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Research Paper

SCREENING OF POLYHYDROXYALKONATES (PHA) ACCUMULATING BACTERIA FROM DIVERSE HABITATS

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Abstract

The exponential growth of the human population has led to the accumulation of huge amounts of plastic across our planet. Bioplastics are considered good substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties and biodegradable nature. Polyhydroxyalkanoates (PHAs) are biodegradable plastics produced by various bacteria. This work has been undertaken to screen PHA accumulating microorganisms from diverse habitats such as soil from oil mills, machine oil contaminated areas, mangroves and Lonar Lake. And waste from sugar industry (baggase), textile industry effluent, and crude honey samples from Rudraprayag were also used for screening. A total of 120 isolates were collected and purified. Screening for bioplastic production was done by Sudan Black B staining to detect lipid cellular inclusions. The positive lipid accumulators were further analysed for PHA accumulation by Nile Blue A plate assay and 34 cultures scored positive for PHA accumulation by the above assay. The positive isolates were grown in Sterile E2 medium to promote accumulation of PHA and extracted by the modified Hypochlorite method. The strains accumulating high PHA were analysed for maximum PHA production by the Slepecky and Law method. The promising isolate showing maximum PHA accumulation was identified as *Bacillus megaterium* Strain JHA by morphological, cultural, biochemical characteristics and 16s rRNA sequence analysis.

Key words: *Polyhydroxyalkanoates, bioplastic, Nile Blue A, Modified Hypochlorite method*.

INTRODUCTION

Hydrocarbons are naturally occurring stable compounds that can retain much of their original architecture over very long periods of time [1].The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials [2]. Nature's built-in mechanisms and self-regulation ability cannot

tackle novel pollutants and the increase in the mineral oil prices are prompting many countries to start considering alternatives for synthetic plastics and are beginning to develop biodegradable plastic [3].

Bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of non-degradable plastics [4]. Among these, polyhydroxyalkanoate (PHA) belonging to the group of polyoxoesters has received intensive attention as it possesses biodegradable thermoplastic properties [5, 6].

Lemoigne (1926) first reported the formation of poly(3-hydroxybutyrate) (PHB) inside bacteria [7]. PHAs are synthesized by many living organisms but the main candidates for the large-scale production of PHAs are plants and bacteria. Prokaryotic organisms, including gram positive and gram negative bacteria produce polyhydroxyalkanoates (PHA), a class of storage lipids serving as an endogenous carbon and energy source during starvation periods [8]. PHA also plays a significant role in the survival of the microorganisms under conditions of environmental stress such as osmotic pressure, desiccation and UV irradiation [2, 9]. 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 [10, 11].

The bacteria that accumulate PHAs may either require the limitation of an essential nutrient such as nitrogen, phosphorous, magnesium or sulphur for the synthesis of PHA in presence of excess carbon source like *Alcaligenes eutrophus, Protomonas extorquens and Protomonas oleovorans* or others like *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 [12]. PHAs are produced by many different bacterial cultures like *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) [13], *Bacillus* sp., *Pseudomonas* spp, *Aeromonas hydrophila, Rhodopseudomonas palustris, Burkholderia sacchari, Halomonas boliviensis, Acinetobacter, Sphingobacterium, Brochothrix, Caulobacter, Ralstonia, Burkholderia*, and *Yokenella* [14, 15]. PHB accumulation has been reported in archaea such as *Halobacterium marismortui, Halococcus* sp., *Halorubrum* sp., *Halobacterium noricense* DSM 9758, and haloakaliphiles (*Natronobacterium gregoryi* NCMB 2189T, *Natronococcus occultus* DSM 3396T) [16, 17, 18, 19]. The major advantage of PHAs is that both the physical properties and the rate of degradation of PHAs can be altered by changing the bacterial source of the polymer [20].

The aim of this study is to screen PHA accumulating bacteria from diverse habitats and identification of the isolates showing maximum PHA production.

