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

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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.

Keywords: directed evolution, isobutanol, 2/3-methyl butanol, algae protein bioconversion, cofactor engineering, algal biofuel

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 bioethanol⁵, biohydrogen⁶, biogas⁷, biocrude⁸, terpenes⁹, 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 yield¹⁴. 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 fermentation¹⁴. 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 isobutanol¹⁵. However, under anaerobic fermentation conditions, glycolysis is the sole pathway that can produce 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.

Material and Methods

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.

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).

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 *EcoRI* and *BamHI* 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 study¹⁸. 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 (100µg/mL) as the antibiotics for library screening. According to the previous study¹⁷, 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 previously¹⁸. 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 hours) and induced by 1.0 mM IPTG for another 24 hours 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 (pH7.0) containing 1 mg/mL lysozyme (Sigma, MO), 20 U/mL DNase (NEB, CA) at 4°C for 6 hours 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 (pH7.0), 10µL of isobutyraldehyde, and 40 µL of 50 mM MOPS (pH7.0). 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.

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.

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 5mL of culture was transferred into 150 mL of 1X M9 medium^{14, 19} (in a 160mL 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.

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 with the corresponding aforementioned antibiotics. Then 3 mL of culture was transferred into 30 mL of 1X M9 medium containing 20 g/L amino acid mixtures (Sigma, MO), 5 g/L LB, and antibiotics. The fermentation was performed as described previously. The sequence details of the mutants are listed in the table 1.

Table 1: Primers and mutants of YqhD and IlvC used in this study. The nuclease cutting sites or mutations were highlighted.

Order	Name	Sequence	Demo
1	YqhD-pBbE1a-F	5-CTC AGC GAA TTC ATG AAC AAC TTT AAT CTG CAC ACC CCA AC-3	clone YqhD into pBbE1a
2	YqhD-pBbE1a-R	5-TGACCT GGATCC TTA GCG GGC GGC TTC GTA TAT AC-3	
3	IlvC-pBbE1a-F	5-CTC AGC GAA TTC ATG GCT AAC TAC TTC AAT ACA CTG AAT CTG C-3	clone IlvC into pBbE1a
4	IlvC-pBbE1a-R	5-TGACCT GGATCC TTA ACC CGC AAC AGC AAT ACG TTT C-3	
5	YqhD-S40-F	5-GTATTGATTACCTACGGCGGC GGC NNN GTG AAA AAA ACC GGC GTT CTC-3	create mutant library S40
6	YqhD-S40-R	5-GAG AAC GCC GGT TTT TTT CAC NNN GCC GCC GTA GGT AAT CAA TAC-3	
7	YqhD-G39S40-F	5-GTATTGATTACCTACGGCGGC NNN NNN GTG AAA AAA ACC GGC GTT CTC-3	create mutant library G39S40
8	YqhD-G39S40-R	5-GAG AAC GCC GGT TTT TTT CAC NNN NNN GCC GCC GTA GGT AAT CAA TAC-3	
9	YqhD-S40P-F	5-GTATTGATTACCTACGGCGGC CCG GTG AAA AAA ACC GGC GTT CTC-3	Mutant Y1 (S40P)
10	YqhD-S40P-R	5-GAG AAC GCC GGT TTT TTT CAC CGG GCC GCC GTA GGT AAT CAA TAC-3	
11	YqhD-S40R-F	5-GTATTGATTACCTACGGCGGC CGT GTG AAA AAA ACC GGC GTT CTC-3	Mutant Y2 (S40R)
12	YqhD-S40R-R	5-GAG AAC GCC GGT TTT TTT CAC CGT GCC GCC GTA GGT AAT CAA TAC-3	
13	YqhD-G39I/S40R-F	5-GTATTGATTACCTACGGCGGC ATCCGT GTG AAA AAA ACC GGC GTT CTC-3	Mutant Y3 (G39I/S40R)

