

# De novo biosynthesis of aromatic compounds from carboxymethyl-cellulose by microbial co-culture strategy

**Miao Cai**

Ministry of Education, Nankai University

**Jiayu Liu**

Ministry of Education, Nankai University

**Xiaofei Song**

Zhejiang University of Technology

**Hang Qi**

Ministry of Education, Nankai University

**Yuanzi Li**

Beijing Technology and Business University (BTBU)

**Zhenzhou Wu**

Ministry of Education, Nankai University

**Haijin Xu**

Ministry of Education, Nankai University

**Mingqiang Qiao** (✉ [qiaomq@nankai.edu.cn](mailto:qiaomq@nankai.edu.cn))

Ministry of Education, Nankai University

---

## Research Article

**Keywords:** *Saccharomyces cerevisiae*, co-culture, carboxymethyl-cellulose, p-coumaric acid, caffeic acid

**Posted Date:** February 18th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1334289/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Many aromatic compounds have attracted much interest as value-added chemicals and are widely used as additives in foods and cosmetics. Although there are many reports on heterogeneous synthesis of aromatic compounds in microorganisms, most of these studies used glucose as the sole carbon source. Lignocellulose is the most abundant renewable resource in the world. It is an economical and environmentally friendly alternative when using cellulose as a carbon source to produce high value-added products.

**Results:** In the present study, carboxymethyl-cellulose (CMC) was utilized as the sole carbon source, and a metabolically engineered *Saccharomyces cerevisiae* strain SK10-3, which can degrade CMC, was co-cultured with other metabolically engineered *S. cerevisiae* strains to achieve the *de novo* biosynthesis of multiple aromatic compounds from CMC. After a series of optimization, 71.71 mg/L *p*-coumaric acid (*p*-CA) and 16.91 mg/L caffeic acid were obtained from 30 g/L CMC medium.

**Conclusion:** *De novo* biosynthesis of multi aromatic compounds from lignocellulose, a renewable and inedible carbon source, via a co-cultivation strategy provides favorable support for the biosynthesis of more high value-added products from more economical substrates. Furthermore, the multi-strain co-culture strategy could be an alternative solution to improve the final titer of target products and apply it to industrial production.

## Background

Aromatic compounds, which generally contain one or more benzene rings, including aromatic amino acids and many derivatives, are produced from the shikimate pathway. These compounds have been widely used as additives in pharmaceuticals, dye manufacturing, foods, and cosmetics. Most aromatic compounds are extracted from plants as secondary metabolic compounds or are chemically synthesized. However, the long culture period of plants and complex extraction procedures lead to low production and high cost of the synthesized compounds [1]. To meet market demand, researchers have focused their attention on heterologous biosynthesis of aromatic compounds in multiple microorganisms by synthetic biology and metabolic engineering systems [2–7]. In our previous studies, an engineered strain of *Saccharomyces cerevisiae* was used as a host cell to improve the yield of aromatic amino acids and produce multi aromatic compounds [8, 9]. The use of *S. cerevisiae* as a cellular platform for the biosynthesis of aromatic compounds has many advantages. First, as a eukaryotic model organism, *S. cerevisiae* is genetically tractable, and its biochemical pathways can be easily manipulated by metabolic engineering. Second, it has also been proved to be a robust cell that can be used for industrial-scale fermentation and has been widely used in the brewing industry [10] and pharmaceutical industry [11–13] as GRAS (generally recognized as safe). Third, *S. cerevisiae* is also a suitable vector for expressing exogenous genes of higher eukaryotes.

Although there are many studies on the production of aromatic compounds, most of them have utilized glucose as the sole or main carbon source. Unlike starch materials, which are costly and occupy limited agricultural land, lignocellulose is the most abundant renewable resource in the world [14, 15]. Lignocellulose is environmentally friendly and greatly reduces costs, if the bioconversion of lignocellulose into high value-added products is an efficient process. However, as a biomass with high polymer content, the biodegradation of lignocellulose is restricted unless it is pretreated to reduce cellulose crystallinity and lignin content. Following the advances in metabolic engineering, enzyme engineering, and synthetic biology, several microbial strains were engineered and recombined to possess or enhance the function of cellulose degradation and to further yield industrial products, especially for biofuels. In our previous laboratory studies, we constructed a series of cellulase-expressing yeast strains through a *POT1*-mediated  $\delta$ -integration strategy to yield bioethanol in a cellulose-based medium [16]. Liu et al. improved the cellulolytic ability of a recombinant *S. cerevisiae* strain by optimizing the ratio of four cellulases in a cell surface display system, and the ethanol titer was increased to 60% [17]. Gaida et al. achieved the biotransformation from crystalline cellulose to n-butanol by introducing the n-butanol synthesis pathway into the cellulose-degrading bacteria *Clostridium cellulolyticum* [18].

