

Modification of an Engineered *Escherichia Coli* by a Combinatorial Strategy to Improve 3,4-Dihydroxybutyric Acid Production

Yidi Liu

East China University of Science and Technology

Xinlei Mao

East China University of Science and Technology

Baoqi Zhang

East China University of Science and Technology

Jinping Lin (✉ jplin@ecust.edu.cn)

East China University of Science and Technology <https://orcid.org/0000-0002-6788-1602>

Dongzhi Wei

East China University of Science and Technology

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Abstract

Objectives: 3,4-Dihydroxybutyric acid (3,4-DHBA) is a multi-functional C4 platform compound with wide applications in the synthesis of materials and pharmaceuticals. Currently, although the biosynthetic pathway for the production of 3,4-DHBA has been developed, low productivity still hampers its use on large scales. Here, a non-natural four-steps biosynthetic pathway was established in recombinant *E. coli* with a combinatorial strategy.

Results: Firstly, several aldehyde dehydrogenases (ALDHs) were screened and characterized for catalyzing the dehydrogenation of 3,4-dihydroxybutanal (3,4-DHB) to 3,4-DHBA through *in vitro* enzyme assays. Secondly, a recombinant *E. coli* was successfully constructed to generate 3,4-DHBA from D-xylose by introducing the pathway containing *BsGDH*, *YagF*, *PpMdlC* and ALDH into *E. coli* with 3.04 g/L 3,4-DHBA obtained. Then, disruption of competing pathways by deleting *xyIA*, *ghrA*, *ghrB* and *adhP* genes contributed to increase the accumulation of 3,4-DHBA by 87%. Final, fusion expression of *PpMdlC* and *YagF* resulted in an enhancement of 3,4-DHBA titer (7.71 g/L), as the highest titer reported so far.

Conclusions: These results showed that deleting competing pathways and constructing fusion protein could significantly improve the 3,4-DHBA titer in engineered *E. coli*.

Introduction

3,4-DHBA is an important C4 compound containing hydroxyl and carboxyl groups which could be modified to produce antibiotics(Choi et al.), α - and β -amino acids and peptides(Sang et al. 2010). 3HBL, the lactone of 3,4-DHBA, was also a multi-functional chiral building block for various chiral drugs including antilipemic agent atorvastatin(Brower et al.) (the active ingredients of Lipitor of Pfizer), neurotransmitter L-carnitine(Tetrahedron 1990), HIV proteases inhibitor Amprenavir(Kim E E 1995), dermatological medicine 12-HETE(Corey E J 1978), anti-cancer drug aplystatin(Shieh H M 1982).

Nowadays, 3,4-DHBA has been mainly produced by chemical processes through the reaction between glucose and alkali metal hydroxides, which is catalyzed by H_2O_2 at 70°C for 24 hours(Hollingsworth 1994), or the reaction of hydrocyanation and hydrolyzation from *R*-3-chloro-1,2-propanediol(Inoue et al. 1991). However, these approaches were usually with low yield, harsh reaction conditions, complex purification process of product, high costs as well as environmental pollution. In order to avoid these kinds of problems, efforts have been made to produce 3,4-DHBA by microbial fermentation. Therefore, considerable attentions were attracted by the biosynthesis of 3,4-DHBA from renewable sources.

There is no report on the natural pathway for producing 3,4-DHBA. The first de novo biosynthesis of 3,4-DHBA involved with six-step enzymatic reaction using glucose and donors of acyl-CoA as a substrate was developed in *E. coli*(Liu et al. ; Schweiger and Buckel ; Taguchi et al. 2008). After optimizations by Dhamankar *et al*, 0.7 g/L 3,4-DHBA was achieved(Himanshu et al.). However, too many reaction steps, low catalytic efficiency and yield made it inaccessible to apply to industrial production.

D-xylose has a high proportion in lignocellulose, which is as the most abundant renewable energy in nature(Choi et al. ; Kawaguchi et al. 2016). A novel five-step biosynthetic pathway for generating 3,4-DHBA from D-xylose was constructed in *E. coli*(Wang et al.). In this route, xylose is catalyzed to 3,4-DHBA by dehydrogenations twice, oxidization, dehydration and decarboxylation. In shake flask experiments, the titer of 3,4-DHBA achieved 1.27 g/L, while 0.18 g/L of by-product 1,2,4-butanetriol (BTO) was also obtained. However, this titer was still low for lacking of high-activity enzymes to decarboxylating 2-keto-3-deoxy-D-xylonate and dehydrogenating 3,4-DHB efficiently, and intermediate products were consumed by competing pathways.

