Polish Journal of Microbiology
2015, Vol. 64, No 3, 227–239

ORIGINAL PAPER

Optimization of Culture Parameters for Maximum Polyhydroxybutyrate Production by Selected Bacterial Strains Isolated from Rhizospheric Soils

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Submitted 6 July 2014, accepted 27 February 2015

Abstract

The enormous applications of conventional non-biodegradable plastics have led towards their increased usage and accumulation in the environment. This has become one of the major causes of global environmental concern in the present century. Polyhydroxybutyrate (PHB), a biodegradable plastic is known to have properties similar to conventional plastics, thus exhibiting a potential for replacing conventional non-degradable plastics. In the present study, a total of 303 different bacterial isolates were obtained from soil samples collected from the rhizospheric area of three crops, *viz*., wheat, mustard and sugarcane. All the isolates were screened for PHB (Poly-3-hydroxy butyric acid) production using Sudan Black staining method, and 194 isolates were found to be PHB positive. Based upon the amount of PHB produced, the isolates were divided into three categories: high, medium and low producers. Representative isolates from each category were selected for biochemical characterization; and for optimization of various culture parameters (carbon source, nitrogen source, C/N ratio, different pH, temperature and incubation time periods) for maximizing PHB accumulation. The highest PHB yield was obtained when the culture medium was supplemented with glucose as the carbon source, ammonium sulphate at a concentration of 1.0 g/l as the nitrogen source, and by maintaining the C/N ratio of the medium as 20:1. The physical growth parameters which supported maximum PHB accumulation included a pH of 7.0, and an incubation temperature of 30°C for a period of 48 h. A few isolates exhibited high PHB accumulation under optimized conditions, thus showing a potential for their industrial exploitation.

Key words: biochemical characterization, biodegradable plastic, culture parameters, optimization, PHB production

Introduction

Plastic materials have conquered our lives due to their desirable properties but their extensive uses have caused waste disposable problems for the environment. Improper disposal of plastics has threatened the natural environment worldwide. Conventional petrochemical plastics are recalcitrant to microbial degradation (Fiechter, 1990), and remain persistent in the soil for a long time, thus accumulating in the environment at a rapid rate of approximately 25 million tons per year. Thus, problems such as global environmental pollution and solid waste management associated with these petrochemical-based plastics have created much interest in the development of biodegradable plastics that retain the desirable properties of the conventional plastics. Such biopolymers form a safe alternative to petroleum-based polymers with a wide range of environmental advantages. Most of the biodegradable plastics are made from a compound called polyhydroxyalkanoate (PHA) (Lee, 1996). The family of PHAs includes several polymeric esters such as polyhydroxybutyrates, polyhydroxybutyrate co-hydroxyvalerates (PHBV), polyhydroxybutyrate co-hydroxyhexanoate (PHBHx), polyhydroxybutyrate co-hydroxyoctonoate (PHBO) *etc*. Poly 3-hydroxybutyric acid (PHB), the best known and the most common natural microbial PHA (Verlinden *et al*., 2007), is found to accumulate in a large number of microorganisms as reserve food material (Haywood *et al*., 1988; Peoples and Sinskey, 1989).

PHB is a thermoplastic polymer synthesized by a wide variety of bacteria (*Ralstonia eutropha, Azotobacter beijerinckia, Bacillus megaterium, Pseudomonas oleovorans*, various nitrogen fixing microorganisms found in root nodules of legume plant family, and many more) as intracellular compounds and energy storage materials when the nutritional elements are limited and the carbon source is in excess (Halami, 2008; Wang *et al*., 2009; Hyakutake *et al*., 2011; Rodriguez-Contreras *et al*., 2013). Usually lipid in nature, it is accumulated

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as storage material in the form of mobile, amorphous, liquid granules, allowing microbial survival under stress conditions (Barnard and Sander, 1989; Sudesh *et al.*, 2000). Among terrestrial ecosystems, the rhizospheric soil layer influenced by plant roots, with its high microbial activity is a good habitat for PHB-producers. Indigenous microorganisms in the rhizosphere are adapted to changing conditions of the soil environment, and fluctuations in the concentration of nutrients exuded by plant roots. Bacteria capable of inclusion of storage substances have a competitive advantage over other bacteria, therefore, plants have been found to be colonized by numerous bacteria which are potentially able to accumulate polyhydroxybutyrate as energy and carbon sources.

A remarkable characteristic of P(3HB) is its biodegradability in different environments. Due to its similarity to conventional plastics in the physical properties, and having an additional advantage of being biodegradable; there is a great demand to replace the conventional plastics with that of the biodegradable plastics in order to safe guard our environment. However, the most important limitation of the widespread use and commercial production of PHB is the high cost of production compared to synthetic plastics (Sangkharak and Prasertsan, 2008). Isolation of novel high PHB producing strains, and improvement in the current PHB production strategies involving optimization of culture conditions could lead to cost reduction. Keeping these points in view, the present study was designed to isolate PHB producing bacteria from the rhizospheric soil of three different crops, and to optimize growth and culture conditions such as incubation time, temperature and pH; and medium constituents (carbon and nitrogen sources) for maximizing PHB production by them.

Experimental

Materials and Methods

Sample collection. For the isolation of PHB producing bacteria, rhizospheric soil samples were collected from three different crops, *viz*., wheat, mustard and sugarcane growing in 21 different districts of Haryana State in India, amounting to a total of 63 different soil samples (21 from each crop). Intact root systems along with adhered soil from all the three host plants were collected, and the samples were stored in plastic bags at low (4°C) temperature till further use. For further processing, the collected samples were air dried at room temperature, and then crushed with the help of pestle and mortar for further analysis.

Bacterial isolation from collected soil samples. For isolation of bacterial population, one gram of each collected soil sample was suspended in 10 ml of sterile distilled water, the suspension was shaken vigorously, serial dilutions were made and appropriate dilutions (10^{-4} to 10^{-6}) were plated on nutrient agar plates. After 48 hrs of incubation at 30°C, well- formed colonies were obtained on the plates. The total number of bacterial colony forming units (cfu) of each soil sample was enumerated, and the colonies were then studied for their morphological characteristics on the basis of their physical appearance (colour, size, shape and texture). Colonies showing remarkable differences in their morphology were selected and re-streaked on nutrient agar plates to obtain pure cultures.

Screening of isolates for PHB production using Sudan Black dye. All the representative pure isolates were screened for PHB production using the lipophilic stain Sudan Black B (Murray *et al*., 1994) on agar plates, and under light microscope.

Screening for PHB on solid agar. Individual bacterial isolates were streaked on nutrient agar plates (4–5 isolates on one plate), and the plates were incubated at 30°C for 24 hrs. Ethanolic solution of 0.3% (w/v in 70% ethanol) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. The plates were then destained by washing with ethanol (96%) to remove excess stain from the colonies. The colonies that retained their black colour after destaining were attributed as PHB producing strains (Mohamed *et al*., 2012).

Screening for PHB production under light microscope. For microscopic studies, smears of respective colonies were prepared on glass slides, heat fixed and stained with a 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. The colonies were decolorized by immersing the slides in xylene, and were then counterstained with safranin (5% w/v in sterile distilled water) for 10 sec. Bacterial cells appearing black under the microscope were considered PHB producing strains while others were marked as negative (Legat *et al*., 2010). All the positive isolates were assigned code numbers based on their source of isolation.

