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Review

Biosynthesis of Nicotinamide Mononucleotide: Current Metabolic Engineering Strategies, Challenges, and Prospects

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Abstract: Nicotinamide mononucleotide (NMN) is an essential precursor of nicotinamide adenine dinucleotide (NAD^+), which is widely applied in the pharmaceutical and biotech industries. The biosynthesis of NMN is currently attracting much attention because it has non-toxic reaction conditions and low amounts of isomers, whereas chemical synthesis has low yields and is not environmentally friendly. This review systematically describes the two biosynthetic pathways of NMN in detail for the first time and introduces the latest studies on NMN production through different pathways using metabolic engineering strategies. NMN accumulation can be improved by optimizing the activity of key enzymes, enhancing the supply of precursors and co-factors, inhibiting the synthesis of byproducts, and promoting product export. Finally, we also discuss the current challenges of producing NMN and possible solutions for the future.

Keywords: nicotinamide mononucleotide; nicotinamide phosphoribosyltransferase; biosynthetic pathway

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1. Introduction

Nicotinamide mononucleotide (NMN) is an acid- and water-soluble nucleotide containing a pyridine base. Although it exists in α and β anomeric forms, only the β form is the active anomer, which is also termed β-nicotinamide mononucleotide, β-nicotinamide D-ribonucleotide, and β-nicotinamide ribose monophosphate. Inside the cell, NMN is converted into nicotinamide adenine dinucleotide (NAD^+), which is an essential molecule in cells where it functions as an electron carrier in metabolic redox reactions and as a substrate for NAD-dependent signal transduction [1]. Deficiency of NAD^+ can be compensated for with NMN supplementation, which effectively increases NAD^+ levels in various tissues and prevents related metabolic diseases [2]. Recent studies suggested that NMN could recover cognition in Alzheimer's disease model rats and showed a significant protective effect on neurons [3,4]. In addition to improving numerous neuronal functions, NMN was also found to increase resistance to oxidation, improve immunity, and prevent cardiovascular disease [5]. Consequently, NMN is becoming a promising compound for treating neurological disorders and relieving aging-related symptoms.

NMN has a broad market and great application value in medicine and healthcare. To meet this demand, several chemical and biological methods have been developed to synthesize NMN. Chemical synthesis is mainly accomplished through different reaction steps using adenosine monophosphate (AMP), nicotinamide (NAM), and 1,2,3,5-tetra-O-acetyl-D-ribose as raw materials. Compared to chemical synthesis, biological routes have the advantages of mild reaction conditions, non-toxic ingredients, and low amounts of isomers [6]. The biosynthesis of NMN can be subdivided into enzymatic and fermentation-based methods. In vitro enzyme catalysis technology enables NMN synthesis by creating a

multi-enzyme cascade biosystem. As early as 1957, Preiss and Handler produced NMN using an in vitro enzymatic method with a yield of 113.64 mg/L [7]. However, this process requires the purification of enzymes for the reactions, as well as the addition of ATP and NADH to provide the energy required for enzymatic catalysis [8]. With the development of metabolic engineering, Sinclair et al. first constructed a recombinant *Saccharomyces cerevisiae* for NMN synthesis from a nucleoside comprising NAM via nicotinamide phosphoribosyltransferase (NAMPT) catalysis [9,10]. *Escherichia coli* is a simple model organism compared to *S. cerevisiae* and has become the main chassis for NMN production. Recently, Huang et al. reconstructed the metabolism of *E. coli* and achieved NMN yields of 16,200 mg/L in a 5 L bioreactor, the highest production reported to date [11].

There are two main metabolic pathways for the biosynthesis of NMN from nicotinate and nicotinamide in nature: the de novo pathway and the salvage pathway [12–15]. Shen et al. summarized the synthesis of NMN via the salvage pathway using 5-phosphoribosyl-1-pyrophosphate (PRPP) and NAM as precursors [6]. Here, we systematically describe the two biosynthetic pathways of NMN, which is the first comprehensive summary of current strategies to promote microbial biosynthesis of NMN. Strategies for regulating key components such as key enzymes, precursors, cofactors, byproducts, and transport proteins through metabolic engineering are introduced. Moreover, the challenges and solutions for increasing the accumulation of NMN are also discussed.

2. Metabolism of Nicotinamide Mononucleotide

Although NMN metabolism is a universal process closely related to NAD^+, there are subtle differences in the cellular pathways of NMN synthesis. The two basic pathways of NMN synthesis are known as the de novo pathway and the salvage pathway.

2.1. De Novo Pathway

In fungi and some bacteria, such as *S. cerevisiae* and *Bacillus subtilis*, NMN is derived from NAD^+ via the de novo pathway catalyzed by a single enzyme, downstream of which many enzymes are involved in the production of NAD^+ (Figure 1). Nicotinic acid mononucleotide (NaMN) is synthesized in three reactions from aspartate (Asp) or six reactions from tryptophan (Trp), after which it is converted into NAD^+ via two enzymatic reactions [16]. Asp is the starting amino acid for the de novo synthesis of NMN in most bacteria. The de novo pathway is characterized by the synthesis of quinolinic acid (QA) from Asp via two enzymatic reactions mediated by L-aspartate oxidase, quinolinate synthase, and spontaneous cyclization. The two enzymes are respectively encoded by the *nadB* and *nadA* genes [17,18]. Quinolinate phosphoribosyltransferase (NADC) then transfers the phosphoribosyl moiety of PRPP to quinolinic acid (QA), which produces NaMN [13]. Next, NaMN adenylyltransferase (NADD) converts NaMN into nicotinic acid adenine dinucleotide (NaAD) via the addition of an AMP moiety from ATP [19]. Finally, NAD^+ synthetase (NADE) converts NaAD into NAD^+. Conversely, the Nudix hydrolase (NUDE) or other pyrophosphatase (USHA and MAGZ) with similar hydrolytic activity can degrade NAD^+ into NMN. However, NAD^+ is involved in intracellular redox reactions and is rarely decomposed directly into NMN. Therefore, the de novo pathway received relatively little attention until Sorci et al. combined a comparative genomic approach with in vitro and in vivo experiments to identify the *nadE* gene from *Francisella tularensis* [20]. This gene encodes NMN synthetase (FtNADE*), which is involved in the direct conversion of NaMN into NMN without NAD^+ synthesis. Moreover, in most cases, this activity was not detected in any of the characteristic members of the NADE family. As shown in Figure 1, the de novo pathway is very long, leading to a slow rate of NMN synthesis. Hence, this pathway is hardly ever chosen for the biosynthesis of NMN.

