Cofactor engineering for efficient production of α-farnesene by rational modification of NADPH and ATP regeneration pathway in Pichia pastoris

sheng-ling Chen[1], tingshan Liu[1], weiguo Zhang[1], and Jian-Zhong Xu[2]

[1]Jiangnan University
[2]Affiliation not available

October 3, 2022

Abstract

α-Farnesene, an acyclic volatile sesquiterpene, plays important roles in aircraft fuel, food flavoring, agriculture, pharmaceutical and chemical industries. Here, we enhanced α-farnesene production through reconstructing the biosynthetic pathways of NADPH and ATP in Pichia pastoris. First, the native oxiPPP was reconstructed by over-expressing the key enzymes in oxiPPP or/and inactivating glucose-6-phosphate isomerase (PGI), indicating that combined over-expression of ZWF1 and SOL3 improves NADPH supply and thus increasing α-farnesene production while inactivation of PGI was not because of the decreased cell growth. Next, different expression level of heterologous cPOS5 were introduced into P. pastoris, and found that low intensity expression of cPOS5 facilitated α-farnesene biosynthesis. Finally, ATP was increased by overexpression of APRT and inactivation of GPD1. The resultant strain P. pastoris X33-38 produced 3.09±0.37 g/L of α-farnesene in shake-flask fermentation, which was 41.7% higher than that of the parent strain. These results provide a new perspective to construct industrial-strength α-farnesene producer by rational modification of NADPH and ATP regeneration pathway in P. pastoris.

δφαστορ ενγινεερινγ φορ εφφιςιεντ προδυςτιον οφ α-φαρνεσενε βψ ρατιοναλ μοδιφιςατιον οφ ΝΑΔΠΗ ανδ ΑΤΠ ρεγενερατιον πατηωαψ ιν *Πιςηια παστορις*

Sheng-Ling Chen[a], Ting-Shan Liu [a], Wei-Guo Zhang[a], Jian-Zhong Xu [a, *]

[a]The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800#Lihu Road, WuXi 214122, People's Republic of China

[*] Corresponding authors:

Jian-Zhong Xu; E-mail:*xujianzhong@jiangnan.edu.cn*; Tel: +86-510-85329312; Fax: +86-510-85329312

Abstract

α-Farnesene, an acyclic volatile sesquiterpene, plays important roles in aircraft fuel, food flavoring, agriculture, pharmaceutical and chemical industries. Here, we enhanced α-farnesene production through reconstructing the biosynthetic pathways of NADPH and ATP in*Pichia pastoris* . First, the native oxiPPP was reconstructed by over-expressing the key enzymes in oxiPPP or/and inactivating glucose-6-phosphate isomerase (PGI), indicating that combined over-expression of ZWF1 and SOL3 improves NADPH supply and thus increasing α-farnesene production while inactivation of PGI was not because of the decreased cell growth. Next, different expression level of heterologous cPOS5 were introduced into *P. pastoris* , and found that low intensity expression of cPOS5 facilitated α-farnesene biosynthesis. Finally, ATP was increased by overexpression of APRT and inactivation of GPD1. The resultant strain *P. pastoris* X33-38 produced

3.09±0.37 g/L of α-farnesene in shake-flask fermentation, which was 41.7% higher than that of the parent strain. These results provide a new perspective to construct industrial-strength α-farnesene producer by rational modification of NADPH and ATP regeneration pathway in*P. pastoris* .

Keywords

α-Farnesene; *Pichia pastoris* ; Cofactor engineering; Pentose phosphate pathway; NADH kinase

Introduction

α-Farnesene, one of the simplest sesquiterpenes, has an enormous application in nature and industry. For example, α-farnesene works as chemical signaling molecule to signal danger and to implicate the orientation of aphids and termites in nature.[1] In addition, α-farnesene acts as the intermediate to produce biofuel, vitamin E, vitamin K1, squalane and other high value-added products in industry.[2-4]Therefore, α-farnesene has important economic value in agriculture, chemical, bioenergy, medicine, and cosmetics.[1, 3]Since α-farnesene is abundant in plants (e.g., apple and *Artemisia annua*)[1, 5], plant extraction is the major method for producing α-farnesene.[4] However, the weaknesses of plant extraction limit the application in industry, such as the low yield, the high production cost, the limited feedstock and the serious environmental pollution.[3, 5-6] Therefore, researchers turn their attention to use microbial fermentation to produce α-farnesene,[4] and many effective strategies have been used in modifying microorganisms to enhance the biosynthesis of α-farnesene, including enhancing α-farnesene biosynthesis pathway, blocking the downstream α-farnesene biosynthesis pathway, rewriting the central carbon metabolism, compartmentalizing the supply ways of precursors, relieving the cell growth inhibition and optimizing the medium components and culture conditions.[3-4, 7-9] In the previous study, we constructed a α-farnesene high-producing strain*Pichia pastoris* X33-30 by dual regulation of cytoplasm and peroxisomes.[3] P_{CAT1} promoters were replaced with P_{GAP} promoters in X33-30 to obtain strain X33-30*, which produced 2.18 ± 0.04 g/L of α-farnesene in shake flasks. The strain *P. pastoris* X33-30* was enhanced the supply of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Although there are two pathways for producing IPP and DMAPP, i.e., the mevalonate (MVA) pathway and the methylerythritol-4-phosphate (MEP) pathway,[5] however, no fewer than 6 molecules of ATP and NADPH are need to produce 1 molecule of α-farnesene (Fig. 1). The overall stoichiometry of α-farnesene biosynthesis via the MVA pathway is: 9 acetyl-CoA + 9 ATP + 3 H_2O + 6 NADPH + 6 H^+ - 1 α-farnesene + 9 CoA + 6 $NADP^+$+ 9 ADP + 3 Pi + 3 PPi + 3 CO_2.[10] The above equation indicates that the cofactors ATP and NADPH are also important for increasing α-farnesene production except for the precursor acetyl-CoA. And yet, very little research has addressed ATP and NADPH in α-farnesene production.

NADPH acts as cofactor for catalyzing the formation of mevalonate from 3-hydroxy-3-methyl glutaryl coenzyme A (i.e., HMG-CoA) (Fig. 1). Besides, the extra demand of NADPH has been discussed to be responsible for the heterologous protein production. Previous research indicated that NADPH availability is closely related to the yield of biomass and heterologous proteins.[11-12] In addition, NADPH is also used to protect cells against endoplasmic reticulum (ER) stress and oxidative stress.[11, 13] It should be noted that MVA pathway is the major pathway for producing IPP and DMAPP in *P. pastoris* ,[3] and thus 6 molecules of NADPH and 9 molecules of ATP are need to produce 1 molecule of α-farnesene. However, NADH is the predominant reduced cofactor of catabolism rather than NADPH in yeast and bacteria.[4] Thus, increasing the intracellular NADPH level or eliminating NADPH consumption is a common strategy to facilitate NADPH-dependent products, including terpenoid.[14-15] For example, Liu et al. compared the effect of six native enzymes involved in NAPDH regeneration in*Yarrowia lipolytica* and found that mannitol dehydrogenase benefits to increase squalene production.[16] In addition, introduction of a synthetic version of the Entner-Doudoroff pathway from *Zymomonas mobilis* in *E. coli* MG1655 has been shown to be able to increase the NADPH regeneration rate by 25-fold and thus increasing terpenoid production.[17] In *P. pastoris* , there are two inherent routes for NADPH generation, i.e., the oxidative branch of pentose phosphate pathway (oxiPPP) and the acetate biosynthetic pathway.[9, 11, 18] However, the key enzymes in oxiPPP were negatively controlled by NADPH and ATP at the transcriptional and/or the translational level.[11, 19]Although heterogeneous expression of NADH kinase (i.e., POS5, catalyzed NADH to form NADPH) would increase the NADPH regeneration in *P. pastoris* ,[11] NADH plays pivotal roles in ATP regeneration.[18, 20] ATP acts as a key factor for α-farnesene

biosynthesis (Fig. 1), except for as the energy currency in cells.[21] Therefore, the ATP availability is extremely important for cell growth and α-farnesene biosynthesis so that adequate supplies of NADH are need for cell because ATP is mainly produced by NADH oxidation via electron transport phosphorylation (ETP) under aerobic conditions.[22] For these, how to efficiently supply NADPH and ATP already becomes an important research direction in developing a α-farnesene high-producing strain.

