Appl Microbiol Biotechnol (2017) 101:3153–3161
DOI 10.1007/s00253-017-8127-6

Enhancement of succinate yield by manipulating NADH/NAD$^+$ ratio and ATP generation

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Received: 1 July 2016 / Revised: 4 January 2017 / Accepted: 10 January 2017 / Published online: 20 January 2017

Abstract We previously engineered *Escherichia coli* YL104 to efficiently produce succinate from glucose. In this study, we investigated the relationships between the NADH/NAD$^+$ ratio, ATP level, and overall yield of succinate production by using glucose as the carbon source in YL104. First, the use of sole NADH dehydrogenases increased the overall yield of succinate by 7% and substantially decreased the NADH/NAD$^+$ ratio. Second, the soluble fumarate reductase from *Saccharomyces cerevisiae* was overexpressed to manipulate the anaerobic NADH/NAD$^+$ ratio and ATP level. Third, another strategy for reducing the ATP level was applied by introducing ATP futile cycling for improving succinate production. Finally, a combination of these methods exerted a synergistic effect on improving the overall yield of succinate, which was 39% higher than that of the previously engineered strain YL104. The study results indicated that regulation of the NADH/NAD$^+$ ratio and ATP level is an efficient strategy for succinate production.

Keywords Succinate yield · NADH/NAD$^+$ ratio · ATP generation · NADH dehydrogenases · Soluble fumarate reductases · ATP futile cycling

Electronic supplementary material The online version of this article (doi:10.1007/s00253-017-8127-6) contains supplementary material, which is available to authorized users.

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Introduction

Succinic acid, a C_4-dicarboxylic acid, is a precursor of many important chemicals in the food, chemical, and pharmaceutical industries (Ahn et al. 2016; Olajuyin et al. 2016; Wang et al. 2014). Certainly, the widespread importance of succinate has secured its listing among the top 12 chemical building blocks by the US Department of Energy (Werpy and Petersen 2004). To improve succinate production by using *Escherichia coli*, researchers have explored various metabolic engineering strategies (Ahn et al. 2016; Forster and Gescher 2014; Kang et al. 2010; Liang et al. 2015) such as activating the glycolytic pathway, overexpressing pyruvate-metabolizing enzymes, eliminating competing pathways, and providing reducing equivalents and energy (Li et al. 2016; Liang and Qi 2014; Meng et al. 2016; Vemuri et al. 2002; Vuoristo et al. 2016).

We previously constructed succinate-producing strains by using a series of genetic modifications, and a high overall volumetric productivity and concentration of succinate were achieved (Li et al. 2013). A whole-phase succinate production strategy was developed in this study. In this method, the engineered strain produced succinate under aerobic, microaerobic, and anaerobic conditions. Compared with this whole-phase fermentation, strain growth uncouples product formation in the typical dual-phase fermentation. However, the overall yield from our constructed engineered strain should be increased further. Recently, we explored the relationships between ATP generation, substrate ratio of xylose to glucose, and succinate production (Zhang et al. 2016). We observed that excess energy exerted a negative effect on succinate production in the engineered *E. coli* that could not grow anaerobically. Therefore, regulating ATP generation for improving the overall yield of succinate by using glucose as the carbon source in this engineered *E. coli* is essential.

The NADH/NAD$^+$ ratio is also crucial for succinate production (Singh et al. 2009). Under aerobic growth conditions, NAD$^+$ is regenerated through oxidative phosphorylation. Under anaerobic conditions, NAD$^+$ serves as an electron acceptor during substrate degradation, and NADH provides the reducing power for reductive product formation. A balance of NAD$^+$ reduction and NADH oxidation is a prerequisite for anaerobic fermentation (de Graef et al. 1999). In the engineered *E. coli*, NADH-dependent lactate dehydrogenase and ethanol dehydrogenase were inactivated. Inactivation limits the means by which NAD$^+$ may be regenerated from NADH formed from aerobic to anaerobic growth conditions, thus affecting the redox balance to increase the NADH/NAD$^+$ ratio (Fig. 1a; Li et al. 2013). Therefore, we assumed that the low yield of succinate production was because of the limited NAD$^+$ regeneration rate, which resulted in the increase in the NADH/NAD$^+$ ratio from the aerobic to anaerobic phase; however, NAD$^+$ could be regenerated in the reductive tricarboxylic acid (TCA) cycle.

