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MINI-REVIEW

Microbial production of poly(hydroxybutyrate) from C_1 carbon sources

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Abstract Polyhydroxybutyrate (PHB) is an attractive substitute for petrochemical plastic due to its similar properties, biocompatibility, and biodegradability. The cost of scaled-up PHB production inhibits its widespread usage. Intensive researches are growing to reduce costs and improve thermo-mechanical, physical, and processing properties of this green biopolymer. Among cheap substrates which are used for reducing total cost of PHB production, some C_1 carbon sources, e.g., methane, methanol, and CO_2 have received a great deal of attention due to their serious role in greenhouse problem. This article reviews the fundamentals of strategies for reducing PHA production and moves on to the applications of several cheap substrates with a special emphasis on methane, methanol, and CO_2. Also, some explanation for involved microorganisms including the hydrogen-oxidizing bacteria and methanotrophs, their history, culture condition, and nutritional requirements are given. After description of some important strains among the hydrogen-oxidizing and methanotrophic producers of PHB, the article is focused on limitations, threats, and opportunities for application and their future trends.

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Introduction

Polyhydroxyalkanoates (PHAs) are the biopolymers which accumulate as a carbon/energy or reducing power storage material in various microorganisms usually when there is a growth-limiting component, such as O, N, P, S, or trace elements, e.g., Mg, Ca, and Fe in the presence of excess carbon source (Anderson and Dawes 1990; Doi 1990; Brandi et al. 1990; Braunegg et al. 1998; Lee et al. 1999; Sudesh and Doi 2000; Kessler and Witholt 2001, Zinn 2001; Chanprateep 2010; Kunasundari and Sudesh 2011; Ramadas et al. 2010; Morgan-Sagastume et al. 2010). In some *Bacillus* spp., it supplies energy for sporulation (Slepecky and Law 1961). The low molecular weight polyhydroxybutyrate (PHB) is a part of bacterial Ca^{2+} channels (Lara and Huisman 1999).

Among the various biodegradable polymers, PHAs are attractive substitutes for conventional petrochemical plastics due to their similar properties to common thermoplastics, biocompatibility, and biodegradability under various environments (Lee 1996; Steinbüchel and Füchtenbusch 1998). The use of PHA in a wild range of applications has been hampered mainly by their high production cost (Choi and Lee 1997; Ackermann and Babel 1998; Bormann et al. 1998; Choi and Lee 1999b; Du et al. 2001; Hori et al. 2002; Povolo and Casella 2003; Du et al. 2004; Wang and Yu 2007; Van-Thuoc et al. 2008; Papaneophytou et al. 2009; Chanprateep 2010; Ibrahim and Steinbüchel 2010; Kozhevnikov et al. 2010; Povolo 2010; Povolo et al. 2010; Budde et al. 2011).

More than 150 different monomers can be combined within PHA family to give materials with extremely different properties (Doi and Steinbüchel 2002). The mechanical

and biocompatibility of PHA can also be changed by blending, modifying the surface, or combining PHA with other polymers, enzymes, and inorganic materials, making it possible for a wider range of applications. PHA synthases which are key enzymes of PHA production use the coenzyme A-thioester of (r)-hydroxy fatty acids as substrates. The two classes of PHA synthases differ in the specific use of hydroxyfattyacids of short- (SCL) or medium-chain length (MCL). So, three types of SCL, MCL, and long-chain length (LCL) PHA may result from hydroxy fatty acids including 3–5, 6–14, and more than 15 carbon atoms, respectively. SCL-PHA are synthesized by numerous bacteria, including *Ralstonia eutropha* and *Alcaligenes latus* (PHB), and MCL-PHA can be made for example, by fluorescent *Pseudomonas* like *Pseudomonas putida*. A few bacteria, including *Aeromonas hydrophila* and *Thiococcus pfennigii*, synthesize copolyester, from the SCL- and MCL-PHA.

Copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) is less stiff and tougher, and it may be used as a packaging material. Depending on the monomer composition, PHAs properties can range from thermoplastic to elastomeric materials. Increase in the ratio of 3-hydroxybutanoic acid to 3-hydroxypentanoic acid results in an increase in melting point, water permeability, glass transition temperature and tensile strength. However, impact resistance is reduced (Rudnik 2008; Pilla 2011). Recently, the physicochemical properties of PHAs produced from various carbon sources have been reviewed (Du et al. 2012).

Also, functionalized PHAs have been ductile and showed signs of side-chain crosslinking, resulting in reduced degrees of crystallinity. Incorporation of special monomer into the polymeric chains produced desirable thermal properties with enhanced thermal stability and reduced melting temperatures (Höfer et al. 2011). Hazer et al (2012) summarize the modification reactions, which include functionalization and grafting reactions, to improve the mechanical, thermal, and hydrophilic properties of PHAs.

PHB is the first discovered PHA and also the most widely studied and best characterized one. It is accumulated inside a membrane enclosed inclusion in many bacteria at up to 80 % of the dry cell weight. PHB has mechanical properties very similar to conventional plastics like polypropylene or polyethylene. Although PHB can be extruded, molded, spun into fibers, made into films, and used to make heteropolymer, but typically, SCL-PHAs like PHB are highly crystalline and brittle with poor elastic properties. Whereas, LCL-PHA are more ductile and easier to mold (Kabilan et al. 2012).

The fermentation performances, high cell density, carbon substrate, metabolic and genetic engineering, as well as process optimization, modeling, and recovery methods affect the total cost of PHB (Heinzle and Lafferty 1980; Mulchandani et al. 1989; Haywood et al. 1990; Yoo and Kim 1994; Yamane et al. 1996b; Ryu et al. 1997; Raje and Srivastava 1998; Grothe et al. 1999; Tohyama et al. 2002; Lu et al. 2003; Steinbüchel and Lutke-Eversloh 2003; Reddy et al. 2003; Patwardhan and Srivastava 2004; Khanna and Srivastava 2005a, b, c; Koller et al. 2008; Pantazaki et al. 2009; Papaneophytou et al. 2009).

Also, recombinant organisms (Choi and Lee 1999a; Park et al. 2001; Hofer et al. 2011) as well as co-culture systems have been developed for increased polymer production (Ganduri et al. 2005; Patnaik 2005; Lemos et al. 2006; Jing and Jiaying 2011). The utilization of cheap substrates and the development of different fermentation strategies for PHA production (Khosravi-Darani and Vasheghani-Farahani 2005a, 2005b; Akaraonye et al. 2010) as well as opportunities and threats for their competition in the global market have recently been addressed (Chanprateep 2010). We have also studied on reducing these costs by modeling (Shah-Hosseini et al. 2003), proper experimental design (Khosravi-Darani et al. 2003a, 2004b), and development of a new recovery method (Khosravi-Darani et al. 2003b, 2004a; Khosravi-Darani and Vasheghani-Farahani 2005b).

