A Green Strategy to Produce Potential Substitute Resource for Bear Bile Using Engineered *Saccharomyces Cerevisiae*

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Abstract

Background

Bear bile powder is a precious natural material characterized by high content of tauroursodeoxycholic acid (TUDCA) at a ratio of 1.00–1.50 to taurochenodeoxycholic acid (TCDCA).

Results

In this study, we use the crude enzymes from engineered *Saccharomyces cerevisiae* to directional convert TCDCA from chicken bile powder to TUDCA at the committed ratio *in vitro*. This *S. cerevisiae* strain was modified with heterologous 7 α -hydroxysteroid dehydrogenase (7 α -HSDH) and 7 β -hydroxysteroid dehydrogenase (7 β -HSDH) genes. *S. cerevisiae* host and HSDH gene combinatorial optimization and response surface methodology was applied to get the best engineered strain and the optimal biotransformation condition, respectively, under which 10.99 ± 0.16 g/L of powder products containing 36.73 ± 6.68 % of TUDCA and 28.22 ± 6.05 % of TCDCA were obtained using 12.00 g/L of chicken bile powder as substrate.

Conclusion

This study provides a healthy and environmentally friendly way to produce potential alternative resource for bear bile powder from cheap and readily available chicken bile powder, and also gives a reference for the green manufacturing of other rare and endangered animal-derived valuable resource.

Introduction

Bear bile powder is precious material that has been used as medicine and healthcare supplementary thousands of years ago in Asia area (Yang et al. 2008; Feng et al. 2009). It has multiple pharmacological activities and can be used to treat gallstones, cholecystitis, fatty liver and other hepatobiliary diseases (Feng et al. 2009). At present, bear bile powder is mainly from the drainage bear bile extracted from the farmed living bears including *Selenarctos thibetanus* (Asiatic black bear) or *Ursus arctos* (Brown bear) with “Free-dripping Fistula Technique” by implanting a duct or making an artificial fistula in the liver of the bears (Feng et al. 2009). These methods will seriously endanger the health of bears, thus exploring substitute resources is of great significance to meet the demands of bear bile powder as medicinal and healthcare material.

Bile acids (BAs), a group of steroids with C-17 side chains, are the principal bioactive ingredients of bear bile powder, among which ursodesoxycholic acid (UDCA) or its physiologically active form tauroursodeoxycholic acid (TUDCA) takes a high proportion and is considered as distinct and character of bear bile from other animal bile (Ferrandi et al. 2012; Eggert et al. 2014; Zheng et al. 2017). The authentication and standard quality work from 20 batches of drainage bear bile powder samples in our lab revealed that the average content of TUDCA was 26.50%, the ratio of TUDCA to TCDCA

(TUDCA/TCDCA) was from 1.00:1.00 to 1.50: 1.00 (Wang et al. 2018), which could be regarded as the characteristics of bear bile powder.

Till now, the synthesized TUDCA and UDCA are the only acceptable substitutes for bear bile because it has similar bioactivities as bear bile, including neuroprotective action, promoting pancreatic survival and function, and reducing gallstone formation (Lu et al. 2021; Rosa et al. 2021; Zangerolamo et al. 2021). There are several methods to produce UDCA or TUDCA such as chemical synthesis, whole-cell biocatalysts, and the chemo-enzymatic method (Momose et al. 1997; Liu et al. 2013; Zheng et al. 2015). All these methods required pure and rare compounds as precursors or substrates, which increased the production costs and limited the industrial application. In addition, bile acids other than TUDCA or UDCA also have biological activities. For example, TCDCA had anti-inflammatory effect, could stimulate intestinal cell proliferation, protect against apoptotic cell death, and alleviate pulmonary fibrosis (Toledo et al. 2004; Zhou et al. 2013; Li et al. 2019). Therefore, it would be more feasible to explore alternatives based on both the therapeutic effects and the specific chemical properties of natural bear bile powder.

Different from bear bile, poultry bile such as chicken bile is a cheap and easily available resource. It contains high amount of TCDCA but no TUDCA. In chemical structure, TUDCA is the epimer of TCDCA at C-7 hydroxyl. Bacterial 7 α -hydroxysteroid dehydrogenase (7 α -HSDH) and 7 β -hydroxysteroid dehydrogenase (7 β -HSDH) have both oxidative and reductive activities and can interconvert TCDCA and TUDCA coupling with NADPH or NADH as co-factor (Song et al. 2017) (Fig. 1). We had constructed an engineered *Escherichia coli* with 7 α -HSDH and 7 β -HSDH genes which could convert a certain proportion of TCDCA to TUDCA using chicken bile powder as substrates.

