Develop in vivo and in vitro coupling strategies to produce nicotinamide mononucleotide

Utumporn Ngivprom[1], Praphapan Lasin[1], Panwana Khunnonkwao[2], Suphanida Worakaensai[1], Kaemwich Jantama[2], and Rung-Yi Lai[1,3]*

[1]*School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand*
[2]*Metabolic Engineering Research Unit, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[3]*Center for Biomolecular Structure, Function and Application, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand*

Email: rylai@sut.ac.th

ABSTRACT

Nicotinamide mononucleotide (NMN), a ribonucleotide, is a key intermediate in the biosynthesis of coenzyme, nicotinamide adenine dinucleotide (NAD^+). Recently, NMN has gained lots of attention for self-medication as a nutraceutical. However, the *in vitro* biosynthesis of NMN requires expensive substrates, which makes this approach is difficult for large scale production. Therefore, we tried to develop a pathway with lower cost. In this project, the biosynthesis of NMN could be divided into two modules. The first module is to produce ribose from xylose by engineered *Escherichia coli*. In the first module, we conducted CRISPR-Cas9 to knock out two transketolase genes (*tktA* and *tktB*) and one gene (*ptsG*) encoding glucose-specific PTS enzyme IIBC component in *E. coli* MG1655. The engineered *E. coli* MG1655 could produce 2.47 g/L of ribose from 5g/L of xylose in LB medium after 48 hours. The second module is to construct *in vitro* biosynthetic pathway to convert ribose to NMN. The pathway involves *E. coli* ribose kinase (*Ec*RbsK), *E. coli* PRPP synthase (*Ec*PRPP), and *Chitinophaga pinensis* nicotinamide phosphoribosyl transferase (*Cp*Nampt) to convert ribose in the supernatant of engineered *E. coli* MG1655 medium to NMN with the incubation of excess ATP and stoichiometric nicotinamide. To reduce ATP cost, polyphosphate kinase was incorporated in the reaction to regenerate ATP from AMP and ATP using *Cytophaga hutchinsonii* polyphosphate kinase (PPK2). Furthermore, to improve the yield of NMN, the *Ec*PRPP inhibitor of pyrophosphate was hydrolyzed by the addition of Ppase. With all effort, the developed system could produce NMN from Nam with about 70% yield using the supernatant of engineered *E. coli* MG1655 medium. Currently, we continue to optimize the production protocol.

Introduction

Nicotinamide mononucleotide (NMN), a ribonucleotide, exists in all living species and is a key intermediate in the biosynthesis of coenzyme, nicotinamide adenine dinucleotide (NAD^+). It enhances NAD^+ biosynthesis and improves various symptoms of e.g., diabetes and vascular dysfunction[1]. In this project, we develop a *in vitro* cascade reaction to synthesize NMN from ribose, which can be produced from xylose by an engineered *E. coli*.

Recent studies have shown that the engineered *E. coli* MG1655 can produce *D*-ribose from xylose by knocking out two transketolase genes (*tktA* and *tktB*) and *ptsG* for relieving carbon catabolite repression[2] (Figure 1).

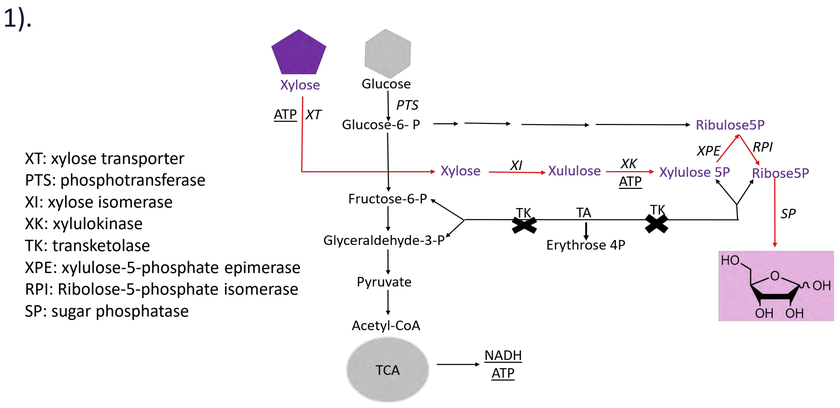


Figure 1 Metabolic pathway for *D*-ribose production from xylose in *E. coli*.

In the biosynthetic pathway, *Ec*RbsK catalyzes the phosphorylation of ribose to form ribose 5-phosphate (R5P)[3]. *Ec*PRPP synthase[4] can catalyze the phosphorylation of R5P to generate PRPP. Lastly, NMN is synthesized from Nam and PRPP catalyzed by Nampt[5]. Recent studies reported that PPK2 can regenerate ATP from AMP and ADP[6]. Pyrophosphatase (PPase) play a key role in the hydrolysis of inorganic pyrophosphate to phosphate (Figure 2).