MATERIALS AND METHODS

1) Collection of sample

Samples were collected such as soil from oil mills, machine oil contaminated areas, mangroves from Mumbai coastline and Lonar Lake. Similarly waste from sugar industry (baggase), textile industry effluent from different areas in Maharashtra, and crude honey samples from Rudraprayag were also obtained. The samples were collected in a sterile screw cap bottle and stored at 4ºC until further use.

2) Enrichment and isolation

The enrichment of the samples was done in Sterile Nutrient Broth. Serial dilutions of the soil sample were prepared in sterile phosphate buffered saline (pH- 7.2). The aliquots were spread on sterile Nutrient Agar medium plates and incubated at 28°C for 24 hrs.

Colonies with different characteristic features were maintained as pure cultures on sterile nutrient agar slants and stored at 4°C.

3) Primary Screening of lipid producing microorganisms

A. Sudan Black B Plate Assay:

All the isolates were qualitatively tested for PHA production using Sudan Black B dye [21]. Ethanolic solution of (0.05% w/v) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHA production [22].

B. Sudan Black B staining:

The isolates that scored positive by the Plate assay were grown and stained for the presence of lipid granules by the Sudan Black B staining method [23, 24]. The organisms showing the presence of black granules were scored as potential lipid producers.

4) Secondary Screening of PHB producing isolates by Nile Blue A plate assay:

The isolates that scored positive for lipid production were further screened for PHA accumulation by the Nile blue A plate assay method. The isolates were cultivated on Sterile modified E2 agar medium containing 3.5g $NaNH_4HPO_4.4H_2O$, 7.5g K_2HPO_4, 3.7g KH_2PO_4, 100 mM $MgSO_4$. $7H_20$, 10ml trace elements with 20 g/Litre (w/v) glucose as carbon source, supplemented with 0.4g Yeast extract and 22gm Agar in 1 Litre of distilled water. The trace element solution consisted of 2.78g $FeSO_4.7H_2O$, 1.98g $MnCl_2.4H_2O$, 2.81g $CoSO_4.7H_2O$, 1.47g $CaCl_2.2H_2O$, 0.17g $CuCl_2$. $2H_2O$ and 0.29g $ZnSO_4.7H_2O$ in 1 Litre of distilled water [25, 26].

The isolates were spot inoculated on the above medium and incubated at 28ºC for 72-96 hours. The plates were stained with ethanolic Nile Blue A solution (1% w/v). Colonies exhibiting fluorescence when exposed to U V light were scored as PHA accumulators [27].

5) Extraction of the accumulated PHA by modified Hypochlorite method

The positive isolates from the Nile Blue A assay were further quantified for PHA accumulation. The isolates were grown in 250 ml Erlenmeyer flask containing 50 ml sterile modified E2 medium. The flasks were inoculated with 2.5% (v/v) (O.D = 0.5 at 530 nm) overnight grown culture; and were incubated at 28ºC for 72 hr. The biopolymer produced was then extracted by the modified Hypochlorite method [28]. The centrifuged cell pellet was suspended in 5 ml of 2% sodium hypochlorite solution, vortexed and incubated at 37°C for 30 min. The white PHA pellet is obtained on centrifugation which is then washed with acetone: alcohol mixture (1:1 v/v). The pellet was vortexed and dissolved in 5 ml of chloroform. The chloroform was allowed to evaporate by pouring the solution in glass Petri plate and placing it at 28ºC. The PHA powder was quantified after evaporation of chloroform [29].

6) Quantification of the extracted PHA

The polymer extracted was assayed quantitatively by UV spectrophotometer at 235 nm using Slepecky and Law method. 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 [30].

7) Identification of the promising isolates

Identification of the promising isolate was done on the basis of morphological, cultural and biochemical test using VITEX 2 systems version 2.1 and the strains were confirmed by 16S rRNA analysis which was carried out at Sai Biosystems Pvt. Ltd., Nagpur, India.