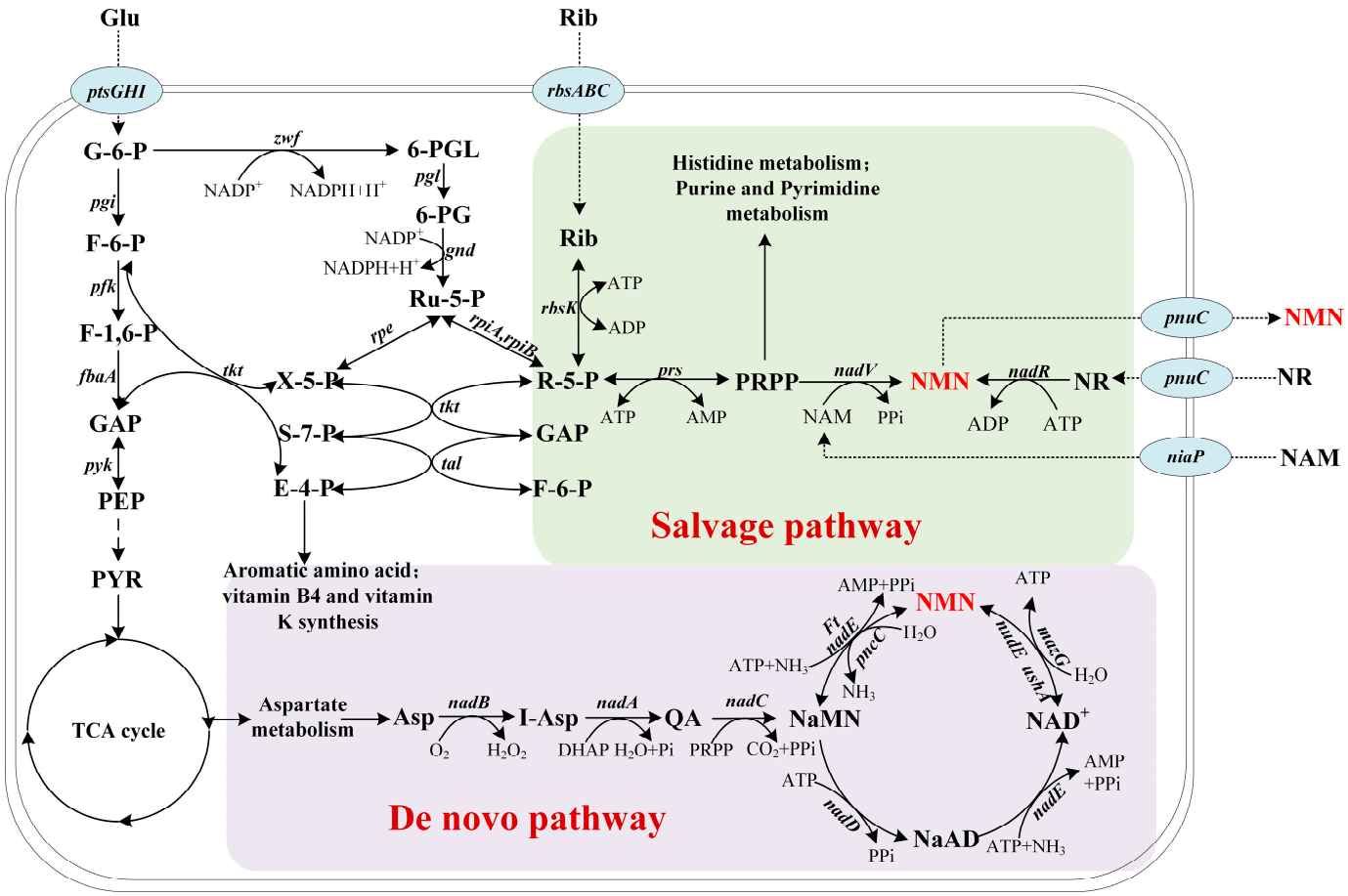


Figure 1. De novo and salvage pathways of nicotinamide mononucleotide synthesis. The de novo synthesis of NMN is mediated by NADB, NADA, and NADC, leading to the production of NaMN. After NAD^+ is generated by the pathway, Nudix hydrolases such as NUDE or other nucleotidases (MAZG and USHA) with similar hydrolytic activity can degrade NAD^+ into NMN. Alternatively, NMN synthetase could directly convert NaMN into NMN. In the salvage pathway, NAM is converted into NMN by NAM phosphoribosyl transferase (NAMPT), whereas NR is converted into NMN by NMN adenylyltransferase (NADR). Abbreviations of NMN intermediates are shown in bold font 12. Asp, aspartate. I-Asp, iminoaspartate. QA, quinolinic acid. NaMN, nicotinic acid mononucleotide. NaAD, deamido-nicotinamide adenine dinucleotide. NAD^+, nicotinamide adenine dinucleotide. NMN, nicotinamide mononucleotide. NAM, nicotinamide. NR, nicotinamide riboside. PRPP, 5′-phosphoribosyl 1-pyrophosphate. ATP, adenosine triphosphate. ADP, adenosine diphosphate. AMP, adenosine monophosphate. DHAP, dihydroxyacetone phosphate. PPi, pyrophosphate. Pi, phosphate. The genes and enzymes associated with the reactions are *nadB*, L-aspartate oxidase; *nadA*, quinolinate synthase; *nadC*, quinolinate phosphoribosyltransferase; *nadD*, NaMN adenylyltransferase; *nadE*, NAD^+ synthetase; *nudE*, NADH hydrolase; *mazG*, nucleoside triphosphate pyrophosphohydrolase; *ushA*, UDP-sugar hydrolase; *pncC*, NMN amidohydrolase; *Ft nadE*, NMN synthase from *Francisella tularensis*; *nadV*, nicotinamide phosphoribosyltransferase; *nadR*, NMN amidohydrolase from *Bacillus subtilis*; *xapA*, xanthosine phosphorylase; and *nadD*, nicotinate nucleotide adenylyltransferase. The production of NMN from NAM and glucose/ribose uses the pentose phosphate pathway or the ribose metabolic pathway. Solid arrows indicate metabolic pathways, whereas dotted arrows indicate the transport directions of transporters. The essential genes and enzymes associated with the reactions are *zwf*, glucose 6-phosphate dehydrogenase; *pgi*, glucose-6-phosphate isomerase; *pgl*, 6-phosphogluconolactonase; *gnd*, 6-phosphogluconate dehydrogenase; *rpiA*, ribose 5-phosphate isomerase A; *rpiB*, ribose 5-phosphate isomerase B; *prs*, ribose-phosphate diphosphokinase; and *rbsK*, ribokinase. Four transporters, *ptsGHI* (glucose transporter), *rbsABC* (D-ribose transporter), *niaP* (niacin

transporter), and *pnuC* (nicotinamide riboside transporter), are shown in blue and purple ovals. Abbreviations of metabolites: Glu, glucose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, 1,6-fructose diphosphate; GAP, glyceraldehyde 2-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvic acid; 6-PGL, 6-phosphogluconolactone; 6-PG, 6-phosphogluconic acid; Ru-5-P, ribulose 5-phosphate; X-5-P, D-xylulose 5-phosphate; S-7-P, sedoheptulose 7-phosphate; E-4-P, erythrose 4-phosphate; Rib, ribose; Ru-5-P, ribulose-5-phosphate; R-5-P, ribulose 5-phosphate.