In this work, the biosynthetic pathways of NADPH and ATP were rationally reconstructed in an α-farnesene high-producing strain *P. pastoris* X33-30*, which was reconstructed the carbon's metabolic pathways in *P. pastoris* X33, to further increase the α-farnesene production. To do this, the native oxiPPP was firstly reconstructed in strain X33-30* by over-expressing the key enzymes in oxiPPP or/and inactivating the glucose-6-phosphate isomerase in glycolysis. Subsequently, the heterologous POS5 from*S. cerevisiae* was introduced into *P. pastoris* and controlled by different intensity of promoters to further optimize the NADPH supply. Finally, the ATP availability was tried to increase by enhancing the supply of adenosine monophosphate (AMP) for the synthesis of ATP and decreasing the consumption of NADH in shunt pathway. As a result, the resultant strain *P. pastoris* X33-38 produced 3.09±0.37 g/L of α-farnesene after 72 h in shake-flask fermentation. These results demonstrate the effectiveness of increasing the availability of NADPH and ATP in *P. pastoris* for increasing the α-farnesene production and provide a new perspective to construct industrial-strength α-farnesene producer by rational modification of NADPH and ATP regeneration pathway in *P. pastoris* .

Materials and Methods

Strains and Medium

After replacing P_{CAT1} promoters of α-farnesene high-producing strain *P. pastoris* X33-30 with promoter P_{GAP}, strain X33-30*, which was reconstructed the carbon's metabolic pathways in *P. pastoris* X33 was used as a host for gene modification. The above *P. pastoris* strains were cultivated in YPD medium at 30°C, and *P. pastoris* engineered recombinant strains were screened with 100 mg/L zeocin or 500 mg/L geneticin, recovery of selectable markers by Cre/LoxP system. The rich YPD medium containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. The medium YPDA for inducing Cre enzyme expression contains: 20 g/L L-galactose, 10 g/L yeast extract and 20 g/L peptone. *E. coli* JM109 was used for gene cloning with antibiotics (25 mg/L of zeocin, 50 mg/L of kanamycin or 100 mg/L of ampicillin).

Construction of plasmids and strains

Some of the constructed strains and recombinant plasmids in this study were listed in Table 1 and Table 2 respectively. The designed primers were listed in Table S1 of Supporting Information. The integration site of the strain was the P_{GAP} promoter site or the *his4* site. The specific strain construction procedures were included in the Supporting Information. The LoxP sites in the Cre/LoxP system were mutated into Lox71 and Lox66 sites, respectively, to prevent repeated cleavage and recombination by Cre enzyme. The details of DNA manipulation and transformation were described in " Supporting Information".

Shake flask culture conditions and biomass analysis

Preserved strains were cultured in YPD medium at 30 ° C for activation. Then the appropriate amount of activated strain was put into 10 ml liquid medium overnight to become seed medium. Initial OD_{600} after inoculation with 50 mL YPD fermentation medium was 0.15, adjusted to pH 6.0 with 100 mM/L potassium phosphate buffer, the upper layer was overlaid with 10% n-dodecane, and the fermentation was completed after 72 h in a reciprocating shaker at 30 ° C with 100 rpm. Optical density (OD) of *P. pastoris* cells was measured at 600 nm with a spectrophotometer. Dry cell weight was measured as described by Tomas et al[11].

Χυαντιφιςατιον οφ α-φαρνεσενε

The fermentation broth was centrifuged at 12,000 rpm for 10 minutes. Then the upper layer of n-dodecane liquid was filtered, and the yield of a-farnesene could be measured. The detection method of a-farnesene is

as described by Liu et al[3]. The standard α-farnesene, antibiotics and chemicals were purchased from Sigma (Sigma Aldrich, USA).

Quantification of intracellular NADH/NAD$^+$, NADPH/NADP$^+$ and ATP

Strains were cultivated in YPD medium for 24 h or 72 h, and cells were harvested by centrifugation at 4°C for 30 min at 10,000×g and re-suspended in cold PBS buffer to OD_{600}=10. The intracellular NADH/NAD and NADPH/NADP were extracted according to the previously described by Faijes et al. [23], and the intracellular ATP were extracted according to Ni et al.'s reports.[24] Their concentration were measured using NADH/NAD$^+$ Quantification Colorimeteric Kit, NADPH/NADP$^+$ Quantification Colorimeteric Kit and ATP Colorimetric/Fluorometric Assay Kit (BioVision, Inc., Milpitas, CA) according to the manufacturer's instructions, respectively.

Results and Discussion

ὂμβινεδ οερ-εξπρεσσιον οφ ΖΩΦ1 ανδ ΣΟΛ3 ιμπροες τηε ΝΑΔΠΗ συππλψ ανδ τηυς ινςρεασινγ α-φαρνεσενε προδυςτιον ιν*P. pastoris*X33-30*

The oxiPPP is the main inherent route for NADPH generation in *P. pastoris* , which catalyzed by glucose-6-phosphate dehydrogenase (ZWF1), 6-gluconolactonase (SOL3), 6-phosphogluconate dehydrogenase (GND2) and D-ribulose-5-phosphate 3-epimerase (RPE1).[11, 18] In order to increase the NADPH availability for α-farnesene biosynthesis, the key enzymes in oxiPPP were optimized to overexpress in a α-farnesene high-producing strain *P. pastoris* X33-30*. Firstly, we respectively overexpressed the single gene *zwf1* (encoding ZWF1),*sol3* (encoding SOL3), *gnd2* (encoding GND2) and *rpe1*(encoding RPE1) in strain X33-30*, resulted strains X33-30*Z, X33-30*S, X33-30*G and X33-30*R. Compared with the strain X33-30*, strains X33-30*Z and X33-30*S showed the increased NADPH concentration whereas the strains X33-30*G and X33-30*R showed no visible difference in NADPH concentration both at 24 h and at 72 h (Fig. 2a). Correspondingly, strains X33-30*Z and X33-30*S also showed the increased α-farnesene production, increased by about 6.5% and 12.0% as compared with strain X33-30* at 72 h, respectively (Fig. 2b). The similar results were also found in previous researches, in which overexpression of ZWF1 or SOL3 increased the foreign protein production in *P. pastoris*[25-27] and the terpenoid production in *S. cerevisiae* [28-29] because of the increased intracellular NADPH level. ZWF1 and SOL3 catalyzed the first and the second steps of the oxiPPP and were inhibited by NADPH and ATP,[11, 25] which catalyzed the rate-limiting steps in oxiPPP.[26] In addition, the native expression level of*sol3* in *P. pastoris* was low.[30]Therefore, these may be why strain X33-30*Z with overexpression ZWF1 and strain X33-30*S with overexpression of SOL3 increased the NADPH availability and thus increasing α-farnesene production. It should be noted that overexpression of GND2 had no positive effects on increasing the NADPH availability and α-farnesene production (Fig. 2), which was similar with the results reported by Kim et al. [31] and Nocon et al. [26] but different to the results reported by Prabhu and Veeranki [25]. Rebnegger et al.[30] pointed out that *gnd2* shows the high expression level while *sol3* shows the low expression level in*P. pastoris* . Based on these, we speculated that GND2 was not a rate-limiting enzyme and only overexpression of GND2 did not enhance the carbon flux in oxiPPP.

To further analyze the synergetic effects of these key genes in oxiPPP on NADPH and α-farnesene production, we tried out different expression combination ways of these genes looking for the highest performing combos. As can be seen from Fig. 3a, the resulting strain X33-30*ZSGR (i.e., combined overexpression of ZWF1, SOL3, GND2 and RPE1) showed the highest intracellular NADPH level, followed by strain X33-30*ZSG (i.e., combined overexpression of ZWF1, SOL3 and GND2). Interestingly, combined overexpression of GND2 and RPE1 (i.e., strain X33-30*GR) had no obvious effect on increasing NADPH level (Fig. 3a), which was similar to single overexpression of GND2 or RPE1 (Fig. 2a). This may be down to the bottlenecks of the rate-limiting step, which catalyzed by ZWF1 and SOL3.[26] However, it should be noted that the α-farnesene production was not increased with the increase of the intracellular NADPH level in the corresponding strain (Fig. 3b). Among the test strains, strain X33-30*ZS (i.e., strain X33-31) produced the highest α-farnesene (i.e., 2.54±0.21 g/L) in spite of the third highest NADPH level (Fig. 3). In contrast, although the strains X33-30*ZSGR and X33-30*ZSG showed the top two NADPH level (Fig. 3a), they showed the worst α-farnesene

production, even lower than the original strain X33-30* (Fig 2b and Fig. 3b). Nocon et al. [26] and Prabhu and Veeranki [25] also found the similar results, in which combined overexpression of ZWF1, SOL3, RPE1 or/and GND2 would be detrimental to foreign protein production. It may be that excess overexpression of the key enzymes in oxiPPP may imbalance the PPP flux[26] or disturb acetyl-CoA biosynthetic pathway[31] and thus negatively impacting on product formation. Thus, the strain X33-31 was selected to further modify to increase α-farnesene production.