Another possible cause of the increased NADH/NAD$^+$ ratio at the initial anaerobic condition is the change in NADH dehydrogenases during the aerobic–anaerobic fermentation process. The aerobic respiratory chain of *E. coli* involves three membrane-bound NADH dehydrogenases (NDH-I, NDH-II, and WrbA) and three ubiquinol oxidases (cytochromes bd-I, bd-II, and bo; Fig. 1b). These enzymes have different efficiencies in coupling electron transfer for the generation of an electrochemical proton gradient (Bekker et al. 2009; Borisov et al. 2011). Under aerobic conditions, *E. coli* mainly uses NADH dehydrogenases II (*ndh*), and under anaerobic conditions, it mainly uses NADH dehydrogenases I (*nuo*). The change in main NADH dehydrogenase in *E. coli* from the aerobic to anaerobic condition may be time-consuming, and this change also resulted in an elevated NADH/NAD$^+$ ratio. Therefore, we speculated that using one type of NADH dehydrogenase during aerobic and anaerobic conditions could reduce the NADH/NAD$^+$ ratio to increase the overall yield of succinate (Singh et al. 2009; Vemuri et al. 2006).

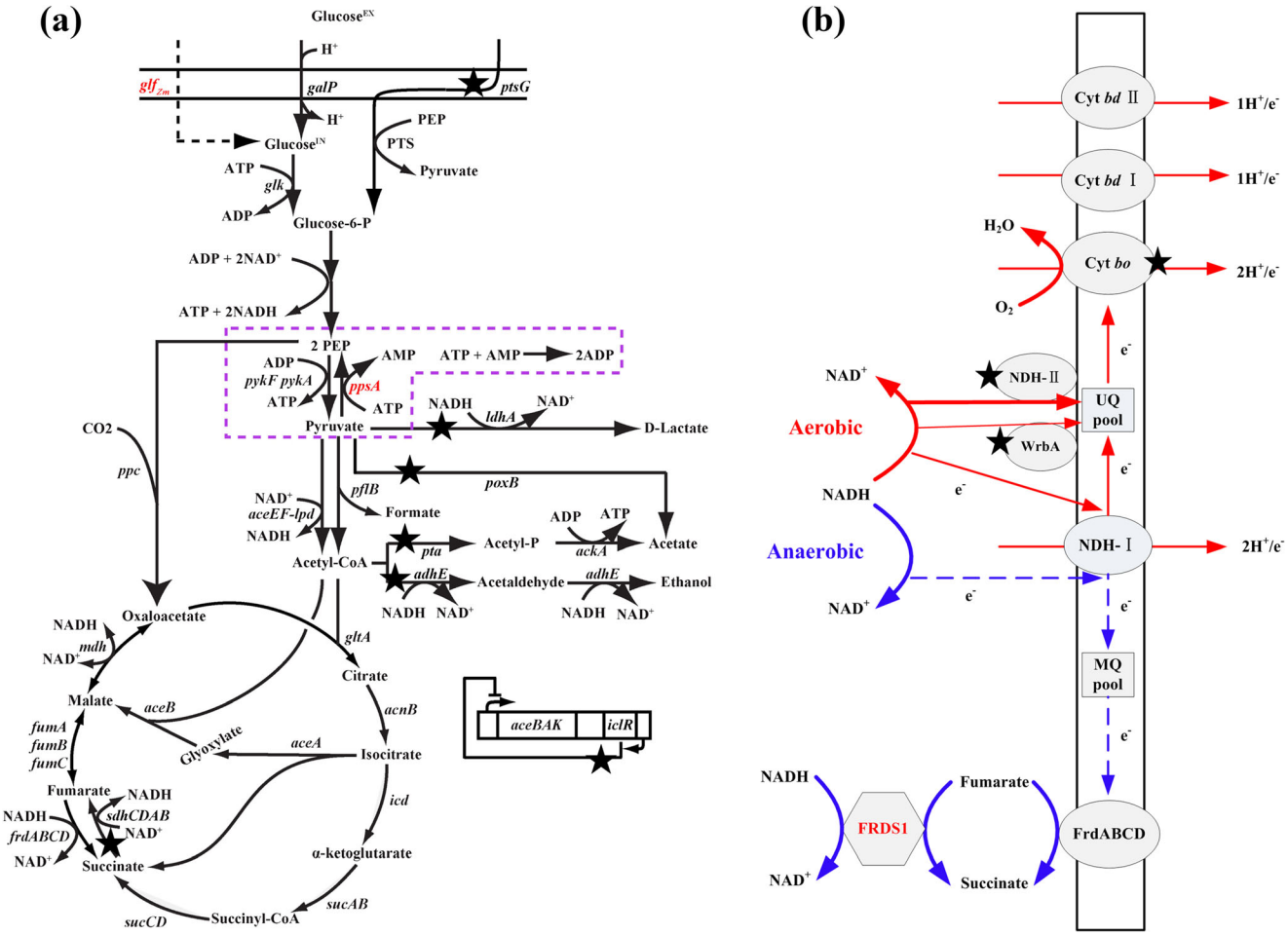

Fig. 1 Central carbon metabolism and electron transport chain in our engineered *E. coli*. **a** Gene deletions in strain YL104 are represented by *black stars*, and gene overexpression is depicted in *red*. ATP futile cycling reactions are denoted by *purple circles*. *PEP* phosphoenolpyruvate. **b** *Red* and *blue lines* represent the aerobic respiratory chain and anaerobic respiratory chain, respectively. *Black stars* represent the gene mutation in YL104. FRDS1 is soluble fumarate reductase from *S. cerevisiae* (color figure online)