While, the complex processes of cellulase expression, cellulose decomposition, and fermentation of high value-added products will increase the metabolic burden of a single strain. Therefore, we used a co-culture system to achieve the bioconversion of carboxymethyl-cellulose (CMC) to aromatic compounds. The simultaneous cultivation of two or more cell populations is termed as co-culture technology. Different from natural mixed culture, co-culture technology is used to study interactions between species, generate new products, or improve yield through a purposeful and conscious use of high-throughput technology and bioinformatics platform [19–21]. For example, Sun et al. constructed a bacterial co-culture of *Rhodococcus* sp. WB9 and *Mycobacterium* sp. WY10; the co-culture showed improved degradation and mineralization of phenanthrene because of the metabolic interactions between these two bacterial species [22]. Oh et al. isolated two new antimicrobial cyclic depsipeptides, namely emericellamides A and B (1 and 2), during co-cultivation of the marine-derived fungus *Emericella* sp. and the marine actinomycete *Salinispora arenicola* [23]. The cellulolytic bacterial species *Clostridium thermocellum* was co-cultured with the butanol-producing strain *Clostridium saccharoperbutylacetonicum* to achieve the conversion from crystalline cellulose to butanol efficiently [24].

Thus, in the present study, to achieve the biotransformation of CMC to multi aromatic compounds, the co-culture system was used that involved the CMC-degrading strain *S. cerevisiae* SK10-3 [25] and an engineered *S. cerevisiae* strain NK-B2 [8], which could produce a high amount of tyrosine, a precursor of many aromatic compounds. Here, *p*-coumaric acid (*p*-CA) was obtained, which is an important precursor of many flavonoids and stilbenes. After a series of optimization, the bioconversion of CMC to *p*-CA was achieved, and the titer of *p*-CA was 71.71 mg/L from 30 g/L CMC. Subsequently, the synthesis of caffeic acid in this co-culture system was attempted. Caffeic acid is a natural phenolic compound and can be derived from *p*-CA. It has received increasing attention because of its many pharmacological activities [26–28]. Moreover, the derivatives of caffeic acid, such as chlorogenic acid [29], rosmarinic acid [30], and caffeic acid phenethyl ester [31], also possess important medicinal values. However, the long growth

cycle of plants, complicated extraction procedures, and inefficient purification process have severely affected the yield of caffeic acid [32, 33]. In the last decade, many studies have reported on heterogeneous biosynthesis of caffeic acid in microorganisms. Rodrigues et al. obtained 280 mg/L caffeic acid from tyrosine in *Escherichia coli* by using tyrosine ammonia lyase (TAL) from *Rhodotorula glutinis* and CYP199A2 from *Rhodospseudomonas palustris* [34]. *S. cerevisiae* is also an excellent host for the heterogeneous biosynthesis of caffeic acid. Li et al. achieved the *de novo* biosynthesis of caffeic acid from glucose in a tyrosine pathway-engineered *S. cerevisiae* [9]. Recently, Zhou et al. maintained a stable production of precursors by the genomic integration of caffeic acid synthetases under the regulation of a modified *GAL* system and further eliminated the feedback inhibition to ensure that a sufficient amount of carbon source is directed toward caffeic acid synthesis. Finally, they reported the highest titer of caffeic acid biosynthesis in *S. cerevisiae* [35]. Besides, Zhang et al. reported the production of caffeic acid from glucose and xylose by engineered *E. coli*, wherein glucose was the main carbon source [36].

In the present study, 16.91 mg/L caffeic acid was synthesized from a medium containing 30 g/L CMC in a multi-strain co-culture system. Despite the production was still low, the possibility of conversion of lignocellulose to aromatic compounds was confirmed. The present study provided the foundation for the bioconversion of lignocellulose, the most abundant renewable resource in the world, to more value-added compounds.

## Results

### Co-culture system construction

In previous studies, we performed genetic manipulation on *S. cerevisiae* wild-type strain BY4741, wherein three codon-optimized cellulase genes encoding *Talaromyces emersonii* CBHI, *Trichoderma reesei* EGII, and *Aspergillus aculeatus* BGLI were integrated into the yeast chromosome by a *POT1*-mediated  $\delta$ -integration strategy [25]. A series of recombinant strains with high cellulolytic activity to degrade different cellulosic substrates [Avicel, CMC, and phosphoric acid swollen cellulose (PASC)] were screened. To achieve the biosynthesis of high value-added products from lignocellulose, a highly efficient CMC-degrading strain, SK10-3, was chosen for further engineering.

The TAL-encoding gene from *Rhodobacter capsulatus* was introduced into SK10-3 to synthesis *p*-CA. However, the degrading pathway of CMC in SK10-3 increased its metabolic burden, and the *p*-CA titer of SK10-3b were 2.14 mg/L in 20 g/L glucose medium and only 0.46 mg/L in 10 g/L CMC medium, which are much lower than the control strain BY4741b (4.98 mg/L) in glucose medium.

To alleviate the metabolic stress of single strain, the *S. cerevisiae* strain NK-B2 without an encoding histone H2A gene *HTZ1* [8] was selected to introduce multi aromatic compound synthetases and co-cultivate with SK10-3. In this co-culture system, CMC was used as the sole carbon source in the medium and was degraded by SK10-3. Following CMC degradation, glucose was released, which was absorbed by SK10-3 and NK-B2, immediately (Fig. 1). To verify whether this strategy is feasible, BY4742a and NK-B2a

strains possessing synthases of betaxanthin were first co-cultured with SK10-3. Due to a longer time period was required for cellulose degradation, we adopted a sequential co-culture strategy. Herein, SK10-3 was first inoculated into the 10 g/L CMC medium for 24 h. Next, BY4742a or NK-B2a was inoculated into the medium in the ratio of 1:1 to SK10-3. After co-incubation of both strains for 48 h, the fermentation broth turned yellow (Fig. 2a), which indicated that BY4742a and NK-B2a were survived in the co-culture system.

