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Review

Use of Nicotinamide Mononucleotide as Non-Natural Cofactor

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Abstract: Nicotinamide mononucleotide (NMN) has emerged as a promising non-natural cofactor with significant potential to transform biocatalysis, synthetic biology, and therapeutic applications. By modulating NAD^+ metabolism, NMN offers unique advantages in enzymatic reactions, metabolic engineering, and regenerative medicine. This review provides a comprehensive analysis of NMN's biochemical properties, mechanisms of action, and diverse applications. Emphasis is placed on its role in addressing challenges in multi-enzyme cascades, biofuel production, and the synthesis of high-value chemicals. The paper also highlights critical research gaps, including the need for scalable NMN synthesis methods, improved integration into enzymatic systems, and comprehensive toxicity studies for therapeutic use. Emerging technologies such as AI-driven enzyme design and CRISPR-based genome engineering are discussed as transformative tools for optimizing NMN-dependent pathways. Furthermore, the synergistic potential of NMN with synthetic biology innovations, such as cell-free systems and dynamic regulatory networks, is explored, paving the way for precise and modular biotechnological solutions. Looking forward, NMN's versatility as a cofactor positions it as a pivotal tool in advancing sustainable bioprocessing and precision medicine. Addressing current limitations through interdisciplinary approaches will enable NMN to redefine the boundaries of metabolic engineering and therapeutic innovation. This review serves as a roadmap for leveraging NMN's potential across diverse scientific and industrial domains.

Keywords: nicotinamide mononucleotide; non-natural cofactors; NAD^+ metabolism; biocatalysis; synthetic biology; metabolic engineering; precision medicine

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1. Introduction

Nicotinamide mononucleotide (NMN) is a nucleotide composed of nicotinamide, ribose, and a phosphate group that serves as an important precursor in the manufacture of nicotinamide adenine dinucleotide (NAD^+). NAD^+ is a coenzyme found in all living cells that is required for several metabolic activities such as oxidative phosphorylation, citric acid cycle, and glycolysis [1]. Through the activity of the nicotinamide phosphoribosyl transferase (NAMPT) enzyme, NMN is produced from nicotinamide via the NAD^+ salvage pathway. Due to the constant consumption and regeneration of NAD^+ through metabolic processes, this route is essential for preserving NAD^+ levels in cells [2].

The biological significance of NMN stems from its role in restoring NAD^+ levels, which might deplete owing to age or stress. An adequate quantity of NAD^+ is required for cellular energy production, DNA repair, and modulation of cellular responses to oxidative stress [3]. As a result, NMN supplementation has been examined for its ability to increases NAD^+ production, promising therapeutic advantages for age-related illnesses, metabolic disorders, and certain neurodegenerative diseases [4].

Non-natural cofactors are molecules that are either modified chemically or synthetically engineered molecules and introduced into enzyme systems to perform similar actions like natural cofactors, but with improved or unique features [5]. Unlike their natural substitutes, which are constrained by evolutionary limitations, non-natural cofactors can be designed to increase reaction rates, broaden substrate ranges, and introduce novel catalytic activities. This provides more versatility while designing biocatalytic processes, particularly in synthetic biology and metabolic engineering, where natural cofactors may not fulfill certain needs [6].

NMN is an important precursor in NAD^+ production, as well as in signaling pathways, redox homeostasis, and energy metabolism that are required for cellular function [7]. Its role in sirtuin activation, DNA repair processes, and age-related pathways emphasizes its physiological importance and possible therapeutic applications [8]. Here, we delve into the molecular basis of these functions, supported by detailed insights into NAD^+ biosynthesis.

Sirtuins are a group of NAD^+-dependent deacetylases that control important processes such cellular stress response, gene expression, and mitochondrial function. Their activity is directly proportional to NAD^+ levels, making NMN an essential component for sustaining their functionality [9]. Sirtuins deplete NAD^+ by removing acetyl groups from lysine residues on histone and non-histone proteins. This process produces nicotinamide, O-acetyl-ADP-ribose, and a deacetylated substrate [10]. NAD^+ depletion caused by aging or metabolic stress impairs sirtuin activity, which has been linked to age-related illnesses [11]. NMN restores intracellular NAD^+ pools, which increases sirtuin activity [12]. According to studies, NMN supplementation improves SIRT1 activity, hence enhances oxidative stress resistance, mitochondrial biogenesis, and insulin sensitivity [13]. As an example, in murine models of aging, NMN treatment restored SIRT1 activity, enhancing cognitive function and reversing vascular aging [14]. Promising solutions for neurodegeneration, metabolic diseases, and cardiovascular diseases are provided by the NMN–sirtuin axis [15]. Increasing sirtuin activity by supplementing with NMN may prolong life and reduce cellular senescence [16].