As a safe and health microbial organism with clear genetic background, *Saccharomyces cerevisiae* has been widely used in food and pharmaceutical industry (Lian et al. 2018). Here, taking the advantage of *S. cerevisiae* and the bi-directional catalytic properties of HSDH, we created an efficient way to produce the potential substitute for bear bile powder using chicken bile powder as raw material through the engineered *S. cerevisiae*. To our knowledge, this is the first report of applying engineered *S. cerevisiae* to make products having certain amount of TUDCA and TCDCA equivalent to that in bear bile powder.

Materials And Methods

Chemicals and reagents

Chicken bile powder (containing 64.78 $\pm$ 0.30 % of TCDCA) were kindly provided by Shanghai Kaibao Pharmaceutica Co. Ltd., China. Resin D101 was purchased from Wuhan Weiqiboxin Biotechnology Co. Ltd., China. All the other chemicals in this study were from Sinopharm Chemical Reagent Co. Ltd., China.

Gene selection, codon optimization and synthesis

Two 7 α -HSDH genes and two 7 β -HSDH genes originating from *Clostridium sardiniense*, *E. coli*, and *Ruminococcus gnavus* (GenBank accession numbers JN191345, D10497, and KF052988) were selected to construct four combinations as our previous work (Shi et al. 2017). Briefly, the coding region of these genes was codon optimized and synthesized by Life Technologies (Shanghai, China), named as Ca7 α -HSDH^{syn}, Ec7 α -HSDH^{syn}, Ca7 β -HSDH^{syn}, and Rg7 β -HSDH^{syn} (GenBank accession numbers KY178305-KY178308) referred to as α 1, α 2, β 1, and β 2, respectively.

Microbial strains, plasmids, and expression vector construction

Two *S. cerevisiae* strains W303-1a (MATa; ade2-1; ura3-1; his3-11; trp1-1; leu2-3; leu2-112; can1-100) and CEN.PK2-1C (MATa; his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2) were used as the hosts to expression the 7 α -HSDH^{syn} and 7 β -HSDH genes. *E. coli* DH5 α was used as hosts for middle expression vectors. Yeast shuttle plasmids pRS424 and pRS426 were separately used to carry 7 α -HSDH^{syn} and 7 β -HSDH^{syn} expression cassettes assembled from P_{PGK1}, 7 α -HSDH^{syn} and T_{PGI}, or P_{TDH3}, 7 β -HSDH^{syn} and T_{PDC1}, respectively. A total of four yeast expression vectors were generated, namely pRS424-P_{PGK1}- α 1-T_{PGI}, pRS424-P_{PGK1}- α 2-T_{PGI}, pRS425-P_{TDH3}- β 1-T_{PDC1}, and pRS425-P_{TDH3}- β 2-T_{PDC1}. One 7 α -HSDH^{syn} combined with one 7 β -HSDH^{syn} expression vectors were co-introduced into *S. cerevisiae* strains W303-1a and CEN.PK2-1C, yielding eight engineered yeast strains, named W303a- α 1 β 1, W303a- α 1 β 2, W303a- α 2 β 1, W303a- α 2 β 2, CEN- α 1 β 1, CEN- α 1 β 2, CEN- α 2 β 1, and CEN- α 2 β 2.

Cultivation of the engineered *S. cerevisiae* strains

The engineered yeast cells were cultured in flasks containing SD-Trp⁻Ura⁻ liquid media at 30°C, 220 rpm on a horizontal shaker. For engineered strain screening, a single colony of each strain was inoculated in 25 ml of liquid media and cultured for 24 h. Then the yeast cell cultures were sub-cultured in 400 mL of liquid media at a proportion of 1:20 and grew for another 24 h. For biotransformation condition optimization, another scale-up sub-culture process was carried out in 8 L of liquid media using the first sub-cultures as seed cells at the same ratio for the third 24 h. The yeast cell cultures were collected to get the cells for crude enzyme preparation.