After the above experimental confirmation, further biosynthesis of aromatic compounds from CMC was performed. Similar to the previous experiment, NK-B2b, which contained the *TAL* gene from *R. capsulatus*, was inoculated after incubation of SK10-3 for 24 h. During the co-culture process, the growth of both these strains was monitored (Fig. 2b). Although the growth of these strains was limited in the CMC medium, *p*-CA was detected in co-culture samples (Fig. 2c). In the co-culture system, 3.41 mg/L and 5.93 mg/L *p*-CA were accumulated by BY4742b and NK-B2b, respectively.

### **Effect of the inoculum ratio and interval time on *p*-CA production**

Considering that cellulose saccharification by SK10-3 is influenced by incubation time, the changes in glucose content for different inoculum amounts of SK10-3 separately incubated in CMC medium were monitored. As shown in Table 1, the higher the amount of SK10-3 inoculated, the faster was the degradation of CMC. CMC was almost fully saccharified after 36 h of incubation, indicating that if the inoculation interval time exceeds 36 h, NK-B2b will not have adequate carbon source for growth. The inoculum ratio is also an important factor to maintain the balance of bacterial growth and product yield in co-culture systems. Thereby, various ratios of SK10-3 to NK-B2b (3:1, 2:1, 1:1, 1:2, and 1:3) and the interval time (0, 12, and 24 h) were investigated simultaneously. The total inoculum OD<sub>600</sub> of the two engineered strains was 0.1. During the co-cultivation period, the growth was recorded and the production of *p*-CA was detected by HPLC after 120 h of co-culture fermentation (Figs. 3 and 4). Although the biomass was lowest when SK10-3 and NK-B2b were simultaneously inoculated, the highest *p*-CA titer (29.2 mg/L) was observed when these two strains inoculated at the same time with the ratio of 1:2. Additionally, when the ratio of SK10-3 to NK-B2b inoculated simultaneously was 1:1 or the ratio was 1:3 and the interval time was 12 h, the production of *p*-CA was considerable (23.4 and 24.3 mg/L, respectively). The glucose content of the co-culture systems was also monitored (Table S1).

Table 1

Glucose content during SK10-3 mono-culture in 10 g/L CMC medium with different inoculum doses.

Inoculation amount of SK10-3 (OD <sub>600</sub> )	Glucose content (mg/L) during mono-cultivation				
	0 h	12 h	24 h	36 h	48 h
0.075	-	31.98 ± 1.28	6.48 ± 2.04	-	-
0.067	-	29.10 ± 2.40	22.32 ± 1.54	4.50 ± 1.44	-
0.050	-	25.38 ± 1.43	25.56 ± 1.88	2.46 ± 0.91	1.38 ± 0.85
0.033	-	15.54 ± 0.75	26.40 ± 0.81	3.36 ± 1.33	3.72 ± 0.81
0.025	-	13.14 ± 1.18	29.10 ± 2.15	10.02 ± 1.26	4.11 ± 0.75
-, indicates no glucose was detected					

### Optimization Of The Total Supply Of The Carbon Source

To further increase the production of *p*-CA in the co-cultivation system, the CMC content in the medium was increased. We increased the final concentration of CMC to 20 and 30 g/L, as adding too much CMC will make the medium thick and almost gelatinous, which is not conducive to medium preparation and fermentation. Subsequently, the three best conditions in the previous result (Fig. 4) were selected to conduct the co-cultivation experiment in the high-carbon source medium, i.e., SK10-3 and NK-B2b inoculated simultaneously at the ratio of 1:1 or 1:2 or inoculated at an interval time of 12 h at the ratio of 1:3. The growth of these strains and the titer of *p*-CA were measured simultaneously (Fig. 5).

After 120 h of co-cultivation, the *p*-CA titer was 46.55 mg/L in 30 g/L CMC medium when SK10-3 and NK-B2b were inoculated simultaneously in the ratio of 1:2 (Fig. 5c), although the lowest level of growth was recorded. This might be probably due to the high consistency of the medium with high CMC content, which limited the efficiency of SK10-3 to degrade CMC. High concentrations of glucose were detected in these co-culture samples after 96 h of culture, and CMC was almost completely decomposed and utilized after 168 h of co-cultivation (Table S2). The *p*-CA titer after 168 h of fermentation was also monitored, and the results were very optimistic. The highest *p*-CA titer was detected in the sample of SK10-3 and NK-B2b simultaneously inoculated at the ratio of 1:2. The production of *p*-CA was increased by 54% (71.71 mg/L) as compared to the titer at 120 h (Fig. 5d). Moreover, to determine the ratio of these two strains after co-culture fermentation, the spotting plate experiment was performed because NK-B2b alone cannot survive in CMC medium. In the optimum co-culture condition, the proportion of NK-B2b was 62% after fermentation.

