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Regulating Cofactor Balance in vivo via Synthetic Flavin Analogue System

Zhuotao Tan, Chenjie Zhu*, Jingwen Fu, Xiaowang Zhang, Ming Li, Wei Zhuang, Hanjie Ying*

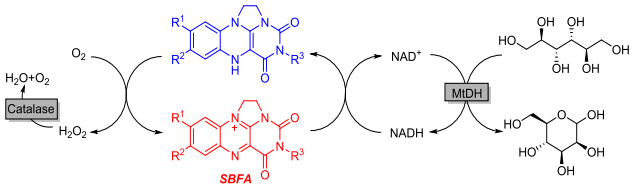
Abstract: A novel strategy to regulate cofactor balance in vivo for whole cell biotransformation using synthetic flavin analogue system was reported. High efficiency, easy operation, and good applicability were observed for the present system. Confocal laser scanning microscopy was employed to verify synthetic flavin analogue can directly permeate into *Escherichia coli* cells without modifying the cell membrane. This work provides a promising intracellular redox regulatory approach to construct more efficient cell factories.

Advanced bio-manufacturing has drawn considerable attention for production of valuable chemicals and biofuels.[1] These processes generally involve multistep biotransformations that require cofactors functioning as the energy carrier for carbon metabolism. In addition, cofactors can also impact reaction thermodynamics and regulate direction of reaction pathways. It has been well known that over 1500 microbial biotransformation reactions need the cofactors NAD(H) or NADP(H), cofactor balance hence plays an important role in manipulating metabolic flux in biosynthesis to favor the production of desired products. Toward that, various cofactor regulation strategies have been reported.[2] Generally, there are three major cofactor regulation strategies: 1) altering endogenous cofactor systems by knocking-down competitive cofactor consumption pathways,[3] strengthening the favorable pathway of cofactor generation,[4] or manipulating global regulatory factors that influence redox pools;[5] 2) supplementing the host with heterologous cofactor regeneration systems based on enzyme cascade[6] or substrate combination;[7] 3) changing cofactor preferences.[8]

More recently a fourth strategy via construction of synthetic cofactor system has emerged as a breakthrough to regulate cofactor balance. As a result of their affordability, stability, designability and sometimes “better than nature” superiority, synthetic cofactors provide an efficient and cost-effective tool for the regulation of cofactor-dependent reactions.[9] However, the applications of these flexible molecules have been restricted to in vitro reactions. Only one in vivo case was reported in which a synthetic nicotinamide cofactor analogue - nicotinamide cytosine dinucleotide (NCD), was uptaken by *Escherichia coli* (*E. coli*) to construct a selective intracellular energy-transfer process.[10] This unique synthetic cofactor system was realized by co-expressing an NAD^+ transporter (Ndt) to enable the NCD uptaking and depended on the mutant enzymes, which however tends to impair cellular activity as overexpressing membrane proteins can become toxic to the host [11] and heavy workload for the construction of mutant library. Thus, the development of a convenient and efficient system that does not require a transporter or modify cell membrane to manipulate cofactor balance in vivo is highly attractive.

Recently, we reported the feasibility of using synthetic bridged flavin analogue (SBFA) for the $NAD(P)^+$ regeneration in vitro.[12] It appeared that further ionization modification of the structure of SBFA might enhance its uptake by cells through free diffusion, establishing a new artificial system to manipulate cofactor balance in vivo without modifying the cell membrane. Such a synthetic flavin analogue system, which has not been reported so far to the best of our knowledge, was examined with *E. coli* to enhance the intracellular nicotinamide cofactor recycling for whole cell biocatalysis.

In vitro experiments were first performed with mannitol-1-dehydrogenase (MtDH)-catalyzed oxidation of D-mannitol into D-mannose to check the efficiency of the SBFA. Recently, sugars like D-mannose was getting increasing attentions for health care and pharmaceutical interests due to its potential biological activities.[13] As shown in the Scheme 1, SBFA were efficient for aerobic NAD^+ regeneration in vitro, and the order of catalytic activity of these SBFA appeared to be **F1** > **F2** > **F4** > **F3**, agreed well with our previous reports.[12] In the presence of 0.1 mol% **F1** and 2 mol% NAD^+, the desired product mannose was obtained in 81% yield in 24 h. Control experiments ruled out the possibility of direct oxidation of the substrate through SBFA. Under this nonoptimized condition, the calculated TOF of **F1** was 33.8 h^{-1}, which is 10-fold higher than that catalyzed by the natural flavin FMN (3.3 h^{-1}), indicating a superior catalytic activity for the synthetic flavin analogue. Recently, an interesting work about photoexcited flavins for the in vitro NAD^+ regeneration was reported, which also exhibited very high activities.[14]