In the de novo pathway, the hydrolysis efficiencies of NAD^+ directly determines the production of NMN. USHA provides most of the pyrophosphatase activity in *E. coli* and showed catalytic efficiencies for the hydrolysis of NAD^+ at 3.7×10^6 $M^{-1} \cdot s^{-1}$, much more than MAGZ and NUDE [21]. However, USHA also has a 5′-nuclease activity that decomposes NMN into NAM. Therefore, the most promising route for the de novo synthesis of NMN is the amidation of NaMN catalyzed by FtNADE*. FtNADE* typically use ammonium as an amine nitrogen donor, and the reaction is driven by ATP hydrolysis. The substrate specificity of FtNADE* is very high, as it catalyzes the conversion of NaMN into NMN with an efficiency of 2.5×10^3 $M^{-1} \cdot s^{-1}$, 60 times higher than with NaAD as substrate [20]. However, the activity of NAD^+ synthase (NADM) is 1000 times higher than that of FtNADE*. This means that the downstream metabolic pathway for the synthesis of NAD^+ must be blocked to achieve high yields of NMN. In addition, the FtNADE* enzyme is a variant of the NADE family with rare functions [22], and no regulation at the transcriptional level has been reported.

2.2. Salvage Pathway

The salvage pathway is a cost-effective way for eukaryotes and prokaryotes to obtain NMN. It is found in mammals, yeast, and Gram-negative bacteria such as *F. tularensis*. There are two main routes of the salvage pathway: (1) direct biosynthesis of NAM and PRPP by NAMPT, and (2) NR phosphorylation (Figure 1).

Most studies of the salvage pathway investigated the salvageable pyridine base, NAM. It is synthesized via NAM and PRPP catalyzed by NAMPT, which is a rate-limiting enzyme for NMN biosynthesis. [15]. The *nadV* genes encoding NAMPT have been identified in mammals such as mice, as well as in phages and bacteria, including *Shewanella oneidensis*, *Xanthomonas translucens*, *Haemophilus ducreyi* [23], *Ralstonia solanacearum*, *Sphingopyxis* sp. C-1, *Chitinophaga pinensis*, and *Vibrio* Phage KVP40 [24]. The activity of enzymes is closely related to their protein structure, but only a few three-dimensional structures of NAMPTs from different strains have been determined, which greatly limits their rational engineering. PRPP is a precursor in this reaction and can be synthesized from glucose as a substrate. However, this conversion requires a long metabolic pathway to obtain PRPP, whereas the conversion of ribose to PRPP requires only two enzymatic reactions (Figure 1). Common model organisms such as *E. coli* and *B. subtilis* have membrane proteins that transport ribose, and ribose phosphate diphosphokinase (PRS) is considered the most prominent limiting factor for PRPP synthesis. The direct contribution of nicotinamide riboside (NR) to NAD^+ metabolism was first recognized by Bieganowski and Brenner in 2004 [25]. NMN is synthesized from NR via NRK-mediated phosphorylation, bypassing the need for NAMPT [15]. Mammals possess specific NRKs that function to synthesize NAD^+ via NMN in addition to the well-known pathways through NaMN [26].