The cost of the carbon source is approximately 40 % of the total operating cost (Yamane 1993; Choi and Lee 1997; Halami 2008). The various cheap carbon sources have been used for PHB production include whey (Wong and Lee 1998; Kim 2000; Koller et al. 2005; Nikel et al. 2006; Koller et al. 2011; Di Donato et al. 2009; López-Cuellar et al. 2011a), wheat bran (Van-Thuoc et al. 2008; Ramadas et al. 2009) and rice bran (Huang et al. 2006), corn steep liquor (Gouda et al. 2001) as well as starch (Quillaguamán et al. 2005; Chen et al. 2006), molasses (Gouda et al. 2001; Omar et al. 2011; Solaiman et al. 2006; Albuquerque et al. 2007; Santimano et al. 2009), waste water from olive mills and starch (Choi and Lee 1999b; Yan et al. 2006; Bengtsson et al. 2007), waste glycerol (João et al. 2009; Dobroth et al. 2011), waste of vegetable (Fukui and Doi 1998; Ribera et al. 2001; Bhubalan et al. 2008; Simon-Colin et al. 2008), sweet sorghum (Kaewkannetra et al. 2008), enzyme hydrolyzate of potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour (Ramadas et al. 2009), waste of potato starch (Haas et al. 2008), canola oil (López-Cuellar et al. 2011b), and waste oil (Wong et al. 2000). Cyanobacteria as the sole photoautotrophs accumulating PHB visualize the desired perspective for reduction of the cost incurred for expensive carbon source and oxygen supply in commercial production of PHB through bacterial fermentation.

This review has been focused on PHB production from some C_1 carbon sources like methane, methanol and CO_2 as low cost substrates which all are serious pollutants in atmosphere of earth).

PHB production from CO_2 by hydrogen-oxidizing bacteria

Main characteristic of hydrogen-oxidizing bacteria or Knallgas bacteria is ability of utilization of H_2 as electron donor and O_2 as electron acceptor to fix CO_2 into cell materials via ribulose biphosphate or reverse tricarboxilic cycle (Aragno and Schlegel 1992). *R. eutropha* is one of the most famous PHB producers of hydrogen-oxidizing bacteria. This bacterium has gone through a series of name changes. At first it was named *Hydrogenomonas eutrophus* due to its ability to utilize hydrogen. Then it was renamed *Alcaligenes eutropha* because of its ability for degeneration peritrichous flagellation. Then its name was changed to *R. eutropha* due to lipid and fatty acid composition as well as 16S rRNA analysis and pheynotype of microbe. The new genus *Wautersia* was created from one of two phenotypically distinct clusters of *R. eutropha*. In turn, *R. eutropha* was renamed *Wautersia eutropha*. Looking at DNA–DNA hybridization and phyenotype comparison with *Cupridavidus necator*, *W. eutropha* was found to be the same species as the previously described *C. necator. C. necator* was named in 1987 far before all other name; it was assigned to *R. eutropha* according to Rule 23a of the International Code of Nomenclature of Bacteria (Vandamme and Coenye 2004).

Hydrogen-oxidizing organisms often inhabit oxic–anoxic interfaces in nature where O_2 and H_2 are supplied. Anaerobic organisms are well known as one of the supplier of O_2 and H_2. The culture of hydrogen-oxidizing bacteria is a matter worth considering as a possible tool for consumption of CO_2. Initial research for growth characteristics and culture method in autotrophic condition of hydrogen-oxidizing bacteria was studied by many researchers (Foster and Litchfield 1964; Repask 1966; Ammann et al. 1968; Schink and Schlegel 1978; Malik and Schlegel 1980; Pinkwart et al. 1983). After their works, many types of the bacteria have been found in several genera and species. Many studies for the autotrophic bacteria have also been reported on taxonomy, physiology, ecology, metabolism, and genetics. However there have not been so many reports about culture technology for autotrophic growth and industrial usage of the bacteria because insoluble and explosive gaseous substrate, H_2 is used as the substrate. Two types of closed culture systems are reported for increasing product yield for PHB production, dead-end (Bongers 1970) and recycled gas culture (Schlegel et al. 1961; Kodama et al. 1975). The dead-end culture system has disadvantages in gas mass transfer due to its lack of aeration. The recycled gas closed circuit culture (RGCCC) system allows many other benefits, such as reducing loss of substrate gas, operation safety for detonation, and continuous process (Ishizaki and Tanaka 1990, 1991; Ishizaki et al. 2001; Nishihara et al. 1991; Takeshita and Ishizaki 1996; Takeshita et al. 1993a, b; Tanaka and Ishizaki 1994; Tanaka et a. 1993; Hayashi et al. 1994; Sugimotoet al. 1999; Bae et al. 2001). In their works, theory, techniques, stoichiometry, and practical fermentation system for PHB production from CO_2 by autotrophic culture of *C. necator* were also investigated. The fermentation technology for this microorganism with industrial aspects was also studied to solve many difficulties like detonation and low efficiency of gas usage due to flow out of exhaust gas from fermenter by using an explosion-proof-type fermentation system (Tanaka et al. 1993, 1995). They achieved also a high cell density cultivation of *C. necator* with production of 91.3 g/L cells and 61.9 g/L PHB in this autotrophic condition using an agitator with very high k_La in the system. Addition of carboxymethyl cellulose to medium in two stage chemolithoautotroph culture of *C. necator* was effective to increase mass transfer coefficient in RGCCC in an air lift type fermentor (Taga et al. 1997). Khosravi-Darani et al. (2006) also has also investigated the bioengineering aspects of RGCCC for industrial application to the culture of hydrogen-oxidizing bacteria as PHB producer from cheap, available, and frequent gas substrate. Their studies on the showed that high concentration of oxygen suppressed the specific growth rate of *C. necator* while limited concentration of gas can promote it. Recently, the cell growth and PHB accumulation in autotrophic batch culture of a newly isolated hydrogen-oxidizing bacterium *Ideonella* spp. strain O-1 in the presence of CO has been reported (Tanaka et al. 2011). This bacterium can grow in high O_2 concentration of 30 % (*v*/*v*), while the growth of *C. necator* and *A. latus* was seriously inhibited. In culture medium containing 1 g/L $(NH_4)_2SO_4$, PHB, and biomass concentration reached to 5.26 and 6.75 g/L, respectively, while PHB content of increased to 77.9 % (*w*/*w*) in dried cell. The strain O-1 was also grew in the present of 70 % (*v*/*v*) CO, while the growth *C. necator* and *A. latus* were seriously inhibited at 5 % (*v*/*v*) CO. Such a CO-resistant hydrogen-oxidizing bacterium is expected to useful for PHB production using directly the industrial exhaust gas containing CO_2, H_2, and also as the substrate.

C. necator B5786 as a CO-resistant strain of the hydrogen bacteria was also able to grow on gas mixtures containing CO at 5–25 % (*v*/*v*), and accumulate up to 70–75 % (*w*/*w*) PHA. No suppressed activity of the key enzymes of PHA synthesis, i.e., acetoacetyl-CoA-reductase, beta-ketothiolase, poly-3-hydroxybutyrate synthase, and butyrate dehydrogenase was reported. Also, no significant difference in crystallinity, molecular weight, and temperature characteristics have been shown compared with polymer produced on electrolytic hydrogen (Volova et al. 2002). In culture of this bacterium, kinetic indices of growth, PHA accumulation, and gas exchange have also been investigated on a gaseous substrate obtained by lignite gasification. Various strategies of gas supply to the culture were studied to increase the gas

consumption. In controlled condition, high polymer yields of 75 % and substrate utilization of up to 90 % was achieved (Volova et al. 2002).

