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Genetic Engineering Strategies for Enhancing Vitamin D3 Production in Saccharomyces cerevisiae Fermentation Systems

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Abstract: Vitamin D is a crucial fat-soluble vitamin that plays a significant role in maintaining human metabolism and normal physiological functions. Vitamin D3, one of the forms of vitamin D, exhibits high biological activity. Saccharomyces cerevisiae, a commonly used microbial host in bioengineering, offers numerous advantages and can serve as a chassis for fermentative production of vitamin D3. The synthesis of vitamin D3 begins with acetyl-CoA, a central carbon metabolism product. Acetyl-CoA proceeds through a series of biochemical reactions, including the mevalonate (MVA) pathway, the squalene pathway and the 7-dehydrocholesterol (7-DHC) pathway. These reactions ultimately yield 7-DHC, which is then converted to vitamin D3 under ultraviolet light irradiation. This process is regulated by various enzymes, such as the ERG series and DHCR24. This article summarizes several genetic engineering strategies to enhance 7-DHC production in Saccharomyces cerevisiae, including increasing the precursor metabolic flux, inhibiting the competing pathways, reconstructing the subcellular structures, and reducing the cytotoxicity of intermediates. These strategies hold significant potential for the industrial production of vitamin D3 and addressing global hidden hunger issues.

Keywords: Vitamin D3, Saccharomyces cerevisiae, 7-DHC, genetic engineering, subcellular structure, cytotoxicity

1. Introduction

Vitamin D is a kind of fat-soluble vitamin, it primarily exists in two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D is essential for human health [1], as it regulates normal bone remodeling cycles [2], enhances the calcium and phosphorus metabolism [3], and plays a role in maintaining the immune system functions [3]. Vitamin D also reduces the risks of many major diseases, such as cancer [4], type 2 diabetes [5] and Parkinson's disease [6]. Holick et al. [7] suggested that a daily intake of approximately 50 μg of vitamin D3 can effectively prevent or treat vitamin D deficiency. Currently, over one billion adults and children worldwide are suffering from varying degrees of vitamin D deficiency [8]. It is a condition referred to as "hidden hunger" [9], which poses a pressing public health challenge. Research into industrial production pathways for vitamin D supplements is a viable solution to address vitamin D shortages, and it has become a hotspot in food engineering and biological sciences. Progress has been made in vitamin D-fortified foods by now. For example, Li et al. [1] expressed vitamin D3 precursors (7-DHC) in leaves, green fruits and ripe

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fruits of tomatoes at significant levels, providing a promising example of vitamin D supplementation food.

A clinical study indicates that vitamin D3 exhibits higher biological activity than vitamin D2 [10], so this review mainly focuses on the biosynthesis of vitamin D3. Traditional chemical synthesis of vitamin D3 involves multiple steps, such as acylation, oxidation, reduction, hydrolysis, and elimination, and it requires organic reagents and solvents like zirconium oxide, acetic anhydride and petroleum ether. Traditional ways are costly and harmful to the environment [11]. In contrast, biosynthesis methods are more sustainable, with lower resource and energy consumption. Obviously, the latter are more suitable for large-scale production of vitamin D3.

Saccharomyces cerevisiae is a model microorganism and is widely used in food, pharmaceutical and biotechnology industries. As a single-celled eukaryote, Saccharomyces cerevisiae offers advantages such as a well-characterized genetic background, high safety and ease of large-scale cultivation [12], making it an ideal chassis for vitamin D3 biosynthesis.

In Saccharomyces cerevisiae, vitamin D3 synthesis can be divided into four stages [13,14]: (1) Central carbon metabolism: Glucose is metabolized via glycolysis and the pyruvate dehydrogenase pathway to produce acetyl-CoA. Nevertheless, acetyl-CoA can also be derived from ethanol oxidation. (2) MVA pathway: Acetyl-CoA is converted to MVA, which is then phosphorylated to form mevalonate-5-phosphate. (3) Squalene pathway: Mevalonate-5-phosphate is transformed into isopentenyl pyrophosphate (IPP), then into farnesyl pyrophosphate (FPP), and subsequently into squalene. (4) 7-DHC pathway: Squalene undergoes a series of reactions to form7-DHC, then is converted to vitamin D3 under UV light irradiation (Fig. 1). In related studies, 7-DHC levels are often used as an indirect measure of vitamin D3 production.

