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REVIEW ARTICLE

Cofactor engineering in cyanobacteria to overcome imbalance between NADPH and NADH: A mini review

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Abstract Cyanobacteria can produce useful renewable fuels and high-value chemicals using sunlight and atmospheric carbon dioxide by photosynthesis. Genetic manipulation has increased the variety of chemicals that cyanobacteria can produce. However, their uniquely abundant NADPH-pool, in other words insufficient supply of NADH, tends to limit their production yields in case of utilizing NADH-dependent enzyme, which is quite common in heterotrophic microbes. To overcome this cofactor imbalance and enhance cyanobacterial fuel and chemical production, various approaches for cofactor engineering have been employed. In this review, we focus on three approaches: (1) utilization of NADPH-dependent enzymes, (2) increasing NADH production, and (3) changing cofactor specificity of NADH-dependent enzymes from NADH to NADPH.

Keywords NADH-dependent enzyme, NADPH-dependent enzyme, transhydrogenase, site-directed mutagenesis, enzyme engineering

1 Introduction

Cyanobacteria can directly convert atmospheric CO_2 into organic compounds, using sunlight as their energy source by photosynthesis. Their simple nutrient requirements, fast cell growth, high photosynthetic activity, and more importantly amenability to genetic engineering enable them to be attractive producers of alternatives to fossil fuel [1–3]. Cyanobacteria that have been genetically engineered by introducing heterologous genes can produce various biofuels and chemicals, such as ethanol [4–7], isopropanol [8], butanol [9–11], lactate [12–16] and polyhydroxybutyrate (PHB) [17,18]. However, the production yield is still not high enough to compete with fossil fuels [19]. Therefore, further technological advancements to enhance their photosynthesis, CO_2 fixation efficiency, redox balance, or enzyme activity to a desired product are required.

In recent studies, cofactor engineering strategies have been widely applied to increase the production yield of cyanobacterial biofuels and chemicals. Cofactors (e.g., ATP, coenzyme A, FAD, NAD(P)H/NAD(P), metal elements) are crucial to numerous biochemical reactions, and their manipulations greatly affect the enzyme and pathway functionalities [20,21]. Particularly, NADPH and NADH as reducing equivalents, have been main targets for cofactor engineering in cyanobacteria, where NADPH produced in photosynthesis is more abundant than NADH [22–24] and is therefore the major reducing equivalent. Conversely, in the heterotrophic microorganisms, NADH is more abundant than NADPH, and most cellular enzymes use NADH as their cofactor solely or preferentially. Accordingly, enzymes from heterotrophic microorganisms often show low activities in cyanobacteria, leading to a low production yield of target product.

As shown Table 1, we herein summarize recent approaches to overcome the imbalance between NADPH and NADH and thus enhance biofuel and chemical productions in cyanobacteria by (1) utilizing NADPH-dependent enzymes, (2) increasing NADH production, and (3) changing co-factor specificity of NADH-dependent enzymes from NADH to NADPH.

2 Utilizing NADPH-dependent enzymes

The most straightforward way to overcome cofactor imbalance is perhaps to replace an NADH-dependent

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Table 1 Co-factor engineering in cyanobacteria to increase biofuel/chemical production

Co-factor engineering	Used enzyme/engineering strategy	Product	Host strains	Reference
Utilizing NADPH-dependent enzyme	Yqhd[a)]	isobutanol	*S. elongatus* 7942	[11]
	Yqhd[a)], PhaB[b)], Bldh[c)]	1-butanol	*S. elongatus* 7942	[10]
	Yqhd[a)]	1,3-propanediol	*S. elongatus* 7942	[25]
	Yqhd[a)] and NADPH-dependent sADH[d)]	1,2-propanediol	*S. elongatus* 7942	[26]
	Yqhd[a)] and *zwf* overexprssion	Ethanol	*Synechocystis* sp.	[7]
	slr1192		*Synechocystis* sp.	[6]
	NADPH-dependent sADH[d)]	2,3-butanediol	*S. elongatus* 7942	[27]
	NADPH-dependent acetoin reductase		*Synechocystis* sp.	[28]
Increasing NADH production	Co-expression of transhydrogenase	L-lactate	*Synechocystis* sp.	[12]
		D-lactate	*S. elongatus* 7942	[29]
			Synechocystis sp.	[13]
		2,3-butanediol	*Synechocystis* sp.	[28]
	Deletion of NADH-consuming pathway	Hydrogen	*S. elongatus* 7002	[30]
	Activation of NAD(P)H generating pathway		*S. elongatus* 7002	[31]
Changing cofactor specificity of enzyme	Site-directed mutagenesis	L-lactate	*Synechocystis* sp.	[15]
		D-lactate	*S. elongatus* 7942	[16]