### De novo biosynthesis of caffeic acid from CMC

To confirm whether the bioconversion of CMC to more high value-added compounds could be achieved under this co-culture strategy, the production of caffeic acid was assessed; caffeic acid has a high medicinal value and is biosynthesized with *p*-CA as a precursor. The strain NK-B2c, which possesses the codon-optimized caffeic acid synthase gene *HpaB* from *Pseudomonas aeruginosa* and *HpaC* from *Salmonella enterica* [35, 37] on the basis of NK-B2b, was co-cultured with SK10-3 under the optimum co-culture condition we screened before. After 168 h of fermentation, 8.33 mg/L caffeic acid was detected by HPLC (Fig. 6) from 30 g/L CMC medium without any precursor addition. Thus, the *de novo* biosynthesis of caffeic acid from lignocellulose was achieved.

### **Improving the production of caffeic acid by a multi-strain co-culture system.**

It has been confirmed that the caffeic acid synthases, *PaHpaB* and *SeHpaC*, are highly catalytically efficient when expressed in *S. cerevisiae* [35, 37]. However, in the co-culture system we studied, the titer of caffeic acid was only 8.33 mg/L, and there was a large amount of *p*-CA residues (Fig. 6c). Accordingly, we speculated that under such low-sugar, unfavorable growth conditions, the caffeic acid biosynthesis pathway increases the growth pressure of the NK-B2c strain, whereby limiting the expression of *PaHpaB* and *SeHpaC*. Therefore, we also split the caffeic acid biosynthesis pathway into two strains, one is the NK-B2b strain, which only expresses the *RcTAL* gene, and the other is the NK-B2d strain, which expresses *PaHpaB* and *SeHpaC* genes. To alleviate the metabolic stress of NK-B2c, a multi-strain co-culture system was constructed (Fig. 7a). In this system, SK10-3 still accounted for one-third of the total biomass to meet the glucose supply. To find a balance between the other two strains to maximize the caffeic acid production, we set different inoculation ratios of NK-B2b to NK-B2d (3:1, 2:1, 1:1, 1:2 and 1:3), and detected the final caffeic acid titers (Fig. 7b). After 168 h fermentation, 16.91 mg/L caffeic acid was accumulated while NK-B2b and NK-B2d was inoculated equally, and the residual amount of *p*-CA was considerably reduced.

## **Discussion**

The co-culture strategy is a very important method in industrial production and research. This approach usually divides a complete biosynthetic pathway into separate serial modules and introduces these modules into different strains, thereby reducing the metabolic pressure on a single strain [38]. This strategy is highly significant in terms of use of cheap substrates, increase in product yield, and development of new substances [39]. Previous studies have reported several applications of co-cultivation using different species of microorganisms [40–44]. Several factors such as environmental conditions, nutrient types, and interaction relationships should be considered during the co-cultivation of strains of different species.

In the present study, we first achieved the bioconversion from lignocellulose to aromatic compounds, like betaxanthin, *p*-CA and caffeic acid. Here, the co-culture system was utilized to alleviate the metabolic burden of a single strain. In pure culture system, SK10-3b degraded CMC to release glucose as well as fermented to produce *p*-CA, the *p*-CA titer of SK10-3b, however, was much lower than the control BY4741b

in glucose medium, and there was even almost no *p*-CA produced in CMC medium. Thus, the co-culture system was considered, we assumed that SK10-3 provides available carbon source for another strains to growth and fermentation (Fig. 1). As shown in Fig. 2a, we can easily see that BY4742b and NK-B2b survived, it indicates that the glucose which SK10-3 released could be absorbed and utilized by other strains.

Obviously, SK10-3 as the carbon source donor strain, the inoculation sequence and ratio are important parameters. However, unexpectedly, the outcome of *p*-CA was the highest when both engineered strains were inoculated simultaneously (Fig. 4). And the production of *p*-CA was proportional to the inoculation amount of NK-B2b, except when SK10-3 and NK-B2b were inoculated simultaneously at the ratio of 1:3. This result could be explained by the fact that SK10-3 also absorbed glucose as the carbon source, which consumes part of the glucose pool. The glucose monitoring data shows that glucose will release in large quantities in a short period of time when inoculate more SK10-3 (Table 2). And the longer the inoculation interval, the less glucose remaining, and the less carbon source NK-B2b can utilize (Table S1). Moreover, when SK10-3 was simultaneously inoculated with NK-B2b, the low concentration of SK10-3 led to the inability to meet the demand of glucose and eventually affected the production. Therefore, finding a balance is crucial. Subsequently, to improve the titer of *p*-CA in the co-culture system, the final concentration of CMC was increased to 20 g/L or 30 g/L. And the result was consistent with our objective, the higher concentration of CMC led to more biomass of the strains and higher production; a high titer of *p*-CA (71.71 mg/L) was obtained after 168 h of co-cultivation in the medium containing 30 g/L CMC. The final proportion of NK-B2b was 62% after co-culture fermentation, which indicates that NK-B2b predominates in the co-culture system under the optimum condition.

Eventually, the synthesis of caffeic acid in this co-culture system was attempted due to its high medicinal value. In the present study, we co-cultured SK10-3 and a recombinant strain NK-B2c, which carried the caffeic acid biosynthesis pathway, and finally achieved *de novo* biosynthesis of caffeic acid from CMC without glucose and other precursors added. The titer of caffeic acid was 8.33 mg/L under the optimum co-culture condition we screened earlier. However, such low caffeic acid production was likely due to the metabolic burden of NK-B2c strain. For this reason, we then split the caffeic acid biosynthesis pathway into two strains (NK-B2b and NK-B2d) and constructed a multi-strain co-culture system, to improve the caffeic acid titer (Fig. 7). As expected, the production of caffeic acid was increased, it was 16.91 mg/L, 2.03-fold higher than two strain co-culture system.

