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Hydrogen oxidising bacteria for production of single-cell protein and other food and feed ingredients

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Abstract: Using hydrogen oxidising bacteria to produce protein and other food and feed ingredients is a form of industrial biotechnology that is gaining traction. The technology fixes carbon dioxide into products without the light requirements of agriculture and biotech that rely on primary producers such as plants and algae while promising higher growth rates, drastically less land, fresh water, and mineral requirements. The significant body of scientific knowledge on hydrogen oxidising bacteria continues to grow and genetic engineering tools are well developed for specific species. The scale-up success of other types of gas- fermentation using carbon monoxide or methane has paved the way for scale-up of a process that uses a mix of hydrogen, oxygen, and carbon dioxide to produce bacteria as a food and feed ingredients in a highly sustainable fashion.

1 Introduction

Bacteria have been part of the human diet for millennia as components of many traditional foods, such as yoghurt, cheese, fermented vegetables, and fermented fish [1, 2]. In the 1960s, initial steps were taken to directly harness bacterial biomass as a source of protein, fats, and vitamins, for which the term single-cell protein (SCP) was subsequently coined [3, 4].

Today multiple factors coincide to drive market interest in SCP, most importantly the increased market demand for protein, and sustainability issues surrounding current protein production. Here, the authors will focus on one of the most sustainable forms of SCP: SCP produced with autotrophic bacteria that fix carbon dioxide (CO_2) into their cellular biomass (Fig. 1).

The United Nations has forecasted the world population to grow from 7.7 billion today to 9.7 billion by 2050 [5]. Inevitably, this has prompted concerns for future global food security, particularly as 2 billion individuals already experienced some level of food insecurity in 2019 [6]. The supply of animal-derived protein is expected to double by 2050 to meet the persistent global consumption demand for high-quality protein. Correspondingly, protein feed sources for livestock and aquaculture must also increase, despite land resources already being stretched [7, 8].

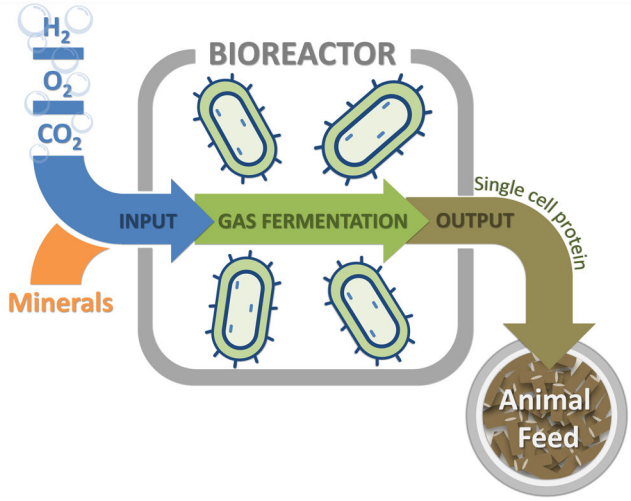

Fig. 1 *General process overview for SCP production using HOB and gaseous substrates*

Agriculture currently utilises ~43% of the land that is not a desert or covered by ice. This percentage will rise if agriculture is to meet the growing protein demand, which in turn will negatively affect biodiversity. Production of the type of SCP we focus on here requires much less land than agricultural protein (Fig. 2). To fix CO_2 energy is required. The basis of our current food supply is formed by plants that use light as their energy source. The energy requirement for the most sustainably produced SCP, as discussed in this review, uses hydrogen (H_2). This H_2 can be produced by electrolysis using sustainably produced electricity. If the required electricity is based on solar power it requires 0.18–0.26 m^2/kg of protein/year, which is considerably less than the 6–16 m^2 of land/kg of protein per year needed for soybean. If wind power is used land-use decreases to around 0.04 m^2/kg of protein/year [9].

2 Micro-organisms

The metabolic diversity of bacteria allows SCP to be produced from a variety of substrates. Most biotechnology and SCP production are based on heterotrophic bacteria grown on substrates such as cheap carbohydrates, lipids or methane and methanol, which inherently release CO_2. In light of climate change, ideally, our food and animal feed would be produced by primary producers capable of fixing CO_2. The CO_2 fixing abilities of autotrophic bacteria make them most suitable for truly sustainable SCP production [10].

The most attractive energy sources to enable CO_2 fixation into SCP are light for photosynthetic cyanobacteria or H_2 for certain chemolithoautotrophic bacteria. The cultivation of photosynthetic bacteria is limited by their light requirement. While natural light has the advantage of being a free energy source, its capture requires a large surface area, furthermore, it is only available half of the day and has a large seasonal variation in many highly populated parts of the world. Using artificial light is a possibility but this increases costs significantly.

