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BIOLOGICAL HYDROGEN PRODUCTION

FINAL ACTIVITY REPORT

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1. PROJECT SUMMARY & SCOPE

1.1 EXECUTIVE SUMMARY

- Successfully optimised a strain of bacteria to utilise simple sugars derived from biomass to produce hydrogen
- Achieved high rates (~10 fold higher than target production rate) and high yields of hydrogen production from glucose (>20%)
- Developed methods for the accurate measurement and control of hydrogen production from the engineered strain of bacteria
- Demonstrated the process and application by powering a small device directly from a small fuel cell powered by the hydrogen (with no storage)
- The bioengineered hydrogen producing strain provides an alternative for low carbon hydrogen production that does not rely on significant electricity infrastructure or storage requirements. The technology to produce hydrogen can be developed in a modular fashion at the site of availability of the waste biomass feedstocks.
- HydGene Renewables Pty Ltd was spun-off as a private company to commercialise the technology and has raised $1.1 million in funding to date.

1.2 PROJECT AIMS

This project aimed to use modern synthetic biology methods to produce a strain of bacteria that produces hydrogen from renewable carbohydrate feedstock such as glucose and sucrose. We aimed to achieve this through an iterative process of engineering in which bacterial cells are optimised for the maximum rate and yield of hydrogen suitable for commercial scale hydrogen production.

To achieve these aims we proposed to:

- Scale up the fermentation production of engineered strain from 2 mL to a 2 L scale to identify the engineering and safety requirements for the safe and efficient production and collection of hydrogen gas using bacterial cultures.
- Optimise the yields of hydrogen from carbohydrate from 2% to 20% by engineering of bacterial strains using synthetic biology methods.
- Optimise the functional expression of the components of the hydrogenase enzyme system and ancillary proteins within the bacteria to maximise the rates of hydrogen production from carbohydrates.
- Produce and collect sufficient amounts of hydrogen (nominally 100g) for testing of purity, and for testing as a fuel in hydrogen fuel cell applications.

The outcomes of these tasks were to:

- Increase the production of hydrogen, through scaling of a fermentation culture system (from 2 mL to 2 L) and capture the produced hydrogen from the genetically engineered bacteria.
- Demonstrate the improved efficiency in the production of hydrogen from carbohydrates through optimisation of a strain of bacteria to increase the yield to a minimum of 2.4 moles of hydrogen

molecules from every 1 mole of glucose equivalent oxidised (2.4 moles of hydrogen represents 20% of the theoretical maximum yield).

- Demonstrate the improved efficiency in the production of hydrogen from carbohydrates through optimisation of a strain of bacteria to achieve a rate of 700 mL hydrogen per hour per litre of bacterial culture at standard temperature and pressure.
- Generate learnings related to the output of the gas composition as well as factors affecting the rate and yield of hydrogen production; and assess the commercial viability of this method for hydrogen production.
- Generate learnings related to cost, design, market need, and implementation to develop a more robust framework for how the technology can be used to generate, collect, and purify hydrogen at the site of use.

Figure 1: Sugar to hydrogen biocatalyst. A bacterial strain is engineered for hydrogen production and grown to scale using conventional fermentation methods. The bacterial strain is encapsulated and used as a biocatalyst to transform sugar to hydrogen.

1.3 SCOPE

The scope of this project involved four key milestones:

- Establishing a 2-L laboratory scale culture system for growing the hydrogen producing bacteria.
- Genetically optimising the bacteria to produce 700 mL hydrogen per hour per litre of culture.
- Genetically engineering the bacterial strain to increase the current hydrogen production yield from 2% to 20% of the theoretical maximum yield (theoretical maximum yield is 12 moles of H_2 per mole of glucose).
- Producing sufficient amounts of hydrogen gas for testing as a renewable fuel in various applications.

