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RESEARCH ARTICLE

De novo biosynthesis and nicotinamide biotransformation of nicotinamide mononucleotide by engineered yeast cells

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Abstract

β-Nicotinamide mononucleotide (NMN) is a precursor of NAD^+ in mammals. Research on NAD^+ has demonstrated its crucial role against aging and disease. Here two technical paths were established for the efficient synthesis of NMN in the yeast *Pichia pastoris*, enabling the production of NMN from the low-cost nicotinamide (NAM) or basic carbon sources. The yeast host was systematically modified to adapt to the biosynthesis and accumulation of NMN. To improve the semi-biosynthesis of NMN from NAM, nicotinamide phosphoribosyltransferases were expressed intracellular to evaluate their catalytic activities. The accumulation of extracellular NMN was further increased by the co-expression of an NMN transporter. Fine-tuning of gene expression level produced 72.1 mg/L NMN from NAM in flasks. To achieve de novo biosynthesis NMN, a heterologous biosynthetic pathway was reassembled in yeast cells. Fine-tuning of pathway nodes by the modification of gene expression level and enhancement of precursor generation allowed efficient NMN synthesis from glucose (36.9 mg/L) or ethanol (57.8 mg/L) in flask. Lastly, cultivations in a bioreactor in fed-batch mode achieved an NMN titre of 1004.6 mg/L at 165 h from 2 g NAM and 868 g glucose and 980.4 mg/L at 91 h from 160 g glucose and 557 g ethanol respectively. This study provides a foundation for future optimization of NMN biosynthesis by engineered yeast cell factories.

INTRODUCTION

The β-nicotinamide mononucleotide (NMN) is a key intermediate of nicotinamide adenine dinucleotide (NAD^+), which is an essential coenzyme for cellular redox reactions in human bodies and other mammals. NAD^+ plays a pivotal role in diverse biological processes, encompassing gene expression, DNA repair, energy generation, etc. It also serves as a regulatory factor in metabolic pathways, specifically the tricarboxylic acid cycle and glycolysis (Yoshino et al., 2021). Recent studies revealed that administering NMN significantly mitigated age-related physiological deterioration in mice and reversed associated mitochondrial dysfunction (Gomes et al., 2013; Mills et al., 2016). Besides, it has been proved to be an efficacious treatment for type II diabetes resulting from a high-fat diet (Yoshino et al., 2011). Recent clinical trials have demonstrated that the addition of NMN can enhance glucose metabolism in human skeletal muscle (Yoshino et al., 2021). Given its profound effects, the NMN has been recognized as a popular raw material for pharmaceutical, food and cosmetic additives. Thus, it is of good value to develop a cost-effective and efficient bioproduction process of NMN that meets biosafety concerns.

The natural biosynthesis of NMN exhibits distinct pathways that can be grouped into: (1) In mammals (Guo et al., 2017; Lin et al., 2016) and some bacteria (Lee et al., 2017), NMN is synthesized by nicotinamide (NAM) phosphoribosyltransferase (Nampt), using NAM and 5-phosphoribosyl 1-pyrophosphate (PRPP) as starting materials; (2) For bacteria lack of Nampt, NMN is predominantly produced via the degradation of NAD^+ (Göckel & Richert, 2015); (3) In bacteria lack of both of the above routes, *Francisella tularensis* as an example, NMN synthase (*Ft*NadE) facilitates the conversion of nicotinic acid mononucleotide (NaMN) to NMN (Sorci et al., 2009); (4) In certain mammals, NMN is synthesized from nicotinamide riboside (NR) via a phosphorylation process catalysed by nicotinamide riboside kinase (Nrk) (Yoshino et al., 2018).

Recently, NMN production by biotransformation from key intermediates has emerged as a preferred strategy as compared to chemical synthesis. Liu et al. (2021) and Maharjan et al. (2021) synthesized NMN of 496.2 and 771.5 mg/L, respectively, from NAM through whole-cell catalysis. Shoji et al. (2021) further identified an NMN transporter (BMpnuC) from *Bacillus mycoides* which improved NMN production to 6.79 g/L from NAM via whole-cell catalysis. Huang et al. (2022) achieved the fermentation semi-synthesis of NMN by metabolic engineering of the semi-biosynthetic pathway in *Escherichia coli*. A nicotinamide phosphoribosyltransferase (Nampt) from *Vibrio bacteriophage* KVP40 (VpNadV) was identified and a remarkable NMN yield of 16.2 g/L was achieved with NAM feedings. Besides, researchers also reported the semi-synthesis of NMN from an alternative substrate nicotinic acid (NA), but it is non-preferred because of the high cost of NA and its low transformation efficiency.

Although high-level NMN production was achieved by semi-biosynthesis in *E. coli*, it poses safety concerns about the potential presence of endotoxins in food applications and entails a strict purification process. Therefore, FDA generally recognized as safe (GRAS) strains could be used as alternative hosts. *Pichia pastoris* (syn. *Komagataella phaffii*), is a GRAS yeast host that has been widely used for the production of drugs and food additives. It has emerged as a versatile industrial strain with the advantages of simple medium, mild post-translational modifications, strong secretion capabilities and good multi-enzyme co-expression capacities in cells (Meehl & Stadheim, 2014; Peña et al., 2018; Safder et al., 2018). In contrast, the reported technical routes relied on semi-biosynthetic production of NMN that needs NAM as the essential substrate. Currently, NAM is mainly produced via chemical synthesis methods, which makes the NMN semi-biosynthesis actually a chem-bio coupling strategy. Furthermore, NMN can be naturally generated via its inherent NAD^+ salvage pathway, and this process can be capitalized upon as an alternative NMN biosynthetic route. The de novo NMN biosynthesis route from aspartate acid (Asp) was explored, and 153 mg/L NMN was obtained from 1.00 g/L-Asp substrate in recent study (Wang et al., 2024). However, the endogenous de novo biosynthesis pathway of NMN is typically initiated from proteinogenic amino acids and tightly regulated in microbial cells (Begley et al., 2001). Most recently, an NAD^+ biosynthetic pathway starting from chorismate has been constructed to achieve high-level NAD(H) in *E. coli* (Ding et al., 2021). Chorismate serves as an exceptional precursor due to its inherent metabolic versatility as a natural branching point for cell metabolism (Dosselaere & Vanderleyden, 2001). This inspires us to construct a heterologous chorismite-quinolinic acid (QA) pathway in yeast cells, followed by QA-NaMN conversion by endogenous QA phosphoribosyl transferase (NadC) (Figure 1), which can be further fused to a downstream NaMN-NMN step catalysed by NMN synthase. As a result, a de novo synthesis of NMN could be realized by engineered cell culture with basic carbon sources.