14	YqhD-G39I/S40R-R	5-GAG AAC GCC GGT TTT TTT CAC ACG GAT GCC GCC GTA GGT AAT CAA TAC-3	
15	YqhD-G39Y/S40H-F	5-GTATTGATTACCTACGGCGGC TATCAT GTG AAA AAA ACC GGC GTT CTC-3	Mutant Y5 (G39Y/S40H)
16	YqhD-G39Y/S40H-R	5-GAG AAC GCC GGT TTT TTT CAC ATG ATA GCC GCC GTA GGT AAT CAA TAC-3	
17	IlvCA71SR76DS78D-F	5-CGT AAA GAA TCG ATT GCC GAG AAG GAT GCG GAT TGG-3	
18	IlvCA71SR76DS78D-R	5-CCA ATC CGC ATC CTT CTC GGC AAT CGA TTC TTT ACG-3	
19	ilvCQ110A-F	5-CGG ACA AGG CGC ACT CTG ATG TAG-3	Mutant A (A71S/R76D/S78D/Q110A)
20	ilvCQ110A-R	5-CTA CAT CAG AGT GCG CCT TGT CCG-3	
21	ilvCQ110V-F	5-CGG ACA AGG TGC ACT CTG ATG TAG-3	Mutant V (A71S/R76D/S78D/Q110V)
22	ilvCQ110V-R	5-CTA CAT CAG AGT GCA CCT TGT CCG-3	
23	ILVC-Pyx90-Gib-F1	5-GAA AGC TCT CTA GGT CGA CGA GGA ATC ACC ATG GCT AAC TAC TTC AAT ACA CTG AAT CTG-3	Replace IlvC with mutant A and V
24	ILVC-Pyx90-Gib-R1	5-GTA CTT AGG CAT GGT ATA TCT CCT TCC GGG TTA ACC CGC AAC AGC AAT ACG TTT CAT ATC-3	
25	ILVD-AvTA-pYX90-Gib-F2	5-GAT ATG AAA CGT ATT GCT GTT GCG GGT TAA CCC GGA AGG AGA TAT ACC ATG CCT AAG TAC-3	
26	ILVD-AvTA-pYX90-Gib-R2	5-GGA TTT GTC CTA CTC AGG AGA GCG TTC ACC GAC AAA CAA CAG ATA AAA CGA AAG GCC CAG-3	
27	Spec-alsS-pYX90-Gib-F3	5-CTG GGC CTT TCG TTT TAT CTG TTG TTT GTC GGT GAA CGC TCT CCT GAG TAG GAC AAA TCC-3	
28	Spec-alsS-pYX90-Gib-R3	5-CAG ATT CAG TGT ATT GAA GTA GTT AGC CAT GGT GAT TCC TCG TCG ACC TAG AGA GCT TTC-3	
29	YqhD-Pyx97-Gib-F1	5-GGA GAA AGG TCA CAT GAA CAA CTT TAA TCT GCA CAC CCC AAC CCG CAT TC-3	Replace YqhD with mutant Y1, Y2, Y3, Y5
30	YqhD-Pyx97-Gib-R1	5-CTC TAG CAC GCG TAC CAT GGG ATC CTT AGC GGG CGG CTT CGT ATA TAC-3	
31	ColE-Amp-pYX97-Gib-F2	5-GTA TAT ACG AAG CCG CCC GCT AAG GAT CCC ATG GTA CGC GTG CTA GAG-3	
32	ColE-Amp-pYX97-Gib-R2	5-CAT GAT AAT AAT GGT TTC TTA GAC GTC AGG TGG CAC TTT TCG GGG AAA TGT GCG CGG AAC-3	
33	LeuDH-KivD-pYX97-F3	5-GTT CCG CGC ACA TTT CCC CGA AAA GTG CCA CCT GAC GTC TAA GAA ACC ATT ATT ATC ATG-3	
34	LeuDH-KivD-pYX97-R3	5-GAA TGC GGG TTG GGG TGT GCA GAT TAA AGT TGT TCA TGT GAC CTT TCT CC-3	

Bioconversion of algal protein hydrolysates into fusel alcohols

Samples of *Nannochloropsis salina* biomass (20% solids) were obtained from Thomas Dempster at AzCATI and pretreated with dilute acid (DA) according to the protocol from the National Renewable Energy Laboratories²⁰ and the flash hydrolysis (FH) protocol described in a previous study^{21, 22}. Whereas DA pretreated biomass contains hydrolyzed carbohydrates in addition to protein, FH concentrates the proteins up to ~70% of the total AFDW of the hydrolysate. Residual hydrolyzed algal carbohydrates were converted into ethanol through alcoholic fermentation using an ethanogenic strain, *E. coli* KO11. The cell mass was removed through centrifugation (6000 rpm, 4°C, 10 mins) following termination of the KO11 fermentation. The supernatant containing ethanol and algal protein was air-bubbled at room temperature for 10 minutes to remove ethanol. Then it was concentrated and digested with 2 mg/mL Pronase (Promega, CA) following the manufacturer's protocol. The protease digested algal protein hydrolysate was filter sterilized using a 0.45 µm PTFE membrane and subsequently used as the fermentation media for fusel alcohol production with no additional supplementation of other nutrient factors. The mutant strain that was identified to produce the highest fusel alcohol yield on the commercial amino acid mixture was employed for subsequent bioconversion studies of the algae hydrolysates. The fermentation was

performed as described previously. Samples were collected at regular intervals for analysis by GC-MS/FID.

