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Optimization of Fermentation Conditions for Elevating Limonene Production with Engineered *Rhodosporidium toruloides*

Di Zhao [1], Qidou Gao [1], Xiaochun Zheng [1], Sasa Liu [1], Qingsheng Qi [2], Xue Wang [3,*] and Xiaobing Yang [1,*]

[1] College of Enology, Northwest A&F University, Yangling, Xianyang 712100, China; zhaodidi@nwafu.edu.cn (D.Z.); gaoqidou@nwafu.edu.cn (Q.G.); zhengxc0425@nwafu.edu.cn (X.Z.); sasaliu@nwafu.edu.cn (S.L.)
[2] State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China; qiqingsheng@sdu.edu.cn
[3] College of Food Science and Engineering, Northwest A&F University, Yangling, Xianyang 712100, China
* Correspondence: wangxue2018@nwafu.edu.cn (X.W.); yangxb@nwafu.edu.cn (X.Y.)

Abstract: Limonene is a valuable monoterpenoid with diverse applications in food, medicine, agriculture, etc. Currently, the commercial production of limonene from citrus pomace cannot meet the evergrowing market demand. With the development of synthetic biology, microbial biosynthesis is offering a sustainable alternative to plant-based extraction. However, the performance of engineered strains can be affected by many factors including the microbial host, the cultivation conditions, etc. Therefore, in the present study, potential factors that influence the limonene production of the engineered strains were investigated through single-factor and orthogonal experiments. The optimized medium was MM, with a working volume of 50 mL, inoculum OD_{600} = 0.6, 22 °C, pH 6, and a dodecane coverage (*v*/*v*) of 20%. The final limonene titer was improved from 52.5 to 358.1 mg/L, a 586% net increase in titer and 34.8-fold improvement in production efficiency (mg/OD_{600}) on 250-mL flasks. The results here demonstrated that *R. toruloides* could be explored as an efficient limonene producer and could facilitate the study of the biosynthesis of other terpenes using *R. toruloides*.

Keywords: limonene; *Rhodosporidium toruloides*; fermentation conditions optimization; orthogonal experiment

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Citation: Zhao, D.; Gao, Q.; Zheng, X.; Liu, S.; Qi, Q.; Wang, X.; Yang, X. Optimization of Fermentation Conditions for Elevating Limonene Production with Engineered *Rhodosporidium toruloides*. *Fermentation* **2023**, *9*, 431. https://doi.org/10.3390/fermentation9050431

Academic Editor: Xian Zhang

Received: 30 March 2023
Revised: 20 April 2023
Accepted: 27 April 2023
Published: 28 April 2023

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

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1. Introduction

Limonene is a typical monoterpene with diverse functions in food, medicine, pesticides, solvents, fuel, etc. [1–7]. Due to its wide range of applications, the global demand for limonene has continued to grow over the past decade and is expected to reach USD 248 million by 2028 [8]. The traditional plant-based extraction method is hindered by the limited acreage and vulnerability to seasonal and climatic changes. The chemical synthesis approach is unfavored due to its high energy consumption, low efficiency, environmental concerns, and toxic impurities, which hinder their large-scale applications in certain areas like food and medicine [1]. With the rapid development of synthetic biology and metabolic engineering, microbial cell factories are becoming a sustainable alternative platform for the production of limonene. Importantly, limonene produced via microbial technology is generally regarded as safe (GRAS) [9].

To date, various microbes including *Escherichia coli* [10,11], *Saccharomyces cerevisiae* [12,13], *Yarrowia lipolytica* [14,15], and *Rhodosporidium toruloides* [16] have been employed for limonene production. However, the maximum titer was only 3.63 g/L with engineered *E. coli* BL21 (DE3) in a bioreactor [10]. Therefore, the current maximum productivity and carbon yield are economically unfeasible for large-scale production [1]. Generally, there are several aspects that influence the bioproduction of limonene: the choice of an efficient

microbial host, the genetic toolbox for the specific host, the available genetic elements for constructing an efficient heterologous synthetic route, and the cultivation conditions for the recombinant strains. Among which the optimization of fermentation conditions is the last but critical procedure for establishing an industrially profitable process, which is featured with easy operation, low input and high efficiency (titer, yield, and productivity) [17].

As an unconventional yeast, *R. toruloides* can metabolize a wide range of low-cost carbon sources to support its cell growth and lipid accumulation. Most interestingly, it is endowed with a naturally robust mevalonate pathway (MVA), which has been rewired for manufacturing various terpenoids including limonene, ent-kaurene bisabolene, and carotenoids [16,18–21]. As in the case of our previous study, the biosynthesis of limonene was achieved by introducing the neryl pyrophosphate synthase (*Sl*NPPS) from *Solanum lycopersicum*, limonene synthase (*Cl*tLS1) from *Citrus limon*, acetoacetyl-CoA thiolase (*Ef*MvaE) and mevalonate synthase (*Ef*MvaS) from *Enterococcus faecalis*, the mevalonate kinase (*Mm*MK) from *Methanosarcina mazei*, and overexpressing the truncated endogenous hydroxy-methyl-glutaryl-CoA reductase (HMGR). The average titer of only 54.4 mg/L limonene was generated in 250-mL flask [16]. The undesired results might be caused by inappropriate strain and cultivation conditions.