1.4 SUMMARY SINCE MIDTERM MILESTONE REPORTING

The fermentation of the hydrogen-producing bacteria was successfully scaled from a 2mL to 100mL to 2L bench scale volumes to a 20L laboratory scale bioreactor (as shown in Figure 1). Further optimisation of the 20L laboratory scale fermentation process to achieve pilot scale generation of larger volumes of hydrogen gas is continuing past the end date of this grant. Several of the engineered bacterial strains were found to produce more than 6L of hydrogen per hour per litre of bacterial culture. This value represents a 1000-fold improvement on the hydrogen rate since the commencement of the project and nearly 10-fold greater improvement than our anticipated milestone target rate of 700mL per hour per litre of bacterial culture.

During the course of this project, the bacterial cultures produced a total of 49.1 grams (601L at Standard Temperature and Pressure) of hydrogen. The majority of this hydrogen was released/vented for safety reasons, although some was allowed to accumulate and was used directly without further purification in a 30W PEM hydrogen fuel cell to power small devices.

The yield of hydrogen production from the engineered bacteria using glucose as its feedstock is 3.0 +/- 0.1 hydrogen molecules per molecule of glucose. This value represents 25% of the theoretical biochemical yield, as shown in the chemical equation, based on the possibility of making 12 hydrogen molecules per glucose molecule (Woodward, J., Orr, M., Cordray, K., & Greenbaum, E. (2000). Biotechnology - Enzymatic production of biohydrogen. *Nature, 405*(6790), 1014-1015).

$$C_6H_{12}O_6 + 6H_2O \Rightarrow 6CO_2 + 12H_2$$

The 25% yield, which exceeds the milestone of 20% yield, was achieved through two key genomic mutations and through optimising culturing conditions of the bacterial strains.

We have further developed bespoke equipment and methods for measuring hydrogen gas from the optimised bacterial strains. This included construction of a laboratory scale generator with the gas output coupled to a 30W hydrogen fuel-cell. The fuel cell was used to directly consume the hydrogen produced from our engineered strains to generate electricity without requiring hydrogen storage.

3. KEY HIGHLIGHTS AND DIFFICULTIES EXPERIENCED

2.1 ESTABLISHING ROBUST AND RELIABLE HYDROGEN MEASUREMENTS

A key challenge at the commencement of this project was the requirement to establish a robust and accurate measurement of the hydrogen production from our hydrogen producing bacterial strains over both short and long-time scales. Protocols were established where we can now accurately measure hydrogen production over time scales from seconds to hours. Establishing robust protocols for achieving real-time measurements of hydrogen production was also crucial for characterising the relationship between the bacterial strain modifications and the hydrogen production rates and yields.

Hydrogen production on the second-to-minute timescale is measured in solution using a commercial Clarke type oxygen electrode. This enables rapid measurement of the rate of hydrogen production from suspensions of bacteria in solution. This method is limited to examining the rates of hydrogen production on short timescales and with relatively low concentrations of sugars as hydrogen concentrations rapidly reach the hydrogen solubility in solution saturation point of ~0.8 mM.

Hydrogen production on the minute to hour time scales is achieved in bespoke sealed containers with gas sampling ports with temperature control and monitoring of the pressure of the system. Gas samples are periodically taken with gas tight syringes after the addition of sugars. The gas is immediately analysed by gas chromatography which allows separation and quantification of H_2, O_2, N_2, CO, CH_4 and CO_2. Gas mixture standards of H_2, O_2, N_2, CO, and CO_2 provide both relative and absolute concentrations of these gases.

2.2 STRAIN ENGINEERING USING CRISPR FOR INCREASING HYDROGEN PRODUCTION RATES AND YIELDS

In this project, we applied and optimised the CRISPR gene editing technology (see CRISPR: The new tool in the gene editing revolution explained for a general explanation of CRISPR technology) to successfully either delete or modify the expression of targeted genes within our bacterial cells with high precision. CRISPR technology for targeted genome editing in bacteria has only been applied widely in recent years and still considered a premature technology for some applications including in bacteria. In this project, we have now optimised the CRISPR technology for our specific bacterial strains and determined key parameters to achieve high precision targeted gene edits. As a result, our engineered bacterial strains now produce more than 6 L of hydrogen gas per hour per litre of culture at a yield of 25% when using glucose as feedstock as shown in Figure 2. We have also used CRISPR technology to confer the ability of the bacteria to utilise sucrose instead of glucose as a carbohydrate feedstock. Sucrose is an attractive feedstock for renewable hydrogen production as it can be obtained from sugar cane waste such as molasses, an abundant resource in Australia. The CRISPR engineered strains have been reported in our PCT[1] application as listed in section 4.1.

Achieving and exceeding the milestone of 20% yield of hydrogen from glucose (being 2.4 moles of hydrogen produced per mole of glucose) required both optimising culture conditions and re-directing the metabolism of glucose, as specified in our PCT application.

[1] Patent Cooperation Treaty

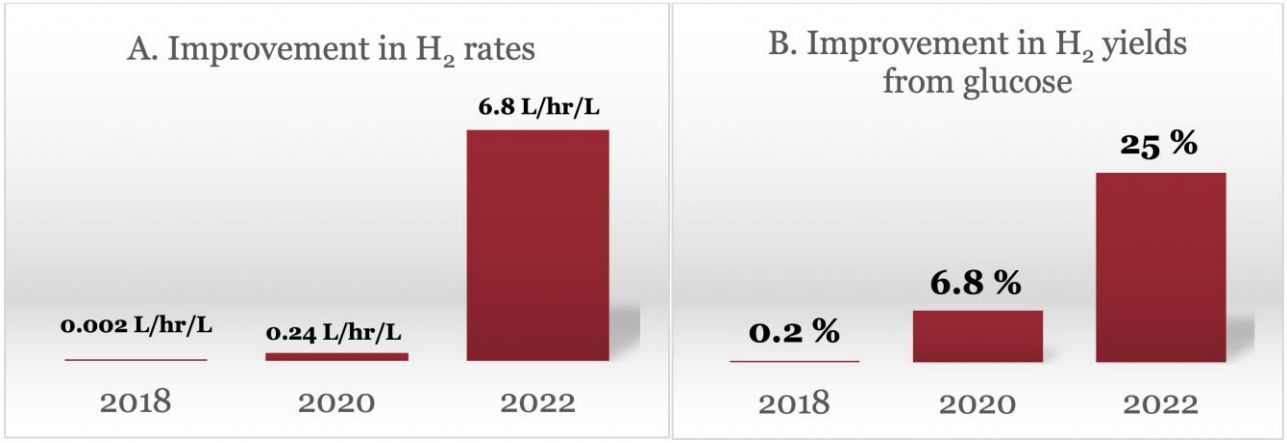

Figure 2: Improvement in rates (A) and yields (B) of H_2 production from the start of the project in 2018, early 2020 to now. Yield is based on 100% corresponding to 12 H_2 molecules produced per glucose.

2.3 SCALE-UP

Since the mid-progress report, we have now established a 20 L laboratory scale bioreactor system to grow our bacterial strains for increased volumes of hydrogen production. This scale has allowed us to reliably and robustly test and optimise conditions for bacterial hydrogen production. Engineered bacteria are highly dependent on a number of tightly regulated conditions including nutrient availability and oxygen levels. We continue to explore these parameters to improve the bacterial cell culture densities in our 20 L scale fermentation trials. Success from these trials will identify ideal conditions for the follow-on pilot scale fermentation experiments at larger scale. The tasks for scaling up to pilot scale production will continue to be a challenge to overcome for this project as it is for other fermentation bioprocess industries.