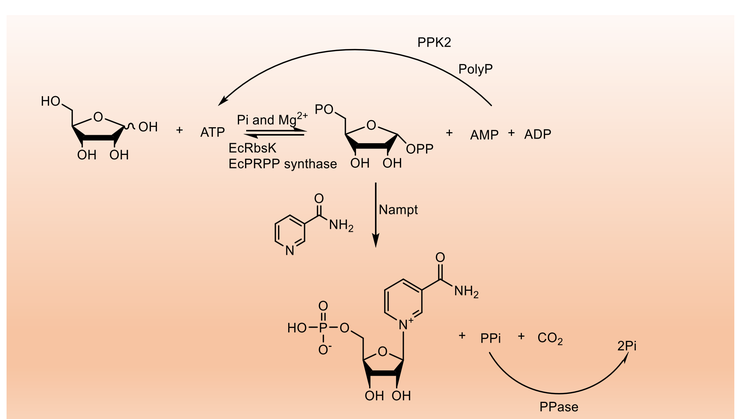
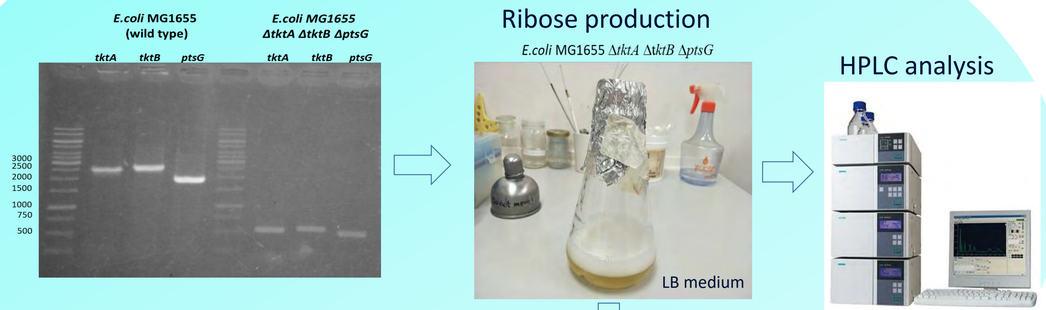


Figure 2. *In vitro* cascade reaction to convert ribose to NMN.

Methodology

Knockout three genes in *E. coli* MG1655 by CRISPR-Cas9 approach



NMN production

Analytical methods for NMN quantification
1. HPLC detection
2. Cyanide adduct formation analyzed by UV-Vis

Results and Discussion

Production of *D*-ribose by *E.coli* MG1655 WT *and E.coli* MG1655 *ΔtktA ΔtktB ΔptsG*

In this study, we conducted CRISPR-Cas9 to knock out two transketolase genes (*tktA* and *tktB*) and one gene (*ptsG*) encoding glucose-specific PTS enzyme IIBC component in *E. coli* MG1655. *E.coli* MG1655 *ΔtktA ΔtktB ΔptsG* can grow on LB medium containing glucose and xylose. It produced 2.47 g/L of ribose from 5 g/L of xylose in LB medium after 48 hours (Table 1).

Table 1. *D*-ribose production in *E.coli* MG1655 WT *and E.coli* MG1655 *ΔtktA ΔtktB ΔptsG*

Strains	Time (hour)	DCW (g/l)	*D*-ribose (g/l)	Glucose (g/l)	Xylose (g/l)
E.coli MG1655 WT	0	-	0.00	4.68	4.57
	24	6.20	0.00	0.19	0.18
	48	5.56	0.00	0.00	0.00
E.coli MG1655 *ΔtktA ΔtktB ΔptsG*	0	-	0.00	4.75	5.01
	24	2.87	1.37	3.02	3.23
	48	4.19	2.47	0.00	2.28

