FINAL REPORT

Optimization of Polyhydroxyalkanoates (PHA) accumulation by bacterium isolated from oil contaminated soil and characterization of the biopolymer

Submitted by

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Project title: Optimization of Polyhydroxyalkanoates (PHA) accumulation by bacterium isolated from oil contaminated soil and characterization of the biopolymer

Abstract

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Bioplastics are considered good substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties. Polyhydroxyalkanoates (PHAs) are energy reserve polymers produced by bacteria to survive periods of starvation in natural habitats. The microflora from the habitat containing oil possesses a high potential to accumulate important polymers such as polyhydroxyalkanoates (PHAs).

Considering the industrial interest of PHA, this work was undertaken to screen PHA producing organisms from oil contaminated habitats. Bacterial isolates from diverse oil containing habitats like oil mills, soil from petrol pumps, baggase were screened for polyhydroxyalkanoates (PHAs). A total of 40 isolates were scored positive for PHA production by Sudan Black B staining. *Bacillus megaterium* spps. showed maximum PHA accumulation. Optimization for maximum accumulation of PHA of 21g/l was achieved when inoculum size of 0.5 at OD 540nm of this isolate was grown in E2 mineral medium containing 2% Glucose and supplemented with 0.04% Yeast extract and pH 8 at 30°C under shaker condition (120rpm) for 72hrs. Partial characterization of PHA was carried out using Fourier transform infrared spectroscopy (FT-IR).

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Introduction

In the recent years, there has been increasing public concern over the harmful effects of petrochemical-derived plastic materials in the environment. Nature's built-in mechanisms and self-regulation ability cannot tackle novel pollutants since these are unfamiliar to it. This has prompted many countries to start developing biodegradable plastic [1].Mineral oil prices will rise substantially in the next century, forcing the world to consider alternatives for petrochemical plastics.

Bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of synthetic plastics [2]. Among these, polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties [3, 4].The renewable nature and biodegradability of PHAs make them suitable materials to replace synthetic plastics in many applications [5].

Bacteria that accumulate PHAs can be divided into two groups based on the culture conditions required for PHA synthesis.

a. The first group of bacteria requires the limitation of an essential nutrient such as nitrogen, phosphorous, magnesium or sulphur for the synthesis of PHA in presence of excess carbon source. *Alcaligenes eutrophus*, *Protomonas extorquens* and *Protomonas oleovorans* are some of the bacteria included in this group.
b. The second group of bacteria, which include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii* and recombinant *E. coli* do not require nutrient limitation for PHA synthesis and can accumulate polymer during growth [6]

PHA is accumulated by a wide range of bacteria when a carbon source is provided in excess and one or more essential nutrient is limited [7, 8].

Significance of the study

With an over increasing population the demand on renewable resources is tremendous. The production of PHA can provide an alternative to the conventional plastic. PHAs have properties similar to synthetic plastics but its property of biodegradability and biocompatibility make them a resourceful contender to replace the conventional plastic available in the market today.

There are three types of biodegradable plastics - photodegradable, semi-biodegradable, and completely biodegradable
In last few decades a lot of work has been done on isolation and characterization of PHAs from various microbial sources. One of the limitations in the popularization of biodegradable plastics is the cost of production. Hence, in order to reduce the cost, different cheaper, locally available wastes can be used for PHA accumulation. The utilization of wastes like textile waste, agro industrial waste, sewage, paper and pulp waste by various microorganisms in order to produce biopolymers can provide a promising alternative thus reducing the cost involved with the accumulation of biopolymers by use of defined media.
This work will provide an alternative biopolymer along with a bioremediation method for waste disposal. The proposed work is therefore of global significance.

Material and methods

i. Collection of samples

Organisms were isolated from machine oil contaminants, soil from petrol pump areas, baggase waste from sugar mill, mangrove samples, and contaminated honey samples. The samples were collected in a sterile screw cap bottle and stored at 4°C until further use.

ii. Enrichment and Isolation of samples

The enrichment of the samples was done in Sterile Nutrient Broth. The enriched samples were then isolated on solid media containing Sterile Nutrient Agar.

iii. Screening of potential PHA producers

The isolated organisms were screened for presence of lipid granules by Sudan Black B staining [**9**]. The organisms which showed the presence of black granules by the staining were scored as potential PHA producers. The isolates that scored positive for lipid production were grown on different minimal media deficient in nitrogen source.

