Advances in Butanol Tolerance Mechanisms and Tolerant Strain Development in Escherichia coli: A Postprint

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Abstract

With global warming and the energy crisis intensifying, bio-butanol has garnered considerable attention as a clean energy source and important chemical commodity. Escherichia coli has emerged as a chassis strain for butanol production due to its superior genetic tractability; however, the toxic effects of butanol on cells have become a bottleneck for enhancing butanol production in engineered strains, making the improvement of butanol tolerance in E. coli a necessary prerequisite for increasing butanol yield. To this end, a comprehensive understanding of the butanol tolerance mechanisms in E. coli is essential. Butanol can disrupt the barrier function of the cell membrane, perturb material transport and signaling functions, and trigger physiological stress responses analogous to those elicited by heat shock, osmotic stress, and other stresses, with cells responding to butanol stress through transcriptional and translational regulation. This review summarizes the butanol tolerance mechanisms in E. coli from the aforementioned perspectives and outlines research advances in obtaining butanol-tolerant strains through rational design via genetic engineering. Nevertheless, the butanol tolerance mechanisms have not yet been fully elucidated, which limits the application of rational design strategies; therefore, this article also summarizes research progress in this area regarding the utilization of directed evolution to obtain butanol-tolerant strains and the reverse metabolic engineering strategy for deciphering functional genes involved in butanol tolerance. Additionally, recent studies on enhancing E. coli butanol tolerance through combinatorial strategies and chemical modification methods are highlighted and discussed. Finally, key strategies for improving butanol tolerance in the chassis strain E. coli are summarized and future prospects are presented.

Full Text

Preamble

Research Progress on Butanol-Tolerant Strain and Tolerance Mechanism of Escherichia coli

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Abstract

Biobutanol has attracted considerable attention as a clean fuel and important chemical feedstock amid escalating global warming and energy crises. *Escherichia coli* has emerged as an ideal chassis organism for butanol production due to its well-characterized genetics and superior amenability to metabolic engineering. However, butanol toxicity represents a critical bottleneck limiting high-titer production, making enhanced butanol tolerance an essential prerequisite for industrial applications. Butanol disrupts the barrier function of cell membranes, impairs material transport and signal transduction, and elicits physiological responses analogous to heat shock and osmotic stress. Cells counteract butanol stress through coordinated transcriptional and translational regulation. This review synthesizes current understanding of *E. coli* butanol tolerance mechanisms across these dimensions and summarizes advances in engineering tolerant strains through rational genetic design (Table 1). Nevertheless, incomplete elucidation of tolerance mechanisms constrains rational design strategies. We therefore discuss inverse metabolic engineering approaches that employ directed evolution to isolate tolerant mutants followed by functional gene identification. Additionally, we examine emerging combinatorial strategies and chemical modification methods for enhancing *E. coli* butanol tolerance, concluding with perspectives on key strategies for developing robust production strains.

Keywords: *Escherichia coli*, butanol tolerance, genetic engineering, directed evolution

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Butanol (C H OH) offers numerous advantages as a next-generation biofuel, including high energy density, low volatility and hygroscopicity, and minimal

corrosiveness [1]. It blends readily with gasoline and finds extensive applications in plastics, printing, and other industries [3]. Biologically produced butanol also serves the food, cosmetics, and pharmaceutical sectors. Native butanol-producing *Clostridium* species achieve titers of 15–18.9 g/L through acetone-butanol-ethanol (ABE) fermentation [4-5]. With advances in metabolic engineering and synthetic biology, heterologous butanol pathways have been constructed in *Saccharomyces cerevisiae*, lactic acid bacteria, and *E. coli*, yielding maximum titers of 130±20 mg/L and 300 mg/L in recombinant yeast and lactic acid bacteria, respectively [5]. *E. coli* stands out as a preferred industrial chassis due to its clear genetic background, rapid growth rate, suitability for high-cell-density cultivation, and facile genetic manipulation [6-7]. Shen et al. [8] engineered a butanol synthesis pathway in *E. coli* achieving 15 g/L titers, which reached 30 g/L when butanol was continuously removed during fermentation. Further pathway optimization enabled recombinant strains to produce 18.3–20 g/L [9-10]. These results demonstrate *E. coli*' s potential as a butanol producer, though cellular toxicity remains the primary limitation to achieving higher concentrations.

Butanol toxicity restricts biological production to approximately 20 g/L [11], creating a major bottleneck that increases process energy consumption, water usage, and waste generation. While in situ recovery can mitigate toxicity by continuously separating butanol, isolating tolerant strains remains crucial for efficient production [11]. *E. coli* growth is completely inhibited at 1% (v/v) butanol [12], underscoring the importance of enhancing tolerance for improving production efficiency. Current efforts focus on engineering *E. coli* strains with improved butanol tolerance through genetic engineering and directed evolution approaches.

1 Genetic Engineering Strategies for Butanol-Tolerant Strains

Butanol stress triggers multifaceted physiological and biochemical changes in bacteria, including compromised membrane function and ion transport, activation of heat shock protein expression, and altered transcriptional and translational regulation. Rational design strategies target these responses by modulating gene expression levels or introducing heterologous genes to modify relevant physiological properties and enhance tolerance [13] (Table 1).

Butanol reduces bacterial membrane surface tension, increasing fluidity and disrupting membrane integrity [14]. This damage impairs the membrane' s barrier function, causing ATP leakage and collapse of pH gradients, which disrupts material transport and energy transduction, ultimately inhibiting growth and causing cell death. Engineering membrane properties offers an effective countermeasure. Increasing outer membrane lipopolysaccharide content reduces cell surface hydrophobicity, hindering entry of toxic hydrophobic compounds and enhancing butanol tolerance [15]. Overexpressing *bioA*, a key enzyme in biotin synthesis, boosts fatty acid biosynthesis and improves tolerance by 30%±4%.

Similarly, overexpressing fatty acid synthesis genes *cfa* and *fabD* significantly enhances tolerance by increasing fatty acid content [14,16]. Expressing the *Pseudomonas aeruginosa cti* gene, which encodes a cis-trans isomerase converting cis-unsaturated fatty acids to their trans-isomers, reduces membrane fluidity while increasing polarity and rigidity. This engineered strain exhibits 16% higher growth rates under 0.6% (v/v) butanol stress compared to control strains [17].

The resistance-nodulation-division (RND) efflux pump, localized in the cytoplasmic membrane, can export toxic compounds. Since native *E. coli* efflux systems cannot recognize butanol, directed evolution was employed to modify AcrB substrate specificity. The evolved variant AcrBv2 showed 37% higher growth rates than wild-type in 0.7% (v/v) butanol [18]. However, AcrB overexpression inhibits cell growth. The *Pgntk* promoter from the gluconate metabolism operon specifically senses membrane stress to autoregulate gene expression. A *Pgntk-acrBv2* negative feedback system increased maximum cell density from 0.5 to 0.7 in 0.7% (v/v) butanol [19]. Expressing the *Pseudomonas putida* efflux pump operon *srpABC* in *E. coli* improved growth by 20–35% in 0.5–1% (v/v) butanol. Notably, expressing only the transmembrane protein gene *srpB* also enhanced tolerance, likely by stabilizing the membrane rather than actively exporting butanol [16].

Iron plays crucial roles in redox reactions, respiration, and DNA precursor synthesis [20]. Overexpressing the iron transporter gene *feoA* increases Fe^{2} uptake and improves tolerance by 21% at 1% (v/v) butanol [16], while overexpressing enterobactin synthesis gene *entC* enhances tolerance by 10%±1% [14].

Proteomic and transcriptomic analyses reveal that *E. coli* responds to butanol stress similarly to osmotic, oxidative, and heat shock stresses, activating stress response genes to enhance tolerance. Heat shock proteins (HSPs) constitute a primary defense system, facilitating protein folding, transport, and repair of damaged or misfolded proteins to maintain native conformation under stress. Overexpressing *groESL* operons from either *Clostridium acetobutylicum* or *E. coli* itself improves butanol tolerance [21-22]. The GroESL system collaborates with ClpB and DnaK systems during stress responses: ClpB specifically disaggregates proteins into polypeptides, while GroESL and DnaK refold these polypeptides. The DnaK system comprises DnaK chaperone and co-chaperones DnaJ and GrpE. Simultaneous overexpression of these key genes (*grpE*, *groESL*, and *clpB*) increased colony-forming units 3.9-fold under 1% (v/v) butanol compared to wild-type [23]. Butanol stress elevates reactive oxygen species (ROS) levels, activating carbon tetrachloride into diffusible radicals that damage macromolecules and peroxidize lipids, directly attacking membranes. Metallothioneins (MTs) serve as antioxidant enzymes that scavenge ROS. Fusing tilapia metallothionein (TMT) with the osmoregulatory membrane protein OmpC and overexpressing this *OmpC-TMT* fusion enhanced tolerance to 1.5% (v/v) butanol [25].

