https://doi.org/10.1595/205651323X16806845172690 *Johnson Matthey Technol. Rev.*, 2023, **67**, (4), 402–413

JOHNSON MATTHEY
TECHNOLOGY REVIEW

technology.matthey.com

# Microbial Production of Hydrogen

**New opportunities for a low-energy source of hydrogen, not reliant on fossil fuels, using bacteria confined in coatings**

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**PEER REVIEWED**

Received 12th December 2022; Revised 3rd April 2023; Accepted 4th April 2023; Online 5th April 2023

Hydrogen offers a source of energy that does not produce any greenhouse gas (GHG) when combusted. However, some hydrogen manufacturing methods consume large amounts of energy and produce carbon dioxide as a byproduct. The production of hydrogen by bacteria is an attractive alternative because it is not energy intensive and, under the right conditions, does not release GHG. In this review, we introduce the five known ways by which bacteria can evolve hydrogen. We then describe methods to encapsulate living bacteria in synthetic layers, called biocoatings, for applications in bioreactors. We review the few examples in which biocoatings have been used to produce hydrogen *via* the photofermentation method. Although not used in biocoatings so far, the dark fermentation method of hydrogen production avoids the need for illumination while offering a high yield with low oxygen evolution. We identify the potential for using genetically-modified bacteria in future research on biocoatings.

## 1. Introduction to Hydrogen Classification and Production Methods

Hydrogen is an attractive energy source because the only product of combustion is water. Unlike the combustion of other fuel sources, there is no production of GHG or negative environmental impact at the point of use. Furthermore, hydrogen has a higher energy value than conventional fuels, as between 120 MJ and 140 MJ of heat is produced from 1 kg, compared to propane (45.60 MJ $kg^{-1}$) and petroleum (44.50 MJ $kg^{-1}$) (1, 2). However, in its liquid state, hydrogen has a lower energy density by volume, which means that larger storage tanks are needed (3). Nevertheless, hydrogen was used as the fuel source for the first-ever jet engine and for NASA's first space shuttle (3). As an indication of future growth, the UK has recently invested £23 million into the Hydrogen for Transport Scheme, aiming to increase the number of vehicles, especially buses, using hydrogen fuel cells (4).

Currently the majority of hydrogen, around 95%, is made from fossil fuels (1), with the most common and cheapest method being steam reformation of natural gas (methane). The traditional way of categorising types of hydrogen is using a colour system based on the input energy source in the production. **Figure 1** shows the four main types of hydrogen. 'Brown' hydrogen is produced from the gasification of coal to yield both $H_2$ and $CO_2$. 'Grey' hydrogen, which is the most common, is produced by steam reforming of natural gas to yield the same two gaseous products. 'Blue' hydrogen is similar to grey hydrogen, but in this method, the $CO_2$ emissions are captured and stored. Finally,



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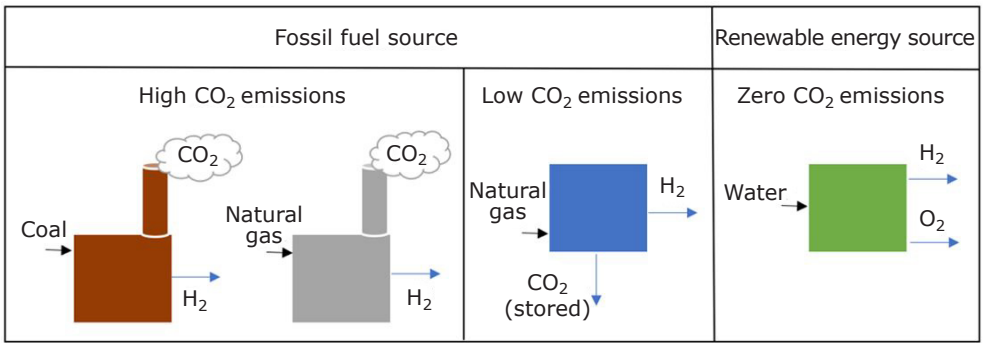


Fig. 1. Colour categorisation of hydrogen production methods, showing the input and outputs for brown, grey, blue and green hydrogen

**Table I Advantages and Disadvantages of Methods for Hydrogen Production (6–12)**

| Method of Production | Advantages | Disadvantages |
|---|---|---|
| **Brown** | Low cost; existing technology | Fossil fuel source; high $CO_2$ emissions; needs high temperature and pressure |
| **Grey** | Most inexpensive method; high conversion efficiency | Fossil fuel source; high $CO_2$ emissions; needs high temperature; needs additional step to purify hydrogen; often impurities can inhibit the reactions |
| **Blue** | Reduced $CO_2$ emissions compared to brown and grey; high conversion efficiency | Fossil fuel source; possibility of fugitive methane emissions; energy cost to separate $CO_2$ |
| **Water hydrolysis (green)** | Potential for zero GHG emissions; no fossil fuel substrate | Expensive; need to separate $H_2$ from $O_2$; electricity might be derived from fossil fuel sources |
| **Biohydrogen** | Reduced environmental burden; possible at ambient temperatures and pressures; less energy intensive; can make other products at the same time | Newer technology, needs improvement; less efficient than grey hydrogen; complex bioreactor design; need to separate gas products |

'green' hydrogen usually refers to any method that uses a renewable energy source and produces zero GHG emissions. The example used in **Figure 1** is the electrolysis of water, which is one of the more common methods of producing green hydrogen (5).