When NAM is used as a precursor, NMN is formed by NAMPT. NAMPT is found in mammals and some pathogenic microorganisms, and studies on its regulation have focused on mammalian cells such as humans and mice [1,27]. The catalytic activity of human NAMPT could be increased from $1.60 \pm 0.06 \times 10^3$ to $1.8 \pm 0.9 \times 10^6$ $M^{-1} \cdot s^{-1}$ by hydrolytic coupling with ATP [28]. Studies on NAMPT in microorganisms have focused on the identification of enzymes [23]. PRS is the key enzyme to produce the essential precursor PRPP. In some bacteria, PRS was complexed with ATP and R-5-P, as well as its four domains for substrate or cofactor recognition (Mg^{2+}, MgATP, Pi, and R5P), and catalytic activity has been studied [29]. In *B. subtilis*, PRS was responsible for the formation of PRPP and purines synthesis, which had K_m values of 4.8×10^{-4} M for R-5-P. The regulation of PRS activity is

multi-dimensional and complex. Its activity is affected by negative feedback regulation by some nucleotides, especially ADP and GDP [30]. Furthermore, PRS is also regulated by metal ions and Pi. Only when these ions are present can PRS show good activity [31]. In addition, the precursor PRPP can bind to the purine repressor *purR* and regulate the transcription of the purine pathway, which may cause a poor effect on the binding of PRPP to NAMPT [32].

When NR is used as a precursor, NMN is derived from the phosphorylation of NR in one enzymatic step. In prokaryotes such as *E. coli* and *Haemophilus influenzae*, the full-size multifunctional NADR protein (encoded by *nadR*) is composed of a central nicotinamide mononucleotide adenylyltransferase (NMNAT) domain and a C-terminal NR kinase (NRK) domain, which endow it with NMNAT and NRK enzymatic activities, respectively [33,34]. A new NRK from *Kluyveromyces marxianus* (Klm-NRK) ranks the highest among reported NRKs. The specific activity of purified Klm-NRK was 7.9 U·mg^{-1} protein, and the catalytic efficiencies (k_{cat}/K_m) toward ATP and NR were 5.74 × 10^4 and 8.44 × 10^4 M^{-1}·s^{-1}, respectively [35]. In eukaryotes such as yeast, NRK1 (encoding by *nrk1*) catalyzes the reaction of NR with ATP to produce NMN. After purification, NRK from *S. cerevisiae* reached an activity of 2252.59 U·mg^{-1} and K_m values of 5.427 × 10^{-2} and 1.869 × 10^{-2} M for ATP and NR, respectively [36]. Human cells have two gene-encoded NRK isoforms: NRK1 and NRK2. Both enzymes convert NR with comparable K_m values of 3.4 × 10^{-6} and 4.6 × 10^{-6} M for NRK1 and NRK2, respectively, but also utilize other nucleoside substrates with lower affinity [37,38]. In particular, NRK1 limits the use of exogenous NR for the synthesis of NMN and NAD$^+$ [39]. It is worth noting that NADR may interact with an integral membrane transporter PnuC protein and directly participate in the uptake of exogenous NAD$^+$ precursors [40]. PnuC is a membrane protein and usually regarded as a transporter protein of NMN, but NMN must be dephosphorylated to NR before it can be transported intracellularly [41]. Fortunately, mutant PnuC*290 can transport NMN directly. PnuC*290 has a duplication of the six-base sequence AAAAGC encoding amino acids 108 (K) and 109 (A), which adds K and A residues just before the fourth membrane-panning region [42]. In *Salmonella typhimurium*, transport of NMN requires two proteins: NADR and PnuC. PnuC undertakes the main function of the transport system, whereas NADR is a regulatory protein. PnuC is often clustered with genes for NAD biosynthesis, and in enterobacteria, *pnuC* forms a manipulator with the gene *nadA* (encoding quinolinic acid synthase) and is regulated by NADR [43]. The activity of PnuC* is regulated by intracellular NAD levels. When high concentrations of NAD are sensed by NADR proteins, the PnuC transport system returns to its inactive condition [40]. It was demonstrated that when the internal concentration was high, the PnuC transporter protein was able to export the substrate [44]. Although NMN transporter proteins have not been identified in eukaryotes, the NAD$^+$ transporter protein Ndt1p [45] and NR transporter protein Nrt1 [46] have been identified in *S. cerevisiae*.

3. Metabolic Engineering Strategies for Nicotinamide Mononucleotide Production

NMN is usually produced by microbial fermentation, for example, by constructing a heterologous pathway in *E. coli*. In the salvage pathway, researchers tried to increase the titer and yield of NMN to a certain extent via multiple metabolic engineering strategies, including overexpressing key enzyme NAMPT [11] and optimizing the activity of NAMPT via directed evolution, increasing the supply of key precursors PRPP and NAM by enhancing the carbon flux to PRPP and expressing NAM transporter proteins [47], improving the energy supply of ATP by building an ATP regeneration system [48], eliminating the production of byproducts by knocking out the byproduct pathway [49], and enhancing the output of NMN by overexpressing NMN transporter proteins [50]. Recent advances in the research on NMN biosynthesis are summarized in Table 1.

Table 1. Metabolic engineering strategies for improving the production of NMN.