Quantification of PHB production and selection of isolates. PHB production was quantified by using the method of Law and Slepecky (1961) and the amount of PHB produced was calculated from the standard curve prepared by using commercial poly-β-hydroxybutyrate (Sigma-Aldrich). The PHB positive bacterial culture growth was pelleted at 10,000 rpm at 4°C for 10 min. The pellet was then washed with acetone and ethanol to remove unwanted materials, resuspended in equal volume of 4% sodium hypochlorite and incubated at room temperature for 30 min. The mixture was then centrifuged at 10,000 rpm for 10 min. to sediment the lipid granules. The supernatant was discarded, and the pellet was washed successively with acetone and etha-

nol. The pelleted polymer granules were dissolved in hot chloroform and filtered through Whatman No 1 filter paper (previously treated with hot chloroform). To the filtrate, 10 ml of hot concentrated H_2SO_4 was added, which converts the polymer to crotonic acid, turning it into a brown colored solution. The solution was cooled and absorbance was read at 235 nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer (Soam *et al*., 2012). The quantity of PHB produced was determined by referring to the standard curve.

Preparation of standard curve: Pure PHB (Sigma, USA) was used to prepare the standard curve of PHB. Two gram of PHB was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min to convert PHB into crotonic acid, which gave 200 mg/ml of crotonic acid. From the above stock, working solutions were prepared by diluting it to obtain different concentrations ranging between 10 mg/ml to 150 mg/ml. Absorbance of all the dilutions was read at 235 nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer, and the standard graph was made by plotting the various concentrations on the x-axis and the respective optical densities on the y-axis. The standard curve was used for estimation of PHB yield of the bacterial isolates.

Selection of isolates: Based on the PHB yield, all the PHB isolates were divided into three categories, *viz*., high producers, medium producers and low producers. Representative isolates from each category (6, 1, and 1 each from high, medium and low producing categories, respectively, for isolates belonging to each of the three crops), amounting to a total of 24 isolates (8 isolates from the rhizospheric area of each of the three crops) were finally selected for further studies.

Morphological, physiological and biochemical characterization of selected isolates. The selected isolates were grown on nutrient agar plates and their colony morphology was recorded. The morphological characteristics of the representative bacterial isolates (from each soil sample) showing differences in their physical appearances were recorded under four major headings, *viz*., size, colour, texture and shape. All these isolates were also studied under the microscope with respect to their cellular morphology and Gram staining properties (Gram, 1884). Biochemical characteristics of the isolates were studied following the standard microbiological methods described by Williams *et al.* (1994). Identification of isolates was carried out on the basis of the results of morphological, cellular and biochemical characters studied. Molecular characterization of the isolates is underway.

Optimization of culture medium constituents and growth conditions for maximum PHB production. Growth conditions such as the presence of different nutrient sources in the media and other physical parameters play an important role in the production rate of PHB. Therefore, the effect of various media ingredients like different carbon and nitrogen sources; and of growth conditions such as pH, temperature, and incubation time on PHB production was determined for the selected isolates.

Optimization of different carbon sources. The effect of different carbon sources on PHB production was determined by raising the cultures of the selected isolates in 100 ml of minimal salt medium (MSM) (Suresh Kumar *et al*., 2004) supplemented with different carbon sources such as glucose, fructose, sucrose, maltose and arabinose at 2% concentration. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB produced by the isolates was quantified spectrophotometerically (as described earlier), and based on the yield, the best carbon source was determined.

Nitrogen source optimization. The selected isolates were inoculated in 100 ml of MSM broth containing the best carbon source and different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract) at 1% concentration. After 48 h of incubation at 30°C, PHB yield was determined for all the isolates, and the best nitrogen source was selected on the basis of their yield. Further, the effect of different concentrations of the best nitrogen source on PHB production was also studied by determining the PHB yield upon growing the isolates in MSM supplemented with the best C-source and different concentrations (0.5, 1.0 and 1.5 g/l) of the best N-source.

Optimization of carbon to nitrogen ratio (C/N ratio). In addition to the determination of the best C and N sources, the effect of different C:N ratios on PHB production was also determined. For this, cultures were inoculated in MSM supplemented with different concentrations of the best C and N source (C/N ratio as 10:1, 15:1, 20:1 and 25:1). Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 h. After incubation, PHB yield was quantified spectrophotometerically, and based on the yields the best C/N ratio was determined.

pH optimization. For pH optimization, cultures of the selected isolates were raised in MSM supplemented with the best C and N source having different pH, *viz*., 6.0, 7.0 and 8.0. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB yield was quantified spectrophotometerically, and the pH exhibiting maximum yield was determined.

Temperature optimization: The effect of different temperatures on PHB production was determined by inoculating the cultures in MSM supplemented with the best C and N source and then incubating at different temperatures *viz*., 25°C, 30°C, 35°C, 40°C, and 45°C. After 48 h of incubation at respective temperatures, PHB yield was quantified spectrophotometerically; based

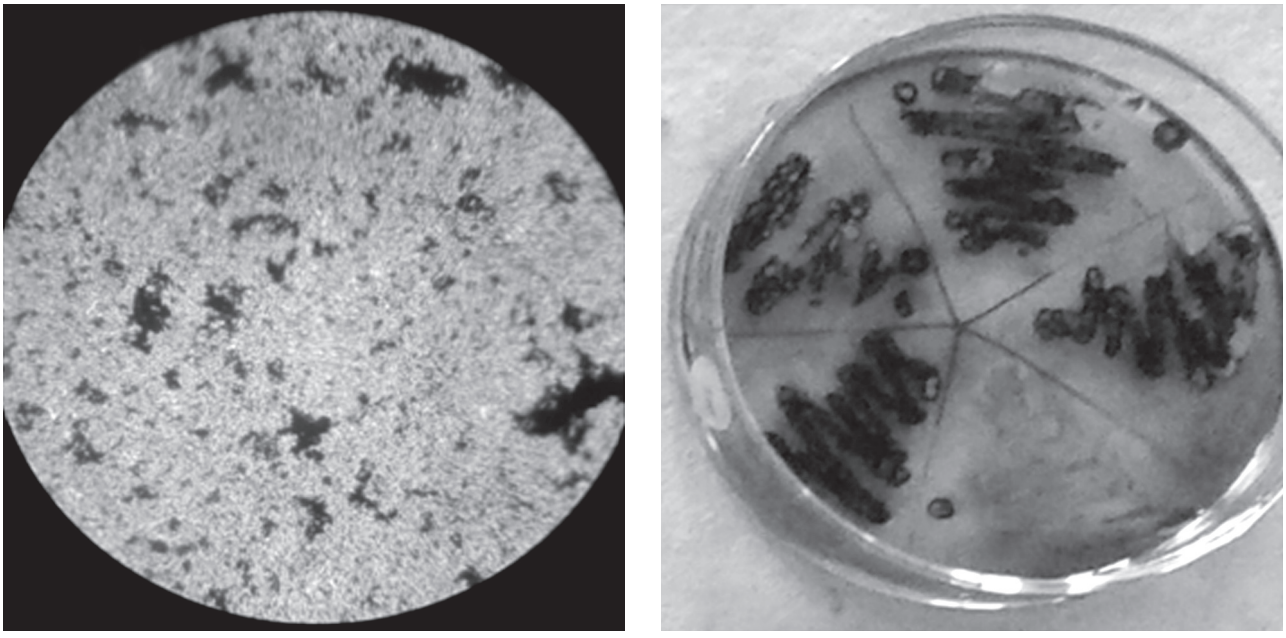

Fig. 1. Sudan Black screening of bacterial isolates, (a) Under the microscope, and (b) On nutrient agar plate

on the yields the optimum temperature for maximum PHB production was determined.

Optimization of incubation time. After the optimization of pH and temperature, the selected isolates were incubated for different time periods ranging from 12 to 72 hrs in optimized conditions. At each time point (12, 24, 36, 48, 60 and 72 h), cultures were tested for growth and PHB production and the PHB yield was recorded.