For genomic stability and cellular survival, DNA integrity is essential [17]. By sustaining NAD^+ levels, which are critical for the activity of poly (ADP-ribose) polymerases (PARPs), NMN plays a vital role in DNA repair [18]. Poly (ADP-ribose) (PAR) chains are synthesized by PARPs using NAD^+ as a substrate when they detect breaks in DNA strands [19]. Repair enzymes are drawn to the injured spot by these PAR chains. This process is hampered by NAD^+ depletion, which results in the accumulation of DNA damage [20]. NAD^+ production is supported by NMN, which ensures PARP function and effective DNA repair. Preclinical research shows that NMN supplementation increases PARP activity, which lowers the buildup of DNA damage in aging tissues and cells [21]. Moreover, NMN's radioprotective potential has been demonstrated by its ability to shield animal models from radiation-induced DNA damage. In oncology, where preserving genomic stability is essential, NMN's function in DNA repair makes it a promising therapeutic agent. Additionally, it has the potential to lessen the aging-related decline in DNA repair ability, which is a major contributing cause to aging and age-related illnesses [13].

Aging is an intricate process defined by molecular and cellular damage buildup, resulting in functional decline and higher disease vulnerability [22]. NMN has an important role in preventing aging-related processes by influencing NAD^+-dependent pathways. Mitochondrial dysfunction is a defining feature of aging. By raising NAD^+ levels, NMN supplementation activates SIRT3 and SIRT4, which control mitochondrial proteins associated with energy production and the detoxification of reactive oxygen species (ROS) [23]. By preventing pro-inflammatory pathways like NF-κB from being activated, NMN regulates inflammatory responses. Also by stimulating sirtuin-mediated resistance to stress and

genomic stability, it decreases cellular senescence [24]. Research on animals has consistently demonstrated that NMN hinders the physiological decline associated with aging [25]. As an illustration of NMN's ability to support healthy aging, mice treated with it show increased muscle strength, improved insulin sensitivity, and decreased inflammatory markers [23].

The structural resemblance of NMN to NAD^+ makes it a particularly interesting non-natural cofactor since it can interact with a wide range of enzymes [26]. NMN, on the other hand, has special qualities including modified reactivity and enhanced stability that make it a viable option for usage in artificial pathways or designed enzymes. It is a useful tool in both industry and research because of its capacity to increase enzyme activity in non-native environments, which suggests the possibility of optimizing biocatalytic processes to find novel therapeutic uses [27].

The purpose of this article is to investigate the new function of NMN as a non-natural cofactor and its possible uses in synthetic biology and biocatalysis. Although NMN's function in NAD^+ production has previously been investigated, more recent research has shown how useful it is for boosting enzymatic activity and stability in non-native systems [28]. A thorough summary of the most recent studies on NMN's incorporation into synthetic metabolic pathways, its effects on enzyme activity, and its potential to enhance biocatalytic processes is what this review seeks to deliver (Figure 1). The review will also highlight the difficulties in employing NMN as a non-natural cofactor and suggest future lines of exploration to fully utilize its potential in creating innovative synthetic biology applications and biocatalytic approaches.

