Fermentation of CO_2 and H_2 for formic acid production by *Thermoanaerobacter kivui*.

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Introduction

Carbon dioxide is currently the most abundant anthropogenically produced greenhouse gas and its concentration in the atmosphere continues to grow rapidly due to fossil fuels combustion for transportation, heating, electricity generation and cooking. The rise in the CO_2 level is considered to be responsible for the severe rise in global temperatures over the last decades (Oelkers and Cole 2008). In recent times, attention has been growing for the conversion of CO_2 into fuels or valuable chemicals in order to limit climate change and to develop sustainable technologies for energy conversion and storage (Appel *et al.* 2013). According to the circular economy concept, the developing of new microbial factories (MFs) can allow the exploitation of the atmospheric or industrial CO_2 to produce value-added chemicals.

A novel enzyme capable of reducing CO_2 directly with molecular hydrogen to formic acid was discovered in *Acetobacterium woodii* and *Thermoanaerobacter kivui*. This enzyme is called hydrogen-dependent CO_2 reductase (HDCR) and is considered more efficient than any chemical catalyst in hydrogenation of CO_2 (Müller 2019). The difficult application conditions required by isolated HDCR can be overcome by exploiting a whole cell system for the conversion of carbon dioxide and hydrogen to formic acid (Kottenhahn *et al.* 2018). Bacteria containing HDCR can have the dual function of hydrogen storage and formic acid production from carbon dioxide (Schuchmann and Müller 2013). In fact, hydrogen presents storage and transport problems that make it not directly usable for economic and safety reasons. These can be overcome by converting the hydrogen into the so-called liquid organic hydrogen carriers (LOHC) (Preuster *et al.* 2017).

The formic acid is liquid, easy to handle and low toxic, so it can be considered a hydrogen carrier. It finds its main applications as substrate for the production of other products containing carbon, preservative, antibacterial agent in livestock feed, in the textile industry and as de-icing agent (Pérez-Fortes *et al.* 2016). Formate can also be used as a hydrogen storage system together with a downstream H_2 release system or act as an electron source for a fuel cell device (Preuster *et al.* 2017).

Material and methods

In this study, a culture of *Thermoanaerobacter kivui* was chosen to develop a microbial cell factory for CO_2 exploitation. The reasons for which this bacterium was chosen instead of the more studied *Acetobacterium woodii* are various:

- *T. kivui* is more versatile than *A. woodi* managing to grow also on syngas or CO (Weghoff and Müller 2016).
- *T. kivui* is a thermophilic bacterium, while *A. woodi* is mesophilic. For the first one the risk of contamination and the cost of cooling are lower, the metabolic and diffusion rate are higher.
- The formic acid production of *T. kivui* is much greater than that of *A. woodi*, even at 30 °C (Müller 2019).

T. kivui is a thermophilic anaerobe acetogenic organism that can sustain heterotrophic or autotrophic metabolism. It grows excellently at 66 ° C and the optimum pH is 6.4 (Leigh *et al.* 1981).
As suggested by the DSMZ-German Collection of Microorganisms and Cell Cultures, from which *T. kivui* was purchased, the growth medium M171 was chosen for the bacterium. The medium M171 does not include expensive vitamin solution as it would not have consequences on *T. kivui* grow rate and maximum optical density reached (Weghoff and Müller 2016); this allows to decrease the growth costs of the process.

A.woodii was routinely cultivated in serum bottles of 160 ml with a liquid volume of 30 ml. Glucose 28 mM with a gas phase of N_2 + CO_2 (80:20 [v/v]) was chosen as organic growth substrate in the serum bottles (Schwarz and Müller 2020), while for the inorganic one in the bottle's headspace an overpressure of 0.4 bar is created by means of H_2 + CO_2 (80:20 [v/v]).

Liquid products (acetate and formic acid) were quantified via high-performance liquid chromatography.

Results and Discussion

The growth curves in heterotrophy in serum bottles showed an exponential growth of the bacteria from optical density (OD) 0.2 to 1. This growth was found to be inhibited by a drop in pH due to the production of acetic acid by *T. kivui*, while the glucose was not fully consumed. Therefore, a concentration of 10 g/l of 4-Morpholineethanesulfonic acid (MES) was added to the medium to buffer the pH and the growth curves were

repeated. In this case, the exponential growth of OD reached values of 1.2 and was not inhibited by the pH but by the complete depletion of the growth substrate. The most formed product is acetic acid, while formic acid is significantly produced only from the beginning of the stationary phase.

The growth curves in autotrophy in serum bottles, with the addition of 10 g/l of MES, showed an exponential growth of the bacteria from OD 0.1 to 0.8. To shorten the lag phase following the inoculation of heterotrophic bacteria in a serum bottle under autotrophic conditions, the glucose concentration in the latter was brought from 0 to 4 mM. In this way the quantity of biomass in the serum bottles increases more rapidly and the bacteria are sooner in the condition of being able to convert high quantities of carbon dioxide. This foresight is particularly important in anticipation of a future scale up from 160 ml serum bottles to a stainless steel stirred tank reactor type of 2 L, with a working volume of 1 L. Glucose is consumed entirely by bacteria in the first 24 hours of growth which raise the OD to 0.28, while the subsequent growth substrate is entirely made up of H_2 + CO_2 (80:20 [v/v]). The most formed product is acetic acid, while formic acid is significantly produced only in the initial lag phase, because there is an imbalance between the food they have and what they can manage, and in the stationary phase. The formic acid produced initially is consumed by *T. kivui* during the exponential phase.

Formic acid is an intermediate product of the Wood–Ljungdahl Pathway (WLP) of CO_2 reduction followed by *T. kivui* under such conditions. Therefore, to obtain formic acid, as major end product, and not acetic acid it is necessary to inhibit the WLP. Recent literature data suggested that a *T. kivui* suspension of resting cell can produce exclusively formic acid by adding 300 mM of $KHCO_3$ to the medium (Schwarz and Müller 2020). As expected, adding to the autotrophic medium 300 mM of $KHCO_3$ the efficient conversion of H_2 + CO_2 to formic acid by a whole-cell of *T. kivui* culture was found.

Conclusions

In this work the efficacy of the thermophilic acetogenic bacterium *T. kivui* as biocatalyst for the direct hydrogenation of carbon dioxide to formic acid was proved. This makes the microorganism suitable for the storage of hydrogen and the conversion of CO_2 into biochemicals.

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