The media used for PHA accumulation:

a) Sterile E 2 agar medium [**10, 11**]
b) Sterile PHA production medium [**12**]

The isolates were spot on the two media and incubated at room temperature for 72-96 hours. The plates were then stained with ethanolic Nile Blue A solution. Colonies exhibiting fluorescence when exposed to U V light were scored as PHA accumulators [**13**].

iv. Extraction of the accumulated PHA by Rapid Hypochlorite method

The positive scored isolates were grown in the respective liquid medium. The isolate were grown in 100 ml Erlenmeyer flask containing 50 ml of the medium. The flask were inoculated with 2.5% (v/v) overnight grown culture; and incubated at Room temperature for 72 hr. The biopolymer produced was then extracted by the modified Rapid hypochlorite method [**14**].The centrifuged cell pellet were suspended in 5 ml of 2% sodium hypochlorite solution and incubated at 37°C for 30 min. The PHA pellet obtained was then washed with acetone. After washing, the pellet was dissolved in 5 ml of chloroform. The chloroform was allowed to evaporate by pouring the solution in glass Petri plate and placing it at Room temperature. The PHA powder was quantified after evaporation of chloroform.

v. Quantification of the extracted PHA

The polymer extracted was assayed quantitatively by UV spectrophotometer at 235 nm using Slepecky and Law method [**15**].The amount of PHA in sample was quantified by comparing the obtained readings with standard crotonic acid assay as PHA on heating in acid is depolymerized to crotonic acid.

vi. Characterizing the promising isolate

Prominent selected isolate(s) were identified on the basis of morphological, cultural and biochemical properties.16S rRNA sequencing was outsourced to confirm identity of the isolate(s). The sequence results were compared using homologous analysis online with NCBI BLAST.

vii. Optimization studies

To increase the yield of PHA accumulation, stepwise modification of various parameters will be done for optimization. For each experiment of optimization, PHA accumulation will be carried out as described above, after adjusting the parameters. The extracted PHA will then be quantified spectrophotometrically at 235 nm. [**15, 16,17**]

- Incubation period: The yield of PHA accumulated will be checked at different time intervals ranging from 12 hr to 96 hr and the withdrawal of the sample will be done after every 12 hr.
- Inoculum size: The inoculum size will be adjusted to 2.5% to 25% and used for PHA accumulation.
- Aeration: The effect of aeration will be examined by using regular incubator for non-agitating flasks and shaker incubator for agitating flasks.
- Carbon source: The effect of various carbon sources such as glucose, lactose, sucrose, maltose, fructose, mannitol and xylose will be examined. Oils like olive oil, groundnut oil and coconut oil also will be used as a carbon source. Carbon source may be substituted with wastes like used cooking oil, fruit wastes and whey.
- Nitrogen source: Organic and inorganic nitrogen sources such as soya meal, peptone, yeast extract, beef extract, urea, sodium nitrate, ammonium sulphate and ammonium nitrate will be used.
- C: N ratio: To test the effects of C/N ratios on PHA production, the concentration of the nitrogen source will be fixed and the concentrations of carbon source will be adjusted to obtain different C/N ratios.
- Temperature: PHA production at various temperatures ranging from 20 to 60°C will be checked.
- pH: The variation in the yield of PHA with respect to the pH will be checked over a pH range of 2-10.
- Parameters on biopolymer extraction: Varying concentration of Sodium hypochlorite will be used to check the effect on biopolymer extraction.

viii. Scale-up studies

The optimized parameter will then be used to scale up the PHA yield. The scale up will be done to 3 liters at laboratory scale.

ix. Microporous Biofilm formation

Microporous biofilm formation will be done using chloroform which will be evaporated slowly by placing in trays in the cold room at 4°C. **[18, 19]**.

x. Polymer Characterization and analysis

The extracted polymer is then analyzed for composition of the biopolymer by FTIR analysis **[20]**

Results

1) Enrichment and Isolation:

Different types of colonies were obtained on the Nutrient agar plates using machine oil contaminants, soil from petrol pump areas, baggase waste from sugar mill, mangrove samples, and contaminated honey samples. The colonies obtained after 24 hrs of incubation were further studied.

2) Staining and Microscopic examination:

70 different isolates were studied for the presence of lipid granules by the Burdon's method. A total of 46 out of 70 isolates showed presence of black lipid granules. Both Gram negative and Gram positive bacteria showed the presence of lipid granules.

3) PHA accumulation in bacteria (plate assay method):

To confirm the presence of PHA, the isolates were grown on suitable media like E2 agar plates (nitrogen deficient medium) and PHA production agar plates (complex medium) at RT for 72 hours. The isolates were then stained with alcoholic Nile blue A solution and observed under transilluminator, Of the 46 isolates under study, 24 were found to accumulate PHA, which was observed by the exhibition of orange fluorescence. The intensity of fluorescence was seen proportional to the amount of PHA.

5) Extraction of PHA by hypochlorite method

The isolates were grown in E2 medium and PHA production media and accumulated PHA in the cell was extracted by Sodium Hypochlorite method. White colored PHA powder was obtained after extraction.

6) Assay of PHA

The PHA extracted from the isolate was estimated quantitatively by Slepecky and Law method. Also a standard graph was plotted using crotonic acid to measure the amount of PHA.

Estimation of standard graph using Crotonic acid

Table V- 6.1: Standard table for assay of crotonic acid.

