Development of a High-Titer Culture Medium for the Production of Cholesterol by Engineered *Saccharomyces cerevisiae* and Its Fed-Batch Cultivation Strategy

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Steroids are a class of compounds with cyclopentane polyhydrophenanthrene as the parent nucleus, and they usually have unique biological and pharmacological activities. Most of the biosynthesis of steroids is completed by a series of enzymatic reactions starting from cholesterol. Synthetic biology can be used to synthesize cholesterol in engineered microorganisms, but the production of cholesterol is too low to further produce other high-value steroids from cholesterol as the raw material and precursor. In this work, combinational strategies were established to increase the production of cholesterol in engineered *Saccharomyces cerevisiae* RH6829. The basic medium for high cholesterol production was selected by screening 8 kinds of culture media. Single-factor optimization of the carbon and nitrogen sources of the culture medium, and the addition of calcium ions, zinc ions and citric acid, further increased the cholesterol production to 192.53 mg/l. In the 5-L bioreactor, through the establishment of strategies for glucose and citric acid feeding and dissolved oxygen regulation, the cholesterol production was further increased to 339.87 mg/l, which was 734% higher than that in the original medium. This is the highest titer of cholesterol produced by microorganisms currently reported. The fermentation program has also been conducted in a 50-L bioreactor to prove its stability and feasibility.

**Keywords:** Cholesterol, *S. cerevisiae*, fermentation optimization, fed-batch culture, citric acid

**Instruction**

Steroids are compounds with cyclopentane phenanthrene as the parent nucleus. Their unique biological structure usually brings rich biological and pharmacological activities, especially of the antibacterial and antiviral type [1], [2]. Steroids are widely used in making immunity inhibitors, hormone drugs, diuretics, cardio tonics, etc. Hydrocortisone, progesterone, diosgenin and dexamethasone are all commonly used clinically steroid drugs [3]. Cholesterol was the first type of steroid discovered. As an essential element of the cell membrane, cholesterol plays an important role in maintaining membrane fluidity, cell growth and proliferation [4]. At the same time, cholesterol is also an important raw material and intermediate for the synthesis of most steroids like bile acids, vitamin D, and some steroid hormones [5]. Biotransformation is more advantageous to the synthesis of some complex or structure-specific compounds, and compared with the traditional preparation methods, the conditions of biotransformation are milder and more environmentally friendly, and most organisms are able to carry out the transformation of steroids [6]. Therefore, in recent years, cholesterol studies looking to obtain active steroids through microbial transformation have been extensive, especially in the side chain cleavage, hydroxylation, dehydrogenation, reduction, isomerization, and esterification reactions of cholesterol [5, 7, 8]. Studies have shown that cytochrome P450 enzymes play a key role in cholesterol synthesis and metabolism [9]. These studies also demonstrated the feasibility of using industrial microorganisms to synthesize valuable steroids. Due to the difficulty of screening strains and enzymes, researchers tend to construct industrial microorganisms to biosynthesize steroids from basic nutrient materials. Therefore, it is necessary to construct an engineered strain to produce cholesterol first.

*S. cerevisiae* is one of the most widely used engineering microorganisms. Compared with prokaryotes, it has a complex downstream structural modification system. Moreover, *S. cerevisiae* itself has a sterol metabolism pathway, and the end product of metabolism is ergosterol [10], which is the fundamental reason why *S. cerevisiae* is employed as a steroid-producing strain for research [11]. Szczepa* et al* introduced 13 genes related to the expression of 8 mammalian proteins into *S. cerevisiae*, optimized two mitochondrial systems and removed harmful side reactions, and successfully achieved the total synthesis of hydrocortisone from glucose in...
recombinant *S. cerevisiae* [12]. Xia *et al.* achieved the biosynthesis of lycopene in *S. cerevisiae*, and through citric acid fed-batch fermentation, the titer of lycopene was increased to 115.64 mg/l [13]. Through inactivating the C-22 sterol dehydrogenase (ERG5) and C24-methyltransferase genes (ERG6) in *S. cerevisiae*, and introducing C-8 sterol isomerase (ERG2), C-5 sterol desaturase (ERG3) and C24-reductase gene (DHCR24) from mice and vertebrates, they successfully synthesized 7-dehydrocholesterol in *S. cerevisiae* [14, 15]. Subsequently, Guo Xiaojing *et al.* further increased the production of 7-dehydrocholesterol to 1.07 g/l in the 5-L bioreactor by overexpressing a series of genes in the mevalonate pathway [16]. By introducing sterol C-7 reductase and sterol C-24 reductase from different sources, Cleiton *et al.* constructed a *S. cerevisiae* strain RH6829 that stably produces cholesterol instead of ergosterol, and obtained a cholesterol titer of 1 mg/g·FW [17]. The biosynthetic route of cholesterol is shown in Fig. 1.

