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Improvement of NADPH bioavailability in *Escherichia coli* by replacing NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase GapA with $NADP^+$-dependent GapB from *Bacillus subtilis* and addition of NAD kinase

Yipeng Wang · Ka-Yiu San · George N. Bennett

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Abstract Enzymatic synthesis of some industrially important compounds depends heavily on cofactor NADPH as the reducing agent. This is especially true in the synthesis of chiral compounds that are often used as pharmaceutical intermediates to generate the correct stereochemistry in bioactive products. The high cost and technical difficulty of cofactor regeneration often pose a challenge for such biocatalytic reactions. In this study, to increase NADPH bioavailability, the native NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *gapA* gene in *Escherichia coli* was replaced with a $NADP^+$-dependent *gapB* from *Bacillus subtilis*. To overcome the limitation of $NADP^+$ availability, *E. coli* NAD kinase, *nadK* was also coexpressed with *gapB*. The recombinant strains were then tested in three reporting systems: biosynthesis of lycopene, oxidation of cyclohexanone with cyclohexanone monooxygenase (CHMO), and an anaerobic system utilizing 2-haloacrylate reductase (CAA43). In all the reporting systems, replacing NAD^+-dependent GapA activity with $NADP^+$-dependent GapB activity increased the synthesis of NADPH-dependent compounds. The increase was more pronounced when NAD kinase was also overexpressed in the case of the one-step reaction catalyzed by CAA43 which approximately doubled the product yield. These results validate this novel approach to improve NADPH bioavailability in *E. coli* and suggest that the strategy can be applied in *E. coli* or other bacterium-based production of NADPH-dependent compounds.

Keywords NADPH bioavailability · NADP-dependent GAPDH · NAD kinase · Reducing equivalent · Cofactor engineering · *E. coli*

Y. Wang · G. N. Bennett (✉)
Department of Biochemistry and Cell Biology, MS-140, Rice University, 6100 Main Street, Houston, TX 77005, USA
e-mail: gbennett@rice.edu

K.-Y. San
Department of Bioengineering, Rice University, Houston, TX 77005, USA

K.-Y. San
Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX 77251, USA

Introduction

The cofactor pair NADPH/$NADP^+$ is essential for all living organisms. Its importance centers mainly on its use as donor and/or acceptor of reducing equivalents in oxidation–reduction reactions in living cells. The NADH/NAD^+ pair is used for catabolic activities of the cell, whereas anabolic metabolism specifically requires NADPH/$NADP^+$. Therefore, these nucleotides have direct impacts on virtually every oxidation–reduction metabolic pathway in the cell. In applications with biocatalysts, many industrially valuable compounds require NADPH for their synthesis. However, because of the high cost and regeneration difficulty of these coenzymes, the enzymatic production of cofactor-dependent compounds has been challenging. There have been a variety of methods developed to regenerate this cofactor such as chemical, electrochemical, photochemical, or enzymatic reactions [8, 40]. Among them enzymatic reactions were favored because of their high specificity and high proficiency. In recent years, whole cell cofactor regeneration systems have made great progress. Strategies such as overexpressing the target enzymes along with a coupled NADPH recycling reaction

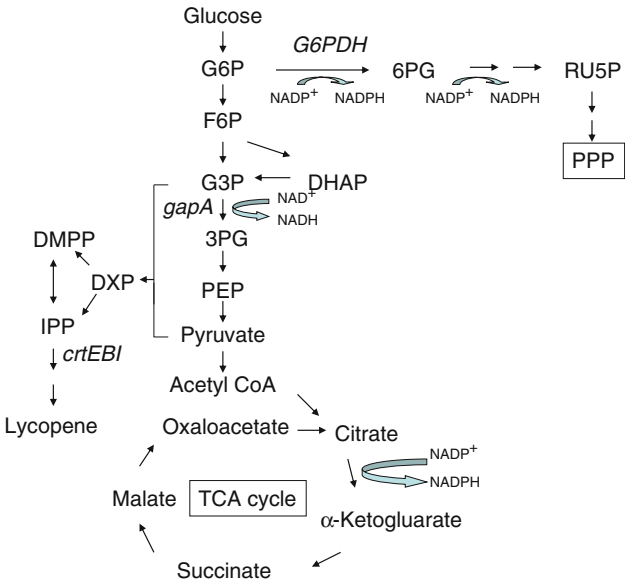

Fig. 1 Simplified diagram of major metabolic pathways and reactions that generate NADPH in *E. coli* including glycolysis, TCA cycle, and pentose phosphate pathway (PPP). Also shown in the figure is the lycopene biosynthesis pathway that was used as a reporting system in this study. *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *G3P* glyceraldehyde-3-phosphate, *DHAP* dihydroxyacetone-3-phosphate, *3PG* 3-phosphoglycerate, *PEP* phosphoenolpyruvate, *6PG* 6-phosphogluconolactone, *RU5P* ribulose-5-phosphate, *DXP* deoxyxylulose-5-phosphate, *DMPP* dimethylallyl pyrophosphate, *IPP* isopentenyl phosphate

have been reported before [11, 12, 53]. Other efforts to overcome this problem include modification of the enzyme's cofactor specificity [41, 58, 59], and more general approaches such as manipulating a host metabolic pathway or introducing novel pathways to increase the availability of NADPH in the host cell [9, 16, 28, 37, 44, 49].