As the main substrates for H_2-based autotrophy are gases, the technology is generally described as gas fermentation. Broadly

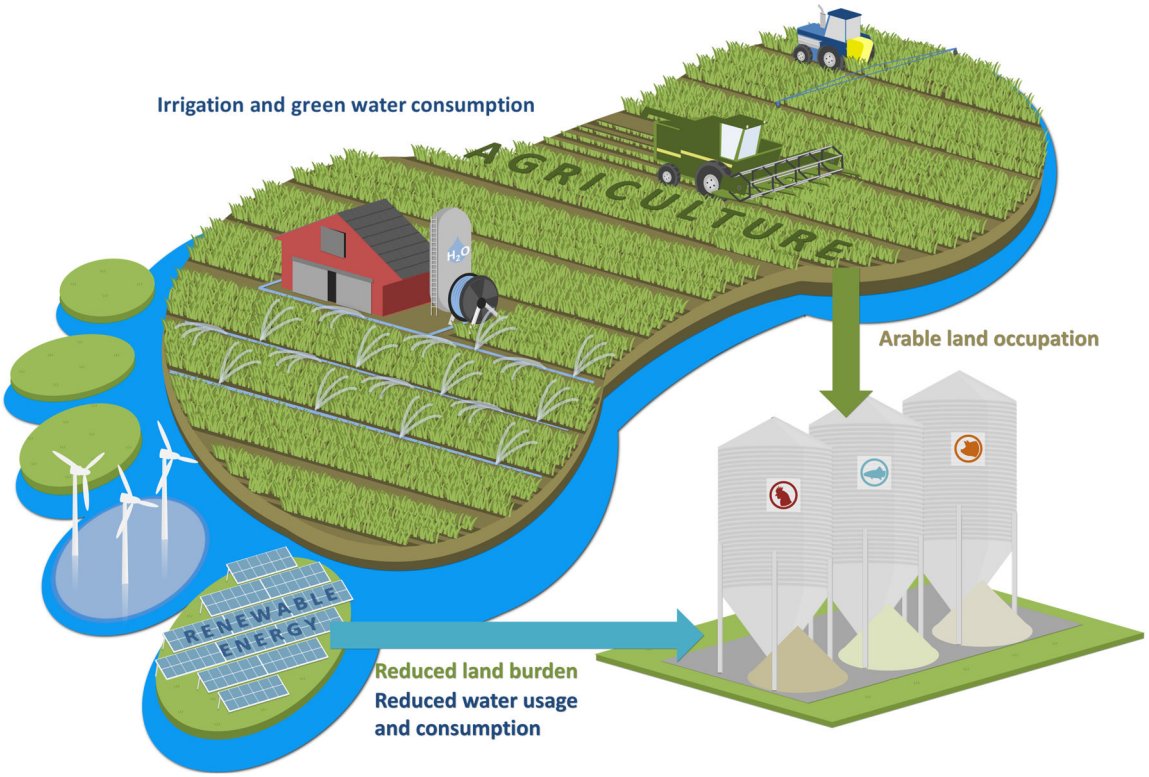

Fig. 2 *Illustrating the advantage of protein production using HOB: reduced land and freshwater use*

speaking the two types of autotrophic gas fermentation are anaerobic and aerobic gas fermentation. Anaerobic metabolism, fed on H_2 and CO_2, is only feasible if 70–80% of the carbon ends up in reduced products such as acetate and roughly 20% as SCP. From the perspective of protein production, this is inefficient unless the produced acetate is used to feed an aerobic second stage process where acetate is used as a carbon source. This has recently been demonstrated on lab-scale [11].

Aerobic H_2-based autotrophy has the capability to produce cell biomass without any significant by-product. This is due to the energy released by the reaction of H_2 with oxygen (O_2). Indeed, a mixture of O_2 and H_2 under optimal conditions can easily react to producing an ear-piercing bang or 'Knall' in German, where the gas mixture is known as Knallgas. Aerobic H_2 autotrophs are therefore known as Knallgas bacteria or the H_2 oxidising bacteria (HOB).

The generalised reaction of aerobic CO_2 fixation via H_2 oxidation can be given as:

$$H_2 + O_2 + CO_2 \rightarrow \text{Biomass} + H_2O$$

If one assumes an overall process where H_2 and O_2 are produced via electrolysis the overall process is analogous to oxygenic photosynthesis:

$$H_2O + CO_2 + \text{Energy} \rightarrow \text{Biomass} + O_2$$

Indeed, most of the industrially relevant HOB use the same metabolic pathway as plants to assimilate the CO_2 into biomass although alternative routes are utilised by certain HOB, such as those of the *Aquificae* phylum [12].