Here, a four-steps biosynthetic pathway using D-xylose as a precursor to produce 3,4-DHBA (Fig. 1) was built. Several aldehyde dehydrogenases (ALDHs) were screened and characterized for 3,4-DHBA accumulation. Secondly, the competing pathways were disrupted by deleting *xyIA*, *ghrA*, *ghrB* and *adhP* genes. Finally, a fusion construct containing *PpMdlC* and *YagF* was created to improve 3,4-DHBA titer. This work lays some strategies for achieving high 3,4-DHBA titer accumulation.

Methods And Materials

Strains, plasmids and culture conditions

The *E. coli* BL21(DE3) was used for expressing, purifying target proteins and producing 3,4-DHBA. Strains used in this study were showed in Table 1. The plasmids of pET28a and pACYC184 were used for cloning and expressing the target genes. *E. coli* cells were cultured at 37°C in Luria-Bertani medium added with appropriate concentration antibiotics. And plasmids in this study were showed in Table S1.

Table 1 *E. coli* strains used in this study

Strains	Description	Source
<i>E. coli</i> BL21(DE3)	Expression, purification and production	Our laboratory
E-C4-01	<i>E. coli</i> BL21(DE3) carrying pE01 (pET28a harboring <i>E. coli yagF</i> and <i>P. putida Ppmd1C</i>) and pA01 (pACYC184 harboring <i>E. coli ynf1</i> and <i>B. subtilis gdh</i>)	This study
E-C4-02	<i>E. coli</i> BL21(DE3) carrying pE01 and pA02 (pACYC184 harboring <i>G. oxydans gox0499</i> and <i>B. subtilis gdh</i>)	This study
E-C4-03	<i>E. coli</i> BL21(DE3) carrying pE01 and pA03 (pACYC184 harboring <i>G. oxydans gox1122</i> and <i>B. subtilis gdh</i>)	This study
E-01-C4	<i>E. coli</i> BL21(DE3) $\Delta xylA$ carrying pE01 and pA02	This study
E-02-C4	<i>E. coli</i> BL21(DE3) $\Delta xylA \Delta ghrA$ carrying pE01 and pA02	This study
E-03-C4	<i>E. coli</i> BL21(DE3) $\Delta xylA \Delta ghrA$ carrying pE01 and pA02	This study
E-04-C4	<i>E. coli</i> BL21(DE3) $\Delta xylA \Delta ghrA \Delta ghrB$ carrying pE01 and pA02	This study
E-05-C4	<i>E. coli</i> BL21(DE3) $\Delta xylA \Delta ghrA \Delta ghrB \Delta adhP$ carrying pE01 and pA02	This study
E-05-F4	<i>E. coli</i> BL21(DE3) $\Delta xylA \Delta ghrA \Delta ghrB \Delta adhP$ carrying pE02 (pET28a harboring fusion gene of <i>E. coli yagF</i> and <i>P. putida Ppmd1C</i>) and pA02	This study

Construction of plasmids

The primers sequences used are listed in Table S2. pE01 harboring *yagF* from *E. coli* and *Ppmd1C* from *P. putida* was constructed. The genes of *yagF* and *Ppmd1C* were fusion expressed by cloning and inserting the gene of linker peptide *ER/K α -helix* (Sivaramakrishnan and Spudich 2011) to create a fusion construct. Similarly, pE04, pE05 and pE06 carrying *gdh* from *B. subtilis* and different ALDH genes, respectively.

The disruption of competing pathways

The disruption of competing pathway was accomplished by CRISPR-Cas9. The donor DNA was connected by overlapping PCR from 500 bp upstream and downstream homologous arm. The *E. coli* BL21(DE3) carrying pCas vector was inoculated in 30°C and then added into 10 mM arabinose for inducing the expression of Cas9. When OD₆₀₀ reached 0.5-0.6, the *E. coli* cells were arranged on ice for 30 min. After washed by 10% glycerol sterile three times, it was split into 100 μ L per tube. The competent cells carrying pCas were mixed with 100 ng pTargetT vector and 400 ng donor DNA to have an electro-transformation (2.5 kV, 200 Ω , 25 μ F) with an electrical shock time within 5 ms. Then they were cultivated into 30°C and confirmed by colony PCR.