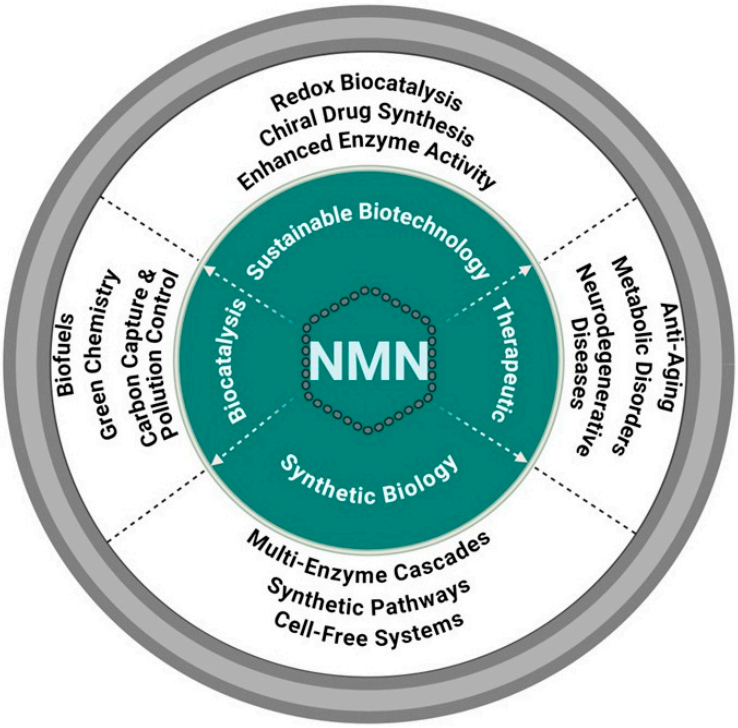

Figure 1. The infographic summary of the review's key findings on nicotinamide mononucleotide (NMN) as a non-natural cofactor.

2. Background

2.1. NMN in Cellular Metabolism: Role in NAD^+ Biosynthesis and Energy Homeostasis

NAD^+ is said to be a prevalent redox cofactor that is involved in metabolic regulation, cellular signaling and the production of energy. There are three pathways on which its biosynthesis depends: the de novo pathway, the Preiss–Handler pathway, and the salvage pathway. All these pathways are served by NMN as a central intermediate (Figure 2) [29].

In the case of the de novo pathway, the synthesis of NAD^+ occurs via a series of enzymatic actions from tryptophan that produce quinolinic acid, which is lastly converted into NMN. Here, oxidation of tryptophan takes place by indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) to generate formylkynurenine. Followed by the conversion of kynurenine by 3-hydroxyanthranilate 3,4-dioxygenase and kynureninase into quinolinic acid [30]. Subsequently, nicotinic acid mononucleotide (NAMN) is formed by the catalyzation of the quinolinic acid phosphoribosyltransferase (QPRT) followed by NMN formation by nicotinamide mononucleotide adenylyltransferase (NMNAT)/nicotinic acid [31]. In the biosynthesis of NAD^+, the de novo pathway highlights the function of NMN as a substrate as well as in high metabolic demand tissue like the liver [32].