In this study, by regulating the NADH/NAD^+ ratio and ATP generation through various strategies, we explored the relationships between the NADH/NAD^+ ratio, ATP level, and overall yield of succinate production with glucose as the carbon source.

Materials and methods

Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 1 and Table S1. All genetic modifications were made in *E. coli* MG1655.

The one-step inactivation of the chromosomal gene method was used for single gene mutants (Datsenko and Wanner 2000). The primers *ndh*-pKD4-F and *ndh*-pKD4-R and the template plasmids pKD4 for *ndh* knockouts were used to obtain linear DNA fragments with antibiotic-resistant gene cassettes flanked by FLP recognition target sites and 39-bp homologous arms for the construction of the single gene mutants. The purified DNA was electroporated into *E. coli* K-12 MG1655 cells harboring the helper plasmid pTKRed expressing Red recombinase enzymes (Kuhlman and Cox 2010). Positive clones were selected using appropriate antibiotics. Subsequently, the helper plasmid and resistance gene were eliminated.

For gene overexpression, pCL1920 was used and digested using *Hin*dIII–*Kpn*I to obtain the plasmid skeleton and resistant gene. A DNA fragment with *frd* sequence (GenBank, KX505965) from *Saccharomyces cerevisiae* S288c was synthesized with codon optimization by Generay Biotechnology (Shanghai, China). This fragment was also digested using *Hin*dIII–*Kpn*I, and the sequence was ligated into the vector pCL1920, thus generating the plasmid pCLfrd. Furthermore, *nuoC* was amplified from wild-type *E. coli* MG1655 by using the primers *nuoC* PF and *nuoC* PR. The vector skeleton and *ppsA* were amplified from the pCL1920 plasmid and *E. coli* MG1655 genome by using the primers pCL1920-F and pCL1920-R and *trc-ppsA*-F and *trc-ppsA*-R, respectively. The plasmid pCLnuoC was obtained through Gibson assembly with the pCL1920 skeleton and *nuoC* fragment. The primers *trc*-RBS-F and *trc*-RBS-R from the pTrc99a plasmid were annealed to obtain the *trc* promoter with RBS. Subsequently, the three fragments, plasmid skeleton, *trc*RBS, and *ppsA*, were assembled together through Gibson assembly. In addition, pCLppsA/frd was constructed using Gibson assembly with the pCLppsA skeleton and *frd* fragment.

Culture medium and growth conditions

Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl; pH 7.2) was used for all DNA manipulations. During the construction of the single-gene-deficient mutants, cultures were grown aerobically at 30, 37, or 42 °C in Super Optimal Broth medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, and 10 mM $MgCl_2$). All cultures were incubated at 37 °C, and the pH was measured using a glass electrode and controlled at 6.4–6.8 by using 5 M K_2CO_3 and 0.5 M NaOH.

Table 1 Bacterial strains and plasmids used in this study

	Relevant description	Reference
E. coli strains		
MG1655	Strain K-12, $F^-\lambda^-$ *rph-1*	ATCC
YL104	MG1655(*ΔptsG ΔpoxB Δpta ΔiclR ΔsdhA ΔarcAΔadhE ldhA::trc-rbs-glf*$_{Zm}$)	(Li et al. 2013)
YL104N	YL104(*Δndh::FRT*)	This study
YL104N/nuoC	YL104N harboring pCLnuoC	This study
YL104NF	YL104N harboring pCLfrd	This study
YL104NP	YL104N harboring pCLppsA	This study
YL104NPF	YL104N harboring pCLppsA/frd	This study
Plasmids		
pTKRed	Helper plasmid, pSC101ori, αβ exo (red recombinase), *spc*	(Kuhlman and Cox 2010)
pKD4	R6K γ ori, FRT-kan-FRT, bla	(Datsenko and Wanner 2000)
pTrc99a	Cloning vector, *trc* promoter, *amp*	Lab stock
pCL1920	Cloning vector, pSC101ori, *spc*	(Kuhlman and Cox 2010)
pCLnuoC	pSC101ori, expression of *nuoC* under the *lac* promoter, *spc*	This study
pCLfrd	pSC101ori, *frd* cloned from *S. cerevisiae* EBY100 under the *lac* promoter, *spc*	This study
pCLppsA	pSC101ori, expression of *ppsA* under the *trc* promoter of pTrc99a, *spc*	This study
pCLppsA/frd	pSC101ori, co-expression of *ppsA* and *frd* under the *trc* promoter of pTrc99a, *spc*	This study