Inactivation of glucose-6-phosphate isomerase and its negative effect on α-farnesene biosynthesis in *P. pastoris* X33-31

Previous research indicated that overexpression of the transcription factor STB5 increased cytosolic NADPH concentrations because STB5 upregulated the expression of most genes in the PPP and repressed the expression of glucose-6-phosphate isomerase-coding gene in glycolysis.[31-32] Maybe since STB5 is a basal regulator of the PPP,[33] overexpression of STB5 did not increase the protopanaxadiol production.[31] PGI (encoded by *pgi*) competes with ZWF1 for glucose-6-phosphate, which catalyzes glucose-6-phosphate to form frucose-6-phosphate (Fig. 1). In order to investigate the effect of PGI on α-farnesene production, the PGI was inactivated in strain X33-31, resulting in strain X33-31ΔP. As a control, strain X33-30*ΔP (i.e., deletion of *pgi* in strain X33-30*) was also done. Unfortunately, inactivation of PGI in strain X33-31 had negative effect on α-farnesene production, in which the resultant strain X33-31ΔP only produced about 5% of α-farnesene compared with the strain X33-31 (0.13±0.06 g/L vs. 2.54±0.21 g/L) (Fig. 4a). In addition, inactivation of PGI increased the intracellular NADPH level but repressed the cell growth and (Fig. 4b, c). In the past, Qin et al. [34]also found that expression of *pgi* controlled by the ultra-low intensity promoter NAT2p in *Saccharomyces cerevisiae* decreased the cell growth and 3-hydroxypropionic acid production. The possible reasons could be that PGI plays an important role in the central carbon metabolism in yeast.[35] In addition, Aguilera & Zimmermann [36] pointed out that inactivation of PGI in*S. cerevisiae* prevents growth on glucose. However, it should be noted that inactivation of PGI in strain X33-30* had little effect on increasing α-farnesene production in spite of the decrease of the cell growth (Fig. 4). There results indicated that inactivation of PGI enforced the carbon flux into PPP and thus increasing the NADPH availability for α-farnesene biosynthesis. Since the surplus NADPH cannot be re-oxidized, the PGI-deficient strain did not grow on glucose.[37] Thus, we speculated the reason the strain X33-30*ΔP did not obviously decrease the cell growth is that more NADPH was used to biosynthesize α-farnesene. The previous results have reinforced this speculation. For example, Fiaux et al.[38] restored the cell growth on glucose of PGI-deficient *S. cerevisiae* mutant by heterologous expression of transhydrogenase UdhA from *E. coli* . However, although the strain X33-30*ΔP showed the increased α-farnesene production as compared with the strain X33-30*, its final titer of α-farnesene was still lower than that of strain X33-31 (2.23±0.18 g/L vs. 2.54±0.21 g/L) (Fig. 4a), indicating that inactivation of glucose-6-phosphate isomerase is not the best strategy to increase α-farnesene production.

Low intensity expression of POS5 from *S. cerevisiae* balances the NADPH/NADH ratio and its promoting α-farnesene biosynthesis in *P.* pastoris

It is well known that the intracellular NADH level is higher than the intracellular NADPH level.[39-41] Previous research indicated that heterologous expression of NADH kinase POS5 provides another source of NADPH except for the oxiPPP in yeast.[11, 28] To further promote the α-farnesene production by optimizing the NADPH supply, we introduced the cPOS5 targeting in the cytosol from *S. cerevisiae* in strain X33-31. Interestingly, the resultant strain X33-32 with gene *cPOS5*controlled by promoter P_{GAP} showed the bad cell growth and α-farnesene production, but it showed the increased productivity of NADPH and α-farnesene (Fig. 5). POS5 catalyzed the NADH to form NADPH, thereby reducing cell energy resources.[11] In addition, excess NADPH in cell would repress the cell growth, glucose consumption and products production.[40, 42] These comments may be an underlying cause of the decreased cell growth and α-farnesene production.

To try to dissolve this problem, we then decreased the expression level of POS5 by replacing P_{GAP} with a series of weak promoters. Based on the previous reports, the relative intensity of the promoters P_{PISI}, P_{GPM1}, P_{MET3}, and P_{PGK1} were 40%, 15˜40%, 13%, and 0˜10% as compared with that of the P_{GAP}, respectively[43-45]. An increased α-farnesene production (i.e., 2.77±0.18 g/L) was obtained in strain X33-35

with gene *cPOS5* controlled by P_{MET3}, which increased by about 9.1% as compared with strain X33-31 (i.e., 2.54±0.21 g/L)(Fig. 5c). Correspondingly, strain X33-35 also exhibited the high intracellular NADPH level (Fig. 5b). In addition, decreasing the expression level of *POS5* restored the cell growth as compared with the strain X33-32 (Fig. 5a), indicating that excess NADPH in cells would be detrimental to cell growth. These results indicated that low intensity expression of cPOS5 in strain X33-31 benefits to maintain the right amount of NADPH for cell growth and α-farnesene production. Although the strain X33-34 exhibited the high NADPH concentration and cell growth (Fig. 5a, b), it should be noted that strain X33-34 did not produce more α-farnesene than that of strain X33-31 (i.e., 2.56±0.26 g/L vs.2.54±0.21 g/L)(Fig. 5c), indicating that another limiting factor hampered the α-farnesene biosynthesis in strain X33-34. As can be seen from Fig. 1, 9 molecules of ATP are need to produce 1 molecule of α-farnesene. ATP is mainly produced by NADH oxidation via ETP under aerobic conditions.[22] Therefore, we speculated that ATP availability is another limiting factor for further increasing α-farnesene production.

Overexpression of adenine phosphoribosyltransferase to increase ATP availability and α-farnesene production in *P. pastoris*

ATP can be synthesized either by substrate level phosphorylation (SLP) or by ETP in aerobic respiring bacteria, and the ETP is the main route for ATP generation using NADH as electron donor.[22, 46]In the process of ETP, AMP or/and ADP was used as the substrate for ATP biosynthesis.[22] Therefore, increasing the AMP or ADP supply could increase the ATP availability in theory. To test this theory, the endogenous adenine phosphoribosyltransferase (APRT) was overexpressed in strain X33-35, resulting in strain X33-37. APRT catalyzes the formation of AMP from adenine and 5-phospho-α-Dribose-1-diphosphate (PRPP),[47] and we found that the intracellular ATP level of strain X33-37 was 9.4% higher than that of strain X33-35 while the intracellular NADH level was slightly lower than that of strain X33-35 (Table 3). Similar results were also found in previous reports, in which the mutated*Corynebacterium glutamicum* with inactivation of AMP nucleosidase showed the increased intracellular ATP level and the decreased intracellular NADH level.[20] The most likely is that more NADH was used for ATP biosynthesis through ETC because of the abundant supply of AMP. Equally unsurprisingly, overexpression of*aprt* gene promoted cell growth and α-farnesene production, the DCW and α-farnesene production of strain X33-37 reached 2.35±0.11 g/L and 2.94±0.25 g/L (Fig. 6), which were 10.3% and 6.1% higher than those of strain X33-35 (i.e., 2.13±0.16 g/L and 2.77±0.18 g/L, respectively), respectively. ATP is a key factor for cell growth and maintenance and controlling intracellular environment,[48] and thus adequate ATP supply could increase biomass. In addition, the biosynthesis of 1 molecule of α-farnesene requires at least 9 molecules of ATP (Fig. 1), thus the increased intracellular ATP level effectively pulled more carbon flux into the α-farnesene biosynthetic pathway, resulting in higher α-farnesene production. These results indicated that overexpression of endogenous APRT is conducive to increase α-farnesene production because it facilitates the AMP biosynthesis and thus increasing the ATP supply.