Crude enzyme preparation

The engineered *S. cerevisiae* cells were collected by centrifugation. The cells were washed with sterile water and phosphate buffer saline (PBS, 100mM) twice, and resuspended in appropriate volume of PBS. Cells were lysed either by 10 cycles of vortex-ice bath (vortex 30 sec, ice bath keeping for 30 sec) after adding certain amount glass beads (diameter: 424-600 μ m), or by homogenization under 600-1000 bar. The lysed cell mixture was centrifuged at 4 °C, 12000 rpm for 5-10 min, and the supernatant that was the crude enzyme solution was transferred to new tubes. The crude enzyme powder was obtained by drying in a freeze dryer. For engineered yeast strain screening, 10 mL of cell cultures were finally resuspended in 150 μ L of PBS. For biotransformation condition optimization, 8 L of cell cultures were finally resuspended

in 200 ml of PBS, and the concentration of total proteins was measured with Bradford method and calculated as 1.08 ± 0.02 mg/ml.

***In vitro* biotransformation of TUDCA**

The biotransformation of TUDCA was conducted in a reaction mixture comprising of 100 mM of PBS, a certain amount of crude enzyme solution and chicken bile powder. For strain screening and biotransformation condition optimization, the reaction was carried out in 1 mL reaction mixture (pH 6.5) with 0.10 g/L of NADP Na₂, 150 μ L of crude enzyme solution, and 4.80 g/L of chicken bile powder at 30 °C for 6 h, refer to our previous work (Shi et al. 2017; Xu et al. 2019). For product preparation, the reaction mixture (pH 7.0) was 1 L containing 170 mL of crude enzyme solution and 12.00 g/L of chicken bile powder and biotransformation condition was 25 °C for 5.23 h. After incubation, the reaction was stopped by keeping in boiling water for 5 min, followed by centrifugation. The supernatant was either analyzed by HPLC or made to powder product. Concentration of TUDCA and the conversion efficiency was used to evaluate the production capacity of engineered *S. cerevisiae* strains. In this study, the conversion efficiency was indicated as the TUDCA yield calculating according to the equation (1) as followed:

$$\text{Conversion efficiency} = \frac{\text{Total amount of TUDCA in products (g)}}{\text{Total amount of TCDCA in substrates (g)}} \times 100\% \quad (1)$$

***In vitro* biotransformation condition optimization**

Based on the catalytic properties of the original 7 α -HSDH and 7 β -HSDH, and our findings on *E. coli* (Shi et al. 2017; Xu et al. 2019), substrate concentration, pH value, temperature, and the fermentation time also have influence on the substrate conversion efficiency. Meanwhile, 7 α -HSDH and 7 β -HSDH are NAD(P)⁺-dependent enzymes. The supply of NADP Na₂ in the reaction mixture would also have effect on the enzyme catalytic activity thereby affecting the conversion efficiency. Taken all these factors into account, *in vitro* biotransformation conditions were optimized through employing single factor exploration and response surface methodology (RSM) by setting a certain range of each factors.

The effects of substrate concentration, ranging from 2.40 g/L to 40.00 g/L, and pH value, covering 6.0 to 7.5, on the conversion were separately investigated. The influence of temperature, incubation time, and the concentration of NADP Na₂ was evaluated using Behnken Design (BBD) of RSM. These three independent factors respectively designed as factor A, B, C were investigated at three different levels. Factor A was divided into 20 °C, 30 °C, and 40 °C, factor B was divided into 1 h, 5 h, and 9 h, and factor C was divided into 0.05 g/L, 0.10 g/L, and 0.15 g/L with five repetitions at the central point using the ratio of TUDCA/TCDCA as response. The data are analyzed by using Design-Expert.V8.0.6.1 software. For RSM experiment, 12.00 g/L of substrates and pH 7.0 was used according to results of single factor exploration.

Preparation of powder products

Isometric resin D101 was used to purify the conversion products. To do that, the supernatant of the reaction mixture was passed through pre-treated isometric resin D101. The impurities on D101 resin were firstly removed by deionized water until the eluent was colorless. Then the resin was washed with 95% ethanol to get the eluent until colorless. The eluent was mixed and concentrated through rotary evaporation at 50 °C and then filtered through 0.45 µm organic filters. The filtrate was evaporated by keeping in water bath at 50 °C to form extractum and then dried to constant weight in vacuum drying oven at 50 °C. After that, the dried solid was taken out and crushed into powder that is the products.

