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Optimization of Fermentation Processes by the Combined Application of Genetically Engineered Strains and Enzymes

Zhewen Li

School of Life Science, Sichuan Agricultural University, Ya'an, China
lizhewen@stu.sicau.edu.cn

Abstract. The efficiency and economic viability of industrial fermentation have always been key concerns in biomanufacturing. Traditional fermentation technologies are limited by the metabolic capacity of microorganisms and the catalytic efficiency of key reaction steps, leaving limited potential for overall improvement. In recent years, however, the rapid advancement of genetic engineering and protein engineering has opened up new opportunities in the field of microbial fermentation. Genetically engineered strains and enzymes are two critical components in industrial fermentation, and the potential for their collaborative optimization is very large. Genetically engineered strains are the "brains" which can be transformed into "cell factories". Enzymes are highly efficient and precise "molecular machines", responsible for driving specific biochemical reactions. The combination of genetic engineering and enzyme engineering is not merely a focus on a single technology; rather, it involves cross-systemal collaborative optimization in terms of function and time-space. This article will comprehensively summarize the latest research progress of the joint strategies of the two, and through this strategy, it will reveal the mechanisms for increasing product yield, high selectivity, and high process efficiency in fields such as biofuels, high-value chemicals, food, and medicine. At the same time, we will objectively discuss the host metabolic burden, difficulties in process scaling-up, and industrial stability issues of this strategy. This article also looks forward to the trend that the fermentation process will gradually become intelligent, integrated, and green under the drive of artificial intelligence and synthetic biology.

Keywords: Genetic engineering strain, Enzymes, Combined application, Fermentation optimization, Metabolic engineering

1. Introduction

As a traditional process method for chemical production using microorganisms, fermentation is an important part of modern industry. From fuel ethanol to amino acids, from organic acids to antibiotics, fermented products have penetrated into the frontier industries of the national economy, such as energy, materials, food and medicine. However, traditional fermentation based on the natural wild-type strain is always limited by the natural metabolism of the microorganism itself. The metabolic pathway of the wild-type strain preferentially maintains cell growth rather than maximizing the accumulation of target products, resulting in low substrate conversion efficiency. On

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the other hand, the wild strain had a very narrow substrate profile and was unable to utilize abundant non-grain biomass. In addition to natural metabolic barriers, most industrial target products or their precursors are toxic, and the tolerance of wild strains is also a barrier to production intensity [1]. These problems make the yield, cost and sustainability of traditional fermentation face serious challenges. In order to break through the limitations of traditional fermentation, genetic engineering strains are obtained by modifying the genes of microbial hosts. Gene editing (such as CRISPR-Cas9) and metabolic engineering are used to direction-change microbial genetic metabolism, delete competing pathways, strengthen target pathways, introduce new heterologous metabolic modules, change metabolic flow, and achieve efficient product accumulation [2].

The enzyme itself has high catalytic activity and substrate specificity, but its disadvantage is that natural enzymes cannot adapt to the harsh conditions such as high temperature, acid-base, and organic solvents in large-scale industrial fermentation. Enzyme engineering has chosen to optimize the enzyme molecule in three ways. At the physical level, rational design, directed evolution and semi-rational design methods are used to strengthen and optimize enzyme molecules to improve their robustness and broad-spectrum applicability [3]. At the molecular level, strategies such as codon optimization, co-expression of molecular chaperones, and screening of high-efficiency signal peptides [4] were used to improve the expression level and secretion efficiency of heterologous enzymes in industrial hosts. At the application level, enzyme immobilization technology is used to improve its operational stability and reuse, and reduce the process cost.