Major sources of NADPH in *Escherichia coli* include the pentose phosphate pathway (PPP), isocitrate dehydrogenase [48] in the tricarboxylic acid (TCA) cycle, and the transhydrogenase system [27]. In this study we developed an approach to increase the NADPH availability in vivo through building a NADPH-generating glycolytic pathway. In this approach we replaced native *E. coli* NAD^+-dependent GAPDH with an overexpressed *Bacillus subtilis* $NADP^+$-dependent GAPDH (Fig. 1) [17]. There are three types of GAPDH, namely $NADP^+$-dependent non-phosphorylating GAPDH that produces 3-phosphoglycerate, NAD^+-dependent phosphorylating GAPDH, and $NADP^+$-dependent phosphorylating GAPDH [57]. The phosphorylating NAD^+/$NADP^+$-dependent GAPDH produces 1,3-diphosphoglycerate along with NADH/NADPH. The *B. subtilis* $NADP^+$-dependent GAPDH catalyzes the following reaction: D-glyceraldehyde-3-phosphate + phosphate + $NADP^+$ $\rightleftharpoons$ 1,3-diphosphateglycerate + NADPH. In the proposed system every mole of glucose passing through the glycolytic pathway generates 2 moles of NADPH instead of 2 moles of NADH for the native pathway. Therefore, the host cell will have more intracellular reducing agent available to support NADPH-dependent biosynthesis processes such as formation of chiral alcohol, carotenoid, and the biodegradable polymer, poly-3-hydroxybutyrate (PHB). Previous work has been reported on modification of the cofactor specificity of GAPDH. Verho et al. [58, 59] found a fungal $NADP^+$-dependent GAPDH from *Kluyveromyces lactis* and expressed it in *Saccharomyces cerevisiae* that led to a higher ethanol production. Previously we reported the use of a *Clostridium acetobutylicum* GAPDH *gapC* to replace native *gapA* of *E. coli* and experiments indicated higher formation of NADPH-dependent products [41]. The current study help expand the candidates of NADPH-utilizing GAPDH enzymes to *Bacillus* GapB. Recent work done on GapB by Kim et al. [30] reported expression of $NADP^+$-dependent malic enzyme and *Bacillus gapB* helped recover growth of phosphoglucose isomerase (*pgi*)-disrupted *E. coli*, but *gapB* alone did not significantly increase the NADPH level in the *pgi* mutant. A number of NADPH-dependent GAPDH enzymes have been isolated from a variety of organisms especially photosynthetic organisms and other bacteria [4, 13, 14, 23, 24, 26, 42, 46]. Many NADPH-utilizing GAPDH enzymes from plants and some other bacteria are ATP coupled and thus provide ATP during the reaction, as is the case with the enzyme we used in this study. This ATP-coupling can help provide energy for the cell. There is also another group of GapN-type, NADPH-dependent GAPDH enzymes that directly form glycerate-3-phosphate (non-phosphorylating) and these have also been used in metabolic engineering experiments particularly with yeast or *Corynebacterium glutamicum* as well as *E. coli* [20, 31, 55, 57, 63]. Such GAPDH enzymes are found in a number of organisms where they have roles in the operation of glycolysis under certain circumstances.

In the course of the study, we found that overexpression of $NADP^+$-dependent GAPDH alone had its own limitations. Cells that overexpressed $NADP^+$-dependent GAPDH consumed less glucose than the wild-type cells possibly owing to the inherent low concentration of $NADP^+$ available in the cell. To solve this problem, *E. coli* NAD kinase encoded by *nadK* was introduced into the system. The NAD kinase converts NAD^+ to $NADP^+$ through phosphorylation using ATP as the phosphoryl donor [29, 62]. Previous studies have shown that overexpression of *nadK* increased the NADPH/$NADP^+$ ratio, but not the overall [NADH + $NADP^+$]/[NADPH + NAD^+] ratio [35]. Several studies have shown that overexpression of *nadK* is beneficial for NADPH-dependent biosynthetic pathways such as isoleucine production in *Corynebacterium glutamicum* [52], PHB production [39, 52], and thymidine production in *E. coli* [35]. In this study, we found that

coexpression of $NADP^+$-dependent *gapB* and *nadK* in combination significantly increased NADPH-dependent 2-chloropropionic acid production catalyzed by 2-haloacrylate reductase.

To test the effectiveness of our proposed NADPH-generating glycolytic pathway, three independent reporting systems were utilized to address this question. These three systems include an established oxygenated biocatalyst assay, an aerobic growth situation where glycolytic intermediate precursors serve two roles both as a direct precursor of the final product and as a means of generating reducing power through oxidation via the TCA cycle, and finally in an anaerobic system where we can quantitate the redox available from glucose and analyze the proportion of reducing power going into the NADPH-derived product accurately. The first system is a one-step Baeyer–Villiger (BV) reaction catalyzed by cyclohexanone monooxygenase CHMO. CHMO catalyzes oxidation of cyclohexanone using molecular oxygen into ε-caprolactone along with oxidation of NADPH to $NADP^+$. Baeyer–Villiger monooxygenase has been found in a variety of organisms; in this study, we used a CHMO from *Acinetobacter* sp. NCIB 9871 which has shown high enantioselectivity [6, 38, 54, 60]. In this system 1 mole of NADPH is consumed to have 1 mole of ε-caprolactone produced. Analysis of NADPH using this system is limited to cultures under aerobic conditions. The second reporting system is lycopene biosynthesis. Lycopene is a commercially important carotenoid with antioxidant activity and other health benefits. Lycopene-producing genes *crtEBI* from *Erwinia herbicola* have been expressed in *E. coli* and lycopene has been successfully synthesized [3, 36, 43, 50, 61]. The biosynthesis of lycopene in *E. coli* follows the non-mevalonate pathway that uses the glycolytic intermediate glyceraldehyde-3-phosphate and pyruvate as precursors, and consumes 16 moles of cofactor NADPH for 1 mole of lycopene production [2]. This reporting system serves as an example where the precursors of the product are directly connected to the formation of NADPH by our proposed modified glycolytic pathway and therefore may allow the application of this process to more complex metabolic engineering situations where the cells are used in a growth mode rather than as a non-growing biocatalyst. The third reporting system we developed is a one-step reaction module using 2-haloacrylate reductase CAA43 from soil bacteria, *Burkholderia* sp. WS [32, 33]. CAA43 catalyzes the reduction of the carbon–carbon double bond of 2-chloroacrylate to (*s*)-2-chloropropionate coupled with NADPH oxidization to $NADP^+$. In this module, 1 mole of NADPH is consumed per mole of 2-chloroacrylate produced. This reaction can be carried out under anaerobic conditions that provide us the opportunity to quantitate and balance the redox flow more precisely, because all the reduced products from NADH- or NADPH-mediated processes can be calculated without competition from the oxidation of NADH by the electron transport chain normally found under aerobic conditions. Secondly, this one-step reaction is independent of cellular metabolism. With enough supply of substrate 2-chloroacrylate, an ample sink should be provided for the cells to oxidize extra NADPH and therefore maintain redox homeostasis. This module is an example for the more practical situation of industrial relevance where a NADPH-dependent product is desired to be formed at high level.

In summary, the current study showed that replacing NAD^+-dependent *E. coli* native GAPDH *gapA* with *B. subtilis* $NADP^+$-dependent GAPDH *gapB* made a NADPH-generating glycolytic pathway and increased NADPH bioavailability in the cell. When NAD kinase *nadK* was coexpressed with *gapB*, the increase of NADPH availability was more pronounced, which suggested it to be a potential strategy for the biosynthesis of industrially valuable chemicals.

Materials and methods

Bacterial strains and plasmids

Table 1 describes the strains and plasmids used in this study. YW4077 and YW4078 were used in the monooxygenase CHMO assay. YW4037, 4033, 4035, and 4036 were used in the lycopene production assay. YW5061, 5073, 5078, and 5079 were used in the 2-haloacrylate reductase assay. pK19-lyco carries the carotenoid biosynthesis genes *crtE, crtI*, and *crtB* from *E. herbicola* [10, 41].