E. coli cells were cultured in 30°C with addition of 0.5 mM IPTG overnight to eliminate pTargetT series because IPTG could induce the other sgRNA, which targets one PAM site of pTargetT. The pCas vector could be cured by cultivating it in 37°C to remove because its temperature sensitivity.

Protein expression and purification

E. coli cells were cultured at 37°C until the OD₆₀₀ was approximately 0.6-0.8, and then 0.2 mM IPTG was used to induce target proteins expression at 20°C for 16 h. Then the cells were collected by centrifuged at 8000 rpm for 10 min, washed by 0.9% NaCl twice and resuspended in appropriate volume PB solution at pH 7.5 (20 mM Na₂HPO₄ and NaH₂PO₄). The resuspended cells were centrifuged to collect the supernatants for purification after ultrasonication. The supernatants were filtered, loaded in the pre-equilibrated (20 mM imidazole, pH 7.5) Ni-chelating column with and then washed with different concentration of imidazole (20 mM and 50mM, pH 7.5). The bound proteins would be eluted by 250 mM imidazole (pH 7.5). The protein samples were then put into dialysate (20 M PB solution, 200 mM NaCl and 5% glycerol, pH 7.5) overnight, and then identified by 12% SDS-PAGE.

Enzymes activity assay

The *in vitro* enzyme assay of YagF and *PpMdlC* under tandem expression and fusion expression was implemented in 50 mM PB solution at pH 7.5 which contains 20 mM D-xylonate, 10 mM MgCl₂, 1 mM TPP, 1 mM NAD⁺. The reaction was incubated at 30°C for 5 min to allow accumulation of 3,4-DHB produced by 5-fold excess of YagF, *PpMdlC* or 5-fold fusion protein of YagF and *PpMdlC*. The enzyme assay was monitored for the production of NADH at 340 nm after 1 mM aldehyde dehydrogenase Ynel was supplied to the reaction.

The *in vitro* enzyme assay of NAD⁺-dependent aldehyde dehydrogenase activity of Ynel, Gox0499 and Gox1122 was tested in 50 mM PB solution at pH 7.5 and contained 20 mM D-xylonate, 10 mM MgCl₂, 1 mM TPP, 1 mM NAD⁺, 5-fold excess of YagF and *PpMdlC*. The reaction was incubated at 30°C for 5 min to allow accumulation of 3,4-DHB before supplying 1 mM aldehyde dehydrogenase of Ynel, Gox0499 and Gox1122 to the reaction, respectively. This reaction was also monitored for the production of NADH at 340 nm, too.

Whole-cell catalysis for 3,4-DHBA production

Whole-cell catalysis was performed in 20 mL scale, containing 20 g/L of D-xylose, 50 mM PB solution (pH 7.5), 50 g/L of *E. coli* cells, 2 mM TPP, 2 mM NAD⁺, 10 mM Mg²⁺ at 30°C. The products were sampled about 1 mL in every 12 h and analyzed via HPLC.

Analytical methods

Metabolite analysis was performed by HPLC on a Transgenomic 87H3 column, using refractive-index detection (RID). The mobile phase was 0.08 N H₂SO₄ with a flow rate of 0.38 mL/min. The temperatures

of the RID detector and column were 35°C. HPLC-MS was used to distinguishing 3,4-DHBA from the supernatants of reaction (Fig. S1). 3,4-DHBA (C₄H₈O₄) was corresponded to the peak of 119.03 Da under the negative ion mode.

Results And Discussion

Selection of enzymes for the dehydrogenation of 3,4-dihydroxybutanal

The biosynthetic pathway of 3,4-DHBA through D-xylose comprises four steps. Firstly, *BsGDH* from *B. subtilis* was showed capable of catalyzing D-xylose to produce D-xylonate(Li et al.). Then, it is reported that *YagF* from *E. coli* and *PpMdlC* from *P. putida* was able to convert D-xylonate to 2-keto-3-deoxy-D-xylonate and produce 3,4-dihydroxybutanal (3,4-DHB) from 2-keto-3-deoxy-D-xylonate, respectively (Synthetic pathway optimization for improved 1,2,4-butanetriol production 2016).

