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Influence of Nutritional and Environmental Conditions on Production of Poly-β-hydroxybutyrate by *Bacillus* sp.

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ABSTRACT

The extensive use and accumulation of synthetic plastics has caused waste disposal problems in the environment. Poly-β-hydroxybutyrate are naturally occurring biodegradable biopolymers which are synthesized by some microorganisms under growth limited conditions. In this study, one hundred and fifteen Gram positive bacterial isolates were obtained from zero tillage and sewage sludge amended soils. Nineteen *Bacillus* isolates showed high fluorescence with Nile blue A dye and three isolates showed significant accumulation of PHB granules which appeared as blue black droplets using Sudan black B staining. Highest amount of PHB was produced at 25 and 30°C by *Bacillus* isolates Ztl-31, Ztl-32 and Sld-110. *Bacillus* isolates started producing PHB after 24 h of growth and it increased till 72 h of incubation. Isolate Sld-110 produced significant amount of PHB (29.3%) within 48 h of incubation. With increase in the concentration of molasses (up to 2%, v/v), the amount of dry cell biomass increased but the amount of PHB produced was maximum at 1% (v/v) concentration of molasses. Cell biomass varied from 522 to 867 mg L^{-1} with addition of urea or ammonium chloride in minimal medium containing 1.0% molasses whereas the amount of PHB obtained was highest in the minimal broth containing ammonium phosphate. *Bacillus* isolate Sld110 produced 64.2% PHB in minimal medium containing 1% molasses as carbon substrate and ammonium phosphate as nitrogen source at 25°C after 48 h of incubation under stationary conditions.

Key words: Poly-β-hydroxybutyrate, *Bacillus* sp., milk whey, molasses, biodegradable plastics

INTRODUCTION

Poly-β-hydroxyalkanoates (PHA) are biodegradable natural biopolymers, synthesized by many prokaryotic organisms as an intracellular carbon and energy storage compound under limited nutrient conditions when carbon source is available in excess (Anderson and Dawes, 1990). Poly-β-hydroxybutyrate (PHB) is an important member of the family PHA and prokaryotic organisms are known to produce PHB amounting to as much as 90% of their cellular weight (Verlinden *et al*., 2007). PHB is accumulated as an energy reserve material by various microorganisms belonging to the genera *Alcaligenes, Azotobacter, Bacillus, Klebsiella, Pseudomonas, Ralstonia, Rhizobium, Staphylococcus* and *Rhodococcus* (Chen and Page, 1997; Ramadas *et al*., 2009; Joshi and Jaysawal,

2010). These PHB biopolymers are biodegradable and biocompatible thermoplastics (Bertrand *et al*., 1990; Vijayendra *et al*., 2007). These biodegradable plastics have emerged as a solution to waste disposal problems resulting from extensive use of synthetic and non-degradable petrochemical based plastics (Full *et al*., 2006).

The production of PHB and its copolymer based plastics on industrial scale is still very costly in comparison to petrochemical based plastics (Reddy *et al*., 2003). This high cost of manufacturing process of this biodegradable plastic is attributed to the cost of carbon source, fermentation process and the downstream processing (Choi and Lee, 1999). The cost of carbon source alone accounts to about 50% of the production costs of the PHA (Halami, 2007) and therefore, use of inexpensive carbon substrates viz. agroindustrial waste or sewage sludge can contribute to as much as 40-50% reduction in the overall production cost (Khardenavis *et al*., 2005; Ramadas *et al*., 2009; Santimano *et al*., 2009). Sucrose, beef extract and diammonium sulphate was found to be the most suitable for growth and PHB accumulation by *B. mycoides* RLJ B-017 (Borah *et al*., 2002). PHB synthesis is made by the bacteria during the stationary phase of growth and these PHB granules facilitate cell survival during stressful conditions (Ramadas *et al*., 2009). Acidic pH conditions were found to result in increased yield of PHB by suppressing sporulation (Valappil *et al*., 2007). Yuksekdag *et al*. (2004) reported that *Bacillus subtilis* 25 and *Bacillus megaterium* 12 produced different amounts of PHB in nutrient broth medium at different incubation times (between 6 and 48 h).