RESULTS

Various samples collected from diverse areas were used to identify the relative occurrence of PHA accumulating bacteria. From the total collected soil samples, 120 different bacterial isolates were obtained on the Nutrient agar plates which were purified and maintained on Sterile Nutrient agar slants for further experiments. 15 isolates were obtained from Crude honey samples from Rudraprayag, 7 isolates from waste from sugar industry (baggase), 10 isolates were obtained from Mangrove soil, 44 isolates were obtained from Machine Oil Contaminants areas, 14 isolates from the Lonar lake, 20 isolates from Textile industry Effluent and 10 isolates were obtained from Soil from oil mills (Table 1).

Viable colony staining technique using Sudan Black B plate assay was performed as a method for rapid screening of lipid accumulating bacteria. The colonies unable to incorporate the Sudan Black B appeared white, while lipid producers appeared bluish black (figure 1). 81 isolates appeared bluish black on staining with Sudan Black B thus, scoring positive for lipid granules (Table 1). The bacterial cells were further purified and stained by Sudan Black B staining and observed microscopically. The microscopic observation showed the presence of lipophilic black granules within pink coloured cells (figure 2).

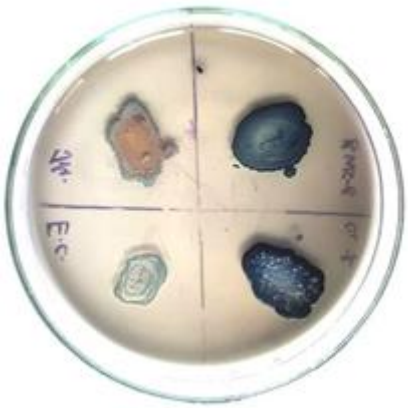

Figure 1: Sudan Black B plate assay showing the presence of blue coloured colonies (lipid positive) and white coloured colonies (lipid negative)

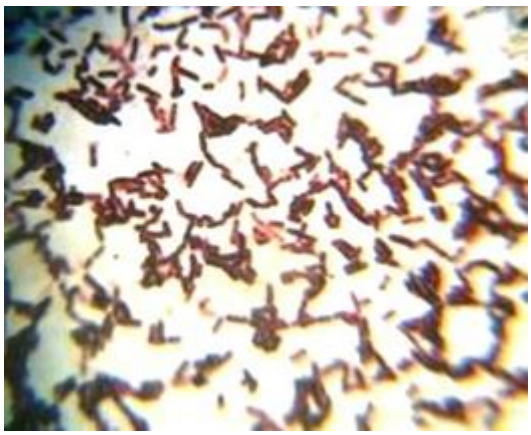

Figure 2: Black lipid granules observed in Isolate CO-1 by staining by Burdon's method

Out of 81 isolates showing the presence of lipophilic granules, the Gram's nature of 56 isolates were Gram positive and 25 isolates were Gram negative in nature. The Gram positive isolates showed rod shape (38 isolates) and cocci shape (18 isolates) morphology. However, all Gram negative organisms showed short rod shape morphology.

The 81 lipid positive isolates were further grown on Sterile E2 Agar medium and checked for PHA accumulation by the Nile Blue A plate assay. 34 isolates were identified to accumulate PHA (Table 1), which was observed by the exhibition of orange fluorescence. The intensity of fluorescence was seen proportional to the amount of PHA accumulated by the organism as shown in figure 3. Table 1 represents the source and number of isolates that have scored positive by Nile Blue A plate assay which was observed as orange fluorescence on exposure to Ultra Violet radiation.

