CRISPR-Cas Technology for Constructing Engineered Strains to Produce High Value-Added Industrial Products

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Abstract. With its ability to make precise, effective, and programmable genetic changes, the CRISPR-Cas system has become a revolutionary tool for microbial genome engineering. This article summarizes the use of CRISPR-Cas technology to create modified strains for the manufacturing of high value-added industrial products. First, the basic mechanisms of CRISPR-Cas systems are explained, including target recognition, Cas-mediated DNA cleavage, and repair pathways including HDR and NHEJ. This article then discusses representative applications in three key microbial hosts: *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Escherichia coli*. In *B. subtilis*, CRISPR-based systems enable multiplex and iterative editing for optimizing hyaluronic acid biosynthesis; in S. cerevisiae, they facilitate marker-free and multi-copy integration for high-value metabolite production; and in E. coli, they improve pathway balance and editing precision for fine chemical synthesis. Collectively, these advances illustrate how CRISPR-Cas technologies provide a versatile and universal platform for rational strain design. The integration of CRISPR-based editing with metabolic engineering continues to drive sustainable, efficient, and scalable biomanufacturing of high value-added products across diverse microbial systems.

1 Introduction

Microorganisms play a vital role in human society by producing a wide range of substances essential for health, industry, and daily life. Early biotechnological applications mainly relied on screening high-yield strains from natural microorganisms through conventional methods such as UV mutagenesis, but these approaches were limited by lack of method diversity, low mutation efficiency, and inability to achieve targeted genetic modifications. The advent of genetic engineering enabled microorganisms to be reprogrammed, providing new metabolic functions and access to products not naturally synthesized. Strains created in this way, often termed engineered strains, have become central to modern biotechnology.

Engineered strains are especially valuable for producing high value-added industrial products, which are characterized by strong market demand and high profitability. Examples include biofuels such as butanol and ethanol, which serve as renewable energy carriers and solvents, and hyaluronic acid, which is widely used in cosmetics, pharmaceuticals, and regenerative medicine. Microbial fermentation allows these compounds to be manufactured sustainably and economically, reducing reliance on petrochemical synthesis or extraction from scarce natural resources. Thus, research on engineered strains not only supports scalable and environmentally friendly production but also improves robustness, efficiency, and product quality in industrial processes.

The construction of efficient engineered strains fundamentally depends on advances in gene-editing technologies. Traditional genetic manipulation techniques were limited by inefficiency and a lack of precision. Although programmability and site-specific modification were made possible by engineered nucleases like ZFNs and TALENs, their high cost, technical complexity, and protein engineering requirements prevented their widespread use, especially for multi-gene or iterative editing.

The discovery and modification of the CRISPR-Cas system, which was initially an adaptive immunological mechanism in bacteria and archaea, was a significant advancement [1]. RNA-guided nucleases are used by CRISPR-Cas to efficiently introduce precise genomic changes. CRISPR is more widely applicable across a variety of organisms, more flexible in target selection, and simpler to construct than ZFNs and TALENs. It enables quick reprogramming with short guide RNAs, multiplex editing, and comparatively easy operation. These benefits have made CRISPR-Cas a revolutionary tool for creating and refining modified strains.

In conclusion, because of its effectiveness, adaptability, and user-friendliness, CRISPR-Cas has essentially replaced previous technologies and is now essential to microbial engineering. Therefore, this article provided an overview of the CRISPR-Cas system, the mechanisms that allow for the creation of altered strains, and example applications. With a focus on strains intended for the biosynthesis of fuels, fine chemicals, and bio-based materials, it also emphasizes current

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developments and prospects in microbial synthetic biology.

2 Mechanism of CRISPR-Cas technology for constructing engineered strains

CRISPR and its related Cas protein, which were originally found in bacteria and archaea, constitute an adaptive immune system that can provide sequence-specific defense against invading viruses. When exogenous DNA enters the cell, a short fragment called “spacers” will be inserted into the CRISPR site. This regional transcription produces crRNA molecules, which guide the Cas protein to identify and cut the complementary sequences in the invader, thus producing acquired immunity [1]. Based on this principle, Jinek et al. developed CRISPR-Cas9 as a programmable genome editing tool: Cas9, guided by chimeric sgRNA, introduces an accurate DSB at targets adjacent to the PAM [2]. Since then, Cas12 (Cpf1) and Cas13 variants have been characterized, providing flexibility in PAM compatibility, cleavage patterns, and RNA targeting. To construct engineered strains, CRISPR-Cas systems generally follow a standardized and modular workflow. The process begins with the definition of the target region and the design of an appropriate sgRNA complementary to the desired genomic locus while satisfying PAM sequence requirements. Subsequently, Cas nuclease genes and sgRNA expression cassettes are assembled into plasmids for transformation into the microbial host, or alternatively integrated into the chromosome to ensure higher stability of expression [2]. Once delivered, the Cas nuclease recognizes the PAM sequence and induces a double-strand break in the target DNA. The host cell then repairs this lesion through one of two main pathways: NHEJ, which often generates small insertions or deletions leading to gene disruption, or HDR, which uses a supplied donor template with homology arms to introduce specific insertions such as new genes, regulatory sequences, or synthetic modules [1]. This HDR-mediated process enables rational metabolic pathway optimization through the introduction of enzymes, promoters, transcription factors, and other regulatory elements. Following editing, modified strains are screened by PCR, sequencing, phenotype observation, or metabolite quantification to confirm successful genomic modification. Because CRISPR systems can be readily reprogrammed, multiple genetic alterations can be performed simultaneously or iteratively in successive cycles, allowing rapid strain optimization for complex biosynthetic pathways and thus accelerating the development of engineered microorganisms with enhanced industrial performance [1].

