Of Polyhydroxybutyrate(PHB) Produced By *Bacillus Cereus* Isolated From Sewage

Sneha Paul[1,]*, Changam Sheela Sasikumar[1], Balakumuran MD[2]

[1]Department of Cellular & Molecular Biochemistry, Frontier Mediville (Affiliated to University of Madras), A unit of Frontier Lifeline & Dr.K. M. Cherian Heart Foundation, India R-30-C, Ambattur Industrial Estate Road, Mogappair, Chennai - 600 101, India
[2]Department of Botany, University of Madras, Guindy, Chennai-600025, India

Abstract : Background of study : The biologically synthesized biodegradable polymers are a suitable alternative to counter the various environmental problems modelled by conventional non-biodegradable plastics. Polyhydroxybutyrate (PHB) is the simplest and commonest member of Polyhydroxyalkonates (PHA).
The present study aimed to investigate the isolating PHB producing bacteria from domestic sewage and its production with cheap sources or waste products.
Materials and Methods : Domestic sewage samples, isolated pure cultures of bacteria were screened for PHB production by Sudan Black staining. The selected isolate was identified by 16S rRNA typing. The efficiency of PHB production by fermentation was increased by optimizing the various associated process parameters and media components like incubation time, temperature, pH, carbon, nitrogen sources and an alternatively Cheap sources was substituted other than commercial salts that gives the maximum yield also were explored. PHB production was confirmed by Fourier transform infrared spectroscopy.
Results : *Bacillus cereus* gives the maximum production rate compared with the other bacterial strains. The PHB has been confirmed by FTIR with the strong peak at 1723cm^{-1} is one of the characteristic peak of Polyhydroxybutyrate along with it various other peaks also confirms the presence of PHB in the sample. Agro and fruit pulp waste was used as alternative substrate for the growth of the bacteria producing polyhydroxylbutyrate, it gave the best result of production.
Conclusion : *Bacillus cereus*sewage isolates, efficiently synthesized PHB using various cheap sources and fruit pulp proves an alternative and better way of synthesizing biodegradable bioplastic.
Keywords : Agro waste, FT-IR, PHB, Optimization, Sudan Black staining.

Introduction:

The drawback of bioplastic is there cost of production that always remains under the higher side. Traditional plasticare a source of petroleum products which remains permanently in the environment. In this peculiar condition, emerging development of bioplastic has great role to play in the environment.

Bio-plastics made up of PHA (polyhydroxyalkonate) are a major substitute to traditional plastics. PHA is produced inside the bacteria naturally under stressful condition. They are found as intracellular granules in

bacteria [28]. PHA based plastics can be synthesized using various renewable sources such as low-molecular weight, fatty acids, alcohols, saccharides etc. Such bioplastics are completely degradable to carbon dioxide and water through natural microbial mineralization.

PHAs have been a polymer producing factories to switch from ecologically harmful end-of-the-pipe production towards sounder technologies [9], still dated all the polyester production has been done in the biotechnology sector from refined raw material such as glucose, sodium propionate etc. but canbe altered or substantiate with cheaper agricultural waste products such as molasses, maltose, glycerol phase from biodiesel production, whey etc. [36] Polyhydroxybutyrate (PHB) is synthesized from acetate or butyrate via beta-hydroxyybutyrl –Co A. It's described as "the first example of a true thermoplastic from biotechnology". PHB can be produced from bacteria under "stressful condition" and substantiated with other "carbon sources". In response, it built up the sugar into granules of PHB essentially in an effort to preserve them for later metabolization [7, 37]. PHB is a stiff &rather brittle polymer of high crystallinity whose mechanical property is not unlike polystyrene which is less brittle and more temperate resistant. PHB-based plastic substitutes are less flexible than traditional plastics; they are completely biodegradable and leave behind no residue [12].

Starch is agro feed stock hydro colloid biopolymer [32]. Amylose is linear polymer comprise approximately 20%w/w starch, all this are used as natural filler component and also incorporated with synthetic plastic matrices as rapidly biodegradable component [9], Glycerol is often used as plastizer in starch blends to create softness &reliability. Cellulose which is also another component got from nature acts as natural fiber instead of bio plastics. These are natural bioplastics of biodegradablility and no chemical properties. Due to lot of limitation towards the production of bioplastics, we have incorporated agricultural waste products towards synthetic nutrients. Thus shows cost effective and environment friendly nature.

Materials and methods:

Collection, processing and isolation of pure cultures:

The bacteria involved in this study were isolated from sewage sample present in Chennai. A total of 7 different sewage samples from Chennai were collected and transported to laboratory for further analysis. The collected samples were placed in sterile container and stored at 4°C. Each gram/ml of the sample was serially diluted up to 10^9 dilutions using sterile saline and the diluted samples from 10^7 were spread on the sterile Nutrient agar plates. The plates were incubated at 37°C for 24 hours. Plates were examined and different isolates were further purified by repeated single colony isolation. The purity of cultures was checked periodically by streaking liquid cultures onto Nutrient agar.

Screening of isolates for the production of PHB:

The isolated bacteria cultures were checked for the presence of PHB by Sudan black staining methods. [10, 11]

Selection of highest PHB production:

7 strains of positive culture are inoculated in the production medium[12]. Cells were harvested and analysed for the maximum production of PHB by law &spleckeymethod[13], the strain giving highest amount of PHB was selected for further studies on the effect of nutrients & environmental condition.

Peptone	- 2.5gm
Sodium chloride	- 2.5gm
Yeast extract	- 1gm
Beef extract	- 0. 5gm
Glucose	- 2%
Distilled water	- 1000ml

Production medium was prepared and sterilized in autoclave at 121°C for 15 minutes.

Optimization studies:

Time course study on PHB production:

Time course on PHB production from selected strain under the optimum condition was studied and their overall production were investigated using production medium [12] to see effect of various temperatures (28, 30, 32, 35, 37), pH (5-9pH), different carbon source (Glucose, Sucrose, Fructose, Maltose, Lactose, Mannitol) and nitrogen source (Peptone, urea, Caesin, Ammonium nitrite, Ammonium Sulphate, Potassium nitrite), effect of carbon and nitrogen ratio (0. 5, 0. 75, 1, 1. 25, 1. 50, 1. 75, 2).

Production of PHB from Agro waste:

Various natural agro products like rice bran, wheat bran, baggase, cassava powder, potato starch, cassava powder, corn waste, coconut oil cake, jack fruit seed powder, toor powder and whey waste water from dairy industry were used (5% concentration) assubstrates (by replacing sugar components in production medium) for effective and alternative PHB production.

Production of PHB from fruit pulp waste:

Various fruit pulp waste like pineapple, fig, grape, and sappota and mixed was used as substrates (by replacing sugar components in production medium) for effective and alternative PHB production.

Pretreatment of agro waste and fruit pulp:

Two types of treatment were followed;First is direct infusion method carried out by drying and pulverizing the agro and fruit pulp waste. The powdered substrate was used as supplement of carbon source along with the minimal salts available. Second is acid treatment method carried out by hydrolyzing the agro and fruit pulp waste using (0. 5-5%)sulphuric acid and autoclaved at 121°C for 30 min. The samples were filtered and supernatant was neutralized by sodium hydroxide(6N). Then hydrolyzates at 5 % concentration were used as suppliments of carbon source. Pure culture of *Bacillus cereus* were added along with suppliments for 36 hours at 30 ºC.

Extraction of PHB: [18]

After every 24hrs of incubation, 10ml of cultured broth was taken and centrifuged at 8000 rpm for 15min. The supernatant was discarded and to pellet obtained, 10ml of sodium hypochlorite was added and then incubated for 1 hr. at 30^0c. After the incubation period the tubes with mixture was again centrifuged at 5000 rpm for 15 min. Serial washes of pellet was done by distilled water, acetone and methanol, then dissolved by 5ml of boiling chloroform. The chloroform was evaporated and PHB powder was collected, stored at RT for further analysis.