Deletion of NADH-dependent dihydroxyacetone phosphate reductase elevates intracellular NADH level and the intracellular ATP level and α-farnesene production in *P. pastoris*

NADH plays an important role in maintaining the redox balance and energy generation NADH,[18] it can used as precursor for the regeneration of NADPH and ATP. In order to maintain the abundant supply of NADH, we tried to decrease the NADH consumption in shunt pathway. To do this, we knocked out the NADH-dependent dihydroxyacetone phosphate reductase. (GPD1)-coding gene *gpd1* in strain X33-37, resulting in strain X33-38. GPD1 catalyzes the biosynthesis of glycerol from dihydroxyacetone phosphate and used the NADH as reducing cofactor (Fig. 1).Previous research indicated that the glycerol biosynthetic pathway plays an important role in maintaining the intracellular NADH and NAD^+ level.[49] So obviously, the intracellular NADH level and NADH/NAD^+ ratio in strain X33-38 increased by 11.6% and 28.6% as compared with strain X33-37, respectively (Table 3). Meanwhile, strain X33-38 had certain improvement in both the intracellular NADPH level and the ATP level (Table 3). The α-farnesene production of strain X33-

38 reached 3.09±0.37 g/L after 72 h in shake-flask fermentation, which was 5.1% higher than that of strain X33-37 (i.e., 2.94±0.25 g/L) (Fig. 7a). The DCW of strain X33-38 was also slightly increased as compared with strain X33-37 (i.e., 2.41±0.23 g/L vs. 2.35±0.11 g/L)(Fig. 7b). It is worth noting that strain X33-38 did not accumulate glycerol throughout the fermentation process, which was different from the strain X33-37 (Fig. 7c). GPD1 is a key enzyme in glycerol biosynthesis [50] and He et al.[49] also found the similar results, in which the strain*S. cerevisiae* DRY01 with silencing GPD1 dramatically decreased the glycerol accumulation. These results indicated that deletion of GPD1 not only improves NADH supply but also decreases the carbon flux in shunt pathway, and thus increasing the α-farnesene production.

Conclusions

α-Farnesene is biosynthesized from farnesyl pyrophosphate (FPP) and dimethylallyl pyrophosphate (DMAPP), which are mainly produced through the mevalonate (MVA) pathway in *P. pastoris* [3, 6], and thus 6 molecules of NADPH and 9 molecules of ATP are need to produce 1 molecule of α-farnesene (Fig. 1). Although many effective strategies have been used to increase the α-farnesene production in yeast, for example, in *P. pastoris* [3],*Saccharomyces cerevisiae* [51-52] and*Yarrowia lipolytica* [53-54], these studies mainly focused on modifying the carbon's metabolism pathway to enhance the carbon flux in α-farnesene biosynthetic pathway. Here, we first tried to rationally reconstruct the biosynthetic pathways of NADPH and ATP to further increase the α-farnesene production in a α-farnesene high-producing strain *P. pastoris* X33-30*. The resultant strain*P. pastoris* X33-38 produced 3.09±0.37 g/L of α-farnesene after 72 h in shake-flask fermentation, which is the highest value ever reported as we know it (Table 4).

In *P. pastoris* , oxiPPP is the main pathway for NADPH generation, but overexpression of the all genes in oxiPPP is not a brilliant choice for increasing α-farnesene production because of the disturbance of the carbon flux in the PPP[26] and acetyl-CoA biosynthetic pathway[31]. Combined over-expression of ZWF1 and SOL3 improves the NADPH supply and thus increasing α-farnesene production. Moreover, the intracellular NADPH level can be further increased by heterologous expression of cPOS5 rather than inactivation of PGI, and thus the α-farnesene production reaches to 2.77±0.18 g/L in strain X33-35. As the other key cofactor in α-farnesene production, increasing the ATP supply also plays an important role in promoting α-farnesene production. The strain X33-38 with overexpression of APRT and deletion of NADH-dependent dihydroxyacetone phosphate reductase. To increase the supply of AMP and NADH for ATP generation shows the obvious increased cell growth and α-farnesene production. Therefore, rational modification of NADPH and ATP regeneration pathway plays a vital role in facilitating α-farnesene biosynthesis in *P. pastoris* . These results also provide a new direction and reference to construct NADPH- and/or ATP-dependent high value-added products producing strains.

Author Information

Author

Sheng-Ling Chen: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China

Ting-Shan Liu: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China

Li-Ming Liu: State Key Laboratory of Food Science and Technology, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China

Wei-Guo Zhang: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China

Jian-Zhong Xu: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China

Author Contribution

L.L., W.Z. and J.X. conceived the experiments. S.C. and T.L. designed and performed the experiments and analyzed the data. S.C. and J.X. wrote the paper. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2021YFC2100900), the Top-Notch Academic Programs Project of Jiangsu Higher Education Institutions, the 111 project (Grant number 111-2-06).

Data availability statement: No data are available

References

1. Yang, X., Nambou, K., Wei, L. J., Hua, Q. (2016) Heterologous production of alpha-farnesene in metabolically engineered strains of Yarrowia lipolytica. *Bioresource Technology* , *216* , 1040-1048.

2. Liu, S.-C., Liu, Z., Wei, L.-J., Hua, Q. (2020) Pathway engineering and medium optimization for α-farnesene biosynthesis in oleaginous yeast Yarrowia lipolytica. *Journal of Biotechnology* ,*319* , 74-81.

3. Liu, H., Chen, S. L., Xu, J. Z., Zhang, W. G. (2021) Dual Regulation of Cytoplasm and Peroxisomes for Improved Ay-Farnesene Production in Recombinant Pichia pastoris. *Acs Synthetic Biology* ,*10* (6), 1563-1573.

4. Liu, Y., Wang, Z., Cui, Z., Qi, Q., Hou, J. (2022) Progress and perspectives for microbial production of farnesene. *Bioresour Technol* , *347* , 126682.

5. You, S. P., Chang, H. X., Zhang, C. Y., Gao, L., Qi, W., Tao, Z. P., Su, R. X., He, Z. M. (2019) Recycling Strategy and Repression Elimination for Lignocellulosic-Based Farnesene Production with an Engineered Escherichia coli. *Journal of Agricultural and Food Chemistry* , *67* (35), 9858-9867.

6. Liu, Y. H., Jiang, X., Cui, Z. Y., Wang, Z. X., Qi, Q. S., Hou, J. (2019) Engineering the oleaginous yeast Yarrowia lipolytica for production of alpha-farnesene. *Biotechnology for Biofuels* , *12* (1).

7. Tang, R. H., Wen, Q. F., Li, M. J., Zhang, W., Wang, Z. B., Yang, J. M. (2021) Recent Advances in the Biosynthesis of Farnesene Using Metabolic Engineering. *Journal of Agricultural and Food Chemistry* , *69* (51), 15468-15483.

8. Liu, G. S., Li, T., Zhou, W., Jiang, M., Tao, X. Y., Liu, M., Zhao, M., Ren, Y. H., Gao, B., Wang, F. Q., Wei, D. Z. (2020) The yeast peroxisome: A dynamic storage depot and subcellular factory for squalene overproduction. *Metabolic Engineering* , *57* , 151-161.

9. Meadows, A. L., Hawkins, K. M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R. H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L. S., Dasika, M. S., Murarka, A., Lenihan, J., Eng, D., Leng, J. S., Liu, C. L., Wenger, J. W., Jiang, H. X., Chao, L. L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C. D., Saija, P. R., Wichmann, G., Holmes, V. F., Benjamin, K., Hill, P. W., Gardner, T. S., Tsong, A. E. (2016) Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* , *537*(7622), 694-+.

10. Sandoval, C. M., Ayson, M., Moss, N., Lieu, B., Jackson, P., Gaucher, S. P., Horning, T., Dahl, R. H., Denery, J. R., Abbott, D. A., Meadows, A. L. (2014) Use of pantothenate as a metabolic switch increases the genetic stability of farnesene producing Saccharomyces cerevisiae. *Metabolic Engineering* , *25* , 215-226.

11. Tomas-Gamisans, M., Andrade, C. C. P., Maresca, F., Monforte, S., Ferrer, P., Albiol, J. (2020) Redox Engineering by Ectopic Overexpression of NADH Kinase in Recombinant Pichia pastoris (Komagataella phaffii): Impact on Cell Physiology and Recombinant Production of Secreted Proteins. *Applied and Environmental Microbiology* , *86* (6).

12. Blank, L. M., Lehmbeck, F., Sauer, U. (2005) Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *Fems Yeast Research* , *5* (6-7), 545-558.

13. Grabowska, D., Chelstowska, A. (2003) The ALD6 gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. *Journal of Biological Chemistry* , *278* (16), 13984-13988.

14. Lu, S. R., Zhou, C. Y., Guo, X. N., Du, Z. D., Cheng, Y. F., Wang, Z. Y., He, X. P. (2022) Enhancing fluxes through the mevalonate pathway in Saccharomyces cerevisiae by engineering the HMGR and beta-alanine metabolism. *Microbial Biotechnology* .