HOB are found in many different bacterial phyla. Once a lab is established to feed mixtures of H_2 and O_2 to bacteria, HOB are fairly easy to isolate. They have been detected in many different environments, such as Antarctic subglacial lakes, temperate soils, and hot hydrothermal vents [13–18].

Notwithstanding the wide range of HOB available only a handful are used and studied in detail. Interestingly many of these more prominent HOB have been mostly studied for reasons other than either their H_2 oxidising capabilities or their quality as a food or feed source.

Examples of HOB with industrial potential that have received significant research attention are *Rhodococcus opacus* and *Xantobacter autotrophicus*.

R. opacus is an attractive HOB for lipid production for nutrition, biofuel, and bio-commodity chemicals [19].

X. autotrophicus produces considerable amounts of zeaxanthin, a common caroteinoid used as a food dye.

X. autotrophicus's most prominent proposed biotechnological use is its capability to degrade chlorinated hydrocarbons [20].

By far the most studied HOB is *Cupriavidus necator*, which we will review in more detail as an example of the possibilities the HOB offer biotechnology.

Multiple studies have been performed on *C. necator* as a SCP source, but it is more widely known for its production of polyhydroxyalkanoate (PHA) bioplastics.

CO_2 fixation in *C. necator* occurs via the Rubisco enzyme as a part of the Calvin-cycle. The reducing power and energy needed to reduce CO_2 and form biomass come from the oxidation of H_2 via [NiFe]-dependent hydrogenases. The nitrogen needed for biomass formation can be supplied in the form of ammonia, urea, or even as nitrogen gas.

Besides the capability of *C. necator* strains to grow on CO_2 as the sole carbon-source they can also grow on a large number of organic compounds found in soil, *e.g.* succinate, fumarate, and malate, while sugar metabolism is often restricted to fructose.

During growth under certain nutrient limitations, the cells accumulate PHA, which can constitute up to 90% of the cells. However, under controlled conditions, the PHA content can be minimised and 75% of the dry matter is protein and the biomass can be used for SCP. It is because of this capability to produce a high protein ratio that the strain was proposed as a protein generator for space stations in the 1960s [21]

The cells of *C. necator* H16 contain two chromosomes and one smaller megaplasmid with a total of 6543 genes [22]. A large number of encoded proteins reflect a diverse and robust metabolism needed to thrive in a complex environment. The key proteins needed for DNA and protein synthesis are located on chromosome I, while chromosome II encodes for many of the enzymes needed for utilising diverse substrates. Two highly similar

gene clusters for the Calvin cycle pathway are present, one on chromosome II while the other is present on the megaplasmid. The NiFe-dependent hydrogenase used for H_2 oxidation is located on the megaplasmid.

The genomic data has guided the creation of a whole-genome-scale metabolic model, which contains 1391 reactions [23]. This model was used to design metabolic engineering strategies to improve the PHA producing potential of the strain and to devise strategies for the production of other chemicals.

The nutritional quality of many HOB is naturally already higher than many plant products however further improvements are desirable. Three routes to improvements in both product quality and process efficiency are process optimisation, directed evolution, and genome editing. The potential of the latter has improved significantly in the recent decade due to the increased knowledge of the genome and metabolism under various growth conditions.

Early studies of *C. necator* made use of Tn5 mutagenesis to develop single gene knock out mutants. This method was used to study the polyhydroxybutyrate (PHB) biosynthetic pathway, the soluble hydrogenase, post-translational modifications to PHA synthase, and other metabolic pathways [24–27]. The availability of suicide vectors employing the *sacB* counter-selection mechanism allows targeted gene deletion and genomic integration via homologous recombination. This method has been exploited to study PHB biosynthesis, 3-hydroxypropionate metabolism, and investigate the production of protein and cyanophycin [28–32].

Plasmid-based expression in *C. necator* has largely relied on the broad-host-range plasmid pBBR1, which was used to introduce pathways for the production of ethyl ketones, fatty acids, isopropanol, and non-native PHA [33–36]. Efforts to further develop the genetic tools have included increasing the number of vectors suitable for use in *C. necator* [37], identifying inducible and constitutive promoters [38, 39], and increasing the stability of those vectors [40, 41]. The latter is important for maximising product formation, as plasmid-based pathways offer a higher gene dosage than those integrated into the genome and plasmid instability is particularly problematic in long-running fermentations with higher selection pressure on the cells. Plasmid addiction systems rely on the deletion of an essential gene from the chromosome, and expression of that gene on a plasmid which contains the desired product synthesis pathway. This method of improving plasmid stability has been adopted for the production of cyanophycin, PHA, and arginine [32, 42, 43].

