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Ethanol production from lignocellulose using novel modified acetivibrio thermocellus

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Abstract. Ignocellulose is an abundant renewable biomass with great potential as a feedstock for biofuel production. In this study, we designed a plasmid through the online tool Benchling to improve the ability of the *acetivibrio thermocellus* to degrade native lignocellulose. By enhancing the expression of the Pyk gene, we will improve the saccharification and fermentation pathways. Our results show that the constructed plasmid has satisfactory theoretical feasibility. This work highlights the potential of synthetic biology in optimizing microbial platforms for sustainable biofuel production.

Keywords: Ignocellulose, Biofuels, Pyk gene.

1. Introduction

The increasing demand for sustainable energy sources has spurred significant interest in biofuels, with ethanol emerging as a promising alternative to fossil fuels[1]. Ethanol production has traditionally relied on food crops and industrial synthesis, competing with food supplies and causing inevitable environmental pollution[2, 3]. Thus, lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin, offers a renewable and non-food-based alternative[4]. Different enzymes facilitate the natural decomposition of lignocellulose by fungi, bacteria, or protozoans[5]. Lignocellulose is ultimately decomposed into three categories of substances: 1. Gaseous products, exemplified by carbon dioxide (CO_2); 2. Liquid products, including acetic acid and ethanol among others; 3. Solid products, such as biochar and similar substances[6, 7]. However, efficient conversion of lignocellulose into bioethanol remains a major challenge.

Acetivibrio thermocellus, a thermophilic anaerobe known by several names *(Hungateiclostridium thermocellum, Clostridium thermocellum, Ruminiclostridium thermocellum*), is well-known for having strong cellulolytic properties[8]. Its cellulase system breaks down lignocellulosic substrates by utilizing both free and cellulosome-bound enzymes. *Acetivibrio thermocellus* efficiently hydrolyzes ethanol, but instead naturally yields acetate and other byproducts[9]. In order to overcome this, metabolic pathways can be rewired with the use of synthetic biology and gene editing, increasing ethanol production while reducing byproducts.

The goal of this research is to maximize *Acetivibrio thermocellus*'s capacity for lignocellulose breakdown and ethanol generation by genetic engineering. We provided particular alterations to promote saccharification, reroute metabolic fluxes towards ethanol synthesis, and increase total yield by using

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the plasmids that were transplanted into *Acetivibrio thermocellus*. In order to provide a more effective platform for the generation of biofuel, this method incorporates cutting-edge synthetic biology techniques with the organism's natural skills.

2. Possible strategies

2.1. Product

In human culture, alcohol, or ethanol (C2H5OH), has significant industrial, social, and economic value. Biofuel and renewable energy for industrial production are two uses for ethanol. The primary conventional method for producing ethanol is fermentation, which works well for converting raw biomass materials on a big scale[10].Chemical synthesis is suitable for the petrochemical industry, producing ethanol through ethylene hydration[11]. Both methods have their own advantages and disadvantages. Fermentation relies on renewable resources and has good sustainability, while chemical synthesis relies on petroleum resources, has high production efficiency but has certain impacts on the environment. Therefore, we will use synthetic biology technology to optimize the production technology route of ethanol to improve the shortcomings of existing technology.

2.2. Approach/Pathway

The metabolic engineering of *acetivibrio thermocellus* was centered on optimizing the glycolytic and fermentation. The glycolysis pathway inside *acetivibrio thermocellus* is the key metabolic pathway for ethanol production. Glycolysis is the basic metabolic pathway for cells to obtain energy, starting from glucose and ultimately producing phosphoenolpyruvate (PEP). In this process, PEP is converted into pyruvate by the catalytic action of pyruvate kinase (Pyk).[12] Pyruvate is a crucial metabolic intermediate that undergoes two distinct routes of subsequent metabolism. First, pyruvate can be carboxylated to yield oxaloacetate; this reaction is frequently linked to the production of amino acids and the citric acid cycle. Second, acetyl-CoA, a key component of several metabolic pathways, can be produced from pyruvate. After that, acetyl-CoA can be reduced to ethanol by first converting to acetaldehyde. This metabolic process demonstrates how, in anaerobic environments, Acetivibrio thermocellus can produce energy via the ethanol fermentation pathway. Its ability to adjust metabolically allows it to live and procreate in a variety of environmental settings.

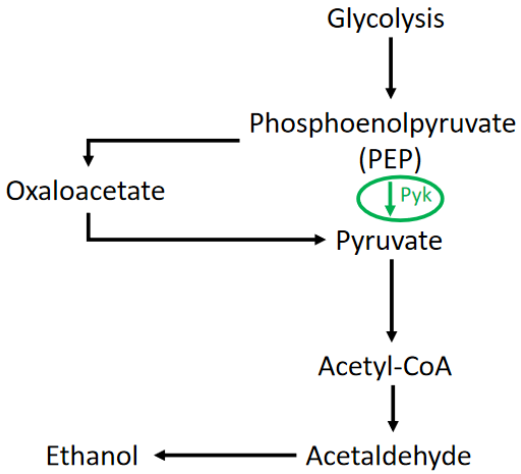

Figure 1. Pathway for ethanol production via modified *acetivibrio thermocellus*.

2.3. Host

The selection of Acetivibrio thermocellus was based on its innate capacity to break down lignocellulose at high temperatures, which is consistent with the thermophilic character of pretreatment procedures for lignocellulosic biomass[9, 13]. Comprising of cellulosomes, the organism's powerful cellulolytic system

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offers an effective mechanism for breaking down complex polysaccharides. Its anaerobic metabolism is also ideal for industrial fermentation settings, which lowers the possibility of air pollution.