In this study, we aim to enable semi-biosynthesis and de novo biosynthesis of NMN in engineered *P. pastoris* and evaluate the production efficiencies of the two different technical routes (Figure 1). Systematic metabolic engineering strategies were involved to deplete the sinking branches of NMN and strengthen the generating pathways. Finally, an equivalent titre of NMN (~1000 mg/L) was obtained for either semi-biosynthesis with NAM addition or de novo biosynthesis from basic carbon sources by yeast cells in 3-L bioreactors. Our study provides insightful references for future advancement in the total biosynthesis of NMN.

EXPERIMENTAL PROCEDURES

Strains, media and growth conditions

The plasmids and strains used in this study are listed in Table S1 and Table S2. *Escherichia coli* Top10 was used for plasmids storage and propagation. The expression vectors of pGAPZ B and pUC18 were stored in our laboratory. All chemical reagents were purchased from Tansoole (Shanghai, China). The *E. coli* and yeast strains were cultured as previously described (Ren et al., 2020). For plate culture, agar of 2% [*w*/*v*] was added. First, 20 μL storage strain (−80°C) was inoculated into 3 mL YPD medium in a 20-mL serum bottle and cultured for 36–48 h. Then 100 μL cell broth was inoculated into 10 mL YPD medium and cultured to an OD_{600} of 6.0. The cells were harvested by centrifugation at 5000*g* for 3 min and resuspended in sterile water, and inoculated in fermentation medium at a final density (OD_{600}) of 1.0. The cell culture was carried out in a 6-well deep plate (10 mL medium in each well of 50 mL) for fermentation production of NMN.

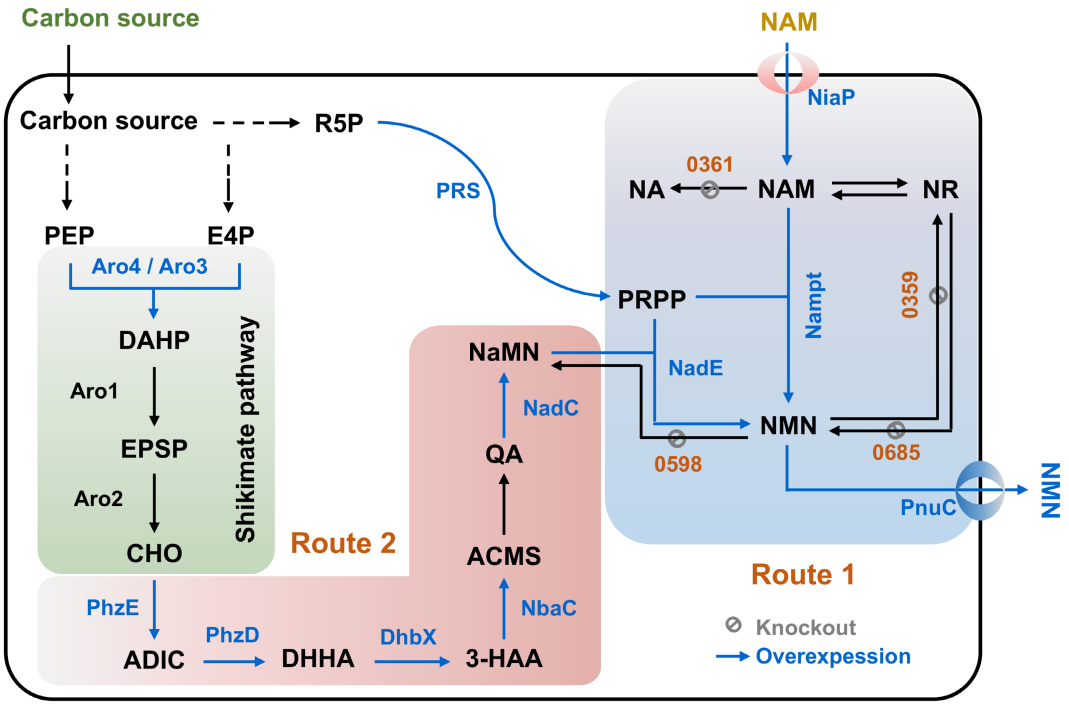


FIGURE 1 Metabolic engineering strategies for the biosynthesis of NMN. Route 1, semi-biosynthesis of NMN by feeding NAM during cell culture; Route 2, de novo biosynthesis of NMN by construction of heterologous NMN synthesis pathway. PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; R5P, ribose-5-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate; CHO, chorismate; AIDC, 2-amino-2-deoxyisochorismate; DHHA, 2,3-dihydro-3-hydroxyanthranilic acid; ACMS, 2-amino-3-carboxymuconate semialdehyde; 3-HAA, 3-hydroxyanthranilic acid; PRPP, 5-phosphoribosyl diphosphate; NaMN, nicotinic acid mononucleotide; QA, quinolinic acid; NA, nicotinic acid; NR, nicotinamide riboside. PhzE, ADIC synthase; PhzD, DHHA synthase; DhbX, DHHA dehydrogenase; NbaC, 3-HAA 3,4-dioxygenase; NadC, QA phosphoribosyl transferase; NadE, NMN synthase; Nampt, nicotinamide phosphoribosyltransferase; NiaP, transporter proteins for NAM; PnuC, transporter proteins for NMN; Aro4 and Aro3, DAHP synthetase; Aro1, pentafunctional aromatic protein; Aro2, chorismate synthase; PRS, PRPP synthase; 0361, nicotinamidase encoded by *PAS_chr3_0361*; 0598, hypothetical protein encoded by *PAS_chr2-1_0598*; 0359, ribosylnicotinamide kinase encoded by *PAS_chr2-2_0359*; 0685, nucleotidase encoded by *PAS_chr2-1_0685*.

