Fusion of Pyruvate Decarboxylase and Alcohol Dehydrogenase Increases Ethanol Production in Escherichia coli

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Fusion of Pyruvate Decarboxylase and Alcohol Dehydrogenase Increases Ethanol Production in *Escherichia coli*

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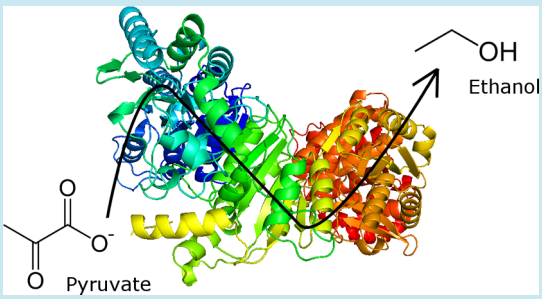
Supporting Information

ABSTRACT: Ethanol is an important biofuel. Heterologous expression of *Zymomonas mobilis* pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) increases ethanol production in *Escherichia coli*. A fusion of PDC and ADH was generated and expressed in *E. coli*. The fusion enzyme was demonstrated to possess both activities. AdhB activity was significantly lower when fused to PDC than when the two enzymes were expressed separately. However, cells expressing the fusion protein generated ethanol more rapidly and to higher levels than cells coexpressing Pdc and AdhB, suggesting a specific rate enhancement due to the fusion of the two enzymes.



Production of ethanol from cellulosic biomass is required to address issues of CO_2 release associated with fossil fuels, and competition with food associated with first generation biofuels derived from grains.[1] *Sacharomyces cerevisiae* is unable to utilize monosacharides generated from hemicellulose. This can be overcome by creation of *Escherichia coli* strains expressing *Zymomonas mobilis* pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB), which increase microbial ethanol production.[2] As ethanol needs to be produced in large quantities to be used as a petrol replacement, any improvements in the production process may make a significant contribution to biofuel commercialization. Here, we report the generation of a translational fusion of *pdc* and *adhB* and characterization of the resulting chimeric protein and the effects of this fusion on ethanol production in *E. coli*.

RESULTS AND DISCUSSION

Genaration of the Pdc-AdhB Fusion. BioBrick BBa_K173003 containing the coding sequences of *Z. mobilis pdc* and *adhB*, codon-optimized for expression in *E. coli* each with ribosome binding site BBa_B0030 (iGEM team Pavia, 2009) in pSB1K2 was used as a template. Forward (GCATCAAGCACCTTTTATATCC) and reverse (CAGCAGTTTATTCACCGGTTTAC) primers were designed to amplify the entire construct with deletion of the stop codon of *pdc*, start codon of *adhB*, and all intervening DNA. The resulting PCR product was self-ligated to generate the final fusion construct, in which the coding sequences of *pdc* and *adhB* are directly apposed. The *lac* promoter and *lacZα′* marker were excised from BBa_J33207 and inserted upstream of the fusion gene to give the final construct BBa_K1122674 in pSB1K2. The same promoter was also introduced upstream of the original unfused construct to give BBa_K1122676. All constructs were verified by sequencing.

Confirmation of Fused Protein State. Recombinant *E. coli* JM109 bearing the fused and unfused constructs were grown overnight in LB with IPTG induction. Cell lysates were prepared by sonication and analyzed by SDS-PAGE and Native-PAGE. SDS-PAGE showed strong bands of the expected size for both fused and unfused proteins (Figure 1a). Native PAGE gels stained for ADH activity showed that the ADH activity band migrated more slowly in the fused state, consistent with a larger protein (Figure 1b). Staining for PDC activity showed that activity was located in the same band (data not shown). ADH activity in the fusion extract was measured as