PHB production from methane

Methane is abundantly available in many oilfields and also can be produced in the biological degradation of organic matter by methanogenes. Approximately 50 % of the total organic carbon which can be degraded by anaerobic microorganisms is converted to methane (Asenjo and Suk 1986). Due to prohibitive cost of storage and transport, this valuable gas is simply flared or released to the atmosphere. In 2009, the USA released about 15 billion pounds as methane to the atmosphere (US Environmental Protection Agency 2011). Production of PHB from waste methane may sequester a potent greenhouse effect gas. Listewnik et al (2007) estimated a price of 6.35 UK pound/kg for produced PHB from natural gas in a two-stage plant producing 500 t PHB/year. Extrapolation for downstream procedures resulted in that the prices will be 11.50–14.00 UK pound/kg. Their estimations showed that a scaled up process of 5,000 t/year may cause a price reduction of approximately 30–35 %. It is interesting that PHB biodegradation in controlled anaerobic environments also leads to methane production (Morse et al. 2011). Some reported methylotrophs which are recognized as PHB producers from CO_2, methane, and methanol as well as the content, yield, and productivity have been summarized in Table 1.

Microorganism and metabolic pathway

Methanotrophic bacteria are able to use oxidize methane as a sole carbon and energy source. (Whittenbury et al. 1970). Methanotrophs as a subset of the methylotrophs can grow on C_1 compounds (Lidstrom 2006) and may be found in different natural ecosystems, bioreactors, gas pipelines, as well as soil near leaky gas pipes and water-treatment systems (Vecherskaya et al. 2001). Their great potential in environmental biotechnology are (1) methane escaping from atmosphere or soil, (2) co-metabolizing of contaminants, such as chlorinated hydrocarbons in the presence of methane by microorganisms, e.g., *Methylocystis* spp. GB 14, and (3) accumulation of PHB. Figures 1 (Van Dien and Lidstrom 2002) and 2 (Yaman 1993) show ribulose monophosphate (RuMP) and serine pathways for production of PHB from methane and methanol. Methanotrophs have been classified into two groups of types I and II; on the basis of their primary carbon assimilation pathway, the RuMP and serine pathway, respectively, different membrane arrangements, and the ability to fix molecular nitrogen (Bowman et al. 1993). Type X methanotrophs, a subset of type I methanotrophs use the RuMP pathway and also possess low levels of serine-pathway enzymes (Hanson and Hanson 1996). The recently discovered acidiphilic Verrucomicrobia methanotrophs of the genus *Methylacidiphilum* possess serine-pathway genes and apparently lack RuMP-pathway genes (Dunfield et al. 2007; Pol et al. 2007). Islam et al. (2008) has reported a methane oxidation at 55 °C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum. Also, Op den Camp et al. (2009) has studied environmental, genomic, and taxonomic perspectives of methanotrophic Verrucomicrobia.

Both types have different survival strategies. Methanotrophs of group I are known by rapid growth under favorable condition and a rapid rate of die-off under stress conditions. While type II methanotrophs grow slower, survive better, and outcompete type I methanotrophs under oxygen- and nitrogen-limiting conditions. The literature contains conflicting evidence for PHB producers of methanotrophs. Both groups of methanotrophs can assimilate reduced C_1 compounds and produce PHB via two pathways of RuMP and the serine pathway (Yamane et al 1996a). Type II bacteria which perform metabolization using the serine route, are more effective than type I bacteria (Wendlandt et al. 2001). To date, pure and mixed cultures of methanotrophs are used for PHB production. Most quantitative PHB production studies are reported from type II genera *Methylocystis* and *Methylosinus*, *Methylococcus*, and *Methylomonas* spp. (Asenjo and Suk 1986; Shah et al. 1996a, b; Wendlandt et al. 2001, 2005; Vecherskaya et al. 2001; Dedysh et al. 2004; Helm et al. 2006, 2008; Xin et al. 2007; Zhang et al. 2008; Pieja et al. 2011b). They and some other researchers (Bowman et al. 1993, 2001; Heyer et al. 2005) also reported also qualitative study for PHB production by type I methanotrophs. Anyway, the majority of PHB producers of methanotrophs type II are, e.g., *Methylocystis paravus*, *Methylosinus trichosporium*, *Methylosinus sporium*, *Methylocystis* spp. GB25, MTS, and *Methylocella tundrae*. Wendlandt et al. (1998, 2001) reported production of PHB with a very high molecular mass above 1 MDa.

At least three genes, phaCAB, are known crucial for PHB production (Madison and Huisman 1999) and have been used to screen for synthesis capacity (Sheu et al. 2000). As Fig. 3 shows that the route for the formation of PHB starts from acetyl-CoA molecules and proceeds via three distinct sequential, enzyme-mediated reactions as follows (Vincenzini and De Philippis 1999): (1) condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA by enzyme of the 3-ketothiolase; (2) reduction of acetoacetyl-CoA to D(-)-3-hydroxybutyryl-CoA catalyzed by enzyme of NADPH-dependent acetoacetyl-CoA reductase; and (3) polymerization of the 3-hydroxybutyrate to yield PHB by enzyme of PHA synthase. Of course the biosynthesis

Table 1 Poly(hydroxybutyrate) producers of methylotrophs from CO_2, methane and methanol

Microorganisms	Carbon source	PHB			References
		Concentration, M_w, and yield	Productivity	Content in the cell (%)	
Cupriavidus necator H16	CO_2	16.0 g/L	0.23 $gL^{-1}h^{-1}$	88.9	Sonnleitner et al. (1979)
C. necator ATCC 17697^T	CO_2	36 g/L	0.6 $gL^{-1}h^{-1}$		Ishizaki and Tanaka (1990, 1991)
C. necator	CO_2	61.9 g/L		67.8	Tanaka et al. (1993, 1995)
Synechococcus sp.	Nitrogen-free inorganic medium +2 % CO_2	–		20–27	Miyake (1996)
Synechococcus sp. MA19[a]	CO_2	27.5		30	Miyake (2000)
C. necator B5786	Gas mixtures containing CO	>90 % substrate utilization		70–75 PHA	Volova et al. (2002, 2004)
Nostoc muscorum[b]	2 % CO_2+0.1 % glucose+acetate‡	662 mg/L		38.9	Sharma and Mallick (2008)
Ideonella spp. strain O-1	CO_2 in presence of CO	5.26 g/L		77.9	Tanaka et al. (2011)
Methylosinus trichosporium	Methane			20–25	Scott et al. (1981)
Methylosinus	Methane	–		25	Dalton (1981)
Methylocystisparvus	Methane		1.17 $mgL^{-1}h^{-1}$	68	Asenjo and Suk (1986)
Methylocystis GB25	Methane	M_w up to 2.5×10^6Da		55	Wendlandt et al. (2001)
M. trichosporium OB3b	Methane	–		0	Doronina et al. (2008)
Pseudomonas methanica	Methanol	92.1 % of lipid phase		29	Kallio and Harrington (1960)
Mycoplana rubra	Methanol	–		80	Lafferty (1979)
Methylobacterium organophilum	Methanol	7.5 g/L		30–70	Powell et al. (1980)
Pseudomonas K	Methanol	–		66	Suzuki et al. (1986a)
Pseudomonas rhodus	Methanol	–		79	Govorukhina and Trotsenko (1991)
Protaminobacter rubrum	Methanol	–		80	
Pseudomonas 135	Methanol		0.925 $gg^{-1-1}h$ in 40	55	Daniel et al. (1992)
Methylobacterium rhodesianum	Methanol	–		50–45	Babel (1992)
Methylobacterium extorquens K	Methanol+*n*-amyl alcohol	–		44 (PHB/V)	Ueda et al. (1992)
Paracoccus denitrificans	Methanol+*n*-amyl alcohol	0.968 g/g methanol		57 (PHB/V)	
M. extorquens	Methanol	Highest M_w values at $0.6–1.7\times10^6$		–	Taidi et al. (1994)
M. extorquens	Methanol	–		33–30	Bourque et al. (1995)
M. organophilum	Methanol	0.19 g/g methanol	1.8–2.0 $gL^{-1}h^{-1}$	52	Kim et al. (1996)
Methylobacterium sp V49	Methanol	–		–	Ghatnekar et al. (2002)
Methylobacterium sp. GW2	Methanol	M_w=2.3 MDa		40	Yezza et al. (2006)
Methanotrophs	Methanol Methane	0.6 g/L and M_w>1.5 MDa		40–12	Zhang et al. (2008)
M. extorquens DSMZ1340	Methanol	9.5 g/L; 15.4 g/L dry cell		65	Mokhtari et al. (2009a)
M. extorquens DSMZ1340	Methanol		0.98 $gL^{-1}h$	35	Mokhtari et al. (2009b)