Chassis	Strategies	Cultivation	Intracellular NMN Production (mg/L)	Extracellular NMN Production (mg/L)	Reference
S. cerevisiae	Overexpression of *nadV*; deletion of *nma1* [1]	Bioreactor [2]	ND [3] (300–350 nmol per g of wet cells)	ND [3]	[9]
E. coli	Overexpression of *nadV*	500 mL benchtop bioreactor	7877.59	10.89	[51]
E. coli	Overexpression of *nadV* and *prs* (with L135I mutation)	500 mL benchtop bioreactor	7563.42	15.42	[51]
E. coli	Overexpression of *nadV* and *nadE*; deletion of *pncC*	2 mL deep-well plate	501.33	ND [3]	[52]
E. coli	Overexpression of *nadV*, *niaP*, *pnuC*, *pig*, *zwf*, *pgl*, *gnd*, *ripA*, *ripB*, and *prs*	2 L fermenter	ND [3]	6.79×10^3	[47]
E. coli	Overexpression of *nadV*, ygcS, *prs* (with L135I mutation), *zwf*, *gnd*, and *ado*1; deletion of *nadR*, *pncC*, *amn*, and *purR* [4]	Flasks [2]	ND [3]	496.2	[53]
E. coli	Overexpression *nadV*, *prs1*, and *prs2*; optimization of the amounts of ribose, Mg^{2+}, phosphate, nicotinamide, and lactose inducer (using response surface methodology)	250 mL Erlenmeyer flask	772.05	ND [3]	[54]
Fructobacillus durionis	Lactic acid bacteria isolated from natural resources cultivated in MRS medium containing 1% D-fructose for 12 h	Flasks [2]	2.1	ND [3]	[55]
E. coli	Overexpression of *nadV*, *prs* (with L135I mutation), and *pnuC*; deletion of *nadR*, *pncC*, *ushA*, and *purR*; controlling the supplementation of nicotinamide and dissolved oxygen level	5 L fermenter	ND [3]	1.62×10^4	[11]
E. coli	Deletion of *tktA* [5], *tktB* [5], and *ptsG* [5]; cascade bioconversion using EcRBSK, EcPRPS, CpNAMPT, CHU0107, and EcPPase	500 mL baffled flask and 10 mL scale bioreactor	ND [3]	284.09	[56]
S. cerevisiae EBY100	Overexpression of *nrk2* [6] (displayed on the cell surface); optimization of the amounts of NR, ATP, and Mg^{2+}; optimization of pH and temperature	30 mL screw vial	ND [3]	1.26×10^4	[57]

[1] *nma1*: encodes nicotinamide mononucleotide adenylyltransferase. [2] Bioreactor, flasks: The size of the vessels used in the literatures are not reported. [3] ND: not determined. [4] *purR*: encodes DNA-binding transcriptional repressor. [5] *tktA* and *tktB*: transketolase; *ptsG*: encodes the glucose-specific IICB component. [6] *nrk2*: NR kinase.

3.1. Selection and Directed Evolution of the Key Enzyme NAMPT

The direct biosynthesis of NAM and PRPP by NAMPT has attracted a lot of attention because of its short metabolic pathway and low metabolic burden. As NAMPT is a key enzyme of the salvage pathway, heterologous metabolic pathways are often constructed in chassis by directly selecting NAMPT derived from different species in nature. Huang et al. first constructed the *E. coli* F004 by deleted four genes (*nadR*, *pncC*, *ushA*, and *purR*) related to the decomposition of NMN, and then they selected eight NAMPT orthologs from different microorganisms and expressed them with the T7 promoter in *E. coli* F004. The results indicated that NAMPT from *Vibrio* bacteriophage KVP4012 (VpNAMPT) showed the highest activity and finally achieved high intracellular NMN production, reaching 81.3 mg/L [11], which made it a potential candidate. Selecting highly active enzymes from nature and directing the carbon flux to the target product could increase the yield of NMN and build a solid foundation for a continuous increase in NMN production [58].

Directed evolution of enzymes is a powerful technology that imitates natural evolution and screens for robust enzymes with mutations, leading to desired properties [59]. The most widely used mutagenesis strategies for NAMPT are error-prone PCR (epPCR) and saturation mutagenesis (SM). NAMPT from *Meiothermus ruber* DSM 1279 (MrNAMPT) was used as the starting enzyme for site-directed mutagenesis. The results suggested that the MrNAMPT carrying the E231Q, D298A, D338E, and D377E mutations showed 6.9 times higher catalytic activity for NAM and PRPP than its parent [60]. However, non-rational strategies such as epPCR are labor-intensive and time-consuming [61]. Therefore, we need to find more efficient methods of modification and selection.

3.2. Improving the Supply of Essential Precursors

The maximum activity of the enzyme is only achieved when the supply of precursors is sufficient. In the salvage pathway, PRPP, NAM, and NR are used as precursors to produce NMN. As the NR-based pathway requires a heterologous kinase that is poorly expressed in recombinant strains, the NAM-based pathway is more commonly chosen in metabolic engineering. Previous studies showed that the accumulation of PRPP in the cytoplasm has a significant impact on the synthesis of NMN [62], and PRPP is mainly synthesized through the pentose phosphate pathway (PPP). As glucose is the most accessible sugar worldwide, it may be an ideal raw material for the synthesis of PRPP if sufficiently high biocatalytic conversion efficacy can be achieved. In order to enhance PRPP synthesis, Shoji et al. selected the genes *pgi*, *zwf*, *pgl*, *gnd*, *rpiA*, *rpiB*, and *prs* from the PPP to construct the artificial operons (Figure 1). The reconstructed strain co-expressing NAMPT from *Chitinophaga pinensis* (CpNAMPT) and the artificial operon achieved 2.5 times higher NMN production (189 mg/L) than the parental strain [47]. Although the construction of artificial operons improved PRPP production, vectors expressing the operons and CpNAMPT constitute a double plasmid system that may cause metabolic burdens on cells [63,64]. Alternatively, PRPP can also be produced from ribose via a short pathway, and many bacteria such as *E. coli* and *B. subtilis* can use ribose as a carbon source [65,66].