Bile acid analysis

For conversion efficiency assay, the supernatant was directly filtered through a 0.22 µm filter and supplied to the ThermoUltiMate3000 HPLC machine. For prepared product assay, methanol was added to resolve the dried bile acids and filtered through a 0.22 µm filter before supplied to HPLC machine. The concentration of individual bile acid was determined according to the curve of each authentic standard.

HPLC condition

HPLC was performed according to our previous work (Xu et al. 2019). Briefly, a ThermoUltiMate3000 HPLC machine equipped with Agilent Poroshell 120 EC-C18 column (2.7 µm, 4.6 mm × 150 mm) and Corona Ultra Detector (CAD) was employed using acetonitrile and water (containing 0.3% formic acid and 5 mM ammonium acetate) as the mobile phase at a flow rate of 0.6 mL/min. HPLC gradient elution program was total 45 min with acetonitrile proportion from 20 to 90%.

Results

High productive engineered *S. cerevisiae* strain screening

To screen a high productive engineered yeast strain, crude enzymes from the eight engineered *S. cerevisiae* strains were incubated with 4.80 g/L of chicken bile powder in PBS at 30 °C. After incubation for 6 h, only two strains, W303a-α1β2 and CEN-α2β2, produced TUDCA (Fig. 2a). The conversion efficiency of the two strains was 51.0% and 56.9% (Fig. 2a) and the ratio of TUDCA to TCDCA was 1.37 and 1.12, respectively. Meanwhile, there was a little of tauro-7-ketone lithocholic acid (T-7K-LCA) intermediate built up in all those constructs. The conversion efficiency of crude enzymes from these two strains was further analyzed to select a more practicable one by swapping the biotransformation condition to 25 °C for 3 h, 6 h and 9 h. As shown in Fig. 2b, the conversion efficiency of CEN-α2β2 was 50.62±5.45%, 48.44±4.79%, and 54.18 ± 3.44% and the ratio of TUDCA to TCDCA was 1.05± 0.23, 0.97 ± 0.18, and 1.22 ± 0.17, respectively; whereas that of W303a-α1β2 was below 40% and 0.2, respectively. These results indicated that α2β2 has higher enzymatic catalytic capability than α1β2, possible due to the enzymatic activity of α2 was more flexible than that of α1 under the given conditions. It also indicated that incubation for 9 h was better. Therefore, CEN-α2β2 was selected in next experiments.

Effect of substrate concentration on the biotransformation of TUDCA

To screen an optimal substrate concentration, the reaction mixture was incubated at 30 °C, pH 6.5 for 9 h. As shown in Fig. 3a, the conversion efficiency ranged from $66.59 \pm 0.93\%$, $60.22 \pm 0.18\%$, $57.31 \pm 0.71\%$, $44.32 \pm 1.63\%$, $27.76 \pm 1.64\%$ to $17.01 \pm 0.71\%$ and the ratio of TUDCA/TCDCA was 2.31 ± 0.03 , 1.63 ± 0.01 , 1.39 ± 0.04 , 0.81 ± 0.06 , 0.39 ± 0.03 , and 0.21 ± 0.01 , when feeding with 2.4 g/L, 4.8 g/L, 8.0 g/L, 16.0 g/L, 24.0 g/L, and 40.0 g/L of substrate, respectively. This result suggested that the conversion efficiency decreased with the increase of substrate concentration under the given conditions, and 8.0-16.0 g/L of chicken bile powder was good for *in vitro* biotransformation.

Effect of pH value on the biotransformation of TUDCA

The effect of pH value on TUDCA formation was assessed by setting the incubation at 25 °C or 30 °C for 9 h and feeding 16.00 g/L of chicken bile powder. As shown in Fig. 3b, with the increase of pH value, the yield of TUDCA increased at first and then decreased under both temperatures. When pH value was 7.0, the conversion efficiency was the highest, $43.05 \pm 4.40\%$ and $50.77 \pm 7.34\%$, and the ratio of TUDCA to TCDCA was 0.77 ± 0.14 and 1.08 ± 0.32 at 25 °C and 30 °C, respectively. These results indicated that 30 °C combined with pH 7.0 was the best condition for the biotransformation, followed by 30 °C with pH 7.5, and 25 °C with pH 7.0, when incubation for 9 h.

