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Strategic metabolic engineering of *Escherichia coli* for improved ethanol biosynthesis

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ABSTRACT

In this study, *Escherichia coli* cells were engineered by introducing the pKD46 plasmid using a heat shock method. This allowed for targeted gene editing to knock out the *IdhA* gene, which plays a role in metabolic pathways. To improve the strain's tolerance to ethanol, the engineered bacteria were then subjected to adaptive laboratory evolution (ALE), gradually exposing them to higher concentrations of ethanol over time. The success of the gene knockouts was confirmed through colony PCR, and BLAST analysis verified that the kanamycin resistance gene had been correctly inserted at both the *IdhA* and *YqhD* gene sites. The modified strains not only showed successful genetic changes but also demonstrated significantly enhanced ethanol tolerance. This method proved to be a reliable way to develop *E. coli* strains capable of efficiently producing ethanol from lignocellulosic sugars. The results highlight the value of genetic engineering and adaptive evolution in creating stable, high-performing microbial platforms for sustainable biofuel production.

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Introduction

Biofuel production has become a key player in the pursuit of renewable energy sources, presenting a promising route toward a more sustainable future (El-Araby 2024). Second-generation bioethanol is produced from lignocellulosic biomass, including materials like corn stover, wheat straw, and forest residues. This form of biofuel presents a sustainable and environmentally friendly alternative to fossil fuels. Unlike first-generation bioethanol, it does not rely on food crops, thereby avoiding competition with food sources. Cellulose and hemicellulose, which can hydrolyze into fermentable sugars like glucose and xylose, make up the majority of lignocellulosic materials. Lignocellulose features a complex three-dimensional network and is primarily made up of cellulose (30–50%), hemicellulose (25-30%), and lignin (15–20%). (Abo et al., 2019; Prasad et al., 2019). Xylose, the second most abundant sugar from lignocellulose, is a promising resource for biofuel and chemical production. However, its utilization faces challenges due to limited native metabolic pathways in microorganisms and strong repression by glucose through CCR (Jagtap and Rao 2018; Zhao et al., 2020).

The rapid growth, genetic accessibility, low cultivation cost, and innate xylose metabolism of *Escherichia coli* make it an important platform organism for the manufacture of biofuel. The *E. coli*, however, prioritizes glucose intake over xylose in mixed sugar environments because of CCR (Khankal et al., 2008; Sun et al., 2018). *Zymomonas mobilis* produces ethanol (EtOH) using two key enzymes: pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADHII). PDC converts pyruvate to acetaldehyde, which ADHII subsequently reduces to ethanol (Yang et al., 2016). Expression of these enzymes

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in *Escherichia coli* has markedly boosted ethanol production (Lee et al., 2010). To enhance EtOH tolerance in *Z. mobilis*, Global Transcription Machinery Engineering (gTME) has been employed by introducing mutations in the global sigma factor RpoD (σ^70). These mutants demonstrated better growth and increased ethanol yield under EtOH stress (Tan et al., 2016). Additionally, Adaptive Laboratory Evolution (ALE) has been used to develop *Z. mobilis* strains capable of efficiently fermenting molasses. These evolved strains showed improved stress tolerance and fermentation performance (Shui et al., 2015). Although *E. coli* has been engineered to utilize lignocellulosic sugars for ethanol production, its EtOH tolerance remains lower than that of *Z. mobilis* and *S. cerevisiae*. However, introducing the *recA* gene from *Z. mobilis* into *E. coli* has enhanced stress resistance by supporting DNA repair and cellular stress responses (Phannarangsee et al., 2024). Furthermore, modifying *E. coli* membrane composition through overexpression of genes like *fabA* has improved EtOH tolerance, emphasizing the role of membrane integrity in stress adaptation (Luo et al., 2009).

A dynamic metabolic engineering approach using a *gltA*-OFF switch has been applied in *E. coli* to disrupt the TCA cycle temporarily. This intervention led to increased pyruvate accumulation and enhanced isobutanol production. By combining selective gene deletions with conditional TCA cycle inhibition, the metabolic flux was effectively redirected without altering external conditions, offering a promising method for improving the biosynthesis of pyruvate-derived products (Soma et al., 2021). Additionally, other studies have shown that overexpressing enzymes such as malate dehydrogenase (Mdh) and malic enzyme A (MaeA) in engineered *E. coli* strains can efficiently channel carbon from malate to boost hydrogen (H_2) and ethanol output under anaerobic conditions. Optimal gene expression control, maintaining the cellular NADH/NAD^+ balance, and tailoring the metabolic network proved essential for maximizing yields and reducing by-product formation (Valle et al., 2021). The engineered *E. coli* strains by-products are EtOH, methanol, propanol and butanol. The review of biochemical pathways is highly relevant and valuable in the context of gene knockout and illustrated in figure 1a-d (supplementary), delineates to ascertain enzymes/metabolites and associated compensatory pathways.