For succinate production, a single clone of each single gene mutant and the parent strain were cultured in a 300-mL Erlenmeyer flask containing 50 mL of LB medium at 37 °C and 250 rpm for 12 h. Subsequently, 4 mL (5% *v*/*v*) of the overnight culture was transferred to a 500-mL Erlenmeyer flask containing 80 mL AM1 [2.63 g/L $(NH_4)_2HPO_4$, 0.87 g/L $NH_4H_2PO_4$, 0.15 g/L KCl, and 20.9 g/L 3-(*N*-morpholino)propanesulfonic acid (MOPS)] with an additional 1 g/L yeast extract and 30 g/L glucose and shaken at 250 rpm for 21 h. Antibiotics were added to provide the selective pressure during the cultivation, if necessary (ampicillin 100 μg/mL, kanamycin 25 μg/mL, spectinomycin 25 μg/mL, and chloromycetin 17 μg/mL).

During the succinate fermentation in a multiple mini-fermenter system (INFORS HT, Switzerland), the aforementioned seed culture (10% *v*/*v*) was transferred into 800 mL AM1 medium (without MOPS) supplemented with 40 g/L glucose in a 1-L fermenter for batch fermentation. The total fermentation time (70 h) included a 28-h aerobic phase (pass into O_2) and a 42-h anaerobic phase (pass into CO_2). The air flow rate and agitation were maintained at 1 vvm and 350 rpm, respectively. During the fermentation, 0.05 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at the beginning of anaerobic fermentation with strains harboring plasmids during the batch fermentation. Samples were taken at intervals of 6 or 12 h.

Analytical methods

Biomass was detected by measuring the density at 600 nm [1 $OD_{600} \approx 0.34$ g cell dry weight (CDW) L^{-1}] by using a spectrophotometer (Shimadzu, Japan). The organic acid concentrations and the residual glucose concentration were measured using high-performance liquid chromatography (HPLC; Shimadzu, Japan) and an Aminex HPX-87H ion exclusion column (Bio-Rad, USA). H_2SO_4 solution (5 mM) was applied as a mobile phase at a flow rate of 0.6 mL/min to the column at 65 °C.

The intracellular ATP concentrations were measured using the BacTiter-Glo™ Microbial Cell Viability Assay Kit (Promega, USA), and the intracellular concentrations of NADH and NAD^+ were assayed using a cycling method (Leonardo et al. 1996).

Results

Use of sole anaerobic NADH dehydrogenases to increase succinate yield

First, we inactivated the NADH dehydrogenase II encoded by *ndh* in the previously constructed YL104 (Fig. 1a; Li et al. 2013), thus generating YL104N. In response to this modification, succinate production was similar but the overall yield of succinate in YL104N increased by 7%, reaching 1.05 mol/mol (Table 2). The biomass of YL104N exhibited no substantial change (Table 2), which suggested that the inactivation of NDH-II dehydrogenase did not have a negative effect on the growth of the engineered strains. In addition, we regulated NADH dehydrogenase I by overexpressing an important subunit (encoded by *nuoCD*) of this enzyme (Singh et al. 2009). However, the titer of succinate decreased, and the overall yield only slightly improved (Table 2). We speculated that excess energy was generated by overexpression of the important subunit of NDH-1 that generates proton gradients across cell membranes (Verkhovskaya et al. 2008). In addition, metabolic burdens resulting from overexpression of enzymes may influence the growth of the engineered strain, leading to decreased succinate production. The aerobic NADH/NAD^+ ratio increased in YL104N compared with YL104 (Fig. 2). Moreover, the NADH/NAD^+ ratio in YL104N increased less from the aerobic to anaerobic condition compared with that in YL104 (94 and 29% increases in YL104 and YL104N, respectively). These results indicated that the inactivation of main aerobic NADH dehydrogenase (*ndh*) in YL104 could alleviate the change in the NADH/NAD^+ ratio, resulting in a more stable physiological state, which may be beneficial for anaerobic succinate production (Liang et al. 2012).