Results

Isolation and screening of PHB producing bacteria. For isolation of PHB producing bacteria, a total of 63 soil samples were collected from the rhizospheric area of three crops, *viz*., wheat, mustard and sugarcane belonging to 21 districts of Haryana State in India. The total bacterial population was isolated from each collected soil sample by making serial dilutions of the soil, and then plating appropriate dilutions on nutrient agar plates. Morphological studies of the isolates obtained revealed large diversity: on an average, 4–5 different types of colonies were obtained from each soil sample. Based upon the morphological differences in their colony characteristics (size, shape, colour and texture), a total of 303 different types of colonies (105 from wheat, 97 from mustard, and 101 from sugarcane) were picked up for further evaluation of PHB production using Sudan Black dye.

The screening was done by staining the isolates with Sudan Black B on petri plates as well as under the microscope. Out of a total of 303 isolates, 194 isolates (72 from wheat, 58 from mustard, and 64 from sugarcane rhizospheric soil) were found to be positive for PHB production; showing PHB granules which appeared as blue/ black droplets in the cells under the microscope, and as blue/black colonies when stained on plates with the Sudan Black stain, indicating PHB accumulation in the cells (Fig. 1 a, b). All the PHB positive isolates were given codes based upon the district and the crop (W for wheat, M for mustard, and S for sugarcane) represented by the soil sample.

Quantification of PHB production and selection of isolates. All the 194 Sudan Black B positive isolates were subjected to quantitative estimation of PHB production using the method described by Law and Slepecky (1961). By referring to the standard curve (Fig. 2), the PHB yield was calculated for all the 194 isolates, and it was found to vary between 6.5 mg/ml (MeS-4 isolate) to 132.58 mg/ml (KW-4 isolate) (Table I).

Based upon the amount of PHB produced, all the 194 PHB positive isolates were divided into three categories: (i) **High PHB producers**: having PHB yield above 80 mg/ml; (ii) **Medium PHB producers**: having PHB yield between 50–80 mg/ml; and (iii) **Low PHB producers**: having a PHB yield lower than 50 mg/ml. Out of a total of 194 isolates, 86 isolates were found to belong to the category of high producers, 81 isolates were medium producers, and 27 isolates were found to be low producers. Maximum number of high PHB producers were found to belong to wheat rhizosphere (39.53%), followed by those from the sugarcane rhizosphere (31.39%) and least from mustard rhizospheric soil (29.06%). The number of medium and low PHB producers was found to be almost similar in the bacterial strains isolated from the rhizospheric area of all the

Table I
PHB production by PHB positive bacterial isolates isolated from the rhizospheric soil of wheat, mustard and sugarcane crop.

Sr. No.	District	Wheat Crop		Mustard Crop		Sugarcane Crop	
		Strain	PHB Yield (mg/ml)	Strain	PHB Yield (mg/ml)	Strain	PHB Yield (mg/ml)
1	Ambala	AW-1	125.93	AM-2	110.31	AS-1	74.79
		AW-2	88.76	AM-4	68.09	AS-3	75.82
		AW-3	83.71			AS-4	105.88
						AS-5	28.40
2	Bhiwani	BW-1	90.98	BM-1	90.98	BS-1	60.00
		BW-3	70.57	BM-3	57.73	BS-3	42.37
				BM-4	94.12	BS-4	96.80
						BS-6	105.21
3	Faridabad	FW-2	47.11	FM-1	117.73	FS-1	116.55
		FW-3	55.52	FM-3	89.02	FS-3	90.82
		FW-5	90.31			FS-4	78.45
4	Fatehabad	FtW-2	78.35	FtM-3	108.97	FtS-1	98.71
		FtW-3	93.76	FtM-5	109.48	FtS-2	64.02
		FtW-5	94.95	FtM-7	58.09	FtS-5	89.12
		FtW-6	94.43	FtM-8	109.64		
5	Gurgaon	GW-2	61.29	GM-1	90.36	GS-2	56.65
		GW-4	65.82	GM-3	39.12	GS-3	68.51
				GM-4	94.95	GS-5	65.77
				GM-5	68.20		
6	Hisar	HW-3	98.51	HM-2	109.64	HS-1	69.12
		HW-4	92.01	HM-4	71.60	HS-2	65.26
						HS-4	47.27
						HS-7	59.07
7	Jind	JW-1	109.28	JM-2	92.78	JS-1	39.79
		JW-2	78.40	JM-3	94.79	JS-3	59.64
		JW-4	48.45			JS-4	70.98
		JW-5	64.18			JS-6	77.17
		JW-7	90.26				
8	Jhajjar	JhW-1	25.98	JhM-2	68.76	JhS-1	47.42
		JhW-2	58.09	JhM-3	68.09	JhS-3	97.11
		JhW-4	42.37			JhS-5	71.86
		JhW-5	70.57			JhS-6	89.12
		JhW-6	47.42			JhS-7	27.42
		JhW-8	57.94				
9	Kaithal	KW-1	80.21	KM-2	74.79	KS-1	126.44
		KW-3	79.18	KM-4	88.87	KS-3	126.24
		KW-4	132.58				
10	Karnal	KaW-1	72.11	KaM-1	61.75	KaS-2	57.84
		KaW-2	64.23	KaM-3	54.33	KaS-3	107.06
		KaW-4	61.60				
11	Kurukshetra	KuW-1	89.38	KuM-2	34.43	KuS-2	72.84
		KuW-2	37.58	KuM-3	33.97	KuS-3	94.18
		KuW-4	74.33	KuM-4	52.58	KuS-4	65.77
		KuW-5	62.73	KuM-6	74.85		

C.d. Table I.

Sr. No.	District	Wheat Crop		Mustard Crop		Sugarcane Crop	
		Strain	PHB Yield (mg/ml)	Strain	PHB Yield (mg/ml)	Strain	PHB Yield (mg/ml)
12	Mahendergarh	MW-2	70.77	MM-1	28.35	MS-4	21.91
		MW-3	77.06			MS-6	130.93
13	Mewat	MeW-1	63.45	MeM-1	73.35	MeS-3	7.17
		MeW-3	69.38	MeM-2	77.37	MeS-4	6.50
		MeW-4	82.22	MeM-3	108.76		
		MeW-5	90.26	MeM-4	77.27		
14	Palwal	PlW-1	71.19	PlM-2	30.41	PlS-2	70.36
		PlW-3	89.64	PlM-3	90.31	PlS-3	94.95
		PlW-4	94.43			PlS-4	89.69
15	Panchkula	PnW-2	95.46	PnM-2	70.67	PnS-1	60.46
		PnW-3	93.04	PnM-3	61.55	PnS-2	77.17
		PnW-4	114.43	PnM-6	52.11		
		PnW-5	47.63				
16	Panipat	PaW-2	87.32	PaM-1	71.60	PaS-2	72.01
		PaW-3	76.08	PaM-2	78.92	PaS-3	105.88
				PaM-3	90.98	PaS-4	37.99
				PaM-4	89.02		
17	Rewari	RW-2	109.48	RM-1	119.59	RS-1	105.21
		RW-3	93.92	RM-2	83.51	RS-3	40.57
		RW-5	52.06	RM-4	53.04		
		RW-6	52.94	RM-5	69.07		
		RW-7	37.84	RM-7	52.27		
		RW-8	52.68	RM-8	59.12		
18	Rohtak	RoW-1	126.19	RoM-1	108.76	RoS-4	120.62
		RoW-2	108.66	RoM-2	69.07	RoS-5	73.92
		RoW-4	110.26	RoM-3	80.41	RoS-6	89.43
19	Sirsa	SiW-1	104.59	SiM-1	68.71	SiS-1	85.77
		SiW-2	90.26	SiM-2	47.89	SiS-3	118.40
		SiW-3	75.93			SiS-4	91.03
		SiW-4	90.82			SiS-5	68.20
20	Sonipat	SW-1	94.18	SM-4	61.75	SS-1	94.18
		SW-3	94.02			SS-2	108.87
		SW-4	83.56			SS-4	94.18
		SW-5	70.15				
21	Yamunanagar	YW-5	38.35	YM-3	108.09	YS-2	92.47
		YW-6	18.92	YM-4	94.95	YS-3	58.76
		YW-8	57.73	YM-6	92.17	YS-5	69.07

three crops. However, bacterial strains isolated from the wheat and sugarcane rhizospheric soil showed a higher accumulation of PHB as compared to the strains isolated from the mustard rhizosphere. But still, the differences in the PHB yield were not significant enough to confirm any correlation between the PHB yield and the rhizospheric crop specificity of a bacterial isolate. One isolate each from the category of low and medium producers, and six from the category of high producers for each of the three different crops (constituting a total of 24 isolates: 8 from each crop) were selected (Table II) for characterization, and for optimization of medium constituents to maximize PHB production.

