Enhanced glyoxylate shunt for efficient production of C4 derivatives using fatty acids feedstocks

Yingchun Miao

Institute of Microbiology, Chinese Academy of Sciences

Jiao liu

Institute of Microbiology, Chinese Academy of Sciences

Xuanlin wang

Institute of Microbiology, Chinese Academy of Sciences

Bo Liu (✉ liubo@im.ac.cn)

CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Microbial Resources

Weifeng Liu

Institute of Microbiology, Chinese Academy of Sciences

Yong Tao

Institute of Microbiology, Chinese Academy of Sciences <https://orcid.org/0000-0003-3106-6953>

Article

Keywords: glyoxylate shunt, C4 derivatives, fatty acids feedstocks

Posted Date: August 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-795330/v1>

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

[Read Full License](#)

Abstract

Biosynthesis of TCA cycle-derived C4 chemicals through glyoxylate shunt is an attractive metabolic route because it can be driven by TCA-glyoxylate cycle force under aerobic conditions. However, yield of this route is low with at least 1/3 carbon loss from glucose. FAs could sufficiently provide acetyl-CoA by β -oxidation without carbon loss and directly enter the TCA-glyoxylate cycle, which is acknowledged as a promising alternative feedstock. Here β -alanine was selected as the target TCA cycle-derived chemical, of which the theoretical yield is 1.391 g/g FAs, much higher than that of glucose (0.49 g/g). By adopting multi-metabolic engineering strategies and relieving the active oxygen damage caused by FAs utilization, β -alanine production reached 78.05 g/L with a yield of 1.2 g/g, about 86% of theoretical yield. Our study establish a promising bioproduction route of β -alanine from waste FAs (such as gutter oil, palm fatty acid distillate etc.), and more importantly, provide an efficient platform for TCA cycle-derived C4 chemicals biosynthesis.

Introduction

TCA cycle provides a large class of biosynthesis precursors. Diverse high value-added chemicals, such as succinate, itaconate, fumarate, L-aspartate, malate, and β -alanine, are biosynthesized from TCA cycle intermediates^{1, 2, 3, 4}. In metabolic engineering, supply of TCA cycle intermediates through glyoxylate shunt and oxidative branch TCA cycle has attracted much attention because it works under aerobic condition and can be favorable for cell growth in most biotechnological microbial hosts, making it easy to adapt to industrial scale. However, this route requires acetyl-CoA as precursor, which is associated with a decreased yield due to the loss of carbon during acetyl-CoA formation from pyruvate. Furthermore, when using glucose as feedstock, metabolic flux diversion exists at several nodes within glycolysis, making it difficult to metabolic control. As an alternative route, Supply of TCA cycle intermediates through PEP anaplerotic pathway and reductive-branch TCA cycle under anaerobic condition usually has higher theoretical yield. Yet the low efficiency of PEP carboxylase, problems of NADH limitation and cell growth limit its application^{5, 6}. An unsolved problem is that, as a most widely used feedstock, glucose has relative lower carbon content per mass (40%) and usually associates with carbon loss in the biosynthesis route^{7, 8}, which limit the theoretical yield of chemicals and decrease the process economics^{9, 10, 11}.

Fatty acids (FAs) are promising alternative feedstocks to glucose and can be easily obtained from various kinds of waste oil, crude oil, and oil by-products.^{12, 13, 14, 15}. In our previously work, we found TCA cycle can be significantly activated when using FAs as feedstocks in an engineering *Escherichia coli* strain¹⁶. We therefore speculated FAs feedstocks might be suitable for the production of TCA cycle-derived chemicals due to favorable metabolic regulation. FAs can generate large amounts of acetyl-CoA served as the primarily precursor of TCA cycle intermediates. Meanwhile, because acetyl-CoA is directly generated by β -oxidation of FAs, the metabolic control will be more direct and convenient. FAs have higher degree of reduction and higher carbon content per mass (75%) compared to glucose, generate more reducing equivalents for the anabolism, and thus produce a lot of chemicals with higher yields.

When biosynthesizing TCA cycle intermediates (OAA as example) from palmitic acids (C16 FAs) feedstocks, the theoretical yield is 15.6 mmol/g materials, higher than that of both PEPC route (theoretical yield of 11.1 mmol/g materials) and glyoxylate shunt route (theoretical yield of 5.55 mmol/g materials) from glucose feedstock. Furthermore, large amounts of energy and reducing power generated in this route could also facilitate cell growth and improve pathway efficiency (Fig.1).

In this study, we sought to construct FAs feedstocks-based glyoxylate-TCA route to achieve highly efficient supply of TCA cycle intermediates. A bioproduction route of β -alanine from FAs carbon source was designed. β -alanine is an important nonprotein amino acid derived from TCA cycle intermediates. It can be used as precursor for pantothenic acid¹⁷, acrylamide, acrylonitrile¹⁸, which are widely applied in the production of food additives, pharmaceuticals¹⁹, and bulk chemicals. Two metabolic routes of β -alanine production from glucose have been developed. By using glyoxylate shunt under aerobic and PEPC pathway under microaerobic conditions, the β -alanine yields can reach to 0.135 g/g glucose⁶ and 0.425 g/g glucose⁵, respectively. Here, we proposed β -alanine could produce from FAs with high theoretical yield. The precursor, OAA or fumarate, could be obtained from the glyoxylate-TCA cycle, and the theoretical yield reached 1.391 g/g raw materials (palmitic acid), which was much higher than any route from glucose (Fig.1).

To develop an efficient metabolic route to produce TCA cycle-derived chemicals based on FAs feedstocks, some important issues should be considered. First, the regulation of both glyoxylate shunt and TCA cycle in FAs is different from in glucose. The metabolic profiles of glyoxylate shunt and TCA cycle under FAs-utilization condition should be systematically investigated^{20, 21, 22}. To establish an efficient FAs feedstocks bioconversion process at large scale is another challenge. Developing an efficient bioconversion strategy should consider cell growth, FAs utilization, energy metabolism, β -alanine biosynthesis, and other important factors. The metabolic flux under large-scale conditions should be explored. In this study, we systematically balanced and optimized the metabolic flux between TCA cycle, glyoxylate shunt, and β -alanine biosynthesis pathway at different scaled-up levels. An efficient route for β -alanine production from FAs feedstocks was thus developed. The principles of TCA cycle regulation elucidated here provide useful information to develop new bioproduction routes in the future.