The fermentation medium consists of 2% tryptone, 1% yeast extract, 2% glucose, 0.1% $MgSO_4{\cdot}7H_2O$, 0.6% KH_2PO_4 and 1.64% K_2HPO_4, 0.1% sodium citrate and 0.05% NAM (*w*/*v* for all). In addition, glucose was added at a final concentration of 2% [*w*/*v*] every 24 h. For fed-batch fermentation, strains were cultured in 200 mL YPD medium in 1-L shake flasks for seed preparation. After incubation for 12–16 h, the seed culture was transferred into a 3-L fermenter containing 2 L fermentation medium. The pH was maintained at 4.5 during the batch phase and shifted to 7.0 during the fed-batch phase by the automated control of NH_4OH feeding (50%, *v*/*v*). Dissolved oxygen (DO) was maintained at above 30% by automatical control of aeration and agitation.

Construction of plasmids and strains

Abundant of plasmids and recombinant strains were constructed in this work. The construction details of plasmids and strains were described in the file of Supporting information. All the plasmids and strains with corresponding genotype information were listed in Table S1 and Table S2, respectively. Primers used for plasmid construction are listed in Table S3. Gene manipulation was carried out by our previously developed CRISPR-Cas9 editing tools (Liu et al., 2019a). Information on enzymes and transporter proteins for expression in *P. pastoris* are listed in Table S4.

Analytical methods

NMN content in a 6-well deep plate was analysed referring to the reported fluorometric derivatization method (Marinescu et al., 2018). The broth sample of 69 μL was dispensed into 96-well plates, followed by addition of 28 μL acetophenone solution (dissolved in DMSO by 20%, *v*/*v*) and 28 μL KOH solution (2 mol/L). After incubating on ice for 2 min, 125 μL of formic acid solution (88%, *v*/*v*) was added into each well. Subsequently, the plates were subjected to a 10-min incubation at 37°C. The UV emission intensity at 445 nm was then measured using microplate reader (BioTek Synergy 2), with an excitation wavelength of 382 nm. NMN production in a bioreactor was analysed by high-performance liquid chromatography as previously described (Huang

et al., 2022). A reverse-phase column (YMC-Triart C18, 4.6 × 250 mm) was eluted by 30 mM KH_2PO_4: methanol (95:5, *v*/*v*) at a flow rate of 1 mL/min, and the detector wavelength was set at 254 nm. Cell wet weight was analysed according to Xu et al. (2019).

Statistical analysis

The data were obtained from three biological replicates assayed in triplicate and presented as mean ± standard deviation. Experiments were carried out at least twice. The independent samples Student's *t*-test was performed to determine the differences among grouped data. Statistical significance was assessed at $p < 0.05$.

RESULTS

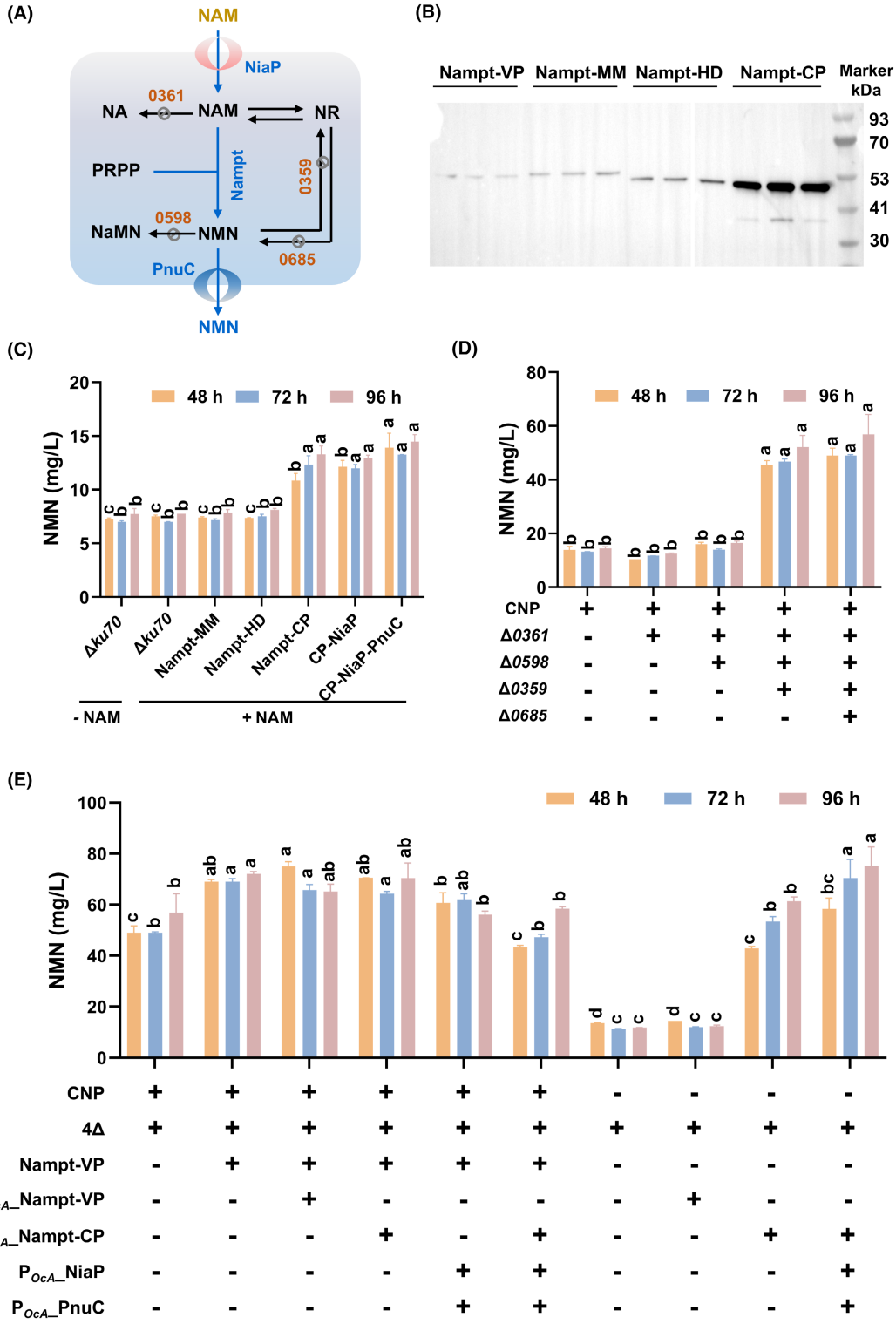
Construction of heterologous pathway for the semi-biosynthesis of NMN