[a] Under nitrogen-deprived conditions

[b] A N_2-fixing cyanobacterium in 5 L photobioreactor from the extracts of pressed mud of brewery and PO_4^{3-} *S*-supplemented fishpond discharges

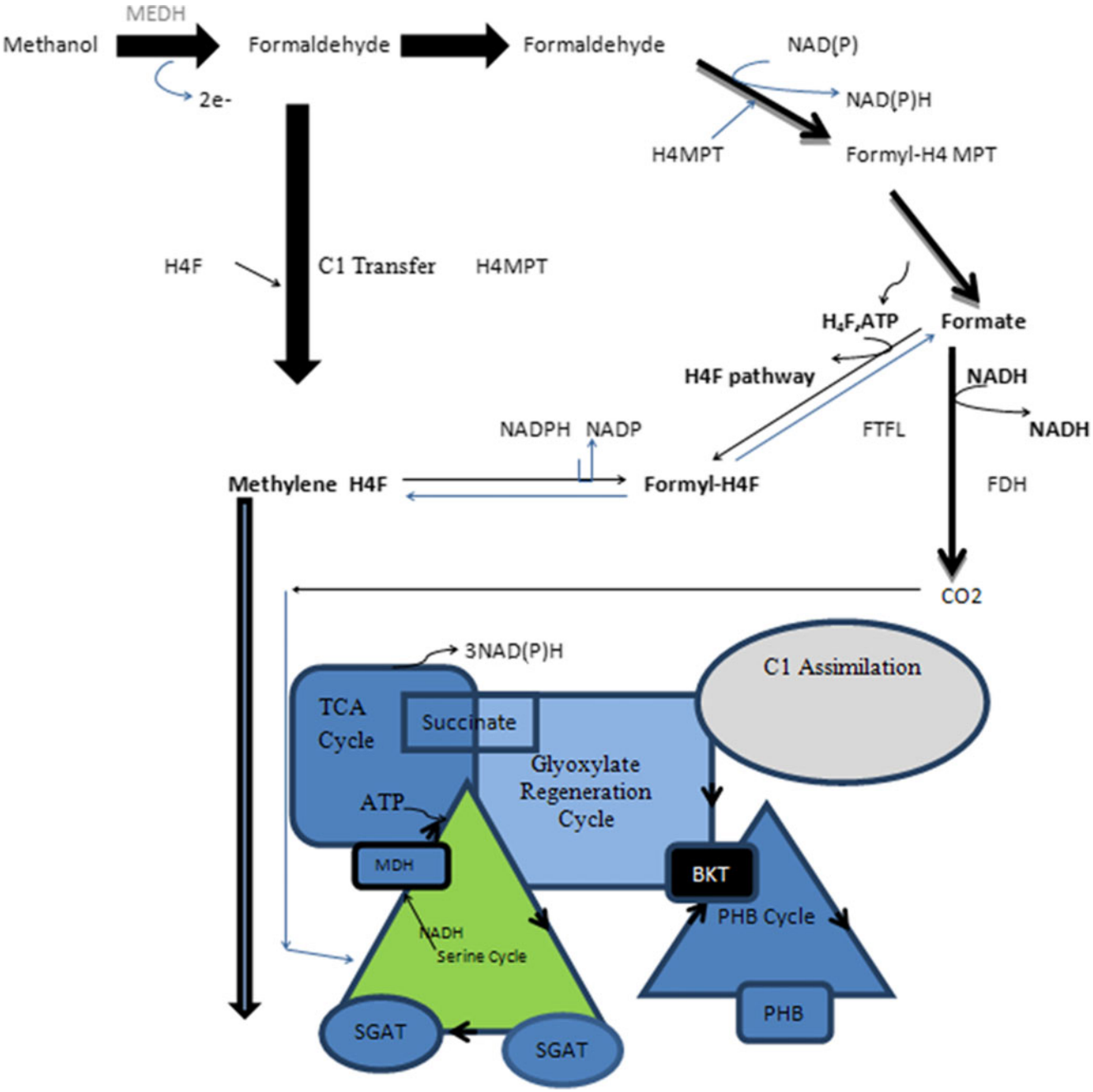

Fig. 1 Metabolism of methanol in *M. extorquens* AM1 (Van Dien and Lidstrom 2002)

pathway for different genera and species of cyanobacteria may differ and further modifications on the pathway will be needed. For PHB production in *Synechococcus* sp. *MA19*, a hypothesized regulation has been reported (Asada et al. 1999). In case of hydrogen-oxidizing bacteria, CO_2 fixation mechanism is mainly Calvin–Benson cycle or reductive TCA cycle (Ishii et al. 1997). In the former case, glyceraldehyde-3-phospahte in Calvin–Benson cycle and EMP is converted to pyruvate then it is changed to acetyl-CoA.

PHB synthesis genes are not reported in type I methanotrophs or in *Methylacidiphilum* strains (Dunfield et al. 2007; Pol et al. 2007). PHB production has been investigated in obligate and facultative methanol-utilizing methylotrophs. Babel hypothesized that under unbalanced growth conditions, RuMP and serine pathways lead to production of PHB and exopolysaccharides, respectively (Babel 1992). In *M. extorquens* AM1 (Korotkova and Lidstrom 2001), linking of the PHB cycle to the serine cycle is reported. In another survey, in several RuMP pathway, methanol-