Each of the categories of hydrogen production offers advantages and disadvantages, as listed in **Table I**. A promising alternative is biohydrogen, which is defined as hydrogen produced biologically using microorganisms *via* either fermentation or photolysis (10). Some bacteria, algae and cyanobacteria produce hydrogen under certain conditions, usually as a way of preventing the build-up of toxic molecules, to regulate internal pH or as a byproduct of metabolic pathways (11, 12).

## 2. Five Types of Biohydrogen Production

Biohydrogen can be divided into five main production methods, as is shown in **Figure 2**. Three of these methods require a visible light source, whereas the other two do not require light and are therefore classified as dark methods.

The microorganisms capable of producing hydrogen all have one thing in common: the use of hydrogenases. Hydrogenases are enzymes that catalyse the reversible oxidation of $H_2$ to protons ($H^+$) and electrons. Some hydrogenases take up hydrogen to oxidise it, and other hydrogenases reduce protons and electrons to produce hydrogen. While there are numerous different types of these enzymes, they can be classified according to the metal(s) found at the enzyme's active sites. [NiFe]-hydrogenases have both a nickel and an iron atom present in the active site and are less sensitive to $CO_2$ and $O_2$ than other hydrogenases. Microorganisms with this hydrogenase include *Wolinella succinogenes, Rhodopseudomonas palustris, Rhodobacter eutropha,* cyanobacteria, archaea and *Escherichia coli.* [FeFe]-hydrogenases contain a complex array of iron atoms at each active site. This group of enzymes is predominantly involved in the production of hydrogen in anaerobic prokaryotes, but it is likely that some of these hydrogenases may also act as hydrogen sensors during anaerobic fermentation (7). The iron-hydrogenase was first discovered in the archaea


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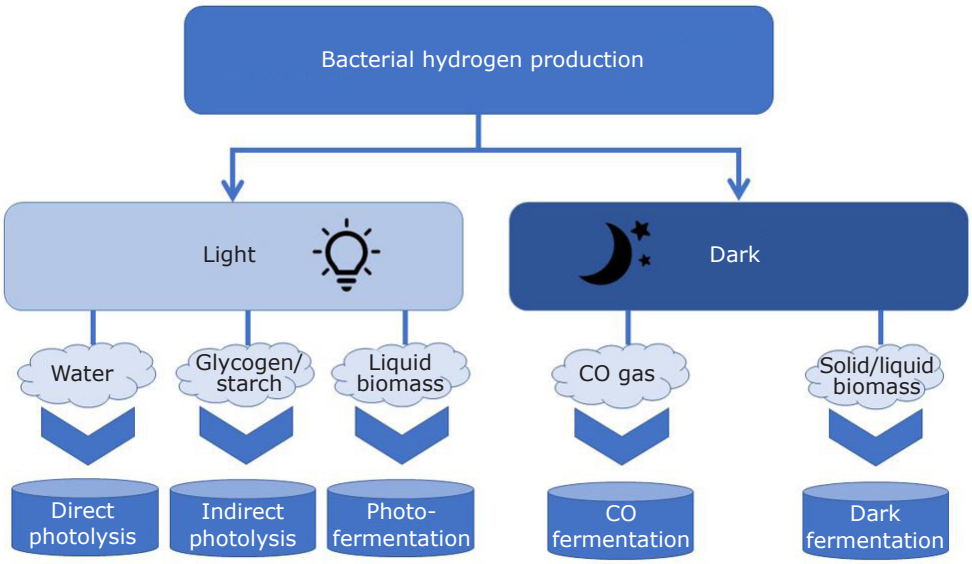


Fig. 2. The five types of biohydrogen production categorised by the type of reaction (either light or dark) and then by the chemical substrate. Adapted from (6)

*Methanothermobacter marburgensis* and was originally thought to be metal-free, but further investigation by Huang *et al.* (8) discovered one iron atom in the active site. Each of the methods illustrated in **Figure 2** will be described in the next sections.

## 2.1 Direct Photolysis

Green microalgae and cyanobacteria can produce hydrogen *via* direct photolysis. These microorganisms contain two photosynthesis systems: Photosystem I (PSI) and Photosystem II (PSII). In PSII, photons derived from light energy split water into oxygen, protons ($H^+$) and electrons (6). PSI then transfers these electrons to ferredoxin (Fd) where, under anaerobic conditions, it is reduced to make $Fd_{(red)}$. [FeFe]-hydrogenase in algae and [NiFe]-hydrogenase and nitrogenase in cyanobacteria then oxidise Fd to make hydrogen (11). The net reactions are Equations (i) and (ii):

$$2H_2O \rightarrow ½O_2 + 2H^+ + 2e^- \quad \text{(i)}$$

$$2H^+ + 2e^- \rightarrow H_2 \quad \text{(ii)}$$

Hydrogen is also made by the donation of electrons from $Fd_{(red)}$ to nicotinamide adenine dinucleotide phosphate (NADP), which then fixes $CO_2$ during the Krebs cycle in cyanobacteria (11).

One of the main issues with direct photolysis is that oxygen is also produced, and so there is a safety risk from combustion. The hydrogen therefore needs to be immediately separated from the oxygen. One way to reduce the amount of oxygen present is to reduce the rate of photosynthesis. This can be done by reducing the amount of sulfur available to the algae, which leads to a 90% reduction in the photosynthesis rate (9).

