Int. Res. J. of Science & Engineering, 2015; Vol. 3 (2): 55-59 ISSN: 2322-0015

RESEARCH ARTICLE

Production and identification of poly – β – hydroxyl - butyrate by microorganisms

Bhusare DU and Kadam Omprakash A

School of Life Sciences, Department of Microbiology, SRTMU, Nanded, (MS) India.

**Corresponding author Email: deep_bhusare@rediffmail.com*

Manuscript Details

Received : 10.09.2014
Revised : 21.10.2014
Re-Revised: 12.1. 2015
Revised Received :11.02.2015
Accepted: 02.03.2015
Published: 25.04.2015

ISSN: 2322-0015

Editor : Dr. Arvind Chavhan

Cite this article as:

Bhusare DU and Kadam OA. Production and identification of Poly – β – hydroxyl - butyrate by microorganisms, *Int. Res. J. of Science & Engineering,* 2015; Vol. 3 (2): 55-59.

ABSTRACT

In the present investigation, production of (poly-β-hydroxy-butyrate) PHB by using *Bacillus megaterium* and *Pseudomonas* spp. was studied. The maximum PHB was produced by *Bacillus megaterium* than *Pseudomonas* spp. having 5.39% yield and 4.66% respectively. The different carbon and nitrogen sources were optimized for PHB production. Higher PHB accumulation was observed in the media containing sucrose as a carbon source and L-Lysine as a nitrogen source by *Bacillus megaterium,* whereas in *Pseudomonas* spp. higher PHB accumulation was in presence of sucrose as a carbon source and potassium nitrate as a nitrogen source. The PHB produced in the medium was extracted by using solvent extraction (chloroform) method and separated by TLC method.

Key words : *Bacillus megaterium, Pseudomonas* sp., PHB, Staining,

INTRODUCTION

Biopolymers are valuable for us because they posses unique properties which cannot be emulate by synthetic polymers, they are available from renewable resources and are biodegradable hence eco-friendly (Khanafari *et al*., 2006). Biopolymers find increasing application as Bioplastic, Biosurfactant, Biodetergent, Bioflocualnt, Biodegradable polysters, Biogum etc. Biopolymers are derived from animals (gelatin) and from plants (gum Arabic, carrageenan), from organisms (PHA, PHB). Biopolymers obtained from growth of micro-organism or from plants which are genetically engineered to produce such polymers are likely to replace currently used plasticts at least in some of the field. PHB and polylactic acid are the kind of polymers which are used as materials of bioplastics. Many species of *Pseudomonas*, *Moraxella, Bacillus, Photobactrium, Spirillum, Azatobacter, Rhizobium* and Alkaligens synthesize PHB (Powar and Daginawala, 2002). Thus, many bacteria of the enteric group and anaerobic spore formers synthesize only glycogen or starch as reserve material. All bacteria capable of PHB

synthesis accumulate PHB granular during the stationary phase of growth when the cells become limited for an essential nutrient but have excess of carbon source. The industrial scale production of PHB has begun by using *Alcaligenes eutophus* and *A. latus.* During the initial balanced growth phase, cell mass is produced but not PHB. Phosphate limitation is imposed in the second phase and PHB accumulates (Bertrand *et al*., 1990).

METHODS AND MATERIAL

Isolation of Pseudomonas *spp*:

The micro-organism was isolated from the garden soil sample, collected from Shri Shivaji College, Parbhani. The serial dilution of sample and streak plate on nutrient agar medium was used for isolation purpose. The plates were incubated at 30°C for 48 hours. The bacterial culture was identified using morphological and biochemical characteristics. The culture was maintained on slant at 4°C (Belam Aslim *et al*., 2002).

PHB Staining Procedure-

Prepare a smear and heat fix. Treat the smear with solution Sudan Black B stain for about 10-15 minutes. Remove solution Sudan Black B and dry it. Do not wash with water. Wash the smear with Xylene. Treat smear with 0.5% saffranin for 5-10 seconds. Wash with water, air dry and examine under oil immersion lens. The lipid granules appear deep blue against light pink cytoplasm (Deshmukh, 1998).

PHB Production:

The enriched bacterial culture was transferred in PHB production medium containing (g/l) KH_2 PO_4:0.25, K_2HPO_4:0.25, $(NH_4)_2SO_4$:0.5, $MgSO_4$:0.1, $NaCl_2$:0.1, $CaCl_2.7H_2O$: 0.02, pH: 7±0.2. Incubate the medium at 37°C for 24 hrs having 120 rpm. After, 24h of cultivation period, cells were harvested by centrifugation at 8000 rpm at 4°C for 10-15min (Kumar and Prabakaran, 2006; Bruce *et al*., 1990; Otari and Ghosh, 2009).

Harvesting the cells:

After incubation, cells were harvested by centrifugation at 8000 rpm for 10-15 min for *Pseudomonas spp.* and at 6000 rpm for 45 min for *B. megaterium*. Then pellets were collected aseptically, dried at 60°C until constant weight and weight was measured (Ugur *et al*., 2002).

Biodegradable polymer extraction:

Alumina was used to crush the cells with mortar and pestle. Form the white powdery mass of the crushed cells, the polymer was extracted with chloroform ($CHCl_3$). Shake chloroform mixtures vigorously and left for 1h. The clear solution was carefully decanted into another clean test tube and evaporated. The powdery mass along the test tube wall was collected, dry weight of the biodegradable polymer (Moreno *et al*., 2007).

Determination of PHB by TLC method:

The 50µl sample was loaded on the TLC plate and allowed to run in the solvent system consisting of Ethyl acetate and Benzene (1:1) mixture for 40 min. Staining was performed by using iodine crystals. TLC plates were kept over the beaker containing iodine crystals for 10-15min for it to get saturated with iodine vapor. After 10min green-black colored spot indicates the presence of PHB. The RF value was measured and compared with the standard chart (Hikmet *et al*., 2003).

Optimization of Nutrients:

Effect of carbon sources on PHB production: The different carbon sources were applied for PHB production such as sucrose, fructose and arabinose having concentration 2% for *B. megaterium* and 0.1% for *Pseudomonas spp.*

Effect of nitrogen sources on PHB production: The different nitrogen sources were applied for PHB production such as potassium nitrate, L-Lysine, L-Cysteine having concentration 2% for *B. megaterium* and 0.1% for *Pseudomonas spp.* (Ojumu, *et al*., 2004).

RESULT AND DISCUSSION

Isolation and Identification of *pseudomonas spp.*:

The micro-organism isolated from the soil sample was identified as *Pseudomonas spp.* on the basis of following morphological and biochemical characteristics.

Table 1 : Morphological characters of *Pseudomonas spp.*

Morphological character	Observation
Size	2mm
Shape	Circular
Morphology	Rod
Colour	Greenish white
Margin	Entire
Elevation	Convex
Surface	Smooth
Opacity	Opaque
Consistency	Sticky
Gram's nature	Rod
Motility	Motile

Table 2: Biochemical tests of *Pseudomonas spp.*

Test	Observation
1] IMViC Test	
a) Indol	Positive
b) Methyl red	Negative
c) Vogus-Proskauer	Negative
d) Citrate utilization	Negative
2] Catalase test	Positive
3] Oxidase test	Positive
4] Sugar fermentation	
Lactose	Acid production without gas
Glucose	Acid production without gas
Maltose	Acid production without gas
5] Mannitol salt sugar	Positive

Table 3: Production of PHB from *Bacillus megaterium* and *Pseudomonas spp.* in Mg/100ml are as follows.

Organism spp.	Dry cell weight (mg)	PHB weight (mg)	Yield of PHB in %
Bacillus megaterium	1390	75	5.39
Pseudomonas spp.	1500	70	4.66

Table 4: production of PHB in *Bacillus megaterium* and *pseudomonas spp.* by using different carbon sources [mg/100ml]:

Organism	Sources of carbon								
	Sucrose			Fructose			Arabinose		
	Dry cell wt.	PHB wt.	Yield of PHB in %	Dry cell wt.	PHB wt.	Yield of PHB in %	Dry cell wt.	PHB wt.	Yield of PHB in %
Bacillus megaterium	500	80	16	1100	85	7.72	1850	15	0.81
Pseudomonas spp.	500	75	15	600	80	13.72	1870	17	0.90

Table no. 5:- production of PHB in *Bacillus megaterium* and *Pseudomonas spp.* by using different nitrogen sources [mg/100ml]:

Organism	Sources of carbon								
	Sucrose			Fructose			Arabinose		
	Dry cell wt.	PHB wt.	Yield of PHB in %	Dry cell wt.	PHB wt.	Yield of PHB in %	Dry cell wt.	PHB wt.	Yield of PHB in %
Bacillus megaterium	2520	15	0.59	300	60	20	3100	90	2.90
Pseudomonas spp.	40	20	50	60	24	40	70	21	30

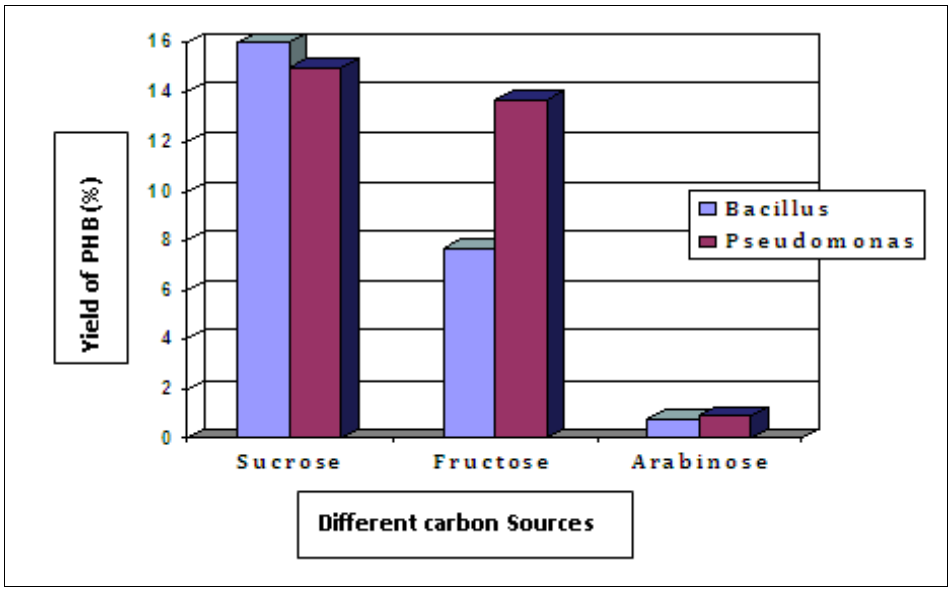

Figure 1: Production of PHB in *Bacillus megaterium* and *Pseudomonas spp.* by using different carbon sources [mg/100ml].

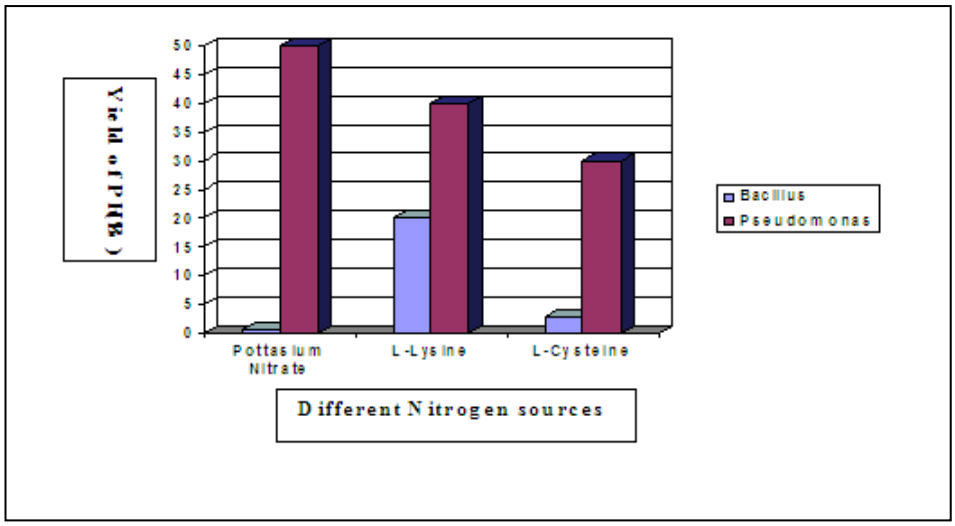

Figure 2: Production of PHB in *Bacillus megaterium* and *pseudomonas spp.* by using different Nitrogen sources [mg/100ml].

PHB Staining:

After PHB staining, pink colored rods in chains were observed with pinkish blue cytoplasm and black colored granules in both *Pseudomonas spp.* and *Bacillus megaterium* (Das *et al*., 2005).

PHB Production:

PHB is a carbon storage polymer widely distributed among prokaryotes including *Bacillus, Pseudomonas, Azatobactor* etc. in recent years, PHB and other PHA have been considered as commercially important because of their possible use biodegradable thermoplastics (Rafael Garcia Ribera *et al*., 2001). Yield of PHB in percentage (%) are calculated as follows:

Yield of PHB (%) = PHB weight × 100 / total dry cell weight

Thus, the table 3 shows the yield of PHB is 5.39% in *Bacillus megaterium* and 4.66% in *P*seudomonas *spp.* The highest PHB accumulated in a *Bacillus megaterium* as compared to *Pseudomonas spp* (Robert *et al*., 1989).

Determination of PHB by TLC method:

Green-black colour bands were observed on TLC plates and RF value was measured and calculated as 0.75 (which is near about of standard value of 0.71), which indicated the presence of PHB in production medium.