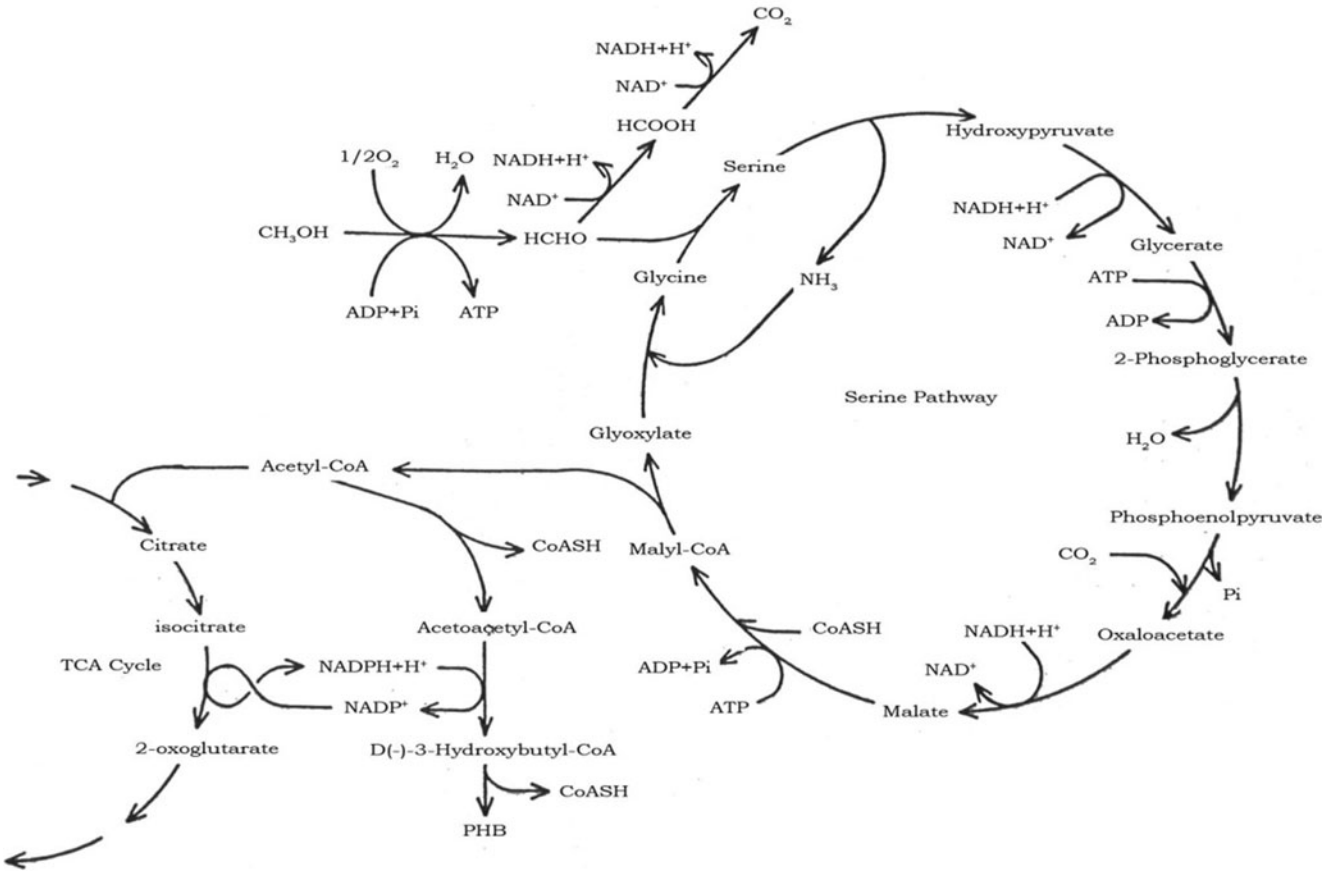

Fig. 2 Serine pathway for production of PHB from methane and methanol (Yamane 1993)

utilizing methylotrophs (containing PHA-biosynthesis genes) (Follner et al. 1993), no PHB production was reported. While, transconjugants of these bacteria could synthesize PHB, suggesting that metabolic limitations do not prevent RuMP pathway. The level of copper in the growth media is one factor that may favor type II over type I methanotrophs due to their enzymatic requirements (Graham et al. 1993; Zahn and DiSpirito 1996; Nguyen et al. 1998; Dunfield et al. 2003; Lidstrom 2006). All type II and some type I genera are able to fix nitrogen; Oakley and Murrell 1988; Hanson and Hanson 1996; Auman et al. 2001; Bowman 2006), but type II methanotrophs may be more useful due to their faster growth on gaseous nitrogen (Murrell and Dalton 1983).

Graham et al. (1993) reported that the provision of N_2 (gaseous) as the sole source of nitrogen favored the type II methanotroph, *M. trichosporium* OB3b, over *Muscodor albus* BG8. They showed that under copper- and nitrate-limited conditions, *M. trichosporium* OB3b was dominant. *M. trichosporium* synthesized soluble methane monooxygenase and nitrogenase under copper and nitrate limitation, respectively. *M. albus* predominated under methane limitation, especially during low-level feeding of methanol. The results imply that nitrogen limitation can be used to select for type II strains, such as *M. trichosporium* OB3b.

Nutrient concentration (Wise et al. 1999; Cebron et al. 2007) and medium pH affect type I methanotrophs, but type II are acidophilic and acid tolerant (Dedysh 2002). Low pH and high concentration of organic carbon source lead to high concentrations of dissolved CO_2, which is a requirement for serine pathway and so favor for type II methanotrophs.

Pieja et al. (2011a) have investigated encoding for PHB synthase (phaC) as well as PHB production under nitrogen-limited conditions in types I and II proteobacterial methanotrophs. All type I and II strains were found as disable and able for both responses. Preferentially, medium conditions for PHB production in type II methanotrophs has been defined as low pH (4–5) medium or culture containing N_2 (gaseous) or dilutes mineral salts in the absence of Cu.

Pieja et al (2011b) have also shown that methanotrophic PHB metabolism is linked to the supply of reducing power as opposed to the supply of C_2 units for synthesis. They showed that type II methanotroph *Methylocystis parvus* OBBP did not replicate using stored PHB in the absence of methane, even in excess amount of other medium components. PHB was a source of reducing equivalents and not a sole growth substrate.

In another study, Pieja et al. (2012) also investigated on favor strategies for PHB accumulation in type II methanotrophic proteobacteria. So, three sequencing batch reactors

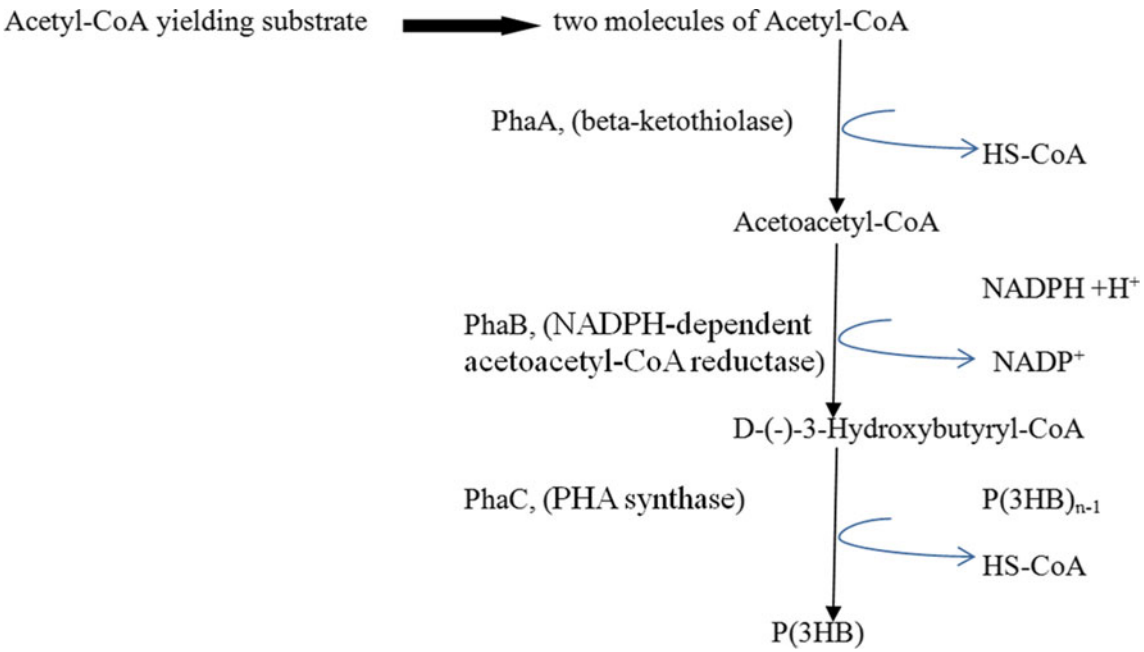

Fig. 3 Metabolic pathway of PHB from acetyl-CoA-yielding substrate (Vincenzini et al. 1999)