Finally, *Ynel* from *E. coli* was demonstrated to convert 3,4-DHB to 3,4-DHBA(Wang et al. 2017). However, *Ynel* generates 3,4-DHBA as well as BTO as a byproduct. In order to obtain high active oxidase to minimize the production of BTO, we found two NAD⁺-dependent aldehyde dehydrogenases (*Gox0499* and *Gox1122*) from *G. oxydans* and compared the catalytic activity with *Ynel* *in vitro* enzyme assays. The enzymatic assays of aldehyde dehydrogenases were implemented on cell lysate to calculate the approximate enzymatic activity. *Gox0499* has a highest specific activity of 4.26 U/mg_{protein} compared with *Ynel* (2.67 U/mg_{protein}) and *Gox1122* (2 U/mg_{protein}).

To evaluate their catalytic activity *in vivo*, plasmids of pE01 (carrying *yagF* and *PpmdlC*), pA01 (carrying *ynel* and *gdh*), pA02 (carrying *gox0499* and *gdh*), pA03 (carrying *gox1122* and *gdh*) were constructed to assemble the whole 3,4-DHBA biosynthetic pathway in *E. coli* BL21(DE3). The SDS-PAGE of target proteins of recombinant strain E-C4-01, E-C4-02 and E-C4-03 was showed in Fig. 2A after cultivating and target inducing. *BsGDH*, *YagF* and *PpMdlC* have same expression quantity in three respective strains, but ALDHs were different. *Gox1122* was higher than *Ynel* and *Gox0499*.

Then, strains E-C4-01, E-C4-02 and E-C4-03 were used for the whole-cell catalysis for 3,4-DHBA production. As seen from Fig. 2B, strain E-C4-02 produced approximately 3.04 g/L 3,4-DHBA after 60 h whole-cell catalytic process with 0.56 g/L byproduct BTO. Compared with E-C4-01 and E-C4-03, E-C4-02 has the highest titer of 3,4-DHBA and the lowest titer of BTO. Since *Gox0499* was shown to be the most efficient enzyme for dehydrogenation of 3,4-DHB under a lower protein expression, it was selected for producing 3,4-DHBA.

Disruptions of the competing pathway to improve 3,4-DHBA titer

In order to improve 3,4-DHBA titer from D-xylose, several competing pathways of *E. coli* was deleted by CRISPR/Cas 9. The xylose isomerase encoded by the *xyIA* catalyzes the production of D-xylulose from D-xylose, flowing to the pentose phosphate pathway for bacteria's growth and metabolism. 2-keto-3-deoxy-d-xylonate aldolases, expressed by *yagE* and *yjhG* of Dahms pathway, always consumed intermediate

product 2-keto-3-deoxy-d-xylonate in *E. coli*(Valdehuesa et al. 2014). However, there are no *yagE* and *yjhG* genes of Dahms pathway but two genes of *ghrA* and *ghrB* which encode glyoxylate reductases play the same roles in *E. coli* BL21(DE3). Besides, alcohol dehydrogenase encoded by *adhP* has a strong reduction activity, which has high preference for producing byproduct BTO from 3,4-DHB(Wang et al. 2017).

After knocking these competing genes out, *xylA*-deficient strain E-01-C4, *xylA* and *ghrA* genes double-deficient strain E-02-C4, *xylA* and *ghrB* genes double-deficient strain E-03-C4, *xylA*, *ghrA* and *ghrB* genes triple-deficient strain E-04-C4, and *xylA*, *ghrA*, *ghrB* and *adhP* quadruple-deficient genes strain E-05-C4 were constructed by CRISPR Cas9, respectively. There is no significant difference of expression of target proteins between these engineered strains (Fig. S2). It means that the deletion of *xylA*, *ghrA*, *ghrB* and *adhP* has showed no effects on the expression of the target proteins in the host cell.

E-01-C4 has a 56% higher of titer of 3,4-DHBA than E-C4-02 (Fig. 3). It shows that the deletion of *xylA* gene could minimize the byproduct formation of D-xylulose and increase the D-xylulose flux toward D-xylonate. The titer of 3,4-DHBA produced by E-02-C4, E-03-C4 and E-04-C4 was 5.05, 5.24 and 5.38 g/L, respectively. This result indicates that the knockout of *ghrA* and *ghrB* has increased the titer of 3,4-DHBA but not obvious. 5.69 g/L 3,4-DHBA was produced by *xylA*, *ghrA*, *ghrB* and *adhP* quadruple-deficient genes strain E-05-C4, and the titer of BTO was decreased 0.56 g/L to 0.33 g/L, proving that the disruption of *adhP* was essential for producing 3,4-DHBA. Above all, E-05-C4 had the highest yield, achieving an average of 5.69 g/L, an 87%-fold improvement on production from E-C4-02. And the disruption of these competing pathway could increase the yield of 3,4-DHBA indeed.