Although it is now feasible to use engineered microorganisms to synthesize cholesterol and other steroids converted from it, the production of cholesterol is too low to further produce other high-value steroids from cholesterol as the raw material and precursor. Moreover, there are currently very few reports on the improvement of cholesterol production in engineered microorganisms. Therefore, this study proposed a combinational fermentation strategy of *S. cerevisiae* RH6829 to increase the production of cholesterol as an intermediate in the synthesis of steroids.

### Materials and Methods

#### Strain and Media

The strain used in this work is *S. cerevisiae* RH6829, provided by Zhang Yan-Sheng’s team from Shanghai University, and stored in 40% (v/v) glycerol at -80°C. The components of each medium used in this work are shown in Table 1.

#### Culture Condition

Fifty microliters of the preserved *S. cerevisiae* RH6829 was added to a serum bottle containing 5 ml of seed culture medium to prepare the inoculum. The first-stage seed was cultured at 30°C and 175 rpm for 48 h on a shaker. To obtain the second-stage seed, 2 ml of the first-stage seed was added into 50 ml of seed culture medium in a 250-ml shake flask and cultured for 24 h under the same condition. Finally, 5 ml of the second-stage seed was added into 50 ml of various media in 250-ml shake flasks and then fermentation was conducted at 30°C and 175 rpm for 120 h on a shaker. Although the cell growth rate differs in various media, considering the time cost, the

### Table 1. Composition of each medium used in this study.

| Medium   | Composition (g/l)                                                                                                                                 |
|----------|-------------------------------------------------------------------------------------------------------------------------------------------------|
| Seed medium | Glucose 20, Yeast Extract 10, Peptone 20                                                                                                        |
| M1 (Original medium) | Same as the seed medium                                                                                                                                 |
| M2 | Glucose 50, Yeast Powder 10, (NH₄)₂SO₄ 15, K₂HPO₄ 2                                                                                       |
| M3 | Sucrose 91.8, (NH₄)₂SO₄ 10.3, KH₂PO₄ 8.7, MgSO₄ 1.5                                                                                     |
| M4 | Glucose 40, Sucrose 20, Peptone 10, Yeast Extract 10, (NH₄)₂SO₄ 5, KH₂PO₄ 1, NaCl 1, MgSO₄ 5, CaCl₂ 0.1, NaNO₃ 2                                      |
| M5 | Glucose 80, Beef Extract 20, K₂HPO₄ 3, MgSO₄ 1.5                                                                                         |
| M6 | Glucose 53.4, Peptone 21.5, Yeast Extract 10, K₂HPO₄ 2.98                                                                                  |
| M7 | Sucrose 40, Peptone 30, KH₂PO₄ 1, NaCl 1                                                                                                  |
| M8 | Glucose 60, Corn steep liquor 20, K₂HPO₄ 3, MgSO₄ 1                                                                                            |
| M9 | Glucose 60, Corn steep liquor 20, K₂HPO₄ 3, MgSO₄ 1.5                                                                                     |
| M10 | Glucose 60, Corn steep liquor 20, K₂HPO₄ 3, MgSO₄ 1.5, CaCl₂ 0.83, ZnSO₄ 0.4                                                                                        |
| M11 | Glucose 60, Corn steep liquor 20, K₂HPO₄ 3, MgSO₄ 1.5, CaCl₂ 0.83, ZnSO₄ 0.4, Citric acid 4                                                                |

Fig. 1. Biosynthesis of cholesterol from glucose in *S. cerevisiae* RH6829.
cholesterol concentration at 120 h of fermentation was uniformly selected as the reference index. Three biological replicates were conducted for each batch of fermentation.

