Productivity and optimization of Poly ß-Hydroxy Butyrate (PHB) by *Alcaligenes eutrophus, Pseudomonas putida* and *Rhizobium meliloti*

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SUMMARY

The PHB production of three test organisms (*Alcaligenes eutrophus, Pseudomonas putida* and *Rhizobium meliloti)* was observed under oil immersion microscope and plate method. The bacteria were estimated in various industrial waste substrates, *viz.* molasses and sesamum oil waste. High PHB productivity was observed in sesamum oil waste containing medium when compared with molasses. High production of PHB was observed in *Alcaligenes eutrophus* (38~g/10ml/40%) when compared with *Pseudomonas putida* (23~g/10ml/40%) and *Rhizobium meliloti* (22~g/10ml/40%). It was concluded that the oil waste based medium and *Alcaligenes eutrophus* be used for PHB production in large scale that minimize the cost of the product. Thus, the use of petroleum-derived plastics can be minimized and by which we can live in a plastic pollution free earth.

Key words : *Alcaligenes eutrophus, Pseudomonas putida* and *Rhizobium meliloti,* Poly β-Hydroxy Butyrate (PHB).

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Plastics are non degradable. The non-degradable plastics accumulate in the environment at a rate of millions of tone per year causing several problems. Recently, issues concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics (Anderson *et al.*, 1990). Poly β-Hydroxy Butyrate (PHB) is an alternative source of the plastics which has similar physical properties like polypropylene and it can be easily biodegradable aerobically and anaerobically (Hankarymer and Jieerdema, 1998). PHB is one of the important storage reservoirs providing energy. It is the cellular inclusion bounded by lipid non-unit membrane separate from cytoplasm. Beta-hydroxy butyrate is connected by ester linking and form PHB (Luzier, 1992).

Poly β-hydroxy butyrate and Poly β-hydroxy valerate (PHV- is a thermoplastic material) belong to PHBV family, which nearly fit to one ecosystem (Brandl *et al.*, 1990). PHV is a thermoplastic material. PHB molecule joined by ester bonds between the carboxyl and the hydroxyl group of the adjacent molecules, in each polymer methyl group attached to the back bane, is present in a single configuration throughout the chain. The structure of the PHB contains the repeating unit of PHB and has a chiral center (Lamoigne, 1924). Molecular structure of PHB does not depend on the features of the strain and condition of carbon nutrition of microorganisms producing PHB (Volova *et al.*, 2000). This bioplastics has many obvious applications in the bone plates, nails, screws (Azehar and Tanisamdin, 2003) and the treatment of asteomylitis (Fusun and Zeynep, 2000).

In the present study indicates, the production of PHB in the important three PHB producers such as *Alcaligenes eutrophus, Pseudomonas putida, Rhizobium meliloti* which can grow on carbon and nitrogen source in both aerobic anaerobic conditions. *Alcaligenes eutrophus* being able to utilize a variety of simple and complex sources as carbon source in both aerobic and anaerobic condition and optical temperature of 30-37°C for PHB synthesis (Doi, 1992). *P. putida* growth occurs from 4-43°C chemo organo trophic, able to use other than one carbon organic compound as sole carbon and energy source for production of PHB (Morris and Roberts, 1959).

R. meliloti cells are able to exist as two distinct entities, the free-living form competes for limiting nutrients with other soil inhabitants. While the symbiotic nitrogen fixing bacteroid forms an intimate association with the host plant from which a steady supply of nutrients is derived. When excess of carbon nutrient is available but a non-carbon nutrient such as N, P, or O_2 is limiting for growth, many bacteria accumulate the intracellular carbon storage compound PHB (Aneja and Charles, 1999).

The current cost of the PHB production is considerly more than that of the synthetic plastics (Byrom, 1987). The aliphatic PHB as

a granular component in bacterial cell proceed without any of the controversies, which marked the recognized as a prototypical biodegradable thermoplastic to solve the waste disposal challenge (Robert *et al.,* 2005). A major drawback of the commercialization of PHB is their much higher of production cost compared with petrochemical based synthetic plastic materials (Lee, 1996).