Morphological, physiological and biochemical characterization of selected isolates. The selected 24 isolates were subjected to morphological and bioche-

Table II
Bacterial isolates selected for optimization of medium constituents for maximum PHB production.

Category (On the basis of PHB yield)	Sr. No.	Strain	PHB Concentration (mg/ml)	Gram Staining	Shape	Identification (on the basis of biochemical characterization)
High PHB Producers	1.	AW-1	125.93	+	Diplococcus	*Micrococcus* sp.
(PHB Yield: Above 80 mg/ml)	2.	KW-4	132.58	+	Bacillus	*Bacillus* sp.
	3.	PnW-4	114.43	+	Coccus	*Micrococcus* sp.
	4.	RoW-1	126.19	+	Coccus	*Micrococcus* sp.
	5.	RoW-4	110.26	+	Coccus	*Micrococcus* sp.
	6.	SW-3	94.02	–	Coccus	*Acinetobacter* sp.
	7.	AM-2	110.31	+	Coccus	*Arthrobacter* sp.
	8.	FM-1	117.73	+	Coccus chains	*Micrococcus* sp.
	9.	FtM-8	109.64	–	Coccus	*Acinetobacter* sp.
	10.	HM-2	109.64	+	Coccus chains	*Micrococcus* sp.
	11.	RM-1	119.59	+	Coccus	*Arthrobacter* sp.
	12.	RoM-1	108.76	–	Coccus	*Acinetobacter* sp.
	13.	KS-1	126.44	+	Coccus	*Micrococcus* sp.
	14.	KS-3	126.24	+	Bacillus	*Geobacillus (Bacillus)*
	15.	MS-6	130.93	+	Coccus	*Arthrobacter* sp.
	16.	RoS-4	120.62	–	Coccus	*Acinetobacter* sp.
	17.	SiS-3	118.40	+	Coccus	*Staphylococcus* sp.
	18.	KaS-3	107.06	–	Bacillus	*Bacillus* sp.
Medium PHB Producers	19.	FtW-2	78.35	–	Coccus chains	*Acinetobacter* sp.
(PHB Yield: Between 50–80 mg/ml)	20.	PaM-2	78.92	+	Coccus	*Micrococcus* sp.
	21.	FS-4	78.45	+	Coccus	*Micrococcus* sp.
Low PHB Producers	22.	JW-4	48.45	–	Coccus	*Acinetobacter* sp.
(PHB Yield: Below 50 mg/ml)	23.	SiM-2	47.89	–	Coccus	*Acinetobacter* sp.
	24.	JhS-1	47.42	+	Coccus	*Streptococcus* sp.

mical characterization. Colony morphology was studied in terms of their size, shape, colour, texture and staining characteristics. Size varied from very small to large, while shapes swirled mainly between circular, ellipses and irregular. Colour varied from white to off-white to slightly yellowish. All types of colony textures were obtained such as raised, flat, convex, wrinkled, grainy, slimy *etc*. Gram staining revealed the presence of both Gram-positive and Gram-negative cells. However, a major section of the isolates was found to be Gram-positive in nature, with only eight Gram-negative isolates among the total 24 selected PHB positive isolates; and

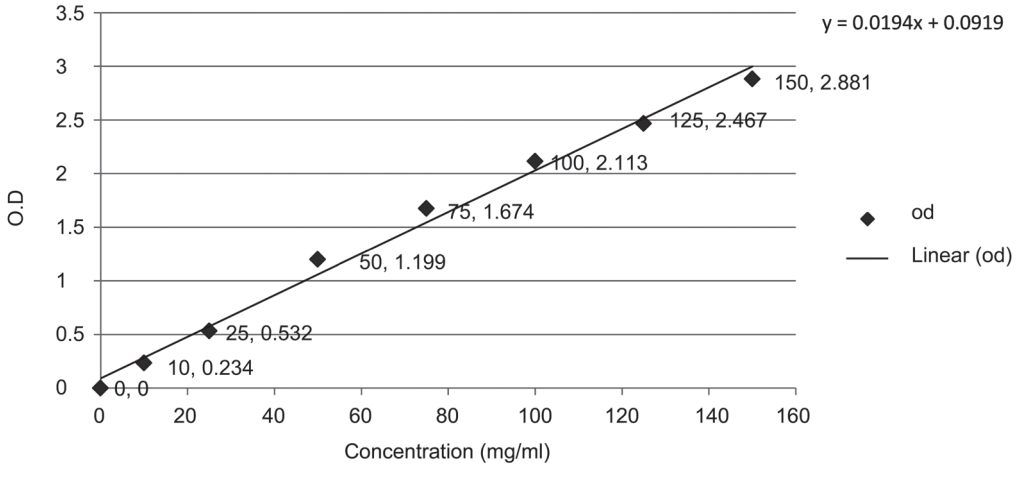

Fig. 2. PHB Standard Curve.

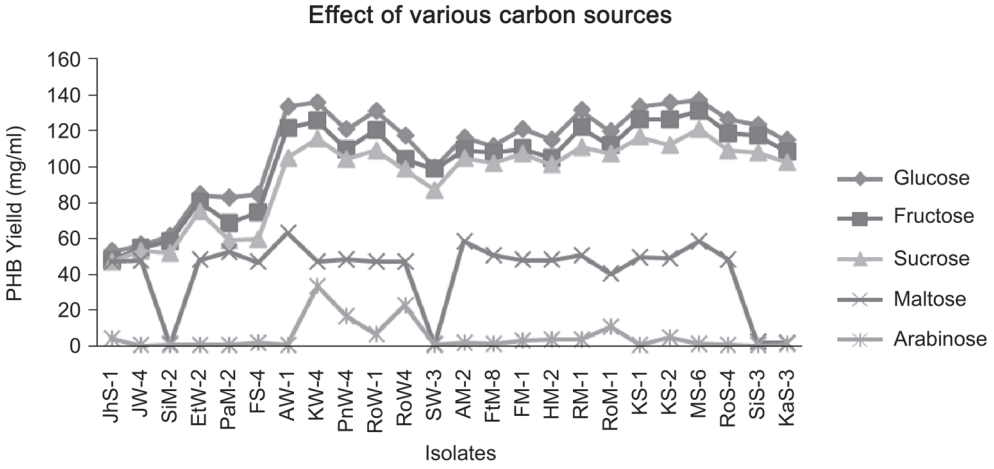

Fig. 3. Effect of various carbon sources on PHB production.

just 17 Gram-negatives among the total 194 PHB positive isolates obtained. Both coccus and bacillus forms were observed and cells could be seen in individual cell forms as well as in diplo- and chain forms (Table II).

All the PHB positive isolates were subjected to the standard biochemical tests and identification was carried out as per the details given in Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1994). On a preliminary basis, the isolates have been found to belong to six genera, namely *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Acinetobacter* and *Arthrobacter.* The molecular identification of the isolates is underway.