15. Zhang, L. T., Zhang, C. H., Xu, R., Yu, W. J., Liu, J. G. (2022) A strategy for promoting carbon flux into fatty acid and astaxanthin biosynthesis by inhibiting the alternative oxidase respiratory pathway in Haematococcus pluvialis. *Bioresource Technology* ,*344* .

16. Liu, H., Wang, F., Deng, L., Xu, P. (2020) Genetic and bioprocess engineering to improve squalene production in Yarrowia lipolytica.*Bioresour Technol* , *317* , 123991.

17. Ng, C. Y., Farasat, I., Maranas, C. D., Salis, H. M. (2015) Rational design of a synthetic Entner-Doudoroff pathway for improved and controllable NADPH regeneration. *Metab Eng* , *29* , 86-96.

18. Nie, Y. S., Huang, M. Z., Lu, J. J., Qian, J. C., Lin, W. L., Chu, J., Zhuang, Y. P., Zhang, S. L. (2014) Impacts of high beta-galactosidase expression on central metabolism of recombinant Pichia pastoris GS115 using glucose as sole carbon source via C-13 metabolic flux analysis. *Journal of Biotechnology* ,*187* , 124-134.

19. Celton, M., Sanchez, I., Goelzer, A., Fromion, V., Camarasa, C., Dequin, S. (2012) A comparative transcriptomic, fluxomic and metabolomic analysis of the response of Saccharomyces cerevisiae to increases in NADPH oxidation. *BMC Genomics* , *13* , 317.

20. Man, Z., Rao, Z., Xu, M., Guo, J., Yang, T., Zhang, X., Xu, Z. (2016) Improvement of the intracellular environment for enhancing l-arginine production of Corynebacterium glutamicum by inactivation of H2O2-forming flavin reductases and optimization of ATP supply.*Metab Eng* , *38* , 310-321.

21. Chung, B. K., Selvarasu, S., Andrea, C., Ryu, J., Lee, H., Ahn, J., Lee, H., Lee, D. Y. (2010) Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast Pichia pastoris for strain improvement. *Microb Cell Fact* , *9* , 50.

22. Zelle, E., Pfelzer, N., Oldiges, M., Koch-Koerfges, A., Bott, M., Noh, K., Wiechert, W. (2021) An energetic profile of Corynebacterium glutamicum underpinned by measured biomass yield on ATP. *Metab Eng* , *65* , 66-78.

23. Faijes, M., Mars, A. E., Smid, E. J. (2007) Comparison of quenching and extraction methodologies for metabolome analysis of Lactobacillus plantarum. *Microb Cell Fact* , *6* , 27.

24. Ni, L., Miao, P., Jiang, J., Wan, F., Li, J., Ai, M., Kong, L., Tu, S. (2022) Glycyrrhiza uralensis promote the metabolism of toxic components of Aconitum carmichaeli by CYP3A and alleviate the development of chronic heart failure. *PLoS One* ,*17* (6), e0270069.

25. Prabhu, A. A., Veeranki, V. D. (2018) Metabolic engineering of Pichia pastoris GS115 for enhanced pentose phosphate pathway (PPP) flux toward recombinant human interferon gamma (hIFN-gamma) production.*Mol Biol Rep* , *45* (5), 961-972.

26. Nocon, J., Steiger, M., Mairinger, T., Hohlweg, J., Russmayer, H., Hann, S., Gasser, B., Mattanovich, D. (2016) Increasing pentose phosphate pathway flux enhances recombinant protein production in Pichia pastoris. *Appl Microbiol Biotechnol* , *100* (13), 5955-63.

27. Nocon, J., Steiger, M. G., Pfeffer, M., Sohn, S. B., Kim, T. Y., Maurer, M., Russmayer, H., Pflugl, S., Ask, M., Haberhauer-Troyer, C., Ortmayr, K., Hann, S., Koellensperger, G., Gasser, B., Lee, S. Y.,

Mattanovich, D. (2014) Model based engineering of Pichia pastoris central metabolism enhances recombinant protein production. *Metab Eng* , *24* , 129-38.

28. Paramasivan, K., Mutturi, S. (2017) Regeneration of NADPH Coupled with HMG-CoA Reductase Activity Increases Squalene Synthesis in Saccharomyces cerevisiae. *J Agric Food Chem* , *65*(37), 8162-8170.

29. Brown, S., Clastre, M., Courdavault, V., O'Connor, S. E. (2015) De novo production of the plant-derived alkaloid strictosidine in yeast.*Proceedings of the National Academy of Sciences of the United States of America* , *112* (11), 3205-3210.

30. Rebnegger, C., Graf, A. B., Valli, M., Steiger, M. G., Gasser, B., Maurer, M., Mattanovich, D. (2014) In Pichia pastoris, growth rate regulates protein synthesis and secretion, mating and stress response.*Biotechnology Journal* , *9* (4), 511-525.

31. Kim, J. E., Jang, I. S., Sung, B. H., Kim, S. C., Lee, J. Y. (2018) Rerouting of NADPH synthetic pathways for increased protopanaxadiol production in Saccharomyces cerevisiae. *Sci Rep* ,*8* (1), 15820.

32. Larochelle, M., Drouin, S., Robert, F., Turcotte, B. (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Molecular and Cellular Biology* , *26* (17), 6690-6701.

33. Cadiere, A., Galeote, V., Dequin, S. (2010) The Saccharomyces cerevisiae zinc factor protein Stb5p is required as a basal regulator of the pentose phosphate pathway. *Fems Yeast Research* ,*10* (7), 819-827.

34. Qin, N., Li, L., Ji, X., Li, X., Zhang, Y., Larsson, C., Chen, Y., Nielsen, J., Liu, Z. (2020) Rewiring Central Carbon Metabolism Ensures Increased Provision of Acetyl-CoA and NADPH Required for 3-OH-Propionic Acid Production. *ACS Synth Biol* , *9* (12), 3236-3244.

35. Zhang, Q., Wang, X., Luo, H., Wang, Y., Wang, Y., Tu, T., Qin, X., Su, X., Huang, H., Yao, B., Bai, Y., Zhang, J. (2022) Metabolic engineering of Pichia pastoris for myo-inositol production by dynamic regulation of central metabolism. *Microb Cell Fact* ,*21* (1), 112.

36. Aguilera, A., Zimmermann, F. K. (1986) Isolation and molecular analysis of the phosphoglucose isomerase structural gene of Saccharomyces cerevisiae. *Mol Gen Genet* , *202* (1), 83-9.

37. Heux, S., Cadiere, A., Dequin, S. (2008) Glucose utilization of strains lacking PGI1 and expressing a transhydrogenase suggests differences in the pentose phosphate capacity among Saccharomyces cerevisiae strains. *FEMS Yeast Res* , *8* (2), 217-24.

38. Fiaux, J., Cakar, Z. P., Sonderegger, M., Wuthrich, K., Szyperski, T., Sauer, U. (2003) Metabolic-flux profiling of the yeasts Saccharomyces cerevisiae and Pichia stipitis. *Eukaryot Cell* , *2* (1), 170-80.

39. Rigoulet, M., Aguilaniu, H., Averet, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X., Larsson, C., Pahlman, I. L., Manon, S., Gustafsson, L. (2004) Organization and regulation of the cytosolic NADH metabolism in the yeast Saccharomyces cerevisiae. *Mol Cell Biochem* , *256-257* (1-2), 73-81.

40. Yukawa, T., Bamba, T., Guirimand, G., Matsuda, M., Hasunuma, T., Kondo, A. (2021) Optimization of 1,2,4-butanetriol production from xylose in Saccharomyces cerevisiae by metabolic engineering of NADH/NADPH balance. *Biotechnol Bioeng* , *118* (1), 175-185.

41. Xu, J. Z., Yang, H. K., Zhang, W. G. (2018) NADPH metabolism: a survey of its theoretical characteristics and manipulation strategies in amino acid biosynthesis. *Critical Reviews in Biotechnology* , *38* (7), 1061-1076.

42. Xu, J. Z., Ruan, H. Z., Chen, X. L., Zhang, F., Zhang, W. G. (2019) Equilibrium of the intracellular redox state for improving cell growth and L-lysine yield of Corynebacterium glutamicum by optimal cofactor swapping. *Microbial Cell Factories* , *18* .