Butanol stress alters transcriptional and translational regulation, affecting carbon metabolism, energy metabolism, membrane modification, transport,

and ion metabolism. Transcriptomic analysis of an evolved tolerant strain revealed decreased Fur regulator activity, leading to increased expression of BasS/BasR regulatory proteins and enhanced lipopolysaccharide modification genes, thereby improving tolerance [15]. Our unpublished results indicate that *rob* transcriptional regulator deletion or mutation also enhances *E. coli* butanol tolerance. With 304 known transcriptional regulators in *E. coli*, only a limited number have been identified as modulating butanol tolerance, and their regulatory mechanisms remain largely uncharacterized, constraining rational design applications.

2 Directed Evolution Strategies for Screening Butanol-Tolerant Strains

Butanol tolerance involves complex, multigenic responses affecting diverse physiological and biochemical parameters. Since the complete set of functional tolerance genes remains unknown, directed evolution offers a powerful alternative for isolating tolerant mutants (Table 2). Random mutagenesis at the genomic level through techniques such as mutagenesis, genome shuffling, and adaptive evolution, followed by phenotypic screening, can yield tolerant strains. Adaptive evolution combined with proton beam irradiation produced a mutant with 2.6-fold higher cell density than the parent under 0.9% (v/v) butanol stress. Genomic analysis revealed mutations in cis-regulatory elements of membrane protein genes *yqjA* and *yabI* and transcriptional regulator *rob*, upregulating their expression and conferring tolerance [7]. Screening an *E. coli* genomic library identified nine tolerant mutants with reduced cyclopropane fatty acids and increased unsaturated fatty acids in their membranes, enhancing membrane rigidity [26]. Real-time visual evolution combined with DNA shuffling yielded a best-performing mutant with 10- to 100-fold improved survival in 2% (v/v) butanol. Genome sequencing and transcriptomic analysis revealed significant alterations in cell wall/membrane synthesis, iron transport, and metabolic genes [14].

Our laboratory isolated two *E. coli* mutants tolerant to 2% (v/v) butanol using error-prone PCR genome shuffling [27]. While adaptive evolution directs microbial improvement under stress, it requires lengthy cultivation periods [28]. Genome shuffling and mutagenesis dramatically accelerate evolution by increasing mutation rates and improving efficiency.

Transcriptional engineering introduces global transcriptional perturbations to generate multiscale phenotypic libraries for directed screening. RNA polymerase comprises , , ', , and subunits, with factors specifically recognizing promoter -35 and -10 regions to initiate transcription. *E. coli* factors regulate over 1,000 genes, and their mutation can globally affect transcription. Two rounds of random mutagenesis of *rpoD* (encoding) produced mutant B8, which tolerates 2% (v/v) butanol [28]. Similarly, error-prone PCR mutagenesis of *rpoA* (encoding the subunit involved in transcription factor interactions and promoter recognition) yielded mutants with approximately 2-fold higher cell density than

wild-type in 0.75% and 0.9% (v/v) butanol [29]. Lee et al. [30] constructed artificial transcription factor libraries by fusing zinc-finger DNA-binding proteins with cAMP receptor protein (CRP), selecting strain BT capable of tolerating 1.5% (v/v) butanol. A random mutagenesis library of the exogenous transcription factor *irrE* produced mutants with 2- to 6-fold higher OD than wild-type across 0.125–0.875% (v/v) butanol and 100-fold higher survival in 2.1% (v/v) butanol shock experiments [31].

Genome and transcription engineering strategies generate genetically diverse libraries for screening butanol-tolerant phenotypes, enabling identification of causal genes and mechanistic elucidation. Subsequent rational design of identified tolerance genes enables "phenotype-genotype-phenotype" inverse metabolic engineering applications in *E. coli* butanol tolerance research [32].

3 Combinatorial Strategies for Butanol-Tolerant Strains

Strain BT, isolated through transcription engineering, tolerates 1.5% (v/v) butanol [30]. Transcriptomic analysis revealed upregulation of inner membrane protein and metabolic genes but no changes in fatty acid synthesis gene expression. A combinatorial strategy overexpressing fatty acid synthesis genes, iron transporter *feoA*, and efflux pump *srpABC* in this background produced a strain tolerant to 2% (v/v) butanol [16] (Table 2). Combinatorial approaches thus enable synergistic integration of beneficial tolerance traits for further enhancement.

4 Chemical Modification Strategies for Enhancing Butanol Tolerance

Butanol-induced membrane fluidization limits tolerance development. Inserting the membrane-intercalating molecule COE1-5C (a pentacyclic polyphenylene conjugated oligoelectrolyte) into the phospholipid bilayer reduces phospholipid diffusion rates while increasing specific growth rates. The modified strain exhibits 3-fold higher specific growth rates than control strains under 3.5% (v/v) butanol. During butanol stress, COE1-5C' s -conjugated aromatic backbone acts as a hydrophobic agent for glycerophospholipid acyl chains, counteracting membrane fluidization, enhancing bilayer integrity, and reducing cellular depolarization. Chemical modification thus complements genetic engineering for constructing stress-tolerant strains [33].

5 Summary and Outlook

Escherichia coli serves as an effective cell factory for butanol production, with optimized engineered strains achieving titers comparable to native producers (20 g/L, ~2.47% v/v). However, butanol toxicity constrains biological production to approximately 20 g/L, creating a critical bottleneck. Breaking this limit requires isolating mutants with enhanced tolerance as a prerequisite for high-

yield strains. Directed evolution and combinatorial strategies have yielded *E. coli* strains tolerant to 2% (v/v) butanol. Nevertheless, the complex, multigenic regulatory mechanisms underlying butanol tolerance remain incompletely understood, limiting further improvements. Future efforts should focus on elucidating transcriptional and translational tolerance mechanisms, constructing comprehensive stress response networks, and employing combinatorial strategies to integrate synergistic tolerance gene effects. Beyond molecular genetic approaches, physicochemical methods that modulate cellular physiology during fermentation offer promising complementary strategies for developing robust, high-butanol-tolerant strains.

References

[1] Wang QL, Liu L, Shi JP, et al. Current status and prospects of the expression of butanol pathway in *Escherichia coli*. China Biotechnology, 2014, 34(06): 90–97.
[2] Liu Y, Liu HJ, Zhang JA, et al. Research progress in new biofuel butanol. Modern Chemical Industry, 2008, 28(06): 28–31+33.
[3] Jiang Y, Liu J, Jiang W, et al. Current status and prospects of industrial bio-production of n-butanol in China. Biotechnol Adv, 2015, 33(7): 1495–1501.
[4] Dong H, Zhao C, Zhang T, et al. Engineering *Escherichia coli* cell factories for n-butanol production. Adv Biochem Eng Biotechnol, 2016, 155: 141–163.
[5] Liu S, Qureshi N, Hughes SR. Progress and perspectives on improving butanol tolerance. World J Microbiol Biotechnol, 2017, 33(3): 51.
[6] Dai ZJ, Dong HJ, Zhu Y, et al. Metabolic engineering for biobutanol production: a review. Chinese Journal of Bioprocess Engineering, 2013, 11(02): 58–64.
[7] Jeong H, Lee SW, Kim SH, et al. Global functional analysis of butanol-sensitive *Escherichia coli* and its evolved butanol-tolerant strain. J Microbiol Biotechnol, 2017, 27(6): 1171–1179.
[8] Shen CR, Lan EI, Dekishima Y, et al. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl Environ Microbiol, 2011, 77(9): 2905–2914.
[9] Ohtake T, Pontrelli S, Laviña WA, et al. Metabolomics-driven approach to solving a CoA imbalance for improved 1-butanol production in *Escherichia coli*. Metab Eng, 2017, 41: 135–143.
[10] Dong H, Zhao C, Zhang T, et al. A systematically chromosomally engineered *Escherichia coli* efficiently produces butanol. Metab Eng, 2017, 44: 284–292.
[11] Jang YS, Lee SY. Recent advances in biobutanol production. Industrial Biotechnol, 2015, 11(6): 316–321.
[12] Knoshau EP, Zhang M. Butanol tolerance in a selection of microorganisms. Appl Biochem Biotechnol, 2009, 153(1-3): 13–20.
[13] Rau MH, Calero P, Lennen RM, et al. Genome-wide *Escherichia coli* stress response and improved tolerance towards industrially relevant chemicals. Microb Cell Fact, 2016, 15(1): 176.