As *P. pastoris* lacks enzymes for NAM-to-NMN transformation in native cells, three different Nampt enzymes derived from mammals and bacteria were first introduced into this host (Figure 2A). Codon-optimized genes encoding Nampt-HD from *Haemophilus ducreyi* (Marinescu et al., 2018), Nampt-MM from *Mus musculus* (Marinescu et al., 2018) and Nampt-CP from *Chitinophaga pinensis* (Shoji et al., 2021) were synthesized and expressed in the Δ*ku70* mutant of *P. pastoris* (partially deficient in non-homologous DNA end-joining, Liu et al., 2019a). Western blot analysis conducted on the cell lysates of each transformant proved successful expression of all the tested Nampt enzymes, albeit with variations in their expression levels (Figure 2B). The NMN production capability of each strain is roughly correlated with its Nampt expression level. The Nampt-CP from *C. pinensis* allowed the highest NMN production (Figure 2C,D), which was also in accordance with the previous study (Shoji et al., 2021). The titre of the Nampt-CP expression strain reached up to 13.3 mg/L at 96 h (fed with 0.5 g/L NAM), which was increased by 71% compared to the parent strain Δ*ku70* (Figure 2C). In addition, cell growth of the Nampt-CP expression strain increased slightly in comparison to the Δ*ku70* (Figure S1). Besides, the parent strain seemed to produce NMN at a very low level by an endogenous de novo synthetic pathway (Δ*ku70* with and without NAM addition in Figure 2C). Overall, the Nampt-CP expression strain with the highest NMN titre was selected for further experiments.

To increase the production of NMN, it is crucial to enhance NAM uptake and NMN effluxion mediated by transporter proteins. Thus codon-optimized genes for effective NAM and NMN transporters, NiaP derived from *Burkholderia cenocepacia* and PnuC from *Bacillus mycoides* (Shoji et al., 2021), were synthesized and co-expressed in the Nampt-CP expression strain, resulting in strains of CP-NiaP (CN) and CP-NiaP-PnuC (CNP), respectively. After 96 h culture, the CN and CNP strains produced 12.9 and 14.5 mg/L NMN, respectively, with 0.5 g/L NAM feeding during culture (Figure 2C). That is, expression of NiaP did not promote the accumulation of NMN in the Nampt-CP strain, while further expression of PnuC (CNP) increased NMN production by 9%. Besides, cell growth of the CNP strain also increased slightly (Figure S1).

Although the CNP strain well transformed NAM to NMN, it was observed that the exogenously added NAM was easily converted into NA (Figure S2). In our engineered cells, NA directly compete with NMN for the substrate of NAM (Figure 2C). Also, microbial cells commonly incorporate complicated metabolic networks to sustain stable levels of intracellular NAD^+/NADH. Therefore, it is necessary to rewire metabolic pathways for the accumulation of NMN. The NAM and NMN metabolic pathway genes in *P. pastoris* were then identified from the KEGG and Genbank databases, that is, *PAS_chr3_0361* (0361), *PAS_chr2-1_0598* (0598), *PAS_chr2-2_0359* (0359) and *PAS_chr2-1_0685* (0685). These genes were knocked out, resulting in strains of CPNΔ*0361*, CPNΔ*0361*Δ*0598*, CPNΔ*0361*Δ*0598*Δ*0359* and CPNΔ*0361*Δ*0598*Δ*0359*Δ*0685* (CPN4Δ). Knockout of these genes did not show growth damage on cells