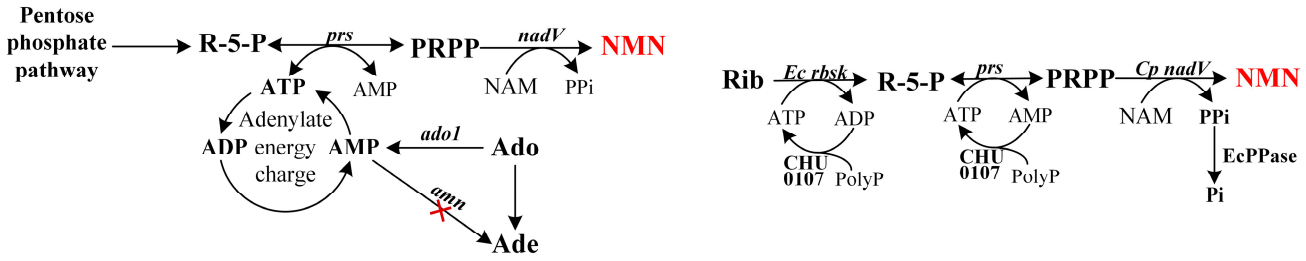
In order to promote PRPP synthesis, the activity of PRS needs to be increased. Firstly, the activity of enzyme can be increased by adding cofactors. PRS1 requires phosphate and Mg^{2+} for its activity since the Mg-ATP complex acts as the actual substrate for the enzyme. Maharjan et al. combined the PRS1, PRS2, and NAMPT genes in sequential order on a plasmid and expressed them in *E. coli* [54]. They found that co-expression of PRS1 and PRS2 resulted in higher NMN production (417.78 mg/L) in the presence of Mg^{2+} than without. When both Mg^{2+} and phosphate were present, the NMN titer increased from 200.53 to 524.73 mg/L. Hence, the idea of adding Mg^{2+} and phosphate proved to be beneficial for improving the activity of PRS. Secondly, relieving the negative feedback inhibition of ADP and GDP on PRS can enhance the PRPP supply. Zakataeva et al. discovered that PRS from *Bacillus amyloliquefaciens* strain IAM1523 with the L135I mutation (BaPRS) effectively resists the inhibitory effects of purine nucleotides [67].

NAM is another important precursor in the salvage pathway; the rate of NAM uptake by cells limits the activity of NAMPT. Many microorganisms such as *E. coli* cannot synthesize NAM and instead need to absorb it from the extracellular environment. Accordingly, the first step in strain engineering is to improve the uptake of NAM into cells. Genetic analysis and experimental validation of the niacin transporter (NiaP) protein family revealed that they function as transporters of NAM analogs [68]. Shoji et al. expressed NiaP homologs from six different species, and the results clearly showed that BC-NiaP derived from *Burkholderia cenocepacia* took up NAM at the fastest rate, resulting in an uptake that was up to 25% faster than without expressing the transporter protein [47]. However, NAM is expensive, and its industrial use is not economically viable.

3.3. Constructing ATP Regeneration Systems

In the salvage pathway, PRS transfers two phosphate groups from ATP to R5P for the synthesis of PRPP (Figure 1) [69]. ATP hydrolysis provides a thermodynamic driving force for the reaction and contributes to efficient production of NMN [70]. In order to increase product synthesis in fermentation, the ATP balance is very important. Microorganisms generally synthesize ATP via the purine pathway, which is energy intensive. In *S. cerevisiae*, adenosine kinase (encoded by *ado1*) catalyzes the phosphorylation of adenosine to produce AMP and ATP (Figure 2a) [71]. In order to increase production of ATP and prevent the hydrolysis of AMP into ribose 5-phosphate and adenine, the *amn* gene (encoding AMP nucleosidase) needs to be knocked out [72]. Therefore, strain NMN08 was constructed by integrating the *ado1* gene at the *amn* locus to delete it at the same time. The results suggested that the intracellular concentration of ATP in strain NMN08 was increased, and its NMN titer reached about 220 mg/L after 8 h, which was 22% higher than in the

unmodified strain [53]. It has been shown that enhancing the ATP supply can further increase NMN production.



(**a**) (**b**)

Figure 2. Two types of ATP regeneration systems. (**a**) ATP synthesis via the purine pathway. The red cross represents a deleted pathway. R-5-P, ribose 5-phosphate. PRPP, 5′-phosphoribosyl 1-pyrophosphate. NAM, nicotinamide. NMN, nicotinamide mononucleotide. ATP, adenosine triphosphate. ADP, adenosine diphosphate. AMP, adenosine monophosphate. PPi, pyrophosphate. Ade, adenine; Ado, adenosine. The genes and enzymes associated with the reactions are *amn*, AMP nucleosidase; *ado1*, adenosine kinase (in *S. cerevisiae*); *prs*, ribose-phosphate diphosphokinase; and *nadV*, nicotinamide phosphoribosyltransferase. (**b**) CHU0107 catalyzes the synthesis of ATP from ADP and AMP. The expression of CHU0107 eliminates ADP and promotes ATP production, EcPPase degrades PPi. Pi, inorganic phosphate. The enzymes associated with the reactions are CHU0107, polyphosphate kinase II from *Cytophaga hutchinsonii*; EcPPase, pyrophosphatase from *E. coli*; *EcRbsK*, ribokinase from *E. coli*; and *Cp nadV*, nicotinamide phosphoribosyltransferase from *Chitinophaga pinensis*.

Ngivprom et al. found that CHU0107 consumes AMP or ADP for the biosynthesis of ATP, and inorganic pyrophosphatase from *E. coli* (EcPPase) could hydrolyze PPi generated by CpNAMPT to help drive the reaction (Figure 2b) [56,73]. They constructed *E. coli* MG1655 Δ*tktA*Δ*tktB*Δ*ptsG*, which can metabolize xylose to generate D-ribose. The fermentation supernatant metabolized by the recombinant strain contained D-ribose and was applied to synthesize NMN in the second module, composed of EcRbsK-EcPRS-CpNAMPT. In this system, CHU0107 enables ATP recycling. Finally, a 10 mL optimal cascade bioconversion generated NMN with a good yield of 84% from 150.13 mg/L D-ribose supplied in the supernatant of *E. coli* MG1655 Δ*tktA*Δ*tktB*Δ*ptsG*. This phenomenon indicates that the construction of an ATP regeneration system not only provides sufficient energy for the enzymatic reactions but also reduces the production of ADP and AMP to prevent the feedback inhibition of PRS activity.