Furthermore, considering that many unnecessary pathways consume a large proportion of glucose in this co-culture system, in the future research, an increase in the production of caffeic acid can be achieved through a series of optimization, including weakening the metabolic capacity of SK10-3 to utilize glucose and the ability of producing ethanol to reduce its consumption of glucose. Besides, the fed-batch fermentation method could also be used to optimize the co-culture procedure.

## Conclusion

In this study, multi aromatic compounds were biosynthesized from the most abundant renewable and inedible resource lignocellulose via a co-culture strategy. After a series of optimization, the optimum co-culture condition was obtained: simultaneous inoculation of SK10-3 and NK-B2b or NK-B2c at the ratio of 1:2, and the multi-strain co-culture system can be used to synthesis more complex compounds. This finding was significant for industrial manufacturing, although the final titer was still low to meet industrial production, the present study has provided the foundation for the application of *de novo* biosynthesis of a variety of high value-added products from lignocellulose. The co-cultivation strategy also creates more possibilities for synthetic biology research, wherein more than two strains can be co-cultured to relieve the metabolic burden and produce multi complexes.

## Materials And Methods

### Strains, media, and mono-culture conditions

The metabolically engineered strains SK10-3 and NK-B2 were constructed in our previous studies [8, 25]. All strains and plasmids used in this study are listed in Table 2. Yeast strains were cultured at 30 °C with shaking at 220 rpm in SC medium (2% glucose, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.17% yeast nitrogen base without amino acids (YNB), and 0.13% amino acid mixture). A drop-out synthetic medium without uracil or histone or uracil-histone (SC-ura or SC-his or SC-ura-his) was used to enrich strains carrying plasmids. These strains were then inoculated into 20 mL fresh SC medium or CMC medium (1% CMC, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.17% YNB, and 0.13% amino acid mixture) in 100 mL flasks with an optical density at OD<sub>600</sub> of 0.1 and fermented continuously for 120 h.

**Table 2.** Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source
<b>Strains</b>		
BY4741	<i>MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0</i>	EUROSCARF, Frankfurt, Germany
BY4742	<i>MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0</i>	EUROSCARF, Frankfurt, Germany
NK-B2	BY4742; <i>htz1Δ</i>	[8]
SK10-3	SK1 [BY4741 ( <i>MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</i> ); <i>tpi1::loxP</i> ]; $\delta$ -integration of BGLI, EGII, CBHI genes	[25]
BY4741b	BY4741; pLC-c1	This study
SK10-3b	SK10-3; pLC-c1	This study
BY4742a	BY4742; pLC84	This study
BY4742b	BY4742; pLC-c1	[8]
NK-B2a	NK-B2; pLC84	[8]
NK-B2b	NK-B2; pLC-c1	[8]
NK-B2c	NK-B2b; pLC-c4	This study
NK-B2d	NK-B2; pLC-c4	This study
<b>Plasmids</b>		
pSP-G1	2 $\mu$ m ori, <i>URA3</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$ , Amp <sup>r</sup>	[45]
pLC84	pSP-G1 :: $P_{PGK1}$ - Tyrosine hydroxylase - $T_{CYC1}$ , $P_{TEF1}$ - DOPA dioxygenase - $T_{ADH1}$	[46]
pLC-c1	pSP-G1 :: $P_{PGK1}$ - <i>RcTAL</i> - $T_{CYC1}$ , $P_{TEF1}$ - $T_{ADH1}$	[9]
pLC41	2 $\mu$ m ori, <i>HIS3</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$ , Amp <sup>r</sup>	[47]
pLC-c4	pLC41 :: $P_{PGK1}$ - <i>PaHpaB</i> - $T_{CYC1}$ , $P_{TEF1}$ - <i>SeHpaC</i> - $T_{ADH1}$	This study

### Co-cultivation method

Before inoculation into the co-culture system, the strains were incubated in SC medium or drop-out medium for 24 h to prepare seed culture. The seed culture was centrifuged and washed, and the cell pellets were suspended in S solution (0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.17% YNB, and 0.13% amino acid mixture) and inoculated into CMC medium. In the experiment for verifying the effect of the inoculum ratio and interval

time on *p*-CA production, the seed culture of SK10-3 was inoculated first and mono-cultured for 12, 24, 36, or 48 h, then the NK-B2b seed culture was inoculated with the total inoculum OD<sub>600</sub> of 0.1 and fermented for another 120 h.

## HPLC analysis

The aromatic compounds in this study were quantified by an HPLC instrument (CoMetro 6000, NJ, USA) equipped with an ultraviolet detector (CoMetro 6000 PVW, NJ, USA) and a C18 column (250 mm × 4.6 mm, 5 μm, Agilent). *p*-CA and caffeic acid were detected at 310 nm wavelength (Fig 6). A mixture of 5% acetonitrile and 0.1% trifluoroacetic acid in pure water was used as mobile phase A, while 0.1% trifluoroacetic acid in acetonitrile was used as mobile phase B. A sample volume of 10 μL was injected into the detector, and the flow rate was 1 mL/min. The samples were detected under a 35-min gradient program using the following conditions: 6% to 50% phase B for 13 min, 50% to 98% phase B for 13 min, 98% phase B for 3 min, 98% to 6% phase B for 12 min, and washing with 6% phase B for 4 min.

