Bioresource Technology Reports 33 (2026) 102596

Contents lists available at ScienceDirect

Bioresource Technology Reports

journal homepage: www.sciencedirect.com/journal/bioresource-technology-reports

Engineering microbial cells for optimal biohydrogen production: a concise review of process control and metabolic engineering

Neeraj Kumar Singh [a], Prashant Bhagwat [a], Ayodeji Amobonye [a,b], Manoj Kumar [c], Santhosh Pillai [a,*]

[a] Department of Biotechnology and Food Science, Durban University of Technology, P.O. BOX 1334, Durban, 4000, South Africa
[b] Department of Polymer Chemistry and Technology, Kaunas University of Technology, Radvilenu Rd. 19, 50254, Kaunas, Lithuania
[c] Department of Hydro and Renewable Energy, Indian Institute of Technology Roorkee, Roorkee, 247667, Uttarakhand, India

ARTICLE INFO

Keywords:
Dark fermentation
CRISPR-Cas technology
Genome engineering
Metabolically engineered strains
Process optimisation
Renewable biomass

ABSTRACT

Increasing global energy demand and heavy dependency on fossil fuels have significantly contributed to global warming. To address these growing challenges, clean energy derived from renewable sources could be an alternative option. Biohydrogen production has attracted significant interest in this context, as it is a clean energy source with a high calorific value and zero emissions during production and utilisation. Among the various biohydrogen production technologies available, microbial biohydrogen has demonstrated great potential in producing high hydrogen yields from renewable biomass through dark fermentation. However, despite significant efforts, many dark fermentation processes fail to achieve desirable hydrogen yields. In this context, metabolic and genome engineering offer powerful tools for manipulating microbes to improve hydrogen productivity during the fermentation process. Therefore, the current review emphasizes advanced metabolic engineering tools for biohydrogen production, such as developing metabolic pathways via homologous/heterologous expression, improving sugar consumption, deleting competitive pathways, and overcoming carbon catabolite repression. It also discusses the sophisticated genome-engineering approach of CRISPR-Cas and its prospects for performing precise, efficient genetic modifications to enable stable biohydrogen production. Furthermore, it highlights key strategies for overcoming product toxicity during biohydrogen production. This review summarises the current state of biohydrogen production and identifies prospective areas for future research.

1. Introduction

The world has witnessed a 700% increase in population since 1800, accompanied by a massive increase in global energy consumption. Over the next twenty years, global energy consumption is expected to increase by 48% due to population growth (Akram et al., 2024). A significant proportion of this global energy demand would be met via the combustion of fossil fuels, a situation that has been identified as the major driver responsible for 86% of carbon dioxide emissions and, consequently, climate change and global warming (Canadell et al., 2023). Despite numerous agreements, communiqués, and policies established over the past decades to reduce global dependency on fossil fuels and address the associated challenges, fossil fuel production is still projected to exceed sustainable levels by 200% by 2030 (van Asselt and Green, 2023). In addition to the environmental damage caused by these non-renewable energy sources, other concerns, such as the depletion of fossil fuel reserves and the volatility of fuel prices, have sparked a global search for renewable energy alternatives (Jayakumar et al., 2023).

Abbreviations: Adhe, Aldehyde dehydrogenase; CCR, Carbon catabolite repression; CRISPR-Cas, Clustered Regularly Interspaced Short Palindromic Repeats system; *DrpiA*, Ribulose-5P-isomerase-deficient mutant; Endo, Endonucleases; ED, Entner-Doudoroff pathway; FHL, Formate hydrogen lyase; *fhlA*, Formate hydrogen lyase activator; frdD, Fumarate reductase; HRT, Hydraulic retention time; H_2, Hydrogen; Ldh, Lactate dehydrogenase; ldhA, Lactate dehydrogenase A; *Mdh*, Malate dehydrogenase; *MaeA*, Malic enzyme A; Mega, Meganucleases; MFA, Metabolic flux analysis; NadE, NAD synthetase; PPP, Pentose phosphate pathway; PEP, Phosphoenolpyruvate; PTS^{Glc}, Glucose phosphotransferase system; TALENs, Effector nucleases resembling transcription activators; VFA, Volatile fatty acids; ZFNs, Zinc-finger nucleases.

* Corresponding author.
E-mail addresses: neerajs@dut.ac.za (N.K. Singh), prashantb@dut.ac.za (P. Bhagwat), ayodeji.amobonye@ktu.lt (A. Amobonye), manojkumar8313@gmail.com (M. Kumar), santhoshk@dut.ac.za (S. Pillai).

https://doi.org/10.1016/j.biteb.2026.102596
Received 28 September 2025; Received in revised form 13 January 2026; Accepted 23 January 2026
Available online 4 February 2026

Given their relative carbon neutrality, environmental compatibility, and readily available feedstocks, biofuels have been identified as viable, renewable alternatives to fossil fuels. They are classified into first, second, and third generation biofuels according to the feedstock utilised: food crops, lignocellulosic biomass, and algal biomass, respectively (Bhagwat et al., 2024). Similarly, biofuels can be classified based on their nature, chemical composition, and functionality into bioethanol, biobutanol, biomethane, and biohydrogen, among others, each with its own merits and demerits. Biohydrogen, which can be potentially produced from food, lignocellulosic, and algal biomass, has gained attention due to its high energy content, low carbon footprint, versatility, efficiency, and scalability (Putatunda et al., 2022). Typically, biohydrogen is produced through bio-photolysis, dark fermentation, photofermentation, microbial electrolysis cells, or a combination of these methods (Bhagwat et al., 2024; Kumar et al., 2024). However, dark fermentation has been noted as the most viable production method due to its low energy requirement and the ability to utilise a wide range of raw materials as microbial feedstock (Zhao et al., 2024).

Despite the huge potential of biohydrogen to ameliorate the current global energy crisis, the industrial applicability of this renewable resource has been encumbered by the low microbial production rate and inefficient bioconversion of biomass, which are borne out of various factors, including substrate composition, pretreatment method, and bioreactor conditions (Ayodele et al., 2023). Hence, attempts to enhance biohydrogen production have been investigated from various angles due to the multifaceted nature of the problem. However, as current biohydrogen production technologies harness the metabolic capabilities of microbes, especially bacteria and microalgae, modifying the metabolic machinery underlying hydrogen production has been identified as one of the most feasible routes to improve the technology (Zhang et al., 2023). To this end, a clear understanding of the molecular fundamentals that drive the production and utilisation of hydrogen in biological systems is considered critical. Thus, it has been established that the stability, efficiency, and yield could be enhanced by engineering the enzymatic systems involved in biohydrogen synthesis, improving both direct and indirect photolysis, the extension of substrate utilisation, enhancement of tolerance against stress as well as engineering of H_2-producing native pathways and incorporation of non-native pathways (Hallenbeck et al., 2009; Jiao et al., 2024; Oh et al., 2011).

The last two decades have witnessed significant progress in engineering microbial metabolism and genomes for enhanced biohydrogen production (Kim et al., 2023; Rollin et al., 2015; Zhang et al., 2020). Among the most important metabolic-engineered hydrogen-producing microorganisms are *Escherichia coli*, *Enterobacter* (*E. aerogenes* and *E. cloacae*), and obligate anaerobes such as *Clostridium*, *Alcaligenes*, and *Bacillus* (Faloye et al., 2013; Goyal et al., 2013; Valle et al., 2019). For example, the overexpression of the glyceraldehyde-3-phosphate dehydrogenase gene was demonstrated to enhance biohydrogen production by 66.3% in *Clostridium acetobutylicum* ATCC 824 (Son et al., 2021). More recently, the overexpression of glucose-6-phosphate dehydrogenase and FeFe hydrogenase was shown to significantly increase hydrogen production in another strain of the same Genus (Lu et al., 2022). The highly efficient CRISPR-Cas9 gene editing system was also employed to overexpress the gene responsible for NAD synthetase (NadE) as a novel approach for enhancing biohydrogen production in *Enterobacter aerogenes* (Zhang et al., 2023). These approaches have also been successfully utilised to enhance hydrogen production in microalgae (Ban et al., 2019). For instance, the light energy utilisation efficiency of the mutant strain of *Chlamydomonas reinhardtii* was enhanced by transforming with light-harvesting complex translation inhibitors, resulting in a 1.8–5.2 times higher hydrogen yield than the wild-type strain (Ban et al., 2019).

Despite the huge potential of biohydrogen in the quest for environmental sustainability, advancements in metabolic and genome engineering for microbial biohydrogen production have been slow. Moreover, most available studies are limited to laboratory-scale investigations using engineered microbes under highly optimised conditions that often inhibit hydrogen production due to substrate toxicity. Consequently, comprehensive, in-depth studies and data are still required to enable the scale-up and commercialisation of these engineered microbes across diverse bioreactor operating conditions. To address this gap, this article aims to highlight the current state of research in this field, thereby increasing the industrial applicability of biohydrogen. In this regard, this paper critically reviews the potential of metabolic and genome engineering to enhance biohydrogen production, with a special focus on manipulating central metabolic pathways, redirecting metabolic flux, and using the CRISPR-Cas9 genome-editing tool. Furthermore, light was shed on other potential strategies to facilitate biohydrogen production, such as recombinant gene expression, process optimisation, and enhanced fermentation efficiency. Finally, the challenges preventing the widespread deployment of these technologies, as well as the probable developmental approaches to address them, are highlighted.

2. Metabolic engineering approaches for biohydrogen production

Studies have shown that wild strains of H_2-producing microorganisms generally produce less H_2 from renewable biomass than their theoretical potential yields (Ersoy et al., 2023; Sivaramakrishnan et al., 2021). Interestingly, higher H_2 productivity can be achieved by developing novel phenotypes with enhanced hydrogen production capabilities through metabolic engineering. Over the last two decades, various studies have employed random genetic modifications via chemical mutagenesis and UV radiation to enhance hydrogen productivity in wild strains (Sanabria, 2011). However, due to limited stability and low success rates, these techniques failed to achieve the desired H_2 yield, prompting researchers to explore metabolic engineering as a more innovative approach. This relatively new approach is largely attributed to cutting-edge technologies, such as metabolomics, transcriptomics, proteomics, and genomics, which enable a deeper understanding and precise manipulation of cellular processes involved in hydrogen production (Hernández-Guisao et al., 2024). Although various strategies for metabolic engineering exist, the most applicable ones, specifically in biohydrogen production, have been identified as the elimination of competitive pathways and carbon catabolite repression for the consumption of less favourable carbon sources, as well as the redirection of metabolic flux (Fig. 1). Various microbial strains have been successfully engineered to enhance their biohydrogen production capabilities (Table 1).

2.1. Elimination of competing pathways

Eliminating competing pathways in microbes is a widely adopted metabolic engineering strategy to facilitate the production of desired metabolites, proteins, or bio-products, especially in the biofuel, agriculture, food, and pharmaceutical industries (Pathade et al., 2024). It is principally based on resource optimisation, flux maximisation and reduced byproducts. Typically, hydrogen-producing bacteria generate competitive byproducts such as acetate, ethanol, lactate, and methane, which reduce overall hydrogen yield by diverting carbon, energy, and electrons away from the hydrogen pathway (García-Depraect et al., 2021; Mohanakrishna and Pengadeth, 2024). Hence, there is a need to eliminate the competitive pathways for producing acetate, ethanol, and methane by modifying the metabolic processes of microbes based on a comprehensive understanding of their native metabolic networks of biochemical reactions. Interestingly, the native metabolic networks of these bacteria can be controlled by a variety of strategies, including pH variation (Li et al., 2019), regulation of redox potential to ensure electron availability for hydrogenase activity (Zhang et al., 2015), modulation of enzyme activity to enhance key enzymatic reactions while suppressing competitive pathways (Ruggeri et al., 2015), and

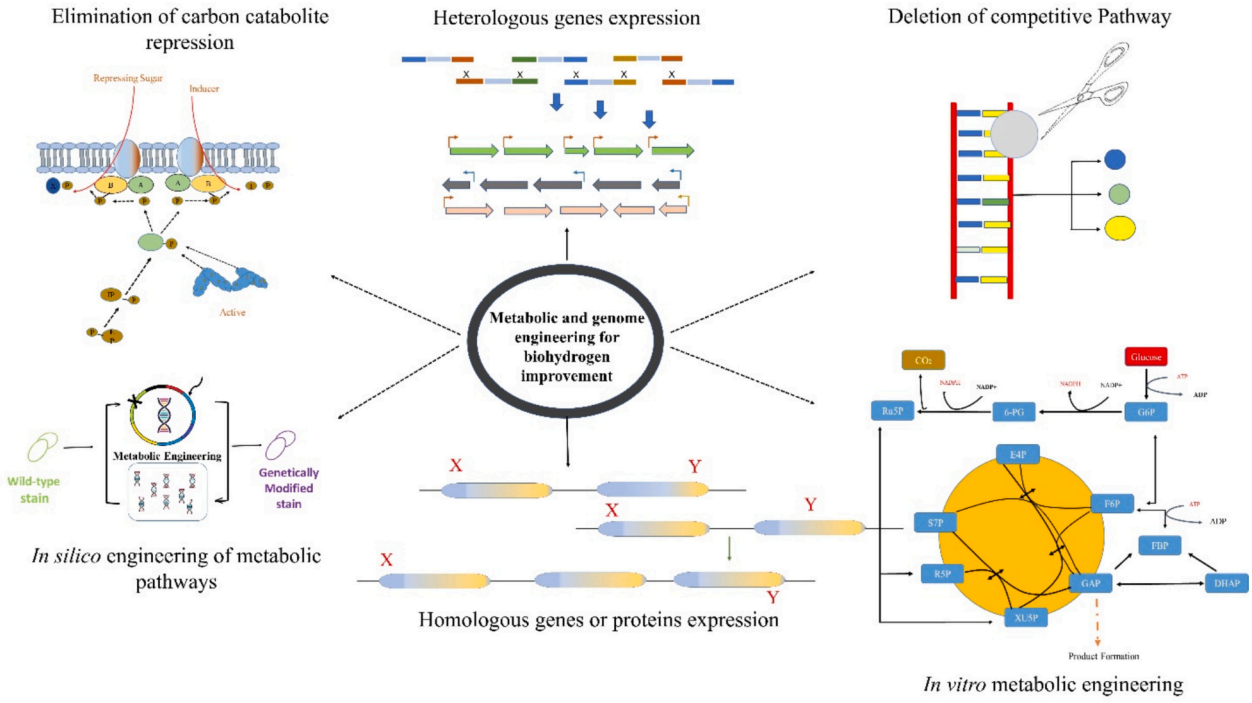

Fig. 1. Schematic overview of metabolic and genome engineering strategies employed for enhancing the fermentative H_2 production. Adapted and modified from (Akaniro et al., 2024).

manipulation of metabolic fluxes to direct carbon and energy resources towards hydrogen production (Dong et al., 2017). These strategies collectively govern the H_2 production pathway.

