JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 1401-1407

Screening, Characterization and Role of Poly-β-hydroxybutyrate (PHB) Production in Nitrogen-Fixing Bacteria

A.S. Yadav* and Sandeep Kumar

Department of Genetics & Plant Breeding, CCS Haryana Agricultural University -125 004, India.

(Received: 14 April 2013; accepted: 13 May 2013)

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates (HAs) synthesized by numerous bacteria as intracellular carbon and energy storage compounds. Poly-β-hydroxybutyrate (PHB) is the most common natural microbial PHA. PHBs, the eco-friendly biopolymers, are gaining importance in curtailing the environmental pollution by replacing the non-biodegradable plastics derived from petroleum. PHBs have sought attention as these possess properties close to polypropylene and have been considered for various agricultural, industrial and medical applications. A large number of nitrogen-fixing microorganisms such as rhizobia, *Azotobacter*, *Azospirrillum*, *Pseudomonas*, etc. are known to produce sufficient amounts of PHB, utilizing varied substances as carbon sources. Synthesis and accumulation of PHB in different nitrogen fixing organisms is found to vary between 1- 84 % of their cell dry biomass. PHB has been implicated as an energy source in the symbiotic nitrogen fixation and is important in maintaining respiratory activities that protect nitrogenase from damage by oxygen and in extending nitrogen fixation to the pod-filling stage.

Key words: Poly-β-hydroxybutyrate (PHB), polyhydroxyalkanoates (PHAs), nitrogen-fixing bacteria, biodegradable plastics, eco-friendly.

Plastic material have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. When these polymers are discarded in the environment, they cause environmental pollution due to their non-biodegradable nature (Anderson *et al*., 1990). So, there is a great public and scientific interest in the use of biopolymer material as an eco-friendly alternative to synthetic plastics (Ojumu *et al*., 2004). These biodegradable plastics have been considered for various agricultural, industrial and medical applications as a partial substitute for non-biodegradable plastics.

A wide variety of microorganisms are known to produce intracellular energy and carbon storage compounds, and also act as a sink for reducing equivalents, generally described as poly-β-hydroxybutyrates (PHBs). It has been found that in most cases, these polymers are polyhydoxyalkanoates (PHAs) comprising co-polymers that contain different alkyl groups at the β-position. These are deposited in many bacteria as insoluble inclusions or granules in the cytoplasm usually when a carbon source is available in excess and growth is limited by another nutrient such as nitrogen, phosphorus, oxygen, sulphur etc. (Ojumu *et al*., 2004). These reserve polymers serve as carbon and energy source when cells are starved of nutrients. The significant feature of PHBs is that these can be synthesized from renewable resources and act as source of potentially useful biodegradable natural plastics, since its physical characteristics are similar to those of petrochemical polyesters such as polypropylene.

After the discovery of poly-β-hydroxybutyrate in *Bacillus megaterium*

* To whom all correspondence should be addressed.
Mob.: +91-9728177574;
E-mail: yadavattar@hau.ernet.in

(Lemoigne, 1923), more than 120 PHAs have been found in prokaryotes from axenic culture or from PHAs isolated from samples such as activated sludge, from domestic sewage plants or estuarine sediments. At present, these are produced by microbial fermentation, but in future the production will also be possible by *in vitro* methods or by agriculture using transgenic plants. But, the production cost of PHB is quite high compared with those of synthetic non-degradable plastics. Hence, it is imperative to search for potential microbial strains with high PHB-accumulating ability. The organisms which have been used for PHB production are *Alcaligens eutrophus*, *A. latus, Pseudomonas, Rhizobium, Baacillus, Azospirrillum, Azotobacter*.