## Determination of glucose concentration

During the co-cultivation process, the glucose concentration in the medium was monitored by a glucose assay kit (Solarbio® BC2500). Samples were collected every 12 or 24 h, and the glucose concentration was then determined according to the manufacturer's protocol. The readings were measured by a UV-vis spectrophotometer (Jinhua 752, Shanghai, China). Every experiment was performed at least three times.

## Determination of the ratio of the two *S. cerevisiae* strains after co-culture fermentation

A spotting plate experiment was performed to determine the ratio of SK10-3 and NK-B2b after co-culture fermentation. SK10-3 can grow on SC medium and CMC medium, while NK-B2b can survive only on SC medium. After co-culture fermentation, the yeast culture suspension was diluted and spread on a fresh SC medium plate. After 48 h of incubation, a count of 100~300 colonies per plate was considered to be appropriate. Next, 100 colonies were randomly selected and spotted on a fresh CMC medium plate. Finally, the number of surviving colonies on the CMC plates was counted; these colonies belonged to the SK10-3 strain, and the remaining colonies that did not grow on the CMC plates were of the NK-B2b strain. The ratio of these two strains was then accordingly calculated. Every experiment was performed at least three times.

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

MC, HX, and MQ designed the experiments. MC and JL performed the experiments and analyzed the data. MC, JL, XS, HQ, YL, ZW, HX, and MQ discussed the results, commented on the manuscript. MC and MQ wrote the manuscript and approved the submission.

## Funding

This work was supported by the Tianjin Key Research Program of Application Foundation and Advanced Technology (17JCZDJC32200), the National Key Research and Development Program (2018YFA0900100), and the Ph.D. Candidate Research Innovation Fund of Nankai University.

## Availability of data and materials

All the data and materials supporting the findings of this article are included within the article and its additional files.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Palma M, Barbero GF, Piñeiro Z, Liazid A, Barroso CG, Rostagno MA, Prado JM, Meireles MAA: **CHAPTER 2 Extraction of Natural Products: Principles and Fundamental Aspects**. In *Natural Product Extraction: Principles and Applications*. The Royal Society of Chemistry; 2013: 58–88.
2. Liu Q, Liu Y, Chen Y, Nielsen J: **Current state of aromatics production using yeast: achievements and challenges**. *Curr Opin Biotechnol* 2020, **65**:65–74.
3. Braga A, Ferreira P, Oliveira J, Rocha I, Faria N: **Heterologous production of resveratrol in bacterial hosts: current status and perspectives**. *World J Microbiol Biotechnol* 2018, **34**:122.
4. Gottardi M, Reifenrath M, Boles E, Tripp J: **Pathway engineering for the production of heterologous aromatic chemicals and their derivatives in *Saccharomyces cerevisiae*: bioconversion from glucose**. *FEMS Yeast Res* 2017, **17**.
5. Krömer JO, Nunez-Bernal D, Aversch NJ, Hampe J, Varela J, Varela C: **Production of aromatics in *Saccharomyces cerevisiae*—a feasibility study**. *J Biotechnol* 2013, **163**:184–193.
6. Liu X, Ding W, Jiang H: **Engineering microbial cell factories for the production of plant natural products: from design principles to industrial-scale production**. *Microb Cell Fact* 2017, **16**:125.

7. Rodriguez A, Martínez JA, Flores N, Escalante A, Gosset G, Bolivar F: **Engineering *Escherichia coli* to overproduce aromatic amino acids and derived compounds.** *Microb Cell Fact* 2014, **13**:126.
8. Cai M, Wu Y, Qi H, He J, Wu Z, Xu H, Qiao M: **Improving the Level of the Tyrosine Biosynthesis Pathway in *Saccharomyces cerevisiae* through HTZ1 Knockout and Atmospheric and Room Temperature Plasma (ARTP) Mutagenesis.** *ACS Synth Biol* 2021, **10**:49–62.
9. Li Y, Mao J, Liu Q, Song X, Wu Y, Cai M, Xu H, Qiao M: **De Novo Biosynthesis of Caffeic Acid from Glucose by Engineered *Saccharomyces cerevisiae*.** *ACS Synth Biol* 2020, **9**:756–765.
10. Gallone B, Steensels J, Prah T, Soriaga L, Saels V, Herrera-Malaver B, Merlevede A, Roncoroni M, Voordeckers K, Miraglia L, et al: **Domestication and Divergence of *Saccharomyces cerevisiae* Beer Yeasts.** *Cell* 2016, **166**:1397–1410.e1316.
11. Schempp FM, Drummond L, Buchhaupt M, Schrader J: **Microbial Cell Factories for the Production of Terpenoid Flavor and Fragrance Compounds.** *J Agric Food Chem* 2018, **66**:2247–2258.
12. Levisson M, Patinios C, Hein S, de Groot PA, Daran JM, Hall RD, Martens S, Beekwilder J: **Engineering de novo anthocyanin production in *Saccharomyces cerevisiae*.** *Microb Cell Fact* 2018, **17**:103.
13. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, et al: **High-level semi-synthetic production of the potent antimalarial artemisinin.** *Nature* 2013, **496**:528–532.
14. Sun C, Zhang S, Xin F, Shanmugam S, Wu YR: **Genomic comparison of *Clostridium* species with the potential of utilizing red algal biomass for biobutanol production.** *Biotechnol Biofuels* 2018, **11**:42.
15. Jiang Y, Liu J, Jiang W, Yang Y, Yang S: **Current status and prospects of industrial bio-production of n-butanol in China.** *Biotechnol Adv* 2015, **33**:1493–1501.
16. Song X, Liu Q, Mao J, Wu Y, Li Y, Gao K, Zhang X, Bai Y, Xu H, Qiao M: **POT1-mediated  $\delta$ -integration strategy for high-copy, stable expression of heterologous proteins in *Saccharomyces cerevisiae*.** *FEMS Yeast Res* 2017, **17**.
17. Liu Z, Inokuma K, Ho SH, den Haan R, van Zyl WH, Hasunuma T, Kondo A: **Improvement of ethanol production from crystalline cellulose via optimizing cellulase ratios in cellulolytic *Saccharomyces cerevisiae*.** *Biotechnol Bioeng* 2017, **114**:1201–1207.
18. Gaida SM, Liedtke A, Jentges AH, Engels B, Jennewein S: **Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose.** *Microb Cell Fact* 2016, **15**:6.
19. Goers L, Freemont P, Polizzi KM: **Co-culture systems and technologies: taking synthetic biology to the next level.** *J R Soc Interface* 2014, **11**.
20. Andrews JS, Rolfe SA, Huang WE, Scholes JD, Banwart SA: **Biofilm formation in environmental bacteria is influenced by different macromolecules depending on genus and species.** *Environ Microbiol* 2010, **12**:2496–2507.
21. Wintermute EH, Silver PA: **Dynamics in the mixed microbial concourse.** *Genes Dev* 2010, **24**:2603–2614.