## 2.2 Indirect Photolysis

In indirect photolysis, cyanobacteria photosynthesise and then convert the starch or glycogen produced to hydrogen to prevent the accumulation of biomass. The reactions are shown in Equations (iii) and (iv):

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \quad \text{(iii)}$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad \text{(iv)}$$

The nitrogenases that catalyse the second reaction are inhibited by oxygen, so it occurs within specialised cells called heterocysts, where the oxygen concentration is kept at a minimum (6). Indirect photolysis offers an advantage over direct photolysis because hydrogen production is separated from photosynthesis, therefore reducing the risk of the hydrogen and oxygen mixing in amounts that would lead to spontaneous combustion. Indirect photolysis also produces more hydrogen than direct photolysis due to the conversion of hydrogen-rich glycogen or starch, not just the reduction of protons (as occurs in direct photolysis).

## 2.3 Photofermentation

Purple non-sulfur (PNS) bacteria, including *R. palustris, Rhodospirillum spp.* and *Rhodobacter*


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*spp.,* can produce hydrogen *via* photofermentation. Unlike algae and cyanobacteria, PNS bacteria do not have PSII so cannot split water to make hydrogen and oxygen (13). Instead, carbon (from a carbon-containing substrate) enters the Krebs cycle and is oxidised to $CO_2$, $H^+$ and $e^-$. Under anaerobic conditions, the protons and electrons are transferred to nitrogenase *via* the carriers Fd and nicotinamide adenine dinucleotide (NAD). A nitrogenase then uses adenosine triphosphate (ATP) sourced from PSI (from light energy) and the electrons to convert protons to hydrogen (11). The ATP is oxidised to become adenosine diphosphate (ADP) and phosphate groups ($P_i$). Ammonia is also produced, as shown in this net reaction (Equation (v)):

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad (v)$$

However, ammonia inhibits the production of hydrogen (11). In the absence of nitrogen, the hydrogen yield is increased because no ammonia is produced (Equation (vi)):

$$8e^- + 8H^+ + 16ATP \rightarrow 4H_2 + 16ADP + 16P_i \quad (vi)$$

A major issue with photofermentation is that a large proportion of the electrons (approximately 30%) are directed to biosynthesis for bacterial growth instead of for hydrogen production (6). When cell growth is inhibited, hydrogen yield increases by 80% (14). Another disadvantage, shared with the other methods of biohydrogen production, is the fact that the nitrogenase is sensitive to oxygen. This means that hydrogen will only be produced under anaerobic conditions; any oxygen will inhibit the nitrogenase. Furthermore, the PNS bacteria also have a hydrogen uptake hydrogenase, which reduces the amount of hydrogen produced by converting hydrogen back to protons and electrons (11).

## 2.4 Carbon Monoxide Gasification

Like photofermentation, carbon monoxide gasification is also carried out by the PNS anaerobic bacteria. In this method, carbon monoxide, a byproduct of coal gasification, is converted to hydrogen (11). Coal gasification is one of the conventional methods of producing hydrogen. The use of bacteria to convert carbon monoxide reduces the emissions from this carbon-intensive process and helps to increase the yield of hydrogen. However, $CO_2$ is produced, so there are still GHG emissions. The bacteria produce hydrogen through Fd hydrogenase as shown in Equations (vii)–(ix):

$$CO + H_2O + 2Fd_{(ox)} \rightarrow CO_2 + 2Fd_{(red)} + 2H^+ + 2e^- \quad (vii)$$

$$2H^+ + 2Fd_{(red)} + 2e^- \rightarrow H_2 + 2Fd_{(ox)} \quad (viii)$$

$$CO + H_2O \leftrightarrow CO_2 + H_2 \quad (ix)$$

## 2.5 Dark Fermentation

Facultative anaerobes and obligate anaerobes perform mixed acid fermentation under anaerobic conditions. The process is called dark fermentation because, unlike photolysis and photofermentation, the reactions do not require light energy. This is an advantage as it means that hydrogen can be produced continuously, unlike the requirements for light-dependant methods (15). Of course, an artificial light source can be used but that increases the cost and the complexity of the bioreactor design.

Obligative anaerobes, including *Clostridium spp.,* convert either sucrose ($C_{12}H_{22}O_{11}$) or glucose ($C_6H_{12}O_6$) to hydrogen, $CO_2$ and acetic acid ($CH_3COOH$) using [FeFe]-hydrogenases. During glycolysis, the hydrogenase catalyses the oxidation of NADH into $NAD^+$ and the electrons are then transferred to Fd. The hydrogenase then helps to transfer the electrons from Fd to $H^+$ in the net reactions, Equations (x) and (xi) (16):

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CH_3COOH + 4CO_2 + 4H_2 \quad (x)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (xi)$$

Both substrates theoretically produce four moles of hydrogen per mole of substrate, but in reality, less hydrogen is produced because glucose and sucrose are used in both cell growth and cell maintenance (5, 11).

Facultative anaerobes, on the other hand, use multi-enzyme complexes to produce hydrogen *via* mixed acid fermentation. The benefit of using facultative anaerobes over obligate anaerobes is that they can be grown aerobically to a high cell density and then switched into an anaerobic environment to begin producing hydrogen, making the process easier to control (15). Since the formate hydrogenlyase (FHL) system of *E. coli* is the most characterised and understood, *E. coli* is used here as a model to explain dark fermentation in facultative anaerobes. The mixed acid fermentation pathway in *E. coli* is shown in **Figure 3**.