To tap the potential of the engineered strains and improve the production efficiency, the cultivation conditions were investigated in the present study through single-factor and orthogonal design experiments (Figure 1). First, the recombinant *R. toruloides* strains were compared for the stability of limonene production. Second, the effects of carbon source, inoculation, working volume, temperature, pH, dodecane coverage, and media on limonene production were investigated. Finally, the cultivation conditions were assessed via orthogonal experiments. The final limonene titer was improved from 52.5 to 358.1 mg/L in 250-mL flask, a 586% net increase. Our results here indicated the possibility of elevating limonene production efficiency via operating at the fermentation level, and the performance of limonene producing strains should be further enhanced at the genetic level.

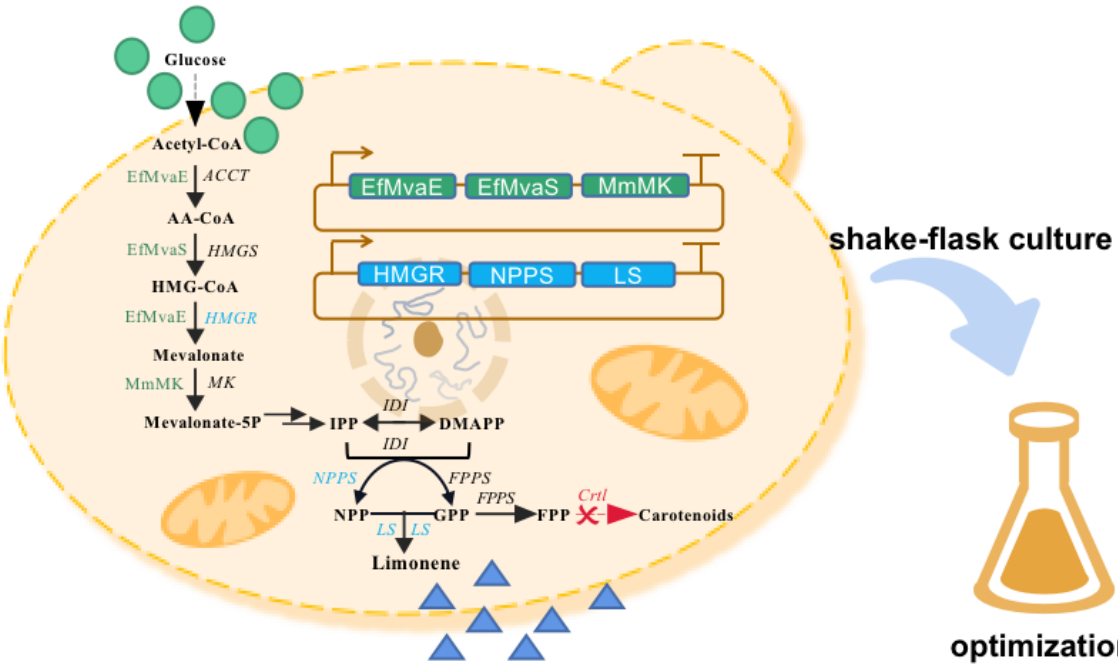


Figure 1. Flow chart of limonene production.

2. Materials and Methods

2.1. Material and Reagents

A limonene standard was purchased from Sigma-Aldrich (Shanghai, China), and 3, 5-dinitrosalicylic acid, hexane, and dodecane were purchased from Shanghai Kefeng

Industry Co., Ltd. (Shanghai, China). All the other reagents were obtained locally. All the genes mentioned in the present study were synthesized in our previous study [16]. The plasmids: P1, PGK-Ble-Tnos-Pxyl-CltLS1-NPPS-tHMGR-Thsp and P2, PGK-Ntc- Tnos-Pxyl-MmMK-EfMvaS-EfMvaE-Thsp were constructed in our previous study [16]. SlNPPS: neryl pyrophosphate synthase from *Solanum lycopersicum*, CltLS1: limonene synthase from *Citrus limon*, the EfMvaE: acetoacetyl-CoA thiolase from *Enterococcus faecalis*, EfMvaS: mevalonate synthase from *Enterococcus faecalis*, MmMK: mevalonate kinase from *Methanosarcina mazei*, tHMGR: truncated endogenous hydroxymethyl glutaryl-CoA reductase [16]. All the genes were under the control of the xylose reductase promoter, and the strains obtained were constructed in our previous study and preserved in the College of Enology, Northwest A&F University (Shanxi, China) [16].

2.2. Microbial Strain, Media, and Fermentations

The carotenogenesis-defcient *R. toruloides* NP11 was kindly provided by Prof. Zongbao kent Zhao from Dalian Institute of Chemical Physics, CAS, and used as the parental strain. The *R. toruloides* engineered strain was constructed through the *Agrobacterium*-mediated transformation (ATMT) [16]. The ATMT experiment was conducted as follows [16]. First, the vector carrying *Agrobacterium tumefaciens* cells were cultivated in LB medium supplemented with 50 μg/mL kanamycin at 28 °C for 15 h, and *R. toruloides* NP11 cells in YPD medium at 30 °C for 15 h. Second, cells were collected by centrifugation at 8000× *g* for 30 s, then washed twice and diluted with sterilized water to an optical density 0.6 (OD_{600}). For transformation, 100 μL of each cell suspension was mixed, and spread onto the IM agar plates containing 200 μM acetosyringone, and incubated at 25 °C for 2 days. Then, the transformant was transferred onto the selection plate to incubate until colony generated. The otained transformants were streaked on selection YPD for five successive generations to verify their phenotypic stability. Finally, the resultant transformants were subjected for testing limonene production efficiency and stability.