43. Delic, M., Mattanovich, D., Gasser, B. (2013) Repressible promoters - a novel tool to generate conditional mutants in Pichia pastoris.*Microb Cell Fact* , *12* , 6.

44. Stadlmayr, G., Mecklenbrauker, A., Rothmuller, M., Maurer, M., Sauer, M., Mattanovich, D., Gasser, B. (2010) Identification and characterisation of novel Pichia pastoris promoters for heterologous protein production. *J Biotechnol* , *150* (4), 519-29.

45. Vogl, T., Glieder, A. (2013) Regulation of Pichia pastoris promoters and its consequences for protein production. *N Biotechnol* , *30* (4), 385-404.

46. Koch-Koerfges, A., Kabus, A., Ochrombel, I., Marin, K., Bott, M. (2012) Physiology and global gene expression of a Corynebacterium glutamicum DeltaF(1)F(O)-ATP synthase mutant devoid of oxidative phosphorylation. *Biochim Biophys Acta* , *1817* (2), 370-80.

47. Glockzin, K., Meek, T. D., Katzfuss, A. (2022) Characterization of adenine phosphoribosyltransferase (APRT) activity in Trypanosoma brucei brucei: Only one of the two isoforms is kinetically active. *Plos Neglected Tropical Diseases* , *16* (2).

48. Ebert, B. E., Kurth, F., Grund, M., Blank, L. M., Schmid, A. (2011) Response of Pseudomonas putida KT2440 to Increased NADH and ATP Demand.*Applied and Environmental Microbiology* , *77* (18), 6597-6605.

49. He, W. J., Ye, S. C., Xue, T., Xu, S. Y., Li, W. Y., Lu, J. H., Cao, L. Y., Ye, B. Y., Chen, Y. Q. (2014) Silencing the glycerol-3-phosphate dehydrogenase gene in Saccharomyces cerevisiae results in more ethanol being produced and less glycerol. *Biotechnology Letters* ,*36* (3), 523-529.

50. Albertyn, J., Vantonder, A., Prior, B. A. (1992) Purification and Characterization of Glycerol-3-Phosphate Dehydrogenase of Saccharomyces-Cerevisiae. *Febs Letters* , *308* (2), 130-132.

51. Wang, J. H., Jiang, W., Liang, C. J., Zhu, L. H., Li, Y. R., Mo, Q., Xu, S., Chu, A., Zhang, L., Ding, Z. Y., Shi, G. Y. (2021) Overproduction of alpha-Farnesene in Saccharomyces cerevisiae by Farnesene Synthase Screening and Metabolic Engineering. *Journal of Agricultural and Food Chemistry* , *69* (10), 3103-3113.

52. Yang, X., Liu, J., Zhang, J., Shen, Y., Qi, Q., Bao, X., Hou, J. (2021) Quorum sensing-mediated protein degradation for dynamic metabolic pathway control in Saccharomyces cerevisiae. *Metab Eng* ,*64* , 85-94.

53. Liu, S. C., Liu, Z. J., Wei, L. J., Hua, Q. (2020) Pathway engineering and medium optimization for alpha-farnesene biosynthesis in oleaginous yeast Yarrowia lipolytica. *Journal of Biotechnology* , *319* , 74-81.

54. Liu, Y. H., Wang, Z. X., Cui, Z. Y., Qi, Q. S., Hou, J. (2021) alpha-Farnesene production from lipid by engineered Yarrowia lipolytica.*Bioresources and Bioprocessing* , *8* (1).

55. Lee, H. J., Choi, J. I., Woo, H. M. (2021) Biocontainment of Engineered Synechococcus elongatus PCC 7942 for Photosynthetic Production of alpha-Farnesene from CO2. *J Agric Food Chem* , *69* (2), 698-703.

56. Lim, H., Park, J., Woo, H. M. (2020) Overexpression of the Key Enzymes in the Methylerythritol 4-phosphate Pathway in Corynebacterium glutamicum for Improving Farnesyl Diphosphate-Derived Terpene Production. *J Agric Food Chem* , *68* (39), 10780-10786.

Figure captions

Φιγυρε 1 - Τηε σχηεματις διαγραμ οφ α-φαρνεσενε βιοσψντηετις πατηωαψς ωιτη ΝΑΔ-ΠΗ ανδ ΑΤΠ ρεγενερατιον πατηωαψ ιν *Πιχηια παστορις*. The pathways of NADPH biosynthesis are shown in blue lines, and the pathways of NADPH catabolism are shown in red lines. The pathways of ATP biosynthesis are shown in green lines, and the pathways of ATP catabolism are shown in pink lines. The key genes were listed in ellipses. Abbreviations: IPP (isopentenyl pyrophosphate), DMAPP (dimethylallyl pyrophosphate), GPP (geranyl pyrophosphate), FPP (farnesyl pyrophosphate).

Φιγυρε 2 – Σcρεενινγ τηε βεστ ενζψμε ιν οξιΠΠΠ φορ α-φαρνεσενε βιοσψντηεσις. (a) Overexpression of the key enzymes in oxiPPP affects the intracellular NADPH level. (b) The effects of overexpression of the key enzymes in oxiPPP on α-farnesene production after 72 h cultivation. The strain X33-30* was used as the control (Grey bar), and the best strain is shown in red bar. These data represent average values and standard deviations achieved from three independent experiments.

Φιγυρε 3 – Οπτιμιζατιον οφ τηε εξπρεσσιον cομβινατιον ωαψc οφ τηε ενζψμες ιν οξιΠΠΠ φορ α-φαρνεσενε βιοσψντηεσις. (a) The effects of the different expression combination ways on the intracellular NADPH level. (b) The effects of the different expression combination ways on α-farnesene production after 72 h cultivation. The strain X33-30*S was used as the control (Grey bar), and the best strain is shown in red bar. These data represent average values and standard deviations achieved from three independent experiments.

Φιγυρε 4 – Ιναcτιατιον οφ ΠΓΙ1 νεγατιελψ αφφεcτc α-φαρνεσενε βιοσψντηεσις. (a) α-Farnesene titers. (b) Dry cell weight (DCW). (c) The intracellular NADPH level at 24 h and 72 h. The strains X33-30* and X33-31 were used as the control (Grey bar). These data represent average values and standard deviations achieved from three independent experiments.

Φιγυρε 5 - Οπτιμιζατιον οφ τηε εξπρεσσιον λεελ οφ cΠΟΣ5 φορ α-φαρνεσενε βιοσψντηεσις. (a) Dry cell weight (DCW). (b) The intracellular NADPH level at 24 h and 72 h. (c) α-Farnesene titers and productivity. The strain X33-31 was used as the control (Grey bar). These data represent average values and standard deviations achieved from three independent experiments.

Φιγυρε 6 –Οερεξπρεσσιον οφ ΑΠΡΤ το ινcρεασε ΑΤΠ cυππλψ φορ α-φαρνεσενε προδυcτιον. (a) The cell growth of strain X33-37 with overexpression of APRT. (b) The α-farnesene production of strain X33-37 with overexpression of APRT. The strain X33-35 without overexpression of APRT was used as the control. These data represent average values and standard deviations achieved from three independent experiments.

Figure 7 – Inactivation of GPD1 to decrease the NADH consumption in shunt pathway. (a) α-farnesene titers of strains X33-37 and X33-38. (b) DCW of strains X33-37 and X33-38. (c) Glycerol titers of strains X33-37 and X33-38. These data represent average values and standard deviations achieved from three independent experiments.