**Calcium Ion and Zinc Ion Feeding**

The concentration of calcium ions in cells will affect the growth of bacteria and various metabolic activities, and zinc ions can effectively improve the ethanol tolerance of *S. cerevisiae* to reduce the toxic effects of ethanol produced during the fermentation process [18]. Therefore, different final concentrations of CaCl₂ and ZnSO₄ were added at different times in medium M9 during the shaking flask fermentation process to determine the optimal concentration and addition time of calcium ion and zinc ion.

**Citric Acid Feeding and Measurement of Gene Transcriptions by Real-Time Quantitative PCR (qRT-PCR)**

Studies have shown that the addition of citric acid can promote the production of lycopene in engineered *S. cerevisiae* [13], and there are many similar pathways in the synthesis of lycopene and cholesterol, such as the mevalonate pathway. Therefore, different final concentrations of citric acid were added at 0 h and 12 h of fermentation in medium M10 to investigate its effect on cholesterol synthesis.

HMGI, ERG20, DHCR7, DHCR24 were the key enzyme genes in the cholesterol synthesis pathway; CIT1 was the citrate synthase gene, and ACT1 is the gene encoding the actin of *S. cerevisiae*, and is also a commonly used reference gene in PCR experiments to quantitatively characterize the transcription level of other genes. These genes and their primer information are shown in Table 2. The total RNA of *S. cerevisiae* RH6829 was extracted using TriZol solution treated as an extracting solution. RNA concentration was determined using a spectrophotometer at A₂₆₀/₂₈₀. Reverse transcription was achieved using total RNA as the starting material and the Hifair III 1st Strand cDNA Synthesis SuperMix (Yeasen Biotechnology; Shanghai, China). The method of real-time quantitative PCR was the modified method of Liu et al. [19]. *S. cerevisiae* RH6829 with fermentation for 12 h (exponential phase) and 36 h (stationary phase) were selected to investigate the transcription levels of these genes.

**Batch and Fed-Batch Fermentation in 5-L Bioreactor**

The optimized strategy of fed-batch fermentation to increase cholesterol production by feeding additional glucose and citric acid was conducted in a 5-L reactor from medium M11, with an inoculum volume of 10%, aeration rate of 3 l/min, and agitation speed of 300 rpm. During the fermentation process, 100 ml of 500 g/l glucose and 8 g/l citric acid were added every 12 h. At the same time, using the reactor’s stirring speed-dissolved oxygen joint control function, the following dissolved oxygen control strategy was established: when the dissolved oxygen in the fermentation broth was less than 30%, the speed was gradually increased at a step value of 20 rpm to increase the dissolved oxygen level; when the dissolved oxygen reached 80%, the speed was gradually reduced at the same step value. The lower and upper limits of the speed were set to 200 and 500 × g. Through the joint control of speed-dissolved oxygen, the bacteria were always in a stable environment with sufficient dissolved oxygen. During the first 24 h of the fermentation process, samples were taken every 6 h to detect cholesterol production and biomass, and then every 12 h thereafter. In addition, when citric acid was added, to avoid the influence of pH reduction of the medium caused by its acidity, 6 g/l of CaCO₃ was added to maintain a stable pH.

**Fed-Batch Fermentation in 50-L Bioreactor**

Scale-up fermentation with medium M11 in a 50-L reactor was conducted to verify the stability and feasibility of the fermentation scheme. The inoculation amount was 10%, the aeration rate was 30 l/min, and the agitation speed was 150 rpm. During the fermentation process, 1 L of 500 g/l glucose was added every 12 h. Samples were taken every 12 h to determine the cholesterol production.

**Analytical Methods**

**Measurement of Biomass and Residual Glucose**

The biomass of strains was characterized by the fresh weight (FW). First, 25 ml of fermentation broth was collected in a centrifuge tube and centrifuged at 3000 × g for 10 min to obtain the cells. The collected cells were then washed with distilled water and centrifuged three times. The biomass was calculated by the weight difference