F1: R^1 = H, R^2 = CF_3, R^3 = H, Yield = 81%, TOF = 33.8 h^{-1} **F2**: R^1 = H, R^2 = CF_3, R^3 = CH_3, Yield = 71%, TOF = 29.6 h^{-1}
F3: R^1 = H, R^2 = H, R^3 = H, Yield = 43%, TOF = 17.9 h^{-1} **F4**: R^1 = Cl, R^2 = H, R^3 = H, Yield = 45%, TOF = 18.8 h^{-1}
FMN: Yield = 8%, TOF = 3.3 h^{-1}

Scheme 1. SBFA-catalyzed in situ aerobic NAD^+ regeneration in vitro.

Encouraged by these results, further in vivo experiments were then conducted with *E. coli* as a model microorganism. We first tested with parent *E. coli* BL21 (DE3) and made sure that it had no influence on the conversion of mannitol, evident from that no consumption of mannitol or mannose was detected. The recombinant *E. coli* BL21 (DE3) hosting the overexpressed MtDH was then applied for the conversion of mannitol. The yield of mannose was however quite low (Table 1, entry 1). We were

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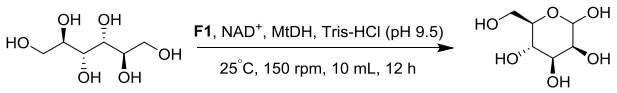
Table 1. Recombinant *E. coli* whole cell catalytic conversion of D-mannitol.



Entry	Mannitol [mM]	NAD^+ [mM]	**F1** [mM]	MtDH [mg/mL][a]	MtDH-NOX [mg/mL][b]	Yield [%][c]
1	50	-	-	10	-	5
2	50	-	0.05	10	-	15
3	50	0.5	-	10	-	33
4	50	1	-	10	-	45
5	50	2	-	10	-	47
6	50	0.5	0.1	10	-	92
7	50	0.5	0.05	10	-	87
8	50	-	-	-	10	3
9	50	0.5	-	-	10	43
10	50	0.5	-	-	20	61

[a] Wet weight of recombinant *E. coli* BL21 (DE3) that expressing MtDH. [b] Wet weight of recombinant *E. coli* BL21 (DE3) that co-expressed MtDH and NOX. [c] The data represents the averages standard deviations for three independent samples.

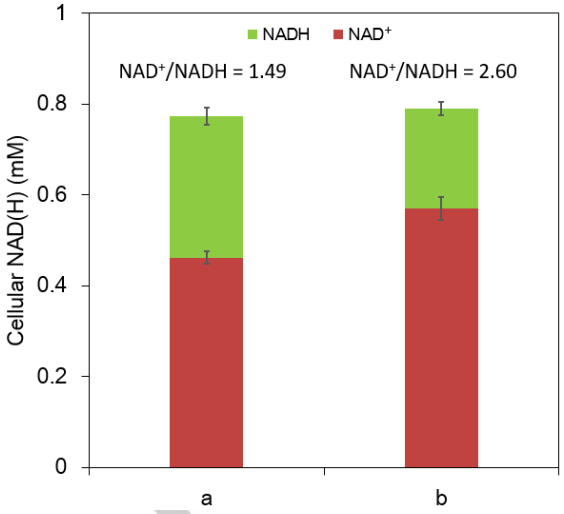


Figure 1. The cellular NAD(H) level for after 12 h reaction time. The data represent the averages standard deviations for three independent samples. a: Only using recombinant cells; b: Using recombinant cells and **F1**.