FIGURE 2 Semi-biosynthesis of NMN by rewiring *P. pastoris* cells with NAM feeding. (A) Basic engineering scheme for NMN semi-biosynthesis; (B) Western blot analysis of Nampt enzymes from different microorganisms expressed in *P. pastoris*. Nampt-MM from *M. musculus*, 56.7 kDa; Nampt-HD from *H. ducreyi*, 55.8 kDa; Nampt-CP from *C. pinensis*, 53.6 kDa; Nampt-VP from *V. bacteriophage*, 55.6 kDa; (C) NMN titres of strains with expression of Nampt enzymes and transporter proteins from different sources; (D) NMN titres of strains constructed from (c) but with NMN / NAM sinking pathways inactivated. (E) NMN titres of strains with improved expression of Nampt enzymes and transporter proteins. Δ*ku70*, deficiency of *KU70* which is a critical gene for non-homologous-end-joining (NHEJ) repairing in *P. pastoris*; Nampt-MM, Nampt-HD, Nampt-CP, Nampt-VP, CP, NiaP and PnuC were generally overexpressed by the constitutive P_{GAP} promoter, unless the stronger P_{OcA} promoter system was used with special illustrations. CP-NiaP, co-expression of Nampt-CP and NiaP; CP-NiaP-PnuC (CNP), co-expression of Nampt-CP, PnuC and NiaP; Δ*0361*, knockout of *PAS_chr3_0361*; Δ*0598*, knockout of *PAS_chr2-1_0598*; Δ*0359*, knockout of *PAS_chr2-2_0359*; Δ*0685*, knockout of *PAS_chr2-1_0685*; 4Δ, knockout of *PAS_chr3_0361, PAS_chr2-1_0598*, *PAS_chr2-2_0359* and *PAS_chr2-1_0685*; Functions of these genes or proteins are described in Figure 1. Statistical significance values of NMN-producing strains at different time intervals are shown; the experiments were performed 3 times as described; the mean values ± SD are presented. Bars with the same letters are not significant ($p < 0.05$) -, not deleted or not expressed; +, deleted or expressed.



(Figure S3). Of note, the titre of CPNΔ*0361*Δ*0598*Δ*0359* and CPN4Δ was achieved as 52.2 and 56.9 mg/L at 96 h, which was 2.6 and 2.9 folds higher than that of the CNP strain respectively (Figure 2D).

To improve the efficiency of NMN synthesis, the expression levels of different genes were further optimized. The native constitutive promoter P_{GAP} and a synthetic constitutive transcriptional device CSAD (7.2 times stronger than P_{GAP} on glucose, Liu et al., 2022) with a hybrid promoter of P_{OcA} were employed to enhance enzyme expression. A Nampt-VP from the *V. bacteriophage* KVP40 well-improved NMN synthesis in *E. coli* (Huang et al., 2022). Thus an Nampt-VP expression cassette under the control of P_{GAP} was introduced into the CPN4Δ to generate a strain of CPNΔ*0361*Δ*0598*Δ*0359*Δ*0685*-VP (CPN4Δ-VP), for

which the NMN titre reached 72.1 mg/L at 96 h and was further increased by 26.7% (Figure 2E). In addition, the expression of Nampt-VP did not affect the cell growth of the recombinant strain (Figure S4).

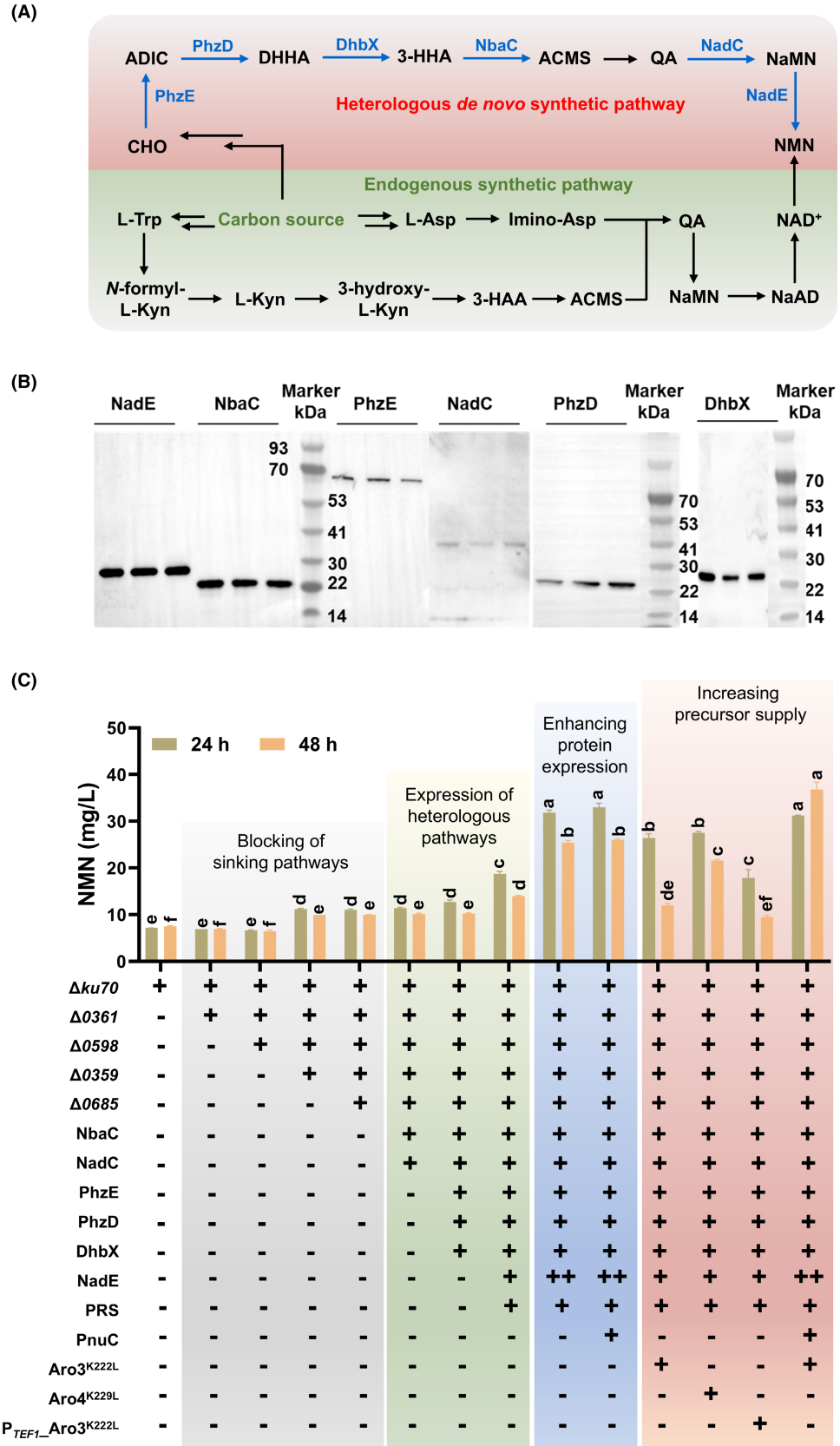
As overexpression of Nampt functioned well, its expression level was further improved by introducing Nampt-CP and Nampt-VP expression cassettes under the control of P_{OcA} into the CPN4Δ-VP strain. Further expression of Nampt-VP showed a minor impact on cell growth, but further expression of Nampt-CP led to biomass decrease (Figure S4). Although efforts were made to promote the expression of Nampt-CP and Nampt-VP further, the NMN synthesis was not improved but weakened as compared to the CPN4Δ-VP strain (Figure 2E). It was speculated that transport of substrates and products might be limited. Therefore, we further enhanced the expression of the transporter proteins of NiaP and PnuC. Unfortunately, the NMN synthesis was still not improved but somehow decreased (Figure 2E). On the contrary, overexpression of NiaP, PnuC and Nampt-CP under the control of P_{OcA} was introduced into Δ*ku70*Δ*0361*Δ*0598*Δ*0359*Δ*0685* to enhance the expression of the functional proteins. The NMN titre of this strain reached up to 75.2 mg/L at 96 h, which was increased by 32.2% in comparison to the CPNΔ*0361*Δ*0598*Δ*0359*Δ*0685* strain (Figure 2E). Nevertheless, the NMN titre was similar to that of the CPN4Δ-VP strain (Figure 2E). In addition, cell growth of this strain became worse (Figure S4) after 72 h culture comparing with the CPN4Δ-VP strain. Besides, overexpression of Nampt-CP or Nampt-VP by the P_{OcA} were tested in the Δ*ku70*Δ*0361*Δ*0598*Δ*0359*Δ*0685*, it seemed only the Nampt-CP positively affected NMN synthesis in contrast to the parent strain (Figure 2E). In total, these results indicated that a moderate expression of the functional proteins was preferred for NMN synthesis and the CPN4Δ-VP was chosen as the final strain for bioreactor fermentation.