Table 1. The main strains used in the study

P. pastoris	Characters
X33-30	A α-farnesene producing strain derived from *P. pastoris* X33 by dual regulation of the carbon's metabolic pat
X33-30*	In the X33-30 strain, P_{CAT1} promoters were replaced with P_{GAP} promoters.
X33-31	Strain X33-30* with overexpression of ZWF1 and SOL3
X33-32	Strain X33-31 with overexpression of cPOS5 under controlled by promoter P_{GAP}
X33-33	Strain X33-31 with overexpression of cPOS5 under controlled by promoter P_{PISI}
X33-34	Strain X33-31 with overexpression of cPOS5 under controlled by promoter P_{GPM1}
X33-35	Strain X33-31 with overexpression of cPOS5 under controlled by promoter P_{MET3}
X33-36	Strain X33-31 with overexpression of cPOS5 under controlled by promoter P_{KEX2}
X33-37	Strain X33-35 with overexpression of APRT
X33-38	Strain X33-37 with inactivation of GPD1

Table 2. The main plasmids used in the study

Plasmids
PGAPZA
Ppic3.5k

Plasmids
PGAP-Z PPISI-Z PGPM1-Z PMET3-Z PPGK1-Z
PGAP-1 PGAP-2 PGAP-3 PGAP-4 PGAP-5 PPISI-1 PGPM1-1 PMET3-1 PPGK1-1 APRT-1 Pcas9-PG1-sg Pcas9-GPD1-

Ταβλε 3. ὃμπαρισον οφ ιντραςελλυλαρ νυςλεοτιδες ςονςεντρατιονς ιν *Π. παστορις* στραινς (μμολ/(γ Δ˜Ω» [a]

Strains	NADH	NAD^+	NADH/NAD^+	NADPH	$NADP^+$	NADPH/$NADP^+$	ATP
X33-30*	4.95±0.12	11.29±0.98	0.44	0.053±0.005	0.217±0.015	0.24	5.98±0.34
X33-31	4.68±0.35	12.03±1.12	0.39	0.073±0.005	0.194±0.017	0.38	5.19±0.45
X33-35	3.19±0.24	13.76±1.24	0.23	0.075±0.007	0.186±0.009	0.40	3.07±0.42
X33-37	2.93±0.31	14.21±1.38	0.21	0.073±0.009	0.199±0.015	0.37	3.36±0.23
X33-38	3.27±0.04	13.63±1.09	0.27	0.077±0.004	0.186±0.018	0.41	4.03±0.37

[a] The cells cultured in YPD medium for 24 h in shake flasks were used for analysis.

All data are meaning values of three determinations of three independent experiments with ± SD.

Ταβλε 4. Οεριεω ον τηε προδυςτιον οφ α-φαρνεσενε

Strains	Strains	Culturing methods	Carbon source	Final titers (g/L)	Productivity (g/L/h) [a]	References
P. pastoris X33-38	*P. pastoris* X33-38	Shake flasks	Glucose	3.09	0.043	This work
S. cerevisiae WH62S	*S. cerevisiae* WH62S	Shake flasks	Glucose	1.48	0.009	[51]
		Fed-batch	Glucose	10.4	0.043	
P. pastoris X33-30	*P. pastoris* X33-30	Shake flasks	Sorbitol+Oleic acid	2.56	0.036	[3]
Synechococcus elongatus SeHL-FN03	*Synechococcus elongatus* SeHL-FN03	Shake flasks	CO_2	5.0×10^{-3}	2.604×10^{-5}	[55]
Yarrowia lipolytica LSC28	*Yarrowia lipolytica LSC28*	Shake flasks	Glycerol	9.0×10^{-2}	7.500×10^{-4}	[53]
		Fed-batch		2.57	0.021	
C. glutamicum JP-2	48-well plates	48-well plates	Glucose	0.28	NA [b]	[56]
Y. lipolytica F5	*Y. lipolytica* F5	Shake flasks	Glucose	1.70	5.903×10^{-3}	[6]
		Fed-batch		25.55	0.089	

[a] Estimated from reference.

[b] NA*:* unavailable.

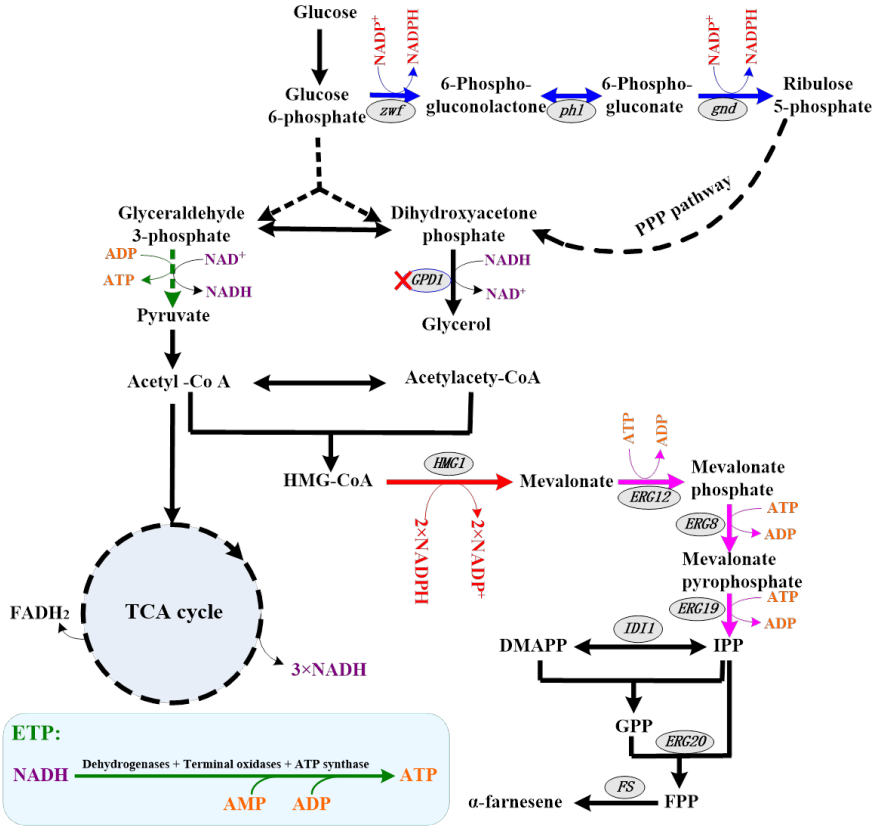


Figure 1

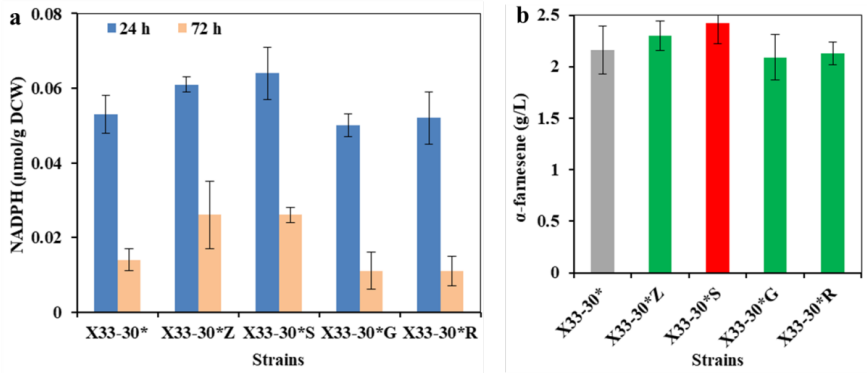
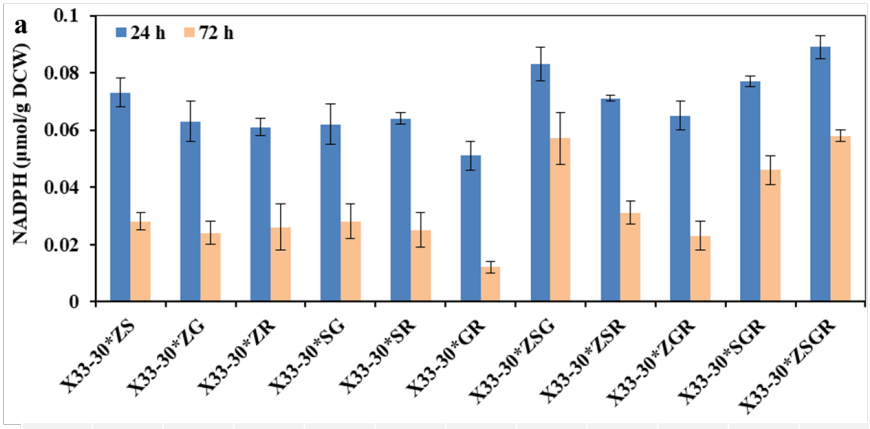
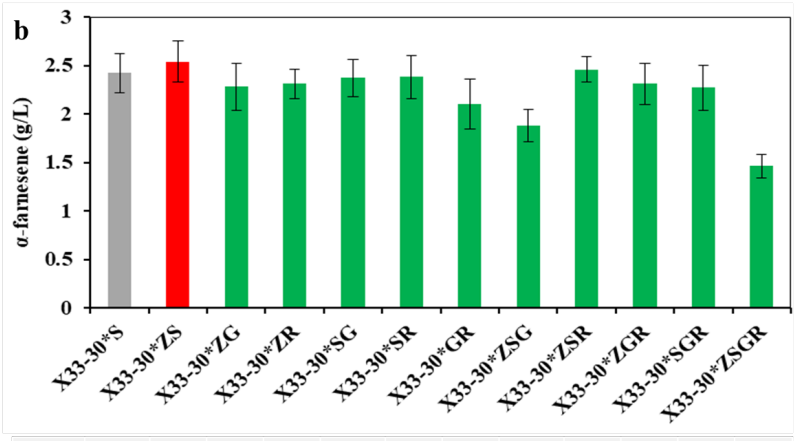