Fusion expression of *PpMdlC* and *YagF* to strengthen carbon flux to 3,4-DHBA pathway

PpMdlC was the rate-limited enzyme in this pathway because of lower catalytic activity(Wang et al. 2017; Weiqun et al. 2019), which causing the accumulation of intermediate products and consumption of competing pathway. Multifunctional enzymes could be constructed through the co-expression of fusion proteins(Chen et al. 2016). It is shown that enhancing the spatial proximity of enzymes by constructing fusion proteins can increase catalytic efficiency and the titer of product in multi-enzyme cascade reaction by previous studies (Albertsen et al. 2011; Lu et al. 2006; Ryosuke et al. 2018). Here, a fusion construct containing *PpMdlC* and *YagF* with a 5-nm rigid α -helical ER/K motif was created, and the obtained recombinant plasmid pE02 was co-transformed into *E. coli* BL(DE3) with pA02, generating the engineered strain E-05-F4.

Compared with the engineered stain E-05-C4 harboring plasmid pE01 and pA02, the protein expressions of *PpMdlC* and *YagF* of E-05-F4 were lower than E-05-C4 (Fig. 4A). Meanwhile, there is a new protein showed over 120 kDa, which means that fusion protein containing *PpMdlC* and *YagF* was constructed successfully. The catalytic activity of *PpMdlC* and *YagF* under tandem and fusion expression was compared by monitoring the change of NADH absorbance at 340 nm with D-xylonate as a substrate and

cascading Gox0499. And the catalytic activity of fusion protein containing *PpMdlC* and YagF was only 1.09 U/g_{wet cell} compared to 2.53 U/g_{wet cell} of *PpMdlC* and YagF under tandem expression.

Further comparison of 3,4-DHBA titer was performed between the engineered strains E-05-C4 and E-05-F4. The consumption rate of the substrate D-xylose of these two engineered strains is similar (Fig. 4B). And the 3,4-DHBA titer of E-05-F4 accumulated was always higher and the reaction ended 12 hours earlier than E-05-C4. Compared to the engineered strain E-05-C4, E-05-F4 exhibited higher 3,4-DHBA titer and productivity at decreased enzymatic activity of *PpMdlC* and YagF. The close spatial proximity of *PpMdlC* and YagF might prevent the diffusion of the intermediate 2-keto-3-deoxy-D-xylonate and effectively regulated catalytic efficiency by constructing variants by fusion enzyme.

Conclusion

In this study, we developed a four-step enzymatic reaction pathway in *E. coli* for producing 3,4-DHBA from D-xylose efficiently. Apart from YagF from *E. coli* and *PpMdlC* from *P. putida* in the 3,4-DHBA biosynthetic pathway reported before (Valdehuesa et al. 2014), *BsGDH* from *B. subtilis* was selected to catalyze the reaction from D-xylose to D-xylonate in our route. Besides, aldehyde dehydrogenase Gox0499 from *G. oxydans* which exhibited higher activity than Ynel from *E. coli*^[21] was chosen to catalyze 3,4-DHB to 3,4-DHBA, resulting in higher 3,4-DHBA titer and lower byproduct BTO titer. Then, the recombinant *E. coli* was modified by a combined strategy of deleting competing pathway, as well as fusion expression of *PpMdlC* and YagF. The whole cells of the obtained strain E-05-F4 could produce 7.71 g/L 3,4-DHBA at productivity rate of 3.864 g/ (L*d), as the highest titer reported so far.