Notably, wild-type Saccharomyces cerevisiae lacks endogenous DHCR24, the key enzyme for converting zymosterol to 7-DHC, so introducing the DHCR24 gene is essential for constructing a vitamin D3 biosynthetic system in Saccharomyces cerevisiae [15-17].

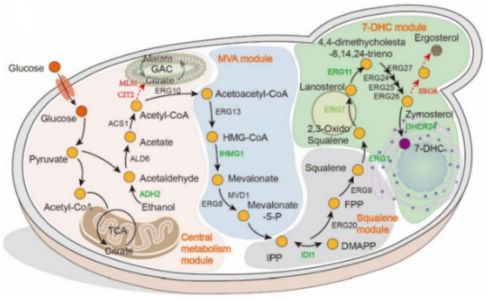

Figure 1: The biosynthetic pathway of 7-DHC in Saccharomyces cerevisiae [13].

Squalene is further converted to zymosterol, and finally to 7-DHC. However, zymosterol is also the precursor for ergosterol (the pre-vitamin of vitamin D2), leading to competition between vitamin D2 and D3 pathways in Saccharomyces cerevisiae.

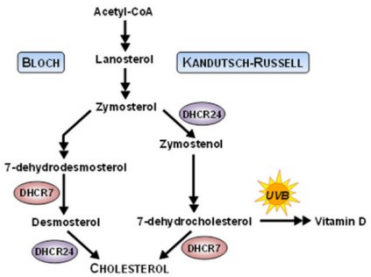

Figure 2: Two pathways for cholesterol synthesis from zymosterol [18].

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In addition to competition with vitamin D2 synthesis, vitamin D3 production also competes with two cholesterol syntheses: the Bloch pathway and the Kandutsch-Russell (K-R) pathway. Both of the two pathways involve DHCR7 catalysis [18]. In the Bloch pathway, lanosterol is converted to 7-dehydrodesmosterol, which is then transformed into desmosterol by DHCR7 and finally into cholesterol by DHCR24. In the K-R pathway, 7-DHC can be converted to cholesterol by DHCR7 instead of forming vitamin D3.

2. Genetic Engineering Strategies for Enhancing Vitamin D3 Production in Saccharomyces cerevisiae

2.1. Increasing the Precursor Metabolic Flux

2.1.1. Central Carbon Metabolism Regulation

Acetyl-CoA, the product of central carbon metabolism, is the raw material of various metabolic reactions, including the vitamin D3 synthesis. Enhancing the efficiency of carbon metabolism can increase cytoplasmic acetyl-CoA levels, thereby boosting the MVA pathway and 7-DHC production. Meadows et al. [19] proposed a strategy to improve acetyl-CoA synthesis. They rebuilt the central carbon metabolic pathway in Saccharomyces cerevisiae by introducing four heterologous enzymes, including phosphoketolase (xPK), phosphotransacetylase (PTA), aldehyde dehydrogenase (ADA), and NADH-dependent HMG-CoA reductase (NADH-HMGr). They also knocked out glycerol-3-phosphate phosphatases (Rhr2p and Hor2p) to reduce ATP demand and CO_2 loss, improving acetyl-CoA production efficiency.

2.1.2. Introduction and Expression Regulation of Exogenous DHCR24

DHCR24 is the only essential exogenous enzyme in Saccharomyces cerevisiae for vitamin D3 synthesis, and it is also a rate-limiting enzyme to the reactions [15]. A series of studies have shown that the source and expression level of DHCR24 significantly affect the 7-DHC titer. Guo et al. [15] compared DHCR24 genes from plants, vertebrates, protists and fungi, finding that the chicken (Gallus gallus) soured DHCR24 gene (GgDHCR24) yielded the highest 7-DHC production (64.1 mg/L). It is speculated that the catalytic substrate of plant-sourced DHCR24 enzymes is not the 7-dehydrodesmosterol but its isomer. They also found that the yield of 7-DHC is related to the promoter and copy number of DHCR24: introducing the GAL1 promoter achieved an increase of 42.1% (to 81.7 mg/L) in shake flasks, and inserting another pair of GgDHCR24 copies at the ERG6 locus increased production by 16.5% (to 169.1 mg/L). Qu et al. [16] introduced Ty1 transposons to elevate DHCR24 and ERG1 expression, boosting 7-DHC production by 120% (to 223.3 mg/L). Bi et al. [17] specially studied the effect of GgDHCR24 copy number on 7-DHC yield, and demonstrated that 7-DHC production plateaued at five DHCR24 copies (to about 106.2 mg/L), indicating that beyond this threshold, DHCR24 expression no longer limited the 7-DHC yield.