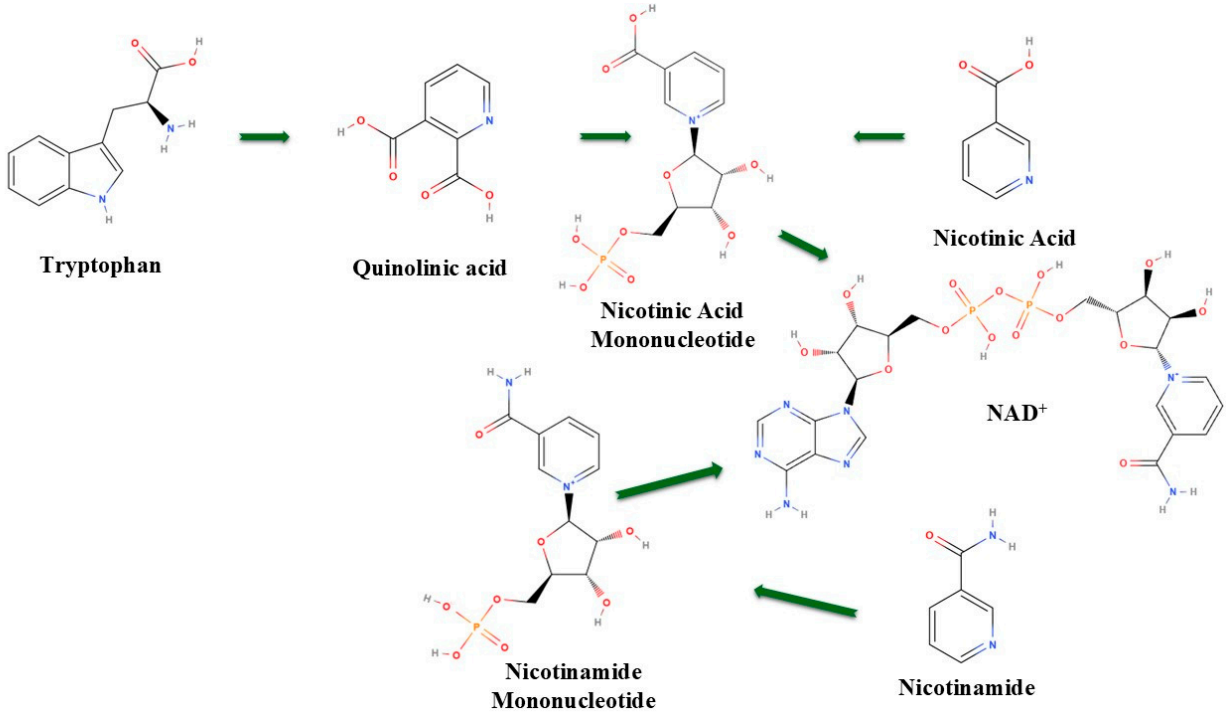

Figure 2. NAD^+ biosynthesis pathways, highlighting NMN's position as a central intermediate.

In the case of the Preiss–Handler pathway, the synthesis of NAD^+ is performed by dietary nicotinic acid (NA). NAMN is formed by the conversion of NA through nicotinic acid phosphoribosyltransferase (NAPRT). Subsequently, the transformation of NAMN by NMNAT leads to the formation of NAD^+ via NMN. The intermediary role of the NMN is highlighted in this pathway, joining the levels of intracellular NAD^+ to dietary sources [33].

In the salvage pathway, the NAD^+ is formed by the recycling of nicotinamide (NAM). This emphasizes the central role of NMN in the regeneration of NAD^+. Here, NMN is produced by the conversion of NAM in the biosynthesis of NAD^+ by a rate-limiting enzyme called nicotinamide phosphoribosyltransferase (NAMPT). Subsequently, adenylation of NMN occurs by NMNAT to generate NAD^+. The majority of tissues use this pathway, which is essential for preserving NAD^+ levels, particularly in aging or stressful situations where the body's need for NAD^+ is higher [34].

2.1.1. NMN's Role in Redox Reactions and ATP Production

NAD^+ acts as a redox cofactor in metabolic pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. NMN supports NAD^+/NADH cycling, enabling electron transfer in oxidation-reduction reactions essential for ATP generation [35]. In glycolysis, NAD^+ accepts electrons to form NADH, facilitating ATP production

via substrate-level phosphorylation. During oxidative phosphorylation, NADH donates electrons to the electron transport chain (ETC), driving proton gradient formation and ATP synthesis [36]. The availability of NMN directly influences NAD^+ levels, impacting cellular energy balance and oxidative metabolism. Declines in NMN during aging or metabolic disorders correlate with reduced ATP production and mitochondrial dysfunction, highlighting NMN's therapeutic potential [37].

2.1.2. Cofactor Functions in Biochemical Reactions

Biocatalysis, the use of natural catalysts such as enzymes in chemical processes, has gained substantial interest for its specificity and environmentally friendly profile. However, many enzymatic reactions are dependent on cofactors, small molecules or ions that enable catalytic activity [38]. Cofactors play a fundamental role in stabilizing reaction intermediates, transferring electrons, and acting as transient carriers of chemical groups [39]. In the context of expanding biocatalysis applications, there is an increasing interest in employing non-natural cofactors to diversify enzyme-catalyzed reactions and improve reaction efficiency [40]. Cofactors are non-protein molecules or ions essential for enzyme activity. They are broadly classified into the following [41]:

- **Inorganic Cofactors**: Include metal ions (e.g., Mg^{2+}, Zn^{2+}) that stabilize enzyme structure or participate in catalysis.
- **Organic Cofactors (Coenzymes)**: Include small organic molecules like NAD^+, FAD, and heme that assist in enzymatic transformations.
- **Prosthetic Groups**: Permanently bound cofactors (e.g., biotin or flavins) integral to enzyme function.

2.1.3. Case Studies of Natural Cofactors

- NAD^+: NAD^+ mediates redox reactions by cycling between oxidized (NAD^+) and reduced (NADH) states. It also serves as a substrate for non-redox enzymes like sirtuins and PARPs. Widely used in biocatalysis for oxidation-reduction reactions. Recent advances include enzymatic regeneration systems for cost-efficient NAD^+ recycling in industrial processes [42].
- Flavin Adenine Dinucleotide (FAD): FAD participates in redox reactions, transferring two electrons and two protons. It acts as a prosthetic group in flavoproteins, such as succinate dehydrogenase in the TCA cycle. FAD-dependent enzymes are exploited in biosensors and biofuel production due to their stability and specificity [43].
- Heme: Heme acts as a prosthetic group in oxygen-binding and electron transfer proteins, such as hemoglobin and cytochromes. Used in synthetic biology to engineer novel oxygen carriers and catalytic systems [44].