Several studies have concentrated on channelling metabolic flux towards enhanced H_2 production in the last decade. For instance, Valle et al. (2015) described an *E. coli* mutant strain with altered metabolic pathways that inhibited the pentose phosphate pathway (PPP) and the Entner-Doudoroff (ED) pathway, consequently redirecting carbon flux to the glycolysis pathway to produce more pyruvate, a key substrate for biohydrogen synthesis. A ribulose-5P-isomerase-deficient mutant (*DrpiA*) of *E. coli* was also designed to increase the carbon flux to glycolysis by blocking the PPP pathway, resulting in higher pyruvate and formate levels, which are then converted by hydrogenase and formate hydrogen lyase (FHL), leading to enhanced hydrogen production (Sundara Sekar et al., 2017).

It has since been observed that formate plays a critical role in microbial biohydrogen production, and tuning its metabolism is a crucial step in enhancing biohydrogen yield. Under anaerobic conditions, it gets converted into H_2 and CO_2 through the action of FHL enzymes; unfortunately, the flow of formate to other metabolic pathways results in the production of ethanol, acetate, succinate and lactate, thus impeding hydrogen production (Gevorgyan et al., 2022; Maeda et al., 2008; Valle et al., 2019). In this regard, fumarate reductase (frdD), which catalyses the conversion of fumarate to succinate, thereby reducing the synthesis of phosphoenolpyruvate (PEP) and pyruvate, which would otherwise be directed to the H_2 production pathway, has been identified as a key metabolic regulator (Jin et al., 2023). Lactate dehydrogenase A (ldhA) is another undesirable participant that reduces pyruvate availability by converting it to lactate under anaerobic conditions when the TCA cycle is not fully operational (Fig. 2) (Noblecourt et al., 2018). Hence, the deletion of genes encoding fumarate reductase and lactate dehydrogenase in *E. coli* has significantly enhanced the H_2 yield by 2.5 times compared to the wild type (Balderas-Hernandez et al., 2020). Another study, which used modified *E. coli* lacking fumarate reductase and lactate dehydrogenase genes to co-produce hydrogen and ethanol, confirmed these findings Lopez-Hidalgo et al., 2021b. Besides formate regulation and consumption, the significant roles of the membrane transporter *foc*A protein, which controls the influx and efflux of formate in the cell, have also been highlighted. A recent study by Kammel and Sawers (2022) found that deleting the *focA* genes in mutant *E. coli* strains led to 1.5 times higher H_2 production than in wild-type strains. In addition to the formate pathway, the NADH pathway is the second route for H_2 synthesis in bacterial species such as *Enterobacter aerogenes*. The FHL is a multi-enzyme complex system that can utilise NADH as an electron donor to drive H_2 production. Meanwhile, the FHL repressor protein, encoded by *hycA,* suppresses FHL activity. Hence, H_2 production by NADH pathways can be controlled by modifying the FHL system via removal of the *hycA* gene (Bai et al., 2023; McDowall et al., 2014; Zhao et al., 2009).

2.2. Elimination of carbon catabolite repression for co-sugar utilisation

Feedstock for hydrogen production, such as plant biomass, algal biomass, animal waste, and food waste, consists of heterogeneous and homogeneous complexes of saccharides (sugars), lipids, and proteins, which are hydrolysed during fermentation. Saccharides are particularly beneficial substrates for H_2 production due to their high degradability; however, their utilisation at the industrial level is believed to be hindered by some complex regulatory mechanisms, especially carbon catabolite repression (CCR) (Deutscher, 2008; Fox and Prather, 2020; Zeng et al., 2017). CCR ensures that microbial cells prioritise preferred carbon sources, particularly glucose, over less preferred ones, thereby limiting H_2 production from other carbon sources (Kremling et al., 2015; Sievert et al., 2017; Zhang et al., 2014). Metabolic engineering has since been effectively applied to modify microbial metabolic pathways to utilise multiple sugars for efficient hydrogen production (Sievert et al., 2017). Knocking out genes such as *CcpA,* which encodes the catabolite control protein that regulates CCR in certain hydrogen-producing bacteria, has been identified as a key strategy for utilising other sugars, such as xylose and arabinose, thereby improving hydrogen production (Deutscher, 2008). Generally, controlling the production of enzymes needed to break down specific sugars, or blocking the activation of

Table 1
Metabolic engineering strategies applied in bacteria for enhanced H_2 production.

Metabolic engineering strategy	Metabolic pathways redirected/ description	Bacterial strain	Genotype	Substrate	Yield (Fold increase)	Reference
Carbon catabolite expression	Elimination of the phosphotransferase system; simultaneous consumption of glucose and xylose	*Escherichia coli*	WDH strain	Glucose/Xylose	3	(Balderas-Hernandez et al., 2020)
Co-overexpression of the malate dehydrogenase (Mdh) and the malic enzyme (MaeA)	Metabolites redirection by the overexpression of the citrate lyase enzyme	*Escherichia coli*	*ΔldhΔgndΔfrdBC*:: kan (M4)	Glycerol	1.25	(Valle et al., 2023)
Deletion of the lactate dehydrogenase gene (*ldh*)	Hydrogen production	*Caldicellulosiruptor bescii*	JWCB005	Maltose and cellobiose	21–34%	(Cha et al., 2013)
Deletion of the acetate production genes (*ak* and *pta*)	Heterogeneous expression of strong hydrogenases	*Caldicellulosiruptor bescii*	JWCB018	Barley straw	25% more	(Cha et al., 2023)
Heterologous expression (screening mutant strains)	Synthesis of hydrogen	*Escherichia coli*	SS1/*hydA*	Glycerol	1	(Soo et al., 2017)
			SS1/*hycE*	Glycerol	1	(Soo et al., 2017)
Homologous overexpression	Overexpression of [Fe-Fe] hydrogenase (*hydA*)	*Clostridium acetobutylicum*	CA-zwf (pIMP-zwf)	Glucose	1.15	(Son et al., 2021)
			CA-hydA (pMTL-hydA)	Glucose	1.39	(Son et al., 2021)
	Hydrogen production. Lactic acid inhibition (*hydA* gene)	*Clostridium paraputrificum*	M-21	Glucose	1.7	(Morimoto et al., 2005)
	Overexpression of the [Fe—Fe] hydrogenase (*hydA*)	*Clostridium tyrobutyricum*	JM1	Glucose	1.5	(Jo et al., 2010)
	Overexpression of Mdh and MaeA under the PBAD promoter	*Escherichia coli*	*ΔgltAΔfrdBC and ΔgltAΔfrdABCD*	Glycerol	36%	(Valle et al., 2021)
Homologous recombination	Regulation of the NADH/NAD + pool	*Enterobacter aerogenes*	IAM1183	Glucose	17.2%	(Bai et al., 2023)
Hydrogenases overexpression	Introduction to the sucrose metabolic pathway	*Clostridium tyrobutyricum*	ATCC 25755 (Ct)	Sugarcane molasses	910.37 mmol/L (reported so far using molasses)	(Fu et al., 2024)
Integrational mutagenesis	Hydrogen production	*Clostridium tyrobutyricum*	ATCC 25755	Glucose	50%	(Liu et al., 2006)
In silico metabolic network reconstruction	Modification of NAD or NAD(P)-linked hydrogenase	*Citrobacter amalonaticus*	Y19	Succinate, lactate, formate, acetate, ethanol	1.8 molH_2/mol glucose	(Oh et al., 2008)
Multi-gene engineering	Modification of glycolytic pathways	*Escherichia coli*	BW25113(SH5)	Glucose	1.32 molH_2/mol glucose	(Seol et al., 2016)
Overexpression of *gapC*	Improvement of substrate catabolism	*Clostridium acetobutylicum*	ATCC 824	Glucose	24.4%	(Kim et al., 2023)
Overexpression of *RyhB*	Disruption of lactate dehydrogenase and metabolic regulation via the small RNA RyhB	*Klebsiella* sp.	FSoil 024	Glycerol/ Glucose	40–50%	(Chu et al., 2021)
Protein engineering of the large subunit of hydrogenase 3	Improve hydrogenase activity	*Escherichia coli*	KeI2	Formate	8	(Maeda et al., 2008)
Redirection carbon pathway	Improvement of electron and redox metabolism (cac0764)	*Clostridium acetobutylicum*	ATCC 824	Glucose	54%	(Foulquier et al., 2022)
Random mutagenesis	Sugars	*Escherichia coli*	ycgR	Glycerol	2.4	(Tran et al., 2015)

specific inducers of sugar metabolism, using sugar-specific or global regulatory systems forms the basis of this metabolic engineering approach (Deutscher et al., 2006).

For instance, in hydrogen-producing *E. coli,* the glucose phosphotransferase system (PTS^{Glc}) prioritises glucose uptake by blocking the metabolism of other sugars; thus, PTS^{Glc} inactivation has been shown to facilitate the utilisation of other available sugars (Liang et al., 2015). The PTS^{Glc} system of hydrogen-producing bacteria consists of three major components that work together to transport glucose into the cell and modify it for metabolism. The first two components, Enzyme I (*EI*) and phosphocarrier protein, are encoded by genes *ptsI* and *ptsH*, respectively. While EIIA and EIIBC, the glucose-specific proteins in the membrane are encoded by the *crr* and *ptsG* genes, respectively, and play a critical role in the uptake and phosphorylation of glucose during its transport into the cell (Escalante et al., 2012; Gosset, 2005). The deletion of PTS^{Glc} genes in *E. coli* separates glucose transport from PEP-dependent phosphorylation for its metabolism, while the conserved PEP during this process is further utilised for the metabolism of other sugars, such as xylose and arabinose (Carmona et al., 2015). It was also observed that the removal of *ptsG* and *crr* genes in the *E. coli* WDH strain enabled the bacteria to utilise both glucose and xylose concurrently, thereby enhancing H_2 production by ~1.2-fold in the engineered strain (Balderas-Hernandez et al., 2020).

In another study, genes involved in the PTS^{Glc} phosphotransfer cascade were knocked out to examine the effect of a single PTS^{Glc} mutation on cell growth and substrate consumption (Liang et al., 2015). It was found that each mutant exhibited distinct levels of adaptation when both glucose and xylose were present at varying ratios, and that the *ptsI* mutant could quickly consume a substrate mixture with a high xylose concentration (Liang et al., 2015). The study further demonstrated that the deletion of *ptsH* in the strain YL104H led to a marked improvement in succinate yield under both aerobic and anaerobic conditions. This suggests that the strategic selection of specific genes of PTS^{Glc} deletion mutants can significantly enhance the co-utilisation efficiency of glucose and xylose. Another study examined the PTS system mutation by deleting the *araC* genes in the *E. coli* GX50 strain. These genes encode

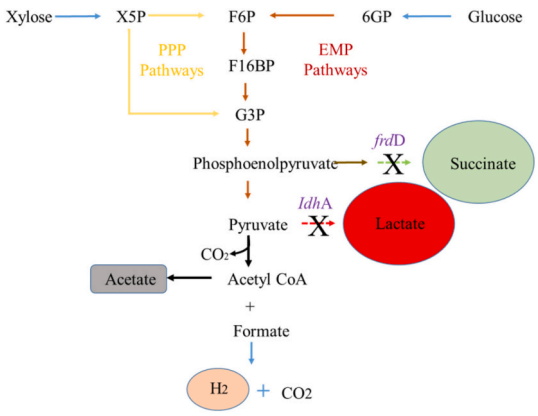

Fig. 2. Carbon flux redirection in *E. coli* via *ldhA* and *frdD* knockout to improve fermentative H_2 production. Adapted and modified from. Lopez-Hidalgo et al., 2021a

transcriptional regulators involved in arabinose catabolism and suppress the transcriptional activator for xylose catabolism. The findings revealed that this mutation enabled the mutant *E. coli* to consume xylose efficiently, even in the presence of glucose (Kim et al., 2015a). A similar study demonstrated that an *araC*-deficient strain of hydrogen-producing bacteria is a successful strategy for overcoming the usual repression of xylose metabolism in the presence of glucose, as it induces simultaneous glucose and xylose uptake (Desai and Rao, 2010; Kim et al., 2015a).

2.3. Redirection of metabolic flux

This strategy involves engineering microbial pathways to channel carbon, energy, and reducing equivalents (e.g., NADH) towards hydrogen production while minimising undesired byproducts such as lactate, ethanol, and acetate. This metabolic engineering approach is a potential novel technique for improving H_2 productivity in certain anaerobic bacteria, such as *Clostridium tyrobutyricum* (Cheng et al., 2013), *C. butyricum* W5 (Cai et al., 2010), and *Desulfovibrio vulgaris* (Marbehan et al., 2024). Metabolic flux techniques are employed to understand and optimise cellular metabolic processes, enabling enhanced hydrogen yield in large-scale bioreactors for commercial applications. These techniques evaluate intracellular changes in response to extracellular perturbations of metabolic and process parameters through stoichiometric analysis and mass balance calculations, providing a comprehensive framework for process optimisation (Cai et al., 2011; Manish et al., 2007). Various wild species of hydrogen-producing bacteria have demonstrated complex metabolic networks for biohydrogen production, highlighting their potential to achieve high hydrogen yields. The advanced development of genetic tools for strict anaerobes has enabled the application of gene deletion studies to metabolic flux analysis in hydrogen-producing bacteria, offering a promising avenue for validating genome-scale models (Heap et al., 2007; Tummala et al., 2003). Incorporating this technique and thermodynamic constraints into genome-scale models enhances our understanding of the behaviour of hydrogen-producing bacteria and may lead to significant improvements in hydrogen production.