Evaluation of experimental design of Box–Behnken Design of RSM

The results of RSM of 17 runs from Box–Behnken Design (BBD) experiments to study the effects of the three independent factors, temperature (factor A), incubation time (factor B), and the concentration of NADP Na₂ (factor C) on the ratio of TUDCA/TCDCA was loaded into Design-Expert.V8.0.6.1 software for regression analysis (Table 1). The quadratic polynomial regression equation describing the response value and independent variables is obtained as below:

$$Y \left(\frac{\text{TUDCA}}{\text{TCDCA}} \right) = + 1.01 - 0.21 * A + 0.28 * B + 0.17 * C - 0.20 * A * B - 7.500E - 003 * A * C + 0.068 * B * C - 0.22 * A * A - 0.22 * B * B - 0.055 * C * C \quad (2)$$

Analysis of variance showed that the *F* value of the model was 20.66, $p < 0.001$, indicating that the model reached a very significant level. There was no significant difference in the Lack of fit of the model ($p > 0.05$). It means that the non-experimental factors had little influence on the experimental results. And the experimental error was small, which could accurately explain the influence of experimental factors on the response value. There was a big difference between $R^2=0.9637$ and $R_{pre}^2=0.7270$ in the regression model. It showed that the error range of TUDCA/TCDCA predicted by the model was large. Adeq-Precision was 12.821 greater than 4, which indicated that the model could be used for prediction. The model Adjusted R-Square is 0.9197, which indicated that the model covers the reason of 91.97% response value change.

Table 1 Experimental designs and the results of the Box–Behnken Design.

No.	A-Temperature[°C]	B-Incubation time[h]	C-NADP Na ₂ [mg]	TUDCA/TCDC
1	30.00	9.00	0.15	1.28
2	20.00	1.00	0.10	0.36
3	30.00	5.00	0.10	0.94
4	30.00	5.00	0.10	1.09
5	30.00	1.00	0.05	0.33
6	30.00	5.00	0.10	0.88
7	40.00	1.00	0.10	0.34
8	40.00	5.00	0.15	0.72
9	30.00	5.00	0.10	1.02
10	30.00	1.00	0.15	0.48
11	20.00	5.00	0.05	0.74
12	30.00	9.00	0.05	0.86
13	40.00	5.00	0.05	0.35
14	40.00	9.00	0.10	0.38
15	30.00	5.00	0.10	1.12
16	20.00	9.00	0.10	1.22
17	20.00	5.00	0.15	1.14

Results of Box–Behnken Design for incubation condition optimization

The *p* values of variables A and B were less than 0.001 (Table 2), which means that factors A and B were extremely significant, indicating that both the incubation time and temperature have significant influence on the biotransformation of TUDCA, according to the principle that the smaller the *p* value, the more dominant the corresponding influencing factor. The *p* value of C is 0.002, far less than 0.01, indicating that NADP Na₂ also plays a highly important role in the conversion. The order of the factors influencing the conversion rate was incubation time > temperature > NADP Na₂.

Table 2 Analysis of variance results of regression simulation

Source	Sum of squares	Degree of freedom	Mean Square	F-value	p-value
Model	1.83	9	0.20	20.66	0.0003
A-Temperature	0.35	1	0.35	35.46	0.0006
B-Incubation time	0.62	1	0.62	63.22	<0.0001
C-NADP Na ₂	0.22	1	0.22	22.83	0.0020
AB	0.17	1	0.17	17.10	0.0044
AC	2.250E-004	1	2.250E-004	0.023	0.8840
BC	0.018	1	0.018	1.85	0.2156
A ²	0.20	1	0.20	20.26	0.0028
B ²	0.20	1	0.20	20.26	0.0028
C ²	0.013	1	0.013	1.30	0.2925
Residual	0.069	7	9.832E-003		
Lack of fit	0.028	3	9.475E-003	0.94	0.5009
Pure error	0.040	4	0.010		
Total	1.90	16			

The significance of the interaction can be reflected by the characteristics of the contour map of the response surface analysis diagram. When the contour map is oval, the interaction is significant; when it is round, it is not significant. Therefore, the importance of the interaction among AB, AC and BC could be intuitively observed. As indicated in Fig. 4, the interaction between temperature and incubation time is the most obvious, which means that the change of temperature significantly affects the incubation time, and vice versa.