2.2. Advances in Non-Natural Cofactors

The engineered molecules having the ability to expand the enzyme's catalytic capability beyond natural reactions are termed non-natural cofactors [45].

- Flavin Analogs: Under non-physiological conditions like organic solvents, the enzyme activity is enhanced by the modified flavins [46].
- Synthetic NAD^+ Mimics: A few of NAD^+'s synthetic analogs, like thionicotinamide adenine dinucleotide (thio-NAD) and nicotinamide riboside (NR), exhibit increased reactivity and selectivity [47].

3. Comparison with NMN

Extensive enzyme engineering is required to produce the mimics of synthetic NAD^+. For the enhancement of efficiency and stability, non-natural cofactors like NR and NMN

are designed [48]. The expansion of reaction capability by the synthetic organometallic cofactors enables the C-H activation, which is a type of complex transformation. The close relation between the natural cofactor NAD^+ and non-natural cofactors like NR and NMN and their engineered properties increases their use in industrial and biotechnological applications [49]. It has also been observed that the functional lifespan of NR and NMN is higher than NAD^+ because of their higher resistance to thermal degradation and hydrolysis [50]. The research has also demonstrated that the improved cellular uptake and bioavailability of NR make it advantageous for its utilization, requiring efficient intracellular delivery. Reduced side reactions and enhanced catalytic turnover were observed with the application of NR and NMN through enzyme engineering [51]. For example, the utilization of NMN in redox biocatalysis to obtain the increased rate of substrate conversion in conditions like high salinity and low pH where the activity of NAD^+ diminishes [52]. In critical stereoselectivity, NR and NMN have been employed for chiral intermediates of drug synthesis like statins and beta-lactam antibiotics [53].

With the introduction of the non-natural reaction mechanism, the enzyme catalytic repertoire was expanded by the organometallic cofactors made up of coordinated ligands and metal centers. The synthetic organometallic cofactors, like iridium and ruthenium complexes, have enabled the traditionally challenging transformation, i.e., C-H activation [54]. Under mild conditions, these cofactors arbitrate the functionalization and the cleavage of the bond, eliminating the requirement for harsh chemical reagents. For the facilitation of the olefin metathesis, cyclopropanation, and cross-coupling reactions, the enzyme system is combined with the organometallic cofactor [55]. For instance, for the catalyzation of the selective C-H hydroxylation, which is a crucial part of the high-value fine chemical production, the engineered enzyme p450 with the incorporation of cofactor ruthenium was used [56]. By integrating natural and artificial cofactors, hybrid enzyme systems enable successive changes, including oxidation followed by organometallic-mediated functionalization, increasing the variety of reactions [57].

Non-natural cofactors allow for tailored biocatalytic processes in industries such as pharmaceuticals and biofuels, optimizing reaction stability and versatility. NMN integrates more seamlessly into biological systems due to its structural similarity to natural cofactors [58]. NMN offers cost advantages due to simplified synthetic pathways. NMN can engage in oxidation–reduction (redox) reactions, enabling electron transfer. NMN shows greater resistance to degradation compared to NAD^+ under certain conditions [59]. Enzymes tailored to recognize NMN can effectively utilize it in reactions. NMN's smaller size allows it to participate in sterically hindered reactions. Its structural characteristics enable precise control over reaction outcomes [60].

4. Relevance to Biotechnology

NMN's integration into synthetic applications bridges fundamental metabolism and industrial innovation. NMN supports engineered pathways for biofuel and bioplastic production. Its role in redox balancing makes it indispensable for optimizing fermentation processes [61]. NMN-dependent systems offer selective redox transformations, with potential applications in pharmaceutical synthesis and green chemistry. NMN supplementation improves NAD^+ levels, with implications for treating metabolic diseases, neurodegeneration, and aging-related dysfunctions [7]. By linking NMN's metabolic roles to its application in synthetic biology and biocatalysis, it emerges as a versatile tool for advancing both fundamental research and industrial processes [62].

5. Use of NMN as a Non-Natural Cofactor

As a non-natural cofactor, the utilization of the NMN shows the shift in paradigm of synthetic biology and biocatalysis. NMN is a promising substitute for traditional cofactors such as NAD^+ because of its distinct functional and structural properties combined with enzyme engineering advancements [26]. We examine NMN's biochemical rationale, incorporation into designed enzymatic systems, and relative benefits over natural cofactors below.