Several species of rhizobia belonging to the genera *Rhizobium, Bradyrhizobium, Azorhizobium* accumulate PHB in free-living or in symbiotic state. The physiological role of these compounds are not completely understood. PHB has been implicated as an energy source in the symbiotic nitrogen fixation. Bergersen *et al*., (1992) proposed that PHB reserve in bacteroids can support nitrogen fixation process during darkness and prolongs the period of N_2 fixation. It has also been suggested that PHB is important in maintaining respiratory activities that protect nitrogenase from damage by oxygen in extending N_2 fixation to the pod filling stage. On the other hand, nodules induced by some of *Rhizobium* mutants unable to synthesize PHB exhibited enhanced N_2 fixation activity. Mandon *et al*., (1998) observed that mutants of structural gene for PHB synthase i.e. *phb* C, were devoid of PHB, totally devoid of nitrogenase activity *ex planta* (Nif^-) and induced nodules devoid of bacteria (Fix^-). So, the present review deals with the production and quantification of PHB by different species of rhizobia and the relationship of PHB produced with the nitrogen fixing ability of bacterial strains.

Screening and quantification of phb production by bacterial strains

Poly- β-hydroxybutyrate (PHB) was first described as an important bacterial product and characterized by Lemoigne in 1923. He identified this material as the homopolyester of hydroxyacid 3-hydroxybutyrate, poly-β-hydroxybutyrate (PHB) and described it tentatively as reserve material (Lemoigne, 1927). The quantitative conversion of poly-β-hydroxybutyrate to crotonic acid by heating in concentrated sulphuric acid and determination of the ultraviolet absorbption of the produced material permits an accurate determination of this material in quantities down to 5 ìg (Law and Slepeckey, 1960). It was reported that PHB granules exhibited strong orange fluorescence when stained with Nile blue A and seen under UV light. They also pointed out that Nile blue A is more specific stain for observing PHB production than Sudan black B (Ostle and Holt, 1982). In *Pseudomonas oleovorans*, there was accumulation of poly-3-hydroxyoctanate (P3HO) units and small amounts of hydroxyhexanoate (P3HHx) units when cultivated on n-octane (De Smet *et al*., 1983). The 4-hydroxyhexadeconoate (4HHXDe) and 3-hydroxyhexadeconoate (3HHXDe) were identified which allowed these thermoplastic materials to have various mechanical properties resembling hard crystalline polymer or elastic rubber depending on the incorporated monomer units.

A five-times faster PHB production was observed in *Azotobacter* UWD (Page and Cornish, 1993) during growth on glucose and NH_4 but PHB synthesis stopped when NH_4 was depleted and nitrogen fixation started. It was stated that fish peptone enhanced PHB synthesis and can be used as a nitrogen source rather than a general growth stimulator by these nitrogen fixing cells. About 80 % of PHB was produced by *A. vinelandii* UWD when it was grown on cell dry weight basis on a medium containing sugar and fish peptone.

In rhizobia, PHB production was studied in active and less active strains of *Rhizobium phaseoli*, *Rhizobium meliloti*, and *Rhizobium trifolii* during their growth on media containing different carbon and nitrogen sources. The less active *R. phaseoli* strain 680 accumulated PHB by 65 % on medium containing sucrose and nitrate as carbon and nitrogen sources, respectively (Bonartseva *et al*., 1994). Page and Manchuk (1994) reported PHB accumulation and copolymers containing PHB and PHV in *A. vinelandii* when grown on a medium containing glucose as the primary source and valerate, pentanoate as precursors. A number of bacteria including *Alcaligenes latus*, *Azotobacter vinelandii*, *Methylotrophus*, *Pseudomonas* and recombinant *Escherichia coli* have been employed for the

production of PHAs and the productivity of greater than 2g PHAs/l/h has been achieved (Lee *et al*., 1995a). Metabolism, molecular biology and genetics of PHAs synthesizing bacteria and cloning of more than 20 different PHA biosynthesis genes allowed construction of various recombinant strains that were able to synthesize polyesters having different monomer units and accumulate much more polymer. Genetically engineered plants harboring the bacterial PHA biosynthesis genes are being developed for the economic production of PHAs. The PHB production in *A. chroococcum* H23 on NH_4^+ medium supplemented with alpechin was up to 50 % of the cell dry weight after 24 h of growth suggesting that these wastes could be utilized as cheap substrates for PHB production (Mertinez *et al*., 1995).

Cho *et al*., (1997) reported 34 % of PHB-Co-HV in *A. vinelandii* UWD with 7.9 mol % of hydroxyl valerate (HV) from two-fold diluted swine waste liquor. Supplementation of glucose to a level of 30 g/l increased cell dry weight to 9.0 g/l with 53.8 % of PHB-Co-HV and 4.3 mol % of HV at a PHBV production rate of 0.11 g PHBV/l. In *Pseudomonas* up to 67 % PHB production was reported (Wang and Bakken, 1998) when grown on a nitrogen-free and carbon-rich medium. Specific enzymes like enoyl-Co A hydratases, 3-ketoacyl CoA-reductase are presumably involved in the conversion of fatty acid â-oxidation intermediates to suitable monomers to be polymerized by PHA synthase. In *Azorhizobium caulinodans*, it was observed that PHB is accumulated in free-living or in symbiotic state (Mandon *et al*., 1998). It was opined that PHB is required for maintaining the reducing power of the cell, therefore the bacterial growth. A majority of forty-two strains of rhizobia were evaluated quatitatively for poly (3-hydroxy-butyric acid) (PHB) production (Manna *et al*., 2003). They suggested that the majority of the strains produced the maximum amount of PHB during the late exponential or stationary phase of growth. Synthesis and accumulation of PHB in different species of rhizobia were found to vary between 1-38 % of their dry biomass. Growth and PHB production by *Rhizobium* strain TAL-640 was greatly influenced by the C-source and D-mannitol was fundamental to both the processes. Cho *et al*., (2001) reported the PHB production in diluted swine waste water by *A. vinelandii* strain and found an increase of 8.6 times in the production of poly-â-hydroxybutyrate. *A. chroococcum* was shown to accumulate PHB up to 68 % of its cell dry mass in nitrogen-free liquid medium containing 2 % glucose. Poly-3-hydroxybutyrate (PHB) production in *Rhizobium japonicum*, *Rhizobium* sp. (*Cicer*), eight species of *Rhizobium* and *Bradyrhizobium japonicum* USDA 110 was studied and PHB accumulation was found as high as 285 mg/l and the percentage yield was 74.03 %. The highest level of PHB accumulation was observed in yeast extract mannitol (YEM) medium with L-cysteine and glycine as nitrogen source. The percentage of PHB yield in *Rhizobium* sp. 640 was 13.40 and 56.67 while with the same nitrogen source in *Rhizobium* spp. 2426, it was 70 and 61.43, respectively (Mercan *et al*., 2002).

In *Methylobacterium* sp. V49 different carbon sources were tested on a minimal salts medium (Ghatnekar *et al*., 2002), the maximum accumulation of PHB was with glucose as a carbon source. The authors also reported that methanol and sugars such as sucrose and lactose also induced PHB accumulation. The optimization of PHB production was done in *Azotobacter beijernckii* under batch culture (Manna and Paul, 2003). The accumulated polymer attained 58 % of cell dry mass during mid-stationary phase with an yield of 0.58 g/l when grown in nitrogen-free medium. In *Azospirillum brasilense*, PHB was used as a carbon and energy source under stress conditions which favoured establishment of this bacterium and its survival in competitive environments (Kadouri *et al*., 2003) but also not provide an advantage in root colonization under the condition tested.

The PHB production was reported in different species of rhizobia viz; *Rhizobium meliloti*, *Rhizobium viciae* and *Bradyrhizobium japonicum* (Mercan and Beyatli, 2005) with different carbon and nitrogen sources. The *R. meliloti* Y11 and *B. japonicum* S Irat Fab strains were producing maximum yields of PHB i.e. 83.75 and 34.31 %, respectively on dry cell mass basis when grown on glucose as carbon source. They also found that *Rhizobium viciae* F 111 produced a high level of PHB (41.22 %) with mannitol as carbon source. The highest PHB accumulation with *R. meliloti* Y11 reached to 56.31 % of dry cell mass basis during its growth in 0.5 % molasses.

Sujatha *et al*., (2005) screened hundreds of indigenous bacterial strains for the accumulation of PHB by Nile red fluorescence microscopy. Based on these studies, two indigenous isolates of *Pseudomonas* LDC-5 and LDC-25 could be potential candidates for bioplastic production. The effect of PhaP, a phasin, on bacterial growth and PHB accumulation from glycerol in bioreactor cultures of recombinant *Escherichia coli* carrying *pha*BAC from *Azotobacter* sp. Strain FA8 (Almeida *et al*., 2007) was studied. It was found that cells expressing *pha*P grew more, and accumulated more PHB, both using glucose and glycerol as carbon sources. When cultures were grown in a bioreactor using glycerol, PhaP-bearing cells produced more polymer (2.6 times) and more biomass (1.9 times) than did those without the phasing.

The *Pseudomonas* sp. RZS1 (isolated from distillery effluent) accumulated optimum amount (703.79 µg/mg of biomass) of poly-β-hydroxybutyrate (PHB) under aerobic process of fermentation and 75 µg/mg of biomass under anaerobic process of fermentation (Sayyed and Gangurde, 2010). Aerobic fermentation yielded 9.3-fold more PHB than semi-aerobic fermentation.

Thirty-eight isolates from pigeonpea [*Cajanus cajan* (L.) Millsp] root nodules were screened for PHB production. Out of these, five isolates produced more than 10 mg PHB per litre of culture medium. The PHB productivity of the isolate 24.6b reached 69 % of cell dry weight when cultured with starch as sole carbon source and the isolate 8.1c synthesized 53 % PHB in dry cell biomass using xylose as sole carbon source (Junior *et al*., 2011).

Twelve polyhydroxyalkanoate (PHA) producing microbes isolated from root nodules of 8 leguminous plants belonging to two phyla: Proteobacteria and Firmicutes. One of the isolate VK-12 of genus *Burkholderia* showed the highest PHA accumulation (42 % wt/wt) as compared to other isolates in the mineral medium (Kumbhakar *et al*.,2012).

Isolation and characterization of PHB mutants

Willis and Walker (1998) observed *phb*C gene in *Sinorhizobium meliloti*, which encodes poly-â-hydroxybutyrate (PHB) synthase. The locus was isolated and subcloned from a genomic library of *R. meliloti* Rm1021 by complementation of a *phb*C mutation of *Alcaligenes eutrophus*. They found no PHB production in *R. meliloti phbC* mutants. In *Azospirillum brasilense*, the strain Sp7 and its mutants (*ntr*A, *ntr*BC, and *ntr*C) were studied for PHB accumulation (Sun *et al*., 2000) in media with high and low ammonia concentrations. The authors reported that the *ntr*BC and *ntr*C mutants can produce PHB in both low- and high-C/N-ratio media, whereas there was no significant PHB production in the wild-type or the *ntr*A mutant on low-C/N-ratio media. They also indicated that *ntr*BC and *ntr*C mutants were able to grow and accumulate PHB simultaneously in the presence of a high concentration of ammonia in the medium, while little PHB was produced in the wild type and *ntr* A (rpoN) mutant during active growth phase. Parshad (2000) screened 37 isolates and mutants of *Azotobacter chroococcum*, out of which one mutant showed maximum PHB production at 36 and 48 hours of growth under submerged and stationary condition, respectively. They further observed that PHB production was higher on sucrose as compared to glucose and mannitol. The single and double mutants of *R. leguminosarum* bv. *viciae* lacked poly-hydroxybutyrate synthase (*pha*C), glycogen synthase (*glg*A), or both (Lodwig *et al*., 2005). For comparison, a single *pha*C mutant was also isolated in a bean-nodulating strain of *R. leguminosarum* bv. *phaseoli*. The authors found that in one large glasshouse trial, the growth of pea plants inoculated with the *R. leguminosarum* bv. *viciae pha*C mutant was significantly reduced as compared to the wild type inoculated plants. Bean plants were unaffected by the loss of polyhydroxybutyrate biosynthesis in bacteroids.

It was reported in *Sinorhizobium meliloti* that PHB granule-associated proteins (phasins) regulate PHB synthesis and granule formation. The PhaP1 and PhaP2 were the two major phasins. Double mutants were defective in PHB production, while single mutants still produced PHB and, unlike PHB synthesis mutants that have reduced exopolysaccharide, the double mutants had higher exopolysaccharide levels (Wang *et al*., 2007). Wang *et al*., (2007) constructed glycogen synthase mutations by in-frame deletion (*glg*A1) or insertion (*glg*A2). These mutations were combined with a *phb*C mutation to make all combinations of double and triple mutants. PHB was not detectable in any

of the mutants containing the *phb*C mutation; glycogen was not detectable in any of the mutants containing *glg*A1 mutation. PHB levels were significantly lower in the *glg*A1 mutant, while glycogen levels increased in the *phb*C mutant. Exopolysaccharide (EPS) was not detected in any of the *phb*C mutants, while the *glg*A1 and *glg*A2 mutants produced levels of EPS similar to the wild type.

Role of PHB in biological nitrogen fixation

It has been suggested that PHB serves as an energy source in the symbiotic nitrogen fixation process between rhizobia and the leguminous plants. The heavy demand for energy for nitrogen fixation is met by the metabolism of photosynthetic carbon compounds transported to the root nodules. It was found that PHB is important in maintaining respiratory activities that protect nitrogenase from damage by oxygen and in extending nitrogen fixation into pod-filling stage (Chohan and Copeland, 1998). The pea nodules infected with the *glg*A mutant accumulated large amounts of starch in II/III interzone (Lodwig *et al*., 2005). This suggests that glycogen may be dominant carbon storage compound in pea bacteroids.

Polyhydroxybutyrate was present in bacteria in the infection thread of pea plants but was broken down during bacteroid formation. In nodules infected with a *pha*C mutant of *R.leguminosarum* bv. *viciae*, there was a drop in the amount of starch in the II/III interzone, where bacteroids form. The authors proposed a carbon burst hypothesis for bacteroid formation, where polyhydroxybutyrate accumulated by bacteria is degraded to fuel bacteroid differentiation. The *Medicago truncatula* plants inoculated with double mutant exhibited reduced shoot dry weight (SDW), although there was no corresponding reduction in nitrogen fixation activity (Wang *et al*., 2007). The glycogen synthase mutants by in-frame deletion (*glg*A1) or insertion (*glg*A2) were tested on *Medicago truncatula* and *Medicago sativa* plants (Wang *et al*., 2007). The authors observed that the ability to synthesize PHB is important for nitrogen fixation in *Medicago truncatula* nodules and younger *M. sativa* nodules, and the blocking of glycogen synthesis resulted in lower levels of nitrogen fixation on *M. truncatula* and older nodules on *M. sativa.*

Future prospects

The demand of biodegradable plastics is increasing day by day. Hence, it is imperative to screen new strains /mutants of nitrogen fixing organisms producing high amount of PHB which could then be used for commercial production of PHB. Further, the correlation between PHB production and nitrogen fixation may be explored in other nitrogen fixing organisms, other than rhizobia.

REFERENCES

1. Almedia, A.D., Nikel, P.I. Giordano, A.M. and Pettinari, M.J. Effect of granule associated protein *pha*P on glycerol dependent growth and polymer production in poly (3-hydroxybutyrate) producing *Escherichia coli*. *Appl. Environ. Microbiol*., 2007;**73**: 7912-7916.
2. Anderson, A.J. and Dawes, E.A. Occurrence, metabolism, metabolic role and industrial uses of bacterial poly-â-hydroxyalkanoates. *Microbiol. Rev*., 1990; **54**: 450-472.
3. Bergersen, F.J. and Turner, G.L. Supply of O_2 regulates O_2 demand during utilization of reserve of poly-â-hydroxybutyrate in N_2-fixing soybean bacteroids. *Proc. R. Soclond. Biol. Sci*.,1992; **249**: 143-148.
4. Bonartseva, G.A., Myshkina, V.L. Zagreba, E.A. Poly-â-hydroxybutyrate content of cells of various *Rhizobium* species during growth with different carbon and nitrogen sources. *J. Microbiol*., 1994; **63**: 78-85.
5. Cho, K.S., Ryu, H.W., Ho, P.C. and Goodrich, P.R. Utilization of swine waste as a feed stock for the production of poly-hydroxy-alkanoates by *Azotobacter vinelandii UWD. J. Bioscience Bioengin*., 2001; **91**: 129-133.
6. Cho, K.S., Ryu, H.W., Park, C.H. and Goodrich, P.R. Poly-(hydroxybutyrate-co-hydroxyvalerate) from swine waste liquor by *A. vinelandii UWD. Biotechnol*., *Lett*. 1997; **19**: 7-10.
7. Chohan, S.N. and Copeland, L. Acetoacetyl coenzyme A reductase and polyhydroxybutyrate synthesis in Rhizobium (*Cicer*) sp. Strain CC1192. *Appl. Environ. Microbiol*., 1998; **64**: 2859-2863.
8. De Smet, M.J., Eggink, G., Witholt, B., Kingma, J. and Wynberg, H. Characterization of intracellular inclusion formed by *Pseudomonas oleovorans* during growth on octane. *J. Bacteriol*., 1983; **154**: 870-878.
9. Ghatnekar, M.S., Pai, J.S. and Ganesh, M.

Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp. V49. *J. Chemical Technol. & Biotechnol*., 2002; **77**: 444-448.

