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Optimization of biohydrogen production by modifying metabolic pathways in *Clostridium Thermocellum*

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Abstract. The ever-escalating energy crisis of disruptions in the supply of fossil fuels suggests the need for alternative means with sustainable energy sources. Of these, biohydrogen stands out as promising because it is clean and efficient. Our research aims to provide strategies for fine-tuning the biohydrogen production process using the dark fermentation pathway of *Clostridium. Thermocellum* since it efficiently degrades cellulose under anaerobic conditions and has high-temperature tolerance, improving hydrogen yield. Sugarcane bagasse enforces this integration by providing abundant resources at little cost, which can support circular economies. However, such problems as ethanol toxicity to *C. Thermocellum* require genetic modifications to enhance production efficiency. The plasmid design overexpresses NADH-dependent [FeFe] hydrogenase, knocks out ethanol production pathways, and hence maximizes biohydrogen output. The design of the plasmid mainly uses Golden gate assembly and homologous recombination to achieve gene overexpression and deletion, and restriction sites were designed for virtual digestion in plasmid verification.

Keywords: Biohydrogen, *Clostridium Thermocellum*, Sugarcane bagasse, Dark Fermentation, Golden gate assembly.

1. Introduction

The energy landscape offers considerable challenges, all dependent on fossil fuels. The current Russia-Ukraine war provided a real-world and slightly sobering example of how vulnerable our conventional energy system has become. Oil and gas supplies from Russia were disrupted, raising the price of energy and crippling economic activity, strongly influencing households worldwide [1]. This crisis thus points to the importance of an urgent shift to more sustainable and secure sources of energy.

Of the many replacements available, one of the most promising alternatives in this regard is biofuels. These consist of liquid or gaseous fuels which are derived from renewable biomasses by-products from agriculture, energy crops, and organic wastes. Compared to their fossil counterparts, there are a number of advantages to using biofuels. First, they are renewable. This means they can be continuously

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replenished in an effort to reduce our reliance on finite resources. Besides, the flexibility of biofuel products suggests the potential to prevent toxic gas to the atmosphere [2].

However, there are many pitfalls in the development of biofuels. First-generation biofuels are obtained from food-producing crops like corn and sugarcane; for that matter, they have been under criticism because of the probability of competing with food production or raising food prices [3]. This has led to the development of second-generation biofuel production using non-food feedstock, mainly from lignocellulosic biomass and agricultural residues. Advanced biofuels such as these can solve the food and fuel dilemma by giving better energy yields with fewer environmental impacts. Even with such improvements, producing biofuels in large quantities is not easy. In history, few technological breakthroughs have been made in converting lignocellulosic biomass into fermentable sugars for producing biofuels without consuming excess energy. Apart from this, the logistics of feedstock collection, transportation, and storage may add cost intensity to the biofuel supply chain.

Research and development continue to fine-tune the production process for better conversion efficiency and new feedstock options so that the biofuel industry will be both viable and sustainable. An even more promising option would be biohydrogen. With the quest for cleaner and more efficient energy sources unabated, unlike the traditional production of biofuels, biohydrogen has a bright future.

2. Possible Optimizations

2.1. Product

In comparison with traditional biofuels, the production of biohydrogen is an increasingly attractive option. Whereas the conversion processes by which lignocellulosic biomass is converted into fermentable sugars for biofuel production are complex and energy-intensive, in the case of biohydrogen a more direct and hence efficient route can be followed [4]. Some microorganisms have acquired the ability to convert the lignocellulosic feedstocks directly into hydrogen gas; through a process called dark fermentation. Such specialized microbes break down cellulose and hemicellulose elements of biomass in the process of dark fermentation and allow the release of this hydrogen gas [5].

There are some specific advantages that set biohydrogen apart from conventional biofuels. First, hydrogen actually has an energy content considerably higher than that contained in liquid biofuels; it has a weight-based higher energy density [6]. That means greater effectiveness in energy storage and, correspondingly, potentially better range for hydrogen-powered cars. In addition, the combustion of hydrogen only results in water having zero direct emissions of GHGs, and indeed is an exceptionally clean energy carrier.

Besides the reduction in environmental impacts, biohydrogen production can also help solve some problems related to traditional biofuels. Production methods unlike first-generation biofuels produced from food crops, include non-food lignocellulosic feedstock sources for biohydrogen, hence avoiding this food-versus-fuel dilemma. This is another added advantage of the switchover to less energy-intensive processing steps that come forth with a more efficient and sustainable bioenergy supply chain.

In a world still racing to meet the demand for clean and secure sources of energy, such is the promise that biohydrogen technology development holds. This would be achieved by exploiting the unique capabilities of specialized microorganisms and the optimization of production processes to satisfy our need for energy on a more sustainable, efficient, and environmentally friendly level in the future.