*E. coli* contains four separate [NiFe]-hydrogenases. Hydrogenase 1 and Hydrogenase 2 are primarily uptake hydrogenases, while Hydrogenase 3


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produces hydrogen. The purpose of Hydrogenase 4 is still unknown, however, it has similar homology to Hydrogenase 3 and has been shown to be expressed during biofilm formation (18). As is shown in **Figure 3**, glucose is converted to phosphoenolpyruvate (PEP) during glycolysis. Since respiration cannot occur under anaerobic fermentative conditions, PEP is converted to oxaloacetate by the enzyme phosphoenolpyruvate carboxylase (PEPC), which is encoded by the gene *ppC*, and to pyruvate by pyruvate kinase (PYK). Oxaloacetate is eventually converted to succinate. Pyruvate is then converted to one of three products: lactate, acetyl-CoA or formate. The enzyme pyruvate-formate lyase (PFL) catalyses the conversion of pyruvate to formate and acetyl-CoA (17). End products from acetyl-CoA are ethanol and acetate.

Hydrogen is produced *via* the FHL pathway. In this pathway, formate is converted to hydrogen and $CO_2$ by the enzyme complex FHL. FHL is formed of two enzymes: formate dehydrogenase-H (FdhH) and Hydrogenase 3 plus electron carriers (16). The production of the large subunit of FdhH, encoded by the gene *fdhF,* which is regulated by the global gene transcriptional regulator narL (itself encoded by the *narL* gene), is inhibited in the presence of oxygen and nitrates, hence why hydrogen is only produced under anaerobic conditions (16). The subunit FdhH is also sensitive to pH. It has been shown that when the pH of growth medium containing bacterial cells is less than 5.0, the FHL is inhibited and there is no production of hydrogen (11). Finally, Hydrogenase 2 then oxidises hydrogen to produce $H^+$ and $e^-$.

In summary, biohydrogen methods could prove to be a better alternative way to produce hydrogen. Some advantages and disadvantages of each biohydrogen method are shown in **Table II**.

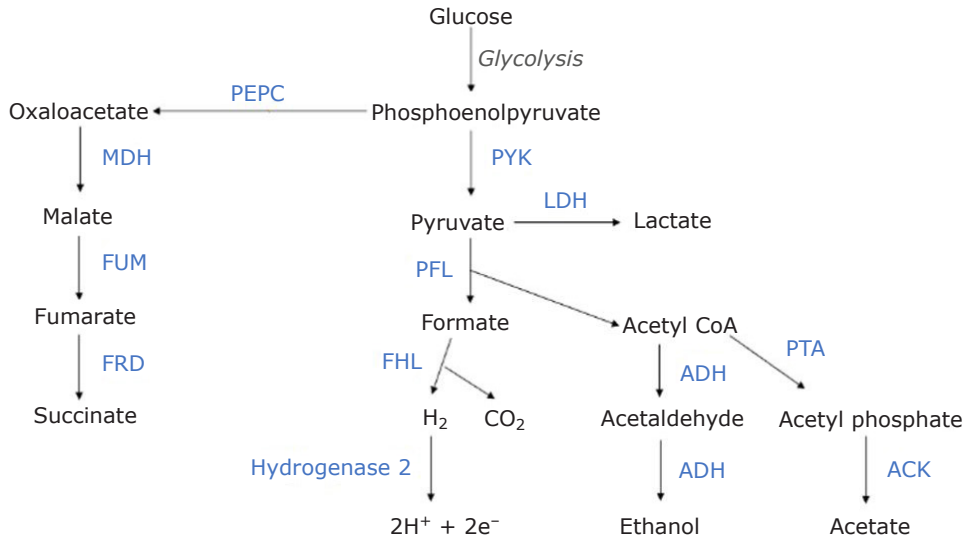


Fig. 3. Mixed acid fermentation pathway of *E. coli*. Enzymes and enzyme complexes are shown in blue font. Adapted from (17)

**Table II Advantages and Disadvantages of the Main Methods of Biohydrogen Production**

| Biohydrogen production method | Advantages | Disadvantages |
|---|---|---|
| **Photolysis** | Simple nutrients needed; microalgae can be cultured in a closed system; energy provided by light and water; no GHG produced | Low light to energy conversion efficiency; complex bioreactor design; low rate of hydrogen production; oxygen also produced |
| **Photo fermentation** | Higher $H_2$ yield than photolysis; energy provided by light; wide range of substrates | Low light to energy conversion efficiency; complex bioreactor design; need to pretreat substrates to allow for light penetration; $O_2$-sensitive nitrogenase; uptake hydrogenase present |
| **Carbon monoxide gasification** | Reduces emissions from carbon gasification | Slow bacterial growth; limitation of mass transfer from gas to liquid; needs constant shaking |
| **Dark fermentation** | High hydrogen yield; wide range of substrates, including waste food; $O_2$ is not produced; $H_2$ produced in a short time | Need to separate $H_2$ and $CO_2$; enzymes are sensitive to $O_2$; uptake hydrogenases reduce $H_2$ yield |


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## 3. Genetic Modification to Improve Hydrogen Yields

One of the biggest limitations of biohydrogen methods is their low yield, which must be improved if biohydrogen is to be economically viable (19). For this reason, much of the current research is using genetic modification to enhance the hydrogen yield from either cyanobacteria or bacteria.