Concentration in ppm	Volume of Stock (ml)	Volume of Diluent (ml)	Total volume (ml)	Absorbance at 235 nm
10	0.5	4.5	5	0.1979
20	1.0	4.0	5	0.4197
30	1.5	3.5	5	0.6361
40	2.0	3.0	5	0.8426
50	2.5	2.5	5	1.0565
60	3.0	2.0	5	1.2740
70	3.5	1.5	5	1.4700
80	4.0	1.0	5	1.6326
90	4.5	0.5	5	1.7912
100	5.0	0	5	1.9348
Blank	0	5	5	0

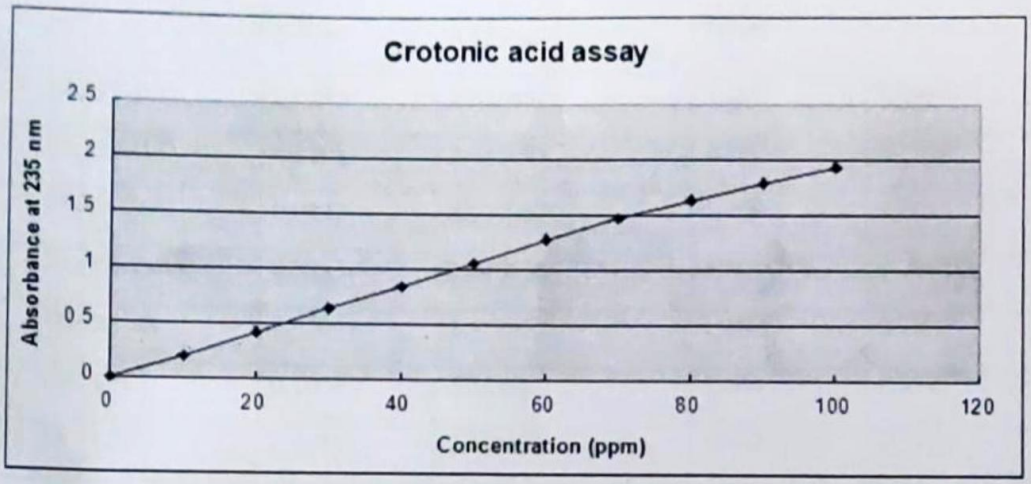


Fig V 6.1: Standard graph of Crotonic acid.

7) Estimation of PHA produced:

Table V- 7.1: Amounts of PHA produced in E2 medium

Sr. No.	Culture no.	Absorbance @ 235 nm	Conc. in ppm obtained from standard graph (A)	Dilution used	PHA in g/L
1.	BWS-6	0.0196	2.459891	1:100	0.0984
2.	HN10	0.0244	2.653869	1:100	0.106
3.	NZeeS	0.0705	4.516872	1:100	0.1086
4.	J1G	0.0588	4.044049	1:100	0.1616
5.	J2S	0.0325	2.981208	1:100	0.1192
6.	Bag1	0.0247	2.665993	1:100	0.1066
7.	Bag3	0.0542	3.858153	1:100	0.1543
8.	BWS-5	0.0758	4.731057	1:100	0.1892
9.	MM2	0.1327	7.030511	1:100	0.2812
10.	MM-4	0.0765	4.759345	1:100	0. 1894

11.	6-IG	0.0957	5.53526	1:100	0.2212
12.	19-ILO	0.0195	2.45585	1:100	0.0984
13.	37-ILO	0.0198	2.467973	1:100	0.0996
14.	9-IMO	0.0877	5.211962	1:100	0.2084
15.	10-IMO	0.0243	2.649828	1:100	0.1065
16.	45-ID	0.0209	2.512427	1:100	0.1005
17.	L1C	0.1038	5.862599	1:100	0.2345
18.	L2C	0.1513	7.782179	1:100	0.3113
19.	PH-9	0.0425	3.38533	1:100	0.1354
20.	PH-10	0.0515	3.74904	1:100	0.1499
21.	L1	0.0928	5.418065	1:100	0.2164
22.	F1	0.0545	3.870277	1:100	0.1548
23.	IN-1	0.0518	3.761164	1:100	0.1504
24.	D2	0.0504	3.704587	1:100	0.1500
25.	42I=Lo	0.0518	3.761164	1:100	0.1504
26.	2I=Mo	0.0195	2.45585	1:100	0.098
27.	36I=Lo	0.0928	5.418065	1:100	0.2164
28.	N1G	0.7040	32.786	1:100	1.311
29.	J1G	0.803	38.5	1:10	0.154
30.	CO-1	0.731	35.6	1:200	2.848