Results

FAs feedstocks enable higher yield of β -alanine production than glucose through glyoxylate-TCA cycle

A biosynthetic route for β -alanine production with a high theoretical yield was designed through glyoxylate-TCA cycle and using FAs as feedstocks. The theoretical yield of this route could reach 1.391 g/g when using C16 palmitic acids as raw material, and 1.433 g/g when using tripalmitin as raw material, which are much higher than that of the routes from glucose feedstock (through the glyoxylate shunt + PEPC (0.659 g/g) or PEPC pathway (0.988 g/g)) (Fig.1).

To verify the efficiency of this new route, chassis strains for β -alanine production from FAs feedstock were developed by introducing L-aspartate- α -decarboxylase encoded by *panD* into the FAs-using chassis FA09. *PanD* from *Tribolium castaneum* (*TcpanD*) was expressed using plasmid pSB1K (pSC101 origin)²⁹. The resulting strain BA20 could produce β -alanine at a concentration of 0.34 g/L after bioconversion for 20 hrs. The expression of *TcpanD* was further optimized using different plasmids and the highest β -alanine production of 2.40 g/L occurred using the medium copy number plasmid pXB1K (p15A origin, strain BA22) (Fig.2A). When additional *panD* from *Bacillus subtilis* (*BspanD*) was chromosomal expressed under P119 promoter, the resulting strain BA52 could produce β -alanine with 2.60 g/L after bioconversion for 20 hrs. The supply of L-aspartate is another major limit step of β -alanine biosynthesis pathway. In this work, we initially expressed aspartate ammonia-lyase encoded by *aspA* catalyzed L-aspartate production from fumarate and resulted in β -alanine production of approximately 3.46 g/L (strain BA62). Plasmid-expression of *aspA* could not improve β -alanine production (supplementary Fig.S1).

In this biosynthetic route, a central work is to maximize the metabolic flux of glyoxylate-TCA cycle. Thus we first knocked out the repressor *iclR* to active glyoxylate shunt pathway. However, after *iclR* was knocked out, the resulting strain BA72 could produce 3.60 g/L of β -alanine, only slightly increased compared with BA62. To test if the β -alanine production yield using FAs feedstocks will be superior to that using glucose feedstock, β -alanine production from FAs and glucose was compared. The yields were determined after bioconversion for 3 hrs (corresponding to fast β -alanine production phase, Fig.2B). It was shown both titer and yield of β -alanine in FAs were much higher those in glucose. The β -alanine production from glucose was only 1.5 g/L (strains BG22). The yield of β -alanine from FAs using BA72 was approximately 0.71 g/g, which was much higher than the 0.33 g/g yield from glucose (Fig.2C).

Auto-upregulation of glyoxylate-TCA cycle under FAs conditions facilitate β -alanine production

However, the yield of β -alanine production was still low, approximately 50% of its theoretical value. We then carried out experiments to determine the factors that limited the yield of β -alanine. First, we suspected that acetyl-CoA generated from β -oxidation of FAs might not efficiently enter TCA cycle. However, no accumulation of acetate, L-lactate or other acetyl-CoA derived by-products were observed during BA72 bioconversion.

Thus we mainly focused on the metabolic balance within glyoxylate-TCA cycle. We suspected the steps of isocitrate converting to α -ketoglutarate and succinyl-CoA within oxidative-branch TCA will cause carbon loss and reduce the metabolic flux towards glyoxylate-TCA cycle. A notable clue was that the yield of β -alanine production under anaerobic condition could reach to near 1.00 g/g, about 50% higher than that under aerobic condition, even if the titer of β -alanine was lower (Fig.3A). This result was coincided with the fact that the activities of oxidative branch of TCA cycle enzymes were down-regulated under oxygen-limited conditions^{30,31}. Therefore, we hypothesized the major bottleneck of β -alanine yield was the metabolic balance between TCA cycle and glyoxylate shunt at isocitrate node. we first carried out several modifications to improve the expression of glyoxylate shunt enzymes to direct metabolic flux

towards glyoxylate-TCA cycle. In addition to *icd* knocked out, the expression of *aceA*, *aceB*, *aceK*, and *glcB* was fine-tuned by either replacement of their native promoters or heterogeneous expression. Unfortunately, no obviously improvement in key production values could be achieved (Fig.3B). As shown in our transcriptional data, the mRNA levels of glyoxylate shunt genes had been significantly up-regulated when FAs were used as feedstocks (Fig.3C), this result was also in coincidence with our previous report that TCA cycle was activated under FAs feedstocks conditions¹⁶. These results clearly indicated the up-regulation of glyoxylate shunt is an automatic response to FAs feedstocks condition and thus result in a favorable metabolic flux toward glyoxylate-TCA cycle and β -alanine biosynthesis, which lead to higher β -alanine production than glucose. Of course, additional modifications are still needed to improve metabolic flux of glyoxylate-TCA cycle.

Modification of TCA cycle targets to improving glyoxylate-TCA cycle metabolic flux.

According to above results, blocking the oxidative branch of TCA cycle might be necessary to improve the metabolic flux towards glyoxylate shunt. In the β -alanine biosynthesis pathway, there are two routes for the supply of L-aspartate, catalyzed by L-aspartate aminotransferase (*aspC*) using OAA as substrate and L-glutamate as amino group donor, or catalyzed by *aspA* using fumarate as substrate and ammonia as amino group donor. The blocking strategies were designed according to either of the two L-aspartate supply routes. First, we paid more attention to the target of *sucA*. As indicated in our previous study, nitrogen metabolism significantly influenced the FAs utilization¹⁶. Knocked out of *sucA* rather than *icd* will be beneficial to the biosynthesis of L-glutamate/L-glutamine, which play central regulation roles in FAs utilization. L-glutamate is also an important cofactor of L-aspartate aminotransferase (*aspC*) during β -alanine biosynthesis. Thus block of TCA cycle at *sucA* node and using *aspC* will facilitate development of strategy to improve β -alanine biosynthesis efficiency.