Both genetic engineering and enzyme engineering have certain application prospects in their respective fields, but the limitations of single technology are gradually emerging in the face of increasingly complex biomanufacturing requirements. Sometimes it is difficult for genetically engineered strains to regulate metabolism to accurately control the rate and balance of all enzymatic reactions, and free enzyme preparations will cause problems such as high production costs, easy inactivation in complex systems, and difficult recycling. Therefore, the integration of engineered microbial hosts and enzyme engineering has become a more competitive choice [4]. Genetic engineering strains can provide a "living reactor" with complete cofactor regeneration and metabolic support, while enzymes are the "catalytic elements" to perform key chemical transformations. The combination of the two will realize the optimization and upgrading of metabolic pathways, as well as the process reengineering of multi-step complex transformations (Figure 1).

From the perspective of system integration, this paper reviews the whole process of genetically engineered strains and enzyme engineering from simple superposition to deep fusion, and systematically reviews the latest research on the combined application of genetically engineered strains and enzymes in fermentation process optimization. Then, the synergy mode and synergy results of the two strategies in different scenarios were analyzed through typical cases, the obstacles still existing in the industrialization process were analyzed objectively, and the development direction was prospected combined with cross technology.

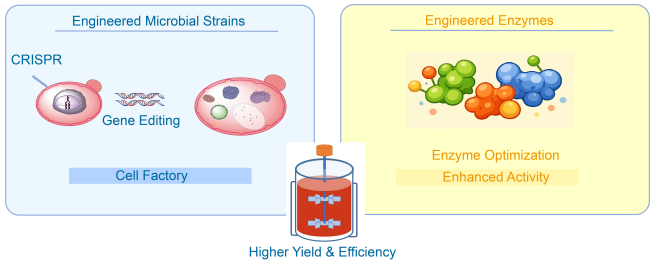

Figure 1. Schematic diagram illustrating the combination of genetic engineering and enzyme engineering to improve fermentation efficiency

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2. Overview of genetically engineered strains and enzyme engineering

2.1. Genetically engineered strains

Excellent genetic engineering strains are the basis for fermentation optimization, and the continuous update of genetic engineering biotechnology is the cornerstone of excellent strain selection.

In terms of editing tools, CRISPR-Cas system has been used for precise genome editing of microorganisms, which can effectively knock out or over-express genes, perform multiple edits, and precisely control gene transcription levels [5]. For example, in the important industrial enzyme producing fungus Trichoderma reesei, the trNA-based CRISPR-Cas multiplex gene editing system was used to improve cellulase and xylanase production by knocking out the transcriptional repressor Cre1 and overexpressing the activator XYr1 [6]. It can be seen that precision gene editing technology has a strong ability to deeply modify the performance of industrial strains, and modify the characteristics of strains at the molecular level.

In the optimization of protein expression and secretion, genetic engineering of the secretion pathway is an important method to improve the production of extracellular products. For example, by knocking down lpp, tolA, tolB, excC and other cell wk-related genes of Escherichia coli to construct "leaky strains", extracellular protein production can be significantly improved [7]. Signal peptide modification is also an important means to improve the secretion efficiency. Using bioinformatics tools such as SignalP, Phobius and PSORTdb to assist in selecting the optimal signal peptide, and modifying its N, H and C regions, the secretion level of ATH35L enzyme has been successfully increased by 3.5 times [8].

At the protein expression level, genes are usually optimized according to host codon preference to improve translation efficiency. To optimize the expression of L-arabinose isomerase (L-AI), the araA gene was optimized according to the codon usage preference of the host Escherichia coli BL21(DE3), and the optimized gene araAo was obtained. When the araAo gene was applied to E. coli, the enzyme activity of L-AI was increased by 79.2%, and the protein expression level was significantly enhanced [9]. Coexpression of molecular chaperones, including DnaKJ-grpE and GroEL-ES systems, can assist recombinant proteins to fold correctly and avoid the formation of inactive inclusion bodies, which can also greatly improve protein expression [7].

In the reconstruction of strain metabolic network, the in-depth modification involves the rational design of the global metabolic network of strains. The core idea is to rationally design complete metabolic pathways according to human economic needs. For example, through rational metabolic design and adaptive laboratory evolution (ALE), the metabolism of Saccharomyces cerevisiae is transformed from alcohol fermentation to stable lipid synthesis, and finally through selection pressure, excellent strains that meet expectations are selected [2].