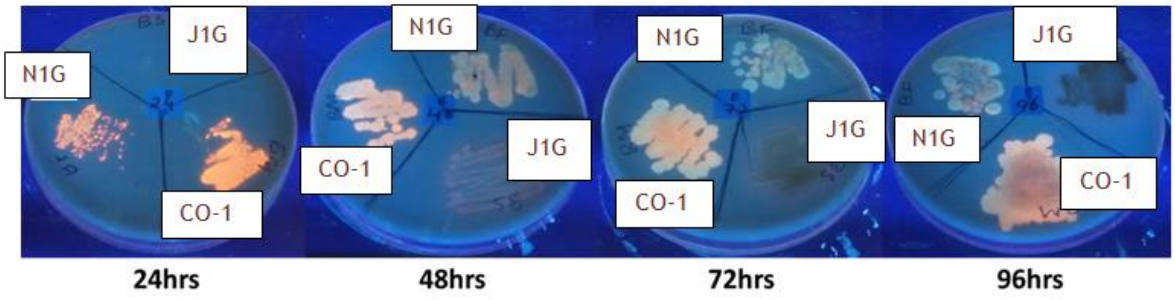

Figure 3: The exhibition of orange fluorescence by the Nile Blue A plate assay by Isolates CO-1, J1G and N1G over a period of 96 hours

Table 1 represents the source and number of isolates that have scored positive by Nile Blue A plate assay which was observed as orange fluorescence on exposure to Ultra Violet radiation.

Table 1: Number of isolates showing positive results with Sudan Black B plate assay, Sudan Black B staining and Nile Blue A plate assay

Sr. No.	Sample	Number of isolates	Isolates scoring positive for Sudan Black B staining		Isolates scoring positive for Nile Blue A plate assay
			Plate assay	Sudan Black B staining	
1	Crude honey samples from Rudraprayag	15	10	10	4
2	Waste from sugar industry (baggase)	7	6	6	2
3	Mangrove soil	10	10	10	2
4	Machine Oil Contaminants	44	32	32	12
5	Soil from Lonar lake	14	5	5	4
6	Textile industry Effluent	20	10	10	2
7	Soil from oil contaminated areas	10	8	8	8

The 34 PHA positive isolates were further grown on a nutrient limiting modified E2 medium with 2% Glucose as a Carbon source, pH 7.2 at 28ºC under shaker conditions (130 rpm) for 72 hours. The PHA was extracted by the modified Rapid hypochlorite method. The white colour PHA powder obtained was further quantified by Slepecky and Law method using crotonic acid as the standard to measure the amount of PHA accumulated at 235nm using the UV-Vis Spectrophotometer. The PHA accumulated by various isolated organisms is reported in table 2.

Table 2: PHA production by 34 isolates on E2 medium

Sr. No.	Culture no.	Absorbance at 235 nm	PHA in g/Litre
1.	BWS-6	0.0196	0.245
2.	BWS-5	0.0758	0.473
3.	HN10	0.0244	0.265
4.	NZeeS	0.0705	0.451
5.	J2S	0.0325	0.404
6.	Bag1	0.0247	0.2666
7.	Bag3	0.0542	0.385
8.	MM2	0.1327	0.703
9.	MM-4	0.0765	0. 475
10.	6I=G	0.0957	0.5535
11.	19I=LO	0.0195	0.245
12.	36I=Lo	0.6329	0.291
13.	37I=LO	0.0198	0.246
14.	42I=Lo	1.9	0.1504
15.	2I=Mo	0.0195	0.2455
16.	9I=MO	0.0877	0.5211
17.	10I=MO	0.0243	0.2649
18.	45I=D	0.0209	0.2512
19.	L1C	0.1038	0.5863
20.	L2C	0.1513	0.7782
21.	PH-9	0.0425	0.3385
22.	PH-10	0.0515	0.3749
23.	L1	0.0928	0.5418
24.	F1	0.0545	0.387
25.	IN-1	0.0518	0.376
26.	D2	0.0504	0.3704
27.	**CO-1**	**0.8364**	**3.983**
28.	N1G	0.6351	2.943
29.	J1G	0.0588	0.385
30.	SB-2	0.070	0.453
31.	SB-3	0.017	0.214
32.	IS-1	0.221	0.956
33.	SS-1	0.020	0.252
34.	SS-2	0.057	0.395

From the result shown in table 2, the isolate CO-1 was identified as a promising PHA accumulating bacterium producing 3.983 g/Litre of PHA. The morphological, cultural and biochemical characteristics of the isolate CO-1 was studied on Sterile Nutrient Agar.