3 Applications of engineered strains by CRISPR-Cas technology

3.1 Application in *Bacillus subtilis* for metabolic engineering and hyaluronic acid production

Bacillus subtilis has been widely used as a chassis microorganism for industrial biotechnology due to its safety, high secretion capacity, and genetic accessibility. The emergence of CRISPR-Cas systems has further empowered its use in metabolic engineering, allowing precise and multiplex genome modifications for the production of high-value biomolecules such as hyaluronic acid (HA). Liu et al. established a CRISPR/Cas9n-based multiplex genome-editing system that significantly improved editing efficiency and reduced cytotoxicity compared with conventional Cas9 [3]. The Cas9n (nickase) variant introduces single-strand breaks instead of double-strand breaks, thereby minimizing off-target effects and reducing cell death associated with repair of DSBs. In the study, the authors reported high deletion efficiencies ($\geqslant$ 80 %) for 1-8 kb fragments, and high insertion efficiencies ($\geqslant$ 90 %) for 1-2 kb fragments, with nearly 100 % efficiency for site-directed mutagenesis in some cases. They also demonstrated multiplex editing by performing three simultaneous point-mutations with $\approx$ 50 % efficiency and showed that disruption of the ligD gene increased the efficiency to about 65 % by modulating the nick - repair pathway [3]. In an applied example, the toolkit was used to remodel three genes within a riboflavin operon in *B. subtilis*, leading to a measurable increase in riboflavin synthesis. This work underscores the power of Cas9n for iterative, multiplexed editing within *B. subtilis*, allowing metabolic engineers to fine-tune entire biosynthetic modules rather than single genes.

More recently, Liu et al. developed a programmable CRISPR/Cas9 toolkit to enhance lycopene production, demonstrating the broader applicability of CRISPR-based systems for metabolic pathway enhancement [4]. Their platform features rapid cloning of gRNA arrays, modular donor-DNA cassettes with homologous arms, and streamlined chromosomal integration workflows that reduce the editing timeline to less than a week. By integrating multiple loci, such as ispG-idi-dxs-ispD, into the chromosome, optimizing promoter strength, and balancing metabolic fluxes, they achieved a substantial increase in lycopene titer. The authors highlight that these design principles (modularity, promoter tuning, pathway balancing) can be readily translated into other high-value biosynthesis platforms, such as HA production in *B. subtilis*. Collectively, these studies underscore the versatility of CRISPR-Cas systems (Cas9, Cas9n, Cpf1, and AsCpf1) in engineering *B. subtilis* as an efficient microbial cell factory. By integrating iterative genome editing, multi-level regulation, and even membrane engineering to improve secretion, CRISPR technologies have become indispensable tools for constructing next-generation *B. subtilis* strains capable of producing high-value compounds such as hyaluronic acid.

3.2 Applications in *Saccharomyces cerevisiae* for high-value bioproduction

Saccharomyces cerevisiae's unambiguous genetics, GRAS safety status, and robust resistance to industrial settings make it one of the most well-known eukaryotic hosts for metabolic engineering. CRISPR-Cas technologies, which enable effective, marker-free, and multiplexed genome editing, have been essential in recent years for creating high-performance yeast strains. These advances have significantly accelerated the "design-build-test-learning" (DBTL) cycle of the yeast cell factory for the development of high value-added products.

Recent reviews have summarized critical improvements in CRISPR tools for yeast, highlighting optimized guide RNA (gRNA) array design, selection of Cas variants, and repair pathway tuning to enhance editing efficiency and precision. These strategies address long-standing challenges such as limited homologous recombination rates and off-target editing. The use of tRNA-linked gRNA arrays and RNA polymerase III promoters now enables rapid multiplex gene editing in a single transformation, greatly reducing strain construction time [5].

One major breakthrough in the past few years has been the development of streamlined vector systems for multi-locus editing. The pCEC-red plasmid, for instance, integrates a Cas9/gRNA module with a visual selection marker, enabling faster identification of correctly edited clones and more reliable multi-target modification. Compared with early CRISPR toolkits, pCEC-red minimizes plasmid assembly steps and improves editing efficiency in both laboratory and industrial polyploid strains [6].