5.1. Biochemical Rationale for NMN as a Non-Natural Cofactor

In the diverse biochemical processes, the NMN is a compelling candidate as a non-natural cofactor because of its functional and structural properties. NMN is made up of a backbone of ribose phosphate linked with the nicotinamide group; when compared with NAD^+, its simple structure reduces the molecular complexity by eliminating the requirement of the adenine nucleotide moiety. The critical binding affinity for various NAD^+-dependent enzymes is retained by its ribose phosphate moiety, which enables the functional integration in catalysis [63].

Unique Properties Compared to NAD^+:

- **Reduced Molecular Complexity**: The integration and the synthesis of the modified enzymes is facilitated by the simple structure of the NMN, which aids in the production cost reduction and its application in the industry [64].
- **Potential Cost Advantages**: Because of fewer processing steps and chemical intermediates, the extensive enzymatic and chemical synthesis of NMN is cheaper [65].
- **Enhanced Stability**: Utilization of the NMN is advantageous for robust cofactors requiring processes because of its greater stability under conditions like extreme pH or high temperature [66].

5.2. Enzyme Engineering for NMN Utilization

It is very important for the potential use of the non-natural cofactor to develop NMN-specific enzymes. This have been made easier by the advancement of the enzyme engineering.

- **Site-Directed Mutagenesis**: For the accommodation of the NMN, mutations targeting the NAD^+-binding pocket of enzymes have been employed. Substitutions that increase affinity for the ribose phosphate moiety while reducing dependence on the adenine group are particularly effective [67].
- **Directed Evolution**: High-throughput screening techniques have been used to evolve enzymes with enhanced NMN specificity. Libraries of mutants are generated and screened for improved catalytic efficiency with NMN [68].
- **Computational Modeling**: In silico docking and molecular dynamics simulations have provided insights into NMN binding, guiding rational design strategies for enzyme modification [69].

A notable study engineered a lactate dehydrogenase variant that utilizes NMN as its primary cofactor. The enzyme achieved catalytic efficiencies comparable to the wild-type enzyme using NAD^+, demonstrating the feasibility of NMN substitution [70]. NMN-specific glucose dehydrogenase has been developed for use in biosensors, offering cost-effective alternatives to NAD^+-based systems [71]. Table 1 summarizes key mechanisms by which NMN is integrated into biocatalytic systems, highlighting specific enzymes, approaches, and their advantages. As shown in Table 1, active site modification has been a critical approach for integrating NMN into alcohol dehydrogenase systems, significantly enhancing substrate specificity and catalytic efficiency.

Table 1. Mechanisms of NMN Integration into Biocatalytic Systems.

Mechanism	Description	Example Enzymes	Advantages
Active Site Modification	Redesigning binding sites for NMN compatibility	Alcohol dehydrogenase, glucose dehydrogenase	Enhances substrate specificity and activity
Cascade Reaction Design	Integrating NMN in multi-step enzymatic pathways	Pyruvate decarboxylase with NMN redox pair	Enables sequential reactions, reducing byproducts
Cofactor Mimicry	Designing NMN analogs for novel reactions	Artificial oxidoreductases	Expands the reaction scope of existing enzymes
Cofactor Recycling Systems	Using NMN-specific enzymes for regeneration	Glucose oxidase with NMN	Improves efficiency in redox biocatalysis

5.3. Comparison with Natural Cofactors

The efficacy of NMN as a non-natural cofactor can be assessed by comparing it to natural cofactors like NAD^+, FAD, and ATP in terms of thermodynamic stability, catalytic efficiency, and enzyme specificity (Table 2 [72]).

Table 2. Enhanced Comparative Analysis of NMN, NAD^+, and FAD.

Property	NMN	NAD^+	FAD
Structure	Ribose phosphate backbone, nicotinamide group	Dinucleotide with adenine and nicotinamide	Flavin nucleotide bound to ribitol
Primary Role	Precursor in NAD^+ biosynthesis; supports redox reactions	Central cofactor in energy production and redox balance	Redox cofactor for oxidases and reductases
Thermodynamic Stability	Moderate under physiological conditions	High stability in native systems; sensitive to oxidative stress	High stability in oxidative environments
Catalytic Efficiency	Comparable in engineered systems; emerging potential	High in natural systems	High for multi-electron transfers
Enzyme Compