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REVIEW

Fixation of carbon dioxide by a hydrogen-oxidizing bacterium for value-added products

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Abstract

With rapid technology progress and cost reduction, clean hydrogen from water electrolysis driven by renewable powers becomes a potential feedstock for CO_2 fixation by hydrogen-oxidizing bacteria. *Cupriavidus necator* (formally *Ralstonia eutropha*), a representative member of the lithoautotrophic prokaryotes, is a promising producer of polyhydroxyalkanoates and single cell proteins. This paper reviews the fundamental properties of the hydrogen-oxidizing bacterium, the metabolic activities under limitation of individual gases and nutrients, and the value-added products from CO_2, including the products with large potential markets. Gas fermentation and bioreactor safety are discussed for achieving high cell density and high productivity of desired products under chemolithotrophic conditions. The review also updates the recent research activities in metabolic engineering of *C. necator* to produce novel metabolites from CO_2.

Keywords Carbon dioxide fixation · Bioproducts · Cell factory · Gas fermentation · *Cupriavidus necator* · Hydrogen-oxidizing bacteria

Introduction

Carbon dioxide (CO_2) from fossil fuel combustion has caused concerns over climate change and ocean acidification. Production of value-added products from CO_2, especially those with large potential markets, could help reducing the greenhouse gas emission (Hunt et al. 2010). Hydrogen-oxidizing bacteria are primary biomass producers and play a role in the natural carbon cycle (Huber and Eder 2006). The chemolithotrophic prokaryotes use hydrogen (H_2) as energy source to fix CO_2 and produce value-added products including bioplastics, hydrogenases, animal feed additives, functional chemicals, liquid fuels, and so on. Clean hydrogen, in contrast to the hydrogen derived from fossil resources, is generated from water electrolysis driven by renewable powers (Ziogou et al. 2012). With rapid technology progress and cost reduction, clean hydrogen could be an inexpensive feedstock of a novel biorefinery that produces value-added products from CO_2 (Nowotny et al. 2014).

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Hydrogen-oxidizing bacteria (Knallgas bacteria) is a physiologically defined but phylogenetically diverse group of bacteria that can utilize H_2 as electron donor and O_2 as electron accepter for CO_2 fixation (Aragno and Schlegel 1981). They are found in diverse habitats including marine sediments, hot springs and soils (Bae et al. 2001; Spear et al. 2005; Florentino et al. 2012). Synergistic relationship based on hydrogen exchange has been suggested to explain the presence of hydrogen-oxidizing bacteria in the environments (Florentino et al. 2012). Most of H_2 produced in the anoxic habitats is immediately consumed and only little H_2 gas enters the oxic environment where hydrogen-oxidizing bacteria thrive (Conrad 1996). The chemoautotrophs have therefore developed an ability to use the very little amount of hydrogen and become facultative to use organic carbon as the carbon and energy source.

Cupriavidus necator

Cupriavidus necator is a Gram-negative facultative hydrogen-oxidizing bacterium that populates soil and fresh water habitats at the aerobic-anaerobic interface and belongs to the class Betaproteobacteria, order Burkholderiales. The

taxonomic position and the name of this taxon have been changed several times

Hydrogenomonas eutropha, Alcaligenes eutrophus, Ralstonia eutropha and *Wautersia eutropha* (Vandamme and Coeyne 2004). *Ralstonia eutropha* and *C. necator* are now used interchangeably. A type strain (H16) of *C. necator* was isolated from soil almost 60 years ago (Schlegel et al. 1961). Since then, it has become the most-studied hydrogen-oxidizing bacterium with the best characterized genome (Pohlmann et al. 2006). Proteomics and metabolomics analysis have been conducted under heterotrophic conditions (Lee et al. 2006; Schwartz et al. 2009; Peplinski et al. 2010), while less information is available under autotrophic conditions (Alagesan et al. 2018).

Cupriavidus necator H16 fixes CO_2 via the Calvin–Benson–Bassham (CBB) cycle under aerobic conditions with hydrogen as the sole source of energy and reducing equivalents (Badger and Bek 2008). Genes for the CBB cycle are found in two operons located in chromosome 2 and megaplasmid (Bowien and Kusian 2002). The transcription of both cbb operons is controlled by a transcription regulator CbbR in response to the intracellular level of phosphoenolpyruvate as the carbon-state of the cell (Grzeszik et al. 2000). Under mixotrophic conditions, the carbon flux through the CBB cycle is affected by the organic substrates, ranging from complete repression in the presence of pyruvate and malate to a significant carbon flux in the presence of glycerol (Alagesan et al. 2018). A recent study reveals that the transcription control of both cbb operons appears to be more complex and, in addition to CbbR, involves transcription of regulator RegA as part of the global transcription regulation system (RegA/RegB) (Gruber et al. 2017).