At the metabolic-engineering level, iterative genome integration has proven particularly effective for enhancing biosynthetic yields. Chen et al. established an iterative multi-copy CRISPR/Cas9 integration strategy that allows stepwise accumulation of pathway genes within the yeast genome. This approach has been successfully applied to increase titers of aromatic compounds, terpenoids, and 2-phenylethanol — demonstrating how genome-scale precision can directly translate into high-value chemical production [7]. By coupling CRISPR-driven multi-copy integration with promoter optimization, metabolic flux can be redistributed to maximize product yield while maintaining cell growth stability.

Furthermore, broader system adaptations have expanded the CRISPR toolkit beyond Cas9. Variants such as Cas12a (Cpf1), base editors, and prime editors have been implemented for fine-tuning expression and single-nucleotide modification. These new platforms are particularly useful for eliminating the need for inducers in regulated pathways—for instance, achieving inducer-free artemisinic acid synthesis or enhancing 2-phenylethanol production via subtle promoter edits. As summarized by Wu et al., recent innovations in Cas9, Cas12a, and CRISPRi/a systems are propelling yeast cell factories toward greater precision, scalability, and sustainability in industrial biomanufacturing [8].

In summary, CRISPR-Cas technologies have transformed S. cerevisiae from a model organism into a flexible cell-factory chassis. Marker-free, multiplex, and iterative editing systems—supported by recent advances in plasmid design, pathway integration, and regulatory precision—collectively enable efficient production of high-value bio-based compounds under industrial conditions.

3.3 Applications in *Escherichia coli* for construction of engineered strains

Due to its rapid growth, full genetic understanding and mature culture technology, *Escherichia coli* is still one of the most powerful microbial hosts in metabolic engineering. In recent years, the CRISPR-Cas method has significantly improved the accuracy and efficiency of E. coli genome engineering, making it easier to build strains that can produce a variety of high value-added industrial products. Early CRISPR-based systems for E. coli editing established RNA-guided DNA cleavage as a reliable means for genome modification. However, these early systems often suffered from cytotoxicity and low survival rates. Recent studies have optimized Cas9 expression levels, guide RNA design, and repair templates to balance editing efficiency with cell viability. For example, a study systematically analyzed the trade-off between CRISPR-Cas9 toxicity and genome-editing efficiency in E. coli, providing an optimized protocol for maintaining high editing accuracy while minimizing cellular stress [9]. This optimization has become essential for large-scale or multi-locus genome engineering projects.

Further innovation focuses on the use of CRISPR tools to strengthen metabolic pathway reconstruction. Ye et al. demonstrated a high-efficiency formula for metabolic engineering of E. coli BW25113 to produce ALA, which is a high-value compound used in medicine and agriculture. By using CRISPR/Cas9-mediated gene knockout and pathway modification, the author significantly improved the metabolic flux pointing to ALA synthesis and achieved scale-up production in the defined culture medium [10]. This study highlights how CRISPR-Cas systems can directly link genomic precision with measurable increases in product yield.

In parallel, recent research has highlighted continuing innovations in CRISPR-based bacterial engineering, particularly in improving DNA delivery systems, homologous recombination efficiency, and genome editing fidelity. Developments in Cas9 optimization and the introduction of alternative nucleases such as Cas12a have expanded editing flexibility and enabled dynamic regulation of gene expression in E. coli. These advances now allow researchers not only to delete or insert genes but also to fine-tune expression and perform multiplex editing of metabolic networks.

Overall, CRISPR-Cas technologies have transformed E. coli into a highly programmable chassis for the production of bio-based chemicals. By integrating optimized editing systems and metabolic pathway enhancement strategies, E. coli strains can now achieve greater metabolic balance, stability, and efficiency. These advances enable sustainable biosynthesis of fine chemicals, biofuels, and therapeutic precursors—firmly establishing CRISPR-Cas systems as indispensable tools in microbial biotechnology.

4 Conclusion

The CRISPR/Cas9 technology has fundamentally reshaped the strategies for microbial genome engineering. By enabling precise, efficient, and programmable genetic modification, it has overcome the limitations of traditional mutagenesis and earlier genome-editing tools, greatly accelerating the construction of engineered strains for industrial applications. In *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Escherichia coli*, CRISPR/Cas9-based systems have proven effective for pathway optimization, metabolic balance, and the synthesis of high value-added products. These cases collectively demonstrate that CRISPR/Cas9 is not only a molecular editing tool but also a versatile framework for rational strain design, adaptable to both prokaryotic and eukaryotic hosts. Looking ahead, continued refinement of CRISPR/Cas9 platforms and their integration with systems biology will further enhance the precision, efficiency, and scalability of microbial engineering. As research deepens and industrial translation advances, CRISPR/Cas9 is expected to remain a cornerstone technology driving sustainable biomanufacturing and innovation in high value-added product development.

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