22. Sun S, Wang H, Yan K, Lou J, Ding J, Snyder SA, Wu L, Xu J: **Metabolic interactions in a bacterial co-culture accelerate phenanthrene degradation.** J Hazard Mater 2021, **403**:123825.
23. Oh DC, Kauffman CA, Jensen PR, Fenical W: **Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture.** J Nat Prod 2007, **70**:515–520.
24. Nakayama S, Kiyoshi K, Kadokura T, Nakazato A: **Butanol production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4.** Appl Environ Microbiol 2011, **77**:6470–6475.
25. Song X, Li Y, Wu Y, Cai M, Liu Q, Gao K, Zhang X, Bai Y, Xu H, Qiao M: **Metabolic engineering strategies for improvement of ethanol production in cellulolytic *Saccharomyces cerevisiae*.** FEMS Yeast Res 2018, **18**.
26. Rajendra Prasad N, Karthikeyan A, Karthikeyan S, Reddy BV: **Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line.** Mol Cell Biochem 2011, **349**:11–19.
27. Kępa M, Mikłasińska-Majdanik M, Wojtyczka RD, Idzik D, Korzeniowski K, Smoleń-Dzirba J, Wąsik TJ: **Antimicrobial Potential of Caffeic Acid against *Staphylococcus aureus* Clinical Strains.** Biomed Res Int 2018, **2018**:7413504.
28. Ikeda K, Tsujimoto K, Uozaki M, Nishide M, Suzuki Y, Koyama AH, Yamasaki H: **Inhibition of multiplication of herpes simplex virus by caffeic acid.** Int J Mol Med 2011, **28**:595–598.
29. Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, Ahmad F, Babazadeh D, FangFang X, Modarresi-Ghazani F, et al: **Chlorogenic acid (CGA): A pharmacological review and call for further research.** Biomed Pharmacother 2018, **97**:67–74.
30. Petersen M, Simmonds MS: **Rosmarinic acid.** Phytochemistry 2003, **62**:121–125.
31. Murtaza G, Karim S, Akram MR, Khan SA, Azhar S, Mumtaz A, Bin Asad MH: **Caffeic acid phenethyl ester and therapeutic potentials.** Biomed Res Int 2014, **2014**:145342.
32. Xing Y, Peng HY, Zhang MX, Li X, Zeng WW, Yang XE: **Caffeic acid product from the highly copper-tolerant plant *Elsholtzia splendens* post-phytoremediation: its extraction, purification, and identification.** J Zhejiang Univ Sci B 2012, **13**:487–493.
33. Ye JC, Hsiao MW, Hsieh CH, Wu WC, Hung YC, Chang WC: **Analysis of caffeic acid extraction from *Ocimum gratissimum* Linn. by high performance liquid chromatography and its effects on a cervical cancer cell line.** Taiwan J Obstet Gynecol 2010, **49**:266–271.
34. Rodrigues JL, Araújo RG, Prather KL, Kluskens LD, Rodrigues LR: **Heterologous production of caffeic acid from tyrosine in *Escherichia coli*.** Enzyme Microb Technol 2015, **71**:36–44.
35. Zhou P, Yue C, Shen B, Du Y, Xu N, Ye L: **Metabolic engineering of *Saccharomyces cerevisiae* for enhanced production of caffeic acid.** Appl Microbiol Biotechnol 2021, **105**:5809–5819.
36. Zhang H, Stephanopoulos G: **Engineering *E. coli* for caffeic acid biosynthesis from renewable sugars.** Appl Microbiol Biotechnol 2013, **97**:3333–3341.