10. Junior, P.I.F., de Oliveira, P.J., Xavier, N.G.R.G.R., Rumjanek, N.G. and Xavier, G.R. Poly-â-hydroxybutyrate and exopolysaccharide biosynthesis by bacterial isolates from pigeonpea [Cajanus cajan (L.) Millsp] root nodules. *Appl. Biochem. Biotechnol*., 2011; **163**: 473-484.
11. Kadouri, D., Jurkevitch, E. and Okon, Y. Involvement of the reserve material poly-â-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Appl. Environ. Microbiol*., 2003; **69**: 3244-3250.
12. Kumbhakar, S., Singh, P.K. and Vidyarthi, A.S. Screening of root nodule bacteria for the production of polyhydroxyalkanoate (PHA) and the study of parameters influencing the PHA accumulation. *African J. Biotechnol*., 2012; **11**: 7934-7946.
13. Law, J.H. and Slepecky, R.A. Assay poly- â-hydroxybutyrate acid. *J. Bacteriol*., 1960; **82**: 33-36.
14. Lee, E.Y., Jendrossek, D., Schirmer, A., Choi, C.Y. and Steinbuchel, A. Biosynthesis of copolyesters consisting of 3- â-hydroxybutyric acid and medium chain length 3-hydroxyalkanoic acids from 1,3-butanediol or from 3-hydroxybutyrate by *Pseudomonas* sp. A33. Appl. *Environ. Microbiol. Biotechnol*., 1995a; **42**: 901-909.
15. Lemoigne, M. Production d'acid-â-oxybutyrique par certain bacteria du grouped du *B. subtilise* C.R. *Acad. Sci Paris*., 1923; **176**: 1761.
16. Lemoigne, M. Etudes sur I' autolyse microbienne: orinine defendant I' acide- â-oxybutyrique forme par autolyse. *Ann. Inst. Pasteur* 1927; **41**: 148-165.
17. Lodwig, E.M., Leonard, M., Marroqui, S., Wheeler, T.R., Findlay, K., Downie, J.A. and Poole, P.S. Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids. *Mol. Plant Microbe Int*., 2005; **18**.: 67-74.
18. Mandon, K., Redellet, N.M., Encarnacion, S., Kaminski, P.A., Leija, A., Cevallos, M.A., Elmerich, C. and Mora, J. Poly- â-hydroxybutyrate turnover in *Azorhizobium caulinodans* is required for growth and affect *nif*A expression. *J. Bacteriol*., 1998; **18**: 5070-5076.
19. Manna, A. and Paul, A.K. Growth associated production and characterization of poly (3-hydroxybutyric acid) of *Azotobacter beijerinckii* DAR-102. *Indian J. Exp. Biol*., 2003; **41**: 129-134.
20. Mercan, D., Lomaliza, Y. and Mudaliar, S. Production of PHB by *Rhizobium japonicum*, *Rhizobium cicer, Bradyrhizobium japonicum* with different substrates. *Turk. J. Biol*., 2002; **56**: 222-228.
21. Mercan, N. and Beyatli, Y. Production of PHB by *Rhizobium meliloti*, *R. viciae* and *Bradyrhizobium japonicum* with different carbon and nitrogen sources and inexpensive substrates. *Zukerindustrie*., 2005; **130**: 410-415.
22. Mertinez, M.V., Gonzalez, J., Rodelas, B., Pozo, C. and Salmeron, V. Production of poly-β- hydroxybutyrate by *Azotobacter chroococcum* H23 in chemically defined medium and alpechin medium. *J. Appl. Bacteriol*., 1995; **78**: 413-418.
23. Ojumu, T.V., Yu, J. and Solomon, B.O. Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. *African J. Biotechnol*., 2004; **3**: 18-24.
24. Ostle, A.G. and Holt, J.G.. Nile blue A as a fluorescent stain for poly- β- hydroxybutyrate . *Appl. Environ. Microbiol*., 1982; **44**: 238-241.
25. Page, W.J. and Cornish, A. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly- â-hydroxybutyrate. *Appl. Environ. Microbiol*., 1993; **59**: 4236-4244.
26. Page, W.J. and Manchak, J. The role of â-oxidation of short chain alkanoates in polyhydroxyalkanoate copolymer synthesis in *Azotobacter vinelandii* UWD. *Can. J. Microbiol*., 1994; **41**: 106-114.
27. Parshad, J. Production of poly- â-hydroxybutyrate by *Azotobacter chroococcum*. M.Sc., 2000; Thesis submitted to CCS Haryana Agric. University, Hisar.
28. Sayyed, R.Z. and Gangurde, N.S. Poly- â-hydroxybutyrate production by Pseudomonas sp. RZS 1 under aerobic and semi-aerobic condition. *Indian J. Exp. Biol*., 2010; **48**: 942-947.
29. Sujatha, K., Mahalakshmi, A. and Shenbagarathai, R. A study on accumulation of PHB in native *Pseudomonas* isolates LDC-5 and LDC-25. *Indian J. Biotechnol*., 2005; **4**: 216-221.
30. Sun, J., Peng, X., Impe, J.V. and Vanderleyden, J. The *ntr*B and *ntr*C genes are involved in the regulation of poly- â- hydroxybutyrate biosynthesis by ammonia in *Azospirillum brasilense* sp. *Appl. Environ. Microbiol*., 2000; **66**: 113-117.

31. Wang, J.G. and Bakken,L.R. Screening of soil bacteria for poly- β- hydroxybutyric acid production and its role in the survival of starvation *Microbiol. Ecol*., 1998; **35**: 94-101.
32. Wang, C., Saldanha, M., Shing, X., Shelswell, K.J., Walsh, K.T., Sobral, B.W.S. and Chales, T.C. Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago* sp. *Microbiol*., 2007; **153**: 388-398.
33. Wang, C., Sheng, X., Equi, R.C., Trainer, M.A., Charles, T.C. and Sobral, W.S. Influence of poly-â- hydroxybutyrate (PHB) granule associated proteins (PhaP1 and PhaP2) on PHB accumulation and symbiotic nitrogen fixation in *Sinorhizobium meliloti* Rm1021. *J. Bacteriol*., 2007; **189**: 9050-9051.
34. Willis, L.B. and Walker, G.C. The *phb*C (poly-β- hydroxybutyrate synthase) gene of *Rhizobium (Sinorhizobium) meliloti* and characterization of *phb*C mutants. *Can. J. Microbiol*., 1998; **44**: 554-564.