The media compositions were as follows. YPD liquid medium (g/L): glucose 20, peptone 20, yeast extract 10, (solid YPD was supplied with Agar powder 20). SD medium (g/L): glucose 20, YNB 6.7 (yeast unit without amino acids). MM (Minimal medium) (g/L): glucose 20, ammonium sulfate 5, potassium dihydrogen phosphate 3, magnesium sulfate heptahydrate 0.5, 1 mL/L trace element solution, and 1 mL/L vitamin solution. NL (nitrogen-limiting) medium (g/L): glucose 20, yeast powder 0.5, ammonium chloride 0.33, magnesium sulfate heptahydrate 1.5, potassium dihydrogen phosphate 1, disodium hydrogen phosphate 1, pH = 6.0. Trace elements (g/L): calcium chloride dihydrate 4, ferrous sulfate heptahydrate 0.55, hydrated citric acid 0.52, zinc sulfate heptahydrate 0.1, manganese sulfate 0.076, 18 mol/L sulfuric acid 100 μL. Vitamin solution (g/L): biotin 0.05, 4-aminobenzoic acid 0.2, nicotinic acid 1, D-calcium pantothenate 1, pyridoxamine dihydrochloride 1.

For 250-mL flask experiments, the seed culture was prepared by inoculating a single colony into 5 mL YPD and cultured at 28 °C and 180 r/min for 24 h, and were then inoculated into 50 mL YPD at a ratio of 2% (*v*/*v*) and cultured at 28 °C and 180 r/min for 24 h. The seed culture was subsequently inoculated into YPD with an initial OD_{600} of 0.6. If not specified, 20% (*v*/*v*) dodecane was used as the overlay before culturing at 28 °C, 180 rpm/min for a certain time (usually 120 h), and glucose was used as the carbon source. The initial pH of the YPD liquid medium in its natural state was 5.3. and the other media were adjusted to pH 5.3 using 0.5 M NaOH. The cultures were sampled at intervals of 24 h to determine levels of sugars, OD_{600}, and limonene.

2.3. Single-Factor Effects on Limonene Production

For the single-factor test, the influence of the initial cell optic density, working volume, temperature, initial pH, carbon source, medium type, and organic solvent overly ratio were investigated with limonene titer as the index. The initial cell density (OD_{600}) was set as 0.2, 0.4, 0.6, and 0.8; working volume (mL): 25, 50, 75, 100, and 125; temperature (°C): 22, 25, 28,

and 31; initial pH: 4, 5, 6, 7, and 8; medium: YPD, MM, SD, and NL; carbon source: glucose, xylose, fructose, and sucrose; and dodecane coverage ratio (v/v, %): 10, 20, 30, and 40.

2.4. Orthogonal Experiments for Elevating Limonene Production

Based on the single-factor experiments above, an orthogonal array of L_9 (3^4) was conducted considering the four most important factors (working volume, temperature, pH, and dodecane coverage) (Table 1). The limonene titer was used as the evaluation criterion. All experiments were performed in triplicates, and the data were presented as the mean and standard deviation. Statistical significance was determined using one-way ANOVA followed by Tukey multiple ranged tests (SPSS22.0 software, Chicago, IL, USA), and statistical differences between the means were evaluated using least significant difference (LSD) analysis at $p = 0.05$. The histograms were drawn using Datagraph 4.3.0 (Visual Data Tools, Inc, USA).

Table 1. Factors and levels in orthogonal experiments.

Level	Medium Volume (mL)	Temperature (°C)	pH	Dodecane Coverage Ratio (%)
1	25	22	4	20
2	50	25	5	30
3	75	28	6	40

2.5. Analytical Methods

The pH was determined using a pH meter (FiveEasy Plus, METTLER-TOLEDO, Columbus, OH, USA), and biomass was measured using an ultraviolet spectrophotometer (EVOLUTION 220, Beijing Guanyuan Technology Co., Ltd., Beijing, China). The glucose level was estimated using a 3,5-dinitryl-salicylic acid (DNS) colorimetry assay [22]. Limonene was quantified using gas chromatography (GC-2014C, Shimadzu, Kyoto, Japan) as previously described [16]. Briefly, a KB-1 column (60 m × 0.25 mm × 0.25 µm, Kromat Corporation, Fieldsboro, NJ, USA) was used with a column temperature of 145 °C, loading temperature of 240 °C, and detector temperature of 260 °C. Nitrogen was used as the carrier gas, the mobile phase was n-hexane, the flow rate was a constant 1.0 mL/min, and the sample size was 1 µL.

3. Results

3.1. Limonene Production Stability of Engineered R. toruloides Strains

The production stability of engineered *R. toruloides* strains is vital for establishing an efficient bioprocess. As such, eight of the previously constructed transformants were compared for their limonene production stability in the YPD medium for three rounds as described by Liu et al. [16]. The limonene titers were determined at 96 h. As shown in Figure 2a, strain 3# exhibited better observable stability than the others and was employed for the following experiments.

The dynamic evolution of 3# then OD_{600}, glucose consumption, and limonene production was monitored for 120 h. The fermentation was started with an initial OD_{600} of 0.6 and maintained at 28 °C and 180 r/min for 120 h. As shown in Figure 2b, *R. toruloides* grew to an OD_{600} of 26.7 within 96 h, but decreased to an OD_{600} of 25.3 at 120 h; the limonene titer evolved with a similar pattern as the cell density. The limonene biosynthesis was started at the beginning of the fermentation, accelerated from 48 h, and reached the top at 96 h (52.2 mg/L, 2.0 mg/OD_{600}). The glucose was mostly consumed within 72 h and depleted by 96 h. Notably, the limonene biosynthesis and glucose depletion were coupled in opposite directions, and limonene was produced only when glucose was present. According to the results above, the fermentation should be ended when the carbon source is exhausted.