Table 3 Response surface prediction of top ten reaction conditions

No.	Temperature	Incubation time	NADP Na ₂	TUDCA/TCDCA	Desirability	
1	25.00	5.23	0.08	1.00	0.58	Selected
2	24.92	5.23	0.08	1.00	0.58	
3	25.08	5.21	0.08	1.00	0.58	
4	24.83	5.22	0.08	1.00	0.58	
5	25.18	5.20	0.08	1.00	0.58	
6	25.28	5.21	0.08	1.00	0.58	
7	25.23	5.25	0.08	1.00	0.58	
8	25.20	5.27	0.08	1.00	0.58	
9	25.44	5.21	0.08	1.00	0.58	
10	25.40	5.25	0.08	1.00	0.58	

According to the results of RSM (Table 3), when three factors (temperature, incubation time, and NADP Na₂ concentration) were considered together by setting the pH at 7.0 and using 12.00 g/L of substrates, the optimal condition was 25 °C for 5.23 h with 0.08 g/L of NADP Na₂. The selected conditions were evaluated by performing three repetitions of biotransformation in a 1 ml reaction mixture, and the average value of TUDCA/TCDCA was 1.12, which was close to the predicted value of response surface optimization design. This result confirmed that the optimization model predicted the experimental results well.

Preparation and chemical analysis of products

Using the selected optimal condition selected from RSM, 10.99 ± 0.16 g/L of powder product was obtained when incubation of the crude enzymes from CEN-α2β2 with 12.00 g/L of chicken bile powder in 1 L of reaction mixture by addition of 0.08 g/L of NADP Na₂ at 25 °C for 5.23 h. This powder product contains 36.73±6.68 % of TUDCA, and 28.22±6.05 % (Table 4). The ratio of TUDCA to TCDCA was 1.30: 1.00.

Table 4 Bile acids in the products

Substrates/ products (%)	TUDCA	TCDCA	T-7-KLCA	TCA	TUCA
chicken bile	0.00	65.52	0.00	10.21	0.00
powder(CBP)					
products	36.73±6.68	28.22±6.05	2.76±1.24	3.15±0.36	6.34±2.18

Discussion

Previously, we had engineered an *E. coli* strain with 7 α -HSDH and 7 β -HSDH genes that could directional convert TCDCA to a certain ratio of TUDCA. The purpose of this project is to use *S. cerevisiae*, one of the most ideal and biosafe microorganism for the production of food and pharmaceutical products, to produce potential substitute of bear bile powder using chicken bile powder as substrate.

Our study using *E. coli* as host cell demonstrated that all the four combinations of 7 α -HSDH and 7 β -HSDH, α 1 β 1, α 1 β 2, α 2 β 1, and α 2 β 2, have the capability to convert TCDCA to TUDCA, and fermentation condition has important effect on the conversion efficiency (Shi et al. 2017; Xu et al. 2019). Applying the given conditions, we found that CEN- α 2 β 2 is the best combination for TUDCA biotransformation (Fig. 2), which indicates that *S. cerevisiae* CEN.PK2-1C is better than w303-1a as host to express active 7 α -HSDH and 7 β -HSDH enzymes. Both CEN.PK2 and W303 are commonly used haploid strains for bioengineering with good sporulation efficiency, and CEN.PK2 has a faster growth rate with doubling times of about 80 min for haploid strains (Rogowska-Wrzesinska et al. 2001; Bruder et al. 2016). This fast growth feature is possibly one of the reasons why α 2 and β 2 expressed in CEN.PK2-1C is more suitable for TUDCA formation.

Both 7 α -HSDH and 7 β -HSDH have oxidative and reductive properties, and their oxidation or reduction activity is affected by environmental conditions (Yoshimoto et al. 1991; Ferrandi et al. 2012; Lee et al. 2013). Balanced reaction conditions are required to achieve directional biotransformation with a specific ratio of TUDCA to TCDCA. Our results suggested that substrate concentration, reaction time, temperature, the pH value and the NADP Na₂ concentration have integrated impact on the biotransformation. For example, when 16.00 g/L of substrates and 0.100 g/L of NADP Na₂ was used, incubation of the reaction mixture at 30°C for 9 h was good (Fig. 3b). When 12.00 g/L of substrates was used, incubation at 25°C for 5.23 h with 0.08 g/L of NADP Na₂ led to the best result (Table 3).