2.2. Properties and applications of enzymes

The enzyme has the characteristics of substrate specificity, high catalytic efficiency, and mild reaction conditions. However, natural enzymes are easily inactivated in the face of severe conditions such as continuous high temperature and strong acid and base [3]. The main goal of enzyme engineering is to make key enzymes better adapted to industrial production environments and to a wider range of substrates through human intervention.

There are three main approaches to achieve enzyme stability: rational design, directed evolution and semi-rational design. Rational design requires knowing the three-dimensional structure of the enzyme in advance, and performing actual point mutation experiments through computer simulation

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of site-directed mutagenesi [3]. In recent years, the application of artificial intelligence tools such as AlphaFold [10] and FireProtASR [11] can not only achieve accurate prediction of protein structure, but also analyze a large amount of sequsion-function relationship data and predict which mutation combinations are likely to have positive effects, which can well guide the direction of directed evolution experiments. Improve the efficiency of enzyme development [12] ; By simulating the natural evolution process, in the case of unknown enzyme structure, the enzyme gene is randomly mutated by error-prone PCR and other techniques, and then excellent variants are obtained through high-throughput screening [3] . A typical case is the combination of computer-assisted directed evolution and site-directed mutagenesis. It significantly improved the stability and activity of α-amylase from Bacillus cereus at 70°C and pH 4-11 [4]. Semi-rational design combines the above two ideas, finds evolutionary conserved sites based on multiple sequence alignment, and conducts mutations at these sites to obtain excellent results with less screening scale [3]. For example, site-directed mutagenesis of conserved sites based on multiple sequence alignment has significantly improved the thermostability and adaptability to acidic environment of Rhizopus oryzae α-amylase [4].

The application of enzymes in fermentation consists of two aspects. In terms of the mode of use, the enzyme is fixed on the solid phase carrier, which can be repeatedly separated from the reaction system and reused in industrial production, with the advantages of good operation stability and good process economy [3]. In the application of joint regulation of enzyme reactions, a complete artificial metabolic pathway requires the expression of multiple functionally related enzymes in the host bacteria at the same time, fine adjustment of the relative expression amount and catalytic kinetics of enzymes, and sometimes real-time monitoring and feedback regulation of metabolite concentrations with the help of biosensors [13]. At the fermentation process level, strategies such as low-temperature induction at 20-25 °C, replacement of IPTG with lactose, and use of high-density media such as TB/SB can maintain cell activity and promote continuous expression and secretion of recombinase without changing the genetic background of the strain [4].

2.3. Combined application of genetic engineering and enzymes

The combined application of genetic engineering and enzymes yields a synergistic effect where "1+1>2." The integration of genetically engineered strains with enzymes can generate a wide range of benefits in various ways and at multiple levels.

The first is to enhance the single catalytic performance of the enzyme. Genetic engineering is very good at establishing complex metabolic networks and providing a complete cellular environment. However, its endogenous enzymes may have low activity or selectivity in a specific transformation step. Therefore, it is more important to improve the efficiency of rate-limiting enzymes [4]. For example, in Bacillus subtilis, cloning the multi-copy plasmid pUB110 and expressing the α-amylase gene from Bacillus amyloliquefaciens, the introduction of the highly efficient exogenous enzyme increased the yield by 2,500 times and enhanced enzyme activity by 5 times [1]. The codons of Bacillus licheniformis α-amylase were optimized in Pichia pastoris, and the GC content was increased from 47.6% to 49.2%. Through the introduction of an exogenous high-efficiency enzyme and optimization of the enzyme's catalytic performance, methanol-induced expression levels increased by 2.31-fold and 2.62-fold in 5-L and 50-L bioreactors, respectively, with a maximum activity of 11,000 U/mL [1]. Conversely, the function of heterologous enzymes also depends on protein folding, post-translational modifications, and cofactor regeneration provided by the host; an optimized host chassis is the foundation for the effectiveness of exogenous enzymes [7].