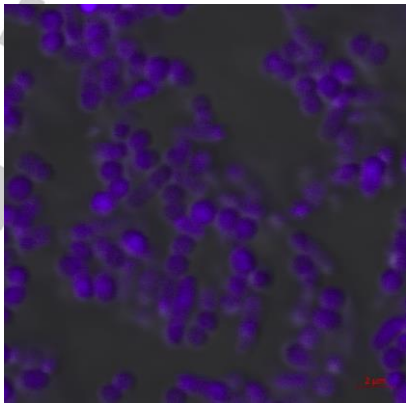


Figure 2. Confocal microscopic images of *E. coli* living cells labbled with **F1**. Blue signals represent the fluorescence from **F1** ($\lambda_{em.}$ = 500 nm).

pleased to find that in the case of the exogenous addition of 0.1 mol% **F1**, the yield was boomed up by three-fold (Table 1, entry 2). Based on this inspiring result, we further evaluated the efficiency under the exogenous addition of NAD^+. Numerous studies have shown that whole cell bioconversions were improved with extracellular supplement of NAD^+ due to the insufficient cellular NAD^+ level.[15] Our results also verified that the yield of mannose was improved with the addition of NAD^+ (Table1, entries 3 and 4). However, further increasing the amount of NAD^+ to 4 mol% did not show much improvement (Table1, entry 5), that means enhancing cellular NAD^+/NADH ratio merely by exogenous addition of NAD^+ has quite limited effectiveness. Excitingly, the yield was dramatically increased to 92% in the presence of 0.2 mol% **F1** when only using 1 mol% exogenous NAD^+ (Table 1, entry 6), which was about 3-fold higher than that obtained in the absence of **F1** under the same condition (Table 1, entry 3). With regard to the dosage of **F1**, it was possible to decrease the amount of **F1** to as low as 0.1 mol% without a significant loss in catalytic efficiency (Table 1, entry 7).

Cellular redox state as indicated by the NAD^+/NADH ratio is important for whole-cell biocatalysis. To confirm the intracellular function of **F1**, the intracellular NAD(H) level was analyzed using fast filtration approach according to the Sauer's method.[16] As shown in Figure 1, in the absence of **F1**, the cellular NAD^+/NADH ratio was 1.49. With 0.1 mM **F1** supplementation, the intracellular NADH concentration was dropped by 31% while NAD^+ concentration increased by 21%, and the corresponding NAD^+/NADH ratio increased to 2.60. We intend to believe that **F1** could enter into *E. coli* cell and accelerate the NAD^+ recycling in vivo.

To further confirm the functioning mode of **F1**, confocal laser scanning microscopy (CLSM) experiments were conducted to verify that F1 could permeate into the E. coli cells. As natural flavin has fluorescence activity due to its isoalloxazine structure, we then determined the auto-fluorescence spectrum of **F1**. Maximum excitation and emission wavelengths of **F1** could be found at about 400 nm and 500 nm, respectively (Figure S5). The CLSM experiment was then processed at 405 nm to get the fluorescence images of *E. coli* living cells. It was evident from Figure 2 that the fluorescence emission signal of **F1** (visible as blue spots) appeared when scanning the **F1**-labelled cells. We could even detect the motions of the fluorescent live cells under the CLSM (See videos in Supporting Information). These exciting images and videos illustrate that **F1** can permeate into *E. Coli* cells and enhance intracellular NAD^+ recycling.

Since it has been proved that the H_2O_2-forming **F1** could permeated into the cell, the intracellular ROS (reactive oxygen species) levels were analyzed during the reaction (Figure 3). ROS production was measured based on intracellular deacylation and oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent molecule 2',7'-dichlorofluorescein (DCF). It was observed that ROS level in **F1**-addition system was almost two-fold higher than that in only using recombinant cells at the early stage of the reaction. The

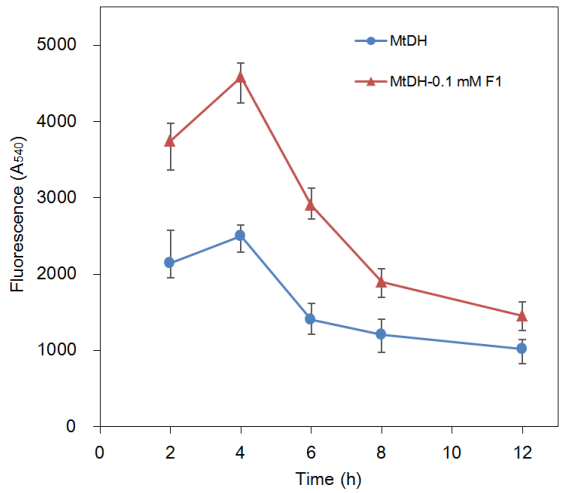


Figure 3. ROS levels during the reaction. Values are averages of three replicate experiments.