a) NADPH-aldehyde reductase; b) acetoacetyl-Coa reductase; c) butyraldehyde dehydrogenase; d) secondary alcohol dehydrogenase

enzyme with an NADPH-dependent enzyme. In the model unicellular cyanobacterium, *Synechococcus elongates* PCC 7942 (hereafter *S. elongatus* 7942), intracellular NADPH concentration is approximately 4 and 6.5 times higher than that of NADH under dark and light conditions, respectively [22]. Thus, utilization of NADPH-dependent enzymes is a smart strategy to directly take advantage of abundant NADPH pool in cyanobacteria (Fig. 1).

Yqhd is an NADPH-dependent aldehyde reductase (NADP-dependent alcohol dehydrogenase) from *Escherichia coli*, and has reductase activity for a broad range of aldehydes [32]. Yqhd was introduced into cyanobacteria for production of isobutanol [11], 1-butanol [10], 1,3-propanediol (1,3-PDO) [25], 1,2-propanediol (1,2-PDO) [26] and ethanol [7]. Yqhd, which was first utilized in *S. elongatus* 7942 for isobutanol production, resulted in higher production of isobutanol than other NADH-dependent enzymes, suggesting that the intracellular NADH was insufficient for isobutanol synthesis in the NADH-dependent manner [11]. 1-Butanol production was also quadrupled by replacing with NADPH utilizing enzymes including Yqhd, PhaB (*Ralstonia eutropha*) and Bldh (*Clostridium saccarobutylacetonicum* NI-4) [10]. Similarly, Yqhd was chosen to convert 3-hydroxypropionaldehyde (3-HPA) to 1,3-PDO in an NADPH-dependent manner, instead of using NADH-dependent 1,3-PDO oxidoreductase that is utilized by a natural 1,3-PDO producer such as *Klebsiella pneumoniae* [25]. The synthetic pathway of 1,2-PDO was also engineered to be NADPH-dependent by employing not only Yqhd but also NADPH-dependent secondary alcohol dehydrogenase (sADH); as a result, 1,2-PDO production was increased [26]. An ethanol production pathway composed of pyruvate decarboxylase from *Zymomonas mobilis* and Yqhd was introduced into *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* sp.), and glucose-6-phosphate dehydrogenase encoded by the endogenous *zwf* gene was also overexpressed [7]. Increased NADPH production by *zwf* overexpression could result in increased production of both biomass and ethanol.

Production of 2,3-butanediol was increased by utilizing NADPH-dependent sADHin *S. elongatus* 7942 [27] and NADPH-dependent acetoin reductase in *Synechocystis* sp [28]. Moreover, endogenous alcohol dehydrogenase slr1192 of *Synechocystis* sp. could significantly increase ethanol production [6], compared with the previous studies using NADH-dependent alcohol dehydrogenase [4,5]. Among different alcohol dehydrogenases tested, the activity of slr1192 was much higher than other enzymes when NADPH was provided as cofactor, indicating that enzymes using NADPH are preferable to those using NADH in cyanobacteria.

3 Increasing NADH production in cyanobacteria

Although utilizing NADPH-dependent enzymes is the most straightforward way to exploit the abundant NADPH-pool in cyanobacteria, it is not always easy and sometimes even impossible to replace NADH-dependent enzymes with the corresponding NADPH-dependent enzymes.