Optimization of culture medium constituents and growth conditions for maximum PHB production. PHB accumulation by different bacteria is affected by the carbon (C) and nitrogen (N) sources being used for bacterial growth. The accumulation has been found to be enhanced if the bacterial cells are cultivated in the presence of an excess carbon source, and when their growth is impaired or restricted by lack of other nutrients, such as nitrogen, phosphorous and sulphur, or also dissolved oxygen (Reddy *et al.*, 2009). Therefore, it is important to find an optimum C and N source, and also to optimize their ratios for getting the maximum accumulation of PHB. In view of this, a total of twenty four isolates were selected for optimization of different medium constituents (carbon source, nitrogen source, and C/N ratio) for maximization of PHB production.

Effect of different carbon sources on PHB production. The selected isolates were grown in the presence of five carbon sources: glucose, fructose, sucrose, maltose, and arabinose. After incubation, the PHB produced by the isolates was quantified spectrophotometrically for selection of parameters showing highest PHB production. Figure 3 depicts the effect of different carbon sources on PHB yield. It was observed that different isolates utilize the various carbon sources differently. However, following a common trend, all of them showed maximum PHB accumulation when the minimal medium was supplemented with glucose, followed by supplementation with fructose and sucrose as C-sources. PHB yield was very low when maltose was used as the C-source; and with arabinose, none of the isolates could accumulate sufficient PHB. The mean PHB production values of all the 24 isolates were found to be 105.52 mg/ml, 102.52 mg/ml, 94.64 mg/ml, 41.89 mg/ml, and 5.23 mg/ml with glucose, fructose, sucrose, maltose and arabinose, respectively, as C-sources. Thus, among the different carbon sources tested to evaluate their effects on PHB yield, glucose was found to be the best carbon source, and least PHB production was obtained with arabinose. The isolate MS-6 recorded the highest PHB yield of 137.2 mg/ml, followed by KW-4, KS-3 and AW-1 with a PHB yield of 136.1, 135.6 and 133.7 mg/ml, respectively.

Effect of different nitrogen sources. PHB yields produced by the selected isolates when grown on different nitrogen sources in presence of the best carbon source (glucose) and four different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate, and yeast extract) are presented in Fig. 4. Out of the four sources, ammonium sulphate was found to support highest PHB production; maximum PHB accumulation being observed by KS-3 (138.7 mg/ml), followed by AW-1 (138.2 mg/ml), MS-6 (137.9 mg/ml) and KW-4 (136.4 mg/ml). The mean PHB yields of all the 24 isolates was found to be 115.30 mg/ml, 81.32 mg/ml, 59.46 mg/ml and 25.93 mg/ml with ammonium sulphate, ammonium chloride, ammonium nitrate, and yeast extract, respectively.

The presence of high concentrations of nitrogen in the culture medium has been reported to be an inhibitor for the accumulation of PHB. Hence, the concentration of the best nitrogen source also needs to be optimized. Different concentrations (0.5, 1.0 and 1.5 g/l) of ammonium sulphate were therefore amended to the

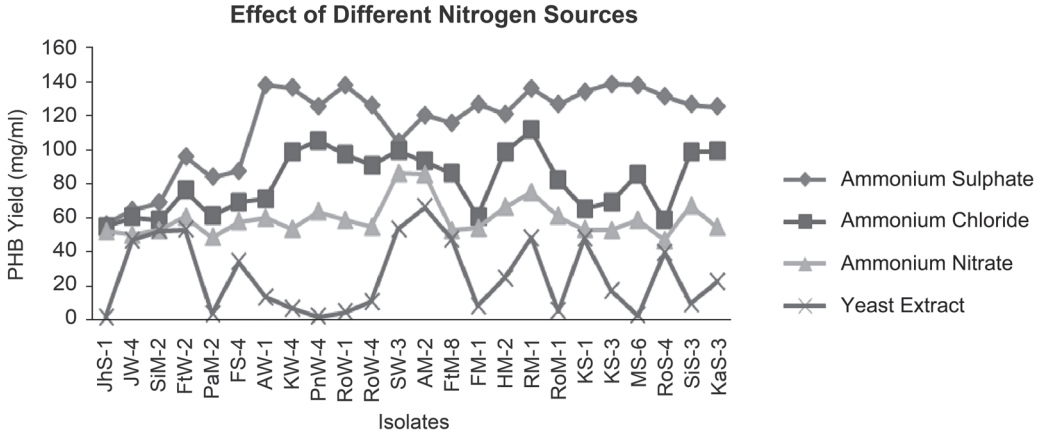

Fig. 4. Effect of different nitrogen sources on PHB production.

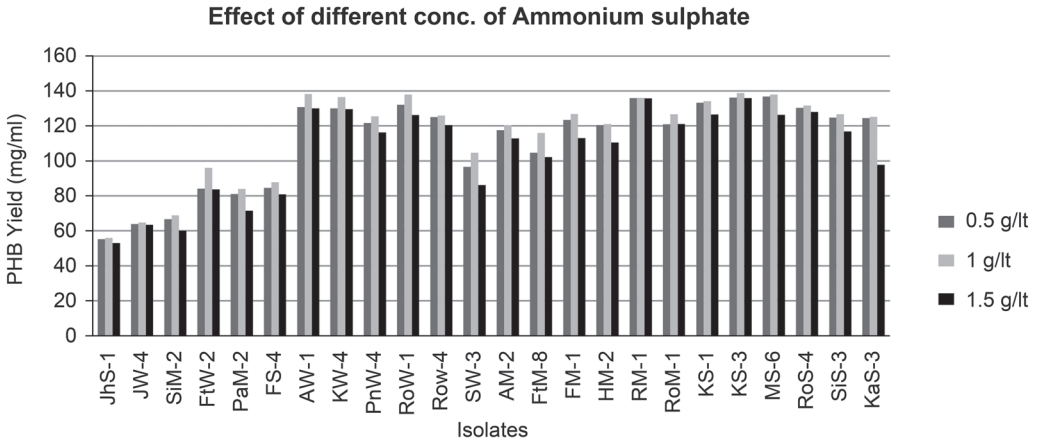

Fig. 5. Effect of different concentrations of ammonium sulphate on PHB production.

medium containing glucose as the best carbon source. From the studies, it was revealed that ammonium sulphate at 1.0 g/l was the optimum concentration for maximum PHB accumulation (Fig. 5). When ammonium sulphate concentration was increased from 0.5 to 1.00 g/l, the PHB accumulation also increased; but, concentrations above 1.0 g/l resulted into a decrease in PHB production by all the isolates.

Effect of relative concentration of carbon and nitrogen sources on PHB production. Under normal conditions, bacteria synthesize their cell materials like proteins and grow. But, in nutrient limiting conditions, bacteria may shift their protein synthesis to PHB synthesis for survival. To exploit this, the C:N ratios in the growth medium were adjusted to10:1, 15:1, 20:1 and 25:1 using glucose and ammonium sulphate as C and N sources, respectively; and the effect of these different concentrations on PHB production was studied (Fig. 6). As the carbon content was increased in the media keeping N as constant; up to a certain limit (*i.e.*, 20:1) PHB accumulation was found to increase, but thereafter (25:1) it showed a decline. This was probably due to the substrate inhibition.

The mean PHB yield with the four different C:N ratios (10:01, 15:01, 20:01 and 25:01) was found to be 107.74 mg/ml, 111.50 mg/ml, 119.86 mg/ml & 115.73 mg/ml, respectively, thus, showing that the C/N ratio 20:01 supported the maximum PHB productivity.

Effect of pH on PHB production. The effect of pH of the medium on PHB production was assessed, for which three different pH conditions (pH-6, pH-7 and pH-8) were maintained in the media prepared using the best carbon and nitrogen sources (Fig. 7). The results obtained revealed the mean PHB yields to be 3.76 mg/ml, 118.73 mg/ml and 100.56 mg/ml at pH 6.0, 7.0 and 8.0, respectively. Maximum PHB accumulation was observed for KS-3 and KW-4 (139.7 mg/ml by both), followed by MS-6 (137.3 mg/ml) and AW-1 (136.6 mg/ml) at pH 7.0. All the isolates were found to produce a very low PHB yield at pH 6. However, at pH 8.0, although the PHB yield was not too low, but still it was found to be less as compared to the accumulation at pH 7.0. It was thus concluded that out of the different media pH tested, pH 7.0 was optimum for PHB production.