results also showed that the ROS level in both system displayed a trend of rise first then fall along with time, which probably due to the fact that microorganisms create a series of antioxidant enzymes, including catalases, superoxide dismutases, and glutathione peroxidase to decrease ROS-generated oxidative damage.[17] And it has also been reported that *E. coli* naturally has multiply intercellular response to remove H_2O_2.[18]

Previously study showed that co-expressing NADH oxidase (NOX) was an efficient method to manipulate redox cofactor level, and has been widely used to enhance NAD^+-dependent biosynthesis in both resting cells[19] and growing cells[20]. Therefore, the present SBFA system was then compared with the classical NOX system. NADH oxidase from *Streptococcus pyogenes* was co-expressed with MtDH in *E. coli* BL21 (DE3) to convert mannitol to mannose. As shown in Table 1, without exogenous NAD^+, extremely low yield was observed only using the MtDH-NOX system (Table 1, entry 8). After exogenous addition of 1 mol% NAD^+, the yield increased to 43% (Table 1, entry 9). However, this value was only about half of that achieved with **F1** system (Table 1, entries 6 and 9). Even when the cell concentration was increased to 20 $g \cdot L^{-1}$, the yield achieved with the MtDH-NOX system (61%) was still much lower than that of **F1** system (92%) (Table 1, entry 10). It has been suspected that overexpressed NADH oxidase might hamper cell growth and affect the metabolism.[21] To check this point, the growth curves of the *E. coli* BL21 (DE3) that only expressed MtDH with that of co-expressed MtDH and NOX were compared. Figure 4 shows that both strains reached stationary phase in about 24 h after induction with isopropyl-β-D-thiogalactoside (IPTG). In the exponential growth phase, the NOX co-expressing cells grew slower than the cells only expressing MtDH. The OD_{600} value of the co-expressed NOX cells stabilized at about 3, which was about half of that of MtDH-only cells (OD_{600} value was about 6). To check the impact of **F1** on the cell growth, the OD_{600} value of the MtDH recombinant *E. coli* incubated with 0.1 mM **F1** was also measured. It is noteworthy that addition of **F1** almost had no influence on cell growth, OD_{600} value of the **F1** system was nearly the same with that of MtDH-only cells.

Reactions with higher mannitol concentrations were further examined for considerations in reaction intensity for future industrial scale production. This was done with mannitol concentration varied from 50 – 1000 mM while keeping all other parameters constant. Under this nonoptimized condition, the reaction was not negatively affected by mannitol concentration up to 250 mM, the relative and space-time yield were 77% and 71 $g \cdot L^{-1} \cdot D^{-1}$, respectively (Figure 5). The TON and TOF of **F1** over the reaction were calculated to be 1925 and 160 h^{-1}, respectively.

With the aim to verify the applicability of the present system, this SBFA system was further examined for whole cell biotransformation of glycerol into 1,3-dihydroxyacetone (DHA) using recombinant *E. coli* BL21 (DE3) that was overexpressed with glycerol dehydrogenase (GlyDH). Ways to convert glycerol, an inexpensive and readily available byproduct of biodiesel industry, to DHA has received considerable interest due to DHA is widely used as an important building block in the cosmetic,

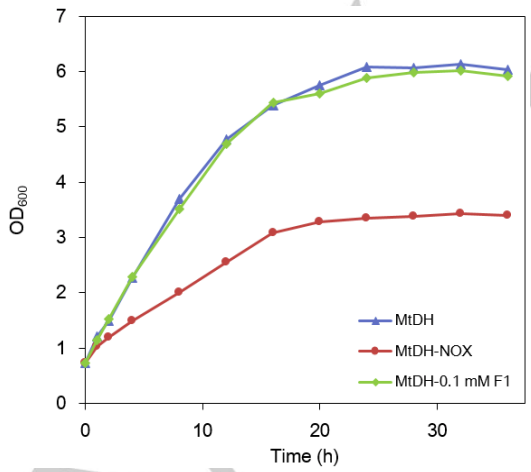


Figure 4. Comparison of growth curves of MtDH, MtDH-NOX, and MtDH-F1 system.