The main aim of the work is the production of PHB by *Alcaligenes eutrophus, Pseudomonas putida* and *Rhizobium meliloti* by using various industrial waste as a cheep substrate (molasses, sesamum oil waste) to minimize the production cost of the PHB and the differentiations between the dry cell weight of the three Gram negative bacteria and by which making a the pollution free environment from non-biodegradable plastics.

MATERIALS AND METHODS

Alcaligenes eutrophus (MTCC 1285), *Pseudomonas putida* (MTCC 1194) and *Rhizobium meliloti* (MTCC 2426) cultures were collected from the Microbial Type Culture Collection (MTCC) of Chandigarh for the production of PHB. The culture purity was tested as per the guidelines of Bergey's Manual of Bacteriology (Holt, 1997). Indole test, Methyl red test, Vogus-Prokaeur test, Citrate utilization test, Oxidase test, Catalase test, Starch hydrolysis, Gelatin hydrolysis, Carbohydrate fermentation tests were done to identify the test organisms. Identification of *rhizobium* was made by using glucose peptone agar (GPA) test.

In the PHB production, different types of media were used such as nitrogen limited medium and industrial waste based medium. In the nitrogen limited medium, glucose was used as standard carbon source, in the waste based medium waste raw materials was used as a carbon source in various concentration such as 10%, 20%, 30% and 40%. Sesamum oil waste and molasses were collected from relevant industry and sesamum oil waste was dried and finally powdered and molasses diluted with sterile water.

The test organism was grown on Nutrient broth medium at 37°C for 24hrs. The broth culture was centrifuged at 5000rpm for 10min. The culture pellets were transferred to nitrogen limited mineral medium and various industrial waste based medium as a sole carbon source, incubated at 37ºC for 72hrs in an incubatory shaker. The fermentative medium was maintained at pH 7 to 7.2.

After incubation, 10ml of culture was taken and centrifuged at 8000rpm for 15min. The supernatant was discarded and the pellet was treated with 10ml of sodium hypochloride and the mixture was incubated at 37ºC for 2hrs. After incubation, the mixture was centrifuged at 5000rpm for 15min and then washed with distilled water, acetone, diethyl ether for washing and extraction. After washing, the pellets dissolved in 5ml of boiling chloroform and then centrifuged at 1500rpm for 10min. The bottom phase containing PHB with chloroform collected and was used for assay of PHB.

The chloroform containing PHB was treated with 2ml of concentration sulphuric acid and boiled at 100ºC for 10min. The different concentrations of crotonic acid was prepared with concentrated sulphuric acid and boiled using water bath. The optical density of crotonic acid was read at 230nm and the standard graph was prepared (Law *et al.*, 1960). The optical density of samples was read at 230nm using UVspectro photometer. The concentration of PHB was determined by plotting the OD with the standard graph of crotonic acid (Lee *et al.*, 1995).

The pure form of PHB was collected using the standard method. The culture a pellet was transferred to nitrogen limited mineral medium and various industrial waste based medium as a sole carbon source, incubated at 37ºC for 72hrs in an incubatory shaker. After incubation, 10ml of culture was centrifuged at 8000rpm for 15min. The supernatant was discarded. The pellet was treated with 10ml of sodium hypochloride and the mixture was incubated at 37ºC for 2hrs. After incubation, the mixture was centrifuged at 5000rpm for 15min and then washed with distilled water, acetone, methanol for washing and extraction and centrifuged. The pellet was resuspened in 5ml of chloroform and evaporated the chloroform by pouring the solution on sterile tray and kept in hot air oven at 4ºC. After evaporation, the powder was collected for infrared analysis (Rawate and Mavnkurve, 2002).