Table V- 7.1: Amounts of PHA produced in PHA production medium

Sr. No.	Culture no.	Absorbance @ 235 nm	Conc. in ppm obtained from standard graph (A)	Dilution used	PHA in g/l
1.	BWS-6	0.099	5.652	1:100	0.225
2.	HN10	0.047	3.581	1:100	0.1432
3.	NZeeS	0.043	3.416	1:100	0.1366
4.	J1G	0.100	5.709	1:100	0.2283
5.	J2S	0.073	4.632	1:100	0.1852
6.	Bag1	0.015	2.266	1:100	0.0906
7.	Bag3	0.043	3.426	1:100	0.1370
8.	BWS-5	0.117	6.416	1:100	0.2566
9.	MM2	0.097	5.604	1:100	0.2242
10.	MM-4	0.094	5.445	1:100	0.2178
11.	6-IG	0.030	2.892	1:100	0.1156
12.	19-ILO	0.064	4.270	1:100	0.1708
13.	37-ILO	0.012	2.153	1:100	0.0861
14.	9-IMO	0.034	3.030	1:100	0.1212
15.	10-IMO	0.035	3.094	1:100	0.1238
16.	45-ID	0.075	4.679	1:100	0.1871
17.	L1C	0.093	5.442	1:100	0.2177
18.	L2C	0.085	5.103	1:100	0.2052
19.	PH-9	0.117	6.380	1:100	0.2552
20.	PH-10	0.032	2.961	1:100	0.1184
21.	L1	0.063	4.230	1:100	0.1710

22.	F1	0.020	2.492	1:100	0.0997
23.	IN-1	0.081	4.957	1:100	0.1982
24.	D2	0.069	4.472	1:100	0.1788
25.	42I=Lo	0.042	3.0	1:100	0.12
26.	2I=Mo	0.011	0.5	1:100	0.02
27.	36I=Lo	0.020	2.492	1:10	0.0099
28.	N1G	0.3229	13.156	1:100	0.526
29.	J2S	0.3757	16.255	1:100	0.6502
30.	CO-1	0.176	14	1:100	0.56

8) Identification of the isolate:

The isolate that showed maximum PHA production was characterized on the basis of 16S r RNA sequencing and was identified as *Bacillus megaterium* JHA (CO-1).

9) Optimization studies:

The isolate showed maximum accumulation in E2 mineral medium. Thus this medium was used for further optimization for various physico-chemical parameters.

a) Incubation period:
The optimum time for PHA accumulation was checked after every 24hrs. The maximum PHA production was obtained at 72hrs and production was decreased after 72hrs.

Sr. No.	Time (hours)	Absorbance @ 235 nm	PHA in g/l
1.	24	0.562	4.5
2.	48	0.750	11.3
3.	**72**	**0.762**	**11.9**
4.	96	0.542	9.1
5.	120	0.450	7.5

b) Inoculum size:
Optical density of the culture also plays an important role in PHA production. It is necessary to find out the optimum culture density required for maximum PHA production. The optical densities of the culture used were 0.1 – 0.9 (OD540nm). The maximum PHA production was observed with 0.5 O.D.

Sr. No.	Inoculum size	Absorbance @ 235 nm	PHA in g/l
1.	0.1	0.759	11.9
2.	0.2	0.783	12.3
3.	0.3	0.812	12.8
4.	0.4	0.840	13.3
5.	**0.5**	**0.982**	**15.6**
6.	0.6	0.953	15.4
7.	0.7	0.830	6.35
8.	0.8	0.565	4.4
9.	0.9	0.533	4.15

c) **Aeration**:
The maximum production of PHA was checked by incubating the flask under static and shaker conditions. Results obtained showed that PHA production under shaker condition was more than that under static condition.

Sr. No.	Condition	Absorbance @ 235 nm	PHA in g/l
1.	Static	0.155	1.25
2.	**Shaker**	**0.875**	**13.7**

d) Temperature:

Sr. No.	Temperature (° C)	Absorbance @ 235 nm	PHA in g/l
1.	**28**	**0.810**	**12.8**
2.	37	0.644	10.6
3.	45	0.395	7.6
4.	55	0.380	3.15

e) pH:

Sr. No.	pH	Absorbance @ 235 nm	PHA in g/l
1.	5	0.176	1.4
2.	6	0.440	3.4
3.	7	0.473	7.3
4.	**8**	**0.568**	**8.8**
5.	9	0.392	6.5

f) Carbon source:

Sr. No.	Carbon source	Absorbance @ 235 nm	PHA in g/l
1.	**Glucose**	**0.682**	**11.3**
2.	Lactose	0.232	2
3.	Sucrose	0.340	5
4.	Fructose	0.935	8
5.	Maltose	0.541	8.1
6.	Mannitol	0.180	1.5

7.	Vegetable oil	0.095	0.7

Concentration of Carbon source using Glucose:

Sr. No.	Concentration of Carbon (%)	Absorbance @ 235 nm	PHA in g/l
1.	1	0.987	16
2.	2	0.875	21
3.	3	0.845	13.3
4.	4	0.807	13.1
5.	5	0.783	12.8

g) Nitrogen source:

Replacement of Yeast extract with inorganic Nitrogen sources

Sr. No.	Inorganic Nitrogen source	Absorbance @ 235 nm	PHA in g/l
1.	Sodium Nitrate	0.450	7.3
2.	**Potassium Nitrate**	**0.986**	**16**
3.	Ammonium chloride	0.623	10.3

Replacement of Yeast extract with organic carbon sources

Sr. No.	Organic Nitrogen source	Absorbance @ 235 nm	PHA in g/l
1.	Yeast extract	0.753	12
2.	**Beef extract**	**0.975**	**15.4**
3.	Peptone	0.955	15.1
4.	Meat extract	0.520	8

h) Concentration of Hypochlorite on biopolymer extraction:

Sr. No.	Concentration of Hypochlorite	Absorbance @ 235 nm	PHA in g/l
1.	1%	0.095	0.7
2.	2%	0.142	1.1
3.	3%	0.415	3.25
4.	**4%**	**0.855**	**6.75**

10) <u>PHA production under optimized conditions</u>:

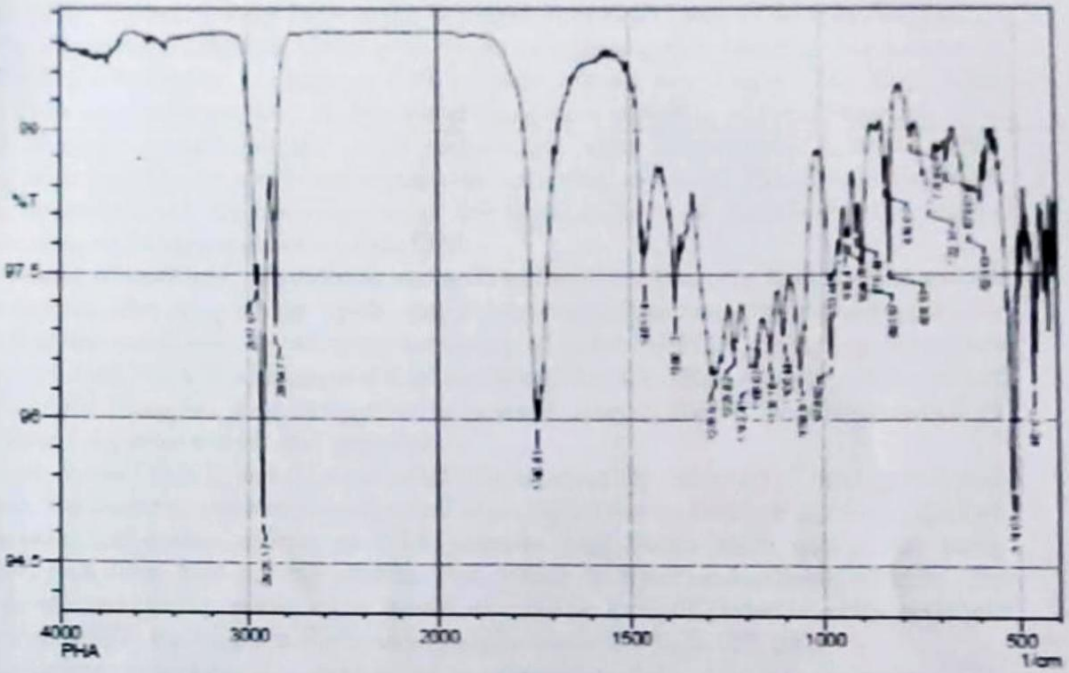
After optimizing all the parameters, maximum PHA accumulation was seen when (0.5 OD 540nm) inoculums size was grown in E2 mineral medium containing 2% Glucose, 0.004% Potassium nitrate and pH 8 at 30°C for 72 hrs under Shaker condition (120rpm). It was seen that the isolate accumulated about **21 g/l** of PHA.

11) <u>PHA film Preparation (Bioplastic)</u>:

PHA was extracted from the isolate by the sodium hypochlorite method. This was used for the preparation of bioplastic. On leaving the chloroform- PHA solution, poured onto Petri plate for about one hour, a film of PHA was obtained.

12) Characterization of PHA by Fouier transform infra-red spectroscopy (FT-IR):

FTIR spectrum of PHA was recorded in the range of 4000-500 cm-1 and spectroscopic analysis showed the presence of broad bands responding to the groups CH, C=O and C-O indicating the structure similar to PHB. The methine (CH) group gave a strong band at 2925.17 which can be exerted by the proteins and PHA. The carbonyl group (C=O) gave a strong band at 1740.83. The (C-O) group showed strong and broad absorption in the range of 1058.97-1225.82. The absorbance peak values obtained were compared with the available literature values and confirmed the product as PHB.



FTIR spectrum obtained for PHA extracted from *Bacillus megaterium JHA.*

Discussion

The use of biological systems for the production of biodegradable materials is becoming important as a solution to the environmental problems concerning non-biodegradable plastic waste [**21**]. The microbial (polyhydroxyalkanoates) (PHAs) family of polyesters is synthesized by a wide variety of bacteria as an intracellular carbon and energy storage materials [**22**]. These polyesters show a higher accumulation in the presence of excess carbon source. When higher amounts of other nutrients such as nitrogen, sulfur, phosphate, iron, magnesium, potassium or oxygen is present; there is a decrease in the growth as well as PHA production [**23**]. The best known PHA is the homopolymer PHB [poly (3-hydroxybutyrate)]. Prokaryotic organisms, including Gram-positive and Gram-negative bacteria, are known to produce PHB amounting to as much as 80% of their cellular dry weight [**24**]. Thus, with respect to PHA accumulation, soil, sludge and effluent from industry, soil and baggasse from sugarcane industry, garage soil and pond soil sample were screened for potential PHA producing organism. The present study focuses on screening, isolation, identification of PHA producing bacterium and the optimization of the physico-chemical parameters to enhance PHA production and characterization of the PHA.