To make pDHC29-*gapB*, *gapB* was amplified by PCR from genomic DNA of *B. subtilis* 168 and inserted into *Bam*HI and *Xba*I digested pDHC29. The primers were *gapB*-R-*Bam*HI: 5′-CCG GGA TCC ACT TAT ACA GCA GAC GGA TGT TTC ATT CGT GC-3′ and *gapB*-F-*Xba*I: 5′-CCG TCT AGA AAT GAA GGT AAA AGT AGC GAT CAA CGG GTT T-3′. To make pDHC29-*gapA*, *gapA* was amplified by PCR from genomic DNA of *E. coli* MG1655 and inserted into *Xba*I and *Bam*HI digested pDHC29. The primers used for cloning were *gapA*-F-*Xba*I: 5′-CCG TCT AGA A ATG ACT ATC AAA GTA GGT ATC AAC GGT TTT GGC-3′ and *gapA*-R-*Bam*HI: 5′-CCG GGA TCC AC TTA TTT GGA GAT GTG AGC GAT CAG GTC CAG AAC-3′. To make pDHC29-*nadK*, *nadK* was amplified by PCR from *E. coli* genomic DNA and inserted into *Cla*I and *Xho*I digested pDHC29. The primers are *nadk*-F-*Cla*I: 5′-CCG ATC GAT AAA CAG AAT TTG CCT GGC GGC AGT-3′ and *nadK*-R-*Xho*I: 5′-AAT CTC GAG AGT ACG CGT TTA GAA TAA TTT TTT TGA CCA-3′. To make pDHC29-*gapB-nadK*, *nadK*

Table 1 List of strains and plasmids used in this study

	Genotype	Reference/ source
Strains		
W3CG	W3110 *gapA*::Tn10	[18]
YW5012	MG1655 *gapA*::Tn10	This study
YW5101	MG1655 pDHC29	This study
YW5102	MG1655 *gapA*::Tn pDHC29	This study
YW5103	MG1655 *gapA*::Tn pDHC29-*gapA*	This study
YW5104	MG1655 *gapA*::Tn pDHC29-*gapB*	This study
YW4077	MG1655 *gapA*::Tn pDHC29-*gapA* pTrc99A-CHMO	This study
YW4078	MG1655 *gapA*::Tn pDHC29-*gapB* pTrc99A-CHMO	This study
YW4079	MG1655 pDHC29-*gapA* pTrc99A-CHMO	This study
YW4080	MG1655 pDHC29-*gapB* pTrc99A-CHMO	This study
YW4037	MG1655 pDHC29 pK19-lyco	This study
YW4033	MG1655 *gapA*::Tn10 pDHC29-*gapB* pK19-lyco	This study
YW4035	MG1655 *gapA*::Tn10 pDHC29-*nadK* pK19-lyco	This study
YW4036	MG1655 *gapA*::Tn10 pDHC29-*gapB-nadK* pK19-lyco	This study
YW5061	MG1655 pDHC29 pTrc99A-CAA43	This study
YW5073	MG1655 *gapA*::Tn10 pDHC29-*gapB* pTrc99A-CAA43	This study
YW5078	MG1655 *gapA*::Tn10 pDHC29-*nadK* pTrc99A-CAA43	This study
YW5079	MG1655 *gapA*::Tn10 pDHC29-*gapB-nadK* pTrc99A-CAA43	This study
Plasmids		
pDHC29	Expression vector	[43]
pDHC29-*gapB*	*gapB* from *B. subtilis* expressed in pDHC29 under the Plac promoter	This study
pDHC29-*gapA*	*gapA* from *E. coli* expressed in pDHC29 under the Plac promoter	This study
pDHC29-*nadK*	*nadK* from *E. coli* expressed in pDHC29 under the Plac promoter	This study
pDHC29-*gapB-nadK*	*gapB* from *B. subtilis* and *nadK* from *E. coli* were coexpressed in pDHC29 under the Plac promoter	This study
pMM4	Monooxygenase CHMO from *Acinetobacter* sp. NCIB 9871 in pET22b(+)	[6, 7]
pTrc99A-CHMO	Monooxygenase CHMO was expressed in pTrc99A under the Ptrc promoter	This study
pK19-lyco	*crtE*, *crtI*, and *crtB* from *E. herbicola* in pK19	[39]
pET101-D-topo-CAA43	CAA43, encoding 2-haloacrylate reductase from *Burkholderia* sp. WS, was expressed in pET101/D-TOPO (Invitrogen), Ap^R	[30, 31]
pTrc99A-CAA43	2-Haloacrylate reductase CAA43 cloned in pTrc99A under the Ptrc promoter	This study

was amplified by PCR from *E. coli* genomic DNA and inserted into *Eco*RI and *Hin*dIII digested pDHC29-gapB. An RBS sequence was inserted upstream of the *nadK* ORF and *gapB* and *nadk* were grouped as a single operon. The primers were pDHC29-*gapB*-RBS-*nadK*-F-*Eco*RI: 5′-CAG GAA TTC AAG AAG GAG ATA TAC ATA CCC ATG AAT AAT CAT TTC AAG TGT ATT GGC-3′ and pDHC29-*gapB*-RBS-*nadK*-R-*Hin*dIII: 5′-GAT AAG CTT TTA GAA TAA TTT TTT TGA CCA GCC-3′.

To make pTrc99A-CHMO, the monooxygenase CHMO gene was amplified by PCR from pMM4 using primers *chnB*-F-*Nco*I: 5′-CCG CCATGG GA ATG TCA CAA AAA ATG GAT TTT GAT GCT ATC GTG-3′ and *chnB*-R-*Xba*I: 5′-CGC TCTAGA TTA GGC ATT GGC AGG TTG CTT GAT ATC TGA-3′. The fragment was then cloned into *Nco*I and *Xba*I digested pTrc99A. To make pTrc99A-CAA43, the gene encoding the 2-haloacrylate reductase, CAA43 was amplified by PCR from pET101-D-Topo-CAA43 using primers CAA43-F-*Nco*I (5′-CCG CCATGG GA ATG GTA ATG GCA GCG GTA ATT CAT AAG-3′) and CAA43-R-*Xba*I (5′-CGC TCTAGA CTA CGC TTG CGG AAG CAA AAC AAT-3′). The PCR fragment was then digested with *Nco*I and *Xba*I and cloned into pTrc99A. All constructs were confirmed by DNA sequencing.