Infrared light radiation can excite a molecule from its lower vibrational state to higher vibrational state. Each compound has characteristics energy of absorption for different types of vibration along the bonds. This is useful to find out the functional groups present in the compound (Silverstein and Morril*,* 1981). One mg of sample was ground well with 10mg of spectral pure anhydrous potassium bromide crystals. The powder was made into a pellet for IR analysis, relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400-4000cm^{-} using JEOL-FT IR-400 plus double beam spectrometer. IR spectra of the samples were measured at ambient conditions. It is used to get accurate value and to estimate of values between various data. By statistical analysis of standard deviation all the obtained data of the experiment were computed by using the appropriate formula.

RESULTS AND DISCUSSION

The cultural character of test organisms were observed based on the colony morphology and microscopic observations. Biochemical character of test organisms was observed (Table 1). Colony grown in nitrogen limited mineral medium appeared as dirty white colour colonies. After staining, it appeared as light gray to blue colour colonies. A blue-black colour droplet was observed within the cell and around the cytoplasm in pink colour.

Table 1 : Biochemical confirmation test

Sr. No	Test	*A. eutrophus*	*P. putida*	*R. meliloti*
1.	Indole	Negative	Negative	-
2.	Methyl red	Negative	Negative	-
3.	Voges proskaeur	Negative	Negative	-
4.	Citrate utilization test	Negative	Positive	-
5.	Oxidase	Positive	Positive	-
6.	Catalase	Positive	Negative	-
7.	Starch	Negative	Negative	-
8.	Gelatin	Negative	Negative	-
9.	Carbohydrate	Negative	Positive	
	Fermentation	Positive	Negative	-
	D-glucose	Negative	Negative	
	D-fructose	Negative	Positive	
	D-manitol			
	D-sucrose			
10.	GPA TEST	-	-	No growth

The thin layer of PHB extracted from test organisms grown in synthetic medium and industrial waste based medium was estimated. The PHB content was measured at 230nm using UV spectro-photometer. The OD was plotted using crotonic acid stand graph. The PHB content was expressed in microgram (µg/10ml) of culture. *Alcaligenes eutrophus* grown in synthetic medium (Nitrogen limited mineral medium) produced 35µg/10ml of culture. In the sesamum oil waste, maximum PHB production was seen in 40% oil waste substrate concentration. In the molasses, maximum PHB production was seen in 30% substrate concentration. When compared with the PHB production in synthetic medium (35µg/10ml) low than PHB production was seen in industrial waste based medium. Particularly maximum PHB production was seen in sesame oil waste medium (Table 2).

Pseudomonas putida grown in synthetic medium (Nitrogen limited mineral medium) produced 24µg/10ml of culture. In the sesamum oil waste, maximum PHB production was seen in 30% oil waste substrate concentration. In the molasses, maximum PHB production was seen in 30% substrate concentration. When compared with the PHB production in synthetic medium (24µg/10ml) low than PHB production was seen in industrial waste based medium. Particularly maximum PHB production was seen in sesame oil waste medium (Table 3).

Table 2 : Production of PHB in different concentration of industrial waste based medium synthetic medium by *Alcaligenes eutrophus*

Sr. No.	Substrate	Concentration	*Alcaligenes eutrophus*	
			Absorbance at 230nm	concentration (µg/ 10ml)
1.	Synthetic medium	Glucose	0.6357±0.12	35
2.	Molasses	10%	0.4723±0.09	26
		20%	0.4941±0.17	27
		30%	0.5862±0.11	32
		40%	0.5499±0.14	30
3.	Sesamum oil waste	10%	0.5343±0.14	29
		20%	0.5629±0.08	31
		30%	0.6127±0.13	34
		40%	0.6898±0.11	38

Note : All the values represent mean ±standard deviation of three determinations.