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Second is synergistic effects and process optimization. A typical example is the use of genetically modified E. coli to produce ethanol. After constructing the coding genes PDC and ADHII of essential enzymes into an artificial operon and introducing it into Escherichia coli, the metabolic direction of Escherichia coli was completely reversed. Ethanol became the absolute dominant fermentation product, with a proportion as high as 95%, and the organic acid by-products such as succinic acid and acetic acid were reduced [14], not only expanding the substrate utilization range but also helping maintain pH stability in the system. A deeper level of synergy is metabolic compartmentalization, where exogenous enzymes or entire synthesis pathways are selectively implanted into a specific organelle of the cell (such as the peroxisome). Through spatial isolation, substrates can be removed and toxins sequestered, greatly enhancing the flux and yield of the target pathway [2]. For example, by integrating an exogenous enzyme into the reaction cavity of fungal type I fatty acid synthase (FAS) to construct a synthetic FAS system, the conversion of long-chain fatty acids into short- and medium-chain fatty acids and methyl ketones was achieved [2].

The third is the simplification and optimization of the fermentation process, that is, it can produce the target product in one stop using complex raw materials. For instance, in Saccharomyces cerevisiae, cellobiohydrolases derived from fungi are expressed and secreted to directly degrade cellulose and ferment it to produce ethanol, which is known as consolidated bioprocessing (CBP) [15]. Although the cellulose degradation efficiency of engineered yeast needs to be improved, it represents the future of integrating multi-step enzymatic hydrolysis and fermentation processes into one, which can greatly simplify the process and reduce production costs.

3. Recent advances in the joint optimization of fermentation processes using genetically engineered strains and enzymes

3.1. Combined applications in biofuel production

Ethanol is the most common biofuel and is typically produced through yeast fermentation. Saccharomyces cerevisiae is the traditional strain used for ethanol production; however, it is sensitive to certain inhibitory factors (such as high concentrations of ethanol) during fermentation, which limits its yield. Ethanol, the second-generation fuel produced from lignocellulose, is an important case of combined fermentation application. The difficulty lies in whether the complex plant biomass can be economically and effectively degraded into fermentable sugars. The traditional stepwise fermentation process has a long cycle and high cost. However, the combined fermentation strategy unifies enzymatic hydrolysis and fermentation in one biological system, greatly enhancing production efficiency and reducing production costs. The successful expression and secretion of fungal-derived cellobiohydrolase in Saccharomyces cerevisiae confirmed its catalytic activity, providing a theoretical basis for the establishment of CBP yeast capable of directly utilizing cellulose [15].

Biodiesel is a fuel produced from vegetable oils or animal fats through a transesterification reaction. Traditional transesterification methods require chemical catalysts and are typically cumbersome and environmentally harmful. In contrast, biocatalytic methods use enzymes as catalysts, making them not only more environmentally friendly but also allowing for milder reaction conditions. For example, Ying Mu et al. used lipase to catalyze the transesterification reaction of triglycerides (fats or oils) with glycerol to generate biodiesel. At the same time, glycerol (including the by-product glycerol) was used as the substrate for Klebsiella pneumoniae to produce 1,3-propanediol [16].

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For higher alcohols such as butanol, the application mode of combined fermentation is also effective. Overexpressing or modifying the enzymes in the butanol synthesis pathway in E. coli or Clostridium, combined with adaptive evolution to enhance the strain's tolerance to butanol toxicity, can increase the final yield and production efficiency [2].

3.2. Combined applications in the food and pharmaceutical industries

"Precision fermentation" in the food industry refers to the process in which genetically engineered microorganisms serve as "cellular factories" for the production of specific functional substances. For instance, Trichoderma reesei can be used to produce ovalbumin as a substitute for egg white, and during the fermentation process, greenhouse gas emissions and land occupation can be reduced [17]; All these processes need to be modified by altering the efficient expression and secretion system of the host bacterial protein.