Moreover, ATP is also an essential activator of the NAMPT reaction. Hara et al. purified recombinant human NAMPT and measured its K_m value for NAM and PRPP in vitro with or without the addition of ATP [74]. The results showed that the addition of 507.18 mg/L ATP reduced the K_m values for these two substrates by 84% and 98%, respectively. When the concentrations of NAM and PRPP were low, the activity of NAMPT was still significant if the ATP concentration reached millimolar levels. Burgos et al. also found that NAMPT drives its reaction through ATP hydrolysis, allowing the enzymatic system to build up 35-fold higher NMN concentrations than in the absence of ATP [28]. Variations in the substrate and enzyme affinities are also influenced by ATP. Understanding the overall regulatory mechanism of ATP is vital for NMN production.

3.4. Blocking the Formation of Byproducts

NaMN, NR, NAD^+, and other byproducts are generated during NMN metabolism (Figure 3). Suppressing the synthesis of these byproducts has been a focus of bioengineering studies. To this end, researchers have constructed mutants with enhanced production of NMN by blocking the metabolic pathways of byproducts such as NaMN, NR, and NAD^+. The deletion of the *nadR* gene in *Lactococcus lactis* NZ9000 enhanced the accumulation of

NMN by 61% and slightly decreased the intracellular levels of NAD^+ [75]. As previously mentioned, Huang et al. constructed the mutant *E. coli* strain F004 by deleting a set of genes (including *nadR*, *pncC*, *ushA*, and *purR*) to avoid the accumulation of NaMN and NR [11]. Expressing NAMPT from *H. ducreyi* in strain F004, the NMN titer increased 5.3-fold compared with the wild-type one (from 3.6 mg/L to 19.1 mg/L). In the de novo pathway, *pncC*, which encodes an enzyme involved in NaMN production, significantly affects the accumulation of NMN. Expressing FtNADE* and NAMPT from *Ralstonia solanacearum* (RsNAMPT) in a Δ*pncC E. coli* strain resulted in an intracellular NMN level of 501.33 mg/L, a 130-fold increase over the wild-type NMN level [52].

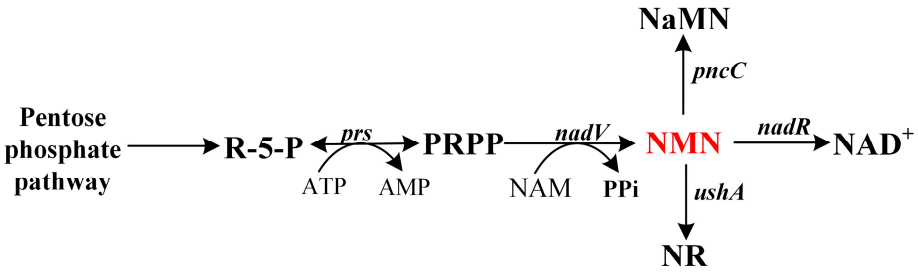


Figure 3. Byproducts of NMN fermentation. During the production of NMN, byproducts such as NAD^+, NR, and NaMN are generated, which influences the final yield. R-5-P, ribose 5-phosphate. PRPP, 5′-phosphoribosyl 1-pyrophosphate. NAM, nicotinamide. NMN, nicotinamide mononucleotide. NR, nicotinamide riboside. NAD^+, nicotinamide adenine dinucleotide. ATP, adenosine triphosphate. AMP, adenosine monophosphate. PPi, pyrophosphate. The enzymes associated with the reactions are *prs*, ribose-phosphate diphosphokinase; *nadV*, nicotinamide phosphoribosyltransferase; *pncC*, NMN amidohydrolase; *nadR*, NMN amidohydrolase; and *ushA*, UDP-sugar hydrolase.

3.5. Expressing Membrane Transporters to Export NMN form the Cell

Excessive intracellular NMN accumulation affects the biological activity of the cells and is detrimental to product synthesis. Even though some model microorganisms such as *E. coli* can spontaneously excrete metabolites out of the cell, additional transporters are needed to accelerate the export of NMN in highly productive engineered strains.

The native substrate of PnuC is generally not NMN but its unphosphorylated precursor NR [76]. However, the transport of NMN was confirmed in a previous study using a PnuC mutant from *Salmonella enterica* [52]. Inspired by this, Shoji et al. screened five PnuC orthologs in *E. coli* and discovered a novel NMN transporter from *Bacillus mycoides*, called BM-PnuC [47]. This transporter was then introduced into recombinant *E. coli* strain NF006, which was derived from *E. coli* strain F004 co-expressing VpNAMPT and PRS from *B. amyloliquefaciens*. After the addition of 200 mg/L NAM, 303.1 mg/L NMN was accumulated extracellularly, which is 2.4 times higher than in the strain without NMN transporter expression. Finally, fine-tuning gene expression and precisely controlling the supplementation of NAM led to an NMN titer of 16,200 mg/L with a conversion ratio of 97.0% from NAM in a 5 L bioreactor, both of which are the highest values reported to date [11]. BM-PnuC can export NMN outside of the cell even when the intracellular production of NMN is low. Therefore, BM-PnuC is the most efficient transporter for NMN reported up to now. Notably, BM-PnuC exhibits the best performance when it is expressed at a comparable or higher level than the enzymes in the corresponding heterologous synthesis pathway.