GAPDH enzyme assay in cell-free extracts

Cells, 50 ml of an overnight growth, were collected by centrifugation at 4 °C. The pellets were washed once with 25 ml of wash buffer containing 25 % of sucrose, 1 mM EDTA, and 50 mM Tris–HCl, pH 8.0, and then resuspended in sonication buffer containing 50 mM EDTA and 500 mM Tris–HCl, pH 8.0 [19]. Sonication was then carried out on ice for 4 × 30 s cycles, power level 5 with a Misonix Sonicator® 3000 (Qsonica, Newtown, CT). After sonication, cell lysates were separated by centrifugation at top speed at 4 °C for 15 min, and the supernatants were saved on ice for further assay. Enzyme assay solution included 40 mM of triethanolamine, 50 mM of Na_2HPO_4, 0.2 mM of EDTA, 1 mM of NAD^+ or $NADP^+$, 1 mM of glyceraldehyde-3-phosphate (G3P), and cell-free supernatant [22]. The blank control reaction contained all the components except the substrate G3P. The reaction was performed at 37 °C and followed for 5 min. Total protein levels for each sample were determined by the Bradford assay. GAPDH activity was calculated on the basis of the increase of the absorbance at 340 nm of the NADH or NADPH produced by the reaction. The molar extinction coefficient of NADH/NADPH at 340 nm is 6,220 l mol^{-1} cm^{-1}. One unit of GAPDH activity is defined as the amount of enzyme required to convert 1 μmol of substrate per min at 37 °C.

Cyclohexanone monooxygenase (CHMO) enzyme assay in cell-free extracts

Cell lysates were obtained using the same protocol as in the GAPDH enzyme assay above. CHMO enzyme activity of different strains was determined on the basis of their ability to oxidize NADPH. The reaction mixture contains 100 mM glycine (pH 9.0), 200 μM NADPH, and 25 mM 100 % cyclohexanone. The mixtures without cyclohexanone were pretreated at 37 °C for 2 min to eliminate endogenous NADPH oxidation, then the substrate cyclohexanone was added to the mixtures. Oxidation of NADPH was then monitored by change of absorbance at OD 340 nm for 400 s [7, 34, 37, 38]. One unit of CHMO activity is defined as the amount of enzyme required to oxidize 1 μmol NADPH at 37 °C/min. Total protein concentration was determined by the Bradford protein assay (Bio-Rad).

Cyclohexanone monooxygenase (CHMO) assay

First, the strains were grown in Luria–Bertani (LB) broth supplemented with 100 μg/ml of ampicillin and 35 μg/ml of chloramphenicol at 37 °C overnight. The overnight seed cultures were diluted at a ratio of 1:200 into fresh LB plus antibiotics, and grown at 30 °C for 5 h until the OD 600 nm reached above 0.6. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to make the final concentration 500 μM in the culture to induce CHMO expression for up to 20 h. The cells were then collected and resuspended in non-growing minimal medium containing 12.8 g/l Na_2HPO_4, 3.0 g/l KH_2PO_4, 0.5 g/l NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4 % glucose, 30 mM cyclohexanone, and antibiotics. The amount of cells collected was determined by the OD 600 nm and the final OD in resuspended cell preparation was around 4 for each sample. The reactions catalyzed by CHMO were then carried out in a shaker at 28 °C and 250 rpm. Samples were taken every 2 h and analyzed by HPLC. Organic acid metabolites were analyzed using a cation-exchange column (HPX-87H, BioRad Labs, CA) as indicated below. Substrate cyclohexanone and product ε-caprolactone were analyzed using a Luna 5 μm C18(2) 100A column (250 × 4.6 mm, Phenomenex, Torrence, CA). Acetonitrile (30 %) was used as the mobile phase at a flow rate of 1 ml/min. Cyclohexanone and ε-caprolactone were detected by a differential refractive index detector (Waters 2410, Milford, MA) and a UV–VIS detector (SPD-10A, Shimadzu Scientific Instruments, Columbia, MD).

Lycopene assay

Strains were first grown in 5 ml of LB broth overnight at 37 °C. Then 500-μl aliquots of the overnight cultures were inoculated into 50 ml of fresh LB supplemented with 2 % glucose, 100 mM phosphate buffer (pH 7.0), and antibiotics, when appropriate, and the cultures were grown in 250 ml flasks in a rotary shaker at 30 °C, 250 rpm. After 24 or 48 h, 10 ml samples were taken, the cells were pelleted and washed once with distilled water. The cell pellets were then resuspended in 5 ml of acetone at 55 °C for 15 min in the dark to extract lycopene from the cells. After extraction, the solutions were centrifuged at 11,000 rpm for 10 min to remove cell debris [41]. The absorbance of the supernatants was then measured at OD 475 nm for the determination of the lycopene concentration. A calibration curve was made using lycopene standard (Sigma, St. Louis, MO). The extinction coefficient used was 3.82 cm^{-1} l mg^{-1}.

2-Haloacrylate reductase (CAA43) assay

Strains were first grown in 5 ml of LB broth overnight in media supplemented with antibiotics such as 100 μg/ml of ampicillin or 35 μg/ml of chloramphenicol if required. Aliquots (1.5 ml) of overnight cultures were then inoculated into 150 ml of fresh LB broth in 500-ml flasks supplemented with antibiotics and grown in a rotary shaker at 250 rpm and 37 °C. After 3–4 h of growth, IPTG was added to make the final concentration of 1 mM in each

flask to induce expression of CAA43 from pTrc99A-CAA43. The induction continued under the same conditions for 18 h before cells were collected for the subsequent experiments.

After 18 h of induction, equal amount of cells were collected on the basis of optical density measurements at OD 600 nm. Cell pellets were then washed with minimal medium without nitrogen source and resuspended in 15 ml of reaction medium. Minimal medium without a nitrogen source was used to keep the cells under non-growing state. Both the CHMO assay and CAA43 assay were done with non-growing cell suspensions as the biocatalysts. This allowed a more complete analysis of the redox utilization without considering the reduced cofactor going into cell mass that would need to be considered in a growing culture. Minimal medium without nitrogen source contains 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, and 0.5 g/l NaCl. The reaction medium is minimal medium without nitrogen source supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mg/ml thiamine, 2 % glucose, 50 mM 2-chloroacrylic acid, and antibiotics when appropriate. The pH of reaction medium was adjusted to 7.0 with 6 N HCl. After cells were resuspended in reaction medium, 14.5-ml aliquots of the suspension of each strain were distributed into 15-ml glass vials. The glass vials were closed and sealed with rubber stoppers for anaerobic fermentation. Samples were taken using sterile needles and syringes at each time point for HPLC analysis.