Table 2 Growth and succinate production of various strains constructed in the study

Strains	Biomass (g/CDW/L)	Glucose consumption (g/L)	Succinate production (g/L)	Overall yield (mol/mol)
YL104	2.70 ± 0.02	43.2 ± 0.5	27.86 ± 0.2	0.98 ± 0.03
YL104N	2.66 ± 0.04	40.53 ± 0.3	27.92 ± 0.2	1.05 ± 0.01
YL104N/nuoC	2.62 ± 0.04	32.90 ± 0.2	22.82 ± 0.4	1.06 ± 0.05

The strains were cultivated at 37 °C in the mineral medium AM1 with an initial glucose concentration of approximately 40 g/L for 70 h in a multiple mini-fermenter system. The agitation rate was maintained at 350 rpm, and the air flow was initially 1 vvm for 30 h and then dropped to 0 vvm for 40 h. When cells with plasmids were transferred into the fermenter, 0.05 mM IPTG was added into the medium at the transition time. Each value is the mean of three parallel replicates ± standard deviation

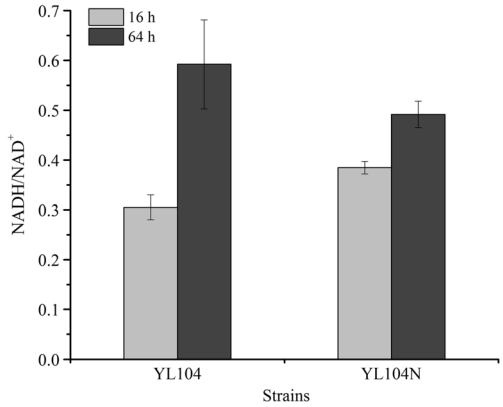

Fig. 2 NADH/NAD$^+$ ratio during aerobic and anaerobic fermentation. Standard deviations were calculated from the results of four independent experiments

Modulating the NADH/NAD$^+$ ratio and ATP level through overexpression of soluble fumarate reductases FRDS1 from *S. cerevisiae*

Fumarate reductases (FRDs), which catalyze the reduction of endogenous or exogenous fumarate into succinate, are key factors in the anaerobic metabolism of many organisms (Camarasa et al. 2007). The *E. coli* FRD is a respiratory enzyme, covalently linked to flavin cofactors (FAD) and membrane-bound enzyme (located in the inner membrane) structurally similar to succinate dehydrogenases. Being associated with the respiratory chain, they are directly involved in the production of ATP through oxidative phosphorylation (Tielens and Van Hellemond 1998). NADH-dependent FRD in *E. coli* can catalyze the metabolic flux flow to succinate and lower the NADH/NAD$^+$ ratio. However, this type of FRD may generate more energy in the interface (a particular period in which the fermentation is switched from the aerobic to the anaerobic condition) from the aerobic to the anaerobic phase because of the connection of the electron transfer chain in YL104N (Fig. 1b), which has a negative effect on succinate production (Zhang et al. 2016). However, inactivating the main aerobic NADH dehydrogenase in YL104 can increase the ATP level at the initial anaerobic phase (Calhoun et al. 1993). The FRDS1 encoded by *frd* from *S. cerevisiae* is a soluble enzyme that noncovalently binds $FADH_2$ (cytoplasmic isozyme) and irreversibly catalyzes the reduction of fumarate independently of the electron transfer chain (Camarasa et al. 2007; Enomoto et al. 1996; Muratsubaki and Enomoto 1998). Thus, we speculated that overexpressing *frd* from *S. cerevisiae* in *E. coli* may change the anaerobic ATP level and NADH/NAD$^+$ ratio of YL104N at the beginning of the anaerobic phase.

We placed *frd* under the *tac* promoter and introduced it into YL104N. The resulting strain YL104NF produced more succinate (28.0 g/L), with a 19% higher yield than that of YL104N (24.4 g/L; Fig. 3a). The overall yield of succinate was also improved in YL104NF (an increase of approximately 13%). The biomass and aerobic glucose consumption of these two strains were similar; however, the anaerobic glucose consumption of YL104NF (9.27 g/L) was faster than that of YL104 (8.71 g/L). This may have been the key factor for the increased succinate production. In addition, after 63 h, YL104NF generated less ATP and a lower NADH/NAD$^+$ ratio during anaerobic fermentation (Fig. 3b). The intracellular ATP level of YL104NF decreased by 10%, and the NADH/NAD$^+$ ratio decreased by 24%. The results indicated that overexpression of soluble FRDS1 could certainly reduce the anaerobic ATP level and NADH/NAD$^+$ ratio. Consequently, both anaerobic succinate production and the total succinate production were improved.