2.2. Inhibiting the Competing Pathways

Competing pathways in Saccharomyces cerevisiae include ergosterol-vitamin D2 synthesis and cholesterol synthesis (via Bloch or K-R pathways). Disrupting these pathways can increase the substrate utilization and redirect the metabolic flux toward 7-DHC. Su et al. [20] introduced DHCR24 and tHMG1 genes to the locus of ERG5 to block the vitamin D2 synthesis, and obtained a 7-DHC yield of 2.61 mg/L. However, ergosterol is a critical component of the yeast cell membrane; abruptly blocking its biosynthetic pathway would impair cellular growth and proliferation, thereby negatively affecting 7-DHC product synthesis. In order to solve this contradiction, Qu et al. [16] replaced the

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ERG6 promoter with glucose-repressible GAL1, allowing ergosterol production during growth (log phase) and cessation during 7-DHC production (stationary phase), yielding 365.5 mg/L 7-DHC with a 43% increase in biomass of Saccharomyces cerevisiae. Prabhu et al. [18] found that the wild-type DHCR7 enzyme is unstable and can be inhibited by cholesterol. Tuckey et al. [21] identified 7-DHC derivatives (8-DHC and 20S(OH)L3) as DHCR7 inhibitors. Both derivatives can be produced in mammalian skin cells using 7-DHC as a substrate upon UV light irradiation. Therefore, introducing relevant genes into yeast to suppress DHCR7 activity may be a feasible strategy. These two studies provide possible ideas for the inhibition of the DHCR7 catalytic reaction.

2.3. Reconstructing the Subcellular Structures

Saccharomyces cerevisiae possesses organelles such as endoplasmic reticulum (ER), lipid droplets (LDs), peroxisomes (PXs), and mitochondria. The biomembrane system formed by these organellar membranes compartmentalizes diverse metabolic reactions within the cell, ensuring their efficient and orderly progression. Compartmentalizing vitamin D3 synthesis pathways into organelles can enhance efficiency. Xiu et al. [13] remodeled six enzymes (ERG7/ERG20/ IDI1/ MVD1/ ERG8/ERG12) by fusing with ER-targeting signal peptide CNE1, and overexpressed INO2 to expand ER size, achieving an increase of 45.8% of 7-DHC titer (to 455.6 mg/L). Guo et al. [22] grouped squalene-related enzymes into four modules (ERG1/11/24, ERG25/26/27, ERG2/3, DHCR24), and targeted these modules to ER and LDs respectively, reducing squalene accumulation by 95.5% and increasing 7-DHC titer by 92.1% (to 360.6 mg/L). Bi et al. [17] divided enzymes into six modules according to the molecular product, and assembled three modules in PXs by fusing with ePTS1, boosting 7-DHC production by about 120% (to 221.67 mg/L). Besides, transcription factors (TFs) OAF1/PIP2 are regulated to promote the proliferation of PXs, and the 7-DHC titer increased by about 82%. Nevertheless, TFs OAF1/PIP2 are also regulated in the strain with four modules assembled in PXs, and compared with the former, a 17.9% increment of 7-DHC titer was observed (to 474.03 mg/L). The reason may be that the proliferation of PXs provides larger space for reactions and storage, thereby enhancing the 7-DHC synthesis. Xiu et al. [23] reconstructed the squalene pathway in LDs by fusing DHCR24/ERG3, ERG2/28, and ERG11/24 with oleosins from Zea mays, further improving production by 50.2% (to 684.1 mg/L). Yee et al. [11] reconstructed the MVA pathway in the mitochondria of Saccharomyces cerevisiae. This approach suggests a potential strategy for 7-DHC synthesis: leveraging the mitochondrial pool of abundant acetyl-CoA to provide sufficient precursor supply for the MVA pathway, thereby enhancing 7-DHC production.