Design and construction of the de novo biosynthesis of NMN

The semi-biosynthesis of NMN needs NAM as the direct substrate, which costs much higher than the basic carbon sources. Also, NAM for the industrial use is mainly obtained by chemical steps. To decrease cost and realize full bioproduction, we also attempted to develop a de novo biosynthetic method for NMN production from basic carbon sources. The endogenous de novo synthetic pathway of NMN in *P. pastoris* starts from amino acids and is tightly regulated by the cells. We, therefore, aim to establish a heterologous de novo synthetic pathway that initiates from the abundant chorismate in cells, and achieves efficient synthesis and accumulation of NMN (Figure 3A).

The Δ*ku70*Δ*0361*Δ*0598*Δ*0359*Δ*0685* strain with four genes knocked out, that is, *PAS_chr3_0361*, *PAS_chr2-1_0598*, *PAS_chr2-2_0359* and *PAS_chr2-1_0685* (Figure 2E), was selected for construction of the de novo synthesis host. Deficiency of the four genes did not affect cell growth of the strain (Figure S5), but increased endogenous NMN production from 7.1 to 11.1 mg/L (Figure 3C). As the previous data revealed the better effects of moderate enzyme expression, the P_{GAP} was then selected for gene expression in this construct. A heterologous pathway from chorismate to QA (Ding et al., 2021) was introduced into *P. pastoris* (Figure 3A). The key genes of this pathway, including *PhzE*, *PhzD*, *DhbX* and *NbaC*, were codon optimized and expressed in the Δ*ku70*Δ*0361*Δ*0598*Δ*0359*Δ*0685* strain. Simultaneously, *P. pastoris* NadC was overexpressed to convert QA to NaMN. Also, NMN synthase (*Ft*NadE) from *F. tularensis* was expressed to convert NaMN to NMN (Black et al., 2020; Sorci et al., 2009). As the other precursor, PRPP reacts with NaMN to synthesize NMN. To improve NMN synthesis, it is necessary to increase the supply of PRPP. In a previous report, BaPRS with an L135I mutation ($BaPRS^{L135I}$) from *Bacillus amyloliquefaciens* was introduced to eliminate the negative feedback allosteric inhibition (Zakataeva et al., 2012). Therefore, the $BaPRS^{L135I}$ was further introduced in *P. pastoris* to improve PRPP supply. Western blot analysis conducted on the cell lysate of each enzyme-expression strain confirmed successful expression of the enzyme in this host (Figure 3B). With these efforts, the NMN titre reached up to 18.7 mg/L, which was increased by 163% and 68.5% in comparison to Δ*ku70* and Δ*ku70*Δ*0361*Δ*0598*Δ*0359*Δ*0685*, respectively (Figure 3C). Also, biomass accumulation was not affected compared with the parent strains (Figure S5).

FIGURE 3 De novo biosynthesis of NMN by equipping *P. pastoris* cells with heterologous synthetic pathways. (A) Endogenous and heterologous biosynthesis pathways of NMN in engineered *P. pastoris* cells; (B) Western blot analysis of enzymes in NMN de novo synthetic pathway expressed in *P. pastoris*. NadE, 29 kDa; NbaC, 21 kDa; PhzE, 69 kDa; NadC, 33 kDa; PhzD, 23 kDa; DhbX, 27 kDa; (C) Improved NMN production systematic metabolic engineering strategy. Δ*ku70*, Δ*0361*, Δ*0598*, Δ*0359* and Δ*0685* were the same as that in Figure 2. PhzE, PhzD, DhbX, NbaC, NadC, NadE, Nampt, PnuC, Aro4, $Aro3^{K222L}$ and PRS were expressed by the constitutive P_{GAP} promoter except for the expression of an additional $Aro3^{K222L}$ illustrated specially. Functions of these genes or proteins are described in Figure 1. Other metabolites were with the same full name as illustrated in Figure 1. Statistical significance values of NMN-producing strains at different time intervals are shown; the experiments were performed 3 times as described; the mean values ± SD are presented. Bars with the same letters are not significant ($p < 0.05$). -, not deleted or not expressed; +, deleted or expressed. L-Try, L-tryptophan, L-Asp, L-aspartate; Imino-Asp, iminoaspartate; *N*-formyl-L-Kyn, *N*-formyl-L-kynurenine; L-Kyn, L-kynurenine; 3-hydroxy-L-Kyn, 3-hydroxy-L-kynurenine; NaAD, deamido-NAD.