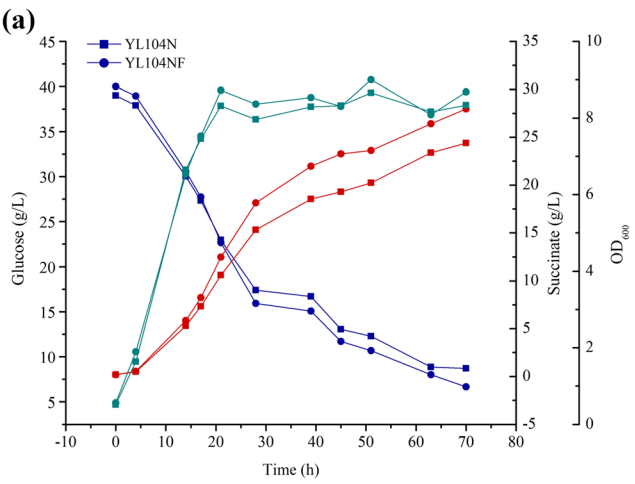

Fig. 3 Growth, succinate production, NADH/NAD$^+$ ratio, and ATP level. **a** Growth, glucose consumption, and succinate production of strains YL104N and YL104NF. Glucose consumption (*dark blue lines*), succinate production (*red lines*), and OD600 (*dark cyan lines*). **b** Measurements of ATP level and NADH/NAD$^+$ ratio of strains YL104N and YL104NF at 63 h. Standard deviations were calculated from the results of four independent experiments (color figure online)

ATP futile cycling to reduce the ATP level under anaerobic conditions

In the glycolytic pathway, pyruvate kinase catalyzes the formation of pyruvate from phosphoenolpyruvate (PEP) with the production of one ATP molecule. The reverse reaction catalyzed by PEP synthase (encoded by *ppsA*) from pyruvate to PEP requires ATP as a cofactor; it is converted into AMP. Thus, a cycle in which one ATP molecule is consumed in net is formed (ATP futile cycling; Fig 1a; Hadicke et al. 2015). We introduced this ATP futile cycling into YL104N to improve the overall yield of succinate by reducing the anaerobic ATP level. The resulting strain YL104NP produced more succinate, with a 7% higher yield than that of YL104N (Fig. 4a), and the overall yield of succinate in YL104NP also increased by 17%. Compared with YL104N, biomass, aerobic succinate production, and glucose consumption decreased, but anaerobic succinate production and glucose consumption were faster than that of YL104N. Moreover, the anaerobic succinate titer of YL104NP was 47% higher than that of YL104N (Fig. 4a). These results suggested that a decreased ATP level resulted in increased succinate production and glucose consumption under anaerobic conditions. The decline in biomass and aerobic succinate of YL104NP may result from the leaky expression of *ppsA* from the *trc* promoter under aerobic conditions, which consumed the available ATP for growth. Regarding the by-products of YL104NP, formate, acetate, and lactate, the quantities produced were nearly half of those produced with YL104N (Fig. 4b).

The anaerobic ATP level of YL104NP decreased by 33% compared with that of YL104N at the end of fermentation (Fig. 5a). In addition, the aerobic ATP level in YL104NP decreased compared with YL104N because of the leaky expression of *ppsA*. Moreover, the anaerobic ATP level in YL104NP decreased more rapidly. The tendency of the regression line (the slope k), which was calculated using the ATP levels throughout the entire fermentation process, is crucial (Stouthamer 1979). In the present study, the slope k_1 of the anaerobic trend line of the ATP curve in YL104N was clearly higher than the slope k_2 of YL104NP (−32.3 and −55.6, respectively), indicating that under the anaerobic condition, YL104NP generated less ATP than YL104N (Fig. 5b; Liu et al. 2013; Zhang et al. 2016).

Coexpression of *frd* and *ppsA* to increase succinate yield

Overexpression of *frd* and *ppsA* could individually reduce the anaerobic intracellular ATP level and increase the overall succinate yield, respectively. Thus, we examined whether coexpression of *frd* and *ppsA* had a synergistic effect on improving the succinate yield and titer. The data illustrated that overexpression of *frd* and *ppsA* individually produced succinate with titers 28.19 and 29.05 g/L, 12 and 9% lower than that produced by YL104NPF (titer 31.87 g/L), respectively (Table 3). Compared with strains YL104NF and YL104NP, the biomass and glucose consumption of YL104NPF were slightly decreased, whereas the overall yield of succinate in YL104NPF was 1.36 mol/mol, which was 13 and 9% higher than those of strains YL104NF and YL104NP, respectively. The final yield of strain YL104NPF was 39% higher than that of the initial strain YL104. Our results suggested that coexpression of *frd* and *ppsA* exhibited a synergistic effect on improving succinate production and yield.