**Table 2. Genes and primer sequences for qRT-PCR.**

| Target gene | Primer name | Primer sequence (5’-3’) |
|-------------|-------------|------------------------|
| CIT1        | CIT1 - forward | TGGCCCATATACAGGTGTCGA   |
|             | CIT1 - reverse | AAACACCTCTCCCTGGGTCA   |
| HMG1        | HMG1 - forward | ACTACTGAGAGGCGTTGCC   |
|             | HMG1 - reverse | ACACAGGCGGCAATACGCC   |
| ERG20       | ERG20 - forward | AAGGACTGAGGGCGAAGC   |
|             | ERG20 - reverse | CACAGGAGTGTACGACCTG   |
| DHCR7       | DHCR7 - forward | TCATAACCGGCTACATGCC   |
|             | DHCR7 - reverse | GCCGGGAAAACCTTCACAAC   |
| DHCR24      | DHCR24 - forward | CCAACAGGGCACAGGTGTC   |
|             | DHCR24 - reverse | ACTGTTGTGGCTTCTTCCG   |
| ACT1        | ACT1 - forward | GAAATGCAAACCGCTGCA   |
|             | ACT1 - reverse | TACCCTCCAGATTCACAC   |
between the centrifuge tube with cells and centrifuge tube. The residual glucose in the broth was measured with an SBA-40E glucose bioanalyzer (Institute of Biology, Shandong Academy of Sciences).

**Cholesterol Extraction and Qualitative Analysis**

The cells were resuspended in a mixture of 30 ml ethanol and 10 ml 60% (w/w) KOH, heated at 85°C for 2 h, cooled and extracted twice with 20 ml petroleum ether. The petroleum ether phase was pooled and evaporated to dryness in a rotary evaporator. The product was dissolved and collected in 10 ml methanol.

The cholesterol concentration was determined by HPLC (Agilent Technologies 1260 Infinity, USA) on an ODS column though external standard method (Eclipse Plus C18, 4.6 mm × 250 mm, 5 μm, 1 ml/min methanol as mobile phase) and the ultraviolet detection was at 205 nm. The standard curve: \( Y = 9.3689X + 58.362 \) \( (R^2 = 0.9994, \text{linearity range: } 31.25-1,000 \text{ mg/l}) \). The formulation of the standard curve was based on the corresponding relationship between the peak area of the cholesterol standard and its concentration. The cholesterol concentration of the sample was calculated according to the standard curve based on its peak area.

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**Fig. 2.** Screening of basic medium for cholesterol production and optimization of carbon and nitrogen sources in medium M6. (A): Cholesterol production and yeast biomass in medium M1-M8; (B): Cholesterol production and biomass in medium M6 under different carbon sources, and the concentration of each carbon source was 80 g/l; (C): Cholesterol production and biomass in medium M6 under different glucose concentrations; (D): Cholesterol production and biomass in medium M6 under different nitrogen sources, and the concentration of each nitrogen source was 20 g/l; (E): Cholesterol production and biomass in medium M6 under different corn steep liquor concentrations. All experiments were repeated three times independently.
Results and Discussion

The media (M1-M8) used in this study are commonly used in yeast culture and production of secondary metabolites in our laboratory. The single-factor investigation of the added concentration and time of the nutrient elements in the selected basal medium was done to obtain the optimal conditions.

Medium Optimization for High Production of Cholesterol

Eight different media were selected to screen out the basic media that can provide high production of cholesterol. As shown in the Fig. 2A, medium M6 has the highest cholesterol titer, so it was selected for subsequent single-factor optimization of carbon and nitrogen sources and further optimization. Figs. 2B and 2C showed that the most suitable carbon source for cholesterol production in the culture medium is 60 g/l glucose, while Figs. 2D and 2E show that the most suitable nitrogen source is 20 g/l corn steep liquor. Through this step, we obtained the new medium M9 (glucose 60 g/l, corn steep liquor 20 g/l, K$_2$HPO$_4$ 3 g/l, MgSO$_4$ 1.5 g/l) for subsequent research.

Different concentrations of CaCl$_2$ from 0 to 10 mM and different concentrations of ZnSO$_4$ from 25 to 400 mg/l were added to the medium M9 to investigate their optimal concentration and time. Figs. 3A and 3B showed that the most suitable calcium ions addition strategy for cholesterol production is adding 7.5 mM CaCl$_2$ at the beginning of fermentation. Figs. 3C and 3D showed that the most suitable zinc ion addition strategy for cholesterol production is adding 0.4 g/l ZnSO$_4$ at the beginning of fermentation. Through this step, we obtained the new medium M10 (glucose 60 g/l, corn steep liquor 20 g/l, K$_2$HPO$_4$ 3 g/l, MgSO$_4$ 1.5 g/l, CaCl$_2$ 0.83 g/l, ZnSO$_4$ 0.4 g/l) for subsequent research.