37. Liu L, Liu H, Zhang W, Yao M, Li B, Liu D, Yuan Y: **Engineering the Biosynthesis of Caffeic Acid in *Saccharomyces cerevisiae* with Heterologous Enzyme Combinations.** *Engineering* 2019, **5**:287–295.
38. Niu FX, He X, Wu YQ, Liu JZ: **Enhancing Production of Pinene in *Escherichia coli* by Using a Combination of Tolerance, Evolution, and Modular Co-culture Engineering.** *Front Microbiol* 2018, **9**:1623.
39. Bader J, Mast-Gerlach E, Popović MK, Bajpai R, Stahl U: **Relevance of microbial coculture fermentations in biotechnology.** *J Appl Microbiol* 2010, **109**:371–387.
40. Ariana M, Hamedi J: **Enhanced production of nisin by co-culture of *Lactococcus lactis* sub sp. *lactis* and *Yarrowia lipolytica* in molasses based medium.** *J Biotechnol* 2017, **256**:21–26.
41. Reis VR, Bassi APG, Cerri BC, Almeida AR, Carvalho IGB, Bastos RG, Ceccato-Antonini SR: **Effects of feedstock and co-culture of *Lactobacillus fermentum* and wild *Saccharomyces cerevisiae* strain during fuel ethanol fermentation by the industrial yeast strain PE-2.** *AMB Express* 2018, **8**:23.
42. Liu C, Hu B, Liu Y, Chen S: **Stimulation of Nisin production from whey by a mixed culture of *Lactococcus lactis* and *Saccharomyces cerevisiae*.** *Appl Biochem Biotechnol* 2006, **131**:751–761.
43. Jiang C, Chen X, Lei S, Shao D, Zhu J, Liu Y, Shi J: **Fungal Spores Promote the Glycerol Production of *Saccharomyces cerevisiae* by Upregulating the Oxidative Balance Pathway.** *J Agric Food Chem* 2018, **66**:3188–3198.
44. Yuan SF, Yi X, Johnston TG, Alper HS: **De novo resveratrol production through modular engineering of an *Escherichia coli*-*Saccharomyces cerevisiae* co-culture.** *Microb Cell Fact* 2020, **19**:143.
45. Partow S, Siewers V, Bjørn S, Nielsen J, Maury J: **Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*.** *Yeast* 2010, **27**:955–964.
46. Mao J, Liu Q, Li Y, Yang J, Song X, Liu X, Xu H, Qiao M: **A high-throughput method for screening of L-tyrosine high-yield strains by *Saccharomyces cerevisiae*.** *J Gen Appl Microbiol* 2018, **64**:198–201.
47. Mao J, Liu Q, Song X, Wang H, Feng H, Xu H, Qiao M: **Combinatorial analysis of enzymatic bottlenecks of L-tyrosine pathway by *p*-coumaric acid production in *Saccharomyces cerevisiae*.** *Biotechnol Lett* 2017, **39**:977–982.

## Figures

### Figure 1

Schematic illustration of the co-culture system of different metabolically engineered *S. cerevisiae* strains for *de novo* biosynthesis of *p*-CA and CA from CMC. In this system, CMC in medium was degraded by cellulolytic enzymes expressed in SK10-3 by the *POT1*-mediated  $\delta$ -integration strategy. The released glucose was assimilated by SK10-3 and another co-culture strain (NK-B2b or NK-B2c) to yield *p*-CA or CA. PEP, phosphoenolpyruvate; EPSP, 5-enolpyruvylshikimate-3-phosphate; Trp, tryptophan; Phe, phenylalanine; Tyr, tyrosine.

## Figure 2

Phenotypes of betaxanthin and *p*-CA production in the co-culture systems in CMC medium. **(a)** Comparison of color phenotypes in mono-culture and co-culture systems. **(b)** Growth curves. **(c)** *p*-CA production in mono-culture and co-culture systems. Three replicates of each sample were used.

## Figure 3

Growth curves of co-culture systems carried out according to different inoculum ratios of SK10-3 to NK-B2b and inoculation at different interval times. Three replicates of each sample were used.

## Figure 4

The *p*-CA production of co-culture systems carried out according to different inoculum ratios of SK10-3 to NK-B2b and inoculation at different interval times. Three replicates of each sample were used.

## Figure 5

Growth curves and *p*-CA production in the carbon source optimization experiment. **(a)** Growth curves in 20 g/L CMC medium. **(b)** Growth curves in 30 g/L CMC medium. **(c)** *p*-CA production after 120 h fermentation. **(d)** *p*-CA production after 168 h fermentation. Three replicates of each sample were used.

## Figure 6

HPLC chromatogram of *p*-CA and caffeic acid. **(a)** Standards of *p*-CA and caffeic acid. **(b)** Co-culture sample of SK10-3 and NK-B2b; peak 1 was *p*-CA obtained from this co-culture system. **(c)** Co-culture sample of SK10-3 and NK-B2c; peak 2 was caffeic acid obtained from this co-culture system.

## Figure 7

**(a)** Schematic illustration of the multi-strain co-culture system. **(b)** CA production and *p*-CA residue after 168 h fermentation in the multi-strain co-culture system with different inoculation ratios of NK-B2b to NK-

B2d. Three replicates of each sample were used.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile.docx](#)