inoculated by type II methanotrophs were subjected to (1) repeated nitrogen limitation, (2) repeated nitrogen and oxygen limitation, and (3) repeated nitrogen and methane limitation. PHB levels were measured over 11 cycles of 24 h. The results showed that repeated nitrogen and methane limitations favored PHB accumulation in strain OBBP and provided it with a competitive advantage under the conditions imposed.

Pfluger et al. (2011) have recently reported the feasibility of growing methanotrophic biomass in a fed batch reactor at ambient temperature and non-sterile conditions. Their results show that by increased concentration of nitrate and dissolved O_2led to a type I methanotroph-dominated biofilm. While decreased O_2 concentrations of 2 mg/L lead to increased growth of type II methanotrophic biofilms of PHB producer.

Stoichiometry

Biochemical pathways in type II methanotrophs have been analyzed firstly by Asenjo and Suk (1986) in order to establish the preliminary kinetic analysis, stoichiometry of PHB synthesis and determination of condition necessary for PHB accumulation from methane. The overall equation of methane to cell mass ($C_4H_8O_2N$) for methanotrophs that use the serine pathway is found:

$$29.5CH_4 + 44.25O_2 + 3NH_3 + FP \rightarrow 3C_4H_8O_2N + 17.5CO_2 + 18ATP + 4FPH_2 \quad (1)$$

where FPH_2 is reduced form of flavoproteins (FP) succinate dehydrogenase. Also, the overall equation obtained for cell mass biosynthesis from nitrate is:

$$41.5CH_4 + 62.25O_2 + 3HNO_3 + FP \rightarrow 3C_4H_8O_2N + 39.5CO_2 + 42ATP + 4FPH_2 \quad (2)$$

The net equation for PHB synthesis is:

$$2CH_4 + 3O_2 + NADH_2 + 2CO_2 + FP \rightarrow C_4H_6O_2(\text{PHB monomer}) + FPH_2 \quad (3)$$

The overall equation for PHB accumulation for methanotrophs that use the serine cycle is found:

$$8CH_4 + 12O_2 + FP \rightarrow C_4H_6O_2(\text{PHB monomer}) + 4CO_2 + 12ATP + FPH_2 \quad (4)$$

When the cells are synthesized using ammonia as a nitrogen source, 0.61 mol ATP/mol CH_4 would be stored. Using nitrate, the value is 1.01 mol ATP/mol CH_4 and for the synthesis of PHB from methane, 1.05 mol ATP/mol CH_4 would be stored. In all the cases, the ratio of O_2/CH_4 metabolized is constant and equal to 1.5, whereas CO_2 evaluation varies. Ueda et al (1992) suggested Eq. 5 for conversion of CH_3OH to PHB in methylotrophic bacteria that accumulate PHB belong to the group having the serine pathway as methanol assimilatory pathway:

$$5CH_3OH + 3O_2 + (5q-3)ADP + (5q-3)pi \rightarrow (1/n)PHB + CO_2 + 4H_2O + (5q-3)ATP \quad (5)$$

which q is moles of ATP and is assumed to be produced through the conversion of CH_3OH to HCHO. However, when

methane is used, the Eq. 6 is added to Eq. 5, and the result was different from that reported by Asenjo and Suk (1986). In fact production of methanol from methane will be primarily added to biosynthesis pathway of conversion of methanol to PHB.

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + NAD^+ \quad (6)$$

Yield

One of the most important factors of PHB production is yield of substrate. Theoretically, yield can be predicted by a stoichiometric reaction scheme (Ueda et al. 1992; Anthony 1982). By using Eq. 4, theoretical yield for bioconversion of methane to PHB can be estimated to be 67 % (86/128 g).

In type II methanotrophic bacteria, however, a large fraction of the methane and oxygen consumed has to be converted to CO_2 in order to generate the $NADP^+$ of acetoacetyl-CoA reductase in the PHB biosynthetic pathway. By using Eqs. 5 and 6, it can be obtained that the theoretical yield for bioconversion of methane to PHB is 54 % without neglect of the regeneration of $NADP^+$ of acetoacetyl-CoA reductase in the PHB biosynthetic pathway.

Empirical PHB yield was calculated on the basis of the PHB content of biomass and methane consumption by Wendlandt et al. (2001). Yield and productivity of PHB produced from methane by *Methylocystis* spp. GB250 SM (type II methanotrophs) were amounted to 0.54 g PHB g^{-1} CH_4 and 2.85 g PHB $L^{-1}h^{-1}$, respectively. This result is very close to theoretical yields reported by Asenjo and Suk (1986) and Ueda et al (1992).

Culture conditions

Many researchers performed cultivation of methanotrophs in a nonsterile manner. A mineral salt medium have been used in a series of researches. Also, controlling of gas composition of head space in culture vessel is also important. In fact, in controlled temperature, pH, and methane concentration, the process of PHB production can be conducted non-sterile due to kind of medium and establishment of a stable microbial community where the dominant species is *Methylocystis* spp. GB 25. Two-stage pilot-scale of PHB production from methane may lead to a maximum polymer content of 52 % of dried cell weight (Wendlant et al. 2010).

Asenjo and Suk (1986) conducted growth of cells in closed shake flask hermitically sealed with a rubber stopper. Two glass tubes were used to flow gas mixture of CH_4/O_2 50 % (*v*/*v*). Cultures were incubated in a shaker with 150–200 rpm at 30 °C. The culture maintained on plates using the same medium including 15 g/L agar and a sealed methane/oxygen chamber 50 % (*v*/*v*).

Wendlandt et al. (2001) investigated for increasing productivity and yield of PHB by a methanotrophic strain *Methylocystis* spp. GB 25 DSMZ 7674 in a 7- and 70-L pressure bioreactor. Cultivation was performed in two stages: a continuous growth phase at dilution rate 0.17 h^{-1} and a PHB accumulation phase under deficiency conditions of an essential nutrient, e.g., ammonium, P, or Mg in batch culture. In their research, the PHB content of biomass reached to 51 %; the maximum PHB yield (relative to consumption of methane) was 0.55 $g g^{-1}$ with high molecular mass of 2.5×10^6 Da.

Zuniga et al. (2011) applied a two-phase partitioning bioreactor in limited nitrogen concentration to promote PHB production. Under this condition, the accumulated PHB in the reactor was 38 % (*w*/*w*) for isolated bacterium *Methylobacterium organophilum* and the highest productivity of 1.61 mg PHB $g^{-1}h^{-1}$ was obtained with PHB content of 57 % (*w*/*w*).