Discussion

Manipulating redox equivalent and energy balance is crucial for the efficient synthesis of target products, particularly for

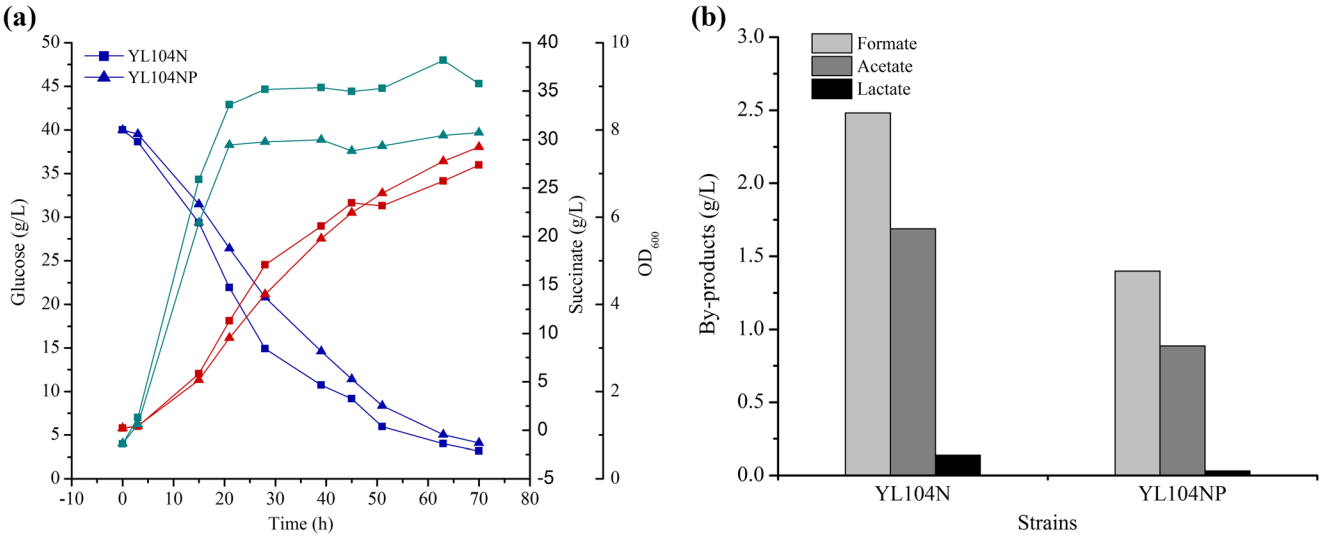

Fig. 4 Growth, succinate production, and by-products. **a** Growth, glucose consumption, and succinate production of strains YL104N and YL104NF. Glucose consumption (*dark blue lines*), succinate production (*red lines*), and OD600 (*dark cyan lines*). **b** By-products of strains YL104N and YL104NP were measured at the end of fermentation (color figure online)

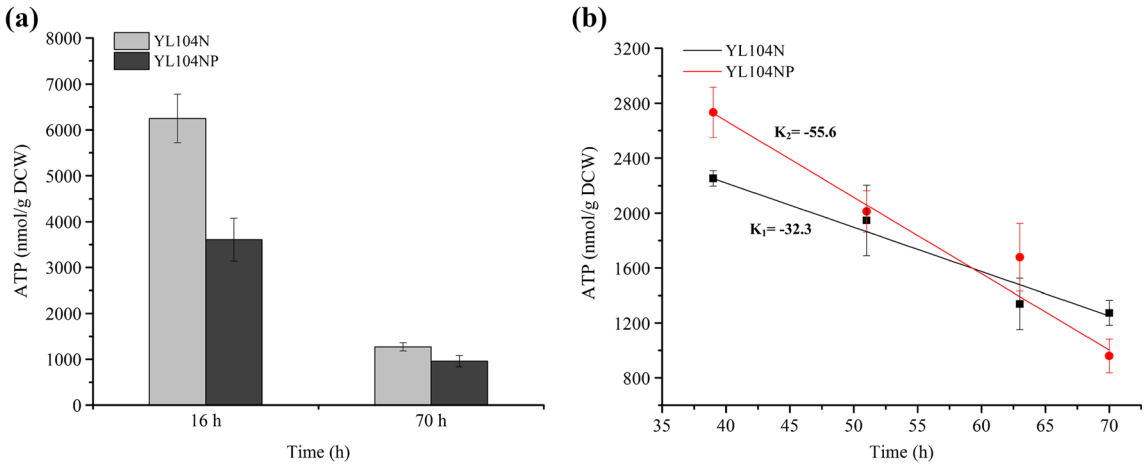

Fig. 5 ATP level and generation in strains YL104N and YL104NP. **a** Measurements of ATP level of strains YL104N and YL104NP at 70 h. **b** Trend line of ATP during the anaerobic fermentation of strains YL104N and YL104NP. Standard deviations were calculated from the results of four independent experiments

succinate biosynthesis (Singh et al. 2011; Zhu et al. 2014). In the present study, we applied the soluble FRDs from *S. cerevisiae* for the first time to modulate the ATP level and NADH/NAD^+ ratio to enhance biochemical production.