Quantitative estimation of PHB: [13]

Approximately 5-50µg of powder obtained after extraction was dissolved in chloroform and transferred to fresh test tube. The chloroform was evaporated and then 10ml of concentrated sulphuric acid was added and then the solution was heated at 100^0c for 10 min. The solution is cooled and the absorbance was measured at 235nm against sulphuric acid blank.

Cell dry weight:

The cell pellet (PHB) that is got from the production medium [19] was compared with newly designed production medium. It gives highest cell dry weight when compared with normal production medium.

Production medium by consortium:

In this study so far, one isolate has been used to produce PHB. The production of PHB by a mixed culture was studied by utilizing two more bacillus strains. The strains *Bacillus subtilis, Bacillus megaterium* utilized for consortium studies were obtained from the culture collection, CAS in Botany [20].

Two isolates were inoculated simultaneously to observe the effect it has on PHB production. Inoculums of the two organisms *Bacillus subtilis* and *Bacillus megaterium* were prepared separately and inoculated into the PHB production medium along with the earlier selected isolate. After 48 hrs, the amount of PHB produced was quantitatively determined by Law and Slepecky method.

Determination of PHB by FT-IR :

PHB was extracted with chloroform and cold methanol, dried and subjected to FT-IR spectroscopy. The samples were prepared in KBr pellet and FT-IR (Bruker) absorption spectrum was measured in room temperature of range 4000–600cm ^{1}. The absorbance peak values obtained were compared with the available literature values and the sample was confirmed for the presence of PHB [21].

Molecular Identification of Bacteria:

Characterization and molecular identification of bacteria, the preliminary characterization of the isolated strain was done using bergey's manual of systemic bacteriology [15]. The identity of the isolate was determined by sequence analysis of the 16s rRNA gene. The overnight cultured bacterial cells were lysed with lysozyme and the DNA was extracted by the phenol: chloroform (1:1) extraction method described by ausubel et al. [16]. The 16s rRNA was amplified in PCR with the primer sequence.

Forward primer: 5' AACGGCTCACCAAGGCGACG 3'
Reverse primer: 5' GTACCGTCAAGGTGCCGCCC 3'.

The amplified region was then sequenced and subject to blast analysis for analysing its phylogeny [17].

Results:

In this study, twenty eight completely different microorganism strains were isolated from different sewerage samples collected in Chennai and microorganism strains were screened by Sudan black staining for dark purple colorgranules that were determined in seven strains. The positive strains were isolated and used for further studies (Figure- 1).

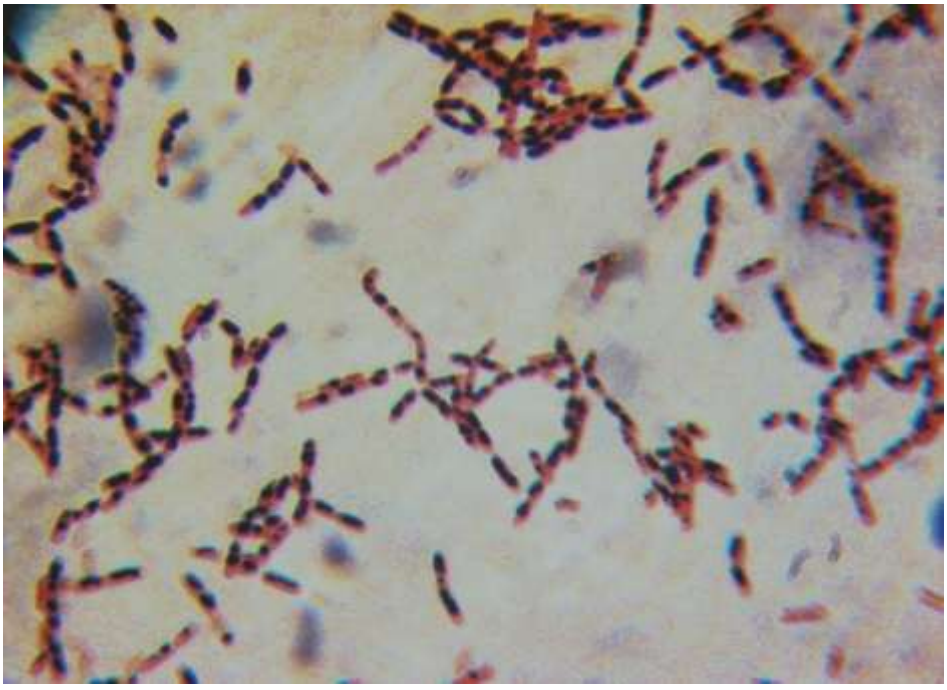

Figure I: Sudan Black B staining of Bacillus cereus

The selected seven isolates were inoculated in production medium [12] for screening of the simplest polyhydroxybutyrate producer. Among the seven isolates the simplest polyhydroxybutyrate producer was hand-picked supported PHB assay victimisation the Law and Slepecky technique and subjected to more improvement studies.

In order to work out the ideal production time for optimum Polyhydroxybutyrate production, the samples were collected at half-dozen hours intervals and analysed for the estimation of polyhydroxybutyrate. In the growth study, we tend to found that choice twelfth hour the assembly of PHB was terribly low and after there's a gradual increase within the production. Most PHB production was ascertained from twenty fourth hour to sixty sixth hour; from there forward gradual decrease within the PHB production was ascertained. the results analysed at different time intervals, it absolutely was determined that the utmost production of PHB was at the thirty sixth hour (Figure-2).

Figure II: Chemical assay for Polyhydroxybutyrate determination

Table – I: Effect of Incubation Time on PHB Production

S.No	Time of culture withdrawal	PHB (µg/ ml)
1	6^{th} hour	44
2	12^{th} hour	102
3	18^{th} hour	158
4	24^{th} hour	220
5	30^{th} hour	240
6	36^{th} hour	340
7	42^{th} hour	280
8	48^{th} hour	270
9	54^{th} hour	262
10	60^{th} hour	250
11	66^{th} hour	240
12	72^{th} hour	228

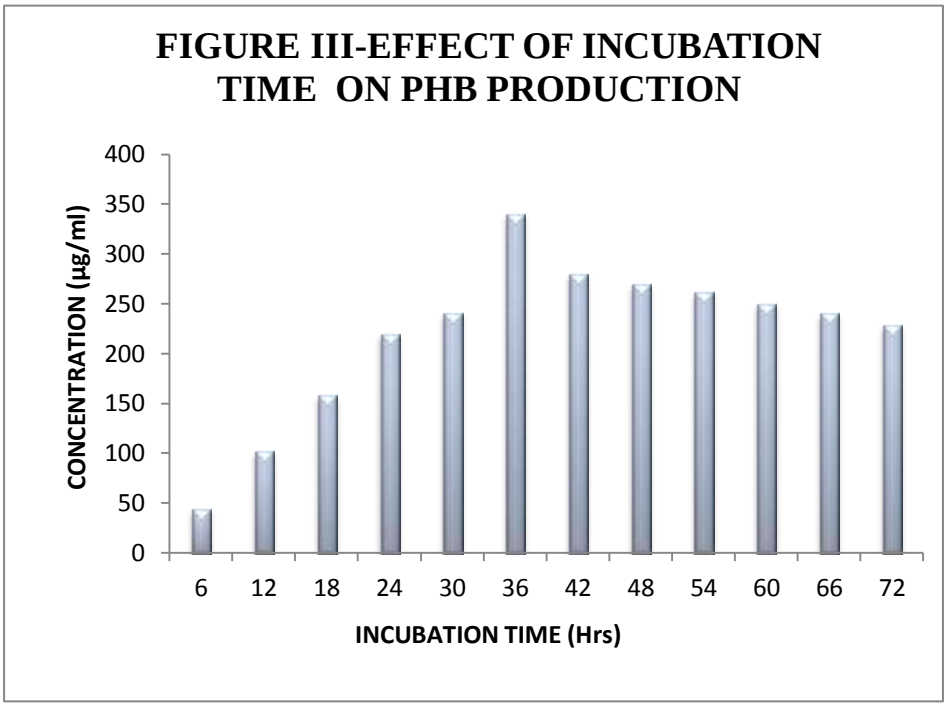

Figure III-Effect of Incubation Time on PHB Production

The environmental parameters like pH, temperature shows nice influence on the development of the organisms and therefore the production of Polyhydroxybutyrate. So as to work out the impact of the incubation temperature, four temperatures were tested: 25, 28, 32, 35 and 37°C for the higher Polyhydroxylbutyrate production, completely different incubation temperatures were maintained within the production method. Supported the readings, it had been ascertained that the chosen strain encompasses an optimum temperature at 37°C (Figure -3).