To achieve biohydrogen production near the theoretical yield, i.e., 12 mol of H_2 per mol of glucose, a combination of dark and photofermentation is required (Das, 2009). However, before integrating these processes, improvements in hydrogen yield during dark fermentation are necessary. Metabolic flux analysis (MFA) is critical in predicting and optimising metabolic pathways through metabolic engineering to enhance hydrogen productivity during dark fermentation. Recent advancements in genetic tools offer opportunities to engineer improved H_2 production efficiency via targeted metabolic modifications. This is achieved by estimating the distribution of intracellular flux using experimental data integrated with models determined for pure cultures (Gonzalez-Garcia et al., 2017). MFA also facilitates the evaluation of electron and material flow under varying conditions to optimise hydrogen production. Studies have shown that parameters such as hydraulic retention time (HRT) significantly influence metabolic flux, directly impacting hydrogen yields in anaerobic mixed cultures (Cheng and Whang, 2023). Additionally, pH regulation has been shown to affect metabolic flux, thereby influencing H_2 production efficiency in *Ethanoligenens harbinense* B49 (Tang et al., 2024). It was found that when metabolic flux was balanced, the highest H_2 production, 10 mol H_2/mol glucose, was achieved with the mixed culture using acetate as the sole end product and biomass (Gonzalez-Garcia et al., 2017). Proper modification of the metabolic network during flux analysis can enhance experimental results; therefore, validating metabolic flux analysis is crucial for achieving high H_2 yields (Scarborough et al., 2020). Additionally, it has been observed that metabolic engineering tools modified specific pathways to enhance hydrogen production at the laboratory scale; however, in this regard, genome editing approaches are more robust for stable hydrogen production under industrial conditions (Mohanraj et al., 2019; Sinha et al., 2016).

3. Genome editing approach: CRISPR-Cas genome editing tool

Compared to traditional metabolic engineering editing tools, genome editing has attracted greater attention due to its high genetic stability, lower technical complexity and strong scale-up stability performance for bioenergy production (Hong et al., 2018; Zhang et al., 2018). Studies have shown that the capacity to alter and improve microbial genomes provides a more effective and sustainable route to biofuel production; thus, genome editing is poised to play a major role in the shift to a more sustainable, renewable energy future (Wei and Li, 2023). The emergence of genome editing techniques, especially CRISPR-Cas9, has revolutionised industrial production across sectors such as agriculture, bioremediation, and industrial biotechnology by enabling rapid and affordable incorporation of sequence-specific alterations into the genomes of various cell types and species. Other genome editing technologies currently utilised industrially include enabling genome editing, Effector nucleases resembling transcription activators (TALENs), Zinc-finger nucleases (ZFNs), and Endonucleases (Endo) or meganucleases (Mega) that are homing (Gaj et al., 2016). The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) system, which is the most advanced tool in the genome-editing arsenal, enables changes in regulatory elements, disruption or replacement of genes, or addition of particular DNA sequences to microbes and this technology has been effectively deployed in enhancing microbial biofuel production (Wei and Li, 2023). Naturally, the CRISPR-Cas system contributes to bacterial adaptability through immunity via RNA-guided DNA cleavage by Cas proteins, thereby protecting bacterial DNA from invasive viruses and plasmids (Sorek et al., 2013; Wiedenheft et al., 2012). According to Javed et al. (2022), CRISPR-Cas involves site-specific genome editing that improves the microbe's biomass conversion process and produces more biofuel of interest. However, achieving the intended editing result largely depends on optimising the Cas protein, sgRNA, and gene regulatory elements required for sgRNA and Cas protein expression (Bruder et al., 2016; Dong et al., 2022).

CRISPR-Cas has been employed in enhancing biohydrogen production via the insertion of synthetic pathways or genetic modifications into the organismal chassis (Lu et al. (2022). Notably, Wu et al. (2017), utilised CRISPR-Cas9 to delete the *nuo*C, *nuo*D, and *nuo*E NADH dehydrogenase subunits of the *Enterobacter aerogenes* strain IAM1183, resulting in enhanced biohydrogen production (Fig. 3). It was observed that the mutant strain IAM1183-CD (Δ*nuo*C/Δ*nuo*D) and IAM1183-CDE (Δ*nuo*C/Δ*nuo*D/Δ*nuo*E) enhanced hydrogen production by 24.5% and 45.6%, respectively (Wu et al., 2017). These findings have been subsequently validated by Lu et al. (2022). The approach has also been noted to increase biohydrogen production by improving the enzymatic

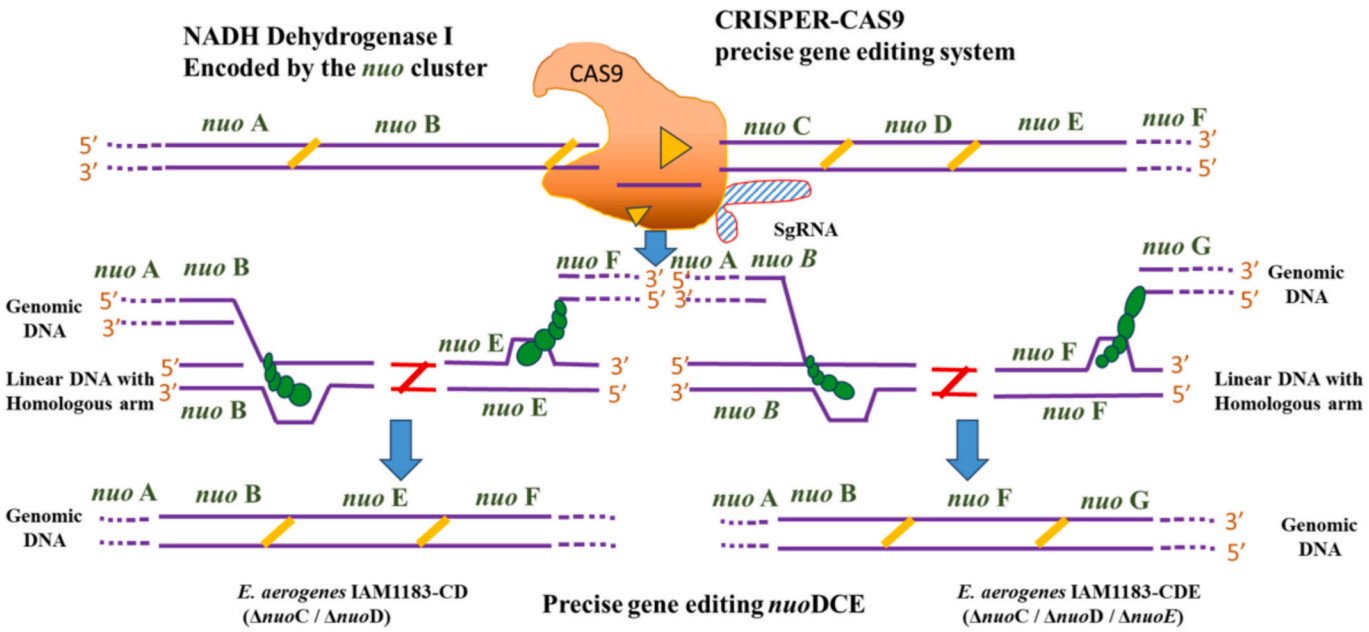

Fig. 3. Construction of Δ*nuo*CD and Δ*nuo*CDE mutants of *Enterobacter aerogenes* IAMII83 by the CRISPR-Cas9 method. Adapted and modified from Wu et al. (2017)

machinery that converts biomass to hydrogen. For instance, CRISPR was used for the overexpression of [FeFe]-Hydrogenase, overexpression of the butyrate pathway, repression of the [NiFe]-Hydrogenase, inactivation/repression of Ldh (lactate dehydrogenase), Adhe (aldehyde dehydrogenase) and Spo0A gene in *Clostridium* spp., which altogether enhanced fermentative biohydrogen production (Husaini et al., 2023). Similarly, improving bioprocess-related genes is equally important. CRISPR-CAS also has the potential to improve microbial tolerance to metabolites and inhibitors, as well as improve the saccharification potential of microorganisms by incorporation of genes for hydrolytic enzymes (Husaini et al., 2023), whereas, in the case of microalgae, following photo-fermentative biohydrogen production can also be enhanced by increasing the photosynthetic efficiency (Zhang et al., 2023). However, a major problem with the application of CRISPR-Cas in some microorganisms is the toxicity of Cas proteins, which can be effectively mitigated by using engineered Cas proteins and small/inducible promoters (Husaini et al., 2023). CRISPR–Cas-based metabolic engineering also poses certain limitations in strict anaerobes and in maintaining the long-term stability of engineered strains during large-scale bioreactor operation for biohydrogen production. Some strict anaerobic species, such as *Clostridium* spp. exhibit low transformation and genome-editing efficiencies, which limit hydrogen productivity during scale-up in industrial bioreactors (Husaini et al., 2023; Pyne et al., 2016).

4. Recombinant gene expression strategies

Recombinant gene expression refers to the production of specific proteins or metabolites via the artificial introduction and expression of a gene of interest in a host organism that is different from its source. It has been identified as a cornerstone of biotechnology, as it has been demonstrated to be quite effective in producing proteins, enzymes, and other biomolecules, such as biohydrogen and other biofuels, at both laboratory and industrial scales (Fan et al., 2020). Recombinant gene expression is primarily governed by two systems: homologous gene expression, which involves the expression of genes native to the host, and heterologous gene expression, which involves the expression of genes foreign to the host organism (Hwang et al., 2021). Both methods are widely applied in microbial biohydrogen production.

4.1. Homologous gene expression

Homologous gene expression entails identifying key hydrogen-producing genes, increasing their expression by altering their promoters or amplifying their genes, or introducing similar genes from related species (Prelich, 2012). Homologous expression can increase the amount and activity of hydrogenase enzymes, the major enzyme responsible for H_2 production, increasing overall hydrogen output in engineered strains (Cao et al., 2022; Girbal et al., 2005; Zhang et al., 2015). For instance, a recent study demonstrated that overexpression of genes encoding hydrogenase enzymes increased hydrogen yield in *Firmicutes* bacteria grown on alkali-based magnetic nanosheets (Cao et al., 2022). Other enzymes, such as malate dehydrogenase (*Mdh*) and malic enzyme A (*MaeA*), also play important roles in enhancing microbial hydrogen production by directing carbon flux towards pyruvate for H_2 production instead of completing the full TCA cycle. A study by Valle et al. (2021) observed that the co-expression of malate dehydrogenase (*Mdh*) and the malic enzyme A (*Mae*A) in *E. coli* mutants derived from the parent *E. coli* BW25113 wild-type significantly enhanced hydrogen production. Specifically, the mutant strains *dcuD* and *frdC-dcuD* exhibited increased H_2 production by 36% and 24%, respectively. In this metabolic engineering strategy, malate plays a crucial role in enhancing the availability of pyruvate, a precursor for H_2 production, and in balancing the NADH/NAD^+ redox ratio, thereby promoting hydrogen production. This finding was supported by various other studies (Dong et al., 2017; Hidese et al., 2020), substantiating the beneficial effects of overexpression of anaplerotic enzymes, which improve carbon redirection towards targeted products. However, in another study, mutant *E. coli* strains such as *gltA* and *frdABCD* failed to achieve the desired hydrogen yield following *Mdh* and *MaeA* overexpression (Valle et al., 2015). Interestingly, it was discovered that co-overexpression of these enzymes only drives cellular growth and cannot induce C-flux for hydrogen production in several mutant *E. coli* strains(Valle et al., 2023; Valle et al., 2021).

In addition to malate metabolic flux, formate metabolic flux is also being investigated, since it is considered the main substrate for hydrogen production (Xiong et al., 2020). Overexpression of the formate hydrogen lyase activator (*fhlA*) gene has been identified as a promising strategy for improving hydrogen production in the facultative anaerobic hydrogen-producing bacterium *Enterobacter cloacae.* For instance, overexpression

of the *fhlA* activator gene in *Enterobacter cloacae* WL1318 resulted in a 188% increase in hydrogen output utilising cotton stalk hydrolysate (Zhang et al., 2020). This yield was achieved by the recombinant strain through a decrease in metabolic flux to the competitive pathways of hydrogen production and an enhancement of FHL activity. A similar metabolic approach of inducing *fhl*A gene overexpression was used for high hydrogen yield in genera such as *Enterobacter* (Zhao et al., 2009), *Escherichia* (Sanchez-Torres et al., 2009), *Clostridia* (Jo et al., 2010) and *Klebsiella* (Jawed et al., 2016).

4.2. Heterologous gene expression

Heterologous gene expression has significantly improved hydrogen productivity via the transfer and overexpression of genes implicated in specific metabolic pathways across different bacterial strains. This method enables the development of bacteria with improved hydrogen productivity by combining new genes and regulatory components. A recent investigation reported the heterologous expression of noncoding RNA *Ryh*B from *E. coli* in *Klebsiella* sp., which improved hydrogen productivity by nearly 50% (Chu et al., 2021). In another study, the *nadE* gene from *K. pneumoniae* was heterologously expressed in *Enterobacter aerogenes*; *nadE* is involved with the NADH pathway, which is critical for anaerobic hydrogen generation; hence, its overexpression resulted in increased availability of NADH, which subsequently diverted the metabolic flux towards hydrogen generation (Wu and Zhao, 2021). Several efforts have been made in the last decade to improve H_2 production by overexpressing the [Fe—Fe] hydrogenases across different bacteria. For instance, certain strains of *Rhodospirillum rubrum* possess inactive *Rrhyd*A genes encoding [Fe—Fe] hydrogenases, which have been successfully expressed in *E. coli* to enhance H_2 production (Abo-Hashesh et al., 2013; Kim et al., 2015b). Similarly, Song et al. (2017) developed a recombinant strain of *E. cloacae*/HPPH by expressing the *hydA* gene, which encodes the hydrogen-promoting protein from *E. cloacae* IIT-BT08, resulting in 50% higher hydrogen output compared to the wild-type strain using pretreated water hyacinth. In another study, in addition to *Rrhyd*A, E.-J. Kim et al. (2015) co-expressed the hydrogenase maturation proteins of *Clostridium acetobutylicum* (*Ca-HydEFG*) to *E. coli*. The study also demonstrates that H_2 production is significantly improved only when the *Rr-HydA* and *Rr-HydB* genes are expressed in a balanced ratio. These findings were corroborated by a similar study, in which the heterogeneous expression of the hydrogenase gene (*RrhydA*) together with the maturation genes from *C. acetobutylicum* in *E. coli* resulted in a two-fold increase in hydrogenase activity and hydrogen production (Abo-Hashesh et al., 2013). Although heterologous gene expression has expanded the options for microbial hydrogen production by transferring non-native genes and pathways to desired strains, the hydrogen yield in many strains remains low (Akaniro et al., 2024). As a result, continued study is required to address the constraints associated with genetic stability and efficiency in various microbial systems.