The cultural characteristics were irregular margin, low convex elevation, opaque with dry consistency and it was observed to be a gram positive organism showing rod shape morphology. For biochemical characterization VITEX 2 systems 2.0 (Table 3) was used and isolate was identified as *Bacillus megaterium* [31]. This was further confirmed on the basis of 16S rRNA sequence analysis as *Bacillus megaterium.* The nucleotide sequence data determined in this study were deposited in GenBank NCBI database under accession number LC201962 for the partial 16S rRNA gene sequences and the strain was named as *Bacillus megaterium* strain JHA.

Table 3: Biochemical characterization of isolate CO-1 using VITEX 2 systems 2.0 [31]

Biochemical Details																	
1	BXYL	-	3	LysA	-	4	AspA	-	5	LeuA	-	7	PhaA	(+)	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	+	18	CDEX	-	19	dGAL	+	21	GLYG	(+)	22	INO	-	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	-	31	dMAN	+
32	dMNE	+	34	dMLZ	+	36	NAG	+	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	(-)	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 5%	+	59	KAN	-
60	OLD	-	61	ESC	+	62	TTZ	-	63	PPLYB_R	-						
Contraindicating Typical Biopattern dMNE(23), LeuA, APPA(29)																	
Installed VITEK2 systems version: 07.01																	
QC Checked report																	

DISCUSSION

Contaminated environments contain essential nutrients and are enriched in conditions for PHA production [32, 33]. In the present study the isolates were obtained from soil from oil mills, machine oil contaminated areas, mangroves from Mumbai coastline, Lonar Lake, waste from sugar industry (baggase), textile industry effluent from different areas in Maharashtra, and crude honey samples from Rudraprayag. The isolates from Mangrove ecosystems are generally rich in organic matter, but deficient in nutrients such as nitrogen and phosphorus. The production of polyesters by halophilic and halotolerant bacterial species isolated from mangrove soil samples in Northern Vietnam has been reported [25, 34]. Kulkarni *et al*. (2010) have isolated haloalkalitolerant *Halomonas campisalis* MCM B-1027 isolated from Lonar Lake that is able to produce of a biodegradable poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer [35]. Isolates have been obtained from soil and rhizosphere samples taken from sugarcane growing farms which have found to accumulate PHB [36, 37]. Many studies have been reported on isolation of polymer producing bacteria from different environments such as soil polluted with organic wastes, heavy metals, oil and munitions of war and also from sludge and water [38]. The contaminated soils have a large diversity of PHA producing bacterial strains and diversity measurements are reflection of the dynamic status of an ecosystem [39]. A striking prevalence of PHA producing bacteria has been observed in waste water samples (including industrial effluents, dairy waste and domestic sewage) and activated sludge samples [22]. However, the presence of organisms accumulating PHA in crude honey samples has not yet been reported.

A wide variety of bacteria are known to accumulate PHA. Today, approximately 150 different hydroxyalkanoic acids are known to be incorporated into polyhydroxyalkanoates [4] with microbial species from over 90 genera have been reported to accumulate these polyesters [40].