2.4. Feedstock & Process

The primary source of biomass is organic matter, particularly animal and plant wastes[14]. With the application of industrial production technologies, biomass may be transformed into biomass energy, which can replace conventional fossil fuels and lower greenhouse gas emissions because of its sustainable and environmentally beneficial qualities. Lignocellulose is one of the main components of plant biomass[15]. In addition, lignocellulose can be obtained from agricultural residues including corn stover and wheat straw, and its abundance and low cost also facilitate large-scale production. The modified *acetivibrio thermocellus* were cultured in anaerobic bioreactors, maintained at 60°C to align with the organism's optimal growth temperature and the thermophilic nature of the pretreatment process[13].

2.5. Design Strategy

We enhanced the formation of phosphoenolpyruvate by overexpressing key genes (Pyk, Tsac_1363) to speed up the rate of glycolysis (Figure 1). These modifications improved the efficiency of lignocellulose saccharification, increasing the availability of fermentable sugars for ethanol production. The design contains promoter sequence and Pyk gene sequence to ensure efficient transcription and translation (Table 1). The plasmid we finally constructed through the online tool Benchling (http://Benchling.com) is shown in Figure 2.

Our constructed plasmid also has many advantages. First, the constructed plasmid contains ampicillin resistance (AmpR) gene[16] and kanamycin resistance (KanR) gene[17], which allows it to be used under two different antibiotic selection conditions. Secondly, ColE1 origin is a high-copy number replication origin[18], which means that this plasmid can exist in the host cell at a higher copy number, increasing the expression of the target gene, which is very beneficial for experiments that require high-level expression.

It should be noted that the results of our study have wider implications. It should be noted that our findings have broader implications. The modified acetivibrio thermocellus can reduce the production cost of biofuels, reduce environmental pollution, promote circular economy and promote the development of related industries.

Table 1. The information of recombinant plasmid.

Primer	Sequence	GC%	Tm
pKIM1-5'-forward	CACCTGCTGCAATCCAGCTTTGGCTAACAC	53.33%	65.4℃
pKIM1-3'-reverse	CACCTGCATTGGGTGTGGCGTAATCATGGT	53.33%	66.0℃
promoter-5'-forword	CACCTGCATTGGGTGAATTGTAATTGCAAT	40%	60.7℃
promoter-3'-reverse	CACCTGCGGCGTGGAAGACAAAAAAATAAA	43.30%	62.5℃
PyK-5'-forward	CACCTGCGGCGTGGAATGCGTAGAACTAAG	56.67%	66.2℃
PyK-3'-reverse	CACCTGCGGCTCGCTCTATTTTATATTCAC	46.67%	61.4℃

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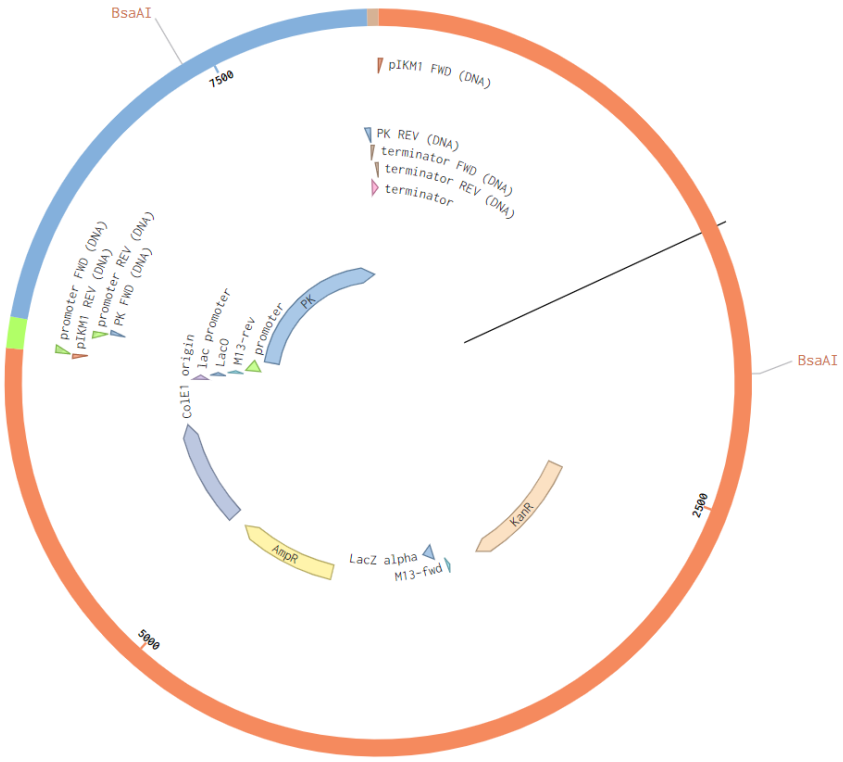

Figure 2. Schematic illustration of plasmids construction.

3. Conclusion

This study was theoretically successful in constructing plasmids with a view to improving *acetivibrio thermocellus*. We use gene editing technology to introduce key genes such as Pyk into cells using plasmids, causing overexpression and thereby enhancing the catalytic action of pyrovate kinase. Ultimately, we can achieve an increase in the rate at which *acetivibrio thermocellus* decomposes lignocellulose to produce ethanol. From this, we can obtain a highly suitable bacterial strain for industrial production of bioethanol fuel, which is beneficial for expanding the production of bioethanol and improving the utilization of cellulose waste. Future work will focus on achieving improved lignocellulose degradation and ethanol production through synthetic biology and gene editing, such as By timely isolating the product, the requirement for ethanol tolerance of the strain can be reduced, and the purity of the product can be improved by knocking out the genes of other products.

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