Table 4 and table 5 shows that yield of PHB using carbon and nitrogen sources. It showed the yield of

PHB is variable with different carbon and nitrogen sources in both organisms. Table 4 and table 5 shows that highest yield of PHB was in *Bacillus megaterium* i.e. 16% than the *Pseudomonas spp. i.e.* 50% than *Bacillus megaterium* when potassium nitrate is used as nitrogen source. Lowest PHB producer spp. is *Bacillus megaterium* when Arabinose and potassium nitrate is used as carbon sources and nitrogen sources respectively, where as in *Pseudomonas spp.* arabinose and L-Cysteine was used as carbon and nitrogen sources respectively.

At conclusive remark, the *Bacillus megaterium* and *Pseudomonas spp.* was collected and PHB granules are separated by chloroform extraction method. *Bacillus megaterium* and *Pseudomonas spp* shows different yield of PHB production when different carbon and sources are used. *Bacillus megaterium* shows maximum yield of PHB when sucrose was used as a carbon sources and L-Lysine was used as a nitrogen sources. *Pseudomonas spp* shows maximum yield of PHB when sucrose was used as a carbon sources and potassium nitrate was used as a nitrogen sources. The carbon and nitrogen sources are not only effect on PHB yield production but also effect on cell mass yield production. Among the different carbon sources used for PHB production, arabinose is responsible for very low production as compared to both sucrose and fructose.

REFERENCES

1. Khanafari A, Akhavan A Sepahei, Mogharab M. Production and recovery of poly-ß-hydroxybutyrate from whey degradation By *Azatobactor.* Iran. J. Environ. Health. Sci. Eng., 2006, 3(3):193-198.
2. Deshmukh AM. Handbook of media strains and reagents in Microbiology, 1998.
3. Aysel Ugur, Nurettin Sahin, Yavuz Beyatli, Accumulation of poly-ß-Sources. *Turk J Biol*, 2002, pp:171-174.
4. Senthil B Kumar and Prabakaran G. Production of PHB (BIOPLASTIC) using bio-effluent as substrate by *Alcaligenus eutophus,* Indian Journal of Biotechnology, 2006, 5: 76-79.
5. Belam Aslim, Zehra Nur Yuksekdag, Yavuz Beyatli, Determination of PHB growth quantities of certain *Bacillus* spp. isolated from soil, *Turkish Electronic Journal of Biotechnology*, 2002: pp 24-30.
6. Bruce A Ramsey, Kanda Lomaliza and *et al*., Production of poly- (ß-hydroxybutyric-co- ß-hydroxyvaleric) acids, *Applied and Environmental Microbiology,* 1990, pp:2093-2098.
7. C.B. Powar and H.F. Daginawala "storage granule" in general microbiology vol II, Himalaya publishing house, Mumbai, 2002, page no. 300-332.
8. Hikmet Katircloglu, Belam Aslim, Zehra Nur Yuksekdag, Yavuz Beyatli, Production of PHB and differentiation of putative *Bacillus* mutant strains by SDS-PAGE of total cell protein, *African Journal of Biotechnology*, 2003; 2:147-149.
9. J.L.Bertrand, B.A.Ramsay, J.A. Ramsey and C. Chavarie, Biosynthesis of poly-ß-hydroxyalkanoates from pentoses by *Pseudomonas pseudoflava, Applied and environmental Microbiology,* 1990, pp-3133-3138.
10. Moreno Nubia and Gutierrez Ivonne, Bioprospecting and characterization of PHAs producing bacteria isolating from Colombian sugarcane producing areas, *African Journal of Biotechnology,* 2007; 6 : 1536-1543.
11. Ojumu TV, Yu J and Solomon BO. Production of poly hydroxyl alkonates, A bacterial biodegradable polymer, African Journal of Biotechnology, 2004; 3: 18-24.
12. Das Q, Chowdhury JU and Anwar MN. Isolation, purification and characterization of biodegradable polymer producing bacteria *Pseudomonas pseudomallei,* International Journal of Agriculture and Biology, 2005-07, pp-114-117.
13. Rafael Garcia Ribera, Mercedes Monteoliva Sanchez, Alberto Ramos-cormenzana, Production of poly hydroxyl alkonates by *Pseudomonas putida* KT 2442 harboring PSK 2665 in wastewater from olive oil mills (alpechin), EJB Electronic Journal of Biotechnology, 2001; 4(2).
14. Robert W Lenz, Richard A Gross and *et al.,* Production of poly-(ß-hydroxyalkanoates): Natural biocompatible and Biodegradable polyesters produced by Bacteria, Chinese Journal of polymer Science, 1989; 7(4):
15. Otari SV and Ghosh JS. Production and characterization of the polymer polyhydroxy Butyrate-co-polyhydroxy valerate by *Bacillus megaterium,* NCIM 2475, *Current research Journal of Biological sciences*, 2009; 1: 23-26.