When *sucA* gene was knocked out, the cell growth of resulting strain YC07 was significantly reduced (date not shown). The β -alanine production in corresponding strain BA81 was decreased at very low level when using *aspA* (Fig.4A). When *aspA* was replaced by *aspC*, especially when NADH-dependent L-glutamate dehydrogenase *rocG* from *B. subtilis* was also introduced to regenerate cofactor L-glutamate, as expected, β -alanine production could be restored to about 1.4g/L, and the yield of β -alanine production increased to 0.85 g/g, higher than BA72 (Fig.4A). However, 1.01 g/L acetate accumulation was observed after bioconversion for 24 hrs. To resolve the problem of low cell growth of Δ *sucA* strains, we then carried out adaptive laboratory evolution using the GREACE method to improve the cell property of YC07 (Fig.4B). After 9 generations of evolution, an evolved strain YC08, which had a high cell growth rate and still retained all genotype, was obtained. The corresponding β -alanine producing strain BA85 could produce β -alanine by about 2.9 g/L, while the yield reached 0.9 g/g (Fig.4C), about 50% higher than their in non- Δ *sucA* strain BA80. And production of acetate decreased from 1.01 g/L to 0.56 g/L (supplementary Fig.S2).

We also optimized the alternative strategy that directly block TCA cycle at *icd* node. In an *icd* knocked-out strain BA91 and using *aspA*, β -alanine production was shown to be approximately 3.0 g/L (3 hrs), and the

yield reached 0.81 g/g (Fig.4D). No acetate accumulation was observed. By compared of these two routes, we hypothesized *aspA-Δicd* route is a better choice for β-alanine production. Differ from our previous speculation, the β-alanine yield of *aspC-ΔsucA* route was just similar to that of *aspA-Δicd* route (Fig.4D). Because the substrate of *aspC*, OAA, is also served as key intermediate to maintain the TCA cycle, making it difficult to regulate during further optimization.

Indeed, the β-alanine yield of *aspA-Δicd* can be further increased by blocking the flux from fumarate towards L-malate by knocking out *fumAC*. The β-alanine production of BA93 was about 3.29 g/L (3 hrs), and the yield could reach to 1.01 g/g. Surprisingly, further knocked out of *fumB* in strain BA94 significantly reduced the β-alanine production to 0.95 g/L and acetate level were increased to 1.6 g/L (Fig.4D). As shown in Fig.3C. the mRNA levels of *fumAC* significantly increased in FAs feedstocks conditions, thus *fumAC* might be the primary enzymes to supply L-malate and maintaining the efficiency of TCA cycle by anaplerotic supply of L-malate from fumarate is essential in FAs conditions.

Significantly improving β-alanine production by efficiently recycling of glyoxylate-TCA cycle at fed-batch level.

Next, we carried out β-alanine bioconversion using a fed-batch strategy with BA93. We first carried out a two-stage fed-batch bioconversion process at 5L level. In the biomass accumulation stage, glucose was used as the main feedstock and the expression of pathway enzymes were induced, then soybean oil (plus 15 mg/L lipase) was added during bioconversion stage. Unfortunately, the cell growth of BA93 was significantly slower at fed-batch level (supplementary table. S2) and total 29 g/L L-glutamate had to added to maintain cell growth. However, the FAs utilization during bioconversion stage was also seriously impeded (supplementary Fig.S5). β-alanine production reached about 35 g/L after total bioconversion time of 50 hrs (Fig.5A). The β-alanine production yield could not be accurately determined because of insoluble oil-source accumulation. When *icd* was knocked out (BA91), citrate was accumulated indicating oxidative-branch of TCA might be still necessary to the efficiency of glyoxylate-TCA cycle (Fig. 6E). on the other hand, TCA cycle plays important roles in sustaining the glyoxylate-TCA cycle. As a control, the FAs can be used in strain BA72 in scaled-up level and the yield of β-alanine production maintained about 1.01 g/g. The β-alanine production gradually increased after 40 hrs and reached 61.97 g/L after total bioconversion time of 84 hrs (Fig.5B).

These results indicated the total block of TCA cycle is not suitable at scaled-up level. However, strain BA92, of which *fumAC* was knocked out but contained normal *icd* gene, even could produce β-alanine with 1.05 g/g yield at flask level (supplementary Fig.S4), but only produced β-alanine with 0.4 g/g yield during fed-batch process. The titer of β-alanine was only about 25.88 g/L in 40 hrs. Meanwhile, large amount of acetate was accumulated to 13.62 g/L (Fig.5C). In an optimized bioprocess, in which the second stage was carried out using collected cells in a fresh medium, the β-alanine production of BA92 could be restored by 1.1g/g in bioconversion 20 hrs (total fermentation 40 hrs). But acetate byproduct was still accumulated and the yield was gradually decreased after 20 hrs (Fig.5D). We suspected acetyl-CoA could not efficiently flow into TCA cycle in BA92. To address the reason, the intracellular levels of

TCA cycle intermediates were determined using UPLC-MS/MS. As shown in Fig.5E, accompanied with the accumulation of acetate, the levels of malate and fumarate were significantly decreased. These results clearly indicated the accumulation of acetate was due to the inefficient of recycle of OAA into citrate caused by the block of reduced-branched TCA cycle.