Recent studies have shown the capability of *C. necator* to produce compounds from CO_2 other than the well-characterised PHB such as methyl ketones, terpenes, carotenoids, acetoin, and isopropanol [33, 44–46]. Furthermore, PHB has been produced using both syngas and real CO_2-rich industrial off-gases emphasising the capability of *C. necator* to thrive and produce compounds in the presence of impure CO_2 sources [47, 48].

The most obvious targets of genetic engineering of strains for SCP use are higher protein content or improved amino acid profiles, but the value can be added to the strains via their production of specific functional proteins, vitamins, valuable fatty acids, carotenoids, terpenoids, flavours, and food dyes or non-toxic valuable co-products. This does require acceptance of the use of genetically modified organisms in addition to the acceptance of SCP. In the last few years, there are trends of growing public and legislative support provided the product is more sustainable. This is illustrated by the popularity of the impossible burger that contains haeme produced by genetically modified organism (GMO) microbes [49]. Public discourse seems to be positive about the idea of incorporating the sustainable versions of biotechnology into our food supply chain [50].

3 Commercialisation of HOB

In recent years, several companies have undertaken the engineering challenge of feeding the highly energetic H_2, O_2, and CO_2 mixture to the HOB to produce food and feed ingredients.

The California-based company Kiverdi, founded in 2011, produces the SCP products Air Protein™ & CO_2 Aquafeed as well as oils and bioplastics [51]. Also based in California is Novonutrients that tailor their SCP to supply aquaculture. Novonutrients' initial feed trials demonstrated the superiority of their SCP compared to control diets that included soy and algae, with respect to fish growth rate [52]. Founded in 2017, the Finnish company Solar Foods produces Solein, their SCP for human food pending EU novel food license approval [53]. The Belgium company Avecom developed several SCPs from low-value substrates. In their Power to Protein project, a collaboration with the Dutch KWR, HOB were used to produce SCP. This project experimented with using ammonium from waste-water as a nitrogen source [54]. Deep Branch Biotechnology was founded in Nottingham, UK in 2018 by a team that has a background in both gas fermentation and synthetic biology. Their first SCP produced by HOB, Proton™ is a protein source with a tailored amino acid profile. In 2019, Deep Branch Biotechnology partnered with the Drax Group to pilot their SCP production technology using CO_2 from Drax flue gas [55].

4 Other commercial gas fermentation

In this review, we have focused on gas fermentation using H_2, O_2, and CO_2 as growth substrates for bacteria. Other types of gas fermentation have been successfully scaled from lab to industrial scale by a handful of companies. Since the engineering challenges are similar, the success of these companies has paved the way for companies utilising HOB.

After the 1990s discovery that some acetogenic bacteria can produce considerable amounts of ethanol when fed carbon monoxide [56], several companies sprung up to use that process, including Lanzatech which remains the most successful example [57]. Lanzatech overcame many technical challenges in gas fermentation. They focus on biofuel and chemical production, however, they also have a patent on using their biomass by-product as SCP.

The German company Electrochaea uses hydrogenotrophic methanogens to produce methane from CO_2 and H_2. Their process is anaerobic and thus does not have flammability issues inside the reactor, however, the large H_2 demands necessitates similar safety precautions as HOB-based biotechnology. Their use of a pure archaeal strain in a biotechnological process is unique [58].

Two examples of companies that have scaled SCP production using gas fermentation are Calysta and Unibio [59, 60]. Both companies use a flammable gas mixture of methane and oxygen as a substrate. This type of gas-fermentation technology has been developing ever since its initial conception in the 1960s. Although the methanotrophic process inherently releases CO_2 while HOB-based gas fermentation captures it, these commercialised technologies are similar enough to HOB fermentation to prove that scaling up gas fermentation processes for commercial SCP production is achievable.

5 Conclusion

Feeding H_2, O_2, and CO_2 to HOB to produce SCP and added value food and feed ingredients is a promising form of biotechnology as is demonstrated by the range of start-up companies involved. There are still process efficiency and scale-up challenges to overcome but both industry and academia are highly motivated. Furthermore, the increased need for sustainability and recent success stories in adjacent technologies suggest that this powerful application of HOB technology will be realised in the short- to medium-term.

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