Effect of incubation temperature on PHB production. Effect of varying the incubation temperature on

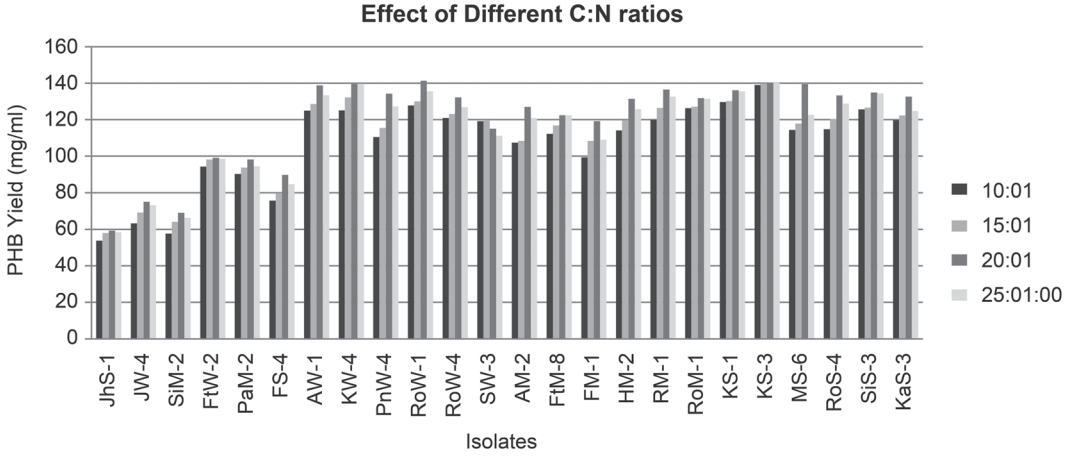

Fig. 6. Effect of different concentrations of carbon and nitrogen on PHB production.

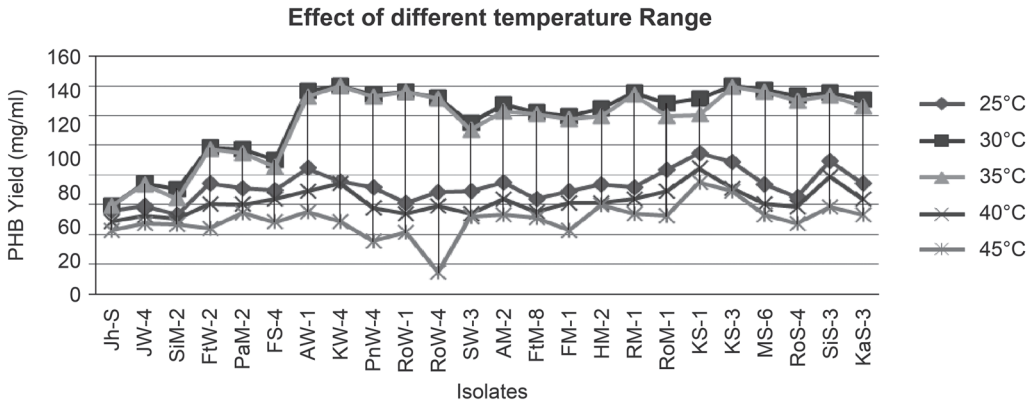

Fig. 7. Effect of incubation temperature on PHB production.

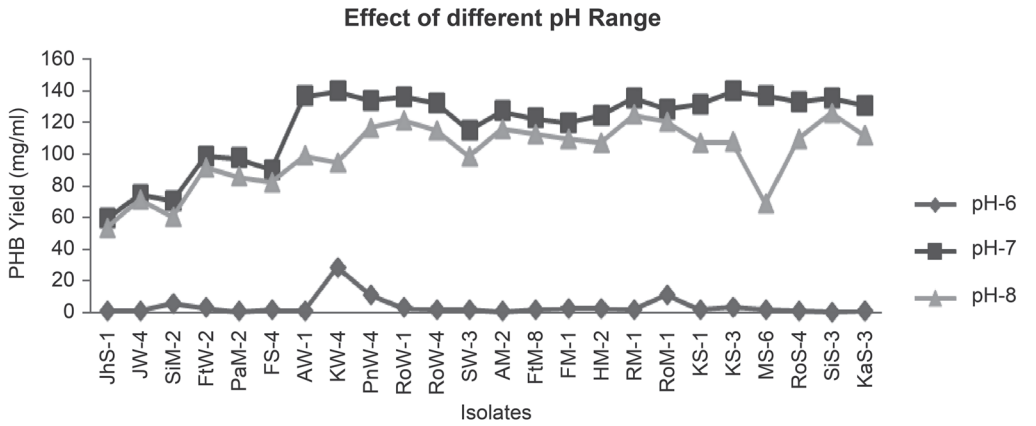

Fig. 8. Effect of pH on PHB production.

PHB production was studied by maintaining different temperature conditions (25°C, 30°C, 35°C, 40°C and 45°C) during incubation of the isolates inoculated in a medium prepared using the best carbon and nitrogen sources. The mean PHB yields of all the 24 isolates with these five different temperatures were found to be 72.47 mg/ml, 118.73 mg/ml, 115.81 mg/ml, 62.40 mg/ml and 50.12 mg/ml, respectively (Fig. 8). The incubation temperature of 30°C was found to be the optimum temperature for maximum PHB production by all the isolates. This was followed by the incubation temperature of 35°C at which the PHB yield was found to be only slightly less than the yield at 30°C. However, below 30°C and beyond 35°C, the PHB yield dropped significantly, suggesting that very low or very high temperatures do not support PHB accumulation.

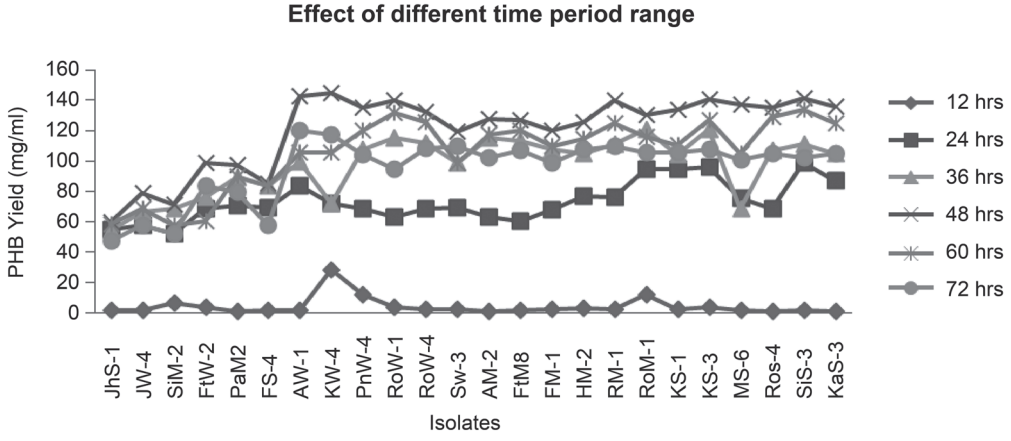

Fig. 9. Effect of incubation time on PHB production.

Effect of incubation time on PHB production. The effect of varying the growth incubation time of isolates on their PHB production was assessed by growing the isolates in a medium prepared using the best C and N sources for different lengths of time (12, 24, 36, 48, 60 and 72 h). The results revealed the mean PHB yields to be 04.08 mg/ml, 73.35 mg/ml, 97.21 mg/ml, 120.88 mg/ml, 105.72 mg/ml, 95.44 mg/ml, after 12, 24, 48, 60 and 72 h of incubation, respectively. It was observed that the PHB accumulation by different isolates increased up to 48 h of incubation, after which there was a decline in PHB production. Thus, an incubation period of 48 h was found to be optimum for maximum PHB production.