5. Enhancement of substrate utilisation

Substrate utilisation is key in hydrogen productivity during the dark fermentation process as optimal substrate concentration enhances biohydrogen production, while higher substrate concentration may lead to hydrogen inhibition (Morsy and Ibrahim, 2016). Many anaerobic microbes have been demonstrated to utilise lignocellulosic biomass for their metabolic processes during dark fermentation. Consequently, microbial H_2 production from lignocellulosic biomass, such as agricultural residues, forest waste, and energy crops, is an economical and environmentally friendly approach (Morsy and Ibrahim, 2016). However, the complex structure of lignocellulosic biomass requires pretreatments, such as acid, alkaline, or enzymatic, to release the maximum amount of fermentable sugars necessary for the biosynthesis of microbial hydrogen, thereby increasing fermentation efficiency (Morsy and Ibrahim, 2016). However, the additional enzymatic pretreatment step can be eliminated by using mutant microbial strains that possess the capability to simultaneously degrade this biomass and produce hydrogen. This approach would reduce the number of biotechnological stages required for the effective utilisation of organic material, thereby decreasing costs, saving time, and increasing the number of industrial cycles in such biotechnological processes. For instance, *E. coli* strain ZH-4 isolated from bovine rumen was engineered with enhanced cellulose and hemicellulose degrading capabilities, with a degradation ratio of 14.30% for cellulose and 11.39% for hemicellulose and biohydrogen production capacity of 4.71 mL H_2 per gram from corn straw (Pang et al., 2017). This engineered strain demonstrates great potential for industrial applications. Further metabolic and regulatory investigations of this strain are needed to explore its enzyme secretion mechanisms for application in single-cell refineries to co-produce multiple value-added products by utilising biomass or waste materials. In addition to lignocellulosic biomass, crude glycerol has also been reported for biohydrogen production. Cofré et al. (2016) studied batch and fed-batch performance on both laboratory and pilot scales using the *E. coli* MG1655 wild-type strain with glycerol from a biodiesel plant as the feedstock. It was found that there was no difference in hydrogen productivity between 5.5 L and 200 L pilot-scale bioreactors using pure and crude glycerol. In another study, researchers developed a glycerol impurity-tolerant co-culture of *E. coli* and *Enterobacter* sp. that produces H_2 from biodiesel waste glycerol. It was observed that the developed co-culture of *E. coli* CECT432 and *Enterobacter* sp. H1 produced 3.1-fold higher productivity than the pure culture, with a yield of 1.53 mol H_2/mol glycerol (Maru et al., 2016). Supplementation of the major carbon sources with other nutrients has also been noted to enhance substrate utilisation to maximise biohydrogen production. A study by Sharma and Melkania (2018b) using anaerobic recombinant *E. coli* and *E. aerogenes* showed the positive effect of amino acids when an organic fraction of municipal solid waste was used as a carbon source. The developed strains produced 685.4 ± 10.1 mL of hydrogen when alanine was supplemented with an organic fraction of municipal solid waste. However, these recombinant strains cannot be used for large-scale hydrogen production with commercial nitrogen sources. Therefore, inexpensive and renewable nitrogen sources must be investigated for future applications in hydrogen production. Another study reported the development of a novel *E. coli* BW25113 strain for renewable H_2 production using brewery spent grains (Poladyan et al., 2018). *E. coli* HD701 was also demonstrated to utilise a mixture of molasses and rotten dates for hydrogen production (Morsy and Ibrahim, 2016). The results from these recombinant strains could pave the way for developing new approaches to hydrogen production combined with simultaneous waste treatment.

6. Optimisation of process engineering parameters to reduce toxicity

One of the major impediments to scaling up microbial biohydrogen production is the generation of various toxic products during dark fermentation. As a result, special focus has been placed on ameliorating product toxicity and inhibition, especially at the dark fermentation stage of biohydrogen production. These inhibitory substrates are also produced during the pretreatment and strategies for their reduction have shown significant effects in increasing the feasibility and economy of scale in biohydrogen production (Mıynat and Argun, 2020b). The process engineering strategies employed in various studies can be classified into dilution of inhibitors, inactivation of inhibitors, removal of inhibitors, and modification of operational parameters (Table 2).

6.1. Dilution and removal of inhibitors

Efficient biohydrogen production is often hindered by the presence of toxic inhibitors generated during substrate pretreatment, making

Table 2
Inhibition strategies to overcome product toxicity during hydrogen production.

Inhibition strategy	Microorganism	Specific strategy	Operational mode	Substrate	pH	Hydrogen yield	Reference
Adjusting the operational parameters	*Clostridium, Enterobacter and Klebsiella*	Maintaining the carbon/nitrogen ratio	Continuous feeding	Synthetic wastewater	6.5	3.5 mol H_2 /mol sucrose	(del Pilar Anzola-Rojas et al., 2015)
	Mesophilic anaerobic sludge	Food waste / anaerobic fermentation with ammonia	Batch fermentation	Food waste	Inoculum (7.70) Food waste (4.75)	Hydrogen yield of 121.4 mL-H_2/gVS	(Pan et al., 2013)
	Mixed anaerobic culture	Maintaining the sCOD/ tCOD ratio of 0.46.	Batch fermentation (35 ± 2 °C)	Municipal solid waste	Feedstock (pH 4.18 ± 0.36)	H_2 yield of 2.05 ± 0.33 M of H_2/M of carbohydrate	(Elsamadony and Tawfik, 2015)
	Sulfate-reducing bacteria	COD/ Sulphate ratio for methane inhibition	Batch fermentation	Glucose	6.0 and 6.75	COD/SO_4^{2-} ratio of 2.18 and pH set at 6.0. Hydrogen production was dominated	(Moon et al., 2013)
	Sulfate-reducing bacteria	Pretreated graphite electrodes completely inhibiting methanogens	Sequential batch fermentation	Glucose	6.2–6.5	3.67 ± 0.31 M/M of glucose was recorded	(Singh and Singh, 2021b)
Dilution	Mesophilic anaerobic culture	Sludge (24.5 times)	Batch fermentation	Food waste	4.75 ± 0.01	60.9 mL-H_2/gVS	(Pan et al., 2013)
	Mixed consortia	New substrate (25% and 50%)	Batch fermentation	Glucose	7.0	2.2 ± 1.3 mM/h	(Srikanth and Mohan, 2014)
	Microbial consortium	Rew substrate (233,466 and 700 mL)	Batch fermentation	Lactose	7.0	148 mmol H_2/L days	(Romão et al., 2019)
	Mixed anaerobic culture	130% of the initial working volume	Batch fermentation	Molasses	6.8	21. 47 mL H_2/h	(Mıynat and Argun, 2020b)
	Clostridium and *Ruminococcaceae*	Detoxifying step for steam-exploded inhibitors	Batch fermentation (37 °C)	Corn stock	6.5	40% higher hydrogen yield	(Liu et al., 2015)
Inactivation of inhibitors	*Anaerobic granular sludge*	Pretreated anaerobic granular sludge (110 °C, 24 h)	Batch fermentation	Lignocellulosic hydrolysates	6.5–6.8	912 Hydrogen production rate (mL H_2/L/d) and Inhibition coefficient (%) was found Zero %	(Muñoz-Páez et al., 2019)
	Domestic biogas digester	Hydrothermal pretreated Degradation and transformation of furfural derivatives	Batch fermentation	Algae and lignocellulose biomass	6.5	188.5 mL/g glucose 100% inhibitor degradation	(Sun et al., 2019)
	Domestic biogas digester	Hydrothermal pretreated algae and lignocellulosic biomass	Batch fermentation	Algae and lignocellulosic biomass	6.5	Inhibitors in the range of 1 to 4 g/L were 100% degraded in 48 h of fermentation	(Sun et al., 2020)
Removal of inhibitors	Sewage sludge	Biochar	Batch fermentation (32 °C)	Food waste	6.4	144.26, increased by 127.8%	(Li et al., 2020; Zhao et al., 2021)
	Thermoanaerobacterium thermosaccharolyticum M18		Batch fermentation (60 °C)	Fungal-pretreated cornstalk	7.5	274.9, increased by 89%	(Zhao et al., 2021)
	River sediment and MJ2		Batch fermentation (55 °C)	Pretreated sugarcane bagasse	7.08	319.5, increased by 79.8%	(Huang et al., 2022)
	Sulfate-reducing bacteria	Heat treatment for removing methanogens	Batch fermentation (37 °C)	Glucose	6.8	2.814 ± 0.091 M/M of glucose	(Singh and Singh, 2021a)
	Sulfate-reducing bacteria	Ferrous and sulphate ions	Batch fermentation (37 °C)	Glucose	6.5	2.523 ± 0.230 H_2 M/M glucose	(Singh et al., 2021)

dilution and removal of these compounds a critical aspect of process engineering to enhance microbial activity and hydrogen yields (Basak et al., 2020). Bacteriocins and volatile fatty acids (VFA) have been identified as the most notorious metabolites which limit biohydrogen production during dark fermentation. The dilution of these metabolites has been demonstrated as a key and cost-effective strategy to counteract their toxic effects during biohydrogen generation (Mıynat and Argun, 2020b; Pan et al., 2013; Srikanth and Mohan, 2014). The main idea behind dilution is to lower the concentration of the VFA formed during the fermentation process for hydrogen production. The reduction in pH caused by the accumulation of VFAs can be effectively managed by maintaining the pH within the range of 6.5 to 6.8. This can be achieved by periodically replacing 30% to 50% of the substrate within the bioreactor with fresh substrate. This will enable the restart of the hydrogen production process without lowering the substrate concentration (Mıynat and Argun, 2020b; Srikanth and Mohan, 2014). Similarly, the reduction of bacteriocins produced by lactic bacteria can be achieved by feeding bioreactors with a fresh nutrient medium containing lactose as a carbon source (Romão et al., 2019). The dilution strategy has also been identified as suitable to regulate VFA levels within the desired concentration range, thereby enhancing hydrogen productivity from molasses and reducing the cost of integrating VFA separation processes (Mıynat and Argun, 2020a). Additionally, studies have shown that a decline in hydrogen production efficiency is correlated with increased methane production efficiency in ammonia-inhibited reactors during the two-phase anaerobic digestion process (Mustafa et al., 2019).

Furthermore, targeted inhibition using sulfate, heavy metals, and ammonia has been identified as an effective approach in other studies (Singh and Singh, 2021b). Inhibitor removal aims to lower the concentration of inhibitors below the threshold level, thus nullifying their toxic effect on hydrogen yield. For instance, metal ions have been identified as a major cause of inhibition of hydrogen producers in anaerobic digesters (Yang et al., 2015). Studies have highlighted the potential of the biosorbent process in removing these ions during dark fermentation (Qin et al., 2020). Optimisation coupled with the supplementation of metal additives (Ni and Fe) and metabolic engineering of *Clostridium pasteurianum* led to a 6.87-fold increase in hydrogen productivity (Li et al., 2025). This dual strategy provides ideal conditions for the genetically modified hydrogen-producing bacteria for efficient hydrogen production. The mechanism of heavy metal inhibition is illustrated in Fig. 4. Electrokinetic and bioleaching are other effective techniques for heavy metal removal from the biohydrogen production substrate (Xu et al., 2017).

6.2. Inactivation of inhibitors

Another important strategy to ameliorate the deleterious effects of inhibitors, such as thiosulfate, bacteriocins, and phenolic compounds, during fermentative biohydrogen production is their inactivation. Studies have demonstrated that these toxic inhibitors can be detoxified or inactivated through acid treatment (Tsai et al., 2021), alkali treatment (Galindo-Hernández et al., 2018), evaporation, or hydrolysis of lignocellulosic biomass (Hu et al., 2018). These methods, when integrated with upstream pretreatment and downstream fermentation processes, significantly enhance hydrogen yield (Tsai et al., 2021). For instance, general heat pretreatment at 100 °C and alkali pretreatment at pH 9.0 were found to inactivate the effect of thiosulphate (Tao et al., 2020). It has also been demonstrated that liquid-solid extraction, liquid-liquid extraction, and heat treatment methods can be highly effective in detoxifying the various inhibitors in bioreactor systems (Chen et al., 2021). However, some consider these treatments to be complex and uneconomical (Chen et al., 2021). In contrast, a method of utilising the furan and phenolic compounds for hydrogen production using specific microorganisms is found to be economical, where genetically modified hydrogen-producing bacteria minimise or inactivate the toxic effects of the phenolic compounds in anaerobic bioreactors (Akobi et al., 2016; Li et al., 2019; Muñoz-Páez et al., 2019). Studies have also reported that enzyme treatment, such as trypsin (a serine protease enzyme), can inactivate the effect of bacteriocins (Godvin Sharmila et al., 2022). Inactivation of inhibitors through process engineering strategies has thus emerged as a promising approach for enhancing hydrogen production. Researchers used genetically modified *Clostridium acetobutylicum* ATCC 824 culture under pre-optimised conditions (37 °C, 200 rpm and pH 6.5), produced 66.3% higher hydrogen, even in the presence of inhibitors such as vanillin, 5-hydroxymethylfufural, acetic acid, and formic acid (Kim et al., 2023). However, effective control of inhibitory factors is still to be explored, essential for establishing this strategy as a sustainable approach within the renewable energy sector (Elbeshbishy et al., 2017).