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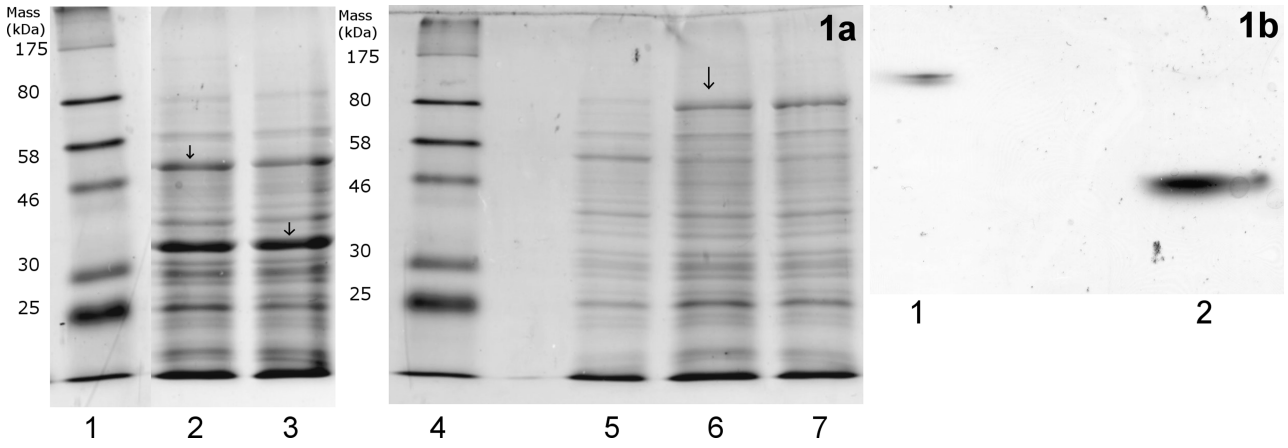

Figure 1. (a) SDS-PAGE analysis. Two intense bands of size corresponding to Pdc (expected 60.93 kDa) and AdhB (expected 40.15 kDa) are present within lysates from cells expressing nonfused enzymes (lanes 2 and 3). Those are replaced with a single band (lanes 6 and 7) of an increased size in cells expressing fused Pdc–AdhB (expected mass: 100.93 kDa). No bands corresponding to Pdc, AdhB, or fused Pdc–AdhB are present in lysates from induced cells transformed with an empty vector (lane 5). Molecular weight of observed protein bands was assessed using NEB broad range protein marker (lanes 1 and 4). (b) Native-PAGE analysis stained for AdhB activity. Activity can be attributed to a peptide of an increased mass in the fused protein state (lane 1) compared to nonfused enzyme (lane 2).

0.091 U/mg, as compared to 1.79 U/mg in the nonfusion extract, indicating that ADH activity might be reduced by the fusion. Specific activity of Pdc was not measured.

Ethanol Production in Bacteria Expressing Fused and Nonfused Enzymes. Recombinant *E. coli* JM109 bearing the fused and unfused constructs was used for ethanol production experiments in LB amended with 8% w/v glucose. Ethanol production was assayed following 24 h incubation of cultures at 37 °C with rotary shaking at 200 rpm. Next the cultures were transferred to sealed 15 mL tubes completely filled to exclude air, and incubated for an additional 48 h. A statistically significant increase in ethanol production for the fusion protein, as compared to the nonfusion system, was observed at both time points (Figure 2; p = 0.004 at 24 h, p = 0.037 at 72 h).

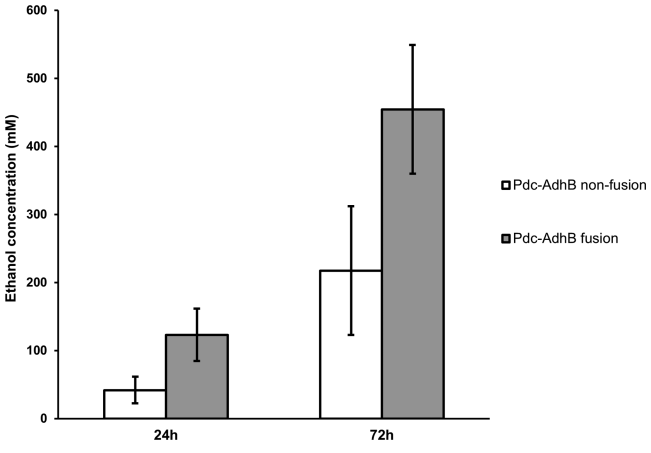

Figure 2. Ethanol yields obtained with fused and unfused enzymes in 24 and 72 h *E. coli* cultures. At 24 h, a mean of 122 mM ethanol concentration was obtained when fused protein was used compared to 41 mM obtained for nonfused enzymes. Following 72 h of incubation, mean ethanol concentrations of 454 mM and 217 mM were obtained for fused and nonfused Pdc–AdhB respectively. Differences in ethanol concentration were statistically significant (p = 0.004 at 24 h, p = 0.037 at 72 h). Results shown are means of six (24 h) or three (72 h) biological replicates; error bars indicate one standard error of the mean.

DISCUSSION

Edinburgh University iGEM team 2013 aimed to remediate and valorise industrial waste. Enzymatic fusions have previously been applied to a variety of multienzyme systems including production of diesel fuel replacements in *E. coli*.[3] We therefore sought to determine whether fusion of PDC and ADH might enhance ethanol production in recombinant *E. coli*. A fusion protein with both activities was successfully generated and this enhanced ethanol production in a simple test system. It remains to be determined whether ethanol production will be similarly enhanced in more realistic systems using existing biocatalyst strains under industrial conditions.