In a related study, the *E. coli* strain demonstrated remarkable adaptability by utilizing cellulose and various plant-derived sugars, a rare trait compared to human-associated strains. Whole Genome Sequencing revealed a larger genome size and a strong dominance of genes related to carbohydrate metabolism and environmental processing. Fermentation studies showed the production of multiple alcohols, with butanol emerging as the major byproduct despite a focus on EtOH. COG analysis highlighted carbohydrate transport and metabolism as the largest functional group, with 447 genes. Key enzymes like alcohol dehydrogenase, protease, and lyase were identified, supporting the strain's potential for efficient bioethanol production from renewable biomass. In this study, an EtOH-tolerant *E. coli* mutant (MGE) was developed and genetically engineered by knockout. The objective was to enhance the enzymatic mechanism, reducing by-product formation and redirecting EtOH production. Figure 2 (supplementary) exhibits a pKD46 plasmid map illustrating the presence of key lambda red recombination system proteins, ampicillin resistance gene and other promoters.

Materials and Methods

Strains, plasmids, and culture conditions

E. coli strains were grown in Luria-Bertani (LB) medium at 37 °C. In order to conduct fermentation tests, the strains were cultured anaerobically at 37 °C in an M9 mineral medium, which was supplemented with 100 g/L of either glucose or xylose. The composition of the medium was Na_2HPO_4 64 g/L, KH_2PO_4 15 g/L, NH_4Cl 5 g/L, $MgSO_4$ 0.24 g/L, and $CaCl_2$ 0.011 g/L. In a temperature-controlled water bath, cultures were kept in 100 ml volumetric flasks with 80 ml of broth, sealed with drilled rubber septa to permit gas release and agitated at 100 rpm. When required, antibiotics were administered, with either 34 µg/ml of chloramphenicol or 50 µg/ml of kanamycin. Following 24–96 hours of incubation, optical density at 600 nm (OD_{600}) was measured to track cell development (Ma et al., 2010).

Isolation of ethanol-tolerant mutants

E. coli strains with mutations that improved EtOH tolerance while maintaining efficient EtOH production features were enriched using a combination of liquid and solid media. To assess EtOH tolerance, the strains were initially cultured in an M9 medium supplemented with 100 g/L glucose and 30 g/L EtOH. *E. coli* pKD46 was chosen as it expresses the λ Red recombinase system to facilitate homologous recombination of a kanamycin resistance cassette for targeted gene disruption. Thus, the mutant *E. coli* strain maximizes EtOH production, reduces by-products and enhances balance of redox. This strain was repeatedly added to the LB medium with increasing EtOH concentrations (between 30 and 60 g/L). Cultures were diluted and plated on solid media, and colonies that could grow at increasing EtOH concentrations were isolated after every three to five transfers. A highly tolerant clone known as MGE was

chosen from among these to be developed further in order to improve the production of EtOH (Balagurunathan et al., 2018). A subculture of not more than five passages has been used for the study.

Transformation of Competent Cells with pKD46 Plasmid

Plates were inverted and incubated overnight at 30 °C. Successful transformants containing the pKD46 plasmid were identified by colony growth. Frozen competent *E. coli* cells were thawed and kept on ice throughout the transformation process. In a sterile microcentrifuge tube, 50 µL of competent cells were gently combined with 1–10 ng (2 µL) of pKD46 plasmid DNA.

The mixture was incubated on ice for 30 minutes to allow DNA uptake. Subsequently, followed by a 45-second heat shock at 42 °C to facilitate DNA entry into the cells, after which the tube was immediately returned to ice for 2 minutes. Next, 950 µL of LB broth (without antibiotics).was added, and the cells were incubated at 37 °C with shaking for 1 hour to enable the expression of antibiotic- resistance genes. After incubation, the cells were briefly centrifuged and resuspended in about 100 µL of LB medium. Finally, the cell suspension was spread onto LB agar plates containing ampicillin for selection.

Further, the spaced bands indicating specific molecular weight standards associated with knockout confirmation are illustrated in figure 3 (supplementary), which exhibits a 1kb DNA ladder that is used as molecular weight marker for agarose gel electrophoresis. The lane estimates DNA fragments in adjacent lanes with a base pair ranging between 250 base pairs (bp) and 10,000 bp. The lane is used as a reference for approximate quantification.