Declarations

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Figures

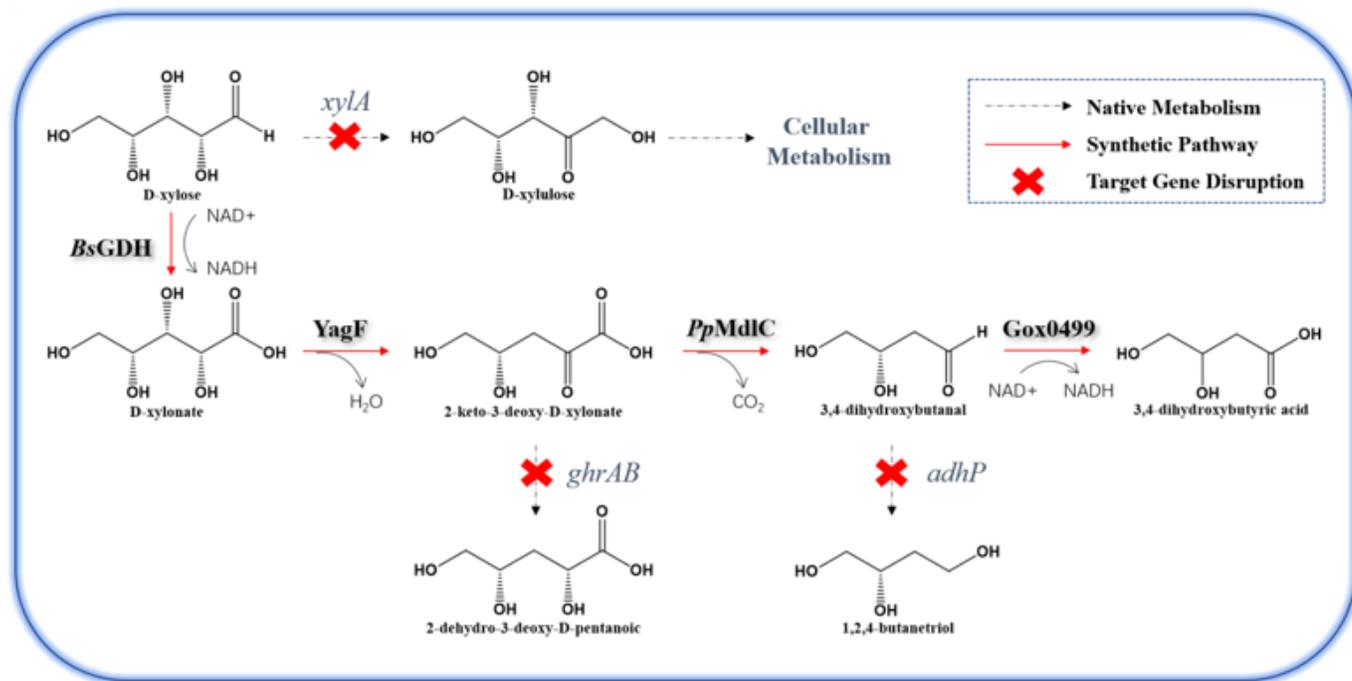


Figure 1

The pathway from xylose to produce 3,4-DHBA in *E. coli*. Enzymes: BsGDH: glucose dehydrogenase; YagF: D-xylonate dehydratase; PpMdIC: decarboxylase; ALDH: aldehyde dehydrogenase. Genes: xylA encoding xylose isomerase; ghrA, ghrB encoding glyoxylate reductases, respectively; adhP encoding ethanol dehydrogenase.