As blocking TCA cycle at *icd* and *fumAC* nodes resulted in serious consequences such as deficiency of FAs utilization and acetate accumulation at scaled-up level, we then systematically investigated other modification targets. First, among the targets within or related to the reductive branch TCA cycle, knocked out of *aspC* is most effective. In *aspC* knocked out strain BA101, β -alanine production could significantly increase to about 3.5 g/L at flask level (Fig.6A). Furthermore, citrate accumulation in BA91 implied the metabolic flux of glyoxylate should be further improved. Thus the effects of glyoxylate shunt-targets were retested and BA104, in which the expression of *glcB* and *aceAK* were enhanced in BA101, could produce β -alanine with high yield at scaled-up level. The titer and yield of BA104 could reach to 42.57g/L and 1.05 g/g respectively in bioconversion 24hrs (total bioconversion 44 hrs) during 5L fed-batch bioconversion. And no acetate accumulation was found (Fig.6B). *GlcB* seems the major enzyme within glyoxylate shunt in this condition, this coincided with the transcription result (Fig.3C). It was also noteworthy that knocked-out of *maeAB* significantly decreased β -alanine production (BA102). This result confirmed the dynamic balance of malate-oxaloacetate-pyruvate play a key role in the regulation of TCA cycle flux during β -alanine production.

Relieve of oxygen damage at fed-batch level to improve cell viability and β -alanine production.

However, during the later stage of bioconversion, the production of β -alanine was no longer increased (Fig.6B). We further tested the cell viability in fed-batch culture. Cells were harvested in either initial or later stage and the β -alanine production was investigated in the flask. It was indicated cells from later stage could not produce β -alanine (Fig.6D). According to the results of intracellular metabolites analysis, the level of glutathione, a key metabolite in oxidative stress, increased about 21.84 folds in fast-FAs utilization stage (Fig.6C). Glutathione plays an important role in maintaining intracellular redox balance and preventing damage caused by reactive oxygen species. This result implied the repression of cell viability and FAs utilization (supplementary Fig.S6) might be related to oxygen damage, most likely caused by the fast FAs metabolism. Thioredoxin/glutathione peroxidase (*btuE*) and glutathione reductase (*gor*), are the main genes involved in the glutathione cycle. Thus, the expression of *btuE* and *gor* genes were enhanced by replacement of native promoters with medium-strong promote P119. The new strain BA105 was thus obtained and fed-batch bioconversion of BA105 was then carried out. The results indicated the final β -alanine production reached approximately 78.05 g/L after bioconversion 40 hrs (total bioconversion time 60 hrs), with a yield and productivity of 1.2 g/g and 1.95 g/L/h, respectively (Fig.6E). And the strain BA105 still retained the β -alanine production ability during the later stage of bioconversion (Fig.6D). Taken together, the results herein provided a novel β -alanine bioproduction route using FAs feedstock, which is the highest titer and yield reported so far.

Discussion

In this study, a carbon efficient glyoxylate-TCA cycle was developed using FAs as feedstocks. By systematical modification of TCA cycle and glyoxylate shunt targets and balancing metabolic flux, β -alanine, an important TCA cycle-derived chemical, could be produced with high yield. Previously, there are two feasible biosynthesis routes for β -alanine from glucose. Song et al. reported the production of β -alanine from glucose through the glyoxylate shunt combined with PEPC pathway to supply L-aspartate resulted in 32.3 g/L and an overall yield of 0.135 g/g glucose⁶. Piao et al. developed a biosynthesis pathway that directly uses PEPC pathway and blocks glycolysis to maximize the supply of OAA and then generate L-aspartate. Through this pathway, 37.7 g/L of β -alanine could be produced with an overall yield of 0.425 g/g glucose⁵. In this work, the β -alanine could be efficiently produced with a titer of 78.05 g/L, yield of 1.2 g/g, and productivity of 1.95 g/L/h at fed-batch level, which are the highest production metrics reported so far.

Our results indicated the primary important work is to improve metabolic flux towards glyoxylate shunt. However, glyoxylate shunt can be auto-activated in the FAs conditions, which is favorable for the optimization of metabolic flux. Improving glyoxylate shunt flux by blocking oxidative-branch of TCA cycle was also evaluated. Knockout of *sucA* or *icd* can achieve higher yield at flask level, but lead to severe growth inhibition and impede FAs utilization (supplementary figS3). Although the strategy of blocking TCA cycle has been successfully applied to improve the production of TCA cycle intermediates-derived chemicals such as succinate, 5-aminolevulinic acid^{32,33}, this strategy seems not suitable in FAs feedstocks. Oxidative-branch of TCA cycle might be essential adapt to the FAs utilization conditions.

In the glyoxylate-TCA cycle, the C4 TCA intermediates served as precursors for both β -alanine production and TCA recycling. The metabolic balance between these two metabolic pathways thus is a key issue. At flask level, β -alanine production could be enhanced by deletion of *fumAC*, but totally deletion of *fumABC* significantly impeded β -alanine production. This is different from that of glucose feedstock route⁶. It was reported *fumAC* are functional in aerobic conditions, thus block of *fumAC* might facilitate metabolic flux towards β -alanine production. Whereas totally block of *fum* might impede the supply of reductive-branch TCA intermediates such as malate and OAA, therefore decrease the efficiency of glyoxylate-TCA cycle. On the other hand, during fed-batch process, large amount of acetate was accumulated in *fumAC* knocked out strain BA92. It seems larger metabolic flux of glyoxylate-TCA cycle than in flask level was needed. This is not surprising considering FAs utilization and metabolic rates were much higher in fed-batch condition. As implied in our metabolite analysis of fed-batch process, the knocked out of *fumAC* lead to decreasing of the reductive-branch TCA cycle metabolites, thus might inhibit the reprogram of TCA cycle and lead to acetate production. As a result, recovering the *fum* genes and further knocked out of *aspC* to increase the supply of reductive-branch TCA intermediates could indeed improve the efficiency of glyoxylate-TCA cycle and β -alanine production.

There still other metabolic regulations and responses affect this metabolic route. Most of these metabolic regulations are related to the FAs metabolic profiles. Because FAs metabolism could generate large amounts of NADH during β -oxidation. Respiration chain and oxygen might be necessary for NADH recycling during fast FAs utilization. Consistent with this, enhanced oxygen stress and significantly cell

damage was observed in fed-batch process. Furthermore, acetyl-CoA is almost the only precursor for all cellular metabolism, including gene expression, supplement of metabolites, energy generation. When using FAs as sole carbon, it is also of great importance to ensure the supplemental biosynthesis of other necessary key metabolic precursors. Indeed, knocked out of *maeAB*, which related to the balance of pyruvate-OAA-malate cycle, lead to the decrease of β -alanine production. In the future, an optimized bioconversion process at large scale level is needed.