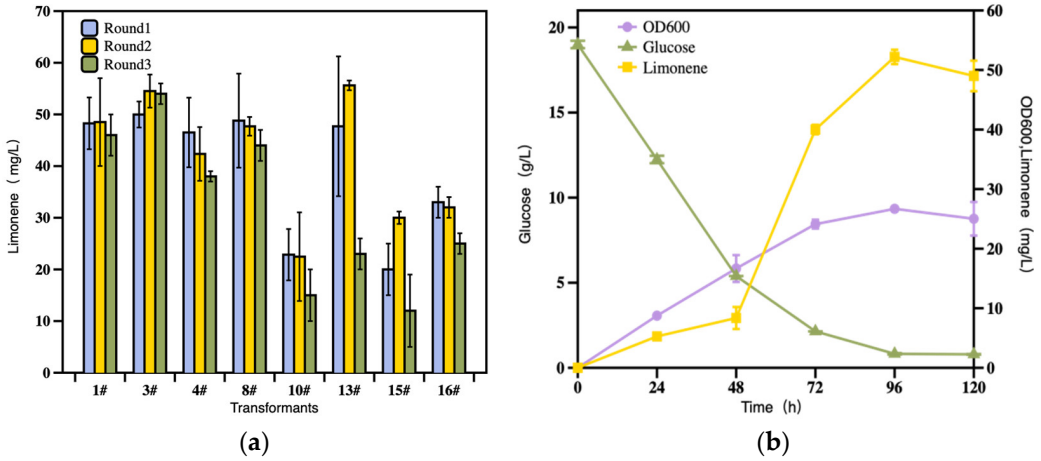


Figure 2. Limonene production using recombinant *R. toruloides* strains. (**a**) Titer of limonene from transformants 1#, 3#, 4#, 8#, 10#, 13#, 15#, 16# at 96 h. (**b**) Strain growth, glucose consumption, and limonene production of strain 3#.

3.2. The Impacts of Fermentation Parameters on Limonene Production

3.2.1. The Effect of Carbon Source on Limonene Production

Carbon source is essential to microorganisms in supporting the biosynthesis of structural macromolecules, the energy generation for cell proliferation and the production of desired products [23,24]. Besides, microbes, especially the engineered ones always prefer varied carbon sources for producing specific products [25]. Thus, it is necessary to optimize the carbon sources when studying the bioproduction of limonene with engineered *R. toruloides* strains. In the present study, xylose, glucose, fructose, and sucrose were used as the candidate carbon sources, and evaluated for their effects on limonene production. As shown in Figure 3a, fructose and glucose were more suitable as carbon sources for supporting the growth of engineered *R. toruloides* than xylose and sucrose. Fructose was the most suitable carbon source, which resulted in the highest cell density of OD_{600} 36.0 and sucrose was not desirable. In terms of limonene titer, the maximum was (75.3 mg/L) achieved with glucose, and the lowest was 10 mg/L with sucrose. When it comes to the limonene production efficiency, the results were 3.4 mg/OD_{600}, 2.0 mg/OD_{600}, 1.3 mg/OD_{600}, and 3.3 mg/OD_{600} for xylose, fructose, sucrose, and glucose, respectively. In summary, glucose was the most viable carbon source for sustaining cell growth and limonene production, followed by xylose and fructose, while sucrose was most unsuitable (Figure 3b). Thus, glucose was used as the carbon source in the following experiments.

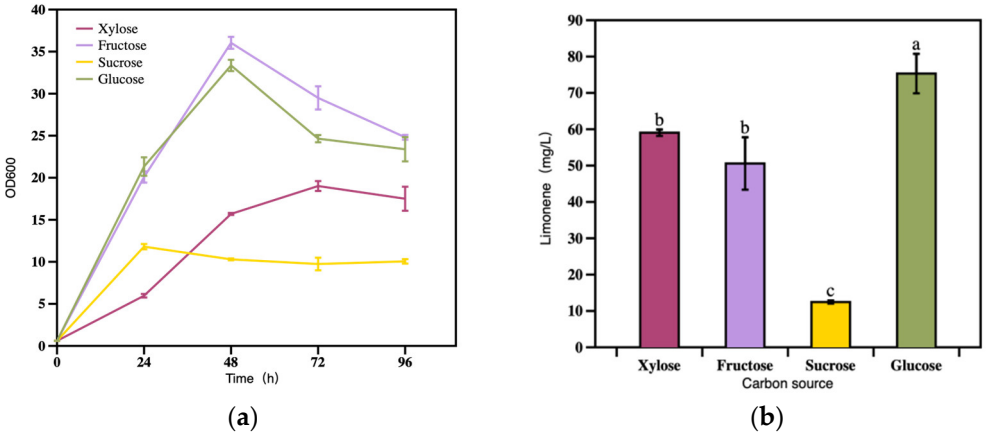


Figure 3. Using different carbon sources (**a**) strain growth and (**b**) limonene production at 96 h. Bars with different letters were significantly different ($p < 0.05$) according to paired *t*-tests.

3.2.2. The Effect of Initial Cell Density on Limonene Production

The concentration of the inoculum affects the start of the fermentation process and product production [26]. Usually, higher inoculum would shorten the lag-phase, allow the product to form earlier, and reduce the chance of microbiological contamination. The optimal inoculum usually depends on the concentration of dissolved oxygen, nutrients in the medium, and the chosen microbial strains [26]. To determine the optimal initial cell density for limonene production, the initial optic densities were set as 0.2, 0.4, 0.6, and 0.8 in 250 mL shake flasks with working volume of 50 mL and cultivated at 28 °C for 96 h. As can be seen from Figure 4, the highest titer was 55.6 mg/L when the initial cell density was set at OD_{600} = 0.6 (p = 0.001 < 0.05, significant difference) (Figure 4). When the initial OD_{600} was 0.2, 0.4, and 0.8, the titers of limonene were significantly lower than that of the cultures with an initial OD_{600} of 0.6, and there was no significant difference among the three groups (p = 0.246, 0.657 > 0.05). Therefore, the optimal initial OD_{600} was set to 0.6 in the following experiments.

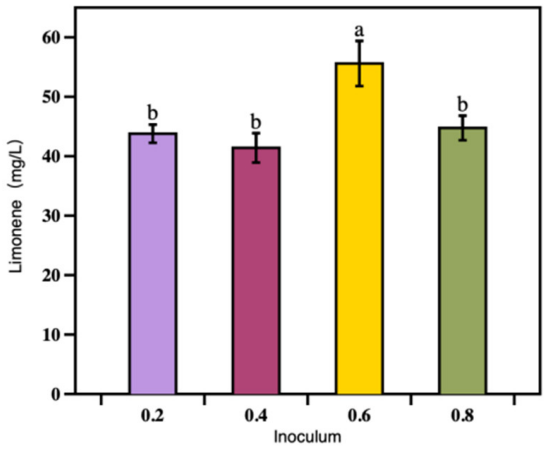


Figure 4. The titer of limonene measured at 96 h under different inoculum concentrations. Bars with different letters were significantly different (p < 0.05) according to paired t-tests.

3.2.3. The Effect of Working Volume on Limonene Production

As a strictly aerobic yeast, the cell growth and metabolism of *R. toruloides* are highly oxygen demanding. Further, terpenoid biosynthesis is also an energy consuming process. Sufficient dissolved oxygen supply is critical to maintain the cell viability and limonene biosynthesis. To investigate the effect of aeration on limonene production, the medium loading volume were set within a range of 25 to 125 mL in 250-mL flasks. It was obvious that the limonene titer decreased when the working volume increased from 25 to 125 mL (Figure 5a). Moreover, the time to reach the maximum cell density increased as the media volume increased (Figure 5b). When the working volume was 25 mL, the biomass of *R. toruloides* reached the maximum and all glucose was depleted within 24 h (Figure 5c), and the limonene reached its maximum of 68.2 mg/L at 96 h. For the group with a loading medium of 50 mL, the titer of limonene reached 56.3 mg/L at 96 h. When the volume was further increased to 75 mL, 100 mL, and 125 mL, the resulting limonene titers were 48.3 mg/L, 26.3 mg/L, and 20.4 mg/L at 96 h, respectively. The results indicated that an excessively high medium loading can lead to insufficient dissolved oxygen, which, in turn, will affect cell growth and the limonene titer [27,28]. Additionally, too high of a media volume might also influence the mass transfer efficiency, which was also an important factor for efficient bioproduction.

