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Short communication

Cofactor engineering of ketol-acid reductoisomerase (IlvC) and alcohol dehydrogenase (YqhD) improves the fusel alcohol yield in algal protein anaerobic fermentation

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ABSTRACT

The feasibility of converting algal protein to mixed alcohols has recently been demonstrated with an engineered *E. coli* strain, enabling comprehensive utilization of the biomass for biofuel applications. However, the yield and titers of mixed alcohol production must be improved for market adoption. A major limiting factor for achieving the necessary yield and titer improvements is cofactor imbalance during the fermentation of algal protein. To resolve this problem, a directed evolution approach was applied to modify the cofactor specificity of two key enzymes (IlvC and YqhD) from NADPH to NADH in the mixed alcohol metabolic pathway. Using high throughput screening, more than 20 YqhD mutants were identified to show activity on NADH as a cofactor. Of these 20 mutants, the four highest activity YqhD mutants were selected for combination with two IlvC mutants, both accepting NADH as a redox cofactor, for modification of the protein conversion strain. The combination of the IlvC and YqhD mutants yielded a refined *E. coli* strain, subtype AY3, with increased fusel alcohol yield of ~60% compared to wild type under anaerobic fermentation on amino acid mixtures. When applied to real algal protein hydrolysates, the strain AY3 produced 100% and 38% more total mixed alcohols than the wild type strain on two different algal hydrolysates, respectively. The results indicate that cofactor engineering is a promising approach to improve the feasibility of bioconversion of algal protein into mixed alcohols as advanced biofuels.

1. Introduction

Increasing concerns about the sustainability of fossil fuels compel the development of sustainable alternatives from renewable resources [1,2]. Algae-based biofuels have been considered as sustainable alternative fuel sources due to several benefits, including reduced recalcitrance and higher biomass productivity than terrestrial (lignocellulosic) biomass, and the ability to cultivate algae using non-arable land and non-fresh water resources [3,4]. Thus far, algae biomass has been reported to be converted into a variety of biofuel candidates, such as bioethanol [5], biohydrogen [6], biogas [7], biocrude [8], terpenes [9], and biodiesel [10,11]. However, the majority of the research and development for algal biofuels has focused on biodiesel production by increasing algae lipid yields under nutrient stress conditions, which results in dramatically reduced net biomass productivity. Under conditions supporting robust algae growth, algal carbohydrates and proteins are typically the two dominant components of the biomass, comprising up to ~80% of the ash-free dry weight of microalgae biomass; with algal protein alone up to 60% [4,12,13]. Recently, conversion of the algal protein fraction to mixed alcohols was demonstrated using an engineered *E. coli* strain, predominantly generating C4 and C5 alcohols from protein hydrolysates at up to 56% of the net theoretical yield [14]. This approach enables a novel path forward for comprehensive utilization of algae biomass for production of promising fuel compounds. Furthermore, the high-value amino acids, methionine, lysine, and tryptophan are not consumed in this conversion pathway, and have potential as a high-value co-product from renewable fuel production for feed and mariculture applications. Through the combination of the native Ehrlich pathway and three exogenous transamination and deamination cycles, the engineered *E. coli* strain reached 0.183 g fusel alcohol/g amino acid mixture under aerobic or microaerobic fermentation [14]. However, anaerobic conditions are preferred for large scale fermentation due to lower operating costs and higher theoretical yield. In the Ehrlich pathway, two key enzymes, ketol-acid reductoisomerase (IlvC) and alcohol dehydrogenase (YqhD) utilize nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, indicating that bioconversion of protein hydrolysates requires two reducing equivalents (in the form of NADPH) as bioconversion of glucose to isobutanol [15]. However, under anaerobic fermentation conditions, glycolysis is the sole pathway that can produce

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nicotinamide adenine dinucleotide (NADH) as an available reducing equivalent because the pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle are not functional (due to the lack of oxygen). These conditions may result in cofactor imbalance leading to unfavorable bioenergetics for amino acid catabolism during anaerobic or oxygen limited fermentation conditions. NADPH limitation resulting in cofactor imbalance has been reported in previous studies for isobutanol production from glucose [16,17]. We therefore hypothesized that improvements in fusel alcohol yield could be achieved by mitigating cofactor imbalance in anaerobic or oxygen-limited fermentation of algal protein. To achieve this, we first tested the hypothesis through supplementation of NADPH in the fermentation media. Then, we conducted protein engineering to switch the cofactor specificity of two isobutanol biosynthesis pathway enzymes, ketol-acid reductoisomerase (IlvC) and alcohol dehydrogenase (YqhD), from NADPH to NADH through directed evolution. Upon combining the beneficial mutations of the two enzymes in the fusel alcohol biosynthesis pathway, the engineered *E. coli* strain with the combination of highest activity mutations improved fusel alcohol yield by ~60% compared to wild type under anaerobic fermentation conditions using amino acid mixtures. When applied to unsupplemented algae hydrolysates, the strain with the best performance produced 100% and 38% more fusel alcohol than the wild type strain on two different algal hydrolysates, respectively.

2. Material and methods

2.1. Strains and plasmids

The mutant *E. coli* strain YH83 (BW25113/F′ [traD36, proAB+, lacIqZΔM15]Δ*glnA*, Δ*gdhA* Δ*luxS* Δ*lsrA*) containing isobutanol biosynthesis pathway genes in three separate plasmids pYX68, pYX90 and pYX97 was generously provided by Professor James C. Liao from University of California, Los Angeles (UCLA) [14]. The strain was engineered for the bioconversion of protein hydrolysates into mixed alcohols, especially isobutanol. The plasmids pYX90 and pYX97 contain IlvC and YqhD, which use NADPH as a redox cofactor. Both plasmids pYX90 and pYX97 containing multiple pathway genes in addition to YqhD and IlvC are induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) under the antibiotics spectinomycin and ampicillin, respectively. The plasmid pBbE1a and the *E. coli* strain DH1 were used for the expression and screening of a mutant library of enzyme YqhD as well as the creation of IlvC mutants.