Taken together, our study extensively characterized the key factors that influence the production of TCA cycle derived chemicals from FAs feedstocks. The higher metrics of β -alanine production from FAs provides a competitive route of β -alanine production, as well as other important chemicals. Furthermore, utilization of all kinds of cheap oils as alternative feedstocks will be greatly helpful for the resolve the environmental and carbon recycling issues.

Methods

DNA manipulations

All chromosomal manipulations were carried out using λ -red mediated homologous recombination²³. All plasmids were constructed using Gibson DNA assembly method²⁴ and sequenced by Tsingke biological technology (Beijing, China). If necessary, heterogeneous genes were synthesized by Genewiz with codon optimization for *E. coli*. The resistance markers were eliminated using the plasmid pSB1s-Cre by adding 0.2% L-arabinose or by using pCP20 according to the Cre-lox or FRT-FLP system. For chromosomal promoter replacement, the CPA1 promoter, 119 promoters were amplified from pSL91k, pSLCPA1k respectively. The fragments were overlapped with about 500 bp of the upstream/downstream region of the target genes. Adaptive laboratory evolution process (GREACE) was performed according to the methods in literature²⁵. Primers used in this study are listed in the supplementary Table S1. DNA polymerase, Gibson kits and T4 DNA ligase purchased from Tsingke biological technology or Fisher Scientific (Pittsburgh, PA), were used for plasmid construction.

Bacterial strains and culture conditions.

E. coli DH5 α were used for plasmids construction. *E. coli* strains were grown at 37 °C, 220 rpm in Luria-Bertani (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) medium. When necessary, antibiotics were added at: ampicillin, 100 μ g/mL; streptomycin, 50 μ g/mL; and kanamycin, 50 μ g/mL. For protein expression, overnight cultures were inoculated into an auto-induction medium (including 1% tryptone, 0.5% yeast extract, 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄, 0.2 $\times$ Trace metals, 0.5% Glycerol, 0.05% Glucose, 0.2% L-arabinose and antibiotics when necessary)²⁶ with 1% inoculum and incubated at 220 rpm, 30 °C for 16 h. The whole-cell bioconversion for β -alanine produced by FAs was carried out using M9 FAs medium containing 12.8 g/L Na₂HPO₄·7H₂O, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, and 5 g/L palmitic acid (emulsified with 4 g/L Brij 58), suspended in 3 ml (OD₆₀₀=30) in 50 ml test tube for aerobic conditions, and 500 μ l (OD₆₀₀=30) in 1.5 ml centrifuge tube for anaerobic

conditions. M9 medium was supplemented with 10 g/L NH₄Cl and 50 μM pyridoxal phosphate (PLP) to provide precursors for the synthesis of β-alanine. The bioconversion reactions were performed at 37 °C, 200 rpm for 3 hrs or 24 hrs. Strains and plasmids used in this study are listed in the table 1. Each bioconversion was repeated at least three times. All other chemicals and reagents were purchased from Shanghai Sheng Gong Bio-chemical Co. Ltd. (Shanghai, China) of analytical grade.

Table 1. Strains and plasmids used in this study.

strains	Relevant genotype	Reference
<i>E. coli</i> BW25113/F ⁺ FA09	<i>rrnBT14 ΔlacZ</i> WJ16 <i>hsdR514 ΔaraBADAH33 ΔrhaBADLD78</i> [F ⁺ <i>proAB lacIqZΔM15 Tn10 (Tetr)</i>]	CGSC
	<i>E. coli</i> BW25113/F ⁺ , Δ <i>fadR</i> , PCPA1- <i>fadD</i> , P ₁₁₉ - <i>fadL</i> , Δ <i>sthA</i> , PCPA1- <i>pntAB</i>	16
BA20	FA09 containing pSB1K- <i>Tcpa</i> n <i>D</i>	This study
BA21	FA09 containing pLB1K- <i>Tcpa</i> n <i>D</i>	This study
BA22	FA09 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA23	FA09 containing pAB1K- <i>Tcpa</i> n <i>D</i>	This study
YC00	FA09 P ₁₁₉ - <i>BspanD</i>	This study
YC01	YC00 Δ <i>iclR</i>	This study
YC02	YC00 PCPA1- <i>aspA</i>	This study
YC03	YC02 Δ <i>iclR</i>	This study
FG00	YC03 Δ <i>ptsG</i> , Δ <i>galR</i> , P ₁₁₉ - <i>glk</i>	This study
YC04	YC02 P ₁₁₉ - <i>glcB</i> -RBS- <i>aceA</i>	This study
YC05	YC04 P ₁₁₉ - <i>aceB</i> -RBS- <i>aceA</i>	This study
YC06	YC05 Δ <i>iclR</i>	This study
YC07	YC01 Δ <i>sucA</i>	This study
YC08	Evolved from YC07	This study
YC09	YC03 Δ <i>icd</i>	This study
YC10	YC03 Δ <i>fumAC</i>	This study
YC11	YC09 Δ <i>fumAC</i>	This study
YC12	YC11 Δ <i>fumB</i>	This study
YC13	YC03 Δ <i>aspC</i>	This study
YC14	YC03 Δ <i>maeAB</i>	This study
YC15	YC13 P ₁₁₉ - <i>btuE</i> P ₁₁₉ - <i>gor</i>	This study
BA52	YC00 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA53	YC00 containing pSB1a- <i>aspA</i> , pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA62	YC02 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA72	YC03 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BG22	FG00 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA73	YC04 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA74	YC05 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study
BA75	YC06 containing pXB1K- <i>Tcpa</i> n <i>D</i>	This study