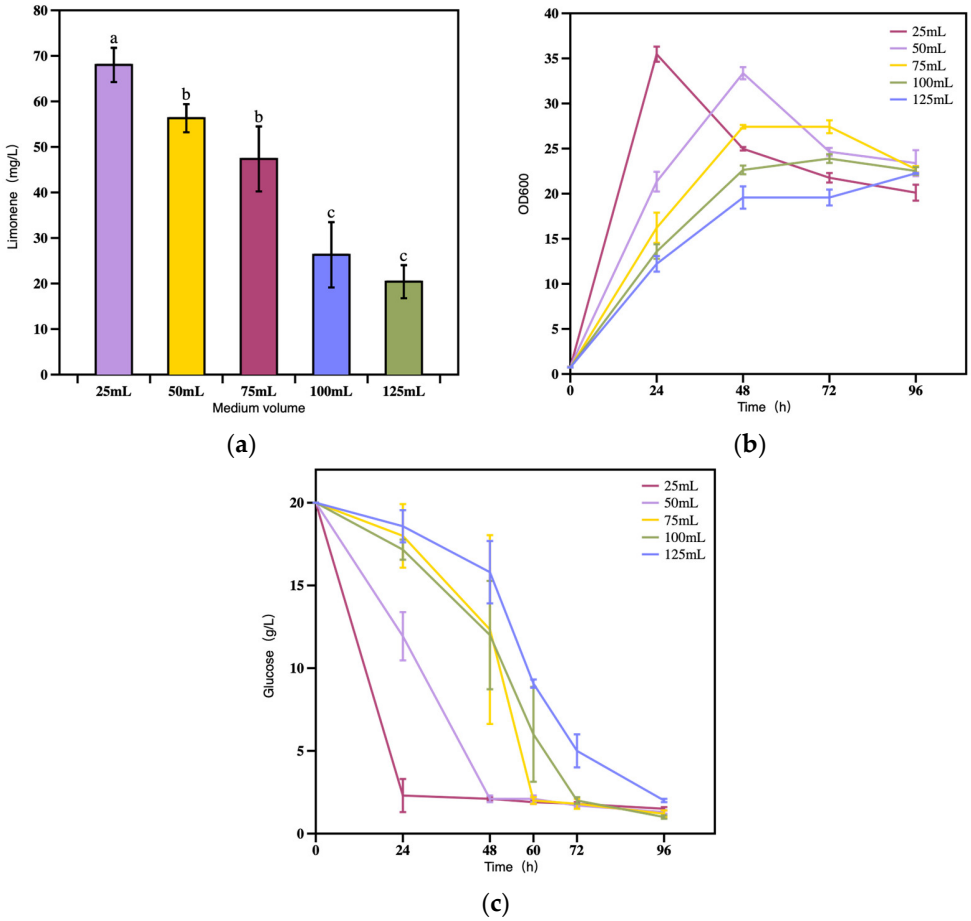


Figure 5. The effects of working volume on limonene production. (**a**) limonene production at 96 h, (**b**) strain growth, and (**c**) glucose consumption. Bars with different letters were significantly different ($p < 0.05$) according to paired *t*-tests.

3.2.4. The Effect of Temperature on Limonene Production

The operation temperature could affect the cell growth via modulating the substrate viscosity, mass transfer, decomposition, and absorption, the dissolved oxygen content, the enzymatic activity in the biochemical process, and finally influence the product synthesis efficiency [17]. Although the optimal fermentation temperature of *R. toruloides* was reported as 28 °C [21], it was probable that it is not the case for limonene production with the engineered strains. Thus, the effects of temperature on limonene production were explored. The results of the present study showed that the cells grew best at 22 °C and 25 °C (Figure 6a). The highest titers were 81.4 mg/L, 78.0 mg/L, and 75.3 mg/L at 22 °C, 25 °C, and 28 °C, respectively (Figure 6b). It is worth noting that the limonene was only 18.1 mg/L at 120 h at 31 °C, which was 70.5% lower than that at 96 h (not shown in figure). Considering the volatility of limonene and the temperature of 31 °C, the significantly lower limonene titer might be attributed to the accelerated evaporation of limonene itself as well as the inadequate enzymatic activity of the yeast cells.

3.2.5. The Effect of Initial pH on Limonene Production

The pH in the cultivation medium impacts cell growth and bioproduction efficiency by affecting the activity of enzymes and changing the cell membrane permeability by altering its charge state, thereby affecting the absorption of nutrients and the efflux of metabolites [28–31]. To investigate its effect on limonene production, the initial pH in the medium was set from 4 to 7 (Figure 7a,b). The results demonstrated that the initial pH of the YPD medium had no obvious effects on cell growth and glucose consumption of

R. toruloides in the first 48 h (Figure 7b,c), but had a significant effect on the production of limonene from 48 h to 72 h (Figure 7a). The highest titer of limonene (85.5 mg/L) was obtained at an initial of pH 4, which was significantly higher than those of the other pH conditions (p = 0.002, 0.001, 0.001, 0.001 < 0.05). Possibly, a lower initial pH facilitated the maintenance of cell viability when the fermentation entered the stationary phase, when the yeast are prone to autophagy and autolysis. The release of intracellular material, especially bioamine, usually causes the increase in the alkalinity of the media, thus affects the yeast viability.

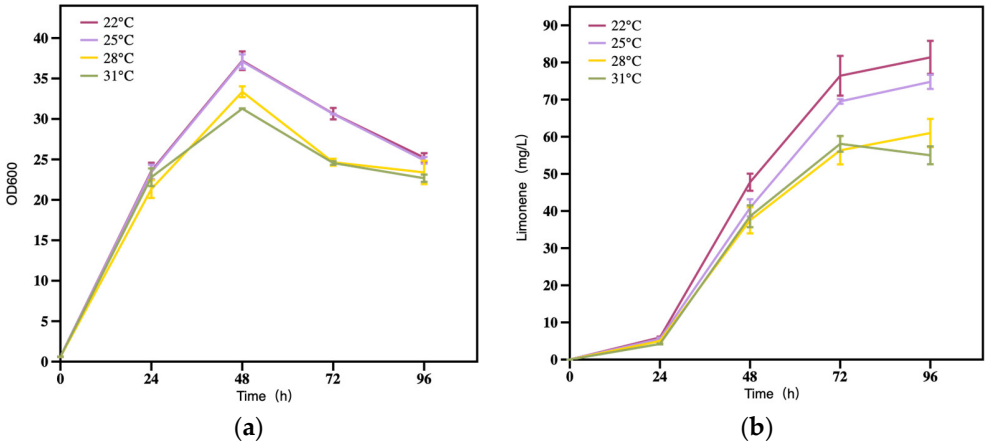


Figure 6. (**a**) Strain growth and (**b**) limonene production at different temperatures.