To improve the efficiency of NMN synthesis, one more copy of NadE was overexpressed. It led to an improved NMN titre of 31.9 mg/L (Figure 3C) at 24 h, which was 70.6% higher in comparison to the previous strain. The results indicated that NadE was a key enzyme and its high-level expression was important

for the NMN synthesis. Moreover, the transport protein PnuC was subsequently co-expressed. However, the NMN titre was not increased further (Figure 3C), which differed from that in the semi-synthesis case (Figure 2E). Then the precursor supply pathway of chorismate was enhanced. Previous reports documented that the overexpression of DAHP synthase and feedback-insensitive mutants of chorismate mutase Aro4^{K229L} increased the yield of derivatives in baker's yeast (Liu et al., 2019b). Moreover, the mutant form of Aro4's isoenzyme Aro3^{K222L} also benefited the synthesis of the downstream products (Liu et al., 2018). These studies provide a good reference for the optimization of the chorismate synthetic pathway. The Aro3^{K222L} was expressed under the control of two constitutive promoters of P_{GAP} and P_{TEF1}. The titre of NMN reached 26.4 mg/L and 17.9 mg/L, respectively (Figure 3C). In contrast, expression of Aro4^{K229L} was also beneficial for the synthesis of NMN and the titre of NMN reached 27.5 mg/L, which was similar to that of the Aro3^{K222L} expression strain. Finally, the Aro3^{K222L} was expressed in the constructed NMN high production strain, and the obtained construct (Δ*0361*Δ*0598*Δ*0359*Δ*0685*-NbaC-NadC-PhzE-PhzD-DhbX-2NadE-PRS-PnuC-Aro3^{K222L}, 4ΔNNPPD2NPPA) produced NMN of 36.9 mg/L at 48 h. It represented 4.9 times higher than that of the Δ*ku70* strain without any NMN pathway modification (Figure 3C). Moreover, ethanol serves as a preferable carbon source to glucose. It promoted NMN production to 57.9 mg/L, which was 60% higher than on glucose (Figure S6).

NMN production by semi-synthesis and de novo biosynthesis in bioreactor

To further evaluate the NMN-producing performance, the semi-biosynthesis strain CPN4Δ-VP and the do novo biosynthesis strain 4ΔNNPPD2NPPA were cultured in a 3-L bioreactor with a fed-batch strategy, respectively.

For the NMN semi-biosynthesis by CPN4Δ-VP, pH was controlled at ~4.5 during batch phase and adjusted to 7.0 during the fed-batch phase subsequently. It was cultured with 40 g/L glucose in the batch culture, and the glucose was fed at a rate of 7.5 g/L/h after the batch culture until the wet cell weight reached ~200 g/L. Then the feeding rate of glucose was reduced to 2.5 g/L/h and NAM was added as 2 g/L in the broth. As a result, NMN gradually accumulated until 165 h, which finally produced 1004.6 mg/L NMN by consuming about 1 g/L NAM and 868 g glucose (Figure 4A).

For the NMN *do novo* biosynthesis by 4ΔNNPPD2NPPA, pH control was kept the same as that in the semi-synthesis culture. It was cultured with 40 g/L glucose in the batch culture, the glucose was fed at a rate of 7.5 g/L/h after the batch culture until wet cell weight reached ~200 g/L. Then the feedstock medium was gradually changed to ethanol and kept at a feeding rate of 4.5 g/L/h thereafter. NMN gradually accumulated until 91 h, which finally produced 980.4 mg/L NMN from 160 g glucose and 557 g ethanol (Figure 4B). At the same time, 120 mg/L NAM was synthesized as a major byproduct. As the price of ethanol lower than glucose and NAM (NAM: ~20,000 $/ton; ethanol: 250–350 $/ton; glucose: 300–400 $/ton, Huang et al., 2022; Sun et al., 2024), the *do novo* biosynthesis of NMN could be a promising technical path in comparison to the semi-biosynthesis of NMN.

DISCUSSION

NMN, a pivotal compound renowned for its physiological significance in furnishing cells in mammals with NAD^+, has garnered widespread recognition, resulting

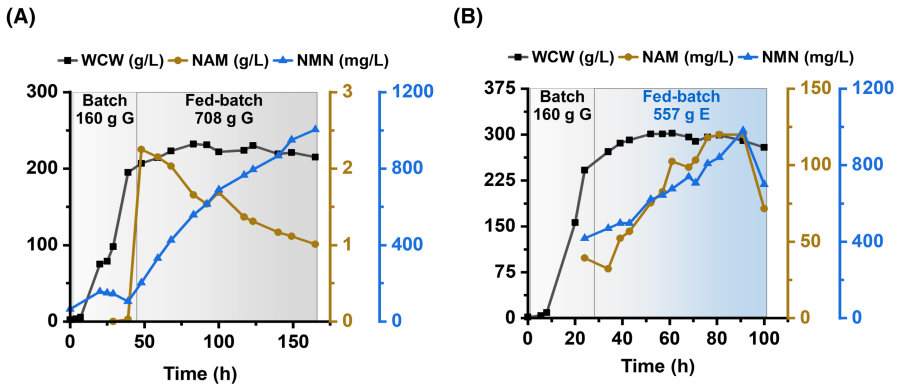


FIGURE 4 Production of NMN by *P. pastoris* in a 3-L bioreactor. (A) Semi-biosynthesis of NMN by the CPN4Δ-VP strain with NAM feeding; (B) De novo biosynthesis of NMN by the 4ΔNNPPD2NPPA strain from basic carbon sources of glucose and ethanol. The broth pH was controlled at 4.5 in the batch phase and adjusted to 7.0 in the fed-batch phase subsequently. E, ethanol; G, glucose; WCW, wet cell weight.