strains	Relevant genotype	Reference
BA76	BA75 containing pSB1a- <i>aceB</i> -RBS- <i>aceA</i>	This study
BA77	BA75 containing pSB1a- <i>glcB</i> -RBS- <i>aceA</i>	This study
BA80	YC01 containing pSB1a- <i>aspC</i> - <i>rocG</i> , pXB1K- <i>TcpanD</i>	This study
BA81	YC07 containing pSB1a- <i>aspA</i> , pXB1K- <i>TcpanD</i>	This study
BA82	YC07 containing pSB1a- <i>aspC</i> pXB1K- <i>TcpanD</i>	This study
BA83	YC07 containing pSB1a- <i>aspC</i> - <i>rocG</i> pXB1K- <i>TcpanD</i>	This study
BA85	YC08 containing pSB1a- <i>aspC</i> - <i>rocG</i> pXB1K- <i>TcpanD</i>	This study
BA91	YC09 containing pXB1K- <i>TcpanD</i>	This study
BA92	YC10 containing pXB1K- <i>TcpanD</i>	This study
BA93	YC11 containing pXB1K- <i>TcpanD</i>	This study
BA94	YC12 containing pXB1K- <i>TcpanD</i>	This study
BA101	YC13 containing pXB1K- <i>TcpanD</i>	This study
BA102	YC14 containing pXB1K- <i>TcpanD</i>	This study
BA104	YC13 containing pSB1a- <i>glcB</i> -RBS- <i>aceA</i> pXB1K- <i>TcpanD</i>	This study
BA105	YC15 containing pSB1a- <i>glcB</i> -RBS- <i>aceA</i> pXB1K- <i>TcpanD</i>	This study
Plasmid	Description	Source
pXB1k	araBAD promoter, p15A ori, Kan ^r	Our laboratory
pSB1k	araBAD promoter, pSC101 ori, Kan ^r	Our laboratory
pSB1a	araBAD promoter, pSC101 ori, amp ^r	Our laboratory
pLB1k	araBAD promoter, r6k ori, Kan ^r	Our laboratory
pAB1k	araBAD promoter, ColA ori, Kan ^r	Our laboratory
pKD46	Temperature sensitive vector carrying λ-Red recombinase, Amp ^r	27
pSL91k	119 promoter, pSC101 ori, Kan ^r selective marker with lox71 and lox66	Our laboratory
pSLCPA1k	CPA1 promoter, pSC101 ori, Kan ^r selective marker with lox71 and lox66	Our laboratory
pXB1k- <i>TcpanD</i>	pXB1k containing <i>Tribolium castaneum panD</i>	This study
pSB1a- <i>aspA</i>	pSB1a containing <i>E. coli aspA</i>	This study
pSB1a- <i>aspC</i>	pSB1a containing <i>E. coli aspC</i>	This study
pSB1a- <i>aspC</i> - <i>rocG</i>	pSB1a containing <i>E. coli aspC</i> and <i>Bacillus subtilis rocG</i>	This study
pSB1s- <i>cre</i>	pSB1s containing <i>Cre</i> recombinase gene, Str ^r	Our laboratory

strains	Relevant genotype	Reference
pCP20	Temperature sensitive vector carrying FLP, Amp ^r	27

Fed-batch bioconversion

The bioconversion mediums include 10 g/L glucose, 14 g/L KH_2PO_4 , 4 g/L $(\text{NH}_4)_2\text{HPO}_4$, and 1.8 g/L citric acid monohydrate. The pH was controlled at 7.0 via automated addition of ammonia, and the dissolved oxygen (DO) concentration was controlled by adjusting the agitation speed and airflow rate during fermentation. For seed preparation, strains were cultivated on LB agar plates using plate streaking at 37 °C overnight and monoclonal colony was inoculated to 5 mL LB medium with antibiotics. After overnight cultivation, the strain was then inoculated to 100 mL LB medium, and was transferred to 1 L fermenter when OD_{600} reached 2. After the initial glucose was nearly exhausted, 2 g/L L-arabinose was added when the OD_{600} reached 30 by expression of *panD* and *glcB-aceA*. The carbon source was switched from glucose to FAs when the OD_{600} reached 60. For fed-batch bioconversion, cultures were fed with FAs supplemented with 1 g/L yeast extract when DO reached 50%, and the total amount of yeast extract did not exceed 5 g/L.

Strain BA91 and BA93 (*icd* knockout) were performed using modified medium containing 29.03 g/L glutamate for cell growth and enzymes induction. Induced cells were collected by centrifugation at 8000 rpm, 4 °C, 30 min. Then suspended in 500 ml bioconversion medium containing 12.8 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L KH_2PO_4 , 0.5 g/L NaCl, 1 g/L NH_4Cl , PH 7.0 via automated addition of ammonia. All results were repeated at three replicates.

Analytical methods

Cell density was estimated by measuring the optical density at 600 nm using an Ultrospec 3000 spectrometer. The expression of recombinant enzymes was analyzed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis. The concentrations of β -alanine were measured by HPLC (Agilent 1260 series, Hewlett-Packard) with an Ascentis Express -C18 2.7 μm column (4.6 $\times$ 250 mm). The analysis was performed at 40 °C with mobile phase 65% A 1% formic acid in water and 35% mobile phase B acetonitrile at a flow rate of 0.5 mL/min. The analytes were detected by derivatization with dinitrofluorobenzene (DNFB) and 0.1 M NaHCO_3 after 60 °C, 1 h and filtered through a 0.22 μm PES membrane (Jinteng, Tianjin, China). The concentrations were determined with a diode array detector (DAD) at 360 nm. Concentrations of glucose and organic acids were measured by HPLC (Agilent 1260 series) with an Aminex HPX-87H column (300 $\times$ 7.8 mm, Bio-Rad, USA). The analysis was performed at 55 °C with a mobile phase comprising 5 mM H_2SO_4 in water at a flow rate of 0.6 mL/min. All data were obtained from three replicates. For the concentrations of FAs, a gas chromatograph (GC, 7890A) was used (Agilent Technologies, USA) with a flame ionization detector (FID). The reagent samples were converted to FAME (FA methyl esters) to analyze by GC, each 300 μL FAs sample with 500 μL 10% (V/V)

H₂SO₄ in methanol at 60 °C for 20 min, then 500 µl hexane was added to the mixture, then vortexed vigorously, and centrifuged at maximum speed to separate organic and aqueous layers, then 300 µl the organic layer containing the FAME was transferred to GC for quantification.. The program was as follows: 150 °C, 5 min, ramping up to 170 °C at a rate of 3 °C/min, held for 5 min at 170 °C, increased up to 210 °C at a rate of 3 °C/min, held for 5 min at 210 °C, with an HP-88 column (60 m × 0.25 mm, 0.2 µm film thickness).

For transcriptomic analysis, strain FA09 was cultured in M9 salt FAs and M9 salt glucose medium separately in 500 ml flasks, cultured to mid-exponential growth phase and harvested by centrifugation, washed three times with 0.85% NaCl solution, then frozen on liquid nitrogen immediately and stored at -80 °C. Samples were sent to Vazyme biotech co., ltd for RNA extraction and transcriptome sequencing analysis (nanjing, China).

The concentrations of intracellular metabolites were determined by UPLC-MS/MS. Cells from late stage and early stage in fermenters (at bioconversion level), for identifying cell viability, cells collected from fast-FAs utilization stage and slowly-FAs stage were chilled on ice, and collected by centrifugation at 13,000 rpm, 4 °C for 10 min, washed twice with ice-cold 0.85% NaCl solution, suspended in 400 µL (OD₆₀₀ = 90) of 80% (vol/vol) methanol (prechilled to -80 °C) for preventing any further metabolism and sonicated²⁸. The UPLC-MS/MS was carried out by MFTC-PS (Metabolomics Facility at the Technology Center for Protein Sciences) of Tsinghua University.

For high-performance liquid chromatography (HPLC) analysis, standards for amino acids (β-alanine) and organic acids were purchased from Sigma-Aldrich (Germany).

Data availability

Data supporting this work are available within the paper and its Supplementary Information files. Source data are provided with this paper.

References

1. Chen X, Dong X, Wang Y, Zhao Z, Liu L. Mitochondrial engineering of the TCA cycle for fumarate production. *Metabolic Engineering* **31**, 62-73 (2015).
2. Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. *Biotechnol Bioeng* **101**, 881-893 (2008).

3. Li Z-J, Hong P-H, Da Y-Y, Li L-K, Stephanopoulos G. Metabolic engineering of *Escherichia coli* for the production of L-malate from xylose. *Metabolic Engineering* **48**, 25-32 (2018).
4. Yuzbasheva EY, *et al.* Engineering *Yarrowia lipolytica* for the selective and high-level production of isocitric acid through manipulation of mitochondrial dicarboxylate–tricarboxylate carriers. *Metabolic Engineering* **65**, 156-166 (2021).
5. Piao X, Wang L, Lin B, Chen H, Liu W, Tao Y. Metabolic engineering of *Escherichia coli* for production of L-aspartate and its derivative β -alanine with high stoichiometric yield. *Metabolic Engineering* **54**, 244-254 (2019).
6. Song CW, Lee J, Ko Y-S, Lee SY. Metabolic engineering of *Escherichia coli* for the production of 3-aminopropionic acid. *Metabolic Engineering* **30**, 121-129 (2015).
7. Lin PP, Rabe KS, Takasumi JL, Kadisch M, Arnold FH, Liao JC. Isobutanol production at elevated temperatures in thermophilic *Geobacillus thermoglucosidasius*. *Metabolic Engineering* **24**, 1-8 (2014).
8. Johnson AO, *et al.* Design and application of genetically-encoded malonyl-CoA biosensors for metabolic engineering of microbial cell factories. *Metabolic Engineering* **44**, 253-264 (2017).
9. Dodds DR, Gross RA. Chemicals from Biomass. *Science* **318**, 1250 (2007).
10. Lee JW, Kim TY, Jang Y-S, Choi S, Lee SY. Systems metabolic engineering for chemicals and materials. *Trends in Biotechnology* **29**, 370-378 (2011).
11. Yu Y, *et al.* Construction of a carbon-conserving pathway for glycolate production by synergetic utilization of acetate and glucose in *Escherichia coli*. *Metabolic Engineering* **61**, 152-159 (2020).

12. Khan MZH, Sultana M, Al-Mamun MR, Hasan MR. Pyrolytic Waste Plastic Oil and Its Diesel Blend: Fuel Characterization. *Journal of Environmental and Public Health* **2016**, 7869080 (2016).
13. Liang S, Liu Z, Xu M, Zhang T. Waste oil derived biofuels in China bring brightness for global GHG mitigation. *Bioresource Technology* **131**, 139-145 (2013).
14. Idris NA, Lau HLN, Wafti NSA, Mustaffa NK, Loh SK. Glycerolysis of Palm Fatty Acid Distillate (PFAD) as Biodiesel Feedstock Using Heterogeneous Catalyst. *Waste and Biomass Valorization* **12**, 735-744 (2021).
15. Meng X, Chen G, Wang Y. Biodiesel production from waste cooking oil via alkali catalyst and its engine test. *Fuel Processing Technology* **89**, 851-857 (2008).
16. Liu B, *et al.* Efficient production of 3-hydroxypropionate from fatty acids feedstock in *Escherichia coli*. *Metabolic Engineering* **51**, 121-130 (2019).
17. Katahira R, Ashihara H. Dual function of pyrimidine metabolism in potato *Solanum tuberosum* plants: pyrimidine salvage and supply of beta-alanine to pantothenic acid synthesis (vol 127, pg 38, 2006). *Physiol Plant* **129**, 830-830 (2007).
18. Könst PM, Franssen MCR, Scott EL, Sanders JPM. A study on the applicability of L-aspartate α -decarboxylase in the biobased production of nitrogen containing chemicals. *Green Chemistry* **11**, 1646-1652 (2009).
19. Hoffman JR, Rathmacher JA, Robinson J, Gepner Y, Cohen H. Effect of β -alanine supplementation on carnosine and histidine content in the hippocampus of 14-month-old rats. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* **44**, 1112-1115 (2019).