In addition to optimising the scale up we are currently limited to growing a maximum of 25 L of cells in a single batch. The primary reason for this is that additional regulatory approval is required when more than 25 L of genetically modified cells are grown in a single container. To grow these larger culture volumes to generate sufficient quantities of biocatalyst requires growth in a OGTR[2] certified Physical Containment Level 2 (PC2) large scale facility. The majority of these facilities are bespoke facilities and only recently has a commercial contract facility become available which can grow the quantities of cells required for further commercial development of our system.

2.4 ON-DEMAND AND ON-SITE HYDROGEN PRODUCTION

The conventional path for producing hydrogen from other approaches such as steam reformation and electrolysis and getting it to the end user requires costly collection, storage and transport. Our approach can overcome the need for large scale storage or transport as it allows for production of hydrogen gas of high purity on site and on demand from the engineered bacterial cells. Through this project, we have demonstrated that the bacterial cells can be harvested (once they reach high density during fermentation) and then stored (for up to 9 months) for hydrogen production at a later time. We have also established conditions to suppress hydrogen production during the high-density fermentation of our bacteria ensuring a

[2] OGTR- Office of the gene technology regulator

safer process. We demonstrated that the harvested bacterial cells produce hydrogen from addition of the carbohydrate feedstock in a simple reactor vessel that can easily be transported and deployed with minimal infrastructure requirement and costs. Our hydrogen production process is tightly controlled which allows for safe, on-demand, and on-site hydrogen production with low infrastructure requirements; negating the need to store or transport hydrogen. These key features of our novel bacterial hydrogen technology give us a significant competitive advantage over centralised green hydrogen production from electrolysis processes which will require additional hydrogen storage and transport technologies to be adopted to move the hydrogen, adding further cost and complexity to the supply chain. This feature of on-site and on-demand production is one component of our provisional patent.

2.5 HYDROGEN SAFETY

Producing hydrogen at high rates and managing the safe operation of our fermenters has been an important component of this project. We have developed safe operation strategies and introduced hydrogen sensors to constantly monitor and adapt to provide safe hydrogen production as our rates and yield increases continued to improve. Further, and as described above, hydrogen production from the engineered cells is a highly controllable process which is achieved by the addition of the carbohydrate feedstock, when needed.

4. COMMERCIALISATION PROSPECTS

Since commencing the ARENA supported project, we have actively engaged with industry partners and various stakeholders from the hydrogen and energy sectors. We have participated in the CSIRO ON Prime and ON Accelerator commercialisation programs. Through these programs, we identified specific market needs for the on-site and on-demand production of hydrogen. Specifically, opportunities for meeting energy demands in remote and off-grid communities using our technology have been identified as well as decarbonisation of chemical manufacturing processes that use hydrogen.

Hydrogen is an excellent renewable fuel due to its high energy density, zero carbon emissions upon use, and high versatility. While the majority of hydrogen is used today for industrial processes such as ammonia and oil-refining, it can also be used for transportation, to generate electricity, heat, or to supplement natural gas to decarbonise our economy and decrease greenhouse gas emissions. According to the CSIRO national hydrogen roadmap report[3], the global hydrogen market is USD155 billion in 2021 and expected to grow, with the European Union, Japan and South Korea, heavily investing in hydrogen R&D and infrastructure.

Currently, nearly all of global hydrogen supply today is produced non-renewably from fossil fuels while less than 1% is from electrolysis, with some produced by renewable energy such as solar and wind energy. The commercial readiness and capacity of electrolysers is currently low with total worldwide installed capacity currently at 0.5 GW. It requires a significant acceleration of installation to reach the 700 GW goal that is required in order to reach the scale and economics required for broader market adoption and replacement of hydrogen production from fossil fuels by 2030[4]. In addition, high costs associated with development of the

[3] https://www.csiro.au/en/work-with-us/services/consultancy-strategic-advice-services/CSIRO-futures/Energy-and-Resources/National-Hydrogen-Roadmap

[4] https://www.iea.org/reports/electrolysers

storage and transport requirements for a green hydrogen are a substantial hurdle for transitioning to a green hydrogen economy.