4. Discussion

Microbial fermentation systems for the synthesis of NMN offer advantages such as greater stability, fewer external additives, and simpler operation. Although significant progress has been made in recent years, further studies are needed to realize the full potential for microbial NMN production. As such, there are several remaining challenges, including: (1) The catalytic activity of the key enzyme NAMPT is insufficient for high

production of NMN, (2) the supply of the important precursor PRPP is difficult to enhance through metabolic engineering due to its long and complex synthetic pathway, and (3) the de novo synthesis of NMN in a single-cell system creates a heavy metabolic burden. In order to address these problems, we propose the following potential solutions.

4.1. Utilizing in Silico Analysis and High-throughput Screening to Evolve Key Enzymes

High activity of enzymes could dramatically increase the rate of reactions in the desired pathway [77], and the activity of natural enzymes is generally low. The characterization of more members from the different NAMPT families with the aid of site-directed mutagenesis and in silico methods would provide more insight into the mechanism behind NMN metabolism and would contribute to enhancing the NAMPT titer [78]. However, research on NAMPT from microorganisms is still lacking [79]; in particular, the three-dimensional structure of this enzyme has rarely been reported. With the development of bioinformatics and computational tools, in silico analysis technology provides a more effective approach for predicting the structures of proteins and making the semi-rational design of NAMPT become a reality.

Deep learning-based computational tools such as trRosetta and AlphaFold can be used to predict three-dimensional protein structures of NAMPT based only on amino acid sequence information [80]. To identify protein structures with improved enzymatic activity, Monte Carlo sampling is carried out in the amino acid sequence space to obtain the protein structure of the network-generated protein, which closely matches the real three-dimensional structure [81]. Once the spatial structure of NAMPT is obtained, molecular docking and surface analysis methods are used to analyze the transition state or product binding in the interaction of NAMPT with precursors [82]. The active sites of NAMPT are obtained by analysis of these interactions. After selecting sites based on key amino acid residues, the gene library consisting of NAMPT candidates is constructed by saturation mutagenesis. Notably, Zhang et al. found that the derivatives generated from NMN by simple chemical treatment could emit fluorescence [83]. In droplet-based high-throughput assays, each of the different variants is dispersed into a small droplet, and rapid selection is achieved by detecting the fluorescence of substrates [61]. Perhaps a combination of these two methods could be used for the high-throughput screening of NAMPT variants.

4.2. Enhancing Synergetic Carbon Utilization to Improve Precursor Supply

The supply of precursors is the limiting factor for the maximum activity of enzymes. Therefore, enhancing the flux to precursor synthesis is a key step to improving NMN production. The co-utilization of glucose and ribose makes it possible to increase the carbon flux for the production of PRPP, but the undesirable effects of carbon catabolite repression (CCR) need to be relieved. The main components of CCR include the catabolite control protein A (CcpA) and the catabolite responsive element (CRE), a 14 bp palindromic DNA sequence. Simultaneous mutation of *ccpA* and *spo0A* maximized the intracellular transcription level of the *rbs* operon when glucose and ribose were both added to the medium [66]. CRE is in the upstream region of the *rbs* operon, and synonymous mutation of two bases in this sequence can also alleviate the CCR effect [84]. In addition, it is possible to circumvent CCR. By establishing a nutrient deficient strain, the co-utilization of glucose and ribose could be achieved. As shown in Figure 1, truncating the pathway of glucose into glycolysis by knocking *zwf* and *pfk* results in cells failing to grow [85]. The addition of ribose enables the cell to produce GAP via PPP, allowing the glycolysis pathway to carry on normally. Meanwhile, R-5-P, produced from ribose, synthesizes amounts of PRPP for NMN production via a very short pathway. Moreover, the new optimization algorithm SIMUP was able to predict nutritionally deficient mutants that can be forced to co-utilize glucose and ribose [86]. The analytical algorithm opt-yield-FBA has been applied to describe the dynamic behaviors of microorganisms in a medium comprising a mixture of carbon sources [87]. With the help of the genome-scale metabolic network model, it is possible

to better regulate the co-utilization of multiple carbon sources in cells and to increase the carbon flux for NMN production.

4.3. Constructing Artificial Microbial Consortia to Reduce the Metabolic Burden

The direct addition of NAM to fermentation broth is expensive, and the synthesis of NAM using glucose as the substrate can reduce the cost. However, the production of both NAM and NMN in a single-cell system increases the metabolic burden of the cell and suppresses cell growth. A division of labor using microbial consortia could spatially separate different synthetic pathways and distribute metabolic loads [88]. The first step is to construct a strain that can produce NAM. NAD^+ is decomposed into NAM by NAD-dependent protein deacetylase (encoded by *yhdZ*) [89]. If a high NAD^+-producing strain is used as the chassis, expressing the *yhdZ* gene in that strain will produce NAM [19]. Based on this, it is possible to use one recombinant *E. coli* to produce NAM and another strain that produces PRPP and absorbs the NAM to produce NMN.

In constructing this system, attention is first paid to the interactions between the different strains and to identifying the optimal inoculation ratio and product induction time. The separation of microorganisms into independent droplets allows for mixed cultures without knowledge of microbial interactions. A recent paper reported on the design of an invert emulsion system by dispersing the culture medium in a mixture of sunflower oil and surfactant PGPR, which could remain stable for at least 24 h [90]. Secondly, it is important to ensure that NAM can be transported smoothly between cell membranes and absorbed by the NMN-producing strains in a timely way. It becomes important to find a more efficient NAM transporter protein or to select and optimize the already reported transporter proteins. Thirdly, microbial species may naturally evolve into distinct phenotypic subpopulations, and it is necessary to prevent low-yield cheaters from becoming the dominant strain of the colony. Quorum sensing creates an environment more conducive to the survival of cooperative individuals and inhibits the growth of cheaters [91].

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