In order to enhance PHB production of the chosen isolate, the influence of pH scale was studied. Results of PHB production ar shown in (Figure - 4), pH scale affects each growth and PHB production. There was a general increase in PHB production and growth of bacterium with increasing pH scale.

Table –II: Effect of Temperature on PHB production

S.No	Temperature (°C)	PHB (µg/ ml)
1	28	310
2	30	360
3	32	320
4	35	312
5	37	300

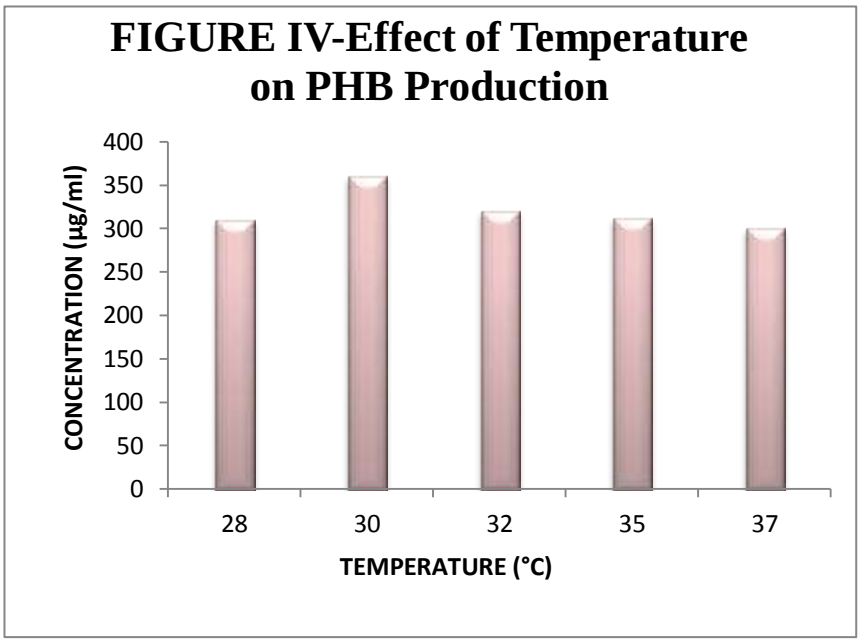

Figure IV-Effect of Temperature on PHB Production

Table - III: Effect of pH on PHB Production

S.No	Ph	PHB (µg/ ml)
1	5	310
2	6	324
3	7	342
4	8	382
5	9	320

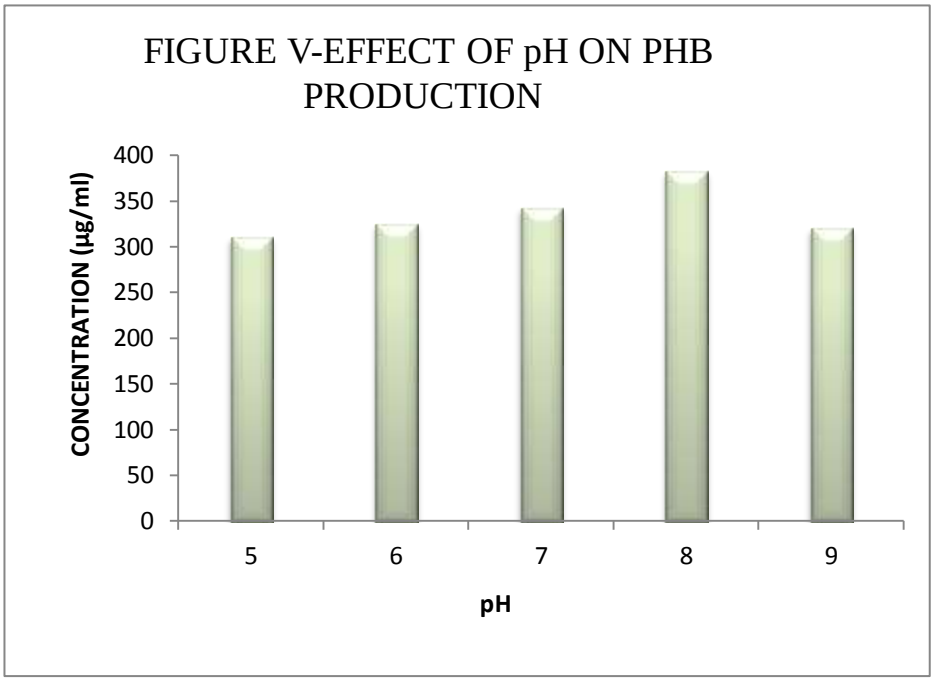

Figure V-Effect of pH on PHB Production

Different carbon sources were screened for optimum production of Polyhydroxylbutyrate for the chosen isolate. Aside from sucrose, the remainder of the carbon sources gave a satisfactory production of PHB. Its most productivity shown in fructose was taken as best carbon supply (Figure -5) besides glucose and maltose was found to be a lot of appropriate for PHB accumulation.

The nitrogen sources, unit the secondary energy sources for the organisms and that they play a very important role within the growth of the organism. Completely different nitrogen sources were screened for optimum production of Polyhydroxylbutyrate for the chosen isolates. All-time low PHB production was obtained with casein and urea. Ammonium sulphate and potassium nitrate gave far more satisfactory result among the chosen element supply. As far as productivity concerned, peptone shows the utmost PHB production during this study (Figure -6).

Table – IV: Effect of Different Carbon Sources on PHB Production

S.No	Carbon source	PHB (µg/ ml)
1	Glucose	350
2	Fructose	368
3	Sucrose	294
4	Maltose	344
5	Lactose	340
6	Mannitol	320

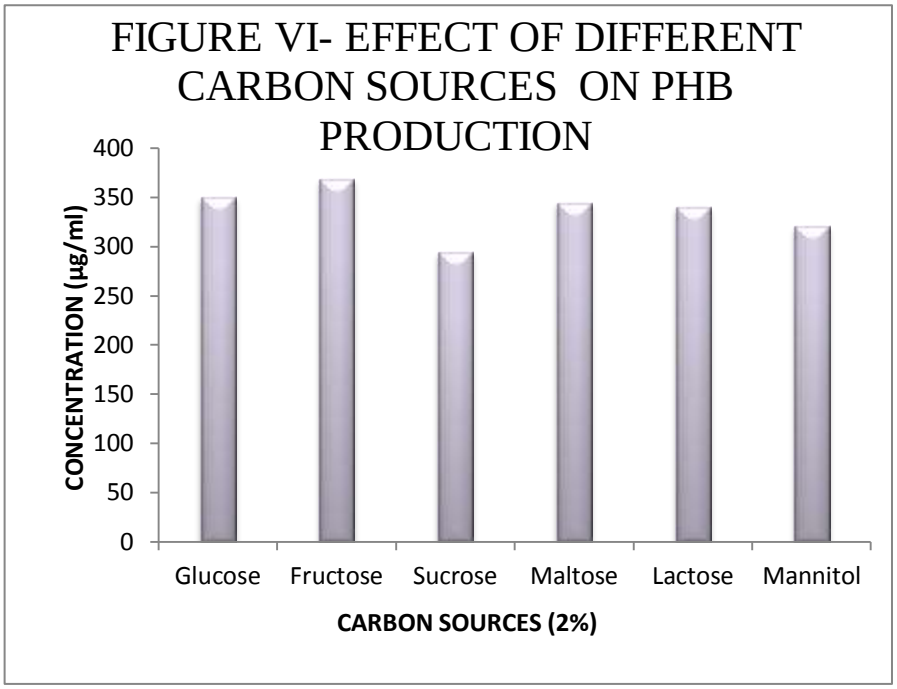

Figure VI- Effect of Different Carbon Sources on PHB Production

Table – V: Effect of Different Nitrogen Sources on PHB Production

S.No	Nitrogen sources	PHB (µg/ ml)
1.	Peptone	382
2.	Potassium nitrate	324
3.	Ammonium sulphate	328
4.	Urea	224
5.	Casein	254
6.	Ammonium nitrate	342

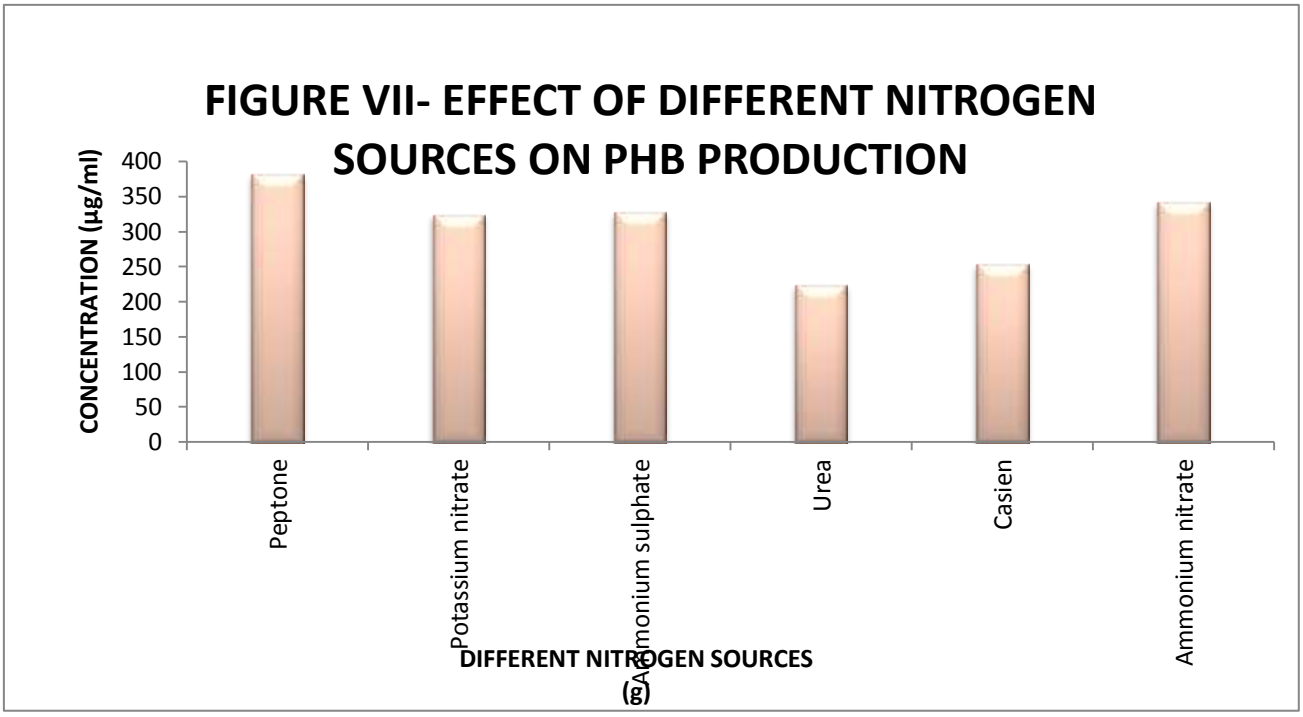

Figure VII- Effect of Different Nitrogen Sources on PHB Production

Table –VI: Effect of Different Carbon Nitrogen ratio On PHB Production

S.No	Carbon - Nitrogen ratio	PHB (µg/ ml)
1.	0.25%	224
2.	0.5%	380
3.	0.75%	326
4.	1%	302
5.	1.25%	242
6.	1.5%	230
7.	1.75%	200
8.	2%	180

Different Carbon - Nitrogen ratio were performed for optimum production of Polyhydroxy- butyrate for the chosen isolate. 0. 5% shows most productivity as so much because the production of PHB worries (Figure - 7).

Alternative to artificial substrate, completely different low-cost substrates like rice bran, wheat bran, baggase, cassava powder, potato starch, cassava powder, corn waste, copra oil cake, jack fruit powder, toor powder, fruit waste and whey waste water from farm industry(Figure –8-9) were used for PHB production. Among a budget sources, wheat bran shows the most effective low-cost substrate for PHB production. Next to agro waste, Fruit pulp wastes gone unused was collected and used as substrates for the assembly of microorganism generating PHB, the sugars gift within the fruit pulp was used as media substitute. *B. cereus* was studied for the accumulation of PHB by sappota, pineapple, fig, grape, papaya and mixed fruit pulp. PHB yields obtained are shown in Figure-11. It had been ascertained that sappota and mixed fruit pulp were best feedstock among different sources wherever as grape pulp didn't yield outstanding quantity of PHB.

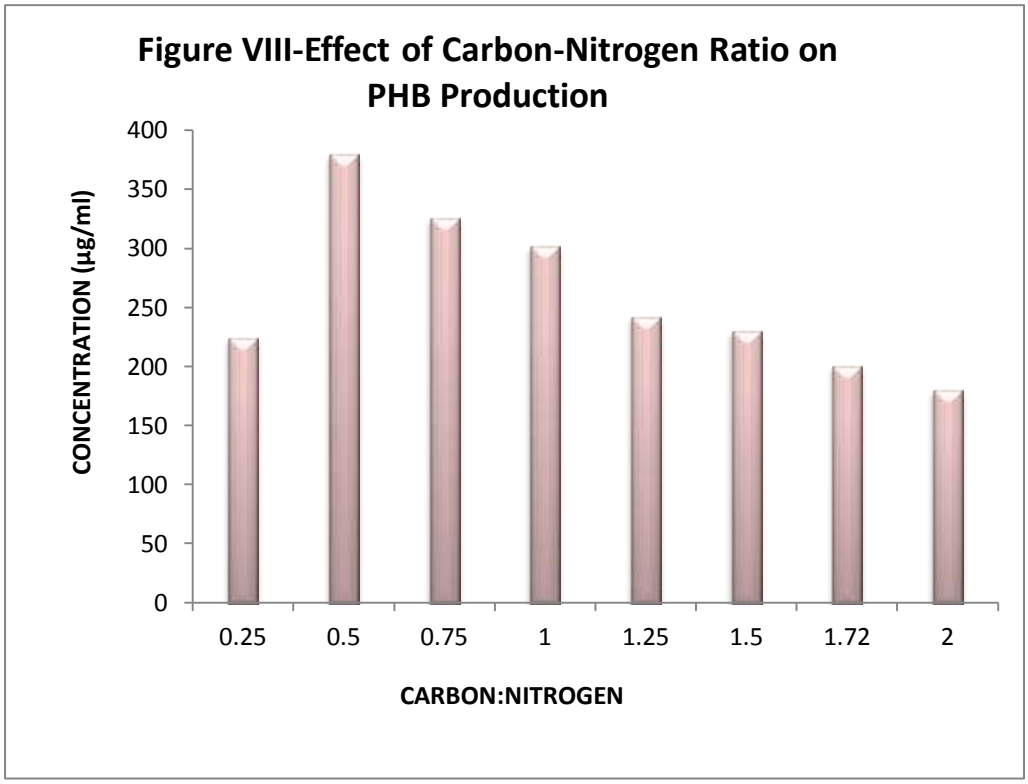

Figure VIII-Effect of Carbon-Nitrogen Ratio on PHB Production

Table – VII: Production of PHB from Agro products

S.No	Cheap sources	PHB (µg/ ml)
1.	Rice Bran	222
2.	Wheat Bran	268
3.	Baggase	120
4.	Potato starch	228
5.	Cassava powder	234
6.	Coconut oil cake	142
7.	Jack fruit powder	128
8.	Corn waste	168
9.	Toor powder	158
10.	Fruit waste	182
11.	Whey waste water	148