2.2. Feedstock

In our effort to optimize biofuel production's efficiency, sugarcane bagasse was chosen as a promising feedstock. Sugarcane bagasse, a fibrous material having cellulose as its main component, is produced as a waste material in large quantities throughout the world. It is most commonly produced in the sugar and paper industry and is a large by-product in Asian countries [7]. Its cellulose abundance and richness make it an ideal raw material for biohydrogen production [8].

There are several factors that we have considered in choosing sugarcane bagasse as our feedstock. (1) Sugarcane bagasse, through the process of dark fermentation, supports sustainability. This process

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helps in managing agricultural waste through promoting a circular economy. Moreover, it also reduces fossil fuel reliance [9]. (2) Sugarcane bagasse is a large byproduct of the sugarcane industry making it easily accessible and cost-effective in its fermentation process. (3) The feedstock has high levels of carbohydrate content such as hemicellulose. Higher levels of these organic contents provide a larger source of fermentable sugars for the process of biofuel production [10].

2.3. Host

Clostridium Thermocellum is the host bacterium that we have chosen specializing in the production of biohydrogen through the utilization of its pathways. While we have considered utilizing its more widely studied counterparts such as Saccharomyces cerevisiae and Escherichia Coli, due to their facultative anaerobic composition, the nature of our research will prevent these organisms from being utilized effectively. Dark fermentation requires organisms to be anaerobic as being optimized for strictly anaerobic conditions will yield higher levels of efficiency [11].

Many organisms in the biofuel industry are optimized for converting Glucose into usable fuels [12]. However, while the utilization of glucose is essential, many plant-based products that get discarded are made up of cellulose. Converting cellulose into glucose requires substantial amounts of energy which we can spare by directly utilizing cellulose. C. Thermocellum is a bacteria configured to live in anaerobic conditions and is able to process cellulosic substances effectively. In fact, it is the best lab bacteria that possess the ability to efficiently use cellulose in creating biohydrogen [13] This trait is essential for optimizing the creation of biohydrogen through the use of sugarcane bagasse. Fittingly, sugarcane bagasse has one of the greatest cellulosic abundance. Their compatibility is the core of our investigation and the main reason we chose C. Thermocellum as the host for our paper.

Temperature considerations are also crucial in the host organism chosen. With the production of biohydrogen in our system pressure, in the system, will increase. For example, shown by a study from David B. Levin et al, the higher the pressure the less efficient the production of hydrogen [14]. To combat this, by increasing our temperature levels, the amount of hydrogen produced will not prevent the organism from its full potential, increasing overall efficiency. C. Thermocellum has the highest temperature tolerance at 60°C compared to the other effective organisms that can only tolerate an average of 37°C [15]. This makes it prime for being the dark fermentation host.

While it has many positives it has its drawbacks. C. Thermocellum is unable to tolerate high levels of ethanol, implying that with increased ethanol levels it drastically decreases its efficiency [16]. The creation of ethanol is primarily due to its product synthesis pathways through pyruvate in which other acetogenic compounds are produced. Nevertheless, ethanol is the main deterrent in production efficiency as acidic compounds do not substantially influence the organisms [17]. The target of our research will be the removal of the gene responsible for ethanol production, increasing the biohydrogen yield from the organism.

2.4. Pathway and Process

The production of biohydrogen revolves around multiple steps which eventually, when the feedstock is broken into pure cellulose, can be produced through fermentation. Sugarcane Bagasse, a common by-product from sugarcane harvesting, is a major component in the sugar industry. The production process begins with the product (i) boiled for 4 hours (ii) soaked in sodium hydroxide with a 4% concentration for 4 hours (iii) soaked in sodium hypochlorite and sodium hydroxide with a concentration of 4% and 2% respectively for 4 hours (iv) the product is distilled (v) the result is cellulose in which when put into the fermentation process becomes biohydrogen. Nonetheless, the process of fermentation is contained in an anaerobic bioreactor that has a controlled environment creating biohydrogen and ethanol as its main byproduct.

In the bioreactor, the fermentation pathway is demonstrated through several steps. Firstly, cellulose is broken down into glucose through phosphorolysis via cellodextrin phosphorylase originating in C. Thermocellum. Next, Glycolysis is performed by using different enzymes, so glucose is converted into pyruvate [18]. Through this process, NADH is produced, which can be used later. To be mentioned,