Our technology is in an ideal position to reach maturity independently of current practical limitations with hydrogen storage and transport, electrolysers, and battery technology. Hence, there is a huge promise for alternative technologies such as ours to provide economically viable and sustainable hydrogen production from alternative and renewable resources such as biomass. In fact a recent report from ARENA highlights the enormous potential in Australia to utilize agricultural waste for Bioenergy production[5]. As pointed out in this report Since we can provide on-site and on-demand hydrogen, we see our solution can meet the needs of remote and off-grid communities who can utilise hydrogen for electricity, heat, fuel or also for in-house ammonia production.

Technoeconomic modelling conducted by Hydgene Renewables Pty Ltd as part of a Business Research and Innovation Initiative (BRII) Grant scheme feasibility trial in early 2021 found that a cost of hydrogen approaching $2-3/kg is achievable by further additional improvements to our current engineered strains. This low cost can be achieved by increasing the yield from glucose to five mole of H_2 per mole of glucose, increasing the rate of hydrogen production to 20 L per hr per L of culture, and scaling the system to process several tonnes of biomass per day. The use of low-cost carbohydrates from underutilised biomass sources will also be essential for achieving this low cost of hydrogen production.

HydGene Renewables Pty Ltd was incorporated in September 2020 and is continuing the development of this technology with continued funding from BRII until the end of March 2023[6]. The primary purpose of this funding to HydGene Renewables is to optimise and scale the processing of biomass for hydrogen production from on-farm biomass sources (straw) for use with this engineered bacterial system.

[5] https://arena.gov.au/assets/2021/11/australia-bioenergy-roadmap-report.pdf

[6] https://hydgene.com/

6. SUMMARY OF KNOWLEDGE SHARING ACTIVITES COMPLETED

4.1 IP PROTECTION

Australian Provisional Patent File No: 2020900990
This application specified the bacterial strains and gene modifications necessary for metabolic engineering strains of *E.coli* that have improved hydrogen production.

PCT File number WO/2021/195705 (Published Sept 2021)
This application followed on from the provisional patent application and specified the strains of *E.coli* and gene modifications that were applied to these strains that have improved hydrogen production from sugars. It also specifies some processes necessary for commercial utilisation of the strains.

4.2 CONFERENCE PRESENTATIONS

Conference talk at the Synthetic Biology Australasia conference, Brisbane, 2019. Title: '*Synthetic Biology optimisation of biohydrogen production in bacteria.'* https://synbioaustralasia.org/wp-content/uploads/2019/10/SBA-2019-Abstract-booklet-High-Resolution-1.pdf

Poster presentation at the Synthetic Biology Australasia conference, in Brisbane, 2019. Title: '*Metabolic engineering in Escherichia coli using CRISPR/Cas9.'* https://synbioaustralasia.org/wp-content/uploads/2019/10/SBA-2019-Abstract-booklet-High-Resolution-1.pdf

Poster presentation at the Synthetic Biology Australasia conference, in Brisbane, 2019. Title: *'Identifying High Hydrogen Producing Hydrogenases'* https://synbioaustralasia.org/wp-content/uploads/2019/10/SBA-2019-Abstract-booklet-High-Resolution-1.pdf

Conference talk at the National Cleantech Conference and Exhibition, virtual, 2020. Creating on-demand electricity from plant-based fuel via clean, green hydrogen.

Conference talk at the Synthetic Biology Australasia conference, virtual, 2021. Metabolic Engineering of Escherichia coli for improved hydrogen production.

Poster presentation at the Synthetic Biology Australasia conference, virtual, 2021. Genetic Engineering of Escherichia coli for Sustainable Bioproduction of Hydrogen from Sucrose

Poster presentation at the Synthetic Biology Australasia conference, virtual, 2021. Identifying High Hydrogen Producing Hydrogenases

Poster presentation at the Synthetic Biology Australasia conference, virtual, 2021. Refinements to lambda red aided homologous recombination in E. coli leading to highly efficient recombineering

Poster presentation at AIChE Metabolic Engineering 14 conference, virtual, 2021. Engineering novel hydrogen solutions with synthetic biology.