Fig. 2A

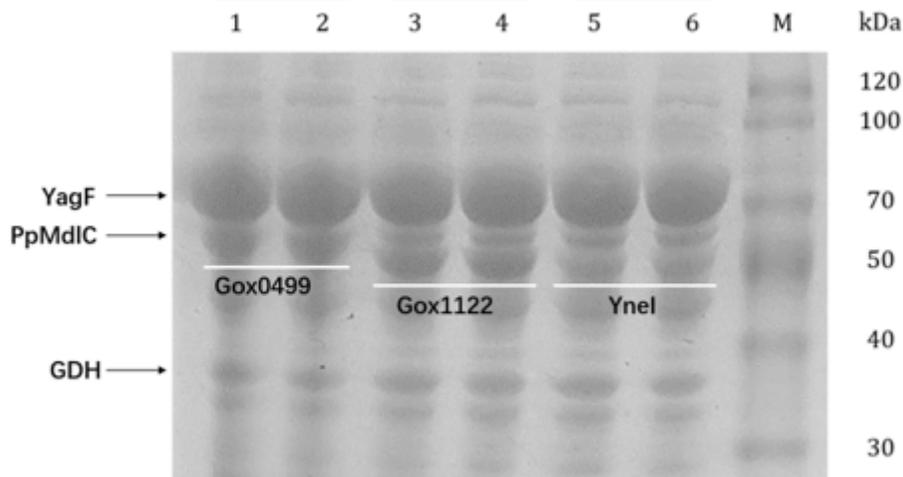


Fig. 2B

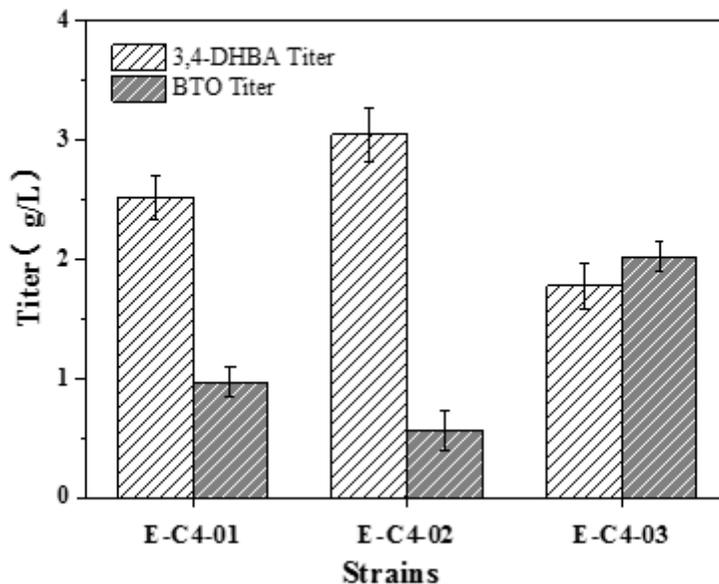


Figure 2

Selection of ALDHs for 3,4-DHBA production. (A) SDS-PAGE analysis of E-C4-01, E-C4-02 and E-C4-03. Lane 1: whole cell of E-C4-02; Lane 2: crude extract of *E. coli* E-C4-02; Lane 3: whole cell of E-C4-03; Lane

4: crude extract of E-C4-03; Lane 5: whole cell of E-C4-01; Lane 6: crude extract of E-C4-01; M: protein molecular weight marker. (B) Several strains were constructed to compare the yield of 3,4-DHBA. The highest 3,4-DHBA yield (3.04 g/L) was produced by E-C4-02 containing BsGDH, YagF, PpMdlC and Gox0499.

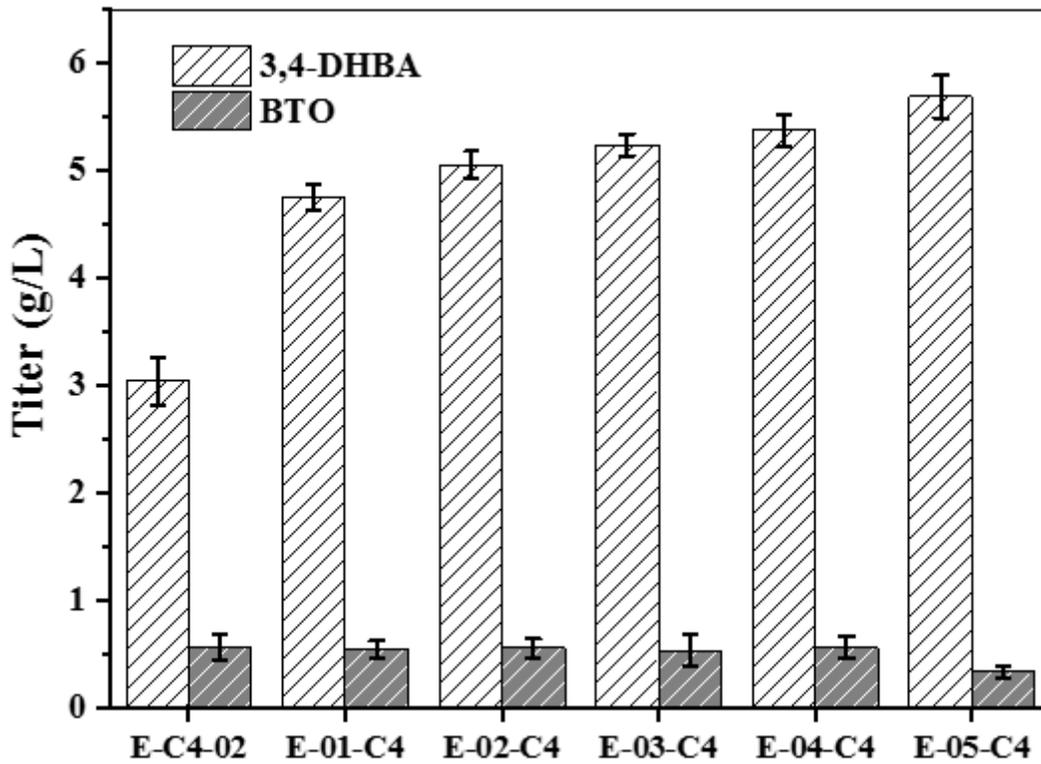


Figure 3

Disruptions of *xylA*, *ghrA*, *ghrB* and *adhP* genes for improving 3,4-DHBA production. Production of 3,4-DHBA by different engineered strains.