Metabolite analysis by HPLC

Samples of 600 µl at each time point were taken and centrifuged at 12,000 rpm for 1 min. The supernatants were then filtered through a 0.2-µm syringe filter and aliquots were subjected to HPLC analysis. To measure extracellular metabolites, CAA43 substrate, 2-chloroacrylic acid, and product, 2-chloropropionic acid, an HPLC system from Shimadzu Scientific Instruments was used. The metabolites were separated by a cation-exchange column (HPX-87H, Bio-Rad Labs, CA) and quantified by a differential refractive index detector (Waters 2410, Milford, MA) and a UV–VIS detector (SPD-10A, Shimadzu Scientific Instruments, Columbia, MD). The mobile phase was 2.5 mM sulfuric acid and the column was operated with a flow rate of 0.5 ml/min at a constant temperature of 55 °C.

Results and discussion

Cloning and characterization of *gapB* from *Bacillus subtilis*

To replace the *E. coli* endogenous NAD^+-dependent GAPDH GapA, we started with a characterized *gapA* null mutant W3CG [18], which has a transposon Tn10 carrying tetracycline resistance cassette inserted in the *gapA* gene. We then used P1 transduction to transfer the *gapA*::Tn10 marker to MG1655 resulting in YW5012 (MG1655 *gapA*::Tn10). To confirm that the *E. coli* native GAPDH activity was eliminated in YW5012, a GAPDH enzyme assay was conducted that demonstrated that the transductant could not use glucose (data not shown). To further analyze the disruption of the *gapA* gene in YW5012 a fragment enclosing the *gapA* gene was amplified by PCR and sequenced. Nucleotide sequencing showed that the Tn10 was inserted in the promoter region of *gapA* between nucleotide positions −24 and −25, which is among the sequence of one of the four promoter sequences upstream of *gapA* [5, 56]. Taken together, the results showed that the endogenous GAPDH activity was effectively removed in YW5012.

Next, we wanted to confirm the cofactor specificity of *B. subtilis* GAPDH GapB compared to *E. coli* native GapA. *gapB* was first cloned into expression vector pDHC29 resulting in plasmid pDHC29-*gapB* under the Plac promoter [45], which was then transformed into YW5012, generating YW5104 (MG1655 *gapA*::Tn10/pDHC29-*gapB*). Similarly, the *gapA* gene from *E. coli* was cloned in pDHC29 in the same manner, resulting in pDHC29-*gapA* which was then transformed into YW5012 to form strain YW5103 (MG1655 *gapA*::Tn/pDHC29-*gapA*). YW5101 (MG1655/pDHC29) and 5102 (MG1655 *gapA*::Tn10/pDHC29) were used as controls. GAPDH enzyme assays for strains YW5101, 5102, 5103, and 5104 were performed on cell lysates obtained by sonication followed by

Table 2 GAPDH enzyme assay with NAD^+ or $NADP^+$ as cofactor

	NAD^+ (IU^a l^{-1} mg total protein)	$NADP^+$ (IU^a l^{-1} mg total protein)
YW5101 (MG1655 pDHC29)	461	18
YW5102 (MG1655 *gapA*::Tn pDHC29)	35	0
YW5103 (MG1655 *gapA*::Tn pDHC29-*gapA*)	8166	32
YW5104 (MG1655 *gapA*::Tn10 pDHC29-*gapB*)	90	314

[a] One IU of GAPDH activity is defined as the amount of enzyme required to convert 1 µmol of substrate per min at 37 °C. Molar extinction coefficient of NADH/NADPH at 340 nm is 6,220 l mol^{-1} cm^{-1}

centrifugation. As shown in Table 2, as expected, overexpression of GapA in the *gapA* null background or the wild-type GapA in native MG1655 showed a strong preference for NAD^+ as the cofactor, whereas GapB showed a higher preference for $NADP^+$ as cofactor. The control *gapA* null strain as expected did not show a significant amount of GAPDH activity with either NAD^+ or $NADP^+$ as cofactor, confirming the disruption of endogenous GAPDH activity in YW5012.

The *gapA* mutation we used originated from strain W3CG from the Coli Genetics Stock Center. An attempt to make a *gapA* neat deletion strain was made using the lambda-rec system; however, this effort initially failed and no Keio strain of *gapA* knockout was available. Previous work with *gapA* strains had shown reversion [21, 25]. GapA deletions were reported [51]; however, although the strain DS112 had only 0.4 % of the GAPDH activity of the parent, the *gapA* mutant was reported to be not readily infected by phage P1 limiting facile gene transfer. A previous report [18] initially tested the earlier DF221 [21]; however, the authors preferred to use a Tn10 transposon insertion strain W3CG they constructed for expression of a foreign GAPDH for biochemical work. We showed that strain W3CG is greatly reduced in GAPDH activity (it has about 7.6 % of the activity of the wild-type parent, Table 2) and can not grow on minimal medium using glucose as the sole carbon source. We later characterized the transposon insertion site to the promoter region of *gapA*. The transposon position in this strain apparently greatly reduces *gapA* expression but allows some useful phenotypes to be present in the strain. So although not with a complete null mutation, we found the host suitable for our purposes.

Effect of *gapB* replacement on NADPH availability

To investigate whether replacing NAD^+-dependent GapA activity with an $NADP^+$-dependent GapB will increase NADPH-dependent biosynthesis, we first compared production of ε-caprolactone in strain YW4077 (MG1655 *gapA*::Tn/pDHC29-*gapA* pTrc99A-CHMO) and YW4078 (MG1655 *gapA*::Tn/pDHC29-*gapB* pTrc99A-CHMO) catalyzed by cyclohexanone monooxygenase. Cells were first grown in rich medium and CHMOs were induced for overexpression, then the cells were collected and used as a non-growing catalyst for the monooxygenase reaction. In these two isogenic strains, glycolytic GAPDHs were provided by GapB or GapA overexpressed from high copy number plasmid pDHC29, while the endogenous NAD^+-dependent *gapA* was disabled by transposon insertion. As shown in Fig. 2a, *gapB*-overexpressed strain had a higher yield for ε-caprolactone compared to *gapA*-overexpressed strain after 2 h of reaction (5.7 product mM/glucose mM vs