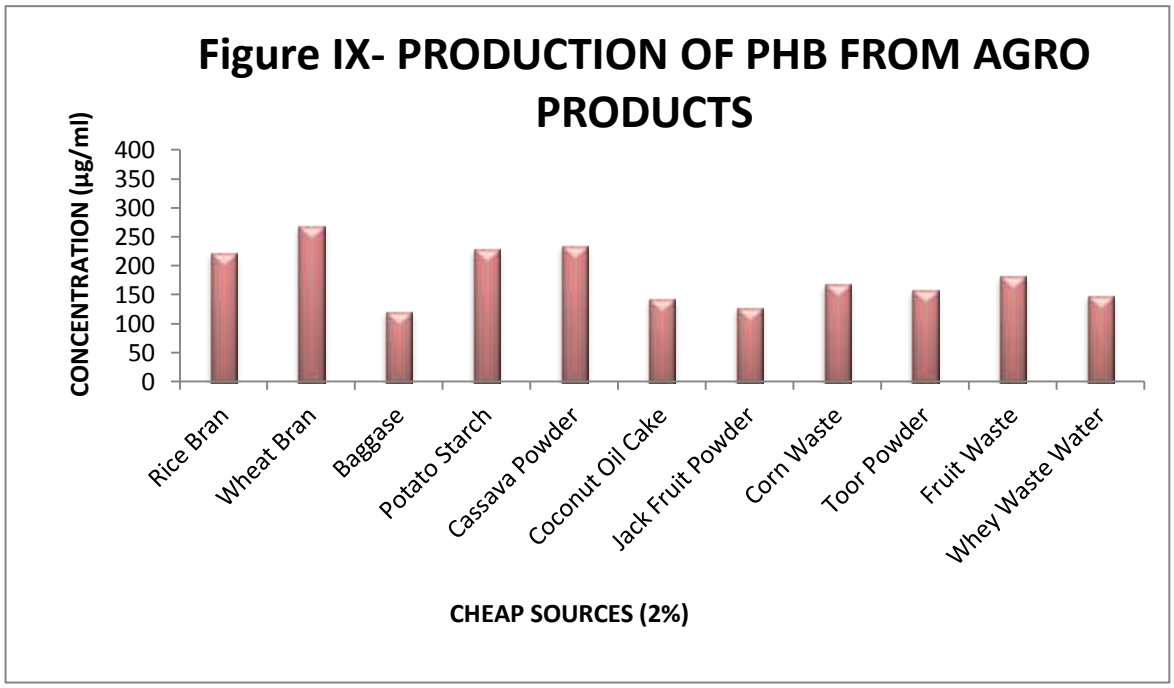

Figure IX- Production Of PHB from Agro Products

Table – VIII: Production of PHB from fruit pulp products

S.No	Fruit pulp	PHB (µg/ ml)
1.	pineapple	220
2.	sappota	268
3.	fig	120
4.	grape	228
5.	pappaya	234
6.	mixed fruit pulp	298

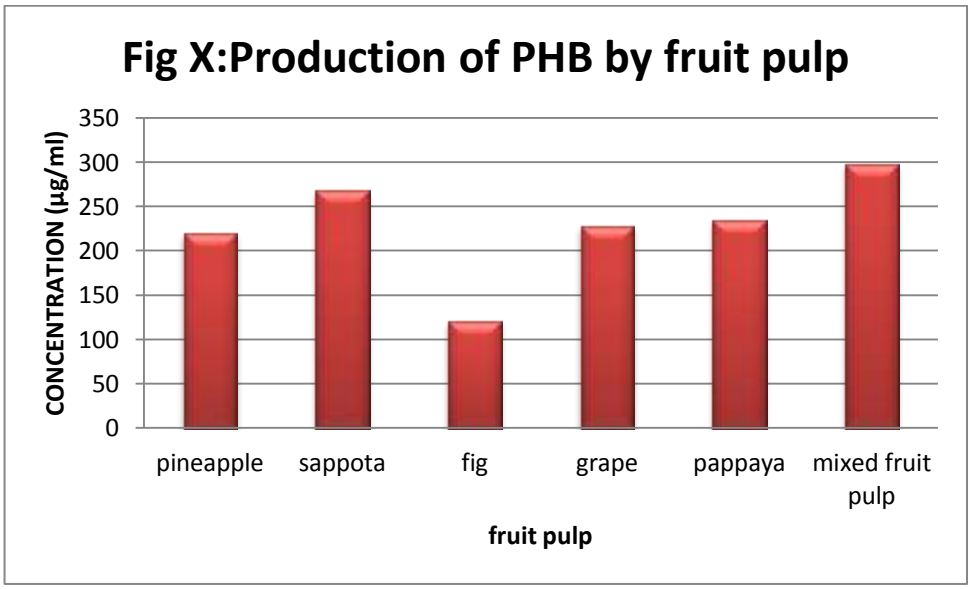

Figure X- Production Of PHB from FRUIT PULP

To characterize the PHB, purified PHB was subjected to FT-IR analysis. FT-IR spectrographic analysis has been demonstratedas a robust tool in deciding the presence of polymers particularly PHB. The FTIR spectrum of PHB is shown in Figure-10. There was a strong absorption phenomenon at 1280 cm-1 and alternative surface assimilation bands at 1730, 1453, 2934, and 1724 were determined. The absorbance peak values obtained were compared with earlier reports to substantiate the compound

Three bacterial strains *Bacillus cereus, Bacillus subtilis* and *Bacillus megaterium* were inoculated into the same Production Broth. The amount of PHB produced was assayed by Law and Slepecky method. 520 µg per ml of PHB was estimated from the assay. Mixed culture gives more prominent amount of PHB production.

The twenty four hours microorganism culture of *Bacillus Cereus* strain PTK PHB 1grown in Luria Bertani medium at 37°C beneath aerobic conditions at one hundred revolutions per minute was collected and also the individual genomic DNAs was isolated. The isolated genomic deoxyribonucleic acid of the *Bacillus Cereus* strain PTK PHB 1 was visualized by UV ligh transmitter, it's shown in figure-11-12.