Methanol

Methanol as one of the building blocks in the chemical industry can be used in bioprocess technology with methylotrophic bacteria for production of value added products. Schrader et al. (2009) have recently reviewed the potential of methylotrophs for the development of economically competitive biotechnological processes based on methanol as an alternative carbon source. Stoichiometric equation for biomass formation on methanol through the RuMP pathway is as Eq. 7, in which, carbon balance shows that 62 % of methanol is used for biomass formation:

$$1.63CH_3OH + 1.39O_2 + 0.23NH_3 + \text{nutrients} \rightarrow CH_{1.69}O_{0.38}N_{0.24}(\text{biomass}) + 0.63CO_2 + 2.76H_2O + 733KJ \quad (7)$$

Methanol also can be used as an alternative substrate for PHB production due to several advantages of low price, moderate requirement of oxygen, complete water miscibility, etc. (Byrom 1987). The maximum theoretical yield of PHB production from methanol is equal to 0.18 g/g in comparison to 0.33 g/g for glucose. In production of "Biopol" (PHA commercially produced by Monsanto Company), methanol was applied as carbon source. But low molecular weight polymer showed difficult extraction. More research showed that methylotrophs can produce high molecular weight PHB (Bourque et al. 1992, 1995) and even its copolymer, poly (3-hydroxybutyrate-3hydroxyvalerate), by addition of n-amyl alcohol or valeric acid while

propionic acid was less effective (Ueda et al. 1992). However, 136 g/L PHB with 66 % of dry weight of the cells was achieved by Suzuki et al. (1986a) using computer-controlled fed-batch fermentation, which is the highest reported yield for any methylotroph. It can be suggested that genetic manipulation of flux control mechanisms may be necessary to match the yields currently achieved with *C. necator*. *Methylobacterium* spp. was the most famous strain for PHB production via the serine pathway (Fidler and Dennis 1992). This bacterium has a complete TCA cycle, unlike restricted facultative methylotrophs which apply the RuMP pathway. Growth-associated production of PHB in *Methylobacterium rhodesianum* (Ackermann and Babel 1997) and possess of two acetoacetyl-CoA reductases in *M. rhodesianum* MB 126 (Babel and Mothes 1994) has been reported. Suzuki et al. (1986a) examined eleven components and found NH_4^+, SO_4^{2-}, Mg^{2+}, Fe^{2+}, and Mn^{2+} ions are crucial deficient ions for PHB accumulation by *Pseudomonas* spp. K. For *Methylobacterium organophilium*, NH_4^+, Mg^{2+}, PO_4^{3-}, and K^+ were found to be crucial deficient ions for the stimulation of PHB accumulation (Choi et al. 1989). Daniel et al. (1992) has examined nutrient-deficient media on PHB accumulation by *Pseudomonas* 135. Among the several nutrient tested, it was found that PHB accumulation was most significantly stimulated by NH_4^+, Mg^{2+}, or PO_4^{3-} deficient medium.

Several studies have been also carried out with various microorganisms and fermentation processes for production of PHB from methanol (Suzuki et al. 1986b, c, 1988, 2000; Byrom 1987; Choi et al. 1989; Haywood et al. 1989; Govorukhina and Trotsenko 1991; Hilger et al. 1991; Anderson et al. 1992; Daniel et al. 1992; Mothes et al. 1997, 1998; Zhao et al. 1993; Taidi et al. 1994; Yezza et al. 2006). Although in some of these studies, media optimization for PHB production from methanol by different methylotrophs were considered but in none of them the statistical design of experiment was applied for screening of all of media components. There are scattered and different concentrations for media components which have been reported in literature and the importance of minerals for maximal biomass and/or PHB production from methanol has been recognized by several investigators (Suzuki et al. 1986a; Choi et al. 1989; Hilger et al. 1991; Daniel et al. 1992; Bourque et al. 1992, 1995). The presence of trace elements is very necessary to achieve high concentration of cell mass (Suzuki et al. 2000). In addition, some of them may have accelerative effect on PHB accumulation. It is desirable to maintain the concentration of every elements at a suitable level at which the cell growth is not limited.

Suzuki et al. (1986a, b, c) studied the effect of type and concentration of nitrogen and phosphorus sources as well as seven trace elements on growth and PHB production of *Protomonas extroquence* spp. K. High concentration of PHB was attained in fed batch culture. Produced polymer showed a high and broad distribution of molecular weight. In another study, Suzuki et al. (1988) tested influence of process variables including temperature, pH, molar ratio of methanol to ammonia, and concentration of methanol on PHB production. They showed impact of methanol concentration on molecular weight of PHB. Daniel et al. (1992) obtained an optimal medium for growth of *Pseudomonas* 135 and reported similar results for nitrogen sources to Suzuki's report (1986a). In their optimal medium, phosphate salts are more influential than phosphoric acid on cell growth. The optimum concentration of $MgSO_4$ was found to be 0.45 g/L. However, increasing of trace elements concentration two fold did not show any significant improvement in cell growth. Bourque et al. (1995) also reported an optimum medium for growth of *Methylobacterium extorquens* ATCC 55366. They made several modification to medium 784 (ATCC 1985) reported by Choi et al. (1989). This medium is richer than medium 784 considering several composing salts but contains no NH_4Cl, K_2HPO_4, $MnCl_2$, $CuCl_2$, or $NiCl_2$. This medium contains less $CaCl_2$ than medium784 in order to prevent significant precipitation problems. They also reported that concentration of trace elements has effect on cell growth in spite of Suzuki et al. (1986a) and Daniel's (1992) opinion.

Mokhtari et al. (2009a) applied Plackett–Burmann design as a screening experimental plan for the first time to assess the relative importance of medium components including carbon and nitrogen sources, phosphate and minerals for maximal biomass and biopolymer production of *M. extorquens* DSMZ 1340 by methanol. Optimization of growth media by CCD resulted in the increased growth with an OD=2.15 compared with an OD=1.35 for Choi's media as control. PHB production media was also optimized by RSM. It was found that a deficiency of $MgSO_4$ and $(NH_4)_2SO_4$ is crucial for PHB accumulation. Fed-batch cultivation at optimum condition resulted in PHB production as high as 62.3 % of DCW, which is higher than those reported in the literature for PHB production from methanol by *Methylobacterium* species. Also, fermentation was conducted with feed composition (639 g/L methanol, 4 g/L $MgSO_4{\cdot}7H_2O$, 41 mL/L trace elements, 5.6 g/L $NaH_2PO_4{\cdot}H_2O$,and 24.3 g/L K_2HPO_4) in limited dissolved oxygen (Mokhtari et al. 2009b). After 35 h, PHB production phase was start and biomass and PHB productivities of 2.8 and 0.98 $gL^{-1}h^{-1}$ were obtained, respectively.

PHB accumulation and growth of *M. trichosporium* IMV3011 on methanol has been investigated (Song et al. 2011; Xin et al. 2011). The carbon source concentration in fermenter is maintained at the lower level by a reasonable feeding strategy in order to reduce the inhibiting effect of substrate in the production of PHB as well as other types of products. The initial concentration of methanol was controlled at 1 g/L. Also, it was added at 0.1 % (*v*/*v*) of the total volume of medium in 5 times separately in the whole fermentation process, which would shorten the lag phase of

growth. The concentration of cells dry weight reached at 2.91 g/L and the PHB concentration was 47.6 % in the fed-batch fermentation of IMV3011. By addition of 0.2 g/L malic acid as optimal organic acid for stimulation of intracellular PHB synthesis, the 3.32 g/L cells dry weight and 58.5 % PHB was obtained.