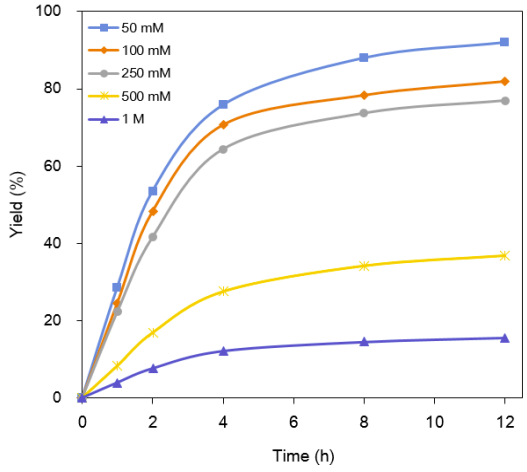


Figure 5. Yield of MtDH whole cell coupled with **F1** under various substrate concentrations. Reaction condition: mannitol (50 mM -1 M), NAD^+ (0.5 mM), F1 (0.1 mM), Tris-HCl (100 mM, pH 9.5), 10 mL.

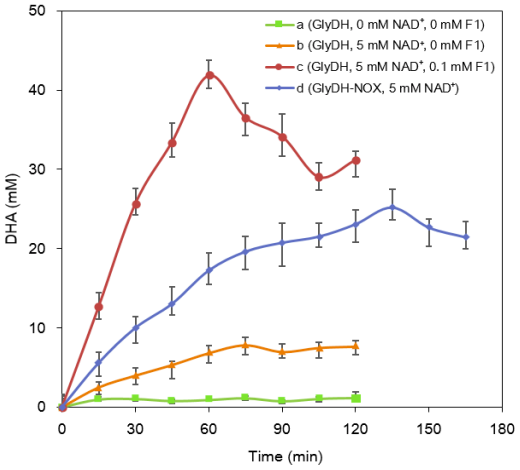


Figure 6. The time course of DHA formation. Reaction condition: glycerol (109 mM), NAD^+, F1, recombinant *E. coli* BL21 (DE3) that only expressing GlyDH or recombinant *E. coli* BL21 (DE3) that co-expressing GlyDH and NOX (10 $g \cdot L^{-1}$), PBS buffer (100 mM, pH 8.0), 10 mL, 30℃, 150 rpm. The data represented the averages standard deviations for three independent samples.

medical, food and agriculture area.[22] To compare the present method with those of previously reported systems, we typically applied the reaction conditions analogous to those reported in previous studies.[15b] Similar to the above study, the catalytic activity of the GlyDH recombinant cells was generally low when no **F1** and NAD^+ were added (Figure 6, Line a). After exogenous addition of 5 mM NAD^+, the reaction rate was slightly improved (Figure 6, Line b). With further addition of 0.1 mM **F1**, the reaction rate was sharply increased, and DHA production was burst to 41.8 mM within 1 h (Figure 6, Line c), which was about 6.1-fold higher than that only adding 5 mM NAD^+. Under the same reaction conditions, using whole cell *E. coli* co-expressed GlyDH and NOX system, only 17.2 mM yield of DHA was achieved within 1 h (Figure 6, Line d), which was about 2.4-fold lower than that of **F1** system, and the highest DHA concentration reached to 25.3 mM after 2.25 h in the GlyDH-NOX system. As also can be seen from the Figure 6, DHA concentration dropped slowly over time in all cases, suggesting that DHA may be further consumed due to the conversion glycerol to DHA is a reversible reaction.[21b]

In conclusion, we have demonstrated a convenient, practical, and efficient method to regulate cofactor balance in vivo based on synthetic flavin analogue system, which does not rely on the modification of the cell membrane through overexpressing channel proteins or increasing cell membrane permeability due to **F1** could straightforward permeated into the cell. This work not only provides a promising green strategy to manipulate redox balance in vivo, but also offers new opportunities to enable pathway-specific energy transfer for engineering cell factories to more efficient produce valuable metabolites. We believe this system is not limited to *E. coli* and can be extended to other eukaryotic or prokaryotic cells. More interesting works towards demonstrating this system in yeast and other organisms are currently under investigation in our laboratory.

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Keywords: cofactors • cofactor regulation • flavin • synthetic cofactor • whole-cell biotransformation

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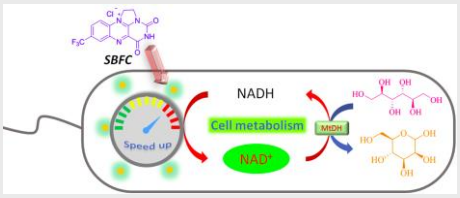

A novel strategy to manipulate intracellular cofactor balance for whole cell biotransformation based on synthetic flavin analogue is constructed. The synthetic flavin analogue can directly permeate into *E. coli* cells accelerating cellular NAD^+ regeneration without requiring the cell membrane modification.

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