Figure 2



ZWF1	+	+	+				+	+	+		+
SOL3	+			+	+		+	+		+	+
GND2		+		+		+	+		+	+	+
RPE1			+		+	+		+	+	+	+



ZWF1	+	+	+	+				+	+	+		+
SOL3		+			+	+		+	+		+	+
GND2			+		+		+	+		+	+	+
RPE1				+		+	+		+	+	+	+

Figure 3

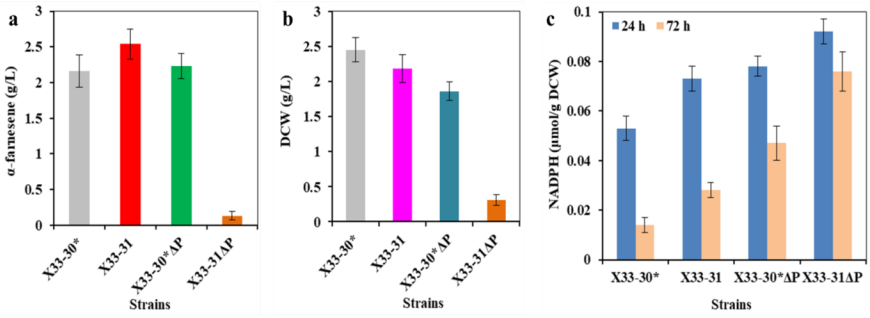
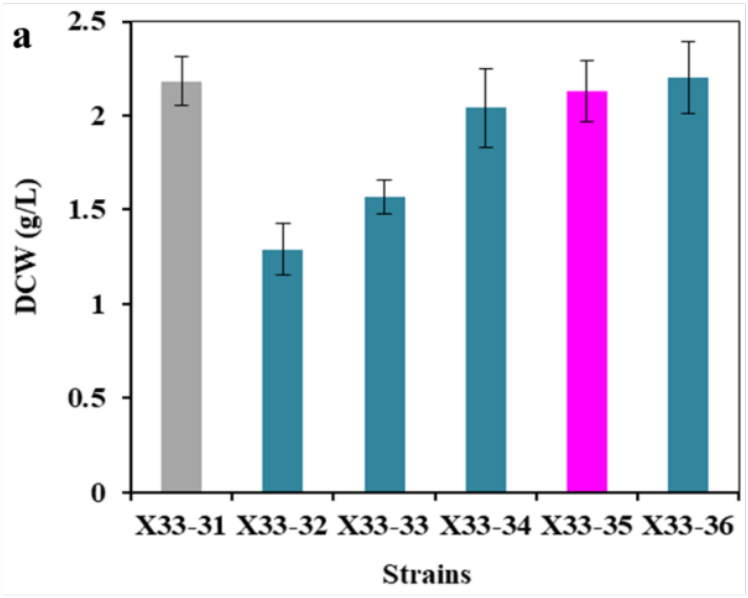
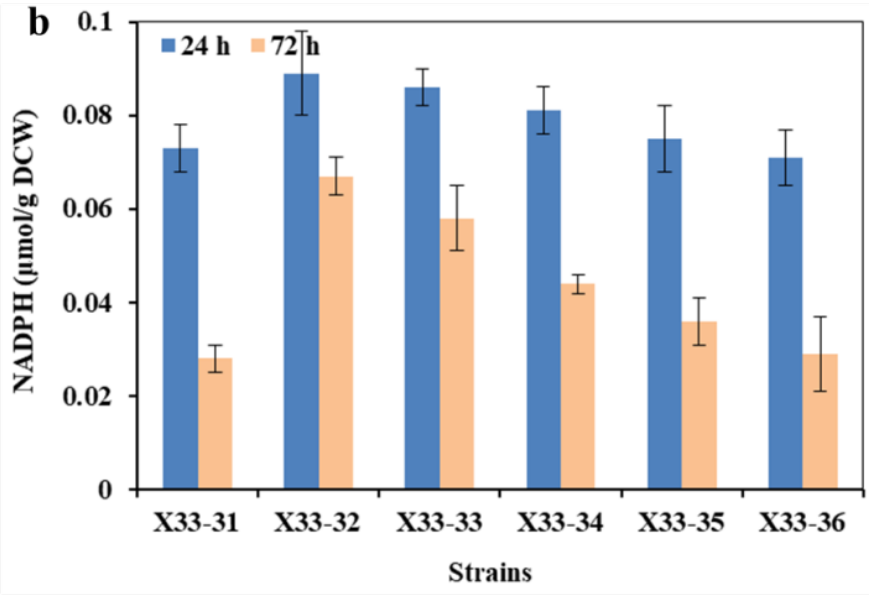


Figure 4





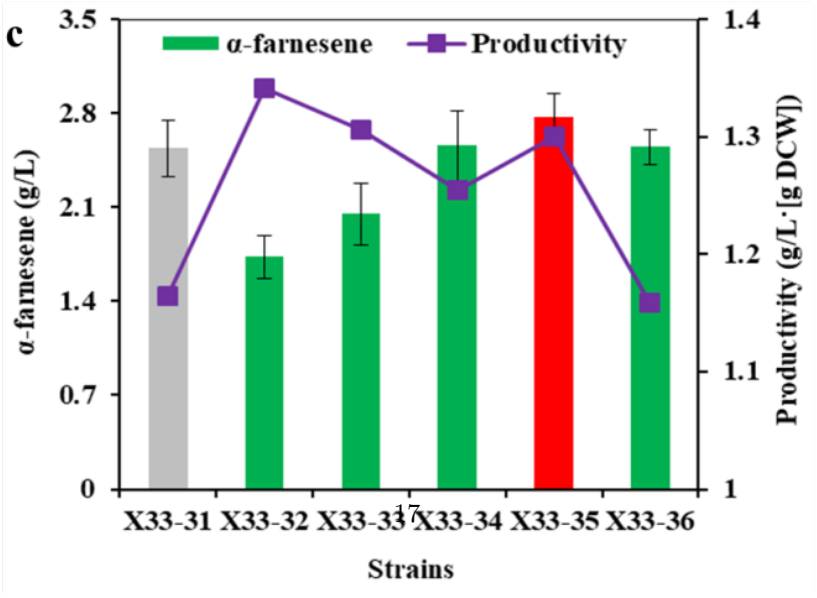


Figure 5

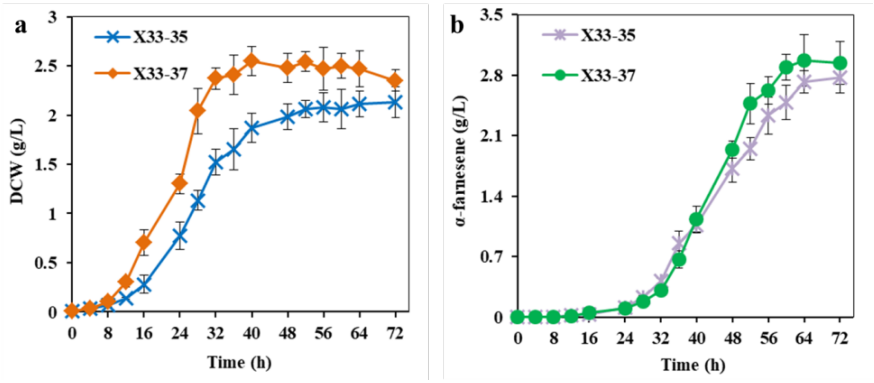


Figure 6

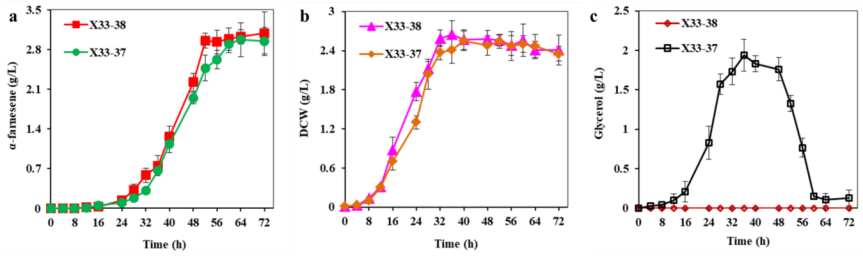


Figure 7