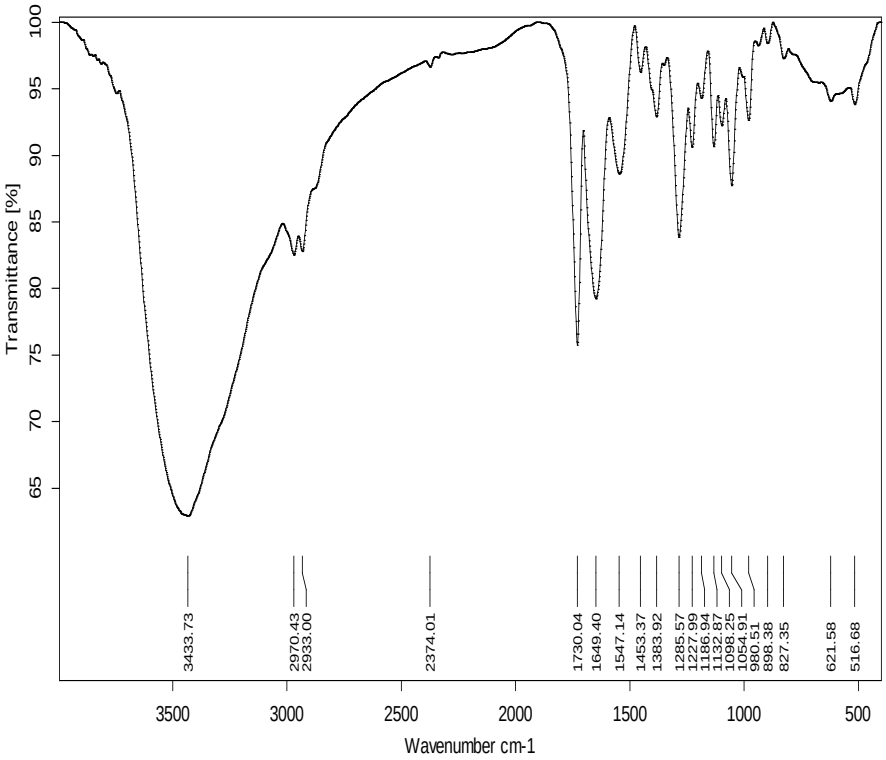

Figure XI: Fourier Transform-Infrared Spectroscopy of Polyhydroxybutyrate

Figure XII: Purified Polyhydroxybutyrate

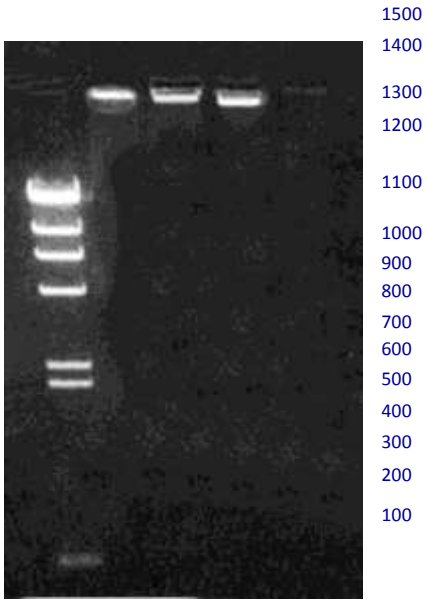

Figure XIII: Genomic DNA

Figure XIV: PCR Amplified DNA

Polymerase chain reaction was performed in Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA) to provide multi-copies of the required deoxyribonucleic acid. The PCR reaction was allowed for thirty cycles for amplification of 16S rRNA gene. Then the PCR product was run on 2 % agarose gel electrophoresis along with 100 bp DNA ladder mix and visualized under UV light. The nucleotide sequence of PCR products of each forward and reverse sequences of the *Bacillus Cereus* strain PTK PHB 1 16S rRNA gene ~ 1476.

The potential PHB is manufacturing bacterium, *Bacillus Cereus* strain PTK PHB 1 was determined by its systematic position supported 16S rRNA sequence analysis and with the help of process program. BLAST homology analysis was carried out to compare with alternative 16S rRNA sequences out there within the GenBank of NCBI. It disclosed that the sequence of *Bacillus Cereus* strain PTK PHB 1. Description of sequences manufacturing important alignmentfor analysing the phylogenetic tree has been shown in figure-13. The sequence analysis of 16S rRNA gene for the isolate *Bacillus Cereus* strain PTK PHB 1 shows the most similarity (99%) with alternative *Bacillus Cereus* from the database.

CGGGGGCGTCCTATACATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAG
TTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACT
CCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAA
AGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT
AAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTC
TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAG
TGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGA
GGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC
TAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCC
TGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATC
TCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGA
ATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCACGAGAGTTGGTACACCGAGTCGTGGGGTAACATTTTGGGA
GGCCAGCCGCCCTTAAGGGTGGGGGACACAGAAAGAAATTGGGGGGTGTAAAC

Figure XV:16S ribosomal RNA gene, partial sequence (1476 bps) for Bacillus cereus strain PTK PHB 1.

Table IX: Sequences Producing Significant Alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ833025.1	Bacillus cereus strain JY7 16S ribosomal RNA gene, partial sequence	2595	2595	97%	0.0	99%
HQ833023.1	Bacillus cereus strain JY2 16S ribosomal RNA gene, partial sequence	2595	2595	97%	0.0	99%
FJ627946.1	Bacillus sp. TR 16S ribosomal RNA gene, partial sequence	2590	2590	97%	0.0	99%
AJ577287.1	Bacillus cereus partial 16S rRNA gene, strain F 3371/93	2590	2590	97%	0.0	99%
CP002508.1	Bacillus thuringiensisserovarfinitimus YBT-020, complete genome	2588	3.612e+04	97%	0.0	99%
HQ833024.1	Bacillus cereus strain JY5 16S ribosomal RNA gene, partial sequence	2588	2588	96%	0.0	99%
GU982920.1	Bacillus cereus strain GXBC-1 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
GU826150.1	Bacillus anthracis strain H13 16S	2588	2588	97%	0.0	99%

	ribosomal RNA gene, partial sequence					
GU566355.1	Bacillus sp. D12(2010) 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
GU297610.1	Bacillus anthracis strain U13 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
GQ280380.1	Bacillus cereus strain Q1 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
CP001598.1	Bacillus anthracis str. A0248, complete genome	2588	2.838e+04	97%	0.0	99%
CP001215.1	Bacillus anthracis str. CDC 684, complete genome	2588	2.836e+04	97%	0.0	99%
FJ868808.1	Bacillus sp. BSRA4 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
CP001407.1	Bacillus cereus 03BB102, complete genome	2588	3.615e+04	97%	0.0	99%
FJ641036.1	Bacillus cereus strain IMAUB1034 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
FJ641017.1	Bacillus cereus strain IMAUB1040 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
CP000227.1	Bacillus cereus Q1, complete genome	2588	3.360e+04	97%	0.0	99%
FJ529033.1	Bacillus sp. S-JS-3 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
FJ462697.1	Bacillus thuringiensis strain GS1 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
CP001177.1	Bacillus cereus AH187, complete genome	2588	3.611e+04	97%	0.0	99%
EU887289.1	Bacillus cereus strain ZB 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
EF062509.2	Bacillus sp. MCCB 101 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AM747221.1	Bacillus cereus partial 16S rRNA gene and ITS1, strain INRA C43	2588	2588	97%	0.0	99%
AM747220.1	Bacillus anthracis partial 16S rRNA gene and ITS1, strain CEB95-0033	2588	2588	97%	0.0	99%
AB295052.1	Bacillus cereus gene for 16S rRNA, strain: NK1	2588	2588	97%	0.0	99%
AM062677.1	Bacillus cereus 16S rRNA gene, isolate AB1A	2588	2588	97%	0.0	99%
AE017355.1	Bacillus thuringiensisserovarkonkukian str. 97-27, complete genome	2588	3.612e+04	97%	0.0	99%
AE017334.2	Bacillus anthracis str. 'Ames	2588	2.838e+04	97%	0.0	99%

	Ancestor', complete genome					
AE017225.1	Bacillus anthracis str. Sterne, complete genome	2588	2.838e+04	97%	0.0	99%
AY425946.1	Bacillus cereus strain G9241 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
CP000001.1	Bacillus cereus E33L, complete genome	2588	3.352e+04	97%	0.0	99%
AY138278.1	Bacillus cereus strain G3317 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AY138277.1	Bacillus cereus strain 2000031503 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AY138276.1	Bacillus cereus strain 2000031491 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AF290562.1	Bacillus sp. AH 526 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AB159768.1	Bacillus sp. A4-20-12 gene for 16S rRNA	2588	2588	97%	0.0	99%
AF176321.1	Bacillus anthracis 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AF155955.1	Bacillus thuringiensis strain B8 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AF155950.1	Bacillus anthracis strain Ames 16S ribosomal RNA gene, partial sequence	2588	2588	97%	0.0	99%
AJ577288.1	Bacillus cereus partial 16S rRNA gene, strain ATCC 4342	2588	2588	97%	0.0	99%
AJ577283.1	Bacillus cereus partial 16S rRNA gene, strain RIVM BC00068	2588	2588	97%	0.0	99%
AE016879.1	Bacillus anthracis str. Ames, complete genome	2588	2.838e+04	97%	0.0	99%
GU325714.1	Bacillus cereus strain MUJ 16S ribosomal RNA gene, partial sequence	2586	2586	96%	0.0	99%
GU113075.1	Bacillus sp. P5(2009) 16S ribosomal RNA gene, partial sequence	2586	2586	97%	0.0	99%

Tree Method - Fast minimum evolution

Figure XVI: Phylogenetic tree

Discussion:

Polyhydroxybutyrates are naturally formed macromolecules throughout the growing cycle of the organisms, and so as said as natural polymers. Polyhydroxybutyrate (PHBs) play necessary roles within the attachment of microorganism cells to a surface and in building and maintaining the three-dimensional, advanced structure of microorganism biofilms. Polyhydroxybutyrate (PHB) made by several microorganism has been investigated by microbiologists, molecular biologists, biochemists, chemical engineers, chemists, chemical

compound specialists and medical researchers over the past a few years. Applications of a PHB are within the field ofbioplastics, fine chemicals, implant biomaterials, medicines, and biofuels are developed.