6.3. Modifying operational parameters

Optimising operational parameters plays a crucial role in biohydrogen production through the dark fermentation process. Engineering processes often involve optimising parameters, such as fermentation temperature, pH, and the carbon-to-nitrogen ratio, which have been noted to significantly affect hydrogen productivity during dark fermentation. In more specific terms, tuning these parameters could facilitate the mitigation of hydrogen inhibition in the bioreactors. For example, a C/N ratio in the range of 47–200 is optimal for the operation of biohydrogen reactors (del Pilar Anzola-Rojas et al., 2015). This ratio can be maintained by increasing the biomass of hydrogen-producing microorganisms in an anaerobic reactor. Notably, an imbalance in the food-to-microorganism (F/M) ratio has been shown to inhibit hydrogen production (Pan et al., 2013). The introduction of sulphate-reducing bacteria into fermentation systems has also been noted to attenuate sulphate inhibition, and this may simply be achieved by maintaining pH in the range of 6.2 to 5.5 to completely stop the sulphate inhibition (Sharma and Melkania, 2018a). Similarly, sulphide toxicity during hydrogen production can also be counteracted by ferrous ion supplementation in the bioreactors, as ferrous ion precipitates the sulphur ions, thus mitigating their toxicity (Dhar et al., 2012). On the other hand, methanogen inhibition can be effectively reduced by heat shock treatment methods (55 °C to 65 °C for 30 min) and the application of 40 mV external voltage (Singh and Singh, 2021a). Like in many other bioproduction processes, the optimisation of agitation speed in the range

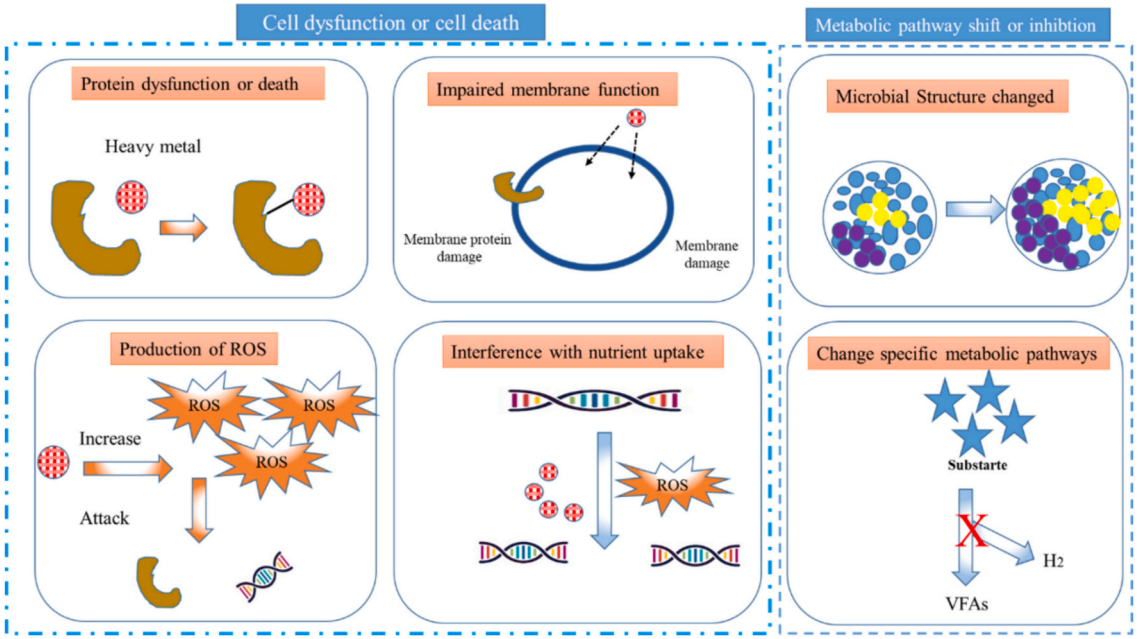

Fig. 4. Systematic mechanism of heavy metal inhibition in microbial cells of hydrogen-producing bacteria. Modified from (Chen et al., 2021).

of 200 to 600 rpm has also been noted to directly affect hydrogen dissolution in the liquid phase and, consequently, productivity in biohydrogen reactors (Beckers et al., 2015; Dreschke et al., 2019). The temperature and agitation are critical parameters for boosting the hydrogen productivity in genetically modified strains. In this context, the study demonstrates that genetic engineering of *Enterobacter aerogenes* IAM1183 (EaΔCΔEF/P strain), under optimised parameters (37 °C, 200 rpm), achieved a 17.2% increase in hydrogen production in a 4.5 L bioreactor in 44 h (Bai et al., 2023). However, an integrated framework of utilising genetically and metabolically engineered organisms with process optimisation conditions for biohydrogen production has not been studied well, but it significantly offers high potential for further enhancing hydrogen productivity.

7. Conclusion and future prospects

Recent advancements in various biohydrogen production technologies have significantly overcome the formidable obstacles of low hydrogen yield, making it feasible at laboratory and pilot scales. Although there is still much to be desired regarding hydrogen production through cellular activity, it is believed that applying metabolic and genetic engineering tools via the dark fermentation process will significantly enhance the applicability of this concept, particularly in terms of economics. There are now various indications that these approaches have the potential to overcome the numerous constraints encountered during the typical fermentation process, thereby increasing the likelihood of achieving hydrogen productivity near the theoretical value (4 mol/mol of glucose). These approaches, which enhance the degradability of hemicellulose and cellulose fermentation activity, have been identified to include the elimination of competing pathways, catabolite repression, as well as both homologous and heterologous gene expression.

Recently, metabolic flux analysis and advanced genome engineering tools, particularly the CRISPR-Cas9 gene editing system, have gained significant traction in facilitating industrial hydrogen production through strain improvement. Although these developed strains undoubtedly produce more biohydrogen, there are some concerns about their stability and safety. Hence, more detailed investigations are needed into the suitability of these genetically modified microbes, especially their performance at the bioreactor level. Biochemical pathways and metabolic engineering can also be coupled to improve H_2 yield. Still, mutant organisms fail to adapt to environmental conditions. Therefore, a precise investigation in this area is needed in future research.

The sustainability of biohydrogen generation can also be enhanced by hybrid pathway engineering, which combines pathways from different organisms to increase synergistic efficiency, as well as modifying the bioprocess to further utilise widely available feedstocks, especially nutrient-rich agricultural residues. An increased understanding of the huge repository of genomics, proteomics, transcriptomics, and metabolomics data currently available in various databases would go a long way in manipulating cellular systems for biohydrogen production. Integrating these systems omics datasets will allow researchers to understand the whole biological system rather than focusing on single genes and pathways. It is also believed that attaining high efficiency in biohydrogen production can be hastened by optimising genetic and metabolic modifications via computational technology, especially machine learning for modelling and simulation networks. Machine learning approach has great potential for improving hydrogen production, as they are based on publicly accessible online databases or previously recorded experimental data. Therefore, future research in advanced machine learning, such as multi-view learning and deep learning driven models, will be expected to predict accurate interpretability of hydrogen production data.

Although recent research findings revealed that synthetic microbial consortia potentially produced high hydrogen yield, it still needs further investigation to understand the population dynamics and shift of metabolic electron flow from butyrate/propionate to acetate pathways for reactor stability during hydrogen production. One of the pertinent issues that needs to be addressed is the undesirable oxygen sensitivity of hydrogenase, as minimal oxygen concentrations have been shown to inactivate the crucial 4Fe—4S cluster of the hydrogenase enzyme, which critically affects biohydrogen productivity.

Despite the success of metabolic and genetic engineering, appropriate, cost-effective strategies to minimise the effect of product toxicity still need to be explored for commercialisation. Future research should also focus on co-fermentation, minimisation of inhibitors, and product toxicity to improve the economy of scale. For example, an integrated approach to mitigate co-existing inhibitors during the hydrogen fermentation process may be explored. Finally, it is hoped that all of these will provoke the development of practical and scalable technologies that can be implemented in real-time for commercial and affordable hydrogen production to enhance the sustainability of our fragile environment.

CRediT authorship contribution statement

Neeraj Kumar Singh: Writing – review & editing, Writing – original draft, Conceptualization. **Prashant Bhagwat:** Writing – review & editing, Writing – original draft, Conceptualization. **Ayodeji Amobonye:** Writing – review & editing. **Manoj Kumar:** Writing – review & editing, Writing – original draft. **Santhosh Pillai:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was supported by the BRICS Joint Science and Technology Research Collaboration grant from the National Research Foundation (NRF), Republic of South Africa, under Grant number 150503.

Data availability

No data was used for the research described in the article.

References

Abo-Hashesh, M., Sabourin-Provost, G., Hallenbeck, P.C., 2013. RrHydA is inactive when overexpressed in *Rhodospirillum rubrum* but can be matured in *Escherichia coli*. Int. J. Hydrog. Energy 3826, 11233–11240. https://doi.org/10.1016/j.ijhydene.2013.06.064.

Akaniro, I.R., Oladipo, A.A., Onwujekwe, E.C., 2024. Metabolic engineering approaches for scale-up of fermentative biohydrogen production–a review. Int. J. Hydrog. Energy 52, 240–264. https://doi.org/10.1016/j.ijhydene.2023.04.328.

Akobi, C., Hafez, H., Nakhla, G., 2016. The impact of furfural concentrations and substrate-to-biomass ratios on biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge. Bioresour. Technol. 221, 598–606. https://doi.org/10.1016/j.biortech.2016.09.067.

Akram, F., Fatima, T., Ibrar, R., ul Haq, I., 2024. Biohydrogen: production, promising progressions and challenges of a green carbon-free energy. Sustain. Energy Technol. Assess. 69, 103893. https://doi.org/10.1016/j.seta.2024.103893.

Ayodele, D.T., Ogunbiyi, O.D., Akamo, D.O., Otun, K.O., Akinpelu, D.A., Adegoke, J.A., Fapojuwo, D.P., Oladoye, P.O., 2023. Factors affecting biohydrogen production: overview and perspectives. Int. J. Hydrog. Energy. https://doi.org/10.1016/j.ijhydene.2023.04.001.

Bai, R., Chu, W., Qiao, Z., Lu, P., Jiang, K., Xu, Y., Yang, J., Gao, T., Xu, F., Zhao, H., 2023. Metabolic regulation of NADH supply and hydrogen production in Enterobacter aerogenes by multi-gene engineering. Int. J. Hydrog. Energy *48*3, 909–920. https://doi.org/10.1016/j.ijhydene.2022.10.015.

Balderas-Hernandez, V.E., Maldonado, K.P.L., Sánchez, A., Smoliński, A., Rodriguez, A. D.L., 2020. Improvement of hydrogen production by metabolic engineering of Escherichia coli: Modification on both the PTS system and central carbon