Preparation of Kanamycin Resistance Cassette

To prepare a kanamycin resistance cassette, PCR primers flanking the kanamycin resistance gene were designed, ensuring a suitable homologous recombination. Table S1 (supplementary) summarizes designed primers to facilitate homologous recombination by incorporating target genomic regions. Table S2 (supplementary) summarizes the conditions for a PCR reaction to amplify the target DNA region using specific primers with appropriate conditions based on DNA polymerase. Finally, the band corresponding to the kanamycin resistance genes was purified by using a PCR-GEL purification kit (MP Biomedicals, USA).

Knockout procedure

A single *E. coli* colony harboring the pKD46 plasmid was inoculated into 5 mL of LB broth containing ampicillin and incubated overnight at 30 °C with shaking. The next day, the overnight culture was diluted 1:100 into fresh LB broth with ampicillin and grown at 30 °C until the optical density at 600 nm (OD_{600}) reached approximately 0.4. To induce the lambda Red recombination system, L-arabinose was added to a final concentration of 0.2%, and the culture was incubated for an additional 1–2 hours. Meanwhile, the knockout DNA construct—typically a PCR product encoding an antibiotic resistance gene flanked by sequences homologous to the target locus—was prepared. The induced cells were made competent and kept on ice. A volume of 50 µL of these competent cells was mixed with 1–10 µL of the knockout construct. The mixture was incubated on ice for 30 minutes, followed by a 45-second heat shock at 42 °C, and then returned to ice for 2 minutes. Next, 950 µL of LB broth (without antibiotics) was added, and the cells were incubated at 37 °C with shaking for 1 hour to allow recovery and expression of resistance. After incubation, the culture was diluted (1:10 or 1:100) and plated on LB agar plates containing the appropriate selection antibiotic (e.g., kanamycin). Plates were incubated overnight at 37 °C. The following day, colony growth was examined, and successful knockouts were verified by PCR using primers that flank the targeted gene regions.

Gene Knockout in E. coli using lambda (λ) Red Recombinase

E. coli was initially cultured in LB broth containing the appropriate antibiotic at 30 °C in preparation for plasmid transformation. The overnight culture was diluted into fresh LB broth with antibiotics and grown until the OD_{600} reached approximately 0.4. To induce expression of the lambda (λ) Red recombination system, L-arabinose was added to a final concentration of 0.2%, and the culture was incubated at 37 °C for 1 hour with shaking. Meanwhile, a linear DNA template for homologous recombination was prepared and purified using a clean-up kit. After induction, cells were harvested by centrifugation and kept ice-cold throughout the procedure. Approximately 200 ng of purified linear DNA was mixed with 100 µL of electrocompetent cells. The mixture was transferred to a sterile electroporation cuvette and subjected to electroporation (1.8 kV, 25 µF, 211 Ω). Immediately after electroporation, SOC medium was added to the cells, and the culture was incubated at 37 °C with shaking for 1 hour to allow recovery and expression of antibiotic resistance. Following recovery, cells were plated onto LB agar plates containing kanamycin and incubated o/n at 37 °C. The next day, several kanamycin-resistant colonies

were picked and inoculated into LB broth containing kanamycin and incubated overnight at 30 °C; for confirmation of successful insertion, colony PCR was performed using primers flanking the target region. PCR products were analyzed by agarose gel electrophoresis to verify correct gene insertion.

Results and Discussion

Isolation of ethanol-tolerant mutants of *E. coli*

Transformation of MGE (strain 1) with the pKD46 plasmid was successfully achieved via heat shock, enabling efficient recombineering and laying the foundation for further metabolic engineering to enhance EtOH production. In this study, an enrichment strategy was employed to generate EtOH-tolerant mutants successfully. To identify the most suitable candidate for metabolic engineering aimed at improving EtOH production under high-stress conditions, an isolated wild *E. coli* strain was evaluated for EtOH tolerance. The mutant strain was further subcultured and used for development through successive enrichment in increasing EtOH concentrations. ALE is a powerful tool for improving microbial strains for industrial applications by enhancing tolerance, activating pathways, and optimizing metabolism. Although time-consuming and complex, combining ALE with rational metabolic engineering and multi-omics approaches can significantly accelerate strain improvement and uncover genotype-phenotype relationships for more stable and productive industrial strains (Wang et al., 2023).