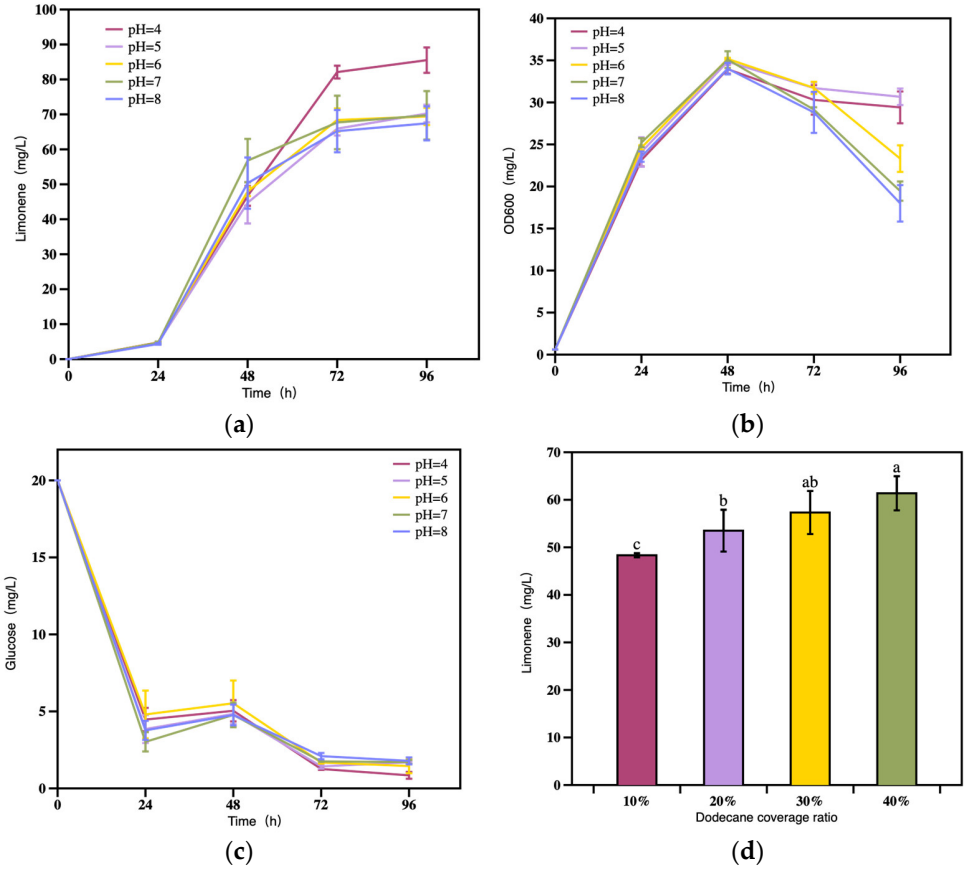


Figure 7. (**a**) Limonene production, (**b**) strain growth, and (**c**) glucose consumption at different pH conditions. (**d**) Limonene production at 96 h using different dodecane coverage ratios. Bars with different letters were significantly different (p < 0.05) according to paired t-tests.

3.2.6. The Effect of Dodecane Coverage Ratio on Limonene Production

Monoterpenes are usually volatile, and can pose certain cytotoxicity on the microbial host. To facilitate the instant extraction of monoterpenes and eliminate its cytotoxicity, a bi-phasic fermentation system is common employed with a biocompatible organic solvent such as diisononyl phthalate, dibutyl phthalate and dodecane as an overlay [1]. Dodecane stands have been extensively used for the in situ production of monoterpenes, including limonene [10–16]. Moreover, the addition of dodecane is also beneficial to increase the dissolved oxygen in the fermentation system [1]. In the present study, the dodecane to water ratios (v/v) were explored for their effects on limonene production. As is shown in Figure 7d, the limonene production increased with the raise of dodecane to water ratio. However, only a 14% increase was observed in terms of limonene titer when the dodecane ratio was elevated from 20% (53.5 mg/L) to 40% (61.4 mg/L) (Figure 7d). The results here indicated that less dodecane coverage would result in the evaporation of limonene during fermentation. In light of the solvent cost in large scale application, the organic overlay maybe combined with a cooling reflux system to achieve a high product recovery efficiency.

3.2.7. The Effect of the Medium on Limonene Production

The composition of the medium employed during fermentation could influence the viability of the yeast inoculated. While rich medium is favored by microbes for cell mass accumulation, the synthetic ones are preferred for assessing the capacity of the microbial host toward a specific product. To facilitate the biosynthesis of limonene with engineered *R. toruloides*, the medium of YPD, SD, MM, and NL were examined (Figure 8).

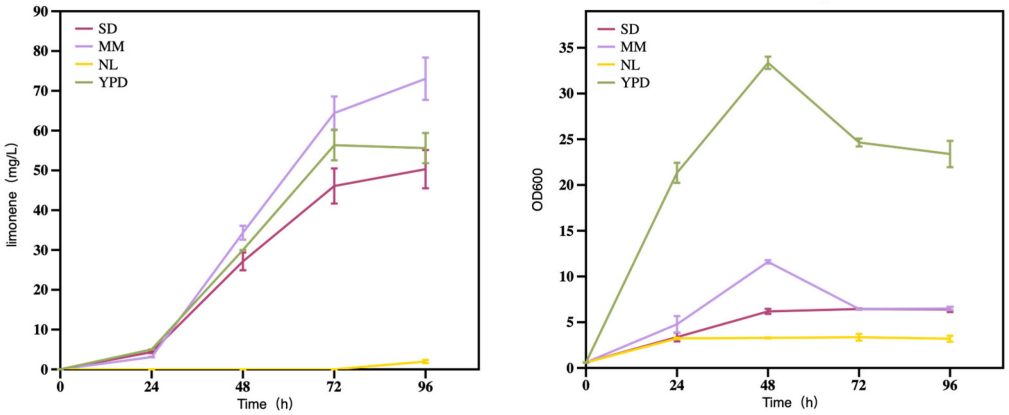


Figure 8. (**a**) Limonene production and (**b**) strain growth using different medium types. SD is SD minimal medium; MM is minimal medium; NL is nitrogen-limiting medium; YPD is yeast extract peptone dextrose medium.