Enhanced activity in enzyme fusions is often attributed to substrate channelling. In our system, specific ADH activity was approximately 20 times higher in the unfused system than in the fusion system. This may be due to steric effects reducing access to the active site, or impaired multimerization; ADH is normally active as a dimer, whereas PDC is normally tetrameric. However, despite decreased activity of at least one of the fusion partners, ethanol production is nevertheless improved. Moreover, we observed a significant (p = 0.039) increase in cell densities of cultures expressing fused Pdc-AdhB (average optical density at 600 nm following 24 h of growth = 5.42; standard error of the mean = 0.17) compared to bacteria expressing nonfused enzymes (average optical density at 600 nm following 24 h of growth = 4.88; standard error of the mean = 0.1). One possible explanation is decreased release of the toxic intermediate acetaldehyde. However, the specific ethanol production, normalized to optical density at 600 nm, was considerably higher for cultures expressing the fusion protein (22.6 mM/OD for fusion compared to 8.6 mM/OD for unfused enzymes after 24 h of incubation), suggesting that increased ethanol levels were not simply due to improved growth. Thus, we postulate that the beneficial effect may be attributed to both enhanced substrate channelling and decreased cellular levels of acetaldehyde, which alleviates cytotoxicity associated with ethanol production. An interesting perspective could be obtained by comparing this system to AdhE, a bifunctional enzyme that catalyzes a two-step reaction converting acetyl-CoA to ethanol via acetaldehyde.[4]

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Our experiments demonstrate that a Pdc-AdhB fusion protein enhances microbial ethanol production in a simple test system. Ethanol yields obtained in this study are lower than those previously reported in literature using strains with multiple beneficial modifications (*e.g.*, deletion of *pta* and *ldh*, integration of *pdc* and *adhB* onto the chromosome). Thus, our results, obtained using a standard laboratory strain of *E. coli*, must now be tested in a more realistic context, using both a superior production strain and a large volume bioreactor, to determine whether commercial ethanol production might be enhanced.

METHODS

Strain and Growth Conditions. *E. coli* JM109 was used in all experiments. Recombinant strains were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl adjusted to pH 7) supplemented with 50 mg/L kanamycin to maintain the pSB1K2 plasmid. For ethanol production experiments, 8% w/v glucose was added. Protein expression was induced with 0.1 mg/mL IPTG.

Ethanol Production Assay. Yeast alcohol dehydrogenase (YADH), NAD^+, phenazine methosulfate (PMS), and iodonitro-tetrazolium violet (INTV) were obtained from Sigma-Aldrich. A reaction mixture containing 25 μg/mL YADH, 13.3 μg/mL NAD^+, 6.1 μg/mL PMS, and 0.5 mg/mL INTV was prepared in 1 mL 50 mM Tris-HCl buffer (pH 7.5). Samples of culture supernatant, following removal of cells by centrifugation, were diluted in this reaction mixture in a 1:30 ratio. Following 10 min incubation at room temperature, absorbance at 500 nm was measured and compared against an ethanol standard curve prepared at the same time.

AdhB Activity Assay. ADH activity was assayed in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM NAD^+ and 0.5% (v/v) ethanol. The reaction was initiated by addition of 5 μL of cell lysate and absorbance at 340 nm was measured every 10 s for 2 min. Protein concentration was measured by Bradford assay (Thermo-Fisher).

PAGE and Activity Stains. Native PAGE analysis was performed using 5% acrylamide stacking gel and 12% acrylamide resolving gel. AdhB activity was visualized by immersing the gel overnight in 20 mL of reaction mixture as described above. Pdc activity was detected by staining overnight in 20 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mg/mL sodium bisulphite and 0.4 mL of 2% (w/v) basic fuchsin dissolved in ethanol.

ASSOCIATED CONTENT

Supporting Information

Chromatogram data confirming the gene fusion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#A.J.L. and J.J.L. contributed equally. C.F. designed the study; A.J.L. and J.J.L. performed the experiments; C.F., A.J.L., and J.J.L. wrote the paper; G.B., C.P., K.R.M., D.T., H.T., H.V., W.X., and J.S. were part of the iGEM team and supported the work; L.H. and A.E. provided academic advice.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Pdc, pyruvate decarboxylase; AdhB, alcohol dehydrogenase B; AdhE, alcohol dehydrogenase E; YADH, yeast alcohol dehydrogenase; PMS, phenazine methosulfate; INTV, iodonitro-tetrazolium violet

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