Biodegradable plastics have a wide range of potential applications in many consumer products, agricultural, marine and medical applications because the polymers can be made into biodegradable razors, shampoo bottles, wrapping films, bags and sheets etc. (Lee, 1996; Chen and Wu, 2005; Arun *et al*., 2006). In this study, *Bacillus* isolates obtained from zero tillage and sewage sludge amended soils were tested for PHB production. Selected strains were used for the production of PHB using milk whey and molasses as carbon substrate to minimize the cost for the production of biodegradable plastics. The effect of incubation conditions, amendment of nitrogen sources and carbon sources as well as aeration conditions was studied on the PHB production in different *Bacillus* isolates.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology, CCS Haryana Agricultural University, Hisar on the *Bacillus* isolates obtained from zero tillage and sewage sludge amended soils during the period March 2006 to November 2008.

Isolation of *Bacillus* species: Soil samples were collected from zero tillage and sewage sludge amended soils from different parts of Haryana in Northern India. Soil suspension was prepared by dissolving 10 g of soil from each composite sample into 90 mL sterilized water and heated at 80°C in a water bath for 30 min to kill the vegetative cells. Serial dilutions were made upto 10^{-5} and 0.1 mL of 10^{-4} and 10^{-5} dilutions was spread on nutrient agar medium plates. After incubation at 28±2°C for 2-3 days, bacterial colonies were picked up, purified and maintained on nutrient agar medium slants at 4°C. Identification of bacteria was done based on Gram staining, spore staining and biochemical tests.

Screening of *Bacillus* isolates for PHB production: Screening of bacterial isolates for PHB production was done by using Sudan black B staining method (Schlegel *et al*., 1970) and Nile blue

A method (Ostle and Holt, 1982). In Sudan black B method, *Bacillus* isolates were stained with Sudan black B (0.3%, w/v) and counter stained with aqueous safranine (0.5%, w/v). The PHB granules in *Bacillus* isolates appeared as blue black droplets and cytoplasmic part of microorganisms appeared as pink under oil immersion objective lens. In Nile blue A method, bacterial strains were spotted on minimal medium plates containing Nile blue (0.05%, w/v) and the plates were incubated at 28±2°C in a BOD incubator for 15 days. Fluorescence was observed under ultraviolet transilluminator at different days of growth. The level of fluorescence in PHB producing bacterial isolates was used as indicator for the amount of PHB produced by the different bacteria.

Quantitative estimation of PHB: PHB production was quantified by using method of Law and Slepecky (1961) and the amount of PHB produced was calculated from the standard curve prepared by using poly-β-hydroxybutyrate. Bacterial culture growth (25 mL) was centrifuged at 8,000 rpm at 4°C for 20 min and pellet of the cells was washed with double distilled water. It was mixed thoroughly in 5 mL of sodium hypochlorite by vortexing. Mixture was incubated at 37°C for 1 h and again centrifuged at 8,000 rpm for 20 min to sediment the lipid granules. Supernatant was decanted, resuspended in 5 mL of distilled water and centrifuged at 8,000 rpm for 20 min. Again the supernatant was decanted and the pellet adhering to walls of centrifuge tube was washed successively with 5 mL of acetone, diethyl ether and ethanol to remove water which interferes with the extraction of polymer.

Pellet was suspended in 5 mL of acetone and centrifuged at 8,000 rpm for 20 min. The sediment obtained after decanting the supernatant was suspended in 5 mL of diethyl ether by vortexing and centrifuged at 8,000 rpm for 20 min at 4°C. Pellet for granules was dissolved in chloroform in boiling water bath for 2 min. It was centrifuged after cooling at 8,000 rpm for 20 min at room temperature and supernatant was saved in 10 mL graduated test tube. Sediment was re-extracted twice with 3 mL of chloroform at 100°C for 2 min. The whole solution was filtered through Whatman No. 1 filter paper (previously treated with hot chloroform). The chloroform extracts were pooled and made to 10 mL with chloroform in clean glass tubes. Chloroform extracts were heated in boiling water bath until all the chloroform gets evaporated. To this, 10 mL of concentrated H_2SO_4 was added and tubes were capped with glass marbles. Tubes were heated for 20 min in boiling water bath, cooled and mixed thoroughly. Absorbance of the solution was read at 235 nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer (Sigma-Aldrich Techware).

Measurement of bacterial growth: Bacterial growth was estimated in the minimal medium containing 1% carbon source in the form of sucrose or sewage sludge. Dry weight of the cells was measured at different days of incubation. Twenty-five mL of culture broth was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant was discarded. Cell pellet was transferred to pre-weighed aluminium foil cup, kept at 80°C in an oven for 2 h and weighed. The amount of dry cells per litre of broth was calculated.

Optimization of growth conditions for PHB production: *Bacillus* isolates were grown for 24 h in the nutrient broth. Minimal medium was inoculated with 1% bacterial inoculum and was incubated at 28±2°C under different conditions of growth. To determine the effect of aeration conditions, experiments were carried out in 150 mL Erlenmeyer flasks incubated for different time

intervals ranging from 24 to 96 h at 28±2°C both under stationary as well as shaking conditions (on a orbital shaking incubator at 120 rpm). For determining the effect of temperature, Erlenmeyer flasks (150 mL capacity) containing 50 mL of the minimal medium broth were inoculated with different bacterial isolates. The flasks were incubated at different temperature (20 to 40°C) for 72 h both under stationary as well as shaking conditions.

For measuring effect of carbon sources, different carbon sources such as milk whey and molasses were tested for PHB production. Bacterial growth and PHB production were monitored at different time intervals to find out the maximum yield of PHB during shaking and stationary conditions. To determine the effect of different concentration of carbon sources, the experiment was conducted with different concentrations of molasses (0.5 to 4.0%) in the minimal medium broth. PHB production was recorded at different time intervals both under shaking and stationary conditions. Nitrogen sources such as urea, ammonium phosphate, ammonium chloride and ammonium acetate were supplemented in the minimal medium broth and compared with the medium lacking the nitrogen source for production of PHB.

RESULTS

A total of 115 bacterial isolates were obtained from zero tillage and sewage sludge amended soils on nutrient agar plates. The bacterial colonies which varied in shape, size and appearance were picked up, further purified by streaking and transferred on nutrient agar medium slants. Bacterial colonies varied from small to large in size, round to irregular in shape and were cream color in appearance.

Screening of *Bacillus* isolates for PHB production: The purified *Bacillus* isolates were screened for PHB production using Nile blue. Only nineteen *Bacillus* isolates showed PHB production and variation in the level of fluorescence was observed with different bacterial isolates. Bacterial isolates which gave significant fluorescence with Nile blue A dye were further verified for PHB production using Sudan black B staining method. Three *Bacillus* isolates also produced large amount of PHB granules which appeared as blue black droplets in the cells when stained with Sudan black B dye.

PHB production in minimal medium broth at different temperatures and at different incubation periods: The amount of cell dry weight obtained was more under shaking conditions as compared to stationary conditions (Table 1) and dry weight of bacterial cells varied from 918 to 1297 mg L^{-1} at different temperatures of growth varying from 20 to 40°C under shaking conditions. The optimum temperature favoring cell biomass was 25°C under both the conditions. Isolates Ztl-32 and Sld-110 produced 1129 and 1022 mg L^{-1} dry cell biomass at 25°C under stationary conditions, respectively. With increase in temperature at 40°C and with decrease in temperature at 20°C, the cell dry weight of all the isolates declined under both the conditions. All the three isolates Ztl-31, Ztl-32 and Sld-110 produced highest amount of PHB at 25 and 30°C. More PHB was produced under stationary conditions. The isolate Sld-110 produced 194.5 mg g^{-1} (w/w) PHB at 20°C which increased to 216.2 mg g^{-1} (w/w) PHB at 25°C and thereafter again declined to 159.7 mg g^{-1} (w/w) at 40°C. Maximum 21.1% of PHB was produced by the *Bacillus* isolate Sld-110 at 25°C under stationary conditions.

Table 1: PHB production by different *Bacillus* isolates in minimal medium broth at different temperatures

		Stationary conditions			Shaking conditions		
Temperature (°C)	Isolate No.	Dry weight of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production	Dry weight of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
20	Ztl-31	898	172.9	19.2	966	123.8	12.8
	Ztl-32	936	170.9	18.2	1012	129.2	12.7
	Sld-110	930	194.5	20.9	933	1228.0	13.1
25	Ztl-31	1019	204.1	20.0	1059	150.1	14.1
	Ztl-32	1129	211.8	18.7	1297	153.9	11.8
	Sld-110	1022	216.2	21.1	1063	160.4	15.0
30	Ztl-31	976	196.7	20.1	1032	148.4	14.3
	Ztl-32	1064	211.4	19.8	1285	152.9	11.8
	Sld-110	1028	204.2	19.8	1083	150.0	13.8
35	Ztl-31	872	161.9	18.5	952	121.6	12.7
	Ztl-32	836	169.9	20.3	986	128.2	13.0
	Sld-110	809	170.5	21.0	928	117.5	12.6
40	Ztl-31	852	155.6	18.2	918	114.5	12.4
	Ztl-32	817	167.6	20.5	998	117.2	11.7
	Sld-110	809	159.7	19.7	943	108.6	11.5
CD at 5% level		23	9		18	11	

PHB production was measured at different time intervals ranging from 24 to 96 h. Three *Bacillus* isolates Sld-110, Ztl-32 and Ztl-31 produced highest cell biomass at 72 h of growth (1333, 1330 and 1066 mg g^{-1}), respectively, but it declined later on both under shaking as well as stationary conditions (Table 2). All the isolates started producing PHB after 24 h of growth and it increased till 72 h of incubation. Isolate Sld-110 produced significant amount of PHB (29.3%) within 48 h and it declined after 84 h. PHB production in isolate Sld-110 declined to 19.7 and 13.2% at 96 h of growth under shaking and stationary conditions, respectively.

PHB production in minimal medium broth containing milk whey and molasses as a carbon source: All the three *Bacillus* isolates showed a poor growth in minimal medium containing milk whey as carbon source and the amount of PHB produced was also lower in milk whey (Table 3). Isolates Ztl-32 and Sld-110 produced 167.0 and 152.4 mg g^{-1} (w/w) PHB after 48 h of growth at 25°C under shaking conditions and produced 187.6 and 167.8 mg g^{-1} (w/w) PHB under stationary conditions. Isolate Ztl-32 produced 40.5 and 50.2% PHB under shaking and stationary conditions, respectively, using milk whey as a carbon source.

When molasses was used as carbon source, all the three isolates produced more cell biomass in minimal broth containing molasses than milk whey (Table 4). The cell biomass varied from 440 to 798 mg L^{-1} at different hours of growth ranging from 24 to 72 hours at shaking conditions. Isolates Sld-110 and Ztl-32 produced 307.0 and 248.8 mg g^{-1} (w/w) PHB under stationary conditions at 48 h of growth and it increased to 322.2 and 281.8 at 72 h of growth. Isolate Sld-110 produced 58.3% PHB after 48 h of growth at 25°C under stationary conditions, whereas isolate Ztl-32 produced 41.7% PHB under stationary conditions using molasses as carbon source.

Cell biomass and PHB produced by all the three isolates was determined in minimal broth containing different concentration of molasses varying from 0.5 to 4.0% (v/v). The amount of dry

Table 2: PHB production by different *Bacillus* isolates in minimal medium broth at different periods of growth

		Stationary conditions			Shaking conditions		
Time (h)	Isolate No.	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
24	Ztl-31	680	165.0	24.2	555	70.0	12.6
	Ztl-32	685	170.0	24.8	565	90.0	15.9
	Sld-110	670	155.0	23.1	500	94.0	18.8
36	Ztl-31	675	167.0	24.7	670	132.7	19.8
	Ztl-32	670	167.0	24.9	612	110.4	18.0
	Sld-110	689	171.0	24.8	622	133.4	21.4
48	Ztl-31	742	206.8	27.8	742	143.5	19.3
	Ztl-32	740	212.1	28.6	750	155.7	20.7
	Sld-110	706	207.2	29.3	713	156.3	21.9
60	Ztl-31	801	207.2	25.8	852	145.5	17.0
	Ztl-32	860	217.0	25.2	860	156.7	18.2
	Sld-110	846	207.4	24.5	849	157.2	18.5
72	Ztl-31	1013	207.9	20.5	1066	149.7	14.0
	Ztl-32	1133	219.1	19.3	1330	158.1	11.8
	Sld-110	1066	209.1	19.6	1333	159.1	11.9
84	Ztl-31	785	171.0	19.6	760	118.4	15.5
	Ztl-32	866	170.0	22.1	873	150.6	17.2
	Sld-110	935	178.0	19.0	966	89.5	9.26
96	Ztl-31	880	167.4	19.0	560	94.6	16.8
	Ztl-32	875	170.0	19.4	512	94.0	18.3
	Sld-110	870	171.4	19.7	560	74.2	13.2
CD at 5% level	21	12		19	9	9	