metabolism. Int. J. Hydrog. Energy 459, 5687–5696. https://doi.org/10.1016/j.ijhydene.2019.01.162.
Ban, S., Lin, W., Luo, Z., Luo, J., 2019. Improving hydrogen production of Chlamydomonas reinhardtii by reducing chlorophyll content via atmospheric and room temperature plasma. Bioresour. Technol. 275, 425–429. https://doi.org/10.1016/j.biortech.2018.12.062.
Basak, B., Jeon, B.-H., Kim, T.H., Lee, J.-C., Chatterjee, P.K., Lim, H., 2020. Dark fermentative hydrogen production from pretreated lignocellulosic biomass: Effects of inhibitory byproducts and recent trends in mitigation strategies. Renew. Sust. Energ. Rev. 133, 110338. https://doi.org/10.1016/j.rser.2020.110338.
Beckers, L., Masset, J., Hamilton, C., Delvigne, F., Toye, D., Crine, M., Thonart, P., Hiligsmann, S., 2015. Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain Clostridium butyricum CWBI1009. Biochem. Eng. J. 98, 18–28. https://doi.org/10.1016/j.bej.2015.01.008.
Bhagwat, P., Permaul, K., Singh, S., Pillai, S., 2024. Feedstocks and cases III: Biohydrogen from Algal Biomass (Macro and Microalgae). In: Soccol, C.R., Brar, S.K., Permaul, K., Pakshirajan, K., de Carvalho, J.C. (Eds.), Biohydrogen - Advances and Processes. Springer Nature Switzerland, pp. 337–364. https://doi.org/10.1007/978-3-031-49818-3_13.
Bruder, M.R., Pyne, M.E., Moo-Young, M., Chung, D.A., Chou, C.P., 2016. Extending CRISPR-Cas9 technology from genome editing to transcriptional engineering in the genus Clostridium. Appl. Environ. Microbiol. 8220, 6109–6119. https://doi.org/10.1128/AEM.02128-16.
Cai, G., Jin, B., Saint, C., & Monis, P. 2010. Metabolic flux analysis of hydrogen production network by *Clostridium butyricum* W5: effect of pH and glucose concentrations. Int. J. Hydrog. Energy, *35*13, 6681–6690. doi:https://doi.org/10.1016/j.ijhydene.2010.04.097.
Cai, G., Jin, B., Monis, P., Saint, C., 2011. Metabolic flux network and analysis of fermentative hydrogen production. Biotechnol. Adv. 294, 375–387. https://doi.org/10.1016/j.biotechadv.2011.02.001.
Canadell, J.G., Monteiro, P.M., Costa, M.H., Cotrim da Cunha, L., Cox, P.M., Eliseev, A. V., Henson, S., Ishii, M., Jaccard, S., Koven, C., 2023. Intergovernmental Panel on climate Change (IPCC). Global carbon and other biogeochemical cycles and feedbacks. In: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, pp. 673–816.
Cao, X., Zhao, L., Dong, W., Mo, H., Ba, T., Li, T., Guan, D., Zhao, W., Wang, N., Ma, Z., 2022. Revealing the mechanisms of alkali-based magnetic nanosheets enhanced hydrogen production from dark fermentation: Comparison between mesophilic and thermophilic conditions. Bioresour. Technol. 343, 126141. https://doi.org/10.1016/j.biortech.2021.126141.
Carmona, S. B., Moreno, F., Bolívar, F., Gosset, G., & Escalante, A. 2015. Inactivation of the PTS as a strategy to engineer the production of aromatic metabolites in *Escherichia coli*. J. Mol. Microbiol. Biotechnol., *25*2-3, 195–208. doi:https://doi.org/10.1159/000380854.
Cha, M., Chung, D., Elkins, J.G., Guss, A.M., Westpheling, J., 2013. Metabolic engineering of Caldicellulosiruptor bescii yields increased hydrogen production from lignocellulosic biomass. Biotechnol. Biofuels 6, 1–8. https://doi.org/10.1186/1754-6834-6-85.
Cha, M., Kim, J.-H., Choi, H.-J., Nho, S.B., Kim, S.-Y., Cha, Y.-L., Song, H., Lee, W.-H., Kim, S.-K., Kim, S.-J., 2023. Hydrogen Production from Barley Straw and Miscanthus by the Hyperthermophilic Bacterium, Cadicellulosirupter bescii. J. Microbiol. Biotechnol. 3310, 1384. https://doi.org/10.4014/jmb.2305.05022.
Chen, Y., Yin, Y., Wang, J., 2021. Recent advance in inhibition of dark fermentative hydrogen production. Int. J. Hydrog. Energy *467*, 5053–5073. https://doi.org/10.1016/j.ijhydene.2020.11.096.
Cheng, H.-H., Whang, L.-M., 2023. Applying metabolic flux analysis to hydrogen fermentation using a metabolic network constructed for anaerobic mixed cultures. Environ. Res. 235, 116636. https://doi.org/10.1016/j.envres.2023.116636.
Cheng, H.-H., Whang, L.-M., Lin, C.-A., Liu, I.C., Wu, C.-W., 2013. Metabolic flux network analysis of fermentative hydrogen production: using Clostridium tyrobutyricum as an example. Bioresour. Technol. 141, 233–239. https://doi.org/10.1016/j.biortech.2013.03.141.
Chu, W., Xu, Y., Yang, J., Wu, Y., Wei, X., Hao, Y., Wang, S., Zhao, H., 2021. Effect of biohydrogen by overexpressing small RNA RyhB combined with ldh impairment in novel Klebsiella sp. FSoil 024. Int. J. Hydrog. Energy *46*37, 19303–19311. https://doi.org/10.1016/j.ijhydene.2021.03.087.
Cofré, O., Ramírez, M., Gómez, J., Cantero, D., 2016. Pilot scale fed-batch fermentation in a closed loop mixed reactor for the biotransformation of crude glycerol into ethanol and hydrogen by Escherichia coli MG1655. Biomass Bioenergy 91, 37–47. https://doi.org/10.1016/j.biombioe.2016.04.015.
Das, D. 2009. Advances in biohydrogen production processes: an approach towards commercialization. Int. J. Hydrog. Energy, *34*17, 7349–7357. doi:https://doi.org/10.1016/j.ijhydene.2008.12.013.
del Pilar Anzola-Rojas, M., da Fonseca, S.G., da Silva, C.C., de Oliveira, V.M., Zaiat, M., 2015. The use of the carbon/nitrogen ratio and specific organic loading rate as tools for improving biohydrogen production in fixed-bed reactors. Biotechnol. Rep. 5, 46–54. https://doi.org/10.1016/j.btre.2014.10.010.
Desai, T.A., Rao, C.V., 2010. Regulation of arabinose and xylose metabolism in Escherichia coli. Appl. Environ. Microbiol. 765, 1524–1532. https://doi.org/10.1128/AEM.01970-09.
Deutscher, J., 2008. The mechanisms of carbon catabolite repression in bacteria. Curr. Opin. Microbiol. 112, 87–93. https://doi.org/10.1016/j.mib.2008.02.007.
Deutscher, J., Francke, C., Postma, P.W., 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. 704, 939–1031. https://doi.org/10.1128/mmbr.00024-06.
Dhar, B.R., Elbeshbishy, E., Nakhla, G., 2012. Influence of iron on sulfide inhibition in dark biohydrogen fermentation. Bioresour. Technol. 126, 123–130. https://doi.org/10.1016/j.biortech.2012.09.043.
Dong, C., Gou, Y., Lian, J., 2022. SgRNA engineering for improved genome editing and expanded functional assays. Curr. Opin. Biotechnol. 75, 102697. https://doi.org/10.1016/j.copbio.2022.102697.
Dong, X., Chen, X., Qian, Y., Wang, Y., Wang, L., Qiao, W., Liu, L., 2017. Metabolic engineering of Escherichia coli W3110 to produce L-malate. Biotechnol. Bioeng. 1143, 656–664. https://doi.org/10.1002/bit.26190.
Dreschke, G., Papirio, S., Lens, P.N., Esposito, G., 2019. Influence of liquid-phase hydrogen on dark fermentation by Thermotoga neapolitana. Renew. Energy 140, 354–360. https://doi.org/10.1016/j.renene.2019.02.126.
Elbeshbishy, E., Dhar, B.R., Nakhla, G., Lee, H.-S., 2017. A critical review on inhibition of dark biohydrogen fermentation. Renew. Sust. Energ. Rev. 79, 656–668. https://doi.org/10.1016/j.rser.2017.05.075.
Elsamadony, M., Tawfik, A., 2015. Potential of biohydrogen production from organic fraction of municipal solid waste (OFMSW) using pilot-scale dry anaerobic reactor. Bioresour. Technol. 196, 9–16. https://doi.org/10.1016/j.biortech.2015.07.048.
Ersoy, Ş., Akaçin, İ., & Güngörmüşler, M. 2023. Comparative evaluation of the biohydrogen production potential of thermophilic microorganisms isolated from hot springs located in Izmir. Int. J. Hydrog. Energy, *48*60, 22897–22908. doi:https://doi.org/10.1016/j.ijhydene.2023.02.092.
Escalante, A., Salinas Cervantes, A., Gosset, G., Bolívar, F., 2012. Current knowledge of the Escherichia coli phosphoenolpyruvate–carbohydrate phosphotransferase system: peculiarities of regulation and impact on growth and product formation. Appl. Microbiol. Biotechnol. 94, 1483–1494. https://doi.org/10.1007/s00253-012-4101-5.
Faloye, F., Kana, E.G., Schmidt, S., 2013. Optimization of hybrid inoculum development techniques for biohydrogen production and preliminary scale up. Int. J. Hydrog. Energy 3827, 11765–11773. https://doi.org/10.1016/j.ijhydene.2013.06.129.
Fan, Q., Neubauer, P., Lenz, O., Gimpel, M., 2020. Heterologous hydrogenase overproduction systems for biotechnology—an overview. Int. J. Mol. Sci. 2116, 5890. https://doi.org/10.3390/ijms21165890.
Foulquier, C., Rivière, A., Heulot, M., Dos Reis, S., Perdu, C., Girbal, L., Pinault, M., Dusséaux, S., Yoo, M., Soucaille, P., 2022. Molecular characterization of the missing electron pathways for butanol synthesis in Clostridium acetobutylicum. Nat. Commun. 131, 4691. https://doi.org/10.1038/s41467-022-32269-1.
Fox, K.J., Prather, K.L., 2020. Carbon catabolite repression relaxation in Escherichia coli: global and sugar-specific methods for glucose and secondary sugar co-utilization. Curr. Opin. Chem. Eng. 30, 9–16. https://doi.org/10.1016/j.coche.2020.05.005.
Fu, H., Yang, D., Li, X., Guo, X., Mo, Y., Wang, S., Wang, J., 2024. Metabolic and process engineering of Clostridium tyrobutyricum for efficient hydrogen production from sugarcane molasses. Fuel 371, 132075. https://doi.org/10.1016/j.fuel.2024.132075.
Gaj, T., Sirk, S. J., Shui, S.-l., & Liu, J. 2016. Genome-editing technologies: principles and applications. Cold Spring Harb. Perspect. Biol., *8*12, a023754. https://doi.org/10.1101/cshperspect.a023754.
Galindo-Hernández, K.L., Tapia-Rodríguez, A., Alatriste-Mondragón, F., Celis, L.B., Arreola-Vargas, J., Razo-Flores, E., 2018. Enhancing saccharification of Agave tequilana bagasse by oxidative delignification and enzymatic synergism for the production of hydrogen and methane. Int. J. Hydrog. Energy 4349, 22116–22125. https://doi.org/10.1016/j.ijhydene.2018.10.071.
García-Depraect, O., Castro-Muñoz, R., Muñoz, R., Rene, E.R., León-Becerril, E., Valdez-Vazquez, I., Kumar, G., Reyes-Alvarado, L.C., Martínez-Mendoza, L.J., Carrillo-Reyes, J., 2021. A review on the factors influencing biohydrogen production from lactate: the key to unlocking enhanced dark fermentative processes. Bioresour. Technol. 324, 124595. https://doi.org/10.1016/j.biortech.2020.124595.
Gevorgyan, H., Vassilian, A., Poladyan, A., Trchounian, K., 2022. The influence of hydrogen production on the formation of metabolic pathways and regulation of ΔpH in Escherichia coli. Int. J. Hydrog. Energy *47*95, 40264–40274. https://doi.org/10.1016/j.ijhydene.2022.07.227.
Girbal, L., Abendroth, G., v., Winkler, M., Benton, P. M. C., Meynial-Salles, I., Croux, C., Peters, J. W., Happe, T., & Soucaille, P., 2005. Homologous and Heterologous Overexpression in *Clostridium acetobutylicum* and Characterization of Purified Clostridial and Algal Fe-only Hydrogenases with High specific Activities. Appl. Environ. Microbiol. 715, 2777–2781. https://doi.org/10.1128/AEM.71.5.2777-2781.2005.
Godvin Sharmila, V., Tamilarasan, K., Dinesh Kumar, M., Gopalakrishnan, K., Sunita, V., Adish Kumar, S., & Rajesh Banu, J. 2022. Trends in dark biohydrogen production strategy and linkages with transition towards low carbon economy: an outlook, cost-effectiveness, bottlenecks and future scope. Int. J. Hydrog. Energy, *47*34, 15309–15332. doi:https://doi.org/10.1016/j.ijhydene.2021.12.139.
Gonzalez-Garcia, R., Aispuro-Castro, R., Salgado-Manjarrez, E., Aranda-Barradas, J., Garcia-Pena, E., 2017. Metabolic pathway and flux analysis of H2 production by an anaerobic mixed culture. Int. J. Hydrog. Energy *42*7, 4069–4082. https://doi.org/10.1016/j.ijhydene.2017.01.043.
Gosset, G., 2005. Improvement of Escherichia coli production strains by modification of the phosphoenolpyruvate: sugar phosphotransferase system. Microb. Cell Factories 4, 1–11. https://doi.org/10.1186/1475-2859-4-14.
Goyal, Y., Kumar, M., Gayen, K., 2013. Metabolic engineering for enhanced hydrogen production: a review. Can. J. Microbiol. 592, 59–78. https://doi.org/10.1139/cjm-2012-049.

Hallenbeck, P.C., Ghosh, D., Skonieczny, M.T., Yargeau, V., 2009. Microbiological and engineering aspects of biohydrogen production. Indian J. Microbiol. 49, 48–59. https://doi.org/10.1007/s12088-009-0010-4.

Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., Minton, N.P., 2007. The ClosTron: a universal gene knock-out system for the genus Clostridium. J. Microbiol. Methods 703, 452–464. https://doi.org/10.1016/j.mimet.2007.05.021.

Hernández-Guisao, R.E., González-García, R.A., McCubbin, T., Guerra-Blanco, P., Manjarrez, E.S., Aranda-Barradas, J., Velasco, A., García-Peña, E.I., 2024. Proteomic analysis of natural photoheterotrophic mixed consortium for biohydrogen production under nongrowing conditions. Bioresour. Technol., 132023 https://doi.org/10.1016/j.biortech.2024.132023.

Hidese, R., Matsuda, M., Osanai, T., Hasunuma, T., Kondo, A., 2020. Malic enzyme facilitates d-lactate production through increased pyruvate supply during anoxic dark fermentation in Synechocystis sp. PCC 6803. ACS Synth. Biol. 92, 260–268. https://doi.org/10.1021/acssynbio.9b00281.

Hong, W., Zhang, J., Cui, G., Wang, L., Wang, Y., 2018. Multiplexed CRISPR-Cpf1-mediated genome editing in Clostridium difficile toward the understanding of pathogenesis of C. Difficile infection. ACS Synth. Biol. 76, 1588–1600. https://doi.org/10.1021/acssynbio.8b00087.

Hu, B.-B., Li, M.-Y., Wang, Y.-T., & Zhu, M.-J. 2018. Enhanced biohydrogen production from dilute acid pretreated sugarcane bagasse by detoxification and fermentation strategy. Int. J. Hydrog. Energy, *43*42, 19366–19374. doi:https://doi.org/10.1016/j.ijhydene.2018.08.164.

Huang, J.-R., Chen, X., Hu, B.-B., Cheng, J.-R., Zhu, M.-J., 2022. Bioaugmentation combined with biochar to enhance thermophilic hydrogen production from sugarcane bagasse. Bioresour. Technol. 348, 126790. https://doi.org/10.1016/j.biortech.2022.126790.

Husaini, C.U.N., Roslan, R., Ramzi, A.B., Luthfi, A.A.I., Tan, J.P., Lim, S.S., Ding, G.T., Jahim, J.M., Abdul, P.M., 2023. The CRISPR technology: a promising strategy for improving dark fermentative biohydrogen production using *Clostridium* spp. Int. J. Hydrog. Energy. https://doi.org/10.1016/j.ijhydene.2023.03.162.

Hwang, S., Lee, Y., Kim, J.H., Kim, G., Kim, H., Kim, W., Cho, S., Palsson, B.O., Cho, B.-K., 2021. *Streptomyces* as microbial chassis for heterologous protein expression. Front. Bioeng. Biotechnol. 9, 804295. https://doi.org/10.3389/fbioe.2021.804295.

Javed, M.A., Zafar, A.M., Hassan, A.A., Zaidi, A.A., Farooq, M., El Badawy, A., Lundquist, T., Mohamed, M.M.A., Al-Zuhair, S., 2022. The role of oxygen regulation and algal growth parameters in hydrogen production via biophotolysis. J. Environ. Chem. Eng. 101, 107003. https://doi.org/10.1016/j.jece.2021.107003.

Jawed, M., Pi, J., Xu, L., Zhang, H., Hakeem, A., Yan, Y., 2016. Enhanced H 2 production and redirected metabolic flux via overexpression of fhlA and pncB in Klebsiella HQ-3 strain. Appl. Biochem. Biotechnol. 178, 1113–1128. https://doi.org/10.1007/s12010-015-1932-4.

Jayakumar, M., Gebeyehu, K.B., Abo, L.D., Tadesse, A.W., Vivekanandan, B., Sundramurthy, V.P., Bacha, W., Ashokkumar, V., Baskar, G., 2023. A comprehensive outlook on topical processing methods for biofuel production and its thermal applications: current advances, sustainability and challenges. Fuel 349, 128690. https://doi.org/10.1016/j.fuel.2023.128690.

Jiao, H., Tsigkou, K., Elsamahy, T., Pispas, K., Sun, J., Manthos, G., Schagerl, M., Sventzouri, E., Al-Tohamy, R., Kornaros, M., 2024. Recent advances in sustainable hydrogen production from microalgae: mechanisms, challenges, and future perspectives. Ecotoxicol. Environ. Saf. 270, 115908. https://doi.org/10.1016/j.ecoenv.2023.115908.