Analytic determination of concentrations of fusel alcohols and amino acids

The concentrations of amino acids were analyzed using amino acid analyzer (Hitachi, Japan) at the Genome Center of University of California, Davis. The fusel alcohols were extracted using ethyl acetate with 2-methyl-pentanol as internal reference at the ratio of 1:1(ethyl acetate: fermentation broth). The mixture was vortex at 1200 rpm for 20 mins and centrifuged at 14000 rpm, 5 mins. The ethyl acetate layer was collected for further GC-MS analysis. Two μL of sample was injected into the injection port (220°C) of an Agilent gas chromatograph 7890A containing a 30m x 0.25mm ID DB wax capillary column with a film thickness 0.25 μm . The column was temperature programmed as follows: 40°C for 4 min, increasing to 65°C at $10^{\circ}\text{C}/\text{min}$ and holding for 10 min, then increasing to 120°C at $10^{\circ}\text{C}/\text{min}$ and holding for 2 min, then increasing to 220°C at $20^{\circ}\text{C}/\text{min}$ and holding for 5 min. The carrier gas was ultra-high purity helium at a constant flow rate of 1.8 mL/min. The initial column head pressure was 16.188 psi with a split ratio of 10. The chromatograph is coupled to a quadrupole MS 5975C. The MSD parameters were EI at 70eV, mass range was 10-650 Da, and the scan speed was 2 scans/sec. Spectral components were searched against the NIST 2015 mass spectral library. Serial of dilutions of isobutanol and 2- and 3-methyl-butanol (henceforth as 2/3-methyl butanol), which were produced as an enantiomeric mixture in the culture, in ethyl acetate were analyzed under same conditions as quantitation standards. The concentrations of fusel alcohols were calculated by referring samples to a standard curve.

Experimental replication and statistical treatment

All the fermentation experiments were performed as biological duplicates. All enzyme activity analyses were performed in sextuple. The analysis of the concentrations of isobutanol and 2/3-methyl-butanol were performed as duplicate. Average values were reported together with standard deviation as error bars in figures.

Results and Discussion

Engineering the cofactor specificity of *E. coli* IlvC and YqhD to NADH

The X-ray crystal structure of YqhD has been previously reported²³, containing the redox cofactor NADP^{+} as a ligand. The protein possesses a GGGS (residues 37-40) motif, which binds the 2-phosphate groups of NADP^{+} through hydrogen bonds, as shown in figure 1(A). These hydrogen bonds provide the preferential binding of NADPH over NADH, making the motif GGGS as major determining factor for

redox cofactor specificity. Based on this observation, the two amino acids Gly39 and Ser40 were chosen for mutation to alter the cofactor specificity from NADPH to NADH through site-saturation mutagenesis. Two mutant libraries (G39S40 and S40) were generated and screened for NADH activity using the aforementioned cell free extract. More than 20 mutants were identified to show activity on NADH as a cofactor. Five of the mutants showed 60-80 times higher activity on NADH than wild-type YqhD, and were down-selected (as showed in figure 1(B)) to replace the wild type YqhD in the plasmid pYX97, including two single mutations of S40 (Y1, Y2) and three double mutations of G39S40 (Y3, Y4, Y5). The *E. coli* IlvC has been previously engineered to switch the cofactor specificity from NADPH to NADH for isobutanol production under anaerobic fermentation¹⁷. Two mutants of IlvC (A71S, R76D, S78D, Q110V/A) with higher activity on NADH were chosen to replace the wild type IlvC in the plasmid pYX90.

Figure 1: Cofactor engineering of *E. coli* YqhD. A): Structural alignment of the NADP⁺-binding pocket of YqhD. B): Mutant enzyme activities using NADH as a redox cofactor. V_{max} was normalized to the total cellular protein. Mean values are shown, and the error bars represent the standard deviation of the mean.

Addition of NADPH improves the fusel alcohol yield under anaerobic fermentation

We hypothesized that cofactor imbalance compromised the fusel alcohol yield from protein bioconversion. To test this hypothesis, the native NADPH cofactor of YqhD and IlvC was added to the fermentation medium as an external source of redox equivalents. The results indicated that the addition of NADPH significantly improved the yields of isobutanol and 2/3-methyl-butanol for protein bioconversion, as shown in the figure 2. Within five days of fermentation, the yields of isobutanol, 2/3-methyl-butanol, and total fusel alcohols increased by 34%, 28%, and 30%, respectively, confirming that cofactor imbalance was a critical factor affecting the yield of fusel alcohol production from protein fermentation. Furthermore, besides these three dominant fusel alcohol products, significant quantities of

additional alcohol and amino acid catabolites were also detected in the fermentation broth, including phenylethanol, butanediol, and acetoin.

Figure 2: NADPH depletion limits fusel alcohol yields. A): the final concentrations of mixed alcohols under anaerobic fermentation from the parent strain with and without addition of NADPH; B): the percentage of fusel alcohol yield improvement compared to the parent strain with NADPH addition at different time points. Mean values are shown, and the error bars represent the standard deviation of the mean.