Discussion

Poly (β-hydroxybutyric acid) (PHB) is an intracellular microbial thermoplastic that is widely produced by many bacteria (Lee and Chang, 1995; Poirier *et al*., 1995; Braunegg *et al*., 1998). Due to its similarity in physical properties with synthetic plastics, it is possible to use PHB in place of conventional plastics. In certain applications, PHB can directly replace the traditional, nonbiodegradable polymers. Use of PHB, primarily as polymer blends, is therefore, becoming quite popular; as such blends greatly increase the spectrum of possible applications by expanding the range of available physical properties. But, the high production cost of PHB restricts its widespread use. However, if microorganisms capable of producing polyhydroxybutyrate are exploited for optimization of PHB production, this may reduce production cost and help in their large scale use for different applications. Plants are colonised by numerous bacteria which are capable of accumulating polyhydroxybutyrates as energy and carbon source; particularly the rhizosphere, which is characterised by temporal and spatial changes in nutrient availability, appears to be a good source for the isolation of PHB producers for biotechnological applications. Keeping this in view, the present study was designed to obtain PHB producing bacterial isolates from the rhizosperic soil of different crops, and to optimize the various culture conditions for maximizing PHB production by them.

Keeping this in view, in the present study, bacteria were isolated from soil samples collected from the rhizospheric area of three different crops on nutrient agar medium. Screening of the bacterial isolates for PHB production was done by using Sudan Black B dye. Sudan Black dye has been used as a screening measure for PHB production by several workers (Pal *et al*., 2009; Reddy *et al*., 2009; Bereka and Thawadi, 2012; Aly *et al*., 2013). Further, PHB production by different isolates was assessed by growing them in MSM supplemented with different sugars; and it was observed that the isolates produced maximum PHB by utilization of glucose as the sugar or C-source. Sujatha *et al*. (2005) reported that Luria Bertani broth supplemented with 2% glucose favours PHB accumulation due to higher C: N ratio. However, working with different carbon sources in MSM broth, Khanna and Srivastava (2005) observed higher PHB yield on fructose by *Alcaligenes eutrophus*. They reported that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, support growth and subsequently PHB production, however, the complex molecules like starch and lactose were not utilized for effective PHB production. In our experiments also, the isolates did not produce PHB on maltose and arabinose indicating that the isolates do not possess enzymes involved in the degradation of arabinose and maltose into glucose. As the complexity of the carbon source increased, PHB yield was found to decrease. Similar conclusions were made by Joshi and Jayaswal (2010). To study the effect of N and to select the best N source for maximum PHB production, different nitrogen sources like ammonium sulphate, ammonium chloride and yeast extract were included in the mineral salts medium (1 g/l), with the best carbon source

(glucose, 2%). It is evident from our results that ammonium sulphate was the best supporter of PHB production. These results are in agreement with the results obtained by Khanna and Srivastava (2005) who also observed the highest PHB production (2.260 g/100 ml) by *R. eutropha* on MSM medium supplemented with ammonium sulphate. Mulchandani *et al.* (1989) and Raje and Srivastav (1998) also worked on the accumulation of PHB by *A. eutrophus* with different salts of ammonium. Similar to the results of the present study, they also obtained highest PHB yield in ammonium sulphate followed by ammonium chloride. Ammonium sulphate being a simple nitrogen source is probably more readily available than the other complex nitrogen sources.

PHB yield was also estimated by growing the isolates in MSM supplemented with different concentrations of the best nitrogen source, *i.e*., ammonium sulphate. It was found that ammonium sulphate at a concentration of 1.0 g/l supported the highest PHB production compared to other concentrations (0.5 and 1.5 g/l) tested. An observed decrease in PHB accumulation upon increasing the ammonium sulphate concentration beyond 1.0 g/l may be attributed to the absence of nitrogen stress condition required for accumulation of PHB. The results of the present study are in accordance with Belal (2013), Khanna and Srivastava (2005), and Shaaban *et al*., (2012) who have also reported maximum PHB accumulation at 1 % concentration of ammonium sulphate. PHB accumulation when assessed by using different C:N ratios maintained using the best carbon and nitrogen sources, it was found that C:N ratio as 20:1 resulted into maximum PHB production. Similar observations have been made by Belal (2013) and Panigrahi and Badveli (2013).

The results of PHB yields at different temperature conditions are in accordance with Grothe *et al*. (1999). They found that 33°C incubation temperature is optimum for PHB synthesis under fermentation condition. The data from our study also concluded that although 30°C was the optimum temperature for PHB production, but high PHB accumulation was also observed at 35°C. The influence of pH of culture media on PHB production was also optimized and highest production was obtained at pH range of 7.0. The obtained results (pH) were in agreement with Aslim *et al*. (2002) who also observed that the PHB in *Rhizobium* sp. strain produced was maximum at pH 7.0. Grothe *et al.* (1999) also reported that pH value ranging from 6.0–7.5 is optimum for PHB production. However, contrary to these, Nakata (1963) reported that PHB production occurs at pH 6.4 and that the lack of polymer accumulation at higher pH value can best be explained by an effect on the degenerative enzymes of polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis.

During optimization studies related to the incubation period, the maximum PHB yield was found to be obtained after 48 h of incubation of the isolates in the medium under stationary conditions of growth and it was found to decrease thereafter. This reduction in PHB production after 48 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production (Flora *et al*., 2010). Studies conducted by Bonartseva *et al*. (1994) are also in consonance with these results wherein the maximal PHB accumulation was observed at 48 h. After 48 h, unfavorable conditions of the medium caused a decrease in PHB yield. This might be because the increase in medium viscosity accompanies exopolysaccharide production resulting in oxygen transfer limitation, causing a decrease in PHB synthesis (Stam *et al*., 1986). The yield decreased at 60 and 72 h; the decrease of PHB indicates that the bacteria use PHB as a source of carbon, causing unsuitable conditions due to inadequate nitrogen and carbon sources in the medium.

Conclusion. The major objective of the present study was to isolate effective polyhydroxybutyrate producing strains and to optimize their culture conditions so as to obtain the maximum PHB yield. According to the results of the present study, the optimum culture conditions for maximum PHB production by a wide range of soil bacteria include supplementation of the culture medium with glucose as C-source, ammonium sulphate at a concentration of 1.0 g/l with the C:N ratio maintained as 20:1 , pH as 7.0 and incubation at 30°C for 48 h. Four promising isolates, *viz.*, KW-4, KS-3, MS-6 and AW-1 were found to accumulate a high level of PHB at the optimized culture conditions; thus, showing a potential for their exploitation in industrial PHB production. The present study has thus provided useful data about the optimized conditions for PHB production that can be utilized for industrial production of PHB, a fast emerging alternative of non biodegradable plastics.

Acknowledgement

We wish to express our sincere gratitude to the University Grants Commission, New Delhi, for providing the financial support for carrying out this research.

Literature

Aly M.M., M.O. Albureikan, H.E.I. Rabey and S.A. Kabli. 2013. Effects of culture conditions on growth and poly-B-hydroxybutyric acid production by *Bacillus cereus* MM7 isolated from soil samples from Saudi Arabia. *Life Science Journal*. 10(4): 1884–1891.

Aslim B., Z.N. Yuksekdag and Y. Beyatli. 2002. Determination of PHB growth quantities of certain *Bacillus* species isolated from soil. *Turkish Electronic J. Biotechnol*. Special Issue: 24–30.

Barnard G.N. and J.K. Sander. 1989. The poly-b-hydroxybutyrate granule *in vivo*. A new insight based on NMR spectroscopy of whole cells. *J. Biol. Chem*. 264: 3286–3291.