### 3.1 Microalgae Modification

The [FeFe]-hydrogenases present in algae cannot produce hydrogen in the presence of oxygen. This is because oxygen binds to iron and competitively inhibits the proton's binding (20). Oxygen can be reduced by removing sulfur and thus reducing the rate of photosynthesis (9), but the hydrogen production is also stopped (20) because without photosynthesis, there is not enough energy being produced to maintain the production of hydrogen. A better method for overcoming the oxygen sensitivity limitation is to decrease the oxygen sensitivity of the hydrogenases, however there is no conclusive evidence that any genetic mutations to the hydrogenase leads to a decrease in hydrogen (20).

### 3.2 Cyanobacterial Modification

The [NiFe]-hydrogenase found in cyanobacteria is more tolerant to oxygen than the [FeFe]-hydrogenase present in algae but is still inhibited due to oxygen binding to the active site between nickel and iron, forming a hydroxyl group which can only be removed using a reducing agent. It has been suggested that cyanobacteria could be genetically modified to contain hydrogenases that are completely oxygen tolerant (21). One such hydrogenase is found in the soil bacterium *Ralstonia eutropha,* which has an active site shape that the oxygen cannot bind to (21).

Another method to increase hydrogen production is to stop other production pathways. McNeely *et al.* (22) produced a mutant strain of *Synechococcus* PCC 7002 which was deficient in lactate dehydrogenase due to a knockout of the gene *ldhA*. This meant that no lactate was produced and instead there was a five-fold increase in hydrogen production (22).

Gene over-expression can also lead to an increase in hydrogen production. As previously described, most of the hydrogen production in cyanobacteria occurs in heterocysts. If the number of heterocysts is increased, the hydrogen yield should, in theory, increase. The formation of heterocysts is controlled by the *hetR* gene (21). One strain that had an over-expression of this gene, and therefore increased numbers of heterocysts, had an increased hydrogen production rate. However, the number of heterocysts was irregular, suggesting that something else controlled the production of heterocysts (21).

### 3.3 Modification of Dark Fermentation Organisms

Laboratory-adapted non-pathogenic *E. coli* strains are often used in genetic manipulation experimentation. This is because *E. coli* grows rapidly, is a facultative anaerobe and can be easily genetically manipulated (21). Furthermore, there is a collection of all non-lethal gene knockouts of *E. coli*, the Keio collection, which allows for rapid analysis of the effect of gene knockouts (23). Obligate anaerobes, on the other hand, remain not as well researched. Many pathways, including the Krebs cycle, are ill-defined (21).

Currently, there are three main ways to increase hydrogen production in *E. coli*: (a) overexpress the FHL complex; (b) eliminate branch pathways; and (c) eliminate uptake hydrogenases (Hydrogenase 1 and Hydrogenase 2) (15).

The FHL complex consists of the subunit Fdh-H and Hydrogenase 3. The gene *narL* inhibits the expression of genes encoding the FHL complex. Fan *et al.* (17) found that after knocking out *narL*, the hydrogen yield significantly increased to 0.96 mol of hydrogen per mole of glucose, which is 1.8 times the amount of hydrogen produced by the wild-type strain. Hydrogenase 3 formation is regulated by the FhlA protein, which is encoded by the gene *fhlA*, which in turn is repressed by the gene *hycA* (24). By knocking out *hycA* and over-expressing *fhlA,* Yoshida *et al.* (25) increased hydrogen production by a factor of 2.8. A similar increase was seen by Maeda *et al.* (26).

Pathways that can be eliminated to increase production include the lactate pathway and succinate pathway. Attempts to eliminate the ethanol pathway led to a decrease in hydrogen production and impaired cell growth, which suggests that ethanol production may be essential to anaerobic bacterial survival (24). Likewise, the elimination of acetate production by preventing the formation of the enzyme pyruvate oxidase (PoxB) or the enzyme acetate kinase (AckA) did not increase hydrogen production as it led to


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reduced cell viability (24). The effect of eliminating the lactate pathway to improve hydrogen yields was only seen in acidic pH, because a low pH is needed to form lactate (24). The most promising pathway to eliminate is the succinate pathway. Fan *et al.* (17) removed this pathway by knocking out the gene *ppC,* which encodes for PEPC. This strain produced 0.73 mols of hydrogen per mole of glucose (0.19 mols more than the wildtype strain).

In *E. coli*, hydrogen is produced from formate, however, excess formate is removed from cells *via* a formate transporter, which is encoded by the gene *focA.* Fan *et al.* (17) also deleted this gene and found that it led to an increase in hydrogen. This knockout had the highest hydrogen yield per milligram of dry cell mass, but when this amount was converted to hydrogen yield per mole of glucose, there was only an increase of 0.09 mol of hydrogen per mole of glucose compared to the wild-type. A similar result was also noted by Maeda *et al.,* (26) who observed that the knockout of *focA* marginally improved the hydrogen yield. Other studies have found that this gene knockout can even lead to a reduction in hydrogen production (24). As the *ppC* and *narL* knockouts in *E. coli* led to the highest increase in hydrogen per mole of glucose (17), we propose that it would be useful to investigate them in a double knockout with potential synergism.

By eliminating the uptake hydrogenases, hydrogen cannot be converted to protons and electrons. Hydrogenase 1 is encoded by the *hya* operon and Hydrogenase 2 is encoded by the *hyb* operon (15). Maeda *et al*. (26) and Tran *et al*. (27) found that by inhibiting the formation of Hydrogenase 1, hydrogen production increased by 10%. By knocking out the *hybC* gene (found in the hyb operon), no hydrogen was produced, suggesting that Hydrogenase 2 is important for producing hydrogen when using glycerol as a substrate (27).