While isolating PHA accumulating bacteria from nature, it is necessary to screen a wide collection of bacteria in a short time by using stains specific to PHA for the detection of the granules. In the current work, isolates showed lipophilic granules were observed by the Sudan Black B Plate assay and staining. Hartman (1940) was the first to suggest the use of Sudan Black B, as a bacterial fat stain [41]. Juan *et al.* (1998) employed viable colony screening method for the rapid detection and isolation of PHA producing *Rhizobium meliloti* strains by using 0.02% alcoholic solution of Sudan Black B [21]. Shlegel *et al.* (1970) used Sudan black B as a dye for the isolation and detection of PHB producing colonies on a nitrogen limiting plate [42]. The lipophilic stain Sudan Black B has long been regarded as a dye with particular high affinity for PHAs [43]. Teeka *et al*. (2010) used this method to screen the potential PHA producing bacteria from soil [44]. Phanse *et al.* (2011) scored 23 isolates for PHA production by Sudan Black B plate assay method based on the intensity of staining [22]. Redzwan *et al*. (1997) reported the use of Sudan Black B staining technique as the first line screening for PHA- producing bacteria [45]. Different bacterial species such as *Bacillus subtilis* NRR-B-941, *Bacillus licheniformis*-B-NRRL 1001, *Bacillus cereus* NRRL-B-3711, *Bacillus megaterium* NRRL-B-3712 and *Bacillus thuringiensis* 798 were screened on the basis of qualitative tests (Sudan black B staining) [46]. The Sudan black B staining was used as first line of qualitative observation of PHB production for the bacterial species as also suggested by certain workers [47].

In this study, the Nile blue A plate assay was used to confirm the presence of PHB granules. Ostle and Holt (1982) advocated the use of Nile Blue A, a water-soluble basic oxazine dye that has a greater affinity and higher specificity than Sudan Black for PHB, and gives a bright orange fluorescence on exposure to ultra violet light [27]. Other inclusion bodies, such as glycogen and polyphosphate, do not stain with Nile Blue A, thus emphasizing its usefulness. Kitamura and Doi (1994) first demonstrated the viable colony method on agar plates; they induced the isolates to accumulate PHA by culturing in E2 medium containing 2% (w/v) glucose before Nile blue A staining. The PHA accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV-light and their fluorescence intensity increased with increase in PHA content of the bacterial cells [48]. Ramachandra and Abdullah (2010) also observed colonies grown on nutrient-rich medium under ultraviolet light (UV) showed fluorescence, which indicated the presence of PHA producers [49]. Rawte and Mavinkurve (1998) isolated PHB producing bacteria from mangroves along the Mandovi estuarine ecosystem by preliminary screening of the isolates grown on tributyrin agar for accumulation of PHBs using the fluorescent dye Nile Blue A [50]. Zribi-Maaloul *et al.* (2013) screened bacteria grown on Luria Bertani agar plates using Nile Blue A staining to investigate their abilities to synthesize PHA granules. The formation of low-molecular weight fluorescent compounds was observed at an emission wavelength of 280 nm. Based on the intensity of the fluorescence observed, 8 strains were identified as potential PHA producers [51]. Shenoy *et al.* (2012) also used the Nile Blue A plate assay to screen for the presence of PHA accumulating organisms which showed orange fluorescence on exposure to Ultraviolet light [33].

In the present work, both gram positive and gram negative organisms showed the presence of PHA. The Gram positive outnumbered the Gram negative. Almost all the isolates were rod shaped either long or short, besides few irregular rods and sometimes rods in chains. Polyhydroxyalkanoic acids (PHA) represent a complex class of storage polyesters that are synthesized by a wide range of different Gram-positive and Gram-negative bacteria [22]. Varieties of Gram-positive and Gram-negative bacteria (over 300

species, examples of which include *Pseudomonas* sp., *Bacillus* sp. and *Methylobacterium* sp.) carry the metabolic ability to biosynthesise PHAs and accumulate them in their cytoplasm as carbon and energy sources in the shape of granules [2, 3, 11].

Currently, E2 medium was used as the accumulation of PHB granules by the isolates. The use of nitrogen deficient medium E2 has been extensively reported for the production of PHA by various bacteria. Thirumala *et al* (2010) inoculated *Bacillus* spps. in nitrogen deficient E2 medium [52]. Several studies have also reported the use of E2 medium for the accumulation and production of PHA by various organisms [45, 53, 54].