Bioconversion of amino acid mixtures and algal protein hydrolysates into fusel alcohols

The fusel alcohol yields of the engineered YH83 strains, which contain both *IlvC* and *YqhD* mutants, were firstly investigated for bioconversion of commercial amino acid mixtures in 1X M9 medium. As expected, the results indicated that the mutant strains produced various amounts of fusel alcohols in the fermentation broth. Similar fusel alcohol profiles were detected from both sets of fermentation media, and 2-methyl-butanol and 3-methyl-butanol were produced as an enantiomeric mixture (henceforth referred to as 2/3-methyl-butanol). Among 7 mutant strains, four strains (AY1, AY3, VY3, and VY5) yielded higher concentrations of fusel alcohols than the wild type, which were more than 30% higher than the parent strain. Particularly, the strain AY3 produced more than 60% higher quantities of fusel alcohols than the wild type, corresponding to 6.0 g/L of total mixed alcohols from 20 g/L amino acids. The wild type produced 3.7 g/L total mixed alcohols, which is similar to the previous report¹⁴. Furthermore, two algal hydrolysates were utilized as substrates to investigate fermentation performance of the mutant strain AY3. As shown in figure 3(B), the mutant strain AY3 produced higher titers of isobutanol, 2/3-methyl-butanol, as well as total fusel alcohols on both the dilute acid (DA) and the flash hydrolysis (FH) pretreated algal hydrolysates than the wild-type strain YH83. Within three days of fermentation, the mutant strain AY3

yielded more than 100% and 38% higher total fusel alcohols titers than wild type on the DA and FH algal hydrolysates, respectively, as shown in figure 3(B). However, the titers of isobutanol, 2/3-methyl-butanol, and total fusel alcohols were significantly lower than the yields on the commercial amino acid mixtures (figure 3(A)) similar to the previous study, in which the wild type strain produced approximately 4 g/L of total fusel alcohol from 20 g/L mixed algal biomass hydrolysates¹⁴. The lower yields of fusel alcohols are likely due to several facts. First, only algal protein hydrolysate was used as the fermentation medium in the studies conducted here, which lacks of all trace nutrient factors included in the LB that added into 1XM9 medium reported previously. Secondly, the pretreated algal protein hydrolysates used in this study lacked the algal carbohydrate which was converted into ethanol in the stage one alcoholic fermentation. Additionally, air bubbling was insufficient to completely remove the ethanol in the algal hydrolysates, which may have inhibited growth of the protein conversion strain. Moreover, the whole algae hydrolysates used in the study have a more complex composition than the mixed algal hydrolysate used in the previous study, likely including fermentation inhibitors such as furfural, fatty acids, and elevated salt loading that are inhibitory to the *E. coli* chassis strain. It is likely that each of these factors, as well as product inhibition, contributes to retarded growth of the fermentation strain and incomplete consumption of the substrate, ultimately reducing the final mixed alcohol yields. Based on the substrate consumption, the strain AY3 yielded 0.2 and 0.18 g fusel alcohol/ g amino acid on the dilute acid pretreated and flash hydrolyzed algal biomass, respectively. The yields of fusel alcohols are similar or slightly higher than the previously reported 0.183 g fusel alcohol/ g amino acid¹⁴ without the need for addition of salts or other prepared media components

Figure 3: Bioconversion of algal protein hydrolysates into fusel alcohols by the engineered protein conversion strain. A): the fermentation performance of the seven select mutant and wild-type strains on

amino acid mixture; B): Bioconversion of two algal protein hydrolysates into fusel alcohols with the AY3 mutant strain. Mean values are shown, and the error bars represent the standard deviation of the mean.

Conclusion

Fusel alcohols are being considered as advanced biofuels because they possess higher energy density and lower vapor pressure, hygroscopicity, and corrosivity than fuel ethanol^{24, 25}. Recently, production of fusel alcohols from algal protein was reported, albeit at a relative low yield and titer¹⁴. One of the potential factors that results in the suboptimal conversion performance is the imbalance of redox cofactors during fermentation. In this study, we investigated engineering of the cofactor binding site for two key enzymes (IlvC and YqhD) in the fusel alcohol²⁶ amino acid catabolic pathway to resolve the cofactor imbalance. By combining beneficial mutations of these two enzymes, the engineered *E. coli* strain AY3 improved fusel alcohol yield by 100% and 38% compared to wild type on algae protein hydrolysates generated by dilute acid pretreatment and flash hydrolysis, respectively. Therefore, this study provides a promising means to improve fusel alcohol production and process feasibility using algae biomass.

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Contributions

W.W.H and R.W.D conceived and designed the study. W.W.H performed the experiments, collected and analyzed the data, wrote and revised the manuscript. R.W.D supervised the study, and revised the manuscript. M.B.T and J.D.J performed the algal biomass hydrolysis and algal hydrolysate bioconversion. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.