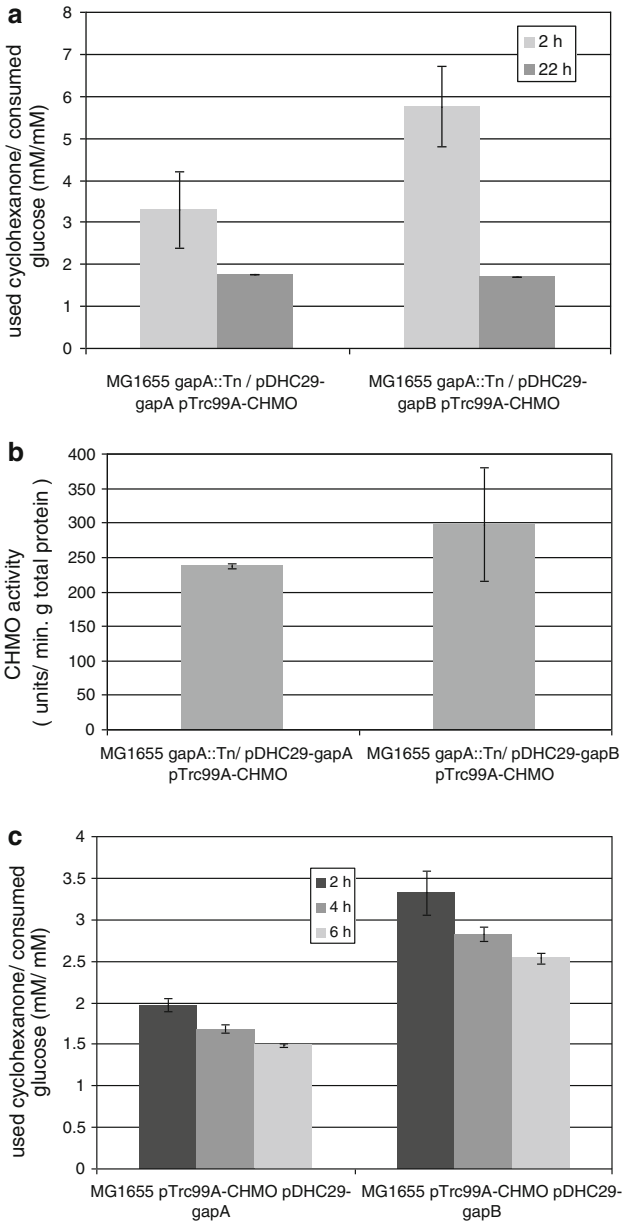

Fig. 2 Overexpression of *gapB* increased NADPH-dependent oxidation of cyclohexanone catalyzed by CHMO. **a** Overexpression of GapB in *gapA* null strain increased cyclohexanone oxidation. Test strains were YW4077 (MG1655 *gapA*::Tn/pDHC29-*gapA* pTrc99A-CHMO) and YW4078 (MG1655 *gapA*::Tn10/pDHC29-*gapB* pTrc99A-CHMO). **b** Test strains YW4077 and 4078 had comparable CHMO enzyme activity under the reaction conditions. CHMO activity is 237 ± 3.94 units in YW4077 and 297 ± 82 units in YW4078. **c** Overexpression of GapB also increased cyclohexanone oxidation in the wild-type background. Test strains are YW4079 (MG1655/pDHC29-*gapA* pTrc99A-CHMO) and YW4080 (MG1655/pDHC29-*gapB* pTrc99A-CHMO). Cultures and analytical methods are as described in the "Materials and methods" section. All the data shown here unless otherwise stated are average of triplicates, and *error bars* represent standard deviation

3.3 product mM/glucose mM). The 22 h sample is a time point that we took as a confirmation of the completion of the reaction. In this assay, we started with 30 mM

substrate. After 2 h, about 15 mM of substrate was converted to product; whereas at 22 h, the substrate concentration was zero and the glucose concentration was also close to zero indicating the completion of the reaction. Therefore, the shorter time period can be considered as a rate experiment whereas the longer time period indicates the completion or final extent of the process and should not be used to reflect accurately the product yield. To confirm that the CHMOs were properly expressed, an enzyme assay was done with cell-free extract from the two test strains. The results (Fig. 2b) confirmed that there was comparable CHMO activity in these two strains. We performed a *t* test on the CHMO activity between the two strains in Fig. 2b. The result showed that there is a statistical difference between the two CHMO activities ($P = 0.272$). For MG1655 *gapA*::Tn pDHC29-*gapA* pTrc99A-CHMO, the mean value is 237 and the standard deviation is 3.94; for MG1655 *gapA*::Tn pDHC29-*gapB* pTrc99A-CHMO, the mean value is 297 and the standard deviation is 82. We analyzed the data, for the *gapB* overexpression strain, among three replicates, one is an outlier and has unusually high activity which is the reason for the large standard deviation. The remaining two replicates are consistent and show no statistical difference from the *gapA* overexpression strain. Under these circumstances, we therefore stated that the two strains have comparable CHMO activities. The difference in cyclohexanone oxidation should stem from the different GAPDH available in the cells but not CHMO activity. We also tested these two GAPDHs in an *E. coli* wild-type background as shown in Fig. 2c. In these settings, cells have GAPDH from two sources, either from genome or from high copy plasmid. This would show the effect of *gapB* when there was a normal NAD^+-dependent GAPDH in the cell. In the wild-type background in strain YW4080 (MG1655 pDHC29-*gapB* pTrc99A-CHMO), when *gapB* was overexpressed from the plasmid, the $NADP^+$-dependent enzyme derived from *gapB* would compete with the NAD^+-dependent GAPDH enzyme formed from the native genomic copy of *gapA*. Our results showed that in the presence of the cell's native GapA protein, the presence of GapB from the overexpression construct still resulted in higher production of NADPH-dependent ε-caprolactone (Fig. 2c) which indicated that GapB functions comparably and competitively as native GapA. These results from the introduction of the *gapB*-plasmid into the wild-type *gapA*+ strain also showed that an increase in NADPH-dependent products could be achieved in an unmodified GAPDH host. The reduction of cyclohexenone to ε-caprolactone is a one-step NADPH-dependent reaction; the yield of the product directly reflects the bioavailability of NADPH in the cells tested. This set of experiments therefore suggested that replacing NAD^+-dependent GapA with $NADP^+$-dependent GapB activity can increase NADPH-dependent biosynthesis. This result is consistent with previous studies in which *E. coli* GapA was replaced by GapC from *C. acetobutylicum* [41] or a fungal $NADP^+$-dependent GAPDH from *K. lactis* was expressed in *S. cerevisiae* [58, 59].