Cupriavidus necator is one of the most prominent producer of poly-3-hydroxybutyrate (P3HB) from CO_2 (Tanaka et al. 1995; Volova et al. 2013a). P3HB is a member of polyhydroxyalkanoates (PHAs) that are formed as inclusion bodies for carbon and energy storage as shown in Fig. 1. Under nutrient limitation such as nitrogen, *C. necator* has a strong ability to direct its carbon flow towards P3HB synthesis (Fig. 2). The biopolyester content can be up to 85% of the dry cell mass (DCM) under nutrient control (Volova et al. 2013a). The phbCAB operon encodes three key enzymes in P3HB synthesis: (i) β-ketothiolase (phaA) that condenses two molecules of acetyl-CoA to acetoacetyl-CoA, (ii) NADPH-dependent acetoacetyl-CoA (phaB) that reduces acetoacetyl-CoA to R(-)-3-hydroxyburyryl-CoA, and (iii) PHB synthase (phaC1) that catalyzes the polymerization of R(-)-3-hydroxybutyryl-CoA (Pohlmann et al. 2006). Induced by sufficient nutrients and limited carbon source, P3HB is hydrolyzed to 3-hydroxybutyrate by depolymerases and the stored carbon is returned to the main metabolism pathway as shown in Fig. 2 (Volova et al. 2013b).

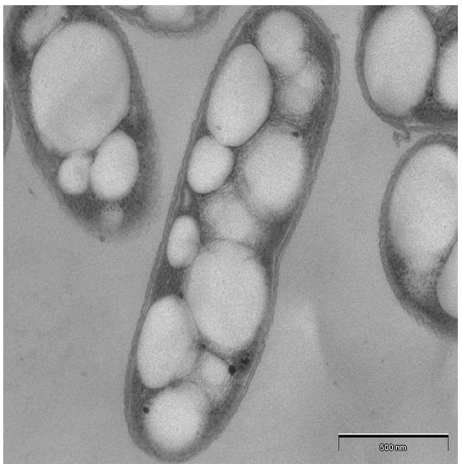

Fig. 1 Electron microscopic image of P3HB granules in *C. necator* (bar is 0.5 µm)

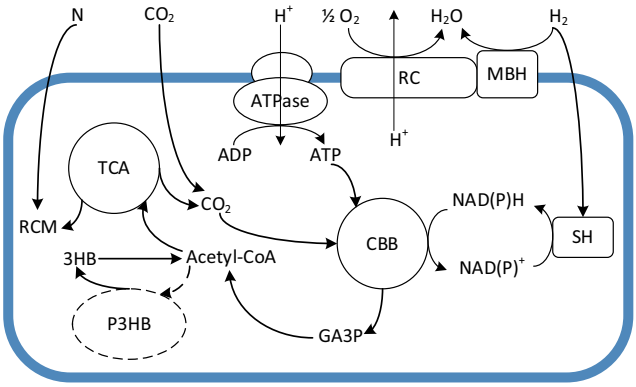

Fig. 2 Carbon flow under chemolithotrophic conditions: biosynthesis of residual cell mass (RCM) with sufficient nutrients (solid line) and formation of P3HB under nutrient limitation (dotted line). P3HB hydrolysis generates 3HB and acetyl-CoA

Value-added products

Polyhydroxyalkanoates

P3HB is a homopolyester of R-(-)-3-hydroxybutyrate and a truly biodegradable polymer that can be completely utilized by microbial species in the environments. The weight-average molecular weight (Mw) of P3HB formed by *C. necator* on CO_2 ranges from 600 to 900 kDa (Volova et al. 2013b). Because of the high stereoregularity in structure, P3HB has a high crystallinity (65–75%), resulting in a rigid plastic (Laycock et al. 2013). Its mechanical properties are similar to those of polypropylene, but with a relatively low ductility. The material ductility can be improved by introducing co-monomers such as

3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 4-hydroxybutyrate (4HB) and 4-hydroxyvalerate (4HV) into the polyester backbones (Volova et al. 2013a; Park et al. 2014; Ghysels et al. 2018). The microbial cells, however, need organic precursors such as valeric acid, hexanoic acid and γ-butyrolactone to form copolymers under the mixotrophic conditions. Because of the excellent biodegradability, PHA bioplastics can find various environmentally friendly applications (Bugnicourt et al. 2014). The biomaterials may also find advanced medical applications, such as in drug delivery systems, wound management, and tissue repair (Luef et al. 2015). In situ and in vivo tests have demonstrated the biocompatibility and low toxicity of P3HB (Valappil et al. 2006).