In our study, the utmost production of PHB productivity was seen at thirty sixth hour. Contradictory effects of incubation time on PHB production by numerous microorganisms are reported. [12] Intheir investigation found that a far better PHB production was determined once forty fifth hour. [22] Indicated that PHB was a growth associated product and its accumulation considerably augmented once all cultures reached the exponential part (after eighteen hrs.) until stationary part (about 48-60 hrs.). The utmost values were achieved at sixty hrs. cultivation [23], assess the result of your time on the assembly of PHB and rumored that Brevibacteriumcasei SRKP2 shows the utmost PHB production (0. 135 g/L) at forty eight hours.

Maximun PHB production productivity will increase by optimum temperature at 37°C and eight pH. Various investigators have concerned their efforts in examining the results of pH on PHB production [24] rumored the optimum growth was determined at 8 pH.Amendment in initial pH of the medium showed a powerful influence on the production of PHB. Even a small difference in pH from the optimum purpose denoted a unexpected reduction in PHB production. Initial pH worth of 7.5, gave the utmost production of PHB of twenty fifth. [25] Though, the optimum pH for PHB production is commonly near 7. 5, [26] was in agreement with our findings.

In subsuiting completely different composition of carbon and nitrogen was varied and therefore the results obtained fructose and peptone were best compared with alternative synthetic chemicals. The biomass and biodegradable polymers were made by QGR on1ce fructose used because the carbon sources and yeast extract used as a nitrogen sources, severally [27] showed accumulation of PHB by *Alcaligenes faecalis* treatment fructose because the carbon supply. Sucrose, besides glucose and fructose was found to be a lot of appropriate for cell growth additionally as PHB accumulation (69. 4 dry out cell weight) by *Bacilli mycoides* RLj B-017 and accumulation of PHB was determined to be growth associated. Similar results were reported for *Rhizobium meliloti* by [28]. Once sucrose was used at optimum level (55 g), most quantities of biomass and PHB yields were found in higher concentration of CSL (25 g). The PHA production was done completely different possible sugars were tested and it absolutely was found that most biomass was made with 2% (w/v) sucrose [29].

The highest worth of PHB concentration for each strain was earned in several media supplemented with ammonia sulfate after ninety six hours incubation. On the other hand, the most effective growth was determined with ammonia salts like chloride, sulphate, salt and phosphate. These results prove that the yield of PHAs isn't associated with the rise in growth. It stated [30] the assembly of PHB by *Alcaligeneseutrophus*during a artificial medium with third glucose supplemented with many ammonia substrates and located that the most effective growth and PHB production were obtained with ammonia sulfate as nitrogen supply. These results are in line with those obtained from [31].

A cultivated [22] *R. sphaeroides*N20 in the culture medium with 4 g/l acetate and 0. 02 g/l (NH4)2SO4 as a carbon and nitrogen source, respectively. The C/N ratios were varied in 12:1, 6:1, 3:1, 0. 3:1 and 0.15:1(mole C/mole N).The optimum C/N ratio was found to be 6:1 in which *R. sphaeroides*N_2O possessed the best PHB content of 73. 2% of DCW and 5.94 ± 0.11 g/l PHB concentration.This confirmed the previous section results (Nitrogen source and concentration). Too high (12:1) and low (<6:1) C: N ratio caused a significant decline of PHB concentration (0. 36-3. 67 g/l) and PHB content (6. 7-47. 0% of DCW) in the cells.

Major drawback of PHB production is the cost of production, so hereby we have come up with the unique idea of synthesizing the PHB by agro waste and fruit pulp waste. In our studies wheat bran and mixed fruit pulp gave the maximum yield. The effect of different concentrations of cane molasses has been studied by [30] which showed that the production of PHB was high between 0.1–0. 3 g/l. However, the percentage of PHB on the basis of cell dry weight was higher with a 0.1% concentration. The maximum production of PHB was obtained with cane molasses and glucose as sole carbon sources (40. 8, 39. 9 per mg cell dry matter, respectively). The best growth was obtained with 2 % wheat bran, while the maximum yield of PHB (46. 2% per mg cell dry matter) was obtained with 2% rice bran [14] which is similar to our studies. Although PHB production was huge in earlier studies, the raw materials used in those studies are costlier. For the production of PHB (PHAs), the cost of the carbon source is supposed to be cheap and yield should be the maximum [32]. According to [33], many carbon sources derived from wastes like whey, cane molasses and sugar beet molasses

was used for production of PHB and for mineral source mixture of different salts was used since. However, Dairy industry effluent, a waste with high organic content, one of the cheapest and easily available resources was supplied as a carbon source. Similar kind of studies with fruit pulp has been obtained by [37].

Synergetic effect of multi bacterial culture gave the promising result while compared with single bacteria. Similiar activities were done by several researchers claimed the integrity and effectiveness system using mixed culture. Unfortunately, they only emphasize the mixed culture using two or three well-known bacterial. The idea of PHB production using mixed culture was ignited owing to the PHB role as a metabolic intermediate of wastewater treatment and as a biodegradable plastic. A laboratory study was conducted to assess the optimal conditions for PolyHydroxybutyrate production using saponified sunflower oil using a mixed culture isolated from sludge. It was also shown that sludge subjected to an aerobic condition in mixed cultures could accumulate high amounts of PHA by manipulating the cycle length (HRT study). [20]

FTIR analysis confirms the PHB and been compared with the earlier reported articles, strong peak at 1723cm^{-1} indicates the stretching of C O ester bond which is the characteristic of Polyhydroxybutyrate. Also the other peaks obtained at 1280cm−1 (corresponding to C H stretch) and 1182cm−1 (corresponding asymmetric C O C bridge), 1383 cm^{-1}, 1453 cm^{-1}, 1730 cm^{-1}, are much closer to the identified characteristic peaks for PHB [35]. Similar results were reported by [36] and [37] showing strong adsorption band at 1279 cm-1 and other adsorption bands at 1379, 1454, 2928, 1724 and 3750 cm-1 for - CH3, -CH2, -CH, C=O, and O-H groups respectively.

16S rRNA factor sequences to check microorganism phylogenesis and taxonomy has been out and away the foremost common work factor used for variety of reasons. These reasons embrace, its presence in the majority microorganism, typically existing as a multigene family, or operons; the perform of the 16S rRNA factor over time has not modified, suggesting that random sequence changes area unit} a a lot of correct measure of your time (evolution) and therefore the 16S rRNA factor (1, 500 bp) is giant enough for information [38]. The sample isolated from waste mattersample confirms *Bacilli Cereus* by 16sRNA sequencing

Conclusion:

Every day hike in fuel price substantially results in the rise of the price for plastics, forcing the world to consider alternatives for petrochemical plastics. The biodegradable in nature, easy availability and cost effective PHB make a suitable material to replace synthetic plastics in many applications. A biotechnological process of synthesizing makes it expensive in culturing bacteria and production of PHAs. Our studies show that Bacillus cereus, a sewage isolates, efficiently synthesized PHB using synthetic mediumcompared with various cheap sources and fruit pulp, shows an alternative and better way to synthesis bioplastics. Agro and fruit pulp waste for cultivating PHB producing bacteria can be commercialized targeting a global market, PHAs are the future bioplastics.