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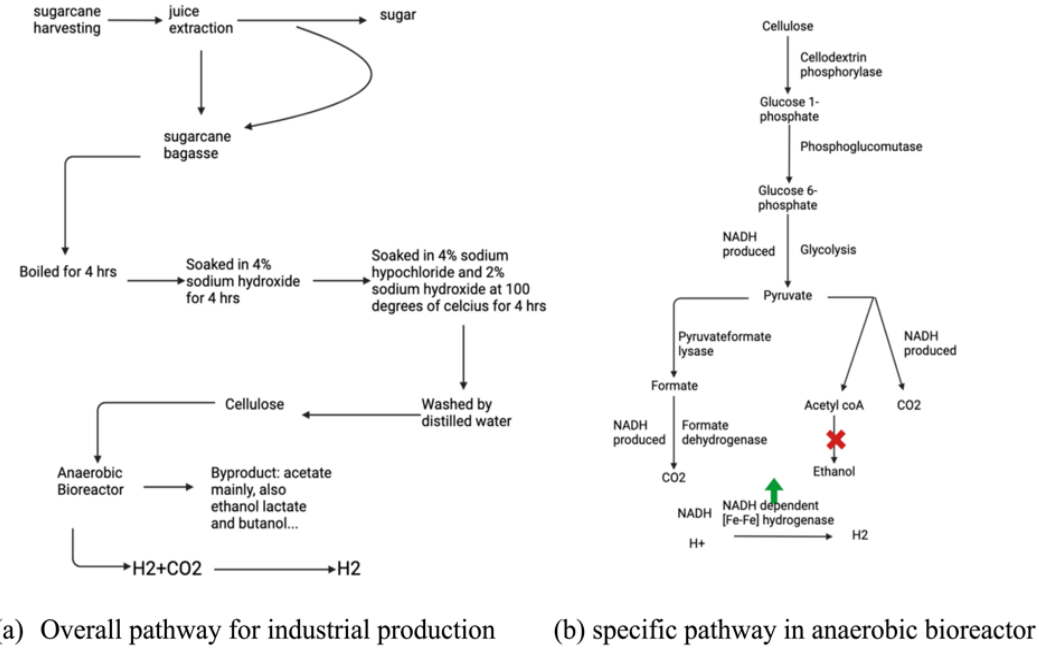
unlike conventional glycolysis, phosphoglucomutase is needed to convert the product of phosphorolysis into the reactants available for glycolysis. Then, the catabolism of pyruvate can be illustrated through 2 significant branches: (1) Lactate/Pyruvate/Formate and (2) Pyruvate/Acetyl-CoA/Ethanol/Acetate. (i) Pyruvate-formate production: With pyruvate production, the pathway deviates into two branches. The first converts pyruvate into formate. The formate is then processed through formate dehydrogenase that catalyzes formate to carbon dioxide with NAD+ reduction into NADH [19]. (ii) Pyruvate-acetyl-CoA production: Pyruvate is also converted into acetyl CoA by ferredoxin oxidoreductase, turning ferredoxin O into ferredoxin R. This process releases hydrogen that attaches to NAD+ converting it into NADH [20]. Lastly, the NADH generated in different branches can be gathered and generate hydrogen using NADH-dependent [Fe-Fe] hydrogenase.



(a) Overall pathway for industrial production (b) specific pathway in anaerobic bioreactor

Figure 1. Pathway for biohydrogen production

2.5. Design Strategy

To optimize the pathway, plasmid design is required as it modifies the host's genome to promote or prevent certain genes from being expressed. In this specific pathway optimization, we selected two certain reactions to modify: 1) increase the rate of reaction from NADH to H2 by overexpressing the NADH-dependent [FeFe] hydrogenase; 2) cut off the pathway from aldehyde to ethanol in consumption of NADH by deleting the expression of Zinc alcohol dehydrogenase.

Several designs can ensure successful gene editing. Firstly, the vector contains the replication site and homologous arms, and hence it can satisfy the needs of replication and homologous recombination in the host bacteria. Apart from that, in terms of gene overexpression, a strong promoter, the gene coding for the targeted protein and the appropriate terminator should be chosen. Lastly, the marker sequence should be inserted to verify the result of plasmid transformation, yet the two markers should be different in the two plasmids.

To be specific, the choice of the different composition of the plasmid is of vital importance. The blank vector used is from a designed temperature-sensitive vector pMU102 used for *C. Thermocellum* that contains a special region for preventing plasmid replicating in high temperature, but to achieve higher efficiency, we removed this part and added two cutting sites for enzyme BsmBI to work [21]. Besides, the selection of homologous arms is associated with the function of the plasmid, and they are chosen from the whole genome of *C. Thermocellum* [22]. The Markers are respectively Kan/NeoR and AmpR since the host is not resistant to these two antibiotics. The promoters used for gene overexpression