Effect of Adding Citric Acid on Cholesterol Production

Different concentrations of citric acid were added at the beginning and at 12 h of fermentation in medium M10. Figs. 4A and 4B showed that adding a certain concentration of citric acid at the beginning of fermentation or at 12 h of fermentation promote the synthesis of cholesterol. Adding 4 g/l of citric acid at the beginning of fermentation has the most obvious promotion effect on cholesterol synthesis, and the cholesterol production was 192.53 mg/l, which was 21.23% higher than that without adding citric acid. Through this step, we obtained the new medium M11 (glucose 60 g/l, corn steep liquor 20 g/l, K$_2$HPO$_4$ 3 g/l, MgSO$_4$ 1.5 g/l, CaCl$_2$ 0.83 g/l, ZnSO$_4$ 0.4 g/l, citric acid 4 g/l) for subsequent research.

Fig. 3. Fermentation under the addition of calcium ions and zinc ions. (A): Cholesterol production and biomass in medium M9 with different concentrations of CaCl$_2$ added at the beginning of fermentation; (B): Cholesterol production and biomass in medium M9 with the addition of 5 mM CaCl$_2$ at different times; (C): Cholesterol production and biomass in medium M9 with different concentrations of ZnSO$_4$ added at the beginning of fermentation; (D): Cholesterol production and biomass in medium M9 with the addition of 400 mg/l ZnSO$_4$ at different times. All experiments were repeated three times independently.
In Figs. 4C and 4D, whether in the exponential phase or the stationary phase, the addition of citric acid reduces the transcription level of the citrate synthase gene (CIT1) of the bacteria itself. On the contrary, the transcription level of these genes (HMG1, ERG20, DHCR7, DHCR24) in the cholesterol synthesis pathway has increased, especially in the stationary phase, so this effect is more significant. Among them, the expression of ERG20 increased by 16.69 times after 36 h of fermentation.

As shown in Fig. 1, citric acid, one of the components of the tricarboxylic acid cycle, is synthesized from acetyl-CoA and oxaloacetate. Based on the results of the gene transcription level determination experiment in Fig. 4, the principle of the increase in cholesterol production caused by the addition of citric acid can be preliminarily analyzed: the addition of citric acid reduces the gene transcription level of its own citrate synthase and hinders the synthesis of citric acid, therefore, the raw material acetyl-CoA can accumulate and enter into the mevalonate pathway to synthesize more cholesterol.

**Batch and Fed-Batch Fermentation in 5-L & 50-L Bioreactors**

Figs. 5A and 5B showed that in batch fermentation, along with the rapid consumption of dissolved oxygen, the residual glucose in the broth decreased to 0 after 12 h of fermentation. Therefore, the follow-up feeding experiment was started after 12 h of fermentation. At the end of batch fermentation, the cholesterol titer obtained was 240.87 mg/l. Fig. 5C showed that after adding glucose every 12 h, the cholesterol production and biomass during the fermentation process were significantly increased. Meanwhile, glucose metabolism brought greater pH changes in the broth. By the end of fed-batch fermentation, the cholesterol titer obtained was 302.72 mg/l, which was 25.68% higher than that of batch fermentation. Fig. 5D showed that when both glucose and citric acid were added every 12 h, the cholesterol production in the fermentation process is further improved. The presence of CaCO3 made the pH of the broth more stable. At the end of fed-batch fermentation, the cholesterol titer obtained was 339.87 mg/l, which was 41.10% higher than that of batch fermentation. Fig. 5E showed that in the glucose fed-
batch fermentation in the 50-L bioreactor, the cholesterol production reached 330.11 mg/l, which was 9.05% higher than that in the 5-L reactor under the same conditions. The successful scale-up of the fermentation proved that the fermentation scheme is stable and reliable.

In this paper, we developed a high-titer culture medium for the production of cholesterol by *S. cerevisiae* RH6829 through basic medium selection, optimization of carbon and nitrogen sources, and investigation of additives. The fed-batch of glucose and citric acid and the regulation of dissolved oxygen were established on a 5-L reactor, which further increased the maximum cholesterol production to 339.87 mg/l. Moreover, the scale-up of glucose fed-batch fermentation was successfully completed in a 50-L bioreactor. This work provides theoretical support for the production of more high-value steroids using cholesterol as a raw material and precursor.

**Conflicts of Interest**

The authors have no financial conflicts of interest to declare.
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