Chemicals and liquid fuels derived from P3HB

P3HB is a platform material from which small functional chemicals can be derived such as 3-hydroxybutyric acid, crotonic acid, acetoacetic acid and 1,3-butanediol (Yu 2014). 3HB can be produced through catalytic hydrolysis of P3HB (Yu et al. 2005) and is an intermediate of lipid metabolism in human body. It may find potential applications in control of eating behavior and neuronal diseases (Laeger et al. 2010). 3HB is also a chemical chaperone capable of protecting enzymes such as lipase and lysozyme from adverse effects of high temperature and oxidation (Obruca et al. 2016). 3HB provides a better enzyme protection than the classical chaperones such as trehalose and hydroxyectoine. Crotonic acid or 3-methylacrylic acid can be obtained with a high yield (> 99 wt%) via catalytic degradation of P3HB (Ariffin et al. 2010). Crotonic acid is a key intermediate in catalytic refining of P3HB into a hydrocarbon oil (Kang and Yu 2015a). On a solid phosphoric acid catalyst, P3HB (87–98 wt% purity) was converted into hydrocarbons (C6–C16) through decarboxylation and deoxygenation in a one-pot reaction (Kang and Yu 2015b). Drop-in liquid fuels can be conveniently obtained from the high quality oil to replace gasoline and biodiesel. The liquid fuels have a very large market and hence benefit to greenhouse gas mitigation. The hydrocarbon oil contains primarily alkenes and benzenes from which organic solvents can be obtained (Kang and Yu 2015a).

Proteins, lipids and bio-oils derived from residual cell mass

Cupriavidus necator H16 was considered as a source of single cell protein in the 1970s (Calloway and Kumar 1969). Almost 93% of bacterial protein is digestible by animals and the concentrations of certain important amino acids are similar to those in casein. There is a renewed interest in using P3HB as a component of animal feed to increase the energy content (Defoirdt et al. 2009; Boon et al. 2010; Kunasundari et al. 2013). The animal feed containing whole bacterial cells and P3HB granules may modulate the gut flora by delivering 3-hydroxybutyric acids (Boon et al. 2010). The short chain fatty acids may be a type of new biocontrol agents for sustainable animal production (Defoirdt et al. 2009). The excretion of P3HB in the fecal pellets was a concern when the primary objective was to increase the energy content of animal feed. The animal digestion of residual cell mass (RCM) is suggested for recovery of P3HB from the bacterial cell mass (Kunasundari et al. 2013).

Cupriavidus necator H16 contains all genes necessary for the de novo fatty acid synthesis via the β-oxidation pathway (Brigham et al. 2010). Under chemolithotrophic conditions, the amount of total lipids is 7.8% of the RCM, excluding P3HB (Zhila et al. 2015). The fatty acid composition of cytoplasmic membrane changes as the cell growth shifts from an active phase to a stationary phase. The mass ratio of saturated FAs to unsaturated FAs increases from 0.8 to 4.9, resulting in a change of membrane fluidity (Zhila et al. 2015). After P3HB was removed from the DCM of *C. necator* growing on CO_2, the RCM contained carbon (45.1 wt%), hydrogen (6.3 wt%), oxygen (27.8 wt%), nitrogen (12.9 wt%) and ash (8.0 wt%) (Kang and Yu 2015c). The RCM was liquefied in subcritical water (300 °C) into two groups of products: a crude oil that was recovered with methylene dichloride and hydrophilic compounds that were left in the aqueous solution. The bacterial crude oil was similar to the bio-crudes derived from microalgae biomass, and had a higher energy content than the bio-crude derived from cellulosic biomass (Kang and Yu 2015c). The hydrophilic hydrolysates solution contained a large amount of organic nitrogen and was found a good source of nutrients for *C. necator* growth under chemolithotrophic conditions.

Oxygen-resistant hydrogenases

Three physiologically different [NiFe]-hydrogenases have been identified in *C. necator* H16: membrane-bound hydrogenase (MBH), cytoplasmic soluble hydrogenase and cytoplasmic soluble regulatory hydrogenase (Burgdorf et al. 2005). They are good source of oxygen-resistant hydrogenases with potential applications in biofuel cells and biosensors (Mertens and Liese 2004). The soluble hydrogenase favors reversible hydrogen oxidation in vivo and its activity has been demonstrated on a polymyxin-coated electrode surface under a potential of 550 mV (Jugder et al. 2016). The enzymes can directly transfer electrons to or from electrodes to reduce the kinetic hindrance in the conventional mediated electron transfer (Rasmussen et al. 2016). The MBH from hydrogen-oxidizing bacteria is a distinctive oxygen-tolerant enzyme that can perform electrocatalytic H_2-oxidation in a high oxygen atmosphere (Yoon et al. 2011).