Some methods of genetic modification involve the use of antibiotic-resistance genes to replace the selected gene knockout. This also helps to selectively identify bacteria that contain the gene knockout. If these genetically-modified bacteria were to escape from a bioreactor into the environment, there is some worry that these genes could pass onto other bacteria in the local environment (21). However, to mitigate the risk, it is possible to resolve these antibiotic-resistance genes to create unmarked genetic mutants.

## 4. Use of Biocoatings in the Production of Hydrogen

To improve process control and prevent contamination (of both the environment and the hydrogen-producing microbes), bacteria should be fully contained within a bioreactor when producing biohydrogen. One promising means to confine living bacteria is in a synthetic biofilm, which is called a biocoating (28), as an abbreviation for a biocatalytic coating.

### 4.1 Introduction to Synthetic Biofilms

A biofilm is a permeable structure created by living bacteria and held together by an extracellular matrix (ECM). The biofilm can contain one species of bacteria or can be a multicellular community (29). Biofilms can adhere to surfaces because the ECM contains adhesion proteins that bind to solids, which is one reason why biofilms are so difficult to remove (29).

A major limitation of biofilms when used in applications is that they take a long time to develop and maintain. They are sensitive to many factors, including pH and chemicals, meaning that biofilm bioreactors are prone to failure (30). Furthermore, bacteria can freely leave a biofilm, which could pose a contamination risk to the wider environment, especially as biofilms promote the spread of antimicrobial resistance genes and promote the existence of antibiotic-resistant persister cells within the population (29). Other microbes can also enter a biofilm, which could influence product yield. For example, if a methanogen (a bacterium that consumes hydrogen) contaminated a biofilm, the yield of hydrogen would decrease and the entire biofilm would have to be removed and grown again (5, 30). Moreover, not all microorganisms are able to form a biofilm.

As biocoatings are artificially made, they can overcome many of the disadvantages of natural biofilms. Biocoatings have been investigated for use in many applications (31), including wastewater treatment (32, 33), environmental remediation (28, 33), abatement of volatile organic compounds in the atmosphere (34, 35), $CO_2$ absorption and fixation (30, 36–38) and biogas production.

Biocoatings are usually cast from a suspension of living microorganism in a waterborne colloidal


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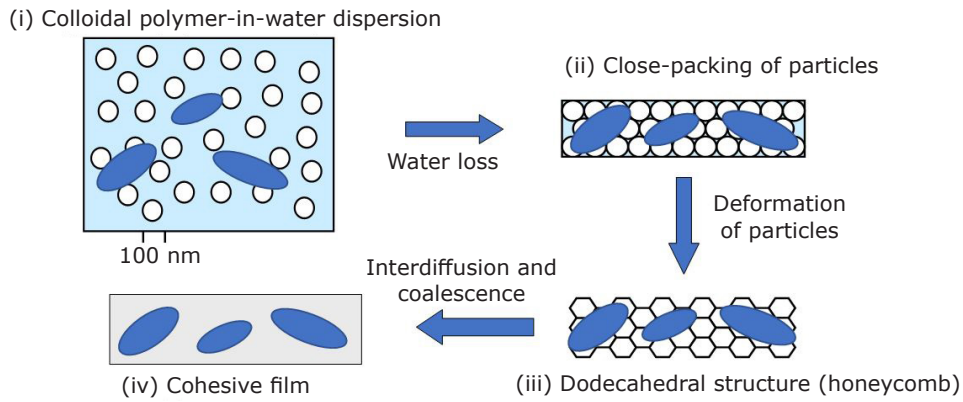


Fig. 4. The steps in the formation of a biocoating from a suspension of microorganisms (shown in blue) in latex

dispersion of polymer particles, called latex (39). **Figure 4** shows that the process of forming a biocoating consists of three main steps: (a) evaporation of water; (b) particle deformation; and (c) polymer molecule interdiffusion between particles leading to coalescence.

Biocoatings have been deposited onto a wide range of surfaces, including plastic (40), chromatography paper (41), textiles such as cotton (14), flax (32), polyurethane foam (34) and loofah sponge (to make a composite) (30, 37, 38). Upon the evaporation of water, the colloidal particles pack into an array around the living cells. To reduce the interfacial energy of the colloids, and under the action of capillary pressure, the spherical particles deform to fill space (39, 42). Film formation is characterised by the onset of optical transparency, occurring when the interparticle void space is closed so that light is not scattered (39, 40, 42).

Particle deformation will not occur unless the temperature is higher than the polymer's glass transition temperature, $T_g$, which is the point where the glass softens. The $T_g$ can be below room temperature to allow film formation under ambient conditions, or the film can be heated to a temperature above the $T_g$ that the bacteria can tolerate (34, 38). Plasticisers have been successfully added to a biocoating formulation to reduce the $T_g$ to allow film formation at room temperature (38). At high concentrations, the plasticisers had only a small detrimental effect on the bacteria viability.

The deformed latex particles coalesce to form a continuous film through the interdiffusion of polymer molecules across the particle/particle boundaries. This diffusion requires contact between particles at the molecular level at temperatures above the $T_g$ of the polymer (39, 42). Particles will produce brittle or even powdery coatings at sub-$T_g$ temperatures. Although the conventional film formation, as described here, requires the complete removal of water, biocoatings are often rehydrated after (partial) film formation to retain the viability of the bacteria (28, 31, 40).