Table 3: PHB production by different *Bacillus* isolates in minimal medium broth using milk whey as a carbon source

		Stationary conditions			Shaking conditions		
Time (h)	Isolate No.	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
24	Ztl-31	326	101.2	31.0	416	37.9	9.1
	Ztl-32	298	108.6	36.0	356	31.7	8.9
	Sld-110	354	115.2	32.5	460	28.6	6.2
48	Ztl-31	480	171.7	35.7	580	150.6	25.9
	Ztl-32	373	187.6	50.2	412	167.0	40.5
	Sld-110	319	167.8	52.6	540	152.4	28.2
72	Ztl-31	360	99.5	27.6	493	97.2	19.7
	Ztl-32	496	178.1	35.9	473	102.7	21.7
	Sld-110	326	106.3	32.6	493	87.4	17.7
CD at 5% level	16	31		18	24		

cell biomass increased, as the concentration of molasses increased up to 2.0% (v/v) (Table 5). However, maximum amount of PHB was produced at 1% (v/v) concentration of molasses in comparison to other concentrations. The isolate Sld-110 and Ztl-32 produced 400.0 and 308.3 mg g^{-1} (w/w) PHB at 1.0% concentration of molasses after 48 h of growth at 25°C under

Table 4: PHB Production by different *Bacillus* isolates in minimal medium broth using molasses as carbon source

		Stationary conditions			Shaking conditions		
Time (h)	Isolate No.	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
24	Ztl-31	386	78.4	20.3	440	27.5	6.25
	Ztl-32	353	98.0	27.7	496	30.0	6.04
	Sld-110	467	75.5	16.1	584	27.0	4.62
48	Ztl-31	604	257.7	42.6	701	178.6	25.40
	Ztl-32	596	248.8	41.7	726	193.2	26.60
	Sld-110	526	307.0	58.3	624	186.5	29.80
72	Ztl-31	640	257.5	40.2	626	154.4	24.60
	Ztl-32	693	281.8	40.6	613	152.3	24.80
	Sld-110	680	322.2	47.3	798	182.4	22.80
CD at 5% level	13	9		11	7		

Table 5: Effect of different concentrations of molasses on PHB production by *Bacillus* sp. under stationary conditions

		Stationary conditions (48 h of growth)		
Concentration of molasses (%, v/v)	Isolate No.	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
0.5 %	Ztl-31	679	200.7	29.5
	Ztl-32	683	259.4	37.9
	Sld-110	698	283.9	40.6
1.0 %	Ztl-31	702	290.7	41.4
	Ztl-32	681	308.3	45.2
	Sld-110	685	400.0	58.3
2.0 %	Ztl-31	886	192.0	21.6
	Ztl-32	844	230.0	27.2
	Sld-110	891	236.5	26.5
3.0 %	Ztl-31	876	192.0	21.9
	Ztl-32	839	216.3	25.7
	Sld-110	884	215.0	24.3
4.0 %	Ztl-31	876	179.3	20.4
	Ztl-32	831	195.1	23.4
	Sld-110	873	195.9	22.4
CD at 5% level		16	11	

stationary conditions. Isolates Sld-110 and Ztl-32 produced 58.3 and 45.2% PHB, respectively at 1.0% (v/v) concentration.

PHB production in minimal medium broth with different nitrogen sources: Different nitrogen sources such as urea, ammonium phosphate, ammonium acetate and ammonium chloride were used along with molasses as carbon source to study their effect on PHB production. With the addition of different nitrogen sources, the cell biomass varied from 522 to 867 mg L^{-1} and maximum cell biomass was obtained with addition of urea and ammonium chloride (Table 6). The amount of PHB obtained was highest in the minimal broth containing 1.0% molasses (v/v) and supplemented with ammonium phosphate. Isolates Sld-110 and Ztl-32 produced 335.2 and 356.4 mg g^{-1} (w/w) PHB. Isolate Sld-110 produced 64.2% PHB in the minimal broth containing molasses with ammonium phosphate as nitrogen source under stationary conditions.