20. Liu M, Lou J, Gu J, Lyu XM, Wang FQ, Wei DZ. Increasing L-homoserine production in *Escherichia coli* by engineering the central metabolic pathways.
21. Shalel-Levanon S, San K-Y, Bennett GN. Effect of oxygen, and ArcA and FNR regulators on the expression of genes related to the electron transfer chain and the TCA cycle in *Escherichia coli*. *Metabolic Engineering* **7**, 364-374 (2005).
22. Valle A, *et al.* A systematic analysis of TCA *Escherichia coli* mutants reveals suitable genetic backgrounds for enhanced hydrogen and ethanol production using glycerol as main carbon source. *Biotechnol J* **10**, 1750-1761 (2015).
23. Tomoya, *et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*, (2006).
24. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**, 343-345 (2009).
25. Luan G, *et al.* Comparative genome analysis of a thermotolerant *Escherichia coli* obtained by Genome Replication Engineering Assisted Continuous Evolution (GREACE) and its parent strain provides new understanding of microbial heat tolerance. *New Biotechnology* **32**, 732-738 (2015).
26. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein expression and purification* **41**, 207-234 (2005).
27. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *P Natl Acad Sci USA* **97**, 6640-6645 (2000).
28. Cui Q, Zhou F, Liu W, Tao Y. Avermectin biosynthesis: stable functional expression of branched chain α -keto acid dehydrogenase complex from *Streptomyces avermitilis* in *Escherichia coli* by selectively

regulating individual subunit gene expression. *Biotechnology Letters* **39**, 1567-1574 (2017).

29. Wang LA-O, Piao X, Cui S, Hu M, Tao Y. Enhanced production of β -alanine through co-expressing two different subtypes of L-aspartate- α -decarboxylase.

30. Phue J-N, Shiloach J. Impact of dissolved oxygen concentration on acetate accumulation and physiology of *E. coli* BL21, evaluating transcription levels of key genes at different dissolved oxygen conditions. *Metabolic Engineering* **7**, 353-363 (2005).

31. Gonzalez JE, Long CP, Antoniewicz MR. Comprehensive analysis of glucose and xylose metabolism in *Escherichia coli* under aerobic and anaerobic conditions by ^{13}C metabolic flux analysis. *Metabolic Engineering* **39**, 9-18 (2017).

32. Noh MH, Lim HG, Park S, Seo SW, Jung GY. Precise flux redistribution to glyoxylate cycle for 5-aminolevulinic acid production in *Escherichia coli*. *Metabolic Engineering* **43**, 1-8 (2017).

33. Lin H, Bennett Gn Fau - San K-Y, San KY. Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate.

Figures

A

Glucose $\rightarrow$ oxaloacetate + 2CO₂ + 2ATP + 6NADH + FADH₂ (5.55 mmol/g materials)

Glucose + 2CO₂ $\rightarrow$ 2oxaloacetate + 2NADH (11.1 mmol/g materials)

C16 + ATP $\rightarrow$ 4oxaloacetate + 15NADH + 11FADH₂ (15.6 mmol/g materials)

B

Figure 1

A. Comparison of metabolic pathway routes of bioproduction of C₄ TCA cycle intermediates (OAA) from FAs or glucose feedstocks. Theoretical yields of each route are shown in gray box. B. Design of glyoxylate-TCA cycle for efficient production of TCA cycle-derived chemicals. Enzymes encoded by genes: *aceA*: Isocitrate lyase; *icd*: isocitrate dehydrogenase; *suc*: 2-oxoglutarate decarboxylase; *fum*: fumarase; *aspA*: aspartate ammonia-lyase; *iclR*: aspartate ammonia-lyase; *panD*: aspartate α -decarboxylase; *aceK*: isocitrate dehydrogenase kinase/phosphatase; *aspC*: aspartate aminotransferase. Blue colors indicate overexpression of the relevant genes through plasmid-based expression or promoter replacement. Red colors indicate deletion of the corresponding gene.

Figure 2

Development of β -alanine production strains using FAs feedstocks. A. Improve β -alanine production by enhancement of *panD*, *aspA* and glyoxylate shunt. B. Time curve of β -alanine production by strain BA72. C. Comparison of production and yield between FAs and glucose feedstocks strategies. Error bars show standard deviation ($n = 3$).

Figure 3

Identify the bottleneck of β -alanine yield. A. β -alanine production in aerobic and anaerobic conditions. B. β -alanine production by modifying the glyoxylate shunt targets. C. mRNA levels of glyoxylate shunt genes of FA09 strain in FAs and glucose conditions. aceB: malate synthase A, aceA: isocitrate lyase, aceK: isocitrate dehydrogenase kinase, glcB: malate synthase G, iclR: glyoxylate bypass operon transcriptional repressor, icd: isocitrate dehydrogenase, fumA: fumarase A, fumC: fumarase C, fumB; fumarase B, sucA; 2-oxoglutarate decarboxylase, gltA: citrate synthase. Error bars show standard deviation (n = 3).

Figure 4

Identify the bottleneck of β -alanine yield. A. β -alanine production in sucA knocked out. B. adaptive laboratory evolution of sucA knocked out strain. C. β -alanine production of BA85 strain. D. β -alanine production in aspA-icd strategy. Error bars show standard deviation (n = 3).

Figure 5

fed-batch β -alanine bioconversion of different strains. A. bioconversion of BA93. B. bioconversion of BA72. C. bioconversion of BA92. D. bioconversion of BA92 using off-site strategy. filled square: β -alanine production, filled circle: acetate production. E. Comparison of metabolites between the late stage and early stage of BA72/91/92 during bioconversion.

Figure 6

A. Modification of TCA targets. BA101: *aspC* knockout; BA102: *maeAB* knockout; BA104: BA101 containing psB1a-glcB-RBS-aceA. B. bioconversion of BA104 strain. C,D. relieve β -alanine production ability in BA105 strain. E. bioconversion of BA105 strain.

Supplementary Files

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

- [SupplementaryInformation.docx](#)