Instead, intracellular NADH level can be increased by co-expression of a soluble transhydrogenase (sth) that catalyzes a conversion between NADPH and NADH, or by manipulating the carbon catabolic pathways that generate NAD(P)H (Fig. 1).

Lactic acid is an important feedstock in various industries such as food, bioplastics, cosmetics and pharmaceuticals; it was traditionally synthesized by lactic acid bacteria [33,34]. Recently, with the aid of genetic engineering, lactate dehydrogenase (ldh) [12,34] or mutated glycerol dehydrogenase (GlyDH) [13] were introduced into cyanobacteria, enabling them to be another producer of lactate. However, the production yield was limited by a low level of intracellular NADH in cyanobacteria, because the heterologous enzymes require NADH as their cofactor. Increasing NADH availability by co-expression of sth from *Pseudomonas aeruginosa* could improve both L-lactate [12] and D-lactate [13] production in *Synechocystis* sp. Similarly, additional expression of the *E. coli* sth encoded by *udhA* gene markedly increased lactate synthesis in *S. elongatus* 7942 expressing NADH-dependent ldh [29]. Besides lactate, co-expression of sth could result in increased productionof 2,3-butanediol from acetoin, which is catalyzed by an NADH-dependent acetoin reductase [28].

Metabolic engineering was conducted to increase NAD(P)H availiability and thus enhance fermentative hydrogen production in cyanobacteria. In *Synechococcus elongatus* PCC 7002 (hereafter *S. elongatus* 7002), reduction of protons to hydrogen by a bidirectional NiFe-hydrogenase is one way to regenerate NAD^+ to enable glycolysis to continue [30,31]. Thus, increasing the NADH/NAD^+ ratio would promote NADH oxidation by the bidirectional hydrogenase, ultimately leading to enhanced hydrogen production. One metabolic engineering approach is to inhibit an NADH-consuming pathway so that excessive NADH can be utilized for hydrogen production. *S. elongatus* 7002 that lacks the enzyme for NADH-dependent reduction of pyruvate to D-lactate, which was the major fermentative reductant sink, could produce five times more hydrogen than the wild type [30].

Another approach to increase intracellular reductant level is to manipulate the carbon flux through particular metabolic pathways. In a recent study [31], glycolysis and oxidative pentose phosphate (OPP) pathway were activated by overexpressing and deleting respectively the gap1 gene that encodes the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH-1) in *S. elongatus* 7002. GAPDH-1 is known as a key enzyme to regulate carbon flow from glycolysis to the OPP pathway, and also a kinetic bottleneck to limit glycolytic catabolism during dark anoxic conditions. Overexpression of GAPDH-1 increased the rate of glycolysis, and resulted in quadrupling of NADH production and tripling of hydrogen production under dark fermentative conditions. Moreover, the elimination of GAPDH-1 could redirect the carbon flow to OPP pathway instead of glycolysis, and increased NADPH production by a factor of 5.7 compared to the wild type, and consequently increased hydrogen production.

4 Changing cofactor specificity from NADH to NADPH

An enzyme's catalytic function and specificity are highly related to its three-dimensional structure, which is basically determined by its amino acid sequence. Because a detailed information on protein structure and function based on its amino acid sequence is becoming available for an increasing number of enzymes, it has been successfully conducted in various studies to alter an enzyme's features by changing one or more amino acids [35–39]. Not surprisingly, cofactor specificity of an enzyme can be also changed from NADH to NADPH or vice versa [40–42]. The site-directed mutagenesis allows to use one or more amino acid residues to replace the crucial ones that are