Effect of *gapB* replacement and NAD kinase coexpression on NADPH availability

When GapB is overexpressed from a plasmid, extra $NADP^+$ will be required for GapB to function on its full capacity. Under normal conditions, $NADP^+$ concentration is inherently lower than that of NAD^+, which the endogenous GAPDH requires as cofactor. $NADP^+$ is synthesized through phosphorylation of NAD^+ by NAD kinase encoded by *nadK* in *E. coli*. To overcome this possible limitation, we coexpressed *nadK* along with *gapB* in pDHC29-*gapB-nadK*. In this plasmid, *gapB* and *nadK* were placed in tandem as one operon, under the control of the Plac promoter, with an extra RBS sequence inserted upstream of the *nadK* open reading frame.

To examine whether coexpression of *gapB* and *nadK* will significantly increase NADPH bioavailability, we tested lycopene production, which is heavily dependent on NADPH availability—1 mole of lycopene needs 16 moles of cofactor NADPH [1]. This experiment examines the effect on formation of a product where the precursors, glyceraldehyde-3-phosphate and pyruvate, are closely tied to the formation of NADPH in the constructed strain. This is the situation where a growing strain is used and where a portion of the glycolytic flux of the glycolytic intermediates is used for product formation in addition to just supplying redox via the TCA cycle and rather than using cells in a non-growing biocatalyst state. The strains we used were YW4037 (MG1655/pDHC29 pK19-lyco), 4033 (MG1655 *gapA*::Tn/pDHC29-*gapB* pk19-lyco), 4035 (MG1655 *gapA*::Tn10/pDHC29-*nadK* pK19-lyco), and 4036 (MG1655 *gapA*::Tn10/pDHC29-*gapB-nadK* pK19-lyco) that overexpress *gapB*, *nadK*, or both *gapB* and *nadK*, respectively. Cells were grown in buffered LB broth supplemented with 2 % glucose at 30 °C. After 24 or 48 h, samples were taken and lycopene was extracted and measured. Figure 3a shows that overexpression of *gapB* alone significantly increased the lycopene yield compared to wild-type control (134 μg lycopene/mmol glucose vs. 42 μg lycopene/mmol glucose). Strains that overexpressed *nadK* or both *gapB* and *nadK* had lower lycopene yield at 75 μg lycopene/mmol glucose and 65 μg lycopene/mmol glucose, respectively. At 48 h, the lycopene yield for this set of four strains showed the same trend, i.e., the *gapB* overexpression strain had the highest yield, whereas *nadK* came in second followed by *gapB* and *nadK* double expression strain, and the lowest yield was generated by the

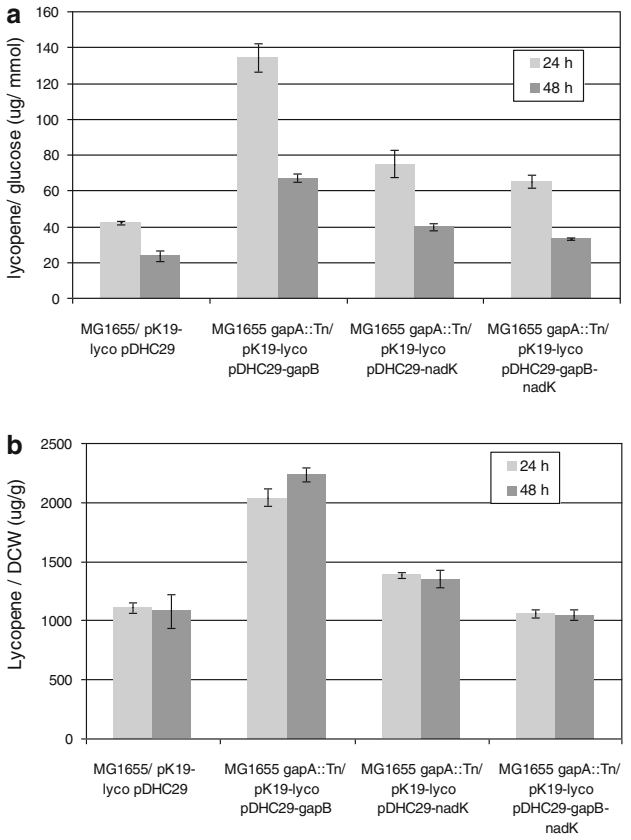

Fig. 3 Lycopene production in *gapB*-, *nadK*-, or both *gapB*- and *nadK*-overexpressed strains. **a** The yield was calculated as the amount of lycopene (μg) produced per mmol glucose consumed. Test strains are YW4037 (MG1655/pK19-lyco pDHC29), YW4033 (MG1655 *gapA*::Tn10/pK19-lyco pDHC29-*gapB*), YW4035 (MG1655 *gapA*::Tn/pK19-lyco pDHC29-*nadK*), and YW4036 (MG1655 *gapA*::Tn10/pK19-lyco pDHC29-*gapB-nadK*). **b** Lycopene production (μg) based on dry cell weight (DCW) (g). 1 OD600 nm = 0.33 g DCW/l. Cultures and analytical methods are as described in the "Materials and methods" section

wild-type control. But compared to yields at 24 h, the lycopene yield for each strain decreased (Fig. 3a). Between 24 and 48 h, the glucose consumption for each strain almost doubled, whereas the increase of the lycopene concentration was small. Therefore, when lycopene yields were corrected for glucose consumption, we saw the decrease which indicates that after 24 h glucose was probably more directed to alternate metabolic flux other than lycopene biosynthesis. We also calculated the lycopene production corrected for dry cell weight (DCW) (Fig. 3b). Overexpression of *gapB* produced the highest yield of 2,042 μg lycopene/g DCW at 24 h. Overexpression of *nadK* alone showed the second highest yield of 1,386 μg lycopene/g DCW at 24 h, whereas coexpression of both *gapB* and *nadK* had a similar yield as the wild-type strain (1,110 vs. 1,061 μg lycopene/g DCW at 24 h). At 48 h, the lycopene yield for each strain was similar without significant change. These results are consistent with previous reports that *nadK* overexpression increased production of NADPH-dependent thymidine [35], isoleucine [52], or PHB [39]. When *nadK* was overexpressed, lycopene yield decreased compared to that of the *gapB*-overexpressed strain, which indicates that the effects of *gapB* and *nadK* are not additive in the case of lycopene biosynthesis. On the basis of previous research [47], in human cells NADK increased the NADPH level instead of the $NADP^+$ level. In the thymidine-overproducing *E. coli* strain that overexpressed *nadK*, the same effect was also observed with NADPH level being increased 1.3-fold while $NADP^+$ level decreased 10 % [35]. With higher concentrations of NADPH in the cell, to compensate the unbalanced redox pool, NADPH/NADH-oxidizing enzymes will be upregulated including enzymes in the TCA cycle. While lycopene biosynthesis in *E. coli* utilizes G3P and pyruvate as precursors [15], with more metabolic flux going to the TCA cycle, the competition for precursors will in the end lower the lycopene yield.