Metabolic engineering for novel metabolites

The utilization of *C. necator* as a single cell factory is not limited to the synthesis of P3HB or derivatives thereof. The ability of the organism to assimilate CO_2 to high cell density and direct the carbon flow to P3HB under nutrient control can be utilized to produce novel metabolites (Müller et al. 2013; Torella et al. 2015; Przybylski et al. 2015). A new host (H16 ΔphaCAB) was generated by deleting the PHA operon from parent strain *C. necator* H16, and a synthetic pathway was rationally designed and expressed in the *C. necator* mutant to efficiently divert carbon flux from P3HB to isopropanol production (Grousseau et al. 2014). Up to 216 mg/L of isopropanol was produced by the engineered strain from CO_2 and H_2 (Torella et al. 2015). With the development of genetic engineering tools in *C. necator*, the number of novel chemicals that can be produced from CO_2 is increased, including: up to 3.2 g/L of 2-hydroxyisobutyric acid (Przybylski et al. 2015), 80 mg/L of methyl ketones (Müller et al. 2013), 4.4 mg/L of alkan(e)s (Crépin et al. 2016), and 100 mg/L of isobutanol with an electromicrobial conversion of CO_2 (Li et al. 2012). One issue that has to be considered when producing the novel metabolites by engineered strains is the toxicity of the chemicals. In order to raise the isopropanol titer, for example, groESL chaperons were searched and identified. Overexpression of the native groEL and groES genes led to a better tolerance of the strains towards exogenous isopropanol (Marc et al. 2017).

Gas fermentation

Gas mass transfer and uptake rates

One major goal of gas fermentation is to obtain high productivity of desired products, which often requires a high cell density (Tanaka et al. 2011). Because the gas substrates, especially hydrogen and oxygen, are poorly soluble in aqueous solution, the concentration of cell mass and/or products is very much dependent on the gas mass transfer rates, or volumetric mass transfer coefficients (k_La) of individual gases. Numerous correlations are available for the value of oxygen k_La (Garcia-Ochoa et al. 2010). In the same bioreactor, hydrogen has a higher k_La than oxygen because of its smaller molecular size and higher diffusivity in water. The ratio of hydrogen k_La to oxygen k_La is 1.47, very close to the square root of the ratio of two gas diffusion coefficients (Lu and Yu 2017b). With sufficient nutrients and little P3HB formation, the maximum DCM concentration of a *C. necator* strain increased with oxygen k_La: 9.5 gDCM/L at 180 h^{-1} and 18.5 gDCM/L at 400 h^{-1} (Lu and Yu 2017b). The DCM concentration was also dependent on the accumulation of P3HB under different nutrient control strategies. It was maximized to 48 g/L at a k_La of 450 h^{-1} with a very high P3HB content (85%) and a low RCM concentration (7.2 g/L) (Volova et al. 2013a). In a laboratory bioreactor with a basket-type agitator, the oxygen k_La could be increased up to 2970 h^{-1}, generating 91 g/L DCM with 68% of P3HB (Tanaka et al. 1995).

During a gas fermentation, the cell density increased with time and one gas became the limiting substrate with its dissolved concentration approaching zero ($dC \approx 0$), while the dissolved concentrations of other gases were kept relatively high (Lu and Yu 2017a). The microbes might be exposed to the conditions of oxygen limitation or hydrogen limitation, depending on the gas composition. Under oxygen limitation ($dO_2 \approx 0$), the hydrogen uptake rate reached a maximum level of 200 mmol H_2 L^{-1} h^{-1}, and the molar ratios of consumed gases H_2/CO_2 and O_2/CO_2 were 7.3 and 2.4, respectively. The numbers indicated that about 66% of H_2 was oxidized for energy supply and 34% used as the reducing agents in CO_2 fixation. The aerobic strain, however, suffered from oxygen limitation and its respiration activity declined quickly with time. Under hydrogen limitation ($dH_2 \approx 0$), the respiration activity was kept at the high level for quite long time, because of very high affinity of hydrogenases to hydrogen. However, the molar ratio of the consumed gases H_2/CO_2 was increased to ca. 10, indicating that more hydrogen was used for energy supply. It might reflect some adverse effect of high dissolved oxygen concentration on CO_2 fixation efficiency (Yu et al. 2013). It is well known that the wasteful oxygenase activity of Rubisco results in a futile cleavage of Ru1,5P into 2-phosphoglycolate and formation of CO_2 (Berg 2011).