2.2. Identification of redox cofactor binding sites

The amino acids for mutation in YqhD were selected through the inspection of the co-factor NADPH binding site. Autodock 4 was used to investigate the cofactor-enzyme binding interaction. The X-ray crystal structure of YqhD (1OJ7) containing the redox cofactor NADPH was extracted from protein data bank (pdb.org).

2.3. Mutant library construction, expression, and high-throughput screening

The plasmid pBbE1a was generously provided by Dr. Taek Soon Lee from Joint BioEnergy Institute. The genes IlvC and YqhD were amplified from plasmids pYX90 and pYX97 and sub-cloned into vector pBbE1a under the restriction cutting sites *Eco*RI and *Bam*HI to achieve vectors pBbE1a-IlvC and pBbE1a-YqhD, respectively. Saturation mutagenesis was applied to create the recombinant mutant library of enzyme YqhD following the description provided in the previous study [18]. For generation of the recombinant library of YqhD, primers with degenerate codons were used to create mutations at two selected amino acid sites. The high-fidelity DNA polymerase fusion Q5 (NEB, CA) was used to generate the library and expressed it in the *E. coli* strain DH1. The strains containing YqhD mutants were diluted and spread onto Luria-Bertani (LB) plates supplemented with ampicillin (100 μg/mL) as the antibiotics for library screening. According to the previous study [17], the two mutant IlvCs (A71S, R76D, S78D Q110V/Q110A) modified the cofactor specificity from NADPH to NADH with high activity for NADH. Site-directed mutagenesis was used to create two mutants of the enzyme IlvC at the four target amino sites mentioned above. An iterative strategy was used to create all four mutations. The high-fidelity DNA polymerase fusion Q5 (NEB, CA) was used to create the point mutations of IlvC in the vector pBbE1a-IlvC as described previously [18]. The mutations were confirmed through DNA sequencing. All the primers used in this study were listed in Table 1.

Single colonies of the YqhD mutant and wild type were picked and cultured into 96-well plates. Each well contained 200 μL LB medium with the corresponding antibiotics. The cultures were incubated at 300 rpm, 37 °C overnight (16 h) and induced by 1.0 mM IPTG for another 24 h at 250 rpm, 30 °C in a humidified shaker. The cells were harvested by centrifugation (3000 rpm, 4 °C, 15 min) and stored at −20 °C prior to the assays. For the high-throughput screening assays, *E. coli* cells were lysed with 200 μL of 50 mM MOPS (pH 7.0) containing 1 mg/mL lysozyme (Sigma, MO), 20 U/mL DNase (NEB, CA) at 4 °C for 6 h under gentle agitation. The lysed cells were spun down and 100 μL of the cell free extract was transferred into another 96 well plate. The activity assay buffer for YqhD and its mutants contained 50 μL of 0.25 mM NADH in 50 mM MOPS (pH 7.0), 10 μL of isobutyraldehye, and 40 μL of 50 mM MOPS (pH 7.0). The oxidation of NADH was monitored by absorbance at 340 nm in a plate reader at 37 °C (Molecular Devices, CA). Additionally, the cell free extract of the wild type was assayed using NADPH or NADH as a cofactor under same conditions as a positive or negative control, respectively. The total protein concentrations in the cell free extract were measured by the bicinchoninic acid assay (Pierce BCA protein assay Kit, Thermo Fisher Scientific, CA) followed the manufacture's protocol. Vmax were calculated after nonlinear regression of the experimental points using a Michaelis-Menten model in Soft Max Pro and normalized by total protein levels of the wild type and each mutant, correspondingly.

2.4. Plasmids and strain construction for anaerobic bioconversion of algal protein hydrolysates

The positive hits with high activity on NADH were selected to replace the wild type YqhD gene in the plasmid pYX97. The two mutant genes of IlvC were selected to replace the wild type IlvC gene in the plasmid pYX90 as well. Gibson assembly was applied to replace the wild type YqhD and IlvC with the mutant genes. The plasmids pYX97, pYX90 with the mutant genes, and pYX68 were co-transformed into strain YH40 for the bioconversion of algal protein hydrolysates into fusel alcohols. All of the primers used in this study were listed in Table 1.

2.5. Hypothesis testing with the addition of NADPH into fermentation media

The wild type strain YH83 was cultured in 20 mL of LB media with 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, and 25 μg/mL spectinomycin, overnight. Then 5 mL of culture was transferred into 150 mL of 1× M9 medium [14,19] (in a 160 mL serum bottle) containing 20 g/L amino acid mixture, 2 g/L LB, and 150 μM NADPH with the corresponding aforementioned antibiotics in a rotary shaker at 220 rpm, 37 °C. The culture was induced by 1 mM IPTG at 37 °C when the OD reached 0.6 to express pathway enzymes for the production of fusel alcohols. Samples were taken at regular time intervals for analysis of fusel alcohol concentrations.

2.6. Fusel alcohol yields of the mutant strains using an amino acid mixture

The engineered YH83 strains containing various combinations of mutant YqhD and IlvC were cultured overnight in 20 mL of LB media