## 4.2 Advantages and Challenges of Biocoatings

Biocoatings can be utilised to overcome the limitations of natural biofilms because biocoatings can be easily controlled. Theoretically, when there are more cells (i.e. a greater number density), then the yield of the metabolite is higher. The cell density is easily increased in biocoatings, meaning that the product yield can be high from the start of production (41). The film thickness can limit the product yields, especially when using photosynthetic bacteria, because of light absorption and scatter. When a film is thicker, less light penetrates to microbes farther from the surface. While it may be true that thicker films contain a greater number of cells per unit area, these microbial cells can shadow cells that are not on the surface layer of the coating, limiting light penetration (31).

The thickness of biocoatings can be adjusted during their production, which offers greater control than available in natural biofilms (41). However, some methods of producing biocoatings limit the thickness of coatings. This issue can be overcome by layering biocoatings on top of each other (36).

Biocoatings are more resistant to environmental pressures, such as pH changes and chemicals, in comparison to biofilms, which means that they do not break down as easily (30). This resistance, along with the small pore size in biocoatings, means that bacterial escape from the coating is less common, thus reducing the risk of contamination to the wider environment. This characteristic is especially important when using genetically-modified bacteria that may contain antimicrobial resistance genes that could pose a threat to other microbes, animals and humans. By preventing the


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escape of microbes, the biocoating also avoids bioreactor plugging, something that is common in bioreactors containing liquid suspensions or natural biofilms and leads to extra costs associated with stopping the reactor and cleaning it (41). A benefit of the biocoatings is that, unlike natural biofilms or liquid suspensions, the bacteria within them are protected from non-target organisms that may decrease product yield (30).

Long-term storage and transport are easy for biocoatings. Gosse *et al.* (43) found that production of hydrogen still occurred in biocoatings after being stored in glycerol at –80ºC for over a year. However, it was discovered that the humidity of the storage conditions and concentration of oxygen affects the biocoatings, with high humidity and oxygen causing damage to them (14).

Numerous studies have found that immobilising bacteria in biocoatings beneficially increases the product yield, sometimes as much as 10% (30, 36, 41). This might be because bacteria are assumed to be non-growing but metabolically active within biocoatings, leading to substrates that would otherwise be used for growth being diverted to the production of a metabolite. This effect was shown by Seol *et al.* (44), who found that when *E. coli* were adapted to be non-growing but metabolically active, the yield of hydrogen increased compared with wildtype *E. coli.* Not only do biocoatings increase yield, they can also extend production life, with Gosse *et al.* (45) showing over 4000 h of hydrogen production when *R. palustris* was in a biocoating.

Two of the major limitations of biocoatings are low permeability and low viability of bacteria. For effective production of metabolites, the latex coating must allow efficient transport for the sufficient exchange of nutrients and products. One way of improving this transport is to increase porosity. Introducing porosity correlates with an increase in the viability of the bacteria, as shown by Chen *et al*. (40), who added halloysites (clay nanotubes) to increase porosity and thus the permeability of the biocoatings. The non-film-forming halloysite particles do not deform and coalesce during film formation so that voids remain within the latex binder. A disadvantage of including these particles is that the increased number of voids in the biocoatings leads to increased light scattering and hence reduced optical transmission. This is a problem if the biocoating contains photosynthetic bacteria because light penetration will be reduced. Another limitation of these non-film forming particles is that they could cause secondary pollution, especially if they are made from microplastics (30) that are not tightly bound to the binder.

One cause of low bacterial viability is the osmotic pressure during full desiccation. To overcome this problem, osmotic regulators have been used with the aim of reducing the osmotic stress (46, 47). Piskorska *et al.* (14), analysed the effect of sucrose, glycerol, trehalose and sorbitol on cell viability. They found that latex suspensions treated with sorbitol led to the greatest survival of bacteria after desiccation.

A new approach was used by Chen *et al.* (32) to avoid the full desiccation of the biocoating during film formation and thereby to reduce associated stresses. They allowed evaporation to proceed to the point of particle close packing and then stopped it. Particle deformation and coalescence occurred in the presence of water, being driven by a reduction of the polymer/water interfacial energy. The bacteria were not subjected to full desiccation, which increased their viability and reactivity.

## 4.3 Examples of Hydrogen Production from Biocoatings

The immobilisation of microbial cells for hydrogen production has not been well studied. There are only a few examples in the literature describing the production of hydrogen from bacteria in standard biocoatings. An alternative method for immobilising hydrogen-producing bacteria is the creation of a biocomposite, in which the cells are mixed with (or deposited on) a porous material to combine the two, rather than spreading on a flat surface using a binder, as is the case for a typical biocoating. A few researchers have immobilised bacteria in biocomposites made of paper, agar or gel beads, as listed in **Table III**. Not all of these biocomposites were successful. For instance, Ishikawa *et al.* (49) immobilised *E. coli* in gel beads but found that hydrogen production was inhibited by inefficient gas exchange. These researchers did not produce a standard biocoating but rather just immobilised bacteria on a surface without the use of a polymer binder.