In another test system where we wished to quantitate the amount of reduction available under anaerobic conditions and examine the proportion going to the NADPH-derived product. For this reason we used the NADPH requiring 2-haloacrylate reductase CAA43 from bacteria *Burkholderia* sp. WS [33]. CAA43 catalyzes the reduction of 2-chloroacrylate to 2-chloropropionic acid with NADPH as cofactor exclusively. This system provided a more robust reducing-power sink that could utilize large amounts of NADPH and allow us to better quantitate the proportion of redox available as NADPH in the cell and we could also assess whether coexpression of *gapB* and *nadK* would aid the biosynthesis of the desired reduced compounds. We cloned CAA43 in pTrc99A under the Ptrc promoter, resulting in pTrc99A-CAA43. This plasmid was then transformed into *gapB*, *nadK*, or *gapB* and *nadK* overexpression strains resulting in the test strains, YW5061 (MG1655/pDHC29 pTrc99A-CAA43), 5073 (MG1655 *gapA*::Tn/pDHC29-*gapB* pTrc99A-CAA43), 5078 (MG1655 *gapA*::Tn/pDHC29-*nadK* pTrc99A-CAA43), and 5079 (MG1655 *gapA*::Tn/pDHC29-*gapB-nadK* pTrc99A-CAA43). In this set of experiments, CAA43 expression was induced by IPTG. The cells were grown, collected, and resuspended in non-growing minimal salts without nitrogen. The substrate of the reaction, 2-chloroacrylate, was added at a concentration of 50 mM, and the reactions were carried out with the resuspended non-growing cells under anaerobic conditions. Anaerobic conditions were used to avoid the use of the NADH or NADPH by the electron transport chain and their removal by oxidation with air. Under anaerobic conditions we were able to analyze the reduced products and determine if they arose from a NADH- or NADPH-dependent route and thus we

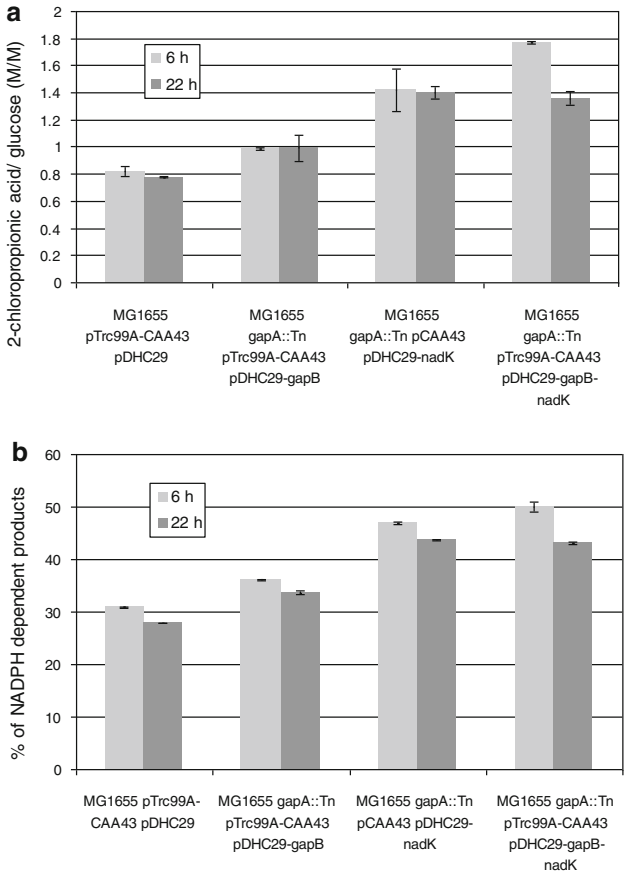

Fig. 4 Coexpression of *gapB* and *nadK* further increased NADPH-dependent 2-chloropropionic acid production in cells with 2-haloacrylate reductase CAA43. **a** Yield of 2-chloropropionic acid based on glucose consumption. **b** Percentage of NADPH-dependent products generated by the test strains. It was calculated as the amount of 2-chloropropionic acid generated vs. all the reduced metabolites resulting from the reaction, including lactate, ethanol, succinate, and 2-chloropropionic acid. The test strains were YW5061 (MG1655/pTrc99A-CAA43 pDHC29), YW5073 (MG1655 *gapA*::Tn10/pTrc99A-CAA43 pDHC29-*gapB*), YW5078 (MG1655 *gapA*::Tn10/pTrc99A-CAA43 pDHC29-*nadK*), and YW5079 (MG1655 *gapA*::Tn10/pTrc99A-CAA43 pDHC29-*gapB-nadK*). Cultures and analytical methods are as described in "Materials and methods"

could determine the proportion of reductant available as NADH or NADPH in the cultures of the engineered strains. Figure 4a shows that *gapB* or *nadK* overexpression alone increased the product yield compared to that of the wild-type parent strain; when *gapB* and *nadK* were combined together, the increase was more pronounced. Compared to cultures of the wild-type strain, cultures of the strain bearing overexpression of *gapB* increased the product yield by 21 %, overexpression of *nadK* increased the product yield by 73 %, and coexpression of *gapB* and *nadK* together increased the product yield by 119 %. We further assessed the amount of reductant formed in the cell and looked at the proportioning of the total NADH + NADPH reductant pool during the transformation of the 2-chloroacrylic acid. The total reducing power generated from the cells that was calculated as the amount of all the reduced products such as lactate, succinate, ethanol, and 2-chloropropionic acid. The metabolites that are detectable under current conditions include glucose, pyruvate, succinate, lactate, formate, acetate, ethanol, fumarate, and malate. The contribution of any other reduced products observed by direct measurement as well as possible changes in intracellular metabolites to the overall redox picture was considered negligible. The proportion of the total reductant available being used for NADPH-dependent production of 2-chloropropionic acid in the wild-type strain is about 30 %, in the *gapB* overexpression strain it is about 36 %, in the *nadK* overexpression strain it is about 46 %, and 49 % of the reductant pool in *gapB* and *nadK* coexpression strains were used for this NADPH-dependent reduction. This test system allowed us to clearly see that in *gapB*- and *nadK*-overexpressed strains more reducing power was generated in the form of NADPH, which should be of great interest for more efficient reductive biosynthesis.

Taken together, the current study showed that replacing a major NADH-generating enzyme in the glycolysis pathway with a NADPH-generating counterpart, along with overexpression of NAD kinase to increase $NADP^+$ availability, is a potential strategy that can be used for biosynthetic reactions.

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