Table 3 : Production of PHB in different concentration of industrial waste based medium synthetic medium by *Pseudomonas putida*

Sr. No.	Substrate	Concentration	*Pseudomonas putida*	
			Absorbance at 230nm	Concentration (µg/ 10ml)
1.	Synthetic medium	Glucose	0.4423±0.11	24
2.	Molasses	10%	0.3197±0.07	17
		20%	0.3584±0.14	19
		30%	0.4012±0.13	22
		40%	0.3612±0.13	20
3.	Sesamum oil waste	10%	0.3544±0.12	19
		20%	0.3659±0.14	20
		30%	0.4627±0.18	25
		40%	0.4232±0.16	23

Note : All the values represent mean ±standard deviation of three determinations.

Rhizobium meliloti grown in synthetic medium (Nitrogen limited mineral medium) produced 22µg/10ml of culture. In the sesamum oil waste, maximum PHB production was seen in 30% oil waste substrate concentration. In the molasses, maximum PHB production was seen in 40% substrate concentration. When compared with the PHB production in synthetic medium (22µg/10ml) low than PHB production was seen in industrial waste based medium (Table 4). Particularly maximum PHB

production was seen in sesamum oil waste medium (Table 4). The Infrared spectrum analysis of the PHB product clearly reveals its purity.

Table 4 : Production of PHB in different concentration of industrial waste based medium synthetic medium by *Rhizobium meliloti*

Sr. No.	Substrate	Concentration	*Rhizobium meliloti* Absorbance at 230nm	Concentration (µg/ 10ml)
1.	Synthetic medium	Glucose	0.4098±0.13	22
2.	Molasses	10%	0.2921±0.12	16
		20%	0.3185±0.17	17
		30%	0.3649±0.09	20
		40%	0.3827±0.12	21
3.	Sesamum oil waste	10%	0.3348±0.14	18
		20%	0.3529±0.09	19
		30%	0.4291±0.07	23
		40%	0.3811±0.10	22

Note : All the values represent mean ±standard devitation of three determinations.

IR spectrum of the compound was recorded in the range of 100-4000cm and it showed characteristic bands for the groups CH, C=O and C-O. The methane (CH) group gave a strong band in range of 2920-3334. The carbonyl group (C=O) gave a strong band in range of 1657.81-1698.31. The (C-O) group showed strong and broad absorption in the range of 1047.25-1089.40. These frequency values were higher than the normal values because polymerization (Table 5).

Table 5 : Infrared analysis in *Alcaligenes eutrophus*

Sr. No.	Peaks	Concentration	Assignment of peaks
1.	3334.18	1.7027	C-H stretching
2.	3401.57	12.1348	C-H stretching
3.	2924.12	10.1328	C-H stretching
4.	1698.31	15.0457	C=O stretching
5.	1657.81	16.2876	C=Ostretching
6.	1413.57	21.7821	C=O stretching
7.	1089.40	10.2785	C-C stretching
8.	1047.25	11.4331	C-O stretching

IR spectrum of the compound was recorded in the range of 100-4000cm- and it showed characteristic bands for the groups CH, C=O and C-O. The methane (CH) group gave a strong band in range of 2924.92-3439.42. The carbonyl group (C=O) gave a strong band in range of 1631.48-1725.01. The (C-O) group showed strong and broad absorption in the range of 1047.59-1281.47. These frequency values were higher than the normal values because polymerization (Table 6).

Table 6 : Infrared Analysis in *Pseudomonas putida*

Sr. No.	Peaks	Concentration	Assignment of peaks
1.	3439.42	1.5018	C-H stretching
2.	2924.52	10.1346	C-H stretching
3.	1725.01	9.1398	C=O stretching
4.	1631.48	13.0456	C=O stretching
5.	1413.57	13.1858	C=O stretching
6.	1281.47	19.4695	C-O stretching
7.	1057.76	9.0384	C-C stretching
8.	1047.59	12.0274	C-O stretching

IR spectrum of the compound was recorded in the range of 100-4000cm- and it showed characteristic bands for the groups CH, C=O and C-O. The methane (CH) group gave a strong band in range of 2914.30-3097.39. The carbonyl group (C=O) gave a strong band in range of 1636.33-1673.37. The (C-O) group showed strong and broad absorption in the range of 1060.43-1089.40. These frequency values were higher than the normal values because polymerization (Table 7).