It had been shown that acetyl-CoA, citric acid, and malic acid caused an increase of PHB production as a key intermediate in the metabolism. The inhibition of TCA cycle increased PHB production of methanotrophic bacteria; 0.2 g/L malic acid was an optimal organic acid addition for intracellular PHB synthesis. The 3.32-g/L cells dry weight and 58.5 % PHB could be obtained under the condition. The corresponding change of M_w would appear in various incubation stages of IMV3011 (Xin et al. 2011)

Cosubstrate of methane and methanol

Methanol can maintain methanotrophic activity in some conditions. Zhang et al. (2008) reported PHB production by methanotrophic strain *M. trichosporium* IMV3011 from methane and methanol in a brief non-sterile process. In this research, methanol at 0.1 % (*v*/*v*) was added to improve the oxidization of methane. The experiments were conducted in two stages cultivation for continuous growth phase and PHB accumulation (with limited concentration of NH_4^+, NO_3^-, P, Cu^{+2}, Fe^{3+}, Mg^{2+}, or ethylenediamine tetraacetate in batch culture. In the most suitable condition, PHB concentration reached to 0.6 g/L. Addition of citric acid as an inhibitor of tricarboxylic acid cycle and simulator of PHB production was also investigated. This strategy caused an increased PHB yield from 12 to 40 % (*w*/*w*). The produced PHB had molecular weight up to 1.5×10^6 Da.

Conclusions

Due to high consumption of plastics and landscape problem in the world, government and industries of developed countries have intensified their efforts in the production and application of PHAs as biodegradable polymers roughly estimated at 50,000 tons/year. The intensive research are going to reduce costs and improve thermo-mechanical, physical and processing properties of these green biopolymers to allow them to become a leading biodegradable polymer in the near future.

Table 2 Economic evaluation for 100,000 ton/year PHB Production by various bacteria and solvent extraction (modified from the report of Choi and Lee (1999a)

Parameters	*Escherichia coli*[a]	*E. coli*[a]	*Methylobacterium organophilium*[a]	*Methylobacterium extroquens*[b]	*M. extroquens*[c]
Characteristics of bioprocess					
Carbon source	Glucose	Glucose	Methanol	Methanol	Methanol
Cost of carbon source (US$/kg)	0.493	0.493	0.18	0.22	0.04
Time of process (h)	41	49	70	48	48
Biomass concentration (g/L)	112	204	250	135	135
PHB concentration (g/L)	81	157	130	70	70
PHB content (%)	72	77	52	38	38
PHB productivity ($gL^{-1}h^{-1}$)	1.98	3.2	1.86	0.98	0.98
Yield of PHB (g/g)	0.29	0.27	0.19	0.15	0.15
Economic evaluation					
Fixed and working capital Investment	1.31	1.00	1.57	2.42	2.42
Labor related cost	0.21	0.16	0.23	0.35	0.25
Upstream and operational costs	0.09	0.08	0.11	0.14	0.14
Cost of carbon source	1.73	1.73	0.94	1.47	0.27
Cost of other raw materials (solvents and mineral salts)	1.26	1.24	2.36	2.81	2.81
Utilities	0.42	0.36	0.46	0.63	0.13
Cost of produced waste	0.35	0.34	1.01	1.22	1.22
Total production cost (US$/kg PHB)	5.37	4.91	6.69	9.04	7.24

[a] Based on Choi and Lee's (1999a, b) cost-estimation calculation

[b] Calculation are based on global price of methanol and energy

[c] Calculation are based on price of methanol and energy in oil field country, e.g., Iran

Of course, at present, PHB has not been commercially produced from gas and the price will differ due to the resource from which is derived. Choi and Lee (1999a) studied on economical consideration of PHB production by various bacteria on some substrates. Table 2 shows the results of their economical evaluation for production of 100,000 ton PHB/year from methane and methanol in compare to glucose. Among the parameters influencing cost of PHB production, constant and current investments, upstream, and labor costs significantly increase with decreased productivity. Comparison of two processes with different productivities for PHB production by recombinant *Escherichia coli* shows the significant impact of productivity on final cost (Table 2). By increasing of productivity from 1.98 to 3.20 $gL^{-1}h^{-1}$, production cost decreases from 5.37 to 4.91 US$/kg PHB. Low cost of methanol and energy in Iran (0.045 US$/kg), as well as high solubility of this gas and possibility of reaching high cell density may lead to decreased product cost. Table 2 shows cost estimation for PHB production from methanol by *M. extroquens* DSMZ 1340 which have been calculated base on global and national (Iran) cost of methanol and energy according to Choi and Lee (1999a). Applying a low-cost efficient process of separation will lead to a more decreased total cost of methylotrophic production of PHB.

Methane could be utilized as the carbon source of mixed culture to grow and produce PHB in a brief non-sterile process. Concentration of nutrient in the medium has great effect on biomass growth and synthesis of PHB. Mixed culture usage for the biotechnological production of PHB is an important step towards the development of a viable large-scale process for the production of PHB using cheap substrates like methane. Some inherent physicochemical properties such as brittleness and hydrophobicity can be improved by various methods, especially blending with another kind of polymer. An important prerequisite for bulk chemical production with methylotrophs will be a robust over expression of anaplerotic key enzymes together with a stable deactivation of oxidative pathways.

Also pulsing of nitrogen into a sequencing batch reactor with a sustained supply of methane has been known as a successful plausible strategy for stimulate the production and utilization of PHB. Further study is needed to examine the effects of repeated nitrogen limitation on methanotrophic cultures and the potential of such strategies to enhance PHB production.

Research on the genetics of biosynthesis of MCL-PHA has led to polymer synthesis in recombinant organisms. Several medium and high cost applications are emerging in new decade. A combination of more efficient metabolically engineered strains capable of utilizing cheap carbon sources and efficient fermentation strategies is certainly a future trend for increase deficiency of PHA production.

Cultivation of hydrogen-oxidizing bacteria from H_2 (with sources of wind, natural gas, nuclear, and biomass) with average price of $2.6/gallons of gasoline equivalent, CO_2 (liquid), and CH_4 (22.0 and $2.169/$m^3$) have also potential for economic production of PHB. Anyway if mentioned gas can be applied in situ as industrial output of a plant, the prices are very low beside great advance of solving greenhouse problem. Anyway, there are no bacteria that can produce copolymers from C1 compounds and only a few strains can produce them even from sugars as sole carbon source. We are studying for construction of recombinant of hydrogen-oxygen bacteria to produce MCL-PHAs from CO_2.

Screening research also will aid to improve and select better candidates for increased biopolymer production. New isolated bacteria should be O_2 and CO tolerant to be useful in the manufacture of MCL-PHA from industrial exhaust gas containing CO_2, H_2, and CO. Technology of autotrophic culture of hydrogen-oxidizing bacteria for production of PHB from CO_2 has been progressed in the recent 20 years as described above however research and innovation is still necessary for practical application of autotrophic culture of the bacteria for the production of MCL-PHA in industrial scale. Structural studies are needed to improve understanding about the mechanism of action of involved enzymes in methanotrophs and hydrogen-oxidizing bacteria. Metabolic engineering thereafter promises to bring a feasible solution for the production of green plastic. Although some C_1 substrates are used for PHB production with several kinds of microorganisms but comparison of yield and structure of produced PHB is yet unknown.

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