

# Beyond the Theoretical Yields of Dark-Fermentative Biohydrogen

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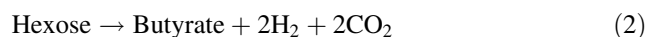
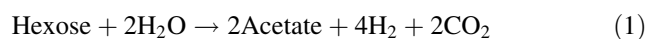
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**Abstract** Theoretical hydrogen (H<sub>2</sub>) yield by dark fermentative route is 12 mol/mol of glucose. Biological H<sub>2</sub> production yields of 3.8 mol/mol of glucose by microbes have been reported. Transient gene inactivation in combination with adaptive laboratory evolution strategy has enabled the H<sub>2</sub> yield to exceed the stoichiometric production values.

**Keywords** Hydrogen · Dark fermentation · Sugar · Microbes · Transient gene inactivation · Adaptive laboratory evolution

Fossil fuel reservoirs are depleting faster than they can be replenished. An associated limitation on the usage of these non-renewable fuels is their high pollution causing capacity. The need is to search for novel fuels, which can be based on renewable resources [1]. In addition, it provides an opportunity to identify an ideal fuel. Here, bioalcohol, biodiesel, biohydrogen and methane are the most competitive and ecofriendly fuels [2–11]. Each of them has its own merits and demerits. Biohydrogen has been assessed to be most energy efficient and non-polluting fuels, easy to transport, and can be converted into other forms of energy [1]. Different H<sub>2</sub> production methods include: (i) Thermochemical, (ii) Electrolytic, (iii) Photolytic and (iv) Biological. Biologically, H<sub>2</sub> can be produced by dark- and

photo-synthetic microbes from pure sugars and biowastes [2]. Theoretically, 12 mol of H<sub>2</sub> can be generated via the complete oxidation of 1 mol of hexose sugar [12]. However, stoichiometrically, a maximum to 4 mol H<sub>2</sub> per mole of hexose sugar can be achieved. Under dark-fermentative conditions, maximum of 4 and 2 mol of H<sub>2</sub> can be produced depending upon the acetate and butyrate as fermentative byproducts, respectively (Eqs. 1 and 2).



Overall, dark-fermentative H<sub>2</sub> production rates are significantly higher than those achieved through photo-fermentative process. In the past three decades, limited number of H<sub>2</sub> producers with little significant improvement in the biological H<sub>2</sub> yields have been reported. The dark-fermentative H<sub>2</sub> production by pure cultures such as: *Bacillus*, *Caldicellulosiruptor*, *Clostridium*, *Thermotoga*, and *Enterobacter* has been reported to be up to 3.80 mol/mol of hexose [12–14]. To achieve higher H<sub>2</sub> yields, various strategies have been adopted, including: (i) identification of novel producers, (ii) optimization of process parameters, and (iii) genetic engineering of native producers. In addition, different approaches have been suggested to improve the overall efficiency of the process by integrating various processes such as: dark- and photo-fermentations, polyhydroxyalkanoate production and biomethanation [15–19].

After a few decades of dedicated efforts, it has now been shown that we can go beyond the physiologic yields of dark-fermentative biohydrogen. It is remarkable that genetically modified extremophile *Thermotoga maritima* under dark-fermentation process showed a 1.9-fold higher H<sub>2</sub> yield of 11.54 mol/mol of maltose compared to the wild

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type [20]. Here, the strategy of transient gene inactivation to disrupt lactate dehydrogenase (*ldh*) to block lactate production was combined with adaptive laboratory evolution. After a few passage, strain Tma200 was evolved, which was slow growing and consumed maltose at a lower rate but could oxidize sugar more efficiency. It was found to be very effective in improving the H<sub>2</sub> yield to 5.77 mol/mol of hexose by competing the needs for cell biomass synthesis with metabolite (H<sub>2</sub>) formation using maltose as feed. This novel strategy can be extended to other H<sub>2</sub> producers to improve the overall efficiency of the process using biowaste as an economical feed for the sustainable development.

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