In the presence of multiple nutrients in the environment the bacteria tend to accumulate reserve materials like intracellular lipids and polyhydroxyalkanoates, which are generally utilized when the conditions are adverse. Screening of potential PHA producing organisms was done by Sudan black B staining and was confirmed by Nile Blue A plate assay method. From the enriched samples pure cultures were isolated. Among the 40 isolates obtained 18 isolates showed the presence of lipid granules.

According to *Burdon* (1946) and *Flora et al* (2010) to detect the presence of lipid granules in the bacteria, the bacterial isolates are subjected to an initial Sudan Black B staining [**25, 26**]. Nile blue stain has greater affinity to PHA granules than Sudan black and is not easily washed off. Nile blue A is a basic oxazine dye, which is water and ethanol soluble. The oxazone or nile red is a lipophilic stain; it will accumulate in lipid globules inside cells and dye is very soluble in the lipids. It fluoresces strongly under UV light [**27, 28**].

Among 18 isolates only 4 isolates were found to accumulate PHA. The isolates showing the characteristic orange colour fluorescence under the UV light of plate assay were selected for further studies. Among of the four isolates showing the presence of PHA, the isolate obtained from garage soil produced higher quantity of PHA (12 g/l) and hence was used for further studies.

A rapid and reproducible sodium hypochlorite method (*Hyp*) was used for extracting PHA from the bacterial cells. *Hyp* was used to extract PHA from all four PHA accumulators. Hypochlorite oxidizes the cellular material, dissolving it completely, while PHA being perfectly insoluble in hypochlorite gets pelleted on centrifugation. PHA extracted was estimated quantitatively by Slepecky and Law method [**29**]. According to *Berger et al.* (1989) 50% reduction in MW of the polymers was observed when the biomass was digested with sodium hypochlorite. Because sodium hypochlorite is a strong oxidant, care has to be taken to select for suitable digestion conditions in order to maintain a high molecular weight of the polymers [**30**]. However, the solvents used in this method are not environmental friendly, thus limiting the use. Other methods based on digestion of non-PHA cell material have also been studied, but by using hypochlorite a degradation of the polymer was observed. Best results were obtained by combining hypochlorite with chloroform or surfactant treatment.

The cultural, morphological and biochemical test were used to identify the isolateas *Bacillus megaterium* and the strain was confirmed using 16S rRNA sequencing. The isolated bacterium is Gram positive and rod shaped. Its colonies are white in colour, irregular and flat.

16S rRNA analysis was done from Yaazh Xenomics, Panvel, Navi Mumbai. The culture was identified as *Bacillus megaterium strain* JHA.

A wide variety of bacterial species are known to accumulate PHA [**31**]. In one study, 29 *Bacillus* strains were assessed for PHB production and found that *B. megaterium* showed maximum production of 0.207g/l and productivity percentage of 48.13%. Lowest PHB was 6.53% in *B. subtilis* K1 [**32**]. PHA producing bacteria has been reported from various environments such as soil, sewage, sludge [**33**] marine sediments, ponds, mangrove environment [**34**] and gas field soil [**35**].

Bacillus megaterium strain JHA showed maximum PHA production in E2 mineral medium (9.5g/l). *Nagamani et al.* (2011) studied the effect of the E2 mineral medium on the growth and PHA production by *Pseudomonas aeruginosa* OU67 isolated from polluted water and found that it was able to produce good amount of PHA (up to 57.77% of dry cell weight) in the presence of excessive starch as carbon source in the E2 mineral medium [**36**]. Use of inexpensive substrates such as starch could contribute to reducing the PHA production cost. *Gowda et al* (2013) studied the effect of nitrogen deficient medium on PHA production by *B. thuringiensis* IAM 12077 [**37**]. The reports showed that under nitrogen limiting conditions, cell biomass reached 2.93 g/l, PHB yield obtained was 1.866g/l amounting to accumulation of 64.16% dry cell weight.
Accumulation of polymer begins in the late log phase of growth and becomes maximum during the late stationary phase of growth, it is therefore important to harvest cells at the optimum time (late stationary phase) to obtain a maximum yield of PHB [**38**]. *Bacillus megaterium strain* JHA showed maximum PHA production at 72hrs of incubation, after which there was a decrease in the PHA accumulation. This time dependent reduction in growth after 72hrs might indicate that the bacteria used PHA as a nutrient source due to inadequate nitrogen and carbon sources in the medium. *Flora et al.* (2010) reported the optimum time required for maximum PHA production in *Bacillus* species [**26**]. The percentage PHB yield was calculated after 24hrs interval. The result showed maximum PHB yield at 72hrs, and there after the PHB yield decreased in time dependent manner. Thus, 72hrs of incubation was selected as optimum incubation time for the PHB production. *Yüksekdağ et al* (2004) studied the effect of time on PHB accumulation by *Bacillus* species [**39**]. In this study, production of PHA by *B. subtilis* 25 and *B. megaterium* 12 strains were detected between 6hrs and 48hrs in nutrient broth medium. It was determined that the PHB yield of the both strains increased (18.03%, 14.79%) until 45thhrs and decreased (7.98%, 6.55%) in 48th hrs. It can be thought that until the sporulation time it produced PHB and then used PHB.