Table 7 : Infrared Analysis in *Rhizobium meliloti*

Sr. No.	Peaks	Concentration	Assignment Of Peaks
1.	3097.39	1.4024	C-H stretching
2.	2948.81	5.4087	C-H stretching
3.	2914.30	9.1763	C-H stretching
4.	1673.37	14.1572	C=O stretching
5.	1636.33	13.2438	C=O stretching
6.	1089.40	12.1858	C-O stretching
7.	1060.43	9.2879	C-C stretching
8.	1037.25	10.4695	C-O stretching

In present studies staining of PHB production of three test organisms were observed under oil immersion microscope and plate method. After staining, blue colour colonies were observed within plate method. A blue-black colour granule was observed with in the cell around the pink colour cytoplasm by slide method. Kitamura and Doi (1994) reported the similar results staining of producing bacteria. PHB appeared as light white to blue colour colonies (plate method). Observed the PHB in oil immersion objectives, a blue-black colour droplet was seen with in the cell and cytoplasm is pink colour.

PHB production of bacteria was estimated in various industrial waste susbrates, *viz* molasses and sesamum oil waste. High PHB productivity was observed in sesamum oil waste containing medium when compared with molasses. Arun *et al*. (2006) reported the similar results of PHB productivity in six substrates, *viz*. soya, malt, sesamum, molasses, bagasse, pharmaceutical waste used for production of PHB by *Alcaligenes eutrophus.* All the substrates supported growth and formation of PHB granules by the culture while seasamum oil waste was proved superior to other substrates.

Three different species such as *Alcaligenes eutrophus, Pseudomonas putida* and *Rhizobium meliloti* were used for PHB production. High production of PHB observed in *Alcaligenes eutrophus* (38µg/10ml/40%) when compared with *Pseudomonas putida* (23µg/10ml/40%) and *Rhizobium meliloti* (22µg/10ml/40%). Kunioka *et al.* (1998) reported the similar results of PHB productivity by *Alcaligenes eutrophus* in various substrates used as PHB production. Cultivation of polyesters content in dry cells was small amount of acetone-soluble polymers (65-75-mol%) with acetone insoluble polymer (11-13-mol%). At same time cultivation of polyesters by *Pseudomonas* sp the P(3HA) productivity was a very small amount (such as 3HB, butyrate, or pentanonate), used as the carbon source for *Pseudomonas oleovorans*. Interestingly, the P(HA) copolymer produced contained 3HA units of C_8-22 mol%,C_{10}-57 mol%,C_{12}-21 mol%. Productivity of PHB by *Rhizobium meliloti* was a 0.285g/l and the percentage yield was 74.03% after 48 hours from the effect of different carbon and nitrogen source. (Nazime mercan *et al.*, 2002).

The IR analysis showed the presence of CH_2-CH_3-C=O methyl ester groups in the extract. These methyl groups confirmed that the extract was PHB. The peaks at 1.7027 as could be assigned to ester carbonyl stretching. The peaks at 1.5018 and 1.4024 cm^- were difficult to assign. So, the IR analysis of PHB film and purified methyl ester were identical. Rawate and Mavinkurve (2002) reported the similar information of infrared analysis of PHA in marine bacteria. The peak at 1.735 as could be assigned to ester carbonyl stretching. The peaks at 1.180 and 1.300 cm^- were difficult to assign. The former band could be assigned to C-O stretching, in alkyl amines or various skeletal carbon stretching and the P=O stretching-H bending or CH2 scissors vibrations. The IR spectra of PHA film and purified methyl esters were identical.

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