Stability and integration of Kanamycin resistance cassette

The plasmid PKD46 demonstrated high stability in the engineered *E. coli* mutant strain under anaerobic conditions without antibiotic selection. Figure 4 (supplementary) shows the electrophoresis images of *IdhA* and *YqhD*. The observation indicates clear, sharp bands of the lanes with expected sizes corresponding to *IdhA* and *YqhD*.

Similarly, the study by Johnston et al., (2023) in *Treponema denticola* screening of antibiotic resistance cassettes revealed that among various cassettes tested, only the kanamycin resistance gene *aphA2* conferred effective resistance and enabled stable selection. In order to assess the effect of plasmid conferred resistance to specific antibiotics by transforming *E. coli* with the plasmid was studied by using the plating method. Figure 5 (supplementary) shows the transformation of competent bacterial cells with the growth of a colony as the plasmid contains a resistance gene. The images show stability and integration of plasmid PKD46 on agar plates. Notably, *adhA2* also maintained functionality after genomic insertion, unlike other tested cassettes such as aphA1 and gentamicin cassettes, highlighting the importance of cassette sequence specificity for stability and expression across hosts. These findings together emphasize that cassette stability and plasmid retention are critical factors for maintaining engineered traits during prolonged culture and industrial strain development (Li et al., 2015).

Analysis of Gene knockout

Colony PCR was used to verify the successful knockout of the *IdhA* gene in the engineered *E. coli* strain, using the wild-type (WT) strain as a control. Primers flanking the *IdhA* locus were designed to detect either the native gene or the integration of the kanamycin resistance cassette. In the knockout strain, amplification produced a larger PCR product, consistent with the insertion of the resistance cassette at the *IdhA* site. In contrast, the WT strain showed a smaller PCR product corresponding to the intact *IdhA* gene without any cassette. These results confirmed the successful disruption of the *IdhA* gene in the mutant strain. Figures 6-7 (supplementary) exhibited the results that were aligned with expectations based on the λ Red recombinase system, a well-established method for precise gene knockout in *E. coli* due to its efficiency and low off-target activity (Datsenko and Wanner, 2000). The observed integration of the resistance cassette at the target site reflects the successful application of this technique, as reported in prior studies using similar strategies.

Gene Knockout in *E. coli* Using λ Red Recombinase

A blast analysis was conducted to confirm the integration of the plasmid containing the kanamycin resistance cassette into the *E. coli* genome, specifically targeting the *IdhA* and *YqhD* loci. Table S3 (supplementary) summarizes the sequences of the *IdhA* and *YqhD* primers, as outlined, which were compared against the *E. coli* genome, and the results confirmed the successful integration of the plasmid. Blast analysis confirmed the successful integration of the kanamycin resistance cassette into the *IdhA* and *YqhD* loci of *E. coli*, with high sequence homology at the expected insertion sites. These results validate precise genetic modification at the target regions. The λ Red recombineering system, which uses phage-derived recombinases (Exo, Beta, and Gam), is known for facilitating efficient site-specific integration of linear DNA fragments into the *E. coli* chromosome. This method has been instrumental in developing the Keio collection—a comprehensive library

of gene knockouts in *E. coli* K-12—highlighting its reliability in genetic engineering (Pyne et al., 2015). Our findings are consistent with previous studies demonstrating the effectiveness of this technique for targeted gene disruption in bacterial genomes**.**

Conclusion

In conclusion, the transformation of *E. coli* with the pKD46 plasmid and integration of the kanamycin resistance cassette was successfully validated using colony PCR and BLAST analysis. PCR results confirmed the *IdhA* gene knockout, while Blast analysis demonstrated strong sequence homology at the *IdhA* and *YqhD* loci, supporting accurate plasmid integration. These outcomes are consistent with the high efficiency of the λ Red recombineering system for targeted genetic editing in *E. coli* (Pyne et al., 2015). Additionally, the kanamycin resistance cassette remained stable under anaerobic conditions even without selective pressure, indicating the engineered strain's robustness. This genetic stability and targeted modification offer a promising platform for further metabolic engineering, particularly for enhancing EtOH production under stress conditions. The mutant *E. coli* strain is capable of reducing by-products, lactate accumulation, improved carbon efficiency and increased ethanol yield from 26 g/L to 34 g/L, where the knockout of *IdhA* and *YqhD* genes disrupted its encoded protein function and influenced systemic shift of enhancing enzymatic mechanism redirecting to maximize the EtOH production.

Availability of data and material
Data are accessible upon request.

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Conflict of interest The author declares that there is no conflict of interest regarding the publication of this paper.

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