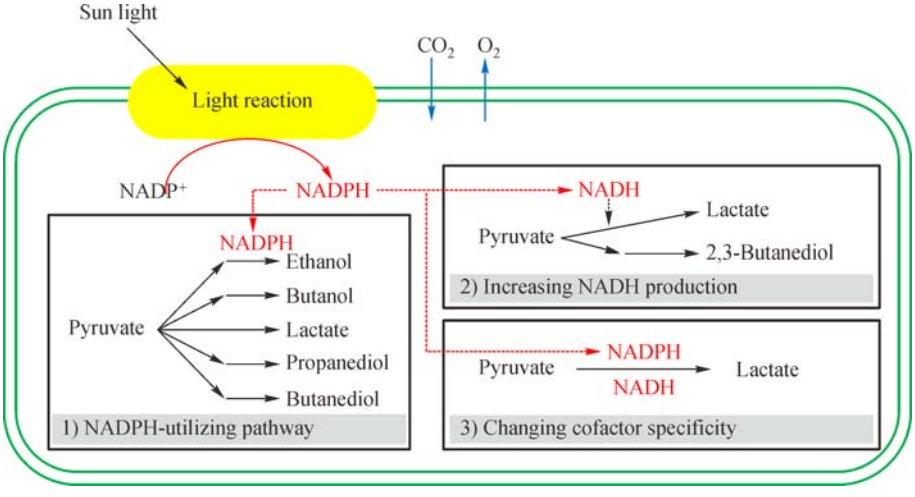

Fig. 1 Schematic representation of cofactor engineering in cyanobacteria

responsible for one cofactor to bind or discriminate from the other (e.g., NADPH from NADH) once they are identified using various protein engineering technologies such as amino acid sequence alignment or homology modeling (Fig. 1).

Lactate dehydrogenase from *Bacillus subtilis* (hereafter *B. subtilis*), which exhibits a high preference for NAD(H), was engineered to co-utilize NAD(H) and NADP(H) with high catalytic efficiency; this change was achieved by single-point mutation at position Val39 [42]. In a recent study, the introduction of the mutated enzyme into cyanobacteria and further codon-optimization increased its affinity to NADPH and lactate production [15]. Another lactate dehydrogenase from *Lactobacillus bulgaricus* ATCC 11842 was also engineered to reverse its cofactor preference from NADH to NADPH [16]. The authors investigated the putative cofactor binding sites of several NADH-dependent dehydrogenases, and found that aspartate, aspargine, and the hydrophobic residues were conserved, presumably because they play a decisive role in discriminaing against NADPH, for instance, by steric hindrance or electrostatic repulsion. Mutations on these amino acids shifted the cofactor specificity of enzyme from NADH to NADPH, and *S. elongatus* 7942 harboring this tailored enzyme produced 3.6 times more D-lactate than did the wild type.

Nevertheless, no definite rule has been established to specify which residues should be replaced to improve or change the cofactor specificity. For instance, aspartate, which has strong negative charge and is relatively large in size, was identified as a key residue in the cofactor binding pocket of NADH-dependent 1,3-propanediol oxidoreductase for recognizing NAD(H) and discriminating against NADP(H). A mutation of Asp41 to Gly enabled the enzyme to accept NADPH and improved its catalytic activity with NADPH [41]. However, for lactate dehydrogenase from *B. subtillis*, a mutation at position Val39 rather than Asp38 increased its affinity to NADPH [42]. In addition, in many cases, the mutations reduced enzyme activity with the original cofactor; or the enzyme activity with new cofactor, NADPH, was still lower than that of original enzyme with NADH. In a recent study [43], a rational engineering strategy successfully broadened the cofactor specificity of D-lactate dehydrogenase to utilize both NADH and NADPH, rather than sacrificing its activity on original cofactor. With well-developed computer tools, rational design will increase the accuracy at which the effects of residue substitution can be prediced, and increase the efficiency with which an optimized enzyme with desirable activity and specificity can be engineered [44,45].

5 Conclusions

Promoting the balanced utilization of reducing equivalants by cofactor engineering is a useful approach to increase cyanobacterial fuel and chemical production and its economic feasibility. Exploitation of NADPH-dependent enzymes or pathways is one way to directly take advantage of abundant NADPH-pool in cyanobacteria. Provided that a suitable candidate is not available, NADH production can be increased by co-expression of transhydrogenase or metabolic engineering, and the enzyme can be engineered to co-utilize NADH and NADPH by protein engineering. Further development in the related technologies, such as enzyme screening, DNA manipulation, or computational design, will overcome cofactor imbalace in cyanobacteria, and change it from a limiting factor to a driving force to increase cyanobacterial fuel and chemical production.

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