PHA is accumulated by organism as storage materials during favorable conditions of growth, generally presence of rich nutrients. Organisms accumulating PHA have the ability to depolymerize and utilize the products as a carbon and energy source, during unfavorable conditions [**40**].The amount of polymer accumulated increases to the maximum during the growth, followed by decline. Optimization of various physical and chemical parameters of growth was done to study their effects on PHA accumulation.

Small variations in oxygen availability, can lead to significant changes in the metabolite distribution of any bacterial cultures. These changes, which reflect the metabolic adjustments that take place in order to optimize cell growth, vary when using different carbon sources, affecting the synthesis of PHB in different ways. By studying the effect of both static and shaker conditions, it was seen that *Bacillus megaterium* strain JHA produced maximum PHA under shaker condition. The PHA produced was 13.7g/l. According to *Kato et al.* (1992) higher PHA accumulation by *Bacillus megaterium* B-124 was observed under shaker condition [**41**]. As per *Nagamani et al.* (2011) growth and PHA production (1.755g/l) by the shaken flask cultures increased upto 30hrs of incubation [**36**].

In most cases, growth increases with increase in temperature but it represses sharply and abruptly at extreme upper and lower limits of temperature. Hence, beyond optimum temperature, PHA production activity decreases because of slower growth and denaturation of enzyme responsible for PHA production. Under different incubation temperature, *Bacillus megaterium* strain JHA in E2 mineral medium gave highest PHA at R.T. i.e. 30°C (12.8 g/l) and 37°C (10.6 g/l). While drastic decrease in PHA production was observed below 30°C and above 37°C respectively. The results are in accordance with *Flora et al.* (2010) who reported maximum PHA production at 30°C and 37°C for *Bacillus* species [**26**]. *Aslim. B. et al.* (2002) reported the growth temperature of *B. megaterium* at 30°C [**32**]. *Singh et al.* (2011) studied the optimum temperature requirement for PHA accumulation by *Bacillus* species [**42**]. In this study maximum PHA accumulation (5.311g/l) was obtained at 30°C.

pH of the medium also plays an important role. Change in initial pH of the medium shows a strong influence on the production of PHB. Even a slight difference in pH from the optimum point results into a sudden reduction in PHB production. *Bacillus megaterium* strain JHAgrew and accumulated maximum PHA (i.e 8.8g/l) at pH 8 of the medium. Although the culture grew at lower and higher pH values, the PHA produced was lesser. The results are nearly in accordance with *Ramadas et al*, (2009) who reported pH 7.5 to be optimum for PHA production by *Bacillus* species [**43**]. *Singh et al.* (2011) reported pH range 6.0-7.0 to be the optimum pH for PHA accumulation [**42**]. *Saleem et al.* (2014) reported maximum yield of 0.983g/l and least yield of 0.015g/l of PHA at pH 7.0 and pH 2.0 respectively by *Bacillus thuringiensis* strain CMBL-BT-6 [**44**].

Carbon source plays very important role in increasing the concentration of PHA. Different bacterial strains utilize different carbon sources. The biosynthesis pathways of PHA production are varied regarding to carbon sources supplied as well as PHA products required. *Bacillus megaterium* strain JHA produced maximum PHA (11.3 g/l) by utilizing glucose as a sole source of carbon. This showed that glucose was the best carbon source for PHA accumulation for this particular strain. Using varying concentrations of glucose, it was seen that 2% conc. of glucose was the best for maximum PHA production. The order of preference of carbon sources by the organism on the basis of PHA production is Glucose, Maltose, Fructose, Sucrose, Arabinose, Lactose and Mannitol. According to the obtained results it seems that the ability of *Bacillus megaterium* strain JHA, to utilize different simple and complex substrates is variable and is dependent on several factors like nature of the substrate used and the type of enzyme produced. According to *Yüksekdağ et al* (2004) and *Hori et al.* (2002) the highest level of PHB accumulation was observed in the medium with glucose as carbon source in *B. subtilis* 25 (19.51%) and *B. megaterium* 12 (19.49%) **[39,45]**. *Singh et al.* (2011) reported raffinose followed by lactose to be the best carbon source for PHA accumulation and obtained 5.07g/l and 5.06g/l of PHB respectively **[42]**. *Bacillus megaterium* strain ASNS13 produced about 0.7g/l of PHA using vegetable oil as the carbon source. *Fernandez et al.* (2005) studied the ability of *Pseudomonas aeruginosa* to feed on fatty acids and frying oil, with a maximum production of 66% (w/w) PHA **[46]**. *Lakshman et al.* (2004) reported the use of different oils as carbon source in PHA production and found that the biomass in *Rhizobium meliloti* 14 were highest in mustard oil (0.39 g/l) and was lowest in soy oil (0.32 g/l) **[47]**.