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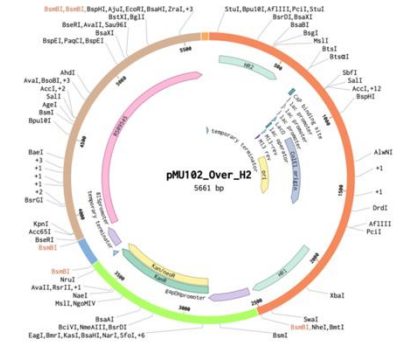
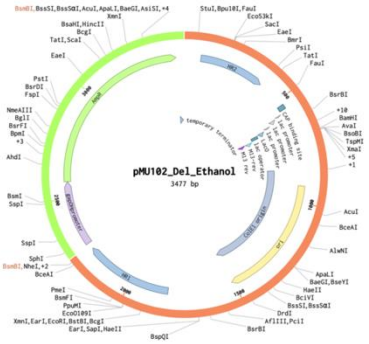
is 815 promoter because of the high efficiency in *C. Thermocellum* [23]. Lastly, the terminator used is a rho-independent terminator of a stem-loop structure that stops transcription [24].

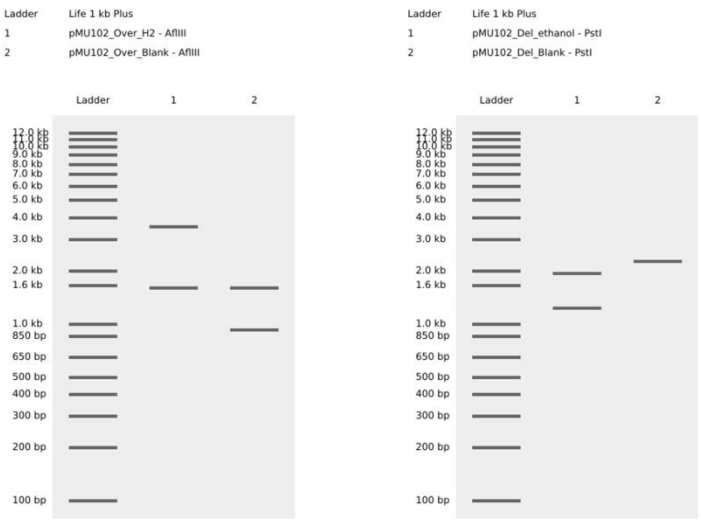
For laboratory design, PCR and Golden Gate assembling are utilized. Primer sequences are listed below in the figure. Moreover, a virtual digest is conducted and the result of using AflIII enzyme to cut the overexpression plasmid as well as that of using PstI enzyme to cut the deletion plasmid are shown respectively.



(a) Plasmid for hydrogenase overexpression



(b) Plasmid for dehydrogenase deletion



(c) Virtual digestion utilizing AflIII (Left) and PstI (Right)

Figure 2. Plasmid design and virtual digestion results (benchling.com)

Table 1. Primer sequences

Name	Sequence	GC	T/C
forward on vector_o	ATTATTTACGCGAGCTAGCCGTCTCAGCGT	50.00%	64.9
forward on KanR/NeoR	GCGTAGAGACGTAATTACTGTATCTCTCTGG CATGCCAGG	50.00%	66.9
reverse on KanR/NeoR	GCTCAGAGACGTTTTTTTTGCGATCGCGTTTT CGC	48.57%	67.2
forward on 815promoter	GAGCGGAGACGATAATATATATCCGT	42.31%	55.7
reverse on 815promoter	TCTCGGAGACGCATATTAAACACCTC	46.15%	58.4
forward on RS09545	GAGAGGAGACGATGCAAATGGTAA	45.83%	56.6
reverse on RS09545	TATGTGAGACGTCATTTAACAGGGT	40.00%	55.9

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Table 1. (continued).

forward on terminator	CATACGAGACGGCGATCGCGAAAACG	57.69%	64.2
reverse on terminator	CCGCCGAGACGTTTTTTTTGCGATCGCG	57.14%	66.9
reverse on vector_o	AAACAATCCTGCATGATCGTCTCTCCGC	50.00%	62.8
forward on vector_d	AACATACGCGAGCTAGCCGTCTCAGCGT	57.14%	66.9
forward on AmpR	GCGTAGAGACGTAATTACTGTATCTCT	40.74%	55.8
reverse on AmpR	CCGCCGAGACGTTTTTTTTGCGATCGC	55.56%	65.4
reverse on vector_d	AATCCTGCATGATCGTCTCTCCGC	54.17%	60.8

3. Conclusion

In conclusion, this research provides possible optimizations for biohydrogen production via modifying the metabolic pathway of *Clostridium Thermocellum*. Taking advantage of sugarcane bagasse as the feedstock not only provides abundant and low-cost resources but also contributes to the potential of the circular economy. Furthermore, biohydrogen production is effectively improved by overexpressing NADH-dependent [FeFe] hydrogenase and knocking out the pathway of ethanol production. This method demonstrates the possibility of achieving clean and efficient energy production without increasing energy consumption, offering a new direction for the development of sustainable energy.

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