Belal E.B. 2013. Production of poly-B-hydroxybutyric acid (PHB) by *Rhizobium elti* and *Pseudomonas stutzeri*. *Current Research Journal of Biological Sciences*. 5(6): 273–284.

Berekaa M. and A.M. Thawadi. 2012. Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by *Bacillus megaterium* SW1-2: Application of Box-Behnken design for optimization of process parameters. *African J. Microbiol. Res*. 6(4): 838–845.

Bonartseva, G.A., V.L. Myshkina and E.D. Zagreba. 1994. Poly-b-hydroxybutyrate content in cells of various *Rhizobium* species during growth with different carbon and nitrogen sources. *Microbiol.* 63(1): 45–48.

Braunegg G., G. Lefebvre and K.F. Genser. 1998. Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and Engineering Aspects. *J. Biotechnol*. 65: 127–161.

Fiechter A. 1990. Plastics from bacteria and for bacteria: Poly (beta-hydroxybutyrate) as natural, biocompatible and biodegradable polyesters. Springer-Verlag, New York, pp. 77–93.

Flora G.D., K. Bhatt and U. Tuteja. 2010. Optimization of culture conditions for poly b-hydroxybutyrate production from isolated *Bacillus* species. *J. Cell and Tissue Res.* 10(2): 2235–2242.

Gram C. 1884. The differential staining of Schizomycetes in tissue sections and in dried preparations. *Fortschitte der Medicin*. 2: 185–189.

Grothe, E., M. Moo-Young and Y. Chisti. 1999. Fermentation optimization for the production of poly-(B-hydroxybutyric acid) microbial thermoplastic. *Enzym. Microbial. Tech*. 25: 132–141.

Halami P.M. 2008. Production of polyhydroxyalkanoate from starch by the native isolates *Bacillus cereus* CFR06. *World J. Microbiol. Biotechnol*. 24(6): 805–812.

Haywood, G.W., A.J. Anderson, L. Chu and E.A. Dawes. 1988. Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. *FEMS Microbiol. Lett*. 52: 91–96.

Hyakutake M., Y.Saito, S. Tomizawa, K. Mizuno and T. Tsuge. 2011. Polyhydroxyalkanoate (PHA) synthesis by class IV PHA synthases employing *Ralstonia eutropha* PH44 as host strain. 75(8): 1615–1617.

Joshi P.A. and S.R. Jaysawal. 2010. Isolation and characterization of poly-B-hydroxyalkanoate producing bacteria from sewage sample. *J. of Cell and Tissue Research*. 10(1): 2165–2168.

Khanna S. and A. K. and Srivastava. 2005. Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Process of Biochemistry*. 4: 2173–2183.

Law J.H. and R.A. Slepecky. 1961. Assay of poly-β-hydroxybutyric acid. *J. Bacteriol*. 82: 33–36.

Lee S.Y. 1996. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* 49: 1–14.

Lee S.Y. and H.N. Chang. 1995. Production of poly(hydroxyalkanoic acid). *Adv. Biochem. Eng. Biotechnol.* 52: 27–58.

Legat A., C. Gruber, K. Zangger, G. Wanner and H. Stanlotter. 2010. Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species. *Appl. Microbiol. Biotechnol.* 87(3): 1119–1127.

Mohamed T., M. Shaaban, A. Turky and E.I. Mowafy. 2012. Production of some biopolymers by some selective Egyptian soil bacterial isolates. *J. Applied Sciences and Research*. 8(1): 94–105.

Mulchandani A., J.H.T. Luong and C. Groom. 1989 Substrate inhibition kinetics for microbial growth and synthesis of poly-β-hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697. *Applied Microbial Biotechnology* 30: 11–17.

Murray R.G.E., R.N. Doetsch and C.F. Rainbow. 1994. Determinative and cytological light microscopy. In: Methods for General and Molecular Bacteriology. Eds., Gerhardt P., R.G.E. Murray, W.A. Wood and N.R. Kreig. Washington DC: American Society for Microbiol. Pp. 21–41.

Nakata H.M. 1963. Effect of pH on intermediates produced during growth and sporulation of *Bacillus cereus. J. Bacteriol*. 86: 577–581.

Pal A., A. Prabhu, A.A. Kumar, B. Rajagopal, K. Dadhe, V. Ponnamma and S. Shivakumar. 2009. Optimization of process parameters for maximum poly(-B-) hydroxybutyrate (PHB) production by *Bacillus thuringiensis* IAM12077. *Polish J. Microbiol*. 58(2): 149–154.

Panigrahi S. and U. Badveli. 2013. Screening, isolation and quantification of PHB-producing soil bacteria. *Int. J. of Engg. Sci. Innovation*. 2(9): 01–06.

Peoples O.P. and A.J. Sinskey. 1989. Poly-3-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). *J. Biol. Chem*. 264: 15298–15303.

Poirier Y., C. Nawrath and C. Somerville. 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Biotechnology* (NY) 13: 142–150.

Raje, P. and A.K. Srivastav. 1998. Updated mathematical model and fed batch strategies for poly-β-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus*. *Bioresource Technology* 64: 185–192.

Reddy S.V., M. Thirumala and S.K. Mahmood. 2009. Production of PHB and P(3HB-co-3HV) biopolymers by *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge. *World J. of Microbiol. and Biotechnol.* 25(3): 391–397.

Rodriguez-Contreras A., M. Koller, M.M. deSousa Dias, M. Calafell, G. Braunegg and M.S. Marques-Calvo. 2013. Novel poly [(R)-3-hydroxybutyrate]- producing bacterium isolated from a Bolivian hypersaline lake. *Food Technol. and Biotechnol*. 51(1): 123–130.

Sangkharak K. and P. Prasertsan. 2008. Nutrient optimization for production of polyhydroxyutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electronic Journal of Biotechnology*. 11(3): 83–94.

Shaaban M.T., M. Attia, A.S. Turky and E.I. Mowafy. 2012. Production of some biopolymers by some selective Egyptian soil bacterial isolates. *J. Appl. Sci. Res*. 8(1): 94–105.

Soam A., A.K. Singh, R. Singh and S.K. Shahi. 2012. Optimization of culture conditions for bio-polymer producing *Bacillus mycoides* (WSS2) bacteria from sewage. *Current Discovery*. 1(1): 27–32.

Stam H., H.W. Verseveld, W.D.E. Van Vries and A.H. Stouthamer. 1986. Utilization of poly-β-hydroxy butyrate in free-living cultures of *Rhizobium* ORS571. *FEMS Microbiol. Lett.* 35: 215–220.

Sudesh K., H. Abe, and Y. Doi. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci.* 25: 1503–1555.

Sujatha K.A., Mahalakshmi and R. Shenbagarathai. 2005. Analysis of PCR products for PHB production in indigenous *Pseudomonas* sp. LDC-5. *Indian J. Biotechnol*. 4: 323–335.

Suresh Kumar M., S.N. Mudaliar, K.M.K. Reddy and J. Chakrabarti. 2004. Production of biodegradable plastics from activated sludge generated from a food processing industrial waste water. *Bioresource Technol*. 95: 327–330.

Verlinden R.A.J., D.J. Hill, M.A. Kenward, C.D. William and Radeckal. 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *J. Appl. Microbiol*. 102: 1437–1449.

Wang H.H., X.T. Li and G.Q. Chen. 2009 Production and characterization of homopolymer polyhydroxyheptanoate by a *fad*BA knockout mutant *Pseudomonas putida* KTOY06 derived from *P. putida* KT2442. *Process Biochemistry* 44(1): 106–111.

Williams S.T., M.E. Sharpe and J.G. Holt. 1994. Bergey's Manual of Systematic Bacteriology. 9th Eds. Williams and Wilkins, Baltimore, USA.