Most FRDs belong to a multimeric complex associated with the respiratory chain and transfer electrons from quinol to fumarate (Van Hellemond and Tielens 1994). However, soluble monomeric FRDs with $FADH_2$/$FMNH_2$ as an electron donor, not linked to the electron transfer chain, have been identified in several *Shewanella* species, *S. cerevisiae*, and the protozoan *Trypanosoma brucei* (Besteiro et al. 2002; Gordon et al. 1998; Muratsubaki and Enomoto 1998). Overexpression of NADH-dependent FRDs from *T. brucei* were employed to maintain the cellular redox balance and enhance the cytosolic NAD^+ and NADPH pools (Besteiro et al. 2002; Coustou et al. 2005; Salusjarvi et al. 2013). Soluble *frds1* from *S. cerevisiae* were inserted into *Aspergillus* for reoxidizing NADH to NAD^+ under anaerobic conditions, thereby increasing the productivity and yield of citrate on glucose (de Jongh and Nielsen 2008; Enomoto et al. 2002). In the present study, the results revealed that expressing soluble FRDS1 from *S. cerevisiae* in *E. coli* could modulate not only the NADH/NAD^+ ratio but also the ATP level under anaerobic conditions for increasing succinate production (Cabrera et al. 2011; Hadicke et al. 2015; Liang et al. 2012; Singh et al. 2009; Vemuri et al. 2006; Zhang et al. 2016).

In this study, sole anaerobic NADH dehydrogenase and ATP futile cycling were employed to regulate the NADH/NAD^+ ratio and ATP level in the interface from the aerobic to anaerobic phase, respectively.

In previous studies, through respiratory chain engineering, combining various NADH dehydrogenases and terminal oxidases with different efficiencies of proton translocation enabled *E. coli* flexibility to provide diverse growth conditions to produce fermentation products (Becker et al. 1997; Portnoy et al. 2008). Yokota et al. observed the highest rate of glucose metabolism in the double mutant (NAD-1 and cytochrome bo3; Kihira et al. 2012). In a glucose-limited chemostat, a branched electron transport chain was adopted rather than a linear one, and the growth behavior and acetate formation were analyzed under this condition (Steinsiek et al. 2014). The quinone synthesis pathway (Wu et al. 2015) and NDH-II dehydrogenase (Liu et al. 2014) were manipulated for enhancing lactate and polyhydroxybutyrate production, respectively. Furthermore, in succinate production, various strategies for reducing the NADH/NAD^+ ratio have been employed. These strategies include regeneration of NAD^+ in the reductive TCA cycle (Goldberg et al. 1983; Liang et al. 2011; Millard et al. 1996; Stols and Donnelly 1997) and provision of additional reducing power (Van der Werf et al. 1997). In the present study, only one type of NADH dehydrogenase of the respiratory chain was

Table 3 Growth and succinate production of overexpression strains

Strains	Biomass (g/CDW/L)	Glucose consumption (g/L)	Succinate production (g/L)	Overall yield (mol/mol)
YL104NF	2.64 ± 0.04	36.11 ± 0.1	28.19 ± 0.2	1.19 ± 0.02
YL104NP	2.30 ± 0.03	35.92 ± 0.3	29.05 ± 0.1	1.23 ± 0.01
YL104NPF	2.37 ± 0.03	35.71 ± 0.4	31.87 ± 0.1	1.36 ± 0.02

Each value is the mean of three parallel replicates ± standard deviation

adopted, thus increasing the overall yield of succinate and alleviating the change in the NADH/NAD^+ ratio during the entire fermentation process.

High ATP levels have been proven to inhibit the activities of key enzymes involved in the central metabolism, thus downregulating the glycolytic pathway and TCA cycle (Kihira et al. 2012; Kim et al. 2008; Weitzman 1981). By using enhanced futile cycling to decrease the ATP supply, production rates and yields of fermentation products were increased, and glucose consumption was elevated under aerobic conditions (Chao and Liao 1994; Patnaik et al. 1992). Recently, the same strategy was also applied for improving the production of lactate under anaerobic conditions (Hadicke et al. 2015). In the present study, we proved that ATP futile cycling is an effective strategy for improving succinate yield.

Finally, our results demonstrated that a combination of these methods exerted a synergistic effect on improving the overall yield of succinate, which was 39% higher than that of the strain constructed in our previous study. Moreover, the results indicate that the regulation of the NADH/NAD^+ ratio and ATP level, which is not involved in the succinate biosynthetic pathway, is an efficient strategy for succinate production.

Acknowledgements This work was financially supported by a grant from the National Basic Research Program of China (2012CB725202), a grant from the National Natural Science Foundation of China (31170097, 31370085), and a grant from the Shandong Science and Technology Development Plan (2015GSF121042).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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