The modified rapid hypochlorite method was used for extraction of PHB, in the present work. There are various solvent and non-solvent based methods used for the extraction of PHA from cells [55, 56]. Rawte and Mavinkurve (2002) reported the use of Rapid Hypochlorite method for PHA extraction from cell [28]. Hypochlorite dissolves the non-PHA cellular material, leaving the insoluble material precipitated in solution [57]. Pramanik *et al* (2014) reported the extraction of copolymer from bacterial cells with chloroform hypochlorite method [58].

In the current study, *Bacillus megaterium* strain JHA accumulated 3.983g/Litre of PHA from the nitrogen deficient E2 medium. Many workers have reported production of PHA by various species of *Bacillus*. *Bacillus* spp. are able to utilize a wide range of substrates such as sugars, volatile fatty acids (VFAs), alcohols and biowaste materials to produce homopolymers such as PHB. PHB yields are highly variable among the different *Bacillus* species: *B. amyloliquefaciens, B. brevis, B. cereus, B. circulans, B. coagulans, B. firmus, B. laterosporus, B. licheniformis, B. macerans, B. megaterium, B. mycoides, B. sphaericus, B. subtilis, B. thuringiensis* and *Bacillus* sp., which depends on either the type of strain or feed used in the process [59, 60, 61, 62, 63, 64, 65, 66].

Gowda *et al.* (2013) reported the effect of nitrogen deficient medium on PHA production by *B. thuringiensis* IAM 12077 which showed that the cell biomass reached 2.93 g/Litre, PHB yield obtained was 1.866g/Litre amounting to accumulation of 64.16% dry cell weight [67]. Yuksekdag *et al.* (2004) have reported PHA production by *Bacillus subtilis* and *B. megaterium* [68]. Production and characterization of PHA produced by *Bacillus megaterium* NCIM 2475 was also reported by Otari *et al.* (2009) [69]. Full *et al.* (2006) have studied production of PHA by *Bacillus* species from industrial wastes [70]. Prasanna *et al* (2011) reported 0.93g/Litre of PHA was extracted from *Bacillus megaterium* isolated from soil [71].

Gram-positive bacteria such as *Bacillus* sp. have been used extensively in industry. However, this organism has not yet been exploited for PHA production at an industrial scale. The Gram negative bacteria are the only commercial source for PHAs but the production from Gram-negative bacteria has been contaminated by liposaccharides (LPS) [52, 72]. The genus *Bacillus* seems to be a potential candidate for production of PHAs due to its better polymer yields under stringent fermentation conditions, nutrient limiting conditions and lack of LPS co-purification with PHAs. However, *Bacillus* sp. has the great ability to produce novel and known PHAs with different ranges of monomeric composition due to their ability to incorporate both short chain length (scl) and medium chain length (mcl). Therefore, the production of PHAs by *Bacillus* genera has distinct features which need intensive study [52].

Gram-positive bacteria have another potential advantage in terms of raw materials for PHA production and are capable of naturally synthesizing commercially important copolymer (poly 3-hydroxybutyrate-co-3-hydroxyvalerate) [P (3HB-co-3HV)] from abundant and inexpensive carbon sources such as glucose. In contrast, gram-negative bacteria need expensive structurally related substrates such as propionic acid, valeric

acid, or other fatty acids with an odd number of carbon atoms to produce 3HV unit. The relatively high expenditure involved is a major hindrance in PHA copolymer production. Hence, gram positive producers could considerably reduce the production cost [73].
Bacillus spps. isolated from oil contaminated soil are capable of producing PHA biopolymer. The yield of accumulated PHA by the *Bacillus* strains has to be optimized for various physico-chemical parameters in order to enhance PHB accumulation. A polymer can be accepted commercially only if it possesses the necessary physical, chemical and mechanical properties, similar to the petrochemical plastics. In order to produce PHA economically, the use of cheap and easily available raw materials is desirable. Hence it is of great importance to study the characterization of PHA to decide their probable commercial use.

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