Limitation of CO_2 and other nutrients

Compared to H_2 and O_2, CO_2 has a relatively high solubility in water. Its predominant form around pH 7, however, is bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). Since free CO_2 is the substrate of Rubisco, it may become the limiting substrate under a low partial pressure (1% mol CO_2). Under CO_2 limitation ($dCO_2 \approx 0$), the molar ratio of consumed gases H_2/CO_2 went up to ca. 20 (Lu and Yu 2017a), indicating that a large portion of hydrogen was wasted because of the lack of CO_2 as the carbon source. The oxygenase activity of Rubisco might become predominant in the absence of dissolved CO_2 (Badger and Bek 2008). *C. necator* uses carbonic anhydrase (CA) to catalyze the interconversion between carbon dioxide and bicarbonate. The genome of H16 strain contains four CA genes: *can, can2, caa* and *cag* (Pohlmann et al. 2006). One CA (Can) has been identified as being essential for growth under atmospheric concentration of CO_2 (Kusian et al.

2002). A recent study reveals that Caa is an α-periplasmic CA and prefers CO_2 as the substrate. Its location in the periplasm of a cell may play a role in transport of CO_2 and supply of bicarbonate to the cell (Gai et al. 2014). Other CAs in cytoplasm prefer bicarbonate as their substrate and hence supply CO_2 to Rubisco.

Under sufficient supply of CO_2, H_2, O_2 and essential mineral nutrients, hydrogen-oxidizing bacteria including *C. necator* can grow to a high cell density with little P3HB accumulation. The limitation of dissolved oxygen ($dO_2 \approx 0$) does not trigger P3HB formation but brings down the overall metabolic activity (Lu and Yu 2017a). Among the essential nutrients for cell growth, including nitrogen, sulfur, phosphorus, potassium, and manganese, nitrogen is the most effective nutrient that can be controlled to direct carbon flow to P3HB synthesis (Volova et al. 2013a). Indeed, nitrogen is one of the major elements of RCM, accounting for 12–13 wt% of DCM (Kang and Yu 2015c).

Bioreactor and safety

Most studies on gas fermentation were conducted in conventional bioreactors with continuous bubbling of a gas stream of H_2, O_2 and CO_2 through aqueous medium solution (Tanaka et al. 1995; Volova et al. 2013a; Garcia-Gonzalez et al. 2014). The exhaust gas was either discharged or recycled in a closed gas system. In the agitated bioreactors, a high gas mass transfer coefficient (k_La) was maintained with high mechanical power dispersion per liquid volume and high gassing rate in terms of gas volume per liquid volume per min (vvm) (Garcia-Ochoa et al. 2010). Recycling the hydrogen-rich exhaust gas is necessary for hydrogen waste minimization and process safety (Tanaka et al. 1995). The equipment and operation costs, however, could be very high, especially with a high gassing rate (1–2 vvm). In a packed bed bioreactor, oxygen k_La up to 400 h^{-1} could be maintained at a very low gassing rate (< 0.2 vvm) (Lu and Yu 2017b).

Hydrogen is a clean, odorless, non-toxic, but highly flammable gas. The lower and upper flammability levels of hydrogen in air are 4 and 75 vol%, respectively. In chemolithotrophic CO_2 fixation, a molar ratio of H_2/O_2 is around 3 to provide a balanced supply of energy and reducing agents, but the gas composition lies in the explosion range (Takeshita and Ishizaki 1996). Several solutions have been proposed to solve the problem of gas explosion risk. The final electron acceptor O_2 was replaced by nitrate (NO_3^-) that could be reduced to nitrite (NO_2^-). This replacement, however, resulted in a substantial decline of cell growth and yield (Tiemeyer et al. 2007). The lower explosion level of O_2 in a hydrogen rich gas is estimated to range from 4.0 vol% (Schröder et al. 2004) to 6.9 vol% (Tanaka et al. 1995). Under a low O_2 partial pressure (< 4 vol%), the dissolved oxygen concentration declines according to the Henry's law. As a result, a low cell density was obtained, giving a low P3HB productivity (Garcia-Gonzalez et al. 2014). A bioreactor setup with a high gas mass transfer rate as well as hydrogen safety is a great technical challenge to industrial gas fermentation of hydrogen-oxidizing bacteria for CO_2 fixation and carbon reuse.

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