Jin, T., Wang, Y., Yao, S., Hu, C., Ma, T., Xia, W., 2023. Bioconversion of carbon dioxide to succinate by Citrobacter. Chem. Eng. J. 452, 139668. https://doi.org/10.1016/j.cej.2022.139668.

Jo, J.H., Jeon, C.O., Lee, S.Y., Lee, D.S., Park, J.M., 2010. Molecular characterization and homologous overexpression of [FeFe]-hydrogenase in Clostridium tyrobutyricum JM1. Int. J. Hydrog. Energy 353, 1065–1073. https://doi.org/10.1016/j.ijhydene.2009.11.102.

Kammel, M., Sawers, R.G., 2022. The FocA channel functions to maintain intracellular formate homeostasis during Escherichia coli fermentation. Microbiology 1684, 001168. https://doi.org/10.1099/mic.0.001168.

Kim, E.-J., Tong, X., & Lee, J. K. 2015b. The ferredoxin Rr-HydB is required for the H2-evolving activity of Rr-HydA, a [FeFe]-hydrogenase of *Rhodospirillum rubrum*. Int. J. Hydrog. Energy, *40*12, 4320–4328. doi:https://doi.org/10.1016/j.ijhydene.2015.01.173.

Kim, S.H., Hwang, J.H., Kim, H.J., Oh, S.J., Kim, H.J., Shin, N., Kim, S.-H., Park, J.-H., Bhatia, S.K., Yang, Y.-H., 2023. Enhancement of biohydrogen production in Clostridium acetobutylicum ATCC 824 by overexpression of glyceraldehyde-3-phosphate dehydrogenase gene. Enzym. Microb. Technol. 168, 110244. https://doi.org/10.1016/j.enzmictec.2023.110244.

Kim, S.M., Choi, B.Y., Ryu, Y.S., Jung, S.H., Park, J.M., Kim, G.-H., Lee, S.K., 2015a. Simultaneous utilization of glucose and xylose via novel mechanisms in engineered Escherichia coli. Metab. Eng. 30, 141–148. https://doi.org/10.1016/j.ymben.2015.05.002.

Kremling, A., Geiselmann, J., Ropers, D., de Jong, H., 2015. Understanding carbon catabolite repression in Escherichia coli using quantitative models. Trends Microbiol. 232, 99–109. https://doi.org/10.1016/j.tim.2014.11.002.

Kumar, M., Singh, N. K., Kumar, R. S., & Singh, R. 2024. Production of Green Hydrogen through Photocatalysis. In Towards Sustainable and Green Hydrogen Production by Photocatalysis: Insights into Design and Development of Efficient Materials (Volume 2) pp. 1–24. ACS Publications. https://doi.org/10.1021/bk-2024-1468.ch001.

Li, W., He, L., Cheng, C., Cao, G., Ren, N., 2020. Effects of biochar on ethanol-type and butyrate-type fermentative hydrogen productions. Bioresour. Technol. 306, 123088. https://doi.org/10.1016/j.biortech.2020.123088.

Li, Y., Qiu, Y., Zhang, X., Zhu, M., Tan, W., 2019. Strain screening and optimization of biohydrogen production by Enterobacter aerogenes EB-06 from glycerol fermentation. Bioresour. Bioprocess. 6, 1–13. https://doi.org/10.1186/s40643-019-0250-z.

Li, Z., He, J., Zhang, S., Xie, Z., Guo, Z., Geng, W., Fan, Y., Liu, F., Xu, D., 2025. Metabolic engineering of Clostridium pasteurianum for enhanced biohydrogen production. Int. J. Hydrog. Energy 151, 150161. https://doi.org/10.1016/j.ijhydene.2025.150161.

Liang, Q., Zhang, F., Li, Y., Zhang, X., Li, J., Yang, P., Qi, Q., 2015. Comparison of individual component deletions in a glucose-specific phosphotransferase system revealed their different applications. Sci. Rep. 51, 13200. https://doi.org/10.1038/srep13200.

Liu, X., Zhu, Y., Yang, S.T., 2006. Construction and characterization of ack deleted mutant of Clostridium tyrobutyricum for enhanced butyric acid and hydrogen production. Biotechnol. Prog. 225, 1265–1275. https://doi.org/10.1021/bp060082g.

Liu, Z., Zhang, C., Wang, L., He, J., Li, B., Zhang, Y., Xing, X.-H., 2015. Effects of furan derivatives on biohydrogen fermentation from wet steam-exploded cornstalk and its microbial community. Bioresour. Technol. 175, 152–159. https://doi.org/10.1016/j.biortech.2014.10.067.

Lopez-Hidalgo, A.M., Balderas Hernandez, V.E., De Leon-Rodriguez, A., 2021a. Scale-up of hydrogen and ethanol co-production by an engineered Escherichia coli. Fuel 300, 121002. https://doi.org/10.1016/j.fuel.2021.121002.

Lopez-Hidalgo, A.M., Magaña, G., Rodriguez, F., De Leon-Rodriguez, A., Sanchez, A., 2021b. Co-production of ethanol-hydrogen by genetically engineered Escherichia coli in sustainable biorefineries for lignocellulosic ethanol production. Chem. Eng. J. 406, 126829. https://doi.org/10.1016/j.cej.2020.126829.

Lu, P., Wu, Y., Bai, R., Jiang, K., Xu, F., Zhao, H., 2022. Integrated strategy of CRISPR-Cas9 gene editing and small RNA RhyB regulation in Enterobacter aerogenes: a novel protocol for improving biohydrogen production. Biotechnology Notes 3, 79–87. https://doi.org/10.1016/j.biotno.2022.10.002.

Maeda, T., Sanchez-Torres, V., Wood, T.K., 2008. Protein engineering of hydrogenase 3 to enhance hydrogen production. Appl. Microbiol. Biotechnol. 79, 77–86. https://doi.org/10.1007/s00253-008-1416-3.

Manish, S., Venkatesh, K., & Banerjee, R. 2007. Metabolic flux analysis of biological hydrogen production by *Escherichia coli*. Int. J. Hydrog. Energy, *32*16, 3820–3830. doi:https://doi.org/10.1016/j.ijhydene.2007.03.033.

Marbehan, X., Roger, M., Fournier, F., Infossi, P., Guedon, E., Delecourt, L., Lebrun, R., Giudici-Orticoni, M.-T., Delaunay, S., 2024. Combining metabolic flux analysis with proteomics to shed light on the metabolic flexibility: the case of Desulfovibrio vulgaris Hildenborough. Front. Microbiol. 15, 1336360. https://doi.org/10.3389/fmicb.2024.1336360.

Maru, B., López, F., Kengen, S., Constantí, M., Medina, F., 2016. Dark fermentative hydrogen and ethanol production from biodiesel waste glycerol using a co-culture of Escherichia coli and Enterobacter sp. Fuel 186, 375–384. https://doi.org/10.1016/j.fuel.2016.08.043.

McDowall, J.S., Murphy, B.J., Haumann, M., Palmer, T., Armstrong, F.A., Sargent, F., 2014. Bacterial formate hydrogenlyase complex. Proc. Natl. Acad. Sci. 11138, E3948–E3956. https://doi.org/10.1073/pnas.1407927111.

Mıynat, E., Argun, H., 2020a. Prevention of substrate and product inhibitions by using a dilution strategy during dark fermentative hydrogen production from molasses. Int. J. Hydrog. Energy *45*60, 34695–34706. https://doi.org/10.1016/j.ijhydene.2020.03.118.

Mıynat, M. E., & Argun, H. 2020b. Prevention of substrate and product inhibitions by using a dilution strategy during dark fermentative hydrogen production from molasses. Int. J. Hydrog. Energy, *45*60, 34695–34706. doi:https://doi.org/10.1016/j.ijhydene.2020.03.118.

Mohanakrishna, G., Pengadeth, D., 2024. Mixed culture biotechnology and its versatility in dark fermentative hydrogen production. Bioresour. Technol., 130286 https://doi.org/10.1016/j.biortech.2023.130286.

Mohanraj, S., Pandey, A., Mohan, S.V., Anbalagan, K., Kodhaiyolii, S., Pugalenthi, V., 2019. Metabolic engineering and molecular biotechnology of biohydrogen production. In: Biohydrogen. Elsevier, pp. 413–434. https://doi.org/10.1016/B978-0-444-64203-5.00017-4.

Moon, C., Singh, R., Chaganti, S.R., Lalman, J.A., 2013. Modeling sulfate removal by inhibited mesophilic mixed anaerobic communities using a statistical approach. Water Res. 477, 2341–2351. https://doi.org/10.1016/j.watres.2013.01.045.

Morimoto, K., Kimura, T., Sakka, K., & Ohmiya, K. 2005. Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. FEMS Microbiol. Lett., *246*2, 229–234. doi:https://doi.org/10.1016/j.femsle.2005.04.014.

Morsy, F.M., Ibrahim, S.H., 2016. Concomitant hydrolysis of sucrose by the long half-life time yeast invertase and hydrogen production by the hydrogen over-producing Escherichia coli HD701. Energy 109, 412–419. https://doi.org/10.1016/j.energy.2016.05.006.

Muñoz-Páez, K.M., Alvarado-Michi, E.L., Buitrón, G., Valdez-Vazquez, I., 2019. Distinct effects of furfural, hydroxymethylfurfural and its mixtures on dark fermentation hydrogen production and microbial structure of a mixed culture. Int. J. Hydrog. Energy 444, 2289–2297. https://doi.org/10.1016/j.ijhydene.2018.04.139.

Mustafa, A.M., Chen, X., Lin, H., Sheng, K., 2019. Effect of ammonia concentration on hythane (H2 and CH4) production in two-phase anaerobic digestion. Int. J. Hydrog. Energy 4450, 27297–27310. https://doi.org/10.1016/j.ijhydene.2019.08.229.

Noblecourt, A., Christophe, G., Larroche, C., Fontanille, P., 2018. Hydrogen production by dark fermentation from pre-fermented depackaging food wastes. Bioresour. Technol. 247, 864–870. https://doi.org/10.1016/j.biortech.2017.09.199.

Oh, Y.-K., Kim, H.-J., Park, S., Kim, M.-S., Ryu, D.D., 2008. Metabolic-flux analysis of hydrogen production pathway in Citrobacter amalonaticus Y19. Int. J. Hydrog. Energy 335, 1471–1482. https://doi.org/10.1016/j.ijhydene.2007.09.032.

Oh, Y.-K., Raj, S. M., Jung, G. Y., & Park, S. 2011. Current status of the metabolic engineering of microorganisms for biohydrogen production. Bioresour. Technol., *102*18, 8357–8367. doi:https://doi.org/10.1016/j.biortech.2011.04.054.

Pan, J., Chen, X., Sheng, K., Yu, Y., Zhang, C., Ying, Y., 2013. Effect of ammonia on biohydrogen production from food waste via anaerobic fermentation. Int. J. Hydrog. Energy *38*29, 12747–12754. https://doi.org/10.1016/j.ijhydene.2013.06.093.

Pang, J., Liu, Z.-Y., Hao, M., Zhang, Y.-F., Qi, Q.-S., 2017. An isolated cellulolytic Escherichia coli from bovine rumen produces ethanol and hydrogen from corn straw. Biotechnol. Biofuels 10, 1–10. https://doi.org/10.1186/s13068-017-0852-7.

Pathade, G.R., Ruikar, S.S., Ghewari, P.G., 2024. Synthetic Biology Approaches in the Development of Biofuels and Biochemicals. NATURALISTA CAMPANO 281, 176–182.

Poladyan, A., Trchounian, K., Vassilian, A., Trchounian, A., 2018. Hydrogen production by *Escherichia coli* using brewery waste: optimal pretreatment of waste and role of different hydrogenases. Renew. Energy 115, 931–936. https://doi.org/10.1016/j.renene.2017.09.022.

Prelich, G., 2012. Gene Overexpression: Uses, Mechanisms, and Interpretation. Genetics *190*3, 841–854. https://doi.org/10.1534/genetics.111.136911.

Putatunda, C., Behl, M., Solanki, P., Sharma, S., Bhatia, S.K., Walia, A., Bhatia, R.K., 2022. Current challenges and future technology in photofermentation-driven biohydrogen production by utilizing algae and bacteria. Int. J. Hydrog. Energy. https://doi.org/10.1016/j.ijhydene.2022.10.042.

Pyne, M.E., Bruder, M.R., Moo-Young, M., Chung, D.A., Chou, C.P., 2016. Harnessing heterologous and endogenous CRISPR-Cas machineries for efficient markerless genome editing in Clostridium. Sci. Rep. 61, 25666. https://doi.org/10.1038/srep25666.

Qin, H., Hu, T., Zhai, Y., Lu, N., Aliyeva, J., 2020. The improved methods of heavy metals removal by biosorbents: a review. Environ. Pollut. 258, 113777. https://doi.org/10.1016/j.envpol.2019.113777.

Rollin, J.A., Martin del Campo, J., Myung, S., Sun, F., You, C., Bakovic, A., Castro, R., Chandrayan, S.K., Wu, C.-H., Adams, M.W., 2015. High-yield hydrogen production from biomass by in vitro metabolic engineering: mixed sugars coutilization and kinetic modeling. Proc. Natl. Acad. Sci. 11216, 4964–4969. https://doi.org/10.1073/pnas.1417719112.

Romão, B.B., Silva, F.T.M., Costa, H.C.d.B., do Carmo, T.S., Cardoso, S.L., Ferreira, J.d.S., Batista, F.R.X., Cardoso, V.L., 2019. Alternative techniques to improve hydrogen production by dark fermentation. 3 Biotech 9, 1–8. https://doi.org/10.1007/s13205-018-1538-y.

Ruggeri, B., Tommasi, T., Sanfilippo, S., 2015. BioH_2 & BioCH_4 through anaerobic digestion: from research to full-scale applications. Springer. https://doi.org/10.1007/978-1-4471-6431-9.

Sanabria, A.J.G., 2011. *Enhanced hydrogen production in Escherichia coli through chemical mutagenesis, gene deletion, and transposon mutagenesis* Texas a & M University. https://hdl.handle.net/1969.1/ETD-TAMU-2010-05-7799.