[14] Sandoval NR, Papoutsakis ET. Engineering membrane and cell-wall programs for tolerance to toxic chemicals: Beyond solo genes. Curr Opin Microbiol, 2016, 33: 56–62.
[15] Reyes LH, Almario MP, Winkler J, et al. Visualizing evolution in real time to determine the molecular mechanisms of n-butanol tolerance in *Escherichia coli*. Metab Eng, 2012, 14(5): 579–590.
[16] Bui LM, Lee JY, Geraldi A, et al. Improved n-butanol tolerance in *Escherichia coli* by controlling membrane related functions. J Biotechnol, 2015, 204: 33–44.
[17] Tan Z, Yoon JM, Nielsen DR, et al. Membrane engineering via trans unsaturated fatty acids production improves *Escherichia coli* robustness and production of biorenewables. Metab Eng, 2016, 35: 105–113.
[18] Fisher MA, Boyarskiy S, Yamada MR, et al. Enhancing tolerance to short-chain alcohols by engineering the *Escherichia coli* AcrB efflux pump to secrete the non-native substrate n-butanol. ACS Synth Biol, 2014, 3(1): 30–40.
[19] Boyarskiy S, López SD, Kong N, et al. Transcriptional feedback regulation of efflux protein expression for increased tolerance to and production of n-butanol. Metab Eng, 2016, 33(1): 130–137.
[20] Feng Y, Liu MF, Cheng AC. Component and functional mechanism of the ferrous iron acquisition system in gram-negative bacteria—A review. Acta Microbiologica Sinica, 2016, 56(7): 1061–1069.
[21] Abdelaal AS, Ageez AM, Abd El-Hadi AE, et al. Genetic improvement of n-butanol tolerance in *Escherichia coli* by heterologous overexpression of *groESL* operon from *Clostridium acetobutylicum*. 3 Biotech, 2015, 5(4): 401–410.
[22] Zingaro KA, Papoutsakis ET. GroESL overexpression imparts *Escherichia coli* tolerance to i-, n-, and 2-butanol, 1,2,4-butanetriol and ethanol with complex and unpredictable patterns. Metab Eng, 2013, 15(1): 196–205.
[23] Zingaro KA, Papoutsakis ET. Toward a semisynthetic stress response system to engineer microbial solvent tolerance. MBio, 2012, 3(5): e00308–12.
[24] Jones DP. Radical-free biology of oxidative stress. Am J Physiol Cell Physiol, 2008, 295(4): C849–C868.
[25] Chin WC, Lin KH, Chang JJ, et al. Improvement of n-butanol tolerance in *Escherichia coli* by membrane-targeted tilapia metallothionein. Biotechnol Biofuels, 2013, 6(1): 130.
[26] Freedman BG, Zu TN, Wallace RS, et al. Raman spectroscopy detects phenotypic differences among *Escherichia coli* enriched for 1-butanol tolerance using a metagenomic DNA library. Biotechnol J, 2016, 11(7): 877–889.
[27] Zhang JY, Xue TL, He XT, et al. Screening butanol tolerant *Escherichia coli* by using error-prone PCR whole genome shuffling. In: Deng ZX, editor. The 13th International Symposium on the Genetics of Industrial Microorganism. Wuhan, China; 2016. p. 313.
[28] Si HM, Zhang F, Wu AN, et al. DNA microarray of global transcription factor mutant reveals membrane-related proteins involved in n-butanol tolerance in *Escherichia coli*. Biotechnol Biofuels, 2016, 9(1): 114.
[29] Klein-Marcuschamer D, Santos CN, Yu H, et al. Mutagenesis of the bacterial RNA polymerase alpha subunit for improvement of complex phenotypes.

Appl Environ Microbiol, 2009, 75(9): 2705-2711.
[30] Lee JY, Yang KS, Jang SA, et al. Engineering butanol-tolerance in *Escherichia coli* with artificial transcription factor libraries. Biotechnol Bioeng, 2011, 108(4): 742-749.
[31] Chen T, Wang J, Yang R, et al. Laboratory-evolved mutants of an exogenous global regulator, IrrE from *Deinococcus radiodurans*, enhance stress tolerances of *Escherichia coli*. PLoS One, 2011, 6(1): e16228.
[32] Zhou XF, Chen TT, Tian Y, et al. Advances on inverse metabolic engineering. Taiwan Agricultural Research, 2015, 01: 73-79.
[33] Hinks J, Wang Y, Matysik A, et al. Increased microbial butanol tolerance by exogenous membrane insertion molecules. ChemSusChem, 2015, 8(21): 3718-3722.

Note: Figure translations are in progress. See original paper for figures.