It is worth mentioning that, unlike *E. coli*, *S. cerevisiae* cell does not allow bile acids to pass through its membrane system freely, so the engineered yeast cell cannot be directly used as a whole-cell factory for TUDCA biotransformation. Glucanase, NaCl, and Tween-80 had been supplemented into the media to improve the permeability of cell membrane, but only Tween 80 had a little effect, yielding a small amount of TUDCA. Modification of yeast cell membrane system will be a potential strategy if whole yeast cell factory is used in the future.

In Conclusion, through optimizing the *S. cerevisiae* host and gene combinations, and *in vitro* biotransformation conditions, we have created a green way to make use of cheap and easily available chicken bile powder to produce potential substitute resource for bear bile powder.

Abbreviations

7 α -HSDH: 7 α -hydroxysteroid dehydrogenase; 7 β -HSDH: 7 β -hydroxysteroid dehydrogenase; BAs: Bile acids; BBD: Behnken Design; CAD: Corona Ultra Detector; PBS: phosphate buffer saline; RSM: response surface methodology; TCDCA: taurochenodeoxycholic acid; TUDCA: tauroursodeoxycholic acid.

Declarations

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Authors' contributions

LJ performed the core experiments and wrote the original manuscript. LY supervised the chemical analysis, acquisition the funding, and revised the manuscript. SZ conceptualized the project, acquisition the funding, and revised the manuscript. ZW conceptualized and supervised the project.

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Availability of data and materials

The data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors approved the consent for publishing the manuscript.

Competing interests

The authors declare that they have no competing interests.

References

- Bruder S, Reifenrath M, Thomik T, et al (2016) Parallelised online biomass monitoring in shake flasks enables efficient strain and carbon source dependent growth characterisation of *Saccharomyces cerevisiae*. *Microb Cell Fact* 15:1–14. doi: 10.1186/s12934-016-0526-3
- Eggert T, Bakonyi D, Hummel W (2014) Enzymatic routes for the synthesis of ursodeoxycholic acid. *J Biotechnol* 191:11–21. doi: 10.1016/j.jbiotec.2014.08.006
- Feng Y, Siu K, Wang N, et al (2009) Bear bile: dilemma of traditional medicinal use and animal protection. *J Ethnobiol Ethnomed* 5:2. doi: 10.1186/1746-4269-5-2
- Ferrandi EE, Bertolesi GM, Polentini F, et al (2012) In search of sustainable chemical processes: cloning, recombinant expression, and functional characterization of the 7 α - and 7 β -hydroxysteroid dehydrogenases from *Clostridium absonum*. *Appl Microbiol Biotechnol* 95:1221–1233. doi: 10.1007/s00253-011-3798-x
- Lee J-Y, Arai H, Nakamura Y, et al (2013) Contribution of the 7 β -hydroxysteroid dehydrogenase from *Ruminococcus gnavus* N53 to ursodeoxycholic acid formation in the human colon. *J Lipid Res* 54:3062–9. doi: 10.1194/jlr.M039834
- Li L, Liu C, Mao W, et al (2019) Taurochenodeoxycholic acid inhibited AP-1 activation via stimulating glucocorticoid receptor. *Molecules* 24:1–10. doi: 10.3390/molecules24244513
- Lian J, Mishra S, Zhao H (2018) Recent advances in metabolic engineering of *Saccharomyces cerevisiae*: New tools and their applications. *Metab Eng* 50:85–108. doi: 10.1016/j.ymben.2018.04.011
- Liu L, Braun M, Gebhardt G, et al (2013) One-step synthesis of 12-ketoursodeoxycholic acid from dehydrocholic acid using a multienzymatic system. *Appl Microbiol Biotechnol* 97:633–639. doi: 10.1007/s00253-012-4340-5
- Lu Q, Jiang Z, Wang Q, et al (2021) The effect of Tauroursodeoxycholic acid (TUDCA) and gut microbiota on murine gallbladder stone formation. *Ann Hepatol* 23:100289. doi: 10.1016/j.aohep.2020.100289
- Momose T, Tsubaki T, Iida T, Nambara T (1997) An improved synthesis of taurine- and glycine-conjugated bile acids. *Lipids* 32:775–778. doi: 10.1007/s11745-997-0099-8