Sanchez-Torres, V., Maeda, T., & Wood, T. K. 2009. Protein engineering of the transcriptional activator FhlA to enhance hydrogen production in *Escherichia coli*. Appl. Environ. Microbiol., *75*17, 5639–5646. doi:https://doi.org/10.1128/AEM.00638-09.

Scarborough, M.J., Hamilton, J.J., Erb, E.A., Donohue, T.J., Noguera, D.R., 2020. Diagnosing and predicting mixed-culture fermentations with unicellular and guild-based metabolic models. MSystems 55. https://doi.org/10.1128/msystems.00755-20, 10.1128/msystems.00755–00720.

Seol, E., Sekar, B.S., Raj, S.M., Park, S., 2016. Co-production of hydrogen and ethanol from glucose by modification of glycolytic pathways in Escherichia coli–from Embden-Meyerhof-Parnas pathway to pentose phosphate pathway. Biotechnol. J. 112, 249–256. https://doi.org/10.1002/biot.201400829.

Sharma, P., Melkania, U., 2018a. Effect of sulfate on hydrogen production from the organic fraction of municipal solid waste using co-culture of E. Coli and Enterobacter aerogenes. Int. J. Hydrog. Energy 432, 676–684. https://doi.org/10.1016/j.ijhydene.2017.11.044.

Sharma, P., Melkania, U., 2018b. Enhancement effect of amino acids on hydrogen production from organic fraction of municipal solid waste using co-culture of Escherichia coli and Enterobacter aerogenes. Energy Convers. Manag. 163, 260–267. https://doi.org/10.1016/j.enconman.2018.02.072.

Sievert, C., Nieves, L.M., Panyon, L.A., Loeffler, T., Morris, C., Cartwright, R.A., Wang, X., 2017. Experimental evolution reveals an effective avenue to release catabolite repression via mutations in XylR. Proc. Natl. Acad. Sci. 11428, 7349–7354. https://doi.org/10.1073/pnas.1700345114.

Singh, N. K., & Singh, R. 2021a. Modeling and statistical analysis of heat-shocked sulfate-reducers and methanogens rich consortiums for hydrogen and methane production in a bio-electrochemical cell. Int. J. Hydrog. Energy, *46*51, 25819–25831. doi:https://doi.org/10.1016/j.ijhydene.2021.05.097.

Singh, N.K., Singh, R., 2021b. A sequential approach to uncapping of theoretical hydrogen production in a sulfate-reducing bacteria-based bio-electrochemical system. Int. J. Hydrog. Energy 4639, 20397–20412. https://doi.org/10.1016/j.ijhydene.2021.03.152.

Singh, N.K., Kumari, P., Singh, R., 2021. Intensified hydrogen yield using hydrogenase rich sulfate-reducing bacteria in bio-electrochemical system. Energy 219, 119583. https://doi.org/10.1016/j.energy.2020.119583.

Sinha, P., Roy, S., Das, D., 2016. Genomic and proteomic approaches for dark fermentative biohydrogen production. Renew. Sust. Energ. Rev. 56, 1308–1321. https://doi.org/10.1016/j.rser.2015.12.035.

Sivaramakrishnan, R., Shanmugam, S., Sekar, M., Mathimani, T., Incharoensakdi, A., Kim, S.-H., Parthiban, A., Geo, V.E., Brindhadevi, K., Pugazhendhi, A., 2021. Insights on biological hydrogen production routes and potential microorganisms for high hydrogen yield. Fuel 291, 120136. https://doi.org/10.1016/j.fuel.2021.120136.

Son, Y.-S., Jeon, J.-M., Kim, D.-H., Yang, Y.-H., Jin, Y.-S., Cho, B.-K., Kim, S.-H., Kumar, S., Lee, B.-D., Yoon, J.-J., 2021. Improved bio-hydrogen production by overexpression of glucose-6-phosphate dehydrogenase and FeFe hydrogenase in Clostridium acetobutylicum. Int. J. Hydrog. Energy *46*74, 36687–36695. https://doi.org/10.1016/j.ijhydene.2021.08.222.

Song, W., Cheng, J., Ding, L., Liu, M., Zhou, J., Cen, K., 2017. Improving fermentative hydrogen production from water hyacinth with genetically modified bacteria. Environ. Prog. Sustain. Energy 365, 1296–1300. https://doi.org/10.1002/ep.12683.

Soo, C.-S., Yap, W.-S., Hon, W.-M., Ramli, N., Shah, U.K.M., Phang, L.-Y., 2017. Improvement of hydrogen yield of ethanol-producing *Escherichia coli* recombinants in acidic conditions. Electron. J. Biotechnol. 26, 27–32. https://doi.org/10.1016/j.ejbt.2016.12.007.

Sorek, R., Lawrence, C.M., Wiedenheft, B., 2013. CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu. Rev. Biochem. 82, 237–266. https://doi.org/10.1146/annurev-biochem-072911-172315.

Srikanth, S., Mohan, S.V., 2014. Regulating feedback inhibition caused by the accumulated acid intermediates during acidogenic hydrogen production through feed replacement. Int. J. Hydrog. Energy 3919, 10028–10040. https://doi.org/10.1016/j.ijhydene.2014.04.152.

Sun, C., Xia, A., Liao, Q., Guo, X., Fu, Q., Huang, Y., Zhu, X., Ding, L., Chen, C., 2019. Inhibitory effects of furfural and vanillin on two-stage gaseous biofuel fermentation. Fuel 252, 350–359. https://doi.org/10.1016/j.fuel.2019.04.068.

Sun, C., Liao, Q., Xia, A., Fu, Q., Huang, Y., Zhu, X., Zhu, X., Wang, Z., 2020. Degradation and transformation of furfural derivatives from hydrothermal pre-treated algae and lignocellulosic biomass during hydrogen fermentation. Renew. Sust. Energ. Rev. 131, 109983. https://doi.org/10.1016/j.rser.2020.109983.

Sundara Sekar, B., Seol, E., Park, S., 2017. Co-production of hydrogen and ethanol from glucose in Escherichia coli by activation of pentose-phosphate pathway through deletion of phosphoglucose isomerase (pgi) and overexpression of glucose-6-phosphate dehydrogenase (zwf) and 6-phosphogluconate dehydrogenase (gnd). Biotechnol. Biofuels 10, 1–12. https://doi.org/10.1186/s13068-017-0768-2.

Tang, J., Li, W., Lin, Z., Yang, J., & Meng, Z. 2024. Metabolic flux analysis of hydrogen production network by *Ethanoligenens harbinense* B49: effect of product inhibition. Environ. Technol., *45*22, 4645–4655. doi:https://doi.org/10.1080/09593330.2023.2283051.

Tao, Z., Wang, D., Yao, F., Huang, X., Wu, Y., Du, M., Chen, Z., An, H., Li, X., Yang, Q., 2020. The effects of thiosulfinates on methane production from anaerobic co-digestion of waste activated sludge and food waste and mitigate method. J. Hazard. Mater. 384, 121363. https://doi.org/10.1016/j.jhazmat.2019.121363.

Tran, K.T., Maeda, T., Sanchez-Torres, V., Wood, T.K., 2015. Beneficial knockouts in Escherichia coli for producing hydrogen from glycerol. Appl. Microbiol. Biotechnol. 99, 2573–2581. https://doi.org/10.1007/s00253-014-6338-7.

Tsai, Y.-C., Du, Y.-Q., Yang, C.-F., 2021. Anaerobic biohydrogen production from biodetoxified rice straw hydrolysate. J. Taiwan Inst. Chem. Eng. 123, 134–140. https://doi.org/10.1016/j.jtice.2021.05.034.

Tummala, S.B., Welker, N.E., Papoutsakis, E.T., 2003. Design of antisense RNA constructs for downregulation of the acetone formation pathway of Clostridium acetobutylicum. J. Bacteriol. 1856, 1923–1934. https://doi.org/10.1128/jb.185.6.1923-1934.2003.

Valle, A., Cabrera, G., Muhamadali, H., Trivedi, D. K., Ratray, N. J., Goodacre, R., Cantero, D., & Bolivar, J. 2015. A systematic analysis of TCA *Escherichia coli* mutants reveals suitable genetic backgrounds for enhanced hydrogen and ethanol production using glycerol as main carbon source. Biotechnol. J., *10*11, 1750–1761. doi:https://doi.org/10.1002/biot.201500005.

Valle, A., Cantero, D., Bolívar, J., 2019. Metabolic engineering for the optimization of hydrogen production in Escherichia coli: a review. Biotechnol. Adv. 375, 616–633. https://doi.org/10.1016/j.biotechadv.2019.03.006.

Valle, A., Haïlaf, A., Ceballos, A., Cantero, D., Bolivar, J., 2021. Co-overexpression of the malate dehydrogenase (Mdh) and the malic enzyme a (MaeA) in several Escherichia coli mutant backgrounds increases malate redirection towards hydrogen production. Int. J. Hydrog. Energy 4629, 15337–15350. https://doi.org/10.1016/j.ijhydene.2021.02.100.

Valle, A., de la Calle, M. E., Muhamadali, H., Hollywood, K. A., Xu, Y., Lloyd, J. R., Goodacre, R., Cantero, D., & Bolivar, J. 2023. Metabolomics of *Escherichia coli* for disclosing novel metabolic engineering strategies for enhancing hydrogen and ethanol production. Int. J. Mol. Sci., *24*14, 11619. doi:https://doi.org/10.3390/ijms241411619.

van Asselt, H., Green, F., 2023. COP26 and the dynamics of anti-fossil fuel norms. Wiley Interdiscip. Rev. Clim. Chang. 143, e816. https://doi.org/10.1002/wcc.816.

Wei, J., Li, Y., 2023. Crispr-based gene editing technology and its application in microbial engineering. Eng. Microbiol., 100101 https://doi.org/10.1016/j.engmic.2023.100101.

Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. 2012. RNA-guided genetic silencing systems in bacteria and archaea. Nature, *482*7385, 331–338. doi:https://doi.org/10.1038/nature10886.

Wu, Y., Zhao, H., 2021. Metabolic engineering of Enterobacter aerogenes for improved 2, 3-Butanediol production by manipulating NADH levels and overexpressing the small RNA RyhB. Front. Microbiol. 12, 754306. https://doi.org/10.3389/fmicb.2021.754306.

Wu, Y., Hao, Y., Wei, X., Shen, Q., Ding, X., Wang, L., Zhao, H., Lu, Y., 2017. Impairment of NADH dehydrogenase and regulation of anaerobic metabolism by the small RNA

RyhB and NadE for improved biohydrogen production in Enterobacter aerogenes. Biotechnol. Biofuels 10, 1–15. https://doi.org/10.1186/s13068-017-0938-2.
Xiong, J., Chan, D., Guo, X., Chang, F., Chen, M., Wang, Q., Song, X., Wu, C., 2020. Hydrogen production driven by formate oxidation in Shewanella oneidensis MR-1. Appl. Microbiol. Biotechnol. 104, 5579–5591. https://doi.org/10.1007/s00253-020-10608-w.
Xu, Y., Zhang, C., Zhao, M., Rong, H., Zhang, K., Chen, Q., 2017. Comparison of bioleaching and electrokinetic remediation processes for removal of heavy metals from wastewater treatment sludge. Chemosphere 168, 1152–1157. https://doi.org/10.1016/j.chemosphere.2016.10.086.
Yang, J., Cao, J., Xing, G., Yuan, H., 2015. Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae Chlorella minutissima UTEX2341. Bioresour. Technol. 175, 537–544. https://doi.org/10.1016/j.biortech.2014.10.124.
Zeng, L., Chakraborty, B., Farivar, T., Burne, R.A., 2017. Coordinated regulation of the EIIMan and fruRKI operons of Streptococcus mutans by global and fructose-specific pathways. Appl. Environ. Microbiol. 8321, e01403–e01417. https://doi.org/10.1128/AEM.01403-17.
Zhang, J., Zong, W., Hong, W., Zhang, Z.-T., Wang, Y., 2018. Exploiting endogenous CRISPR-Cas system for multiplex genome editing in Clostridium tyrobutyricum and engineer the strain for high-level butanol production. Metab. Eng. 47, 49–59. https://doi.org/10.1016/j.ymben.2018.03.007.
Zhang, J., Xue, D., Wang, C., Fang, D., Cao, L., Gong, C., 2023. Genetic engineering for biohydrogen production from microalgae. Iscience. https://doi.org/10.1016/j.isci.2023.107255.
Zhang, L., Zhang, L., & Li, D. 2015. Enhanced dark fermentative hydrogen production by zero-valent iron activated carbon micro-electrolysis. Int. J. Hydrog. Energy, *40*36, 12201–12208. doi:https://doi.org/10.1016/j.ijhydene.2015.07.106.
Zhang, Q., You, S., Li, Y., Qu, X., Jiang, H., 2020. Enhanced biohydrogen production from cotton stalk hydrolysate of Enterobacter cloacae WL1318 by overexpression of the formate hydrogen lyase activator gene. Biotechnol. Biofuels 13, 1–11. https://doi.org/10.1186/s13068-020-01733-9.
Zhang, Z., Aboulwafa, M., Saier, M.H., 2014. Regulation of crp gene expression by the catabolite repressor/activator, Cra, in Escherichia coli. J. Mol. Microbiol. Biotechnol. 243, 135–141. https://doi.org/10.1159/000362722.
Zhao, H., Ma, K., Lu, Y., Zhang, C., Wang, L., Xing, X.-H., 2009. Cloning and knockout of formate hydrogen lyase and H2-uptake hydrogenase genes in Enterobacter aerogenes for enhanced hydrogen production. Int. J. Hydrog. Energy *34*1, 186–194. https://doi.org/10.1016/j.ijhydene.2008.10.025.
Zhao, L., Wang, Z., Ren, H.-Y., Chen, C., Nan, J., Cao, G.-L., Yang, S.-S., Ren, N.-Q., 2021. Residue cornstalk derived biochar promotes direct bio-hydrogen production from anaerobic fermentation of cornstalk. Bioresour. Technol. 320, 124338. https://doi.org/10.1016/j.biortech.2020.124338.
Zhao, Z.-T., Ding, J., Wang, B.-Y., Bao, M.-Y., Liu, B.-F., Pang, J.-W., Ren, N.-Q., Yang, S.-S., 2024. Advances in the biomass valorization in dark fermentation systems: a sustainable approach for biohydrogen production. Chem. Eng. J., 148444 https://doi.org/10.1016/j.cej.2023.148444.