Results showed that *R. toruloides* had the best biomass performance in YPD ($p \approx 0 < 0.05$), and the result was constant with the previous study [32]. However, the MM was the best for limonene production (73.0 mg/L), 31.3% higher than that obtained in YPD (55.6 mg/L). Notably, the limonene production efficiency (11.4 mg/OD_{600}) in the MM was significantly higher than those of other media at 96 h, which represented a much higher conversion from glucose (3.65 mg/g with the YPD medium). In the SD medium, the cell growth was relatively weaker than in MM. This result might be attributed to the absence of amino acids in SD medium, which are essential components for synthesizing proteins within the cell, and thus maintaining the state of cells. The maximum limonene titer was 50.3 mg/L at 96 h with the SD medium (Figure 8a). In the NL medium, the maximum OD_{600} was 3.4 and the limonene was only 2.0 mg/L (Figure 8b). Taken together, the MM was the best choice for limonene production while the NL medium could not be considered further. Thus, the MM was used for subsequent experimental operations.

3.3. Optimization of Limonene Production through Orthogonal Experiments

Based on the single-factor experiment results above, four main influential factors were obtained: working volume, temperature, pH, and the dodecane coverage ratio. To optimize the fermentation system, an L_9 (3^4) orthogonal design was employed. From the range analysis in Table 2, it can be seen that the effect of the various factors on limonene titers were ranked as A > D > C > B (medium volume > dodecane coverage ratio > pH > temperature). The optimal combinations were $A_3D_3C_3B_3$ and $A_2D_3C_3B_1$ according to the K value and the orthogonal results, respectively (Table 2).

Table 2. L_9 (3^4) orthogonal test table and results.

Number	A	B	C	D	Limonene (mg/L)
1	25	22	4	20	114.2
2	25	25	6	30	146.5
3	25	28	5	40	193.0
4	50	22	6	40	358.1
5	50	25	5	20	115.7
6	50	28	4	30	199.5
7	75	22	5	30	235.1
8	75	25	4	40	298.8
9	75	28	6	20	322.3
K1	453.7	707.4	612.5	552.3	
K2	673.3	561.0	543.7	581.0	
K3	856.2	714.7	827.0	849.9	
k1	151.2	235.8	204.2	184.1	
k2	224.4	187.0	181.2	193.7	
k3	285.4	238.2	275.7	283.3	
R	134.2	51.2	94.4	99.2	
Major factor	ADCB				
Optimal combination	$A_3D_3C_3B_3$				

By referring to the single-factor experimental results, a working volume of 75 mL was better than 50 mL. Needless to say, a pH of 6 is optimal, as is a dodecane coverage ratio of 40%. From the point of applicability, a 40% dodecane coverage will encounter serious economic infeasibility. It can be seen from the experimental data of No. 9 that the culture conditions of a 20% (*v*/*v*) dodecane covering ratio, pH = 6, 28 °C, and 75 mL produced an acceptable titer. Therefore, the optimal fermentation conditions were as follows: A (working volume) = 50 mL, B (temperature) = 22 °C, C (pH) = 6, and D (dodecane coverage ratio) = 20%, at the 250 mL flask level (Table 2). Finally, the limonene titer was elevated from 52.5 mg/L to 358.1 mg/L, which was a 586% net increase in titer and 34.8-fold improvement in production efficiency (mg/OD_{600}). Still, it must be noted that the yield of limonene from glucose was only 1.79% (*w*/*w*). The low conversion yield might be attributed to an intrinsic inefficiency of the engineered yeast, which can only be overcome by in-depth metabolic rewiring rather than fermentation optimization [33].

4. Conclusions

The red yeast *R. toruloides* is a versatile platform for producing various products from low-cost feedstock. Our experiments demonstrated that *R. toruloides* can be potentially used as an excellent host for limonene production, and the fermentation conditions greatly impact limonene production. The results of the single-factor and L_9 (3^4) orthogonal experiments showed that the optimal fermentation conditions for limonene production were: initial OD_{600} of 0.6, temperature of 22 °C, initial pH of 6, dodecane coverage ratio of 20%, and working volume of 50 mL in the MM medium on 250-mL flask. Under the optimized conditions, the limonene titer of engineered *R. toruloides* was improved from 52.5 to 358.1 mg/L, a 586% net increase. Collectively, our results lay a good foundation for improving the production of monoterpenes with engineered *R. toruloides* and the performance of limonene producing strains should be further enhanced at the genetic level.

Author Contributions: Conceptualization, methodology, formal analysis, and investigation, D.Z.; Methodology, formal analysis, and data curation, Q.G.; Writing—review and editing, X.Z.; Investigation, S.L.; Project administration and funding acquisition, Q.Q.; Formal analysis, resources, and data curation, X.W.; Writing—review and editing, supervision, project administration, and funding acquisition, X.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the State Key Laboratory of Microbial Technology Open Projects Fund (M2022-13), Chinese Universities Scientific Fund (2452018314), and Natural Science Foundation of Shaanxi Province (2020JM-177).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained within the article.

Acknowledgments: Authors would thank the instrument shared platform of College of Food Sience & Engineering of NWAFU, for the assistance in the GC analysis.

Conflicts of Interest: The authors declare that there are no conflict of interest.

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