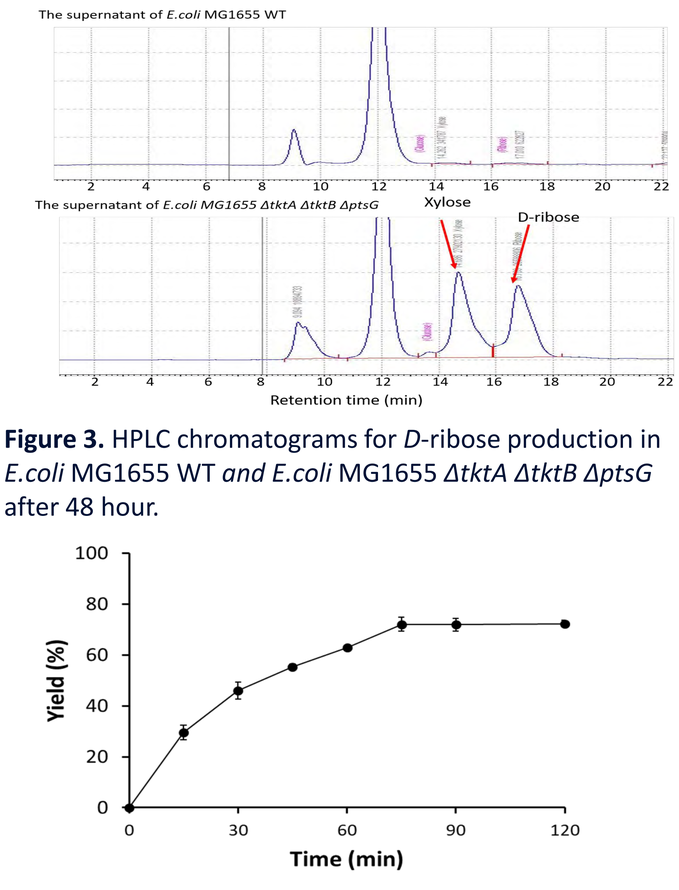


Figure 3. HPLC chromatograms for *D*-ribose production in *E.coli* MG1655 WT *and E.coli* MG1655 *ΔtktA ΔtktB ΔptsG* after 48 hour.

Figure 6. Time course of NMN generated by conversion of ribose in the supernatant of *E.coli* MG1655 *ΔtktA ΔtktB ΔptsG* medium.

In vitro cascade reaction for NMN production

An *In vitro* biosynthetic pathway is developed to convert ribose to NMN. *EcRbsK*, *Ec*PRPP, and *Cp*Nampt can catalyze the cascade reaction to convert ribose in the supernatant of *E. coli* MG1655 *ΔtktA ΔtktB ΔptsG* medium to NMN with the addition of excess ATP and stoichiometric nicotinamide. NMN production in the reaction can be rapidly determined by the cyanide assay (Figure 4). The quantification of NMN can be determined by HPLC analysis (Figure 5). In our study, PPase can help to improve the formation of NMN.

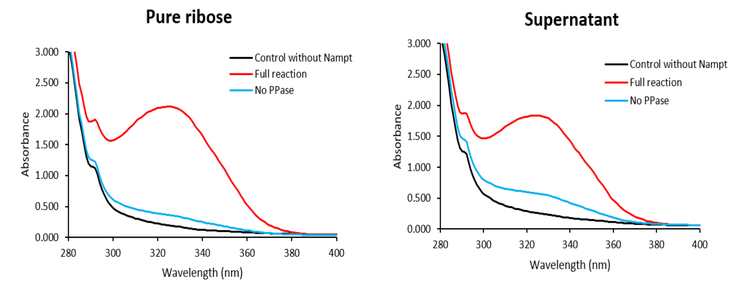


Figure 4. UV-VIS of the cyanide adduct of NMN generated by the conversion of pure ribose and ribose in the supernatant of *E. coli* MG1655 medium.

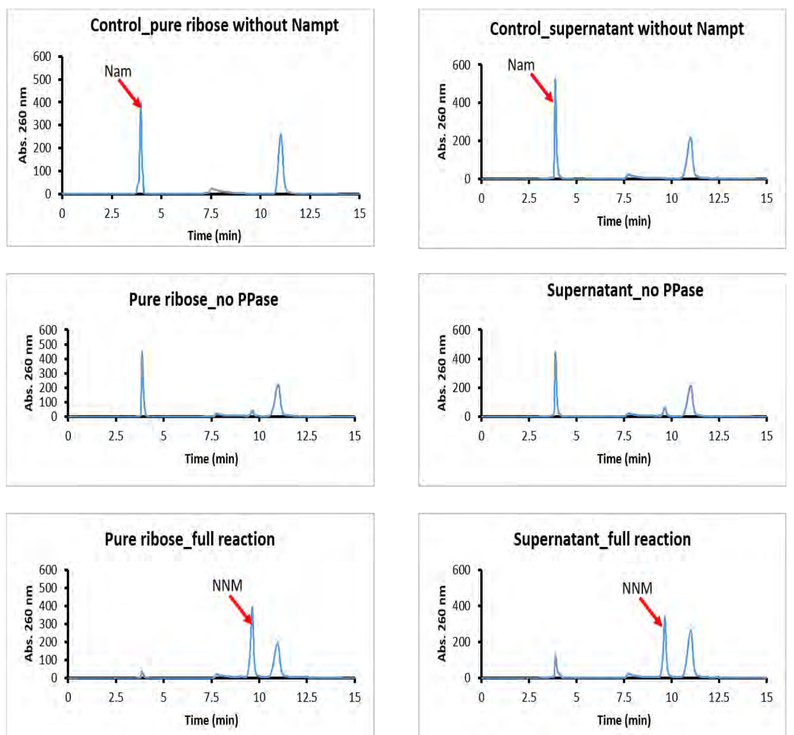


Figure 5. HPLC analysis for NMN generation from the conversion of pure ribose and ribose in the supernatant of *E. coli* MG1655 medium.

Conclusion

- *E. coli* MG1655 is knocked out two transketolase genes and one glucose-specific gene to produce *D*-ribose from xylose.
- *E.coli* MG1655 *ΔtktA ΔtktB ΔptsG* produced 2.47 g/L of ribose from 5 g/L of xylose in LB medium after 48 hours.
- This system could produce NMN from Nam with about 70% yield using the supernatant of engineered *E. coli* MG1655 medium.

Acknowledgements

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