Rogowska-Wrzesinska A, Larsen PM, Blomberg A, et al (2001) Comparison of the proteomes of three yeast wild type strains: CEN.PK2, FY1679 and W303. *Comp Funct Genomics* 2:207–225. doi: 10.1002/cfg.94

Rosa LR de O, Vettorazzi JF, Zangerolamo L, et al (2021) TUDCA receptors and their role on pancreatic beta cells. *Prog Biophys Mol Biol*. doi: 10.1016/j.pbiomolbio.2021.09.003

Shi J, Wang J, Yu L, et al (2017) Rapidly directional biotransformation of tauroursodeoxycholic acid through engineered *Escherichia coli*. *J Ind Microbiol Biotechnol* 44:1073–1082. doi: 10.1007/s10295-017-1935-y

Song C, Wang B, Tan J, et al (2017) Discovery of tauroursodeoxycholic acid biotransformation enzymes from the gut microbiome of black bears using metagenomics. *Sci Rep* 7:1–8. doi: 10.1038/srep45495

Toledo A, Yamaguchi J, Wang JY, et al (2004) Taurodeoxycholate stimulates intestinal cell proliferation and protects against apoptotic cell death through activation of NF- κ B. *Dig Dis Sci* 49:1664–1671. doi: 10.1023/B:DDAS.0000043383.96077.99

Wang J, Xiong A-Z, Cheng R-R, et al (2018) Systematical analysis of multiple components in drainage bear bile powder from different sources. *China J Chinese Mater Medica* 43:2326–2332. doi: 10.19540/j.cnki.cjcmm.20180125.001

Xu Y, Yang L, Zhao S, Wang Z (2019) Large-scale production of tauroursodeoxycholic acid products through fermentation optimization of engineered *Escherichia coli* cell factory. *Microb Cell Fact* 18:34. doi: 10.1186/s12934-019-1076-2

Yang L, Xiong A, He Y, et al (2008) Bile acids metabonomic study on the CCl₄- and alpha-naphthylisothiocyanate-induced animal models: quantitative analysis of 22 bile acids by ultraperformance liquid chromatography-mass spectrometry. *Chem Res Toxicol* 21:2280–2288. doi: 10.1021/tx800225q

Yoshimoto T, Higashi H, Kanatani A, et al (1991) Cloning and sequencing of the 7 alpha-hydroxysteroid dehydrogenase gene from *Escherichia coli* HB101 and characterization of the expressed enzyme. *J Bacteriol* 173:2173–9

Zangerolamo L, Vettorazzi JF, Rosa LRO, et al (2021) The bile acid TUDCA and neurodegenerative disorders: An overview. *Life Sci* 272:119252. doi: 10.1016/j.lfs.2021.119252

Zheng MM, Chen KC, Wang RF, et al (2017) Engineering 7 β -Hydroxysteroid Dehydrogenase for Enhanced Ursodeoxycholic Acid Production by Multiobjective Directed Evolution. *J Agric Food Chem* 65:1178–1185. doi: 10.1021/acs.jafc.6b05428

Zheng MM, Wang RF, Li CX, Xu JH (2015) Two-step enzymatic synthesis of ursodeoxycholic acid with a new 7 β -hydroxysteroid dehydrogenase from *Ruminococcus torques*. *Process Biochem* 50:598–604. doi:

Zhou C, Shi Y, Li J, et al (2013) The effects of taurochenodeoxycholic acid in preventing pulmonary fibrosis in mice. Pak J Pharm Sci 26:761–765

Figures

Figure 1

Reaction catalyzed by 7α-HSDH and 7β-HSDH

Figure 2

Results of high productive engineered *Saccharomyces cerevisiae* screening.

a Results of eight engineered yeast strains and two control yeast strains under 30°C, pH 6.5 for 6 h. b Results of W303a-α1β2 and CEN-α2β2 under 25°C, pH 6.5.

Figure 3

Results of substrate concentration and pH value on the biotransformation of TUDCA.

a Results of substrate concentration when incubation at 30°C, pH 6.5 for 9 h. b Results of pH value when incubation for 9 h.

a

b

c

Figure 4

Results of response surface methodology for TUDCA/TCDCa