The ability of cell to utilize a particular compound depends upon the genetic ability of the organism to synthesize the enzyme. Nitrogen is one of the important parameter for growth and PHA accumulation by the bacterial species. The effect of various nitrogen sources both inorganic and organic (0.004%) on cellular growth and PHA production by the isolate under study was investigated. Out of the various nitrogen sources tested, Beef extract and Potassium nitrate showed maximum PHA yield of 15.4 g/l and 16 g/l respectively. According to *Yüksekdağ et al.* (2004) the highest level of PHB accumulation was observed in the media with proteaz peptone as nitrogen sources in *B. subtilis* 25 (78.69%) and in *B. megaterium* 12 (77.00%) **[39]**. *Singh et al.* (2011) reported that peptone and ammonium chloride are the best nitrogen sources for PHB production, which gave 5.423g/l and 5.191g/l of PHB respectively **[42]**.

Another parameter optimized for maximum PHA, was the Optical density of the isolate under study. It showed that, increase in cell number or optical density of the culture led to decrease in production of PHA by *Bacillus megaterium* strain JHA. The maximum PHA production was observed with 0.5 at O.D540nm of cell suspension i.e. (15.6g/l).

The concentration of sodium hypochlorite used for extraction of PHA from cells also played a major role in the amount of PHA that is been extracted. Maximum amount of PHA i.e. 6.75 g/l was extracted from *Bacillus megaterium* strain ASNS13, at a concentration of 4% hypochlorite (i.e. 4% v/v active chlorine). *Rawte and Mavinkurve* (2002) studied that the modified hypochlorite method was simple, quick and also greater amounts of PHA could be extracted. They studied that the concentration of sodium hypochlorite at which maximum PHA extracted, was 2% (w/v) of active chorine **[14]**.

All the optimized physico-chemical parameters were retested together for their efficacy in producing maximum amount of PHA. The amount of PHA produced by *Bacillus megaterium* strain JHA before optimization was about 12g/l, which increased to 21g/l of PHA after optimization.

Bioplastic film production was carried out. The PHA powder that was extracted from *Bacillus megaterium* strain JHA was used to produce bioplastic film using solvent casting method. The bioplastic film obtained was thin, uniform and white in colour.
The FTIR spectrum analysis of the PHA product was carried out. Analysis showed the presence of broad bands responding to the groups CH, C=O and C-O indicating the structure similar to PHB. The methine (CH) group gave a strong band at 2925.17 which can be exerted by the proteins and PHA. The carbonyl group (C=O) gave a strong band at 1740.83. The (C-O) group showed strong and broad absorption in the range of 1058.97-1225.82. The absorbance peak values obtained were compared with the available literature values and confirmed the product as PHB. The peak values obtained in this study coincides with previous results of *Kalaivani et al.* (2013) [**48**].

Summary and Conclusion:

The isolation and study of PHA producing bacteria was carried out. The primary screening of PHA was done by Sudan black B staining and was confirmed by Nile blue A plate assay method. The maximum PHA producer was detected by extracting the accumulated PHA in it, and quantifying it by UV spectrophotometer. By performing the cultural, morphological, biochemical tests and 16S rRNA sequencing, the isolate was identified as *Bacillus megaterium* ASNS13.
Optimization was carried out to detect the optimum parameters required for the maximum PHA accumulation. Effect of various physico-chemical parameters such as temperature, pH, time of incubation, aeration conditions etc were studied, and it was found that at 30°C, pH8 for 72hrs under shaker conditions the maximum PHA accumulation was observed in E2 mineral medium. The effect of various carbon and nitrogen sources was studied. It was found that 2% (w/v) glucose followed by maltose were the best carbon substrates for PHA accumulation. Also beef extract and potassium nitrate proved to be the best organic and inorganic nitrogen substrates for PHA accumulation. Effect of varying inoculum size on PHA accumulation was checked, and it was found that 0.5 OD at 540nm was the best for PHA production.
After the optimization of all the parameters, the increase in PHA accumulation was recorded from 12g/l to 21g/l. The effect of sodium hypochlorite on PHA extraction was checked and, it was found that 4% conc. (v/v) active chlorine was the best for PHA extraction. The extracted PHA was characterized by FT-IR analysis. The presence of the CH, C=O and C-O groups indicates the structure similar to PHB. A thin, white PHA film was prepared. The physical properties of the polymer under study were as follows, glass transition temperature (Tg) was 150°C, the melting point was around 168°C-170°C and the crystallization was seen around 95°C.
Novel microbial PHA, with desirable physical properties and high productivity are still being discovered in the quest of biodegradable plastics. Since the commercial production of PHA from expensive substrates is untenable, cheap and readily available substrates are now being tried. Researchers focus on improvising the recovery process, studying the molecular biology of bacteria synthesizing PHA, novel monomer constituents of PHA, characterization of the polymer and their biodegradability to enable commercial exploitation.

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