Invited speaker, Future Energy (EF4) Conference, October 18-20, 2021: Optimising Biohydrogen Production in Bacteria using Synthetic Biology – Prof Robert Willows https://www.ausenergyfuture.com/academic-speakers

4.3 LINKS TO PUBLICLY AVAILABLE MEDIA ARTICLES CONCERNING THIS RESEARCH PROJECT

https://lighthouse.mq.edu.au/article/september2/designer-bacteria-could-fuel-the-future-with-cheap-hydrogen

https://www.world-energy.org/article/2581.html

https://www.mq.edu.au/newsroom/2018/09/06/bugs-burps-for-efficient-hydrogen-production/

https://advancedbiofuelsusa.info/bacteria-that-turn-sugar-into-hydrogen-being-engineered-by-researchers/

https://www.gasworld.com/bacteria-that-turn-sugar-into-hydrogen/2015400.article

https://www.biofuelsdigest.com/bdigest/2018/09/10/arena-awards-macquarie-university-researchers-a1-1-million-to-turn-sugar-intro-hydrogen/

https://sciencemeetsbusiness.com.au/future-hydrogen-economy-scaffolded-by-universities/

https://newcastleonhunter.com/2019/09/08/australian-universities-and-hydrogen-research/

https://www.csiro.au/en/Do-business/Hydrogen-Technology-Filter-Landing/Dark-fermentation

https://lighthouse.mq.edu.au/article/please-explain/november-2021/please-explain-how-does-hydrogen-power-work

4.4 LINKS TO PODCAST AND RADIO INTERVIEWS

https://www.listennotes.com/podcasts/science-meets-vc/hydrogen-quantum-vEdFMHIOZct/

https://tinyurl.com/y8qhzj35

https://vimeo.com/425004898

4.5 PUBLICATIONS

King, S. J., Jerkovic, A., Brown, L. J., Petroll, K., & Willows, R. D. (2022). Synthetic biology for improved hydrogen production in *Chlamydomonas reinhardtii*. *Microb Biotechnol, 15*(7), 1946-1965. doi:10.1111/1751-7915.14024

8. Conclusions and Next Steps

The genetic reprogramming of organisms using the synthetic biology cycle of design, build, test and learn, enables rapid optimisation of organisms for particular needs . Using synthetic biology we have engineered a bacteria that can be used as a next generation biocatalyst that is able to convert carbohydrates extracted from biomass into hydrogen at unprecedented rates and yields. This bacterial biocatalyst is extremely robust and tolerant to compounds that are typically toxic to microorganisms as it does not require the organism to grow and divide as in traditional fermentation systems. This bacteria biocatalyst system enables hydrogen production at rates and yields that make it economically viable.

In moving to a low carbon energy future, low carbon green hydrogen will be an important component, and biomass derived hydrogen can fill part of this requirement.

Some of the potential benefits of biohydrogen production using this biocatalyst include:

- hydrogen can be made on-site and on-demand from an appropriate biomass feedstock, which reduces the transport and storage requirements.
- The biocatalyst is stable for several months of continuous usage.
- A biocatalyst is not impacted by grow inhibitors that affect fermentation based solutions.
- The hydrogen production can be regulated by control of feedstock supply.
- The hydrogen produced is carbon neutral or carbon negative as it is derived from renewable biomass.

Some of the challenges faced for future use:

- Demonstrating the system is scalable.
- Commercialising the system.

To realise the potential of our biohydrogen strains and system developed in this project, a pilot scale plant needs to be built to demonstrate that it is scalable from a few litres of hydrogen produced per day to tens of thousands of litres per day. HydGene Renewables has licenced the intellectual property developed in this project and is seeking further investment to build and test such a pilot scale plant.

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