Table 6: Effect of different nitrogen sources on PHB production in minimal medium containing 1.0 % molasses (v/v) as carbon source

	Stationary conditions			
Nitrogen source	Isolate No.	Dry wt. of cells (mg L^{-1})	PHB (mg g^{-1} dry wt. of cells)	% PHB production
Without nitrogen source	Ztl-31	653	240.7	36.8
	Ztl-32	681	277.3	40.7
	Sld-110	675	310.6	46.0
Urea	Ztl-31	846	150.2	17.7
	Ztl-32	861	191.8	22.2
	Sld-110	867	212.9	24.5
Ammonium phosphate	Ztl-31	636	315.4	49.5
	Ztl-32	624	356.4	57.1
	Sld-110	522	335.2	64.2
Ammonium acetate	Ztl-31	696	250.5	35.9
	Ztl-32	725	277.5	38.2
	Sld-110	702	317.3	45.1
Ammonium chloride	Ztl-31	802	173.4	21.6
	Ztl-32	855	199.7	23.3
	Sld-110	863	230.2	26.6
CD at 5% level		22	14	

DISCUSSION

PHB producing *Bacillus* isolates were obtained from soil samples collected from zero tillage and sewage sludge amended soils from different parts of Haryana in Northern India. Screening of different *Bacillus* isolates was done by using Nile blue A dye and fluorescence of PHB producing bacteria was observed under UV transilluminator. Only nineteen *Bacillus* isolates out of 115 isolates tested, showed PHB production and variation in the level of fluorescence was observed in different bacterial isolates. Production of PHB has also been reported in different *Bacillus* species i.e., *B. megaterium* (Pandian *et al*., 2010), *B. subtilis* (Yuksekdag *et al*., 2004) and *Bacillus* sp. (Santimano *et al*., 2009; Joshi and Jaysawal, 2010). Pierce and Schroth (1994) also screened *Pseudomonas* colonies which accumulated poly-β-hydroxybutyrate on Nile blue A incorporated medium. Gerhardt *et al*. (1981) used Sudan black B staining to observe the contents of fatty substance and lipid inclusions in bacteria. Stained PHB granules appeared as blue black droplets inside the pink colored cells after 24 h of growth. Page and Cornish (1993) also observed PHB inclusions in *Azotobacter vinelandii* at 18 to 24 h of growth using Sudan black B dye.

Three *Bacillus* isolates Ztl-31, Ztl-32 and Sld-110 were selected to optimize conditions such as incubation temperature for maximum production of PHB using cost-effective carbon and nitrogen sources. All the three *Bacillus* isolates Ztl-31, Ztl-32 and Sld-110 produced maximum amount of PHB at 25°C and amount of PHB produced decreased at lower and higher temperatures i.e., 20 and 40°C (Table 1). Isolate Sld-110 produced 21.1% more PHB at 25°C under stationary than shaking conditions. Borah *et al*. (2002) observed that maximum values of cell dry weight, PHB yield and productivities were obtained at temperature 30°C in *B. mycoides* RLJ B-017.

It is well recognized that PHB accumulation occurs as an internal reserve of carbon and energy under nutrient limiting conditions during late log and stationary phase of growth. The dry weight of all the three *Bacillus* isolates increased linearly upto 72 h of growth and then declined (Table 2). The amount of cell dry weight was more under shaking conditions than under stationary conditions. However, the production of PHB was more under stationary conditions by utilizing

different carbon sources showing that it is produced under oxygen stress conditions. Chen and Page (1997) also showed that PHB production was suppressed by high aeration of beet molasses medium in *A. vinelandii*. Pandian *et al.* (2010) reported that dissolved oxygen is a major limiting factor that affected PHB synthesis by *B. megaterium* during utilization of dairy waste and sea water.