Only four studies have investigated hydrogen production in polymer colloid (i.e. latex) biocoatings, and all of them have used the same bacteria, *R. palustris,* which produces hydrogen *via* the photofermentation method. **Table III** provides more information. Piskorska *et al.* (14) found that osmotic stabilisers were needed to maintain hydrogen production after coatings were

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**Table III Comparative Summary of Previous Studies of Hydrogen Production Using Biocomposites or Biocoatings**

| Immobilised or biocoating? | Materials | Bacterial species and hydrogen production method | Hydrogen production | Reference |
|---|---|---|---|---|
| **Immobilised** | Alginate gel beads | *E. coli* MC13-4; dark fermentation | 10 ml $h^{-1}$ (production only lasted 20 mins) | (48) |
| **Immobilised** | LB agar | *E. coli* MC13-4; dark fermentation | 1.2 mol $H_2$ $mol^{-1}$ glucose | (49) |
| **Immobilised** | Non-woven cellulose fibres | *R. palustris*; photofermentation | 4.0 ± 0.288 mmol $H_2$ $m^{-2}$ $h^{-1}$ (for over 1000 h) | (50) |
| **Biocoating** | Latex (40% poly(vinyl acetate-co-acylate), 60% poly(butyl acetate-co-vinyl acrylate)) on polymer surface | *R. palustris* CGA009; photofermentation | 6.3 mmol $H_2$ $m^{-2}$ $h^{-1}$ | (43) |
| **Biocoating** | Latex on polyester | *R. palustris* CGA009; photofermentation | 2.08 ± 0.01 mmol $H_2$ $m^{-2}$ $h^{-1}$ (for 4000 h) | (45) |
| **Biocoating** | Acrylate copolymer latex on paper | *R. palustris* CGA009; photofermentation | 0.47 ± 0.04 mmol $H_2$ $m^{-2}$ $h^{-1}$ | (41) |
| **Biocoating** | Latex (vinyl acetate-co-acrylate) on polyester | *R. palustris* CGA009; photofermentation | 0.82 mmol $H_2$ $m^{-2}$ $h^{-1}$ (average over 80 days) | (14) |

stored at 4ºC but maintained dried coatings for over two weeks; hydrogen production still occurred when the coatings were rehydrated. This storage time was shorter than for the coatings produced by Gosse *et al*. (43), who found that hydrated biocoatings maintained activity even after being stored for more than three months in the dark and over one year when stored at –80ºC in glycerol.

These two research teams found differing results when using osmotic stabilisers. Gosse *et al.* (43) found that the best osmotic stabilisers were glycerol and sucrose. Piskorska *et al*. (14) found that sucrose had no effect on cell viability during storage. This difference could be due to the different storage conditions, as Piskorska *et al.* stored the coatings in a low humidity under argon, while Gosse *et al.* stored the coatings in air and used glycerol to protect the coatings during freezing. Gosse *et al*. (45) also investigated the length of time that hydrogen production was maintained in biocoatings. They found that optimum hydrogen production was during the first six days, after which hydrogen production dropped but was maintained at a constant rate for 4000 h, producing 2.08 mmol $H_2$ $m^{-2}$ $h^{-1}$ (45). Later research by Gosse *et al*. (41), used acetate as a substrate for gas evolution, as they were producing multiple metabolites, not just hydrogen. The use of acetate reduced the yield of hydrogen compared to the group's earlier work using glucose as the substrate. In this later work only 0.47 mmol $H_2$ $m^{-2}$ $h^{-1}$ was produced (41).

One major limitation of *R. palustris* is that it produces hydrogen *via* photofermentation, which as was already mentioned, yields less hydrogen than dark fermentation. It also has a low light conversion rate as *R. palustris* only absorbs 20% of the light spectrum (45). Any inclusion of pore-forming particles in a biocoating would decrease the light available to the bacteria further. Industrial scale-up could also be difficult as photobioreactors have a complex design.

The genetic modification of *E. coli* has produced higher hydrogen yields than *R. palustris*, as hydrogenases are 1000 times more efficient than nitrogenase in producing hydrogen (21). Therefore, we conclude the use of *E. coli* in a dark fermentation method is a promising, but unexplored, alternative to produce hydrogen in biocoatings. There is previous work on containing *E. coli* in biocoatings (40, 48, 51, 52), but no prior work on hydrogen production in such materials.

## 5. Concluding Summary

Hydrogen is needed in large quantities for the world's energy needs. Biohydrogen is an attractive source, because it reduces the environmental burden of production (not relying on fossil fuels), is obtained at ambient pressures and temperatures, and uses


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less energy than other sources. Of the methods to produce biohydrogen, dark fermentation has proven to be the most effective. Dark fermentation methods have high yields, are fast, and do not produce produce oxygen, which can react explosively with hydrogen. However, $CO_2$ must be recovered and separated, and the participating enzymes must be protected from oxygen. The yields of hydrogen could be increased *via* genetic modification.

Biocoatings, in which metabolically-active bacteria are contained in a polymer binder, have already been used in applications including bioremediation, carbon capture, wastewater treatment and biofuel production. However, there are only seven examples to date using biocoatings or immobilised bacteria to produce hydrogen. Most of the previous work has used the photofermentation method with *R. palustris*. The dark fermentation process and genetically-modified bacteria have not been yet used for hydrogen production in biocoatings. Both hold promise and are worthy of investigation.

## Acknowledgements

We gratefully acknowledge the Engineering & Physical Sciences Research Council (EPSRC) and Johnson Matthey for a PhD industrial CASE studentship for Kathleen L. Dunbar. We thank Alison Wagland, Beatriz Dominguez and Sarah Montgomery (Johnson Matthey) for helpful discussions.

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