Fig. 4A

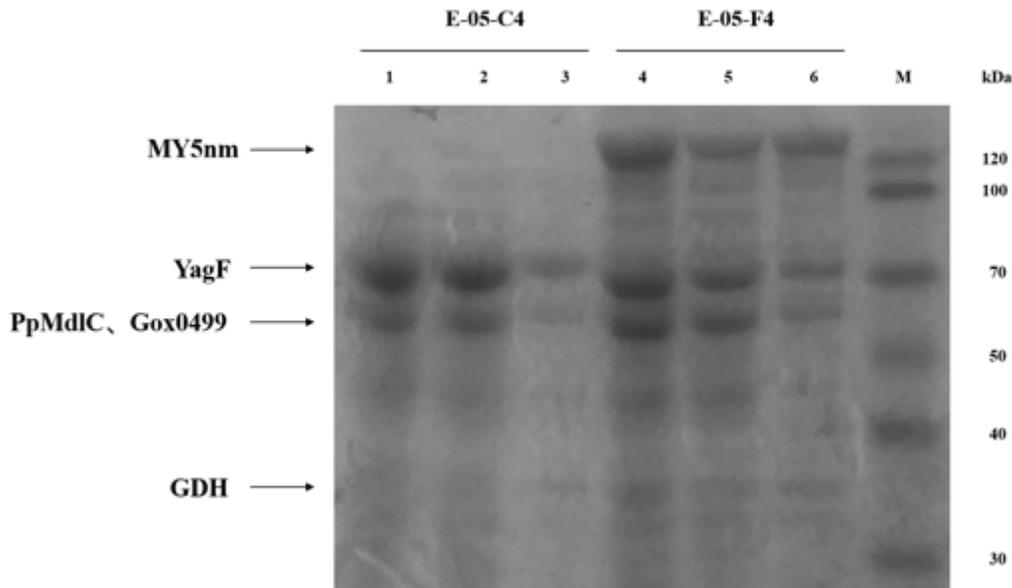


Fig. 4B

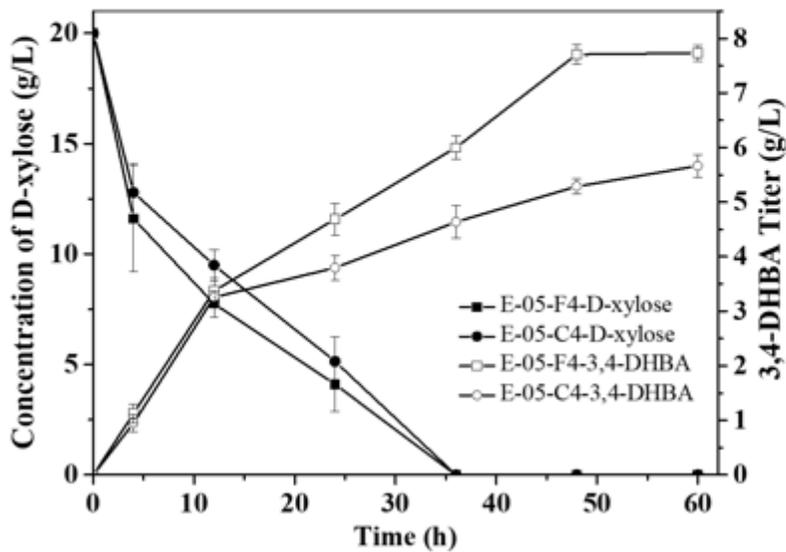


Figure 4

Protein fusion of PpMdlC and YagF to improve 3,4-DHBA production. (A) The SDS-PAGE analysis of E-05-C4 and E-05-F4. Lane 1: whole cell of E-05-C4; Lane 2: crude extract of E-05-C4; Lane 3: precipitate of E-05-C4; Lane 4: whole cell of E-05-F4; Lane 5: crude extract of E-05-F4; Lane 6: precipitate of E-05-F4; M: protein molecular weight marker. (B) E-05-F4 was engineered to have PpMdlC and YagF fusion expression to enhance the reaction efficiency. The highest 3,4-DHBA titer was 7.71 g/L produced by E-05-F4.

Supplementary Files

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