Reference:

1. FM Ausubel, R Brent, RE Kingston, DD Moore, JD Seidman, K Struhl. John Wiley and Sons, New York 1987.
2. Beaulieu M, Beaulieu Y, Melinard J, Pandian S, Goulet J (1995). Influence of ammonium salts and cane molasses on growth of Alcaligeneseutrophus and production of polyhydroxybutyrate. Appl. Environ. Microbiol. 61:165–169.
3. BhairaviGhate, Prashant Pandit, Chandrashekhar Kulkarni, Deepti D Mungi and Twisha S. Patel (2011). PHB production using novel agro-industrial sources from different bacillus species. International Journal of Pharma and Bio Sciences. 2(3):242-49.
4. Braunegg G, Lefebvre G and Genser KF (1998). Polyalkanoates, Biopolyester from Renewable Resources: Physiological and Engineering Aspects. Journal of Biotechnology. 65: 127-161.
5. Burdon KL, Stokes JC, Kimbrough CE (2004). Studies of the common aerobic spore-forming Bacilli staining for fat with Sudan Black B- stain. Journal of Bacteriology. 43:717-24.
6. Chandra and Rustgi R (1998). Biodegradable polymers. Progressive Polymer Science. 23:1273-35.
7. Dawes EA and Senior PJ (1973). The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. 10:135-266.

8. DesoukyAM,Abd-El-Haleem, Sahar A ZakI, AShraf T Abuelhamd, Amro Amara and Gadallah MS Aboelreesh (2007). Biosynthesis of polyhydroxyalkanotes in wildtype yeasts. Journal of Applied Sciences and Environmental Management. 11(3): 5 - 10.
9. GerhartBraunegg, Rodolfo Bona, Florian Schellaue and Elisabeth Wallner (2002). Polyhydroxyalkanoates (PHAs): sustainable biopolyester production. Polimery. 47: 7-8.
10. Grothe C, Otto D, Frotscher M, Unsicker K (1989). A role of basic fibroblast growth factor for rat septal neuron. EXS. 57:251-8.
11. Hong K, Sun S, Tian W, Chen GQ, Huang W (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl. Microbiol. Biot. 51(4):523-526.
12. Indira Mikkili, Abraham P Karlapudi, Venkateswarulu TC, John Babu D, SB Nath, Vidya P. Kodali (2014). Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample. Int. J. PharmTech Res. 6(2): 850-57.
13. Kshamalakshman (2004). Studies on the production of biopolymer by Rhizobium Sps. Their isolation and characterization. [dissertation] Doctor of philosophy in microbiology. CAS Botany, University of Madras.
14. Law John H and Slepecky A Ralph. Assay of poly--hydroxybutyric acid (1960). J. Bacteriol. 82:33-36.
15. Lee SY (1996). Bacterial Polyhydroxyalkanoates. Biotechnology and Bioengineering. 49: 1-14. Microbiology Review, 103, 131-140.
16. Lee SY, Middelberg APJ, Lee YK (1997). Poly (3-hydroxybutyrate) production from whey using recombinant Escherichia coli. Biotechnology Letters. 19: 1033–35.
17. MacidNurbas and TulinKutsal (2004). Production of PHB and P (HB-co-HV) Biopolymers by Using AlcaligenesEutrophus. Iranian Polymer Journal. 13 (1): 45-51.
18. Madison LL, Huisman GW (1999). Metabolic engineering of poly(3 hydroxyalkanoates): from DNA to plastic. Microbiology Molecular Biology Reviews. 63, 21–53.
19. Mahmoud M Berekaa and Ali M Al Thawadi (2012). Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by Bacillus megaterium SW1-2: Application of Box-Behnken design for optimization of process parameters. African Journal of Microbiology Research 6(4): 838-45.
20. Martin O, Schwach E, Averous L, Couturier Y (2001). Properties of biodegradable multilayer films based on plasticized wheat starch. Starch. 53(8): 372-80.
21. MohdFadhilMd Din, ZainiUjang, Mark Van Loosdrecht and MohdAzlan Ahmad (2008). Polyhydroxyalkanoates (PHAs) Production FromSaponified Sunflower Oil In Mixed Cultures Under Aerobic Condition. JurnalTeknologi. 48: 1-19.
22. Mona K. Gouda, Azza E. Swellam, Sanaa H. Omar (2001). "Production of PHB by a Bacillus megaterium strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources". Microbiological Research. 156: 201–207.
23. Nisha V Ramadas, Sudheer Kumar Singh, Carlos Ricardo Soccol and Ashok Pandey (2009). Polyhydroxybutyrate Production using Agro-industrial Residue as Substrate by Bacillus sphaericus NCIM 5149. Brazilian Archives of Biology And Technology. 52: 17-23.
24. NurYuksekdag Z, BelmaAslim, YavuzBeyatli, NazimeMercan (2004). Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by Bacillus subtilis 25 and Bacillus megaterium 12. African Journal of Biotechnology. 3 (1): 63-66.
25. Patel J B (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol. Diagn. 6**:**313-21.
26. Prakash M Halami (2008). Production of polyhydroxyalkanoate from starch by the native isolate Bacillus cereus CFR06. World Journal of Microbiology and Biotechnology. 24: 805–12.
27. NJ Palleroni, NR Krieg, JG Holt (1984). Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore. 141-198.
28. Peters V and Rehm B. H. A (2005). In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS MicrobiolLett. 248: 93–100.
29. Ram Kumar Pandian S, Deepak V, Kalishwaralal K, Muniyandi J, Rameshkumar N, Gurunathan S (2009) . Synthesis of PHB nanoparticles from optimized medium utilizing dairy industrial waste using Brevibacteriumcasei SRKP2: A green chemistry approach. Colloids and Surfaces B: Biointerfaces. 74: 266-73.

30. Ram Kumar Pandian S, Deepak V, Kalishwaralal K, Rameshkumar N, Muniyandi J, Gurunathan S (2010). Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by Bacillus megaterium SRKP-3. Bioresour. Technol. 101: 705-11.
31. Ramadas NV, Singh SK, Soccol CR, PandeyA (2009). Polyhydroxybutyrate production using agro-industrial residue as substrate by Bacillus sphaericus NCIM5149. BrazArchBiolTechno. l52:17–23.
32. Salmoral EM, Gonzalez ME, Mariscal MP (2000). Biodegradable plastic made from bean products. Industrial Crops and Products. 11: 217-25.
33. V Strubel, KH Engesser, P Fischer, HJ Knackmuss (1991). J. Bacteriol. 173: 1932-37.
34. Sangkharak K and Prasertsan P (2008). Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic Journal of Biotechnology. 11(3): 0717-3458.
35. SureshbabuRam KumarPandian, Venkatraman Deepak, Kalimuthu Kalishwaralal, Neelamegam Rameshkumar, MuniyandiJeyaraj and SangiliyandiGurunathan (2010). Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by Bacillus megaterium SRKP-3. Bioresource Technology. 101:705–711.
36. Verlinden RAJ, Hill DJ, Kenward MA, Williams CD and Radecka I (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. Journal of Applied Microbiology. 102:1437-49.
37. Vieira MGA, Da Silva, MA Dos Santos LO and Beppu MM (2011). Natural-based plasticizers and biopolymer films: a review. Eur. Polym. J. 47:254–263.
38. Vishnuvardhan Reddy S, Thirumala M and Mahmood SK (2009). Production of PHB and P (3HB-co-3HV) biopolymers by Bacillus megaterium strain OU303A isolated from municipal sewage sludge. World Journal of Microbiology and Biotechnology. 25: 391–97.

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