The amount of PHB produced was related to the growth phase of bacteria as it was produced in low amount in initial logarithmic phase of growth and increased at 48 and 72 h of growth. In the present studies, PHB production increased linearly during log phase upto 72 h of growth and then declined under both shaking and stationary conditions in minimal medium broth. Yuksekdag *et al.* (2004) also reported that *Bacillus subtilis* 25 and *Bacillus megaterium* 12 produced different amounts of PHB in nutrient broth medium at different incubation times (between 6 and 48 h). The PHB productions were 0.101 and 0.142 g L^{-1}, respectively. The percentage yields were 18.03 and 14.79% after 45 h of growth. Vijayendra *et al.* (2007) observed that *Bacillus* sp. CFR256 produce 8.20 g L^{-1} PHB (51.20% dry cell biomass) after 72 h of fermentation.

PHB production by *Bacillus* isolates was studied in minimal broth using cheap carbon sources such as milk whey and cane molasses. It was found that milk whey poorly supported the growth of all these bacterial isolates and the amount of PHB produced was also lower in milk whey (Table 3). The molasses was found to be best carbon source for the PHB production and cell biomass production by three different *Bacillus* isolates Ztl-31, Ztl-32 and Sld-110 (Table 4), because molasses possess sugars like sucrose, fructose, glucose as well as vitamins. Wen *et al.* (1994) have also studied the PHB production using beet molasses as carbon source by *Alcaligenes eutrophus* H16 at 30°C. In shake flask conditions, the microbial biomass production and PHB were higher (maxima 26 and 13 g L^{-1}), with 20.4% conversion of sugar. The PHB content in cells reached to 50% in both flask and fermentor.

The amount of dry cell biomass increased, as the concentration of molasses increased up to 2.0% (v/v) (Table 5). However, maximum amount of PHB was produced at 1.0% (v/v) concentration of molasses in comparison to other concentrations. Similarly, Wu *et al.* (2001) reported the accumulation of 25 to 35% (w/w) poly-β-hydroxybutyrate by *Bacillus* sp. JMa5 during fermentation in molasses media. Addition of 1% concentration of molasses led to highest production of PHB at 27°C after 48 h of growth under stationary conditions. Borah *et al.* (2002) also observed that sucrose, beef extract and diammonium sulphate was found to be the most suitable for growth and PHB accumulation by *B. mycoides* RLJ B-017. On the other hand, Yilmaz and Beyatli (2005) reported *Bacillus cereus* M5 to produce highest dry cell mass in 4.0% molasses concentration and PHB on dry cell mass in 1.0% molasses concentration. Santimano *et al.* (2009) reported highest production of PHA by *Bacillus* sp. strain COL1/A6 using hydrolyzed wafer residue followed by cane molasses and hydrolyzed citrus pulp.

With the supplementation of different nitrogen sources in molasses containing minimal medium, the cell biomass varied from 522 to 867 mg L^{-1} and maximum cell biomass was obtained with addition of urea and ammonium chloride (Table 6). Highest amount of PHB ranging from 49.5 to 64.2% was obtained in the minimal broth containing 1.0% molasses (v/v) and supplemented with ammonium phosphate under stationary conditions. Yuksekdag *et al.* (2004) reported that complex nitrogen sources increased the yield of PHB whereas Joshi and Jaysawal (2010) observed that better yield of PHA was obtained by *Bacillus, Staphylococcus* and *Pseudomonas* using ammonium sulphate and ammonium phosphate as nitrogen sources than that of yeast extract.

Thus success in production of biodegradable plastic depends on selection of efficient PHB producing bacteria and on optimizing the most favourable conditions for PHB production. This work suggested that *Bacillus* isolates obtained from zero tillage and sewage sludge amended soils could be utilized in the industrial production of PHB.

CONCLUSION

The high cost of biodegradable PHB production is attributed to the cost of carbon source, fermentation process and the downstream processing. Therefore, use of inexpensive agroindustrial waste such as molasses and milk whey for PHB production could reduce 40-50% of the overall production cost. Three *Bacillus* strains were obtained in this study, which showed high fluorescence with Nile blue A and accumulated PHB granules in the cell. Highest amount of PHB was produced at 25 and 30°C till 48-72 h of incubation. The amount of PHB produced was maximum at 1.0% (v/v) concentration of molasses and in minimal broth containing ammonium phosphate. *Bacillus* isolate Sld110 produced 64.2% PHB with ammonium phosphate as nitrogen source in minimal medium containing 1.0% molasses as carbon substrate at 25°C after 48 h under stationary conditions.

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