in the swift commercialization of NMN-based products (Yoshino et al., 2021). This study develops two promising strategies, that is, semi-biosynthesis and de novo biosynthesis, for the production of NMN via one-step living cell fermentation by engineered *P. pastoris*. Systematic metabolic engineering methods were performed on *P. pastoris* for efficient NMN biosynthesis, including block of branch pathways, expression and evaluation of functional enzymes and transporters, construction of heterologous pathways, expression balancing of the synthetic pathway, etc. With these efforts, a NMN titre of ~1000 mg/L was achieved for either semi-biosynthesis with NAM addition or de novo biosynthesis from basic carbon sources in bioreactor fermentation.

Enzyme or whole-cell catalysis is currently a preeminent method for the biosynthesis of NMN, yet the process is inherently intricate and entails considerable costs (Shoji et al., 2021). Most recently, an NAM feeding based fermentation method produced high-level NMN with a simple process, high productivity and high conversion rate was achieved in *E. coli* (Huang et al., 2022), which opened up a new avenue for the semi-biosynthesis of NMN and its derivatives. Nevertheless, the producing host *E. coli* was not a preferable strain applied in food areas. In the present study, a similar semi-biosynthesis method of NMN was achieved in a safe (GRAS) strain *P. pastoris* with a simple process and low-cost substrates. More importantly, we also realized a new technical path of de novo biosynthesis of NMN in an engineered *P. pastoris* strain by fermentation from basic carbon sources like glucose and ethanol. The de novo biosynthesis reached a comparable titre to the semi-biosynthesis by the yeast host but without any NAM feeding and in a much shorter time. Generally, it offers new enlightenment for future improvement in the full biosynthesis of NMN by safe microbial cell factories.

As was reported, Nampts from different sources well supported the construction of *E. coli* strains for the high-level semi-synthesis production of NMN. This inspired us to adapt various Nampts with the yeast host for the efficient conversion of NAM into NMN in this work. The Nampt-CP from *C. pinensis* was well expressed and facilitated NMN production, which was also consistent with the results in other hosts in the previous study (Shoji et al., 2021). However, the Nampt-VP from the *V. bacteriophage* KVP40 that allowed the reported highest level of NMN production (16.2 g/L, Huang et al., 2022) in *E. coli* functioned in *P. pastoris* as well, that a strong promoter P_{OcA} was used for protein expression. It seemed that the Nampt-VP could not adapt well to this yeast host, despite codon-optimized and expression improvements were performed. The translation and folding of this enzyme might be incorrect when expressed in *P. pastoris*. Further enzyme engineering endeavours should be involved to further improve its activity in *P. pastoris*, in view of its impressive enzymatic activity and catalytic efficiency in *E. coli*.

Intracellular accumulation of NMN in cells may hinder physiological processes. Shoji et al. (2021) highly increased extracellular NMN accumulation to 6.79 g/L by introducing an NMN transporter PnuC from *B. mycoides* in engineered *E. coli*. Although overexpression of this PnuC in *P. pastoris* promoted NMN production as well, the improvement effects were somehow limited. In-depth exploration of more efficient transporters will be a useful strategy to further improve NMN biosynthesis. Nonetheless, 1004.6 mg/L NMN was finally obtained at the bioreactor level by semi-biosynthesis strategy, which represents the highest yield of NMN from yeast. Future cell rewiring and culture optimization should be involved to continuously promote its production capacity as it still falls far behind compared with the achievements in *E. coli*.

In contrast, the de novo biosynthesis via a hybrid pathway by yeast fermentation from basic carbon sources represents an attractive path for NMN production. By reassembly of heterologous chorismite-to-NMN pathway, block of branch pathway and improvement of metabolic precursor accumulation, a comparable titre (980.4 mg/L) to semi-biosynthetic production was achieved in bioreactor level but in a much shorter time. Moreover, ethanol serves as a preferable carbon source to glucose. The reasons may lie in the fact that the metabolic pathway of ethanol is inherently simpler than that of glucose, and ethanol exhibits a superior energy density in comparison to glucose, thereby providing more energy for the product synthesis (Sun et al., 2024). Deep metabolic rewiring and bioprocess optimization should be conducted to enhance the production further in future. The endogenous de novo biosynthesis pathway of NMN in *E. coli* from aspartate was improved and 153 mg/L NMN was obtained in a recent study (Wang et al., 2024). However, it still requires the additional supplementation of aspartate as a substrate. Generally, our study reports the first case of the construction of a de novo synthetic pathway for the production of NMN by yeast fermentation from basic carbon sources. It shows the highest yield of NMN by de novo synthesis from basic carbon sources yet reported. It also represents a new production mode of NMN compared with the current strategies, exhibiting good potential for possible industrial use in the future.

AUTHOR CONTRIBUTIONS

Yanna Ren: Writing – original draft; formal analysis; data curation; methodology. **Bei Han:** Data curation; formal analysis. **Shijie Wang:** Data curation; formal analysis. **Xingbin Wang:** Formal analysis. **Qi Liu:** Funding acquisition. **Menghao Cai:** Conceptualization; data curation; formal analysis;

funding acquisition; project administration; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the conclusions of this article will be made available without undue reservation by the authors.

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SUPPORTING INFORMATION

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