One of the most challenging areas in the pharmaceutical industry is plant natural products. Complete microbial synthesis involves long synthetic pathways, many enzymes, and low host enzyme activity, which is unfavorable for modern industrial production and utilization. The vast majority of successful cases result from the in-depth integration of genetically engineered strains and enzymes. For example, when synthesizing the anticancer drug vinblastine in yeast, researchers integrated more than 30 plant-derived enzyme genes and performed dozens of gene edits on the yeast host itself to optimize precursor supply and eliminate metabolic inhibition, ultimately synthesizing the key precursor molecule for vinblastine de novo from glucose [18]. Similarly, by reconstructing complex alkaloid and flavonoid synthesis pathways and introducing or optimizing key rate-limiting enzymes, microbial synthesis of high-value compounds has been achieved, opening up a new path for sustainable and controllable supply of drug raw materials [2]. In addition to the synthesis of natural plant products, traditional antibiotic production is also a major sector in the pharmaceutical field. Scientists edit the production strains to enhance their precursor supply capacity and tolerance to their own products, and combined with fermentation process modifications, the yield is greatly increased. In addition, for protein therapeutics such as insulin and monoclonal antibodies, selecting or engineering efficient eukaryotic expression systems and optimizing protein folding, post-translational modifications, and secretion pathways can improve yield and quality [7]. For chiral drug intermediates, the production goal can also be achieved through enzyme engineering. For instance, carbonyl reductase and glucose dehydrogenase can be co-expressed in Escherichia coli to construct an efficient coenzyme regeneration system for highly selective production of chiral alcohol intermediates, meeting the requirements of green pharmaceuticals [3].

4. Technical challenges of co-optimizing fermentation processes with genetically engineered strains and enzymes

Although the combination of genetically engineered strains and enzymes for fermentation optimization has a broad market prospect, it still faces a large number of complex practical problems that need to be solved in current large-scale industrial applications.

4.1. Enzyme stability and catalytic efficiency

Prolonged exposure to high temperatures leads to irreversible changes in protein structure; additionally, high pH alters the ionization state of key amino acids in the active site; high

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concentrations of substrates or products can cause inhibitory effects, and organic solvents and metal ions in the reaction medium can disrupt the enzyme's three-dimensional structure [3]. Although enzyme engineering offers various modification methods, how to comprehensively modify the long-term production stability of enzymes on the basis of enhancing their catalytic activity will be a challenge [2]. In addition, many heterologous enzymes, especially complex enzymes of eukaryotic origin, rely entirely on the host cell's molecular chaperone system and specific cellular microenvironment for correct folding and functionality. High-level expression of endogenous molecular chaperone systems such as GroEL/ES and DnaK/J may lead to the accumulation of misfolded enzymes, forming inactive inclusion bodies [19]

4.2. Metabolic burden and tolerance of genetically engineered strains

Introducing and overexpressing heterologous genes or reconstructing complex non-natural metabolic pathways in engineered strains imposes a significant metabolic burden on host cells [3]. This consumes ATP, amino acid precursors, and NADPH, thereby slowing cell growth and reducing viability [2]. In addition, many industrial hosts themselves have expression bottlenecks: although Escherichia coli is easy to manipulate and grows rapidly, it lacks appropriate post-translational modification mechanisms (such as glycosylation) and is not suitable for the proper folding of certain eukaryotic-derived enzymes; yeast and filamentous fungi, although possessing post-translational modification capabilities, have unstable expression levels or issues such as excessive glycosylation [3]. On the other hand, many target products (such as butanol and certain alkaloids) or their metabolic intermediates are toxic to the host, inhibit its growth, or even cause its death; this is also one of the bottlenecks in product tolerance [4]. How to balance growth and production and enhance the robustness of the host through regulatory pathway compartmentalization and adaptive evolution is the key to high yield.

4.3. Process scale-up and industrialization

When engineered strains-enzyme combined systems that perform well in small laboratory fermenters are scaled up to industrial production scales of tens to hundreds of cubic meters, they encounter the challenge of the 'scale-up effect' [2]. In large-scale fermenters, factors such as uneven mixing, varying oxygen and nutrient gradients, and differing shear rates can significantly affect the uniformity of microbial growth, the stability of gene expression, and enzyme activity. Economic challenges in large-scale production also include the suitability of expensive inducers for large-scale use, how to carry out low-cost and non-toxic induction and control, and how to handle the difficulty and cost of downstream product separation and purification, etc [3]. In addition, issues such as product inhibition, enzyme self-degradation, and the difficulty in constructing multifunctional enzyme cascade reaction systems (involving regulation of expression ratios, spatial localization, and cofactor regeneration) also restrict the industrialization process [3]. The lack of an intelligent, integrated design and optimization platform capable of synthesizing strain physiological characteristics, enzyme catalytic parameters, fermentation kinetics, and engineering scale-up experience results in uncertainty and high risk in the translation of laboratory findings into industrial applications [7].

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4.4. Safety and regulatory considerations

Public concerns over the environmental release risks and product safety of genetically modified microorganisms (GMO) have persisted, which has also prompted research efforts to shift towards alternative solutions, such as the use of cell-free systems for enzymatic synthesis or the development of strictly biocontained chassis organisms, to address regulatory and public demands [4].

5. Prospects and future development of the combined application of genetically engineered strains and enzymes

In the face of the aforementioned challenges, the future breakthroughs of this combined application will be closely and inseparably integrated with biotechnology, computational science, and engineering principles.

5.1. The deep integration of novel enzymes and genetic engineering technologies

The design of genetically engineered strains will increasingly adhere to the principles of modularity and standardization in synthetic biology, evolving into truly programmable "smart cell factories." CRISPR will evolve from a simple editing tool to a highly complex dynamic regulatory network within cells, enabling timely perception and closed-loop control of fluxes [5]. Artificial intelligence and machine learning will extend to all links from design to optimization: in addition to being used to predict protein structures (AlphaFold) and design new enzyme activities [10], they will also predict optimal gene editing targets, metabolic engineering strategies, and even directly recommend fermentation process parameters through multi-omics data. The machine learning-based "Design–Build–Test–Learn" cycle significantly shortens the trial-and-error phase of engineering and accelerates the establishment of ideal production systems [12].

5.2. Promoting green and sustainable biomanufacturing

Joint application is one of the important engines for biomanufacturing to move towards green, low-carbon and circular development. One important direction is to broaden the sources of raw materials and design a combined system that uses non-grain biomass or one-carbon compounds. Another important direction is biological upgrading and recycling. Biological upcycling refers to the process of using a combination of engineered microorganisms and specific enzymes to depolymerize plastic waste (such as PET and polyurethane) and other environmental pollutants, thereby "upcycling" the monomers into valuable chemicals, such as biodegradable PHA plastics and nylon precursors. This approach offers a new biotechnological solution for addressing white pollution and achieving resource recycling [20].

6. Conclusion

The combined application of genetically engineered strains and enzymes transitions fermentation process optimization from localized modification to systemic integration, integrating the metabolic functions of host cells and the precise catalytic functions of enzyme molecules, thereby enhancing the efficiency, selectivity and economy of biofuel, chemical, food and drug production. It has demonstrated promising advantages and prospects, but this approach has not yet achieved large-scale industrialization. The industrial-grade stability of enzymes, the metabolic burden on hosts, and the scale-up of processes from laboratory to factory are still practical issues that need to be

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addressed. In the future, it will still require multidisciplinary collaboration. Developing more robust and versatile genetically engineered strains, establishing smarter and more efficient methods for enzyme design and evolution, building data-driven, automated "Design–Build–Test–Learn" platforms, and developing green biomanufacturing pathways based on renewable raw materials and waste are not only about economic benefits but also represent the inevitable direction for biotechnology to fulfill its environmental responsibilities and address global resource challenges. The combination of genetically engineered strains and enzymes is leading us towards a new era of more efficient, precise and sustainable biomanufacturing.

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