2.4. Reducing the Intermediate Cytotoxicity

2.4.1. Modulating the Redox Cofactors

Redox cofactors play vital roles in cellular metabolism, and their engineering represents an effective approach for metabolic regulation [24]. Imbalanced cytosolic free NADH/NAD+ ratios can hinder metabolism and consequently inhibit the synthesis of 7-DHC. Su et al. [20] found that knocking out the ergosterol pathway can lead to rising free NADH/NAD+ ratios, so they introduced H_2O-forming NADH oxidase (NOX) and alternative oxidase (AOX1) to consume NADH. It not only reduced the NADH/NAD+ ratios, but also increased the 7-DHC titer by 74.4% (to 8.44 mg/L). Xiu et al. [23] reported AOX1 as more effective in enhancing 7-DHC production than NOX, while the former elevated 7-DHC yield by 9.4% (to 867.6 mg/L). Bi et al. [17] overexpressed IDP2/3 and ALD6 to boost NADPH supply. ENO2p promoter was then introduced to regulate the ALD6 expression levels, and consequently achieved an increase of 35.2% in 7-DHC production (to 640.77 mg/L).

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2.4.2. Reducing Free 7-DHC

For industrial production, accumulation of 7-DHC is essential as it serves as the immediate precursor to vitamin D3. However, excessive free 7-DHC is cytotoxic and may inhibit cell growth. Xiu et al. [23] introduced ARE2 to esterify 7-DHC into triacylglycerols to store it in ER and LDs to avoid the cytotoxicity. They also knocked out TGL3 (related to hydrolysis) to prevent the degradation of triacylglycerols. The production of 7-DHC eventually achieved a 15.9% increase (to 792.9 mg/L) without significant biomass reduction.

3. Conclusions

Vitamin D is an essential nutrient for human health, and its industrial-scale production holds significant potential for addressing global hidden hunger. With advancements in genetic and fermentation engineering, the biosynthetic pathway of vitamin D3 has been progressively optimized. Strategies like pathway optimization, competing pathway inhibition, subcellular compartmentalization and redox balance modulation collectively enhance yields. Existing researches rarely employ a single modification approach in isolation, as combinatorial approaches demonstrate higher efficiency for 7-DHC production.

Despite these advancements, several critical gaps remain to be addressed: (1) Photochemical conversion inefficiency: The conversion of 7-DHC to vitamin D3 involves photochemical reactions, which determine the efficiency of the transformation process [25]. Current industrial production still lacks a suitable enzyme to efficiently catalyze this reaction. With the advancement of protein structure prediction technology, the artificial design of enzymes for photochemical reactions may become a viable solution. (2) DHCR24 specificity: Since the synthesis of vitamin D3 in Saccharomyces cerevisiae can only be achieved by introducing the exogenous DHCR24 gene, and the substrate specificity of exogenous DHCR24 enzymes may be different from that of the yeast's endogenous substrates, side reactions may occur. Thereby, the yield [15] and purity [23] of 7-DHC will be reduced. Future research could focus on enzyme engineering of DHCR24 to enhance its specificity for catalyzing the synthesis of 7-DHC. (3) Holistic pathway engineering: Currently, most genetic engineering approaches for modifying Saccharomyces cerevisiae regulate the expression of only a few specific genes to control intermediate products and side reactions. However, the vitamin D3 biosynthesis pathway involves a series of enzymes and dynamic changes in intermediate levels. Single-gene modifications may cause excessive fluctuations in intermediate concentrations, leading to metabolic imbalances—for example, excessive squalene accumulation can block upstream reactions [22]. Moreover, most studies adopt a linear approach, where engineered genetic sites are introduced into the target strain one by one. In each step, the strain with the highest 7-DHC yield is selected for subsequent experiments. However, such stepwise strategy may not be the optimal solution, as each modification can globally impact cellular metabolism, rendering previously identified high-yield strategies ineffective in the new strain background. For example, in the study by Bi et al. [17], regulating the TFs OAF1/PIP2 in the strain with three modules resulted in lower 7-DHC production than the strain with four modules, despite the former exhibiting higher 7-DHC yields before the TFs regulations. Integrating metagenomics and computational modeling to achieve holistic optimization of cellular metabolic pathways may represent a promising direction for future research. Future research should integrate multi-omics and synthetic biology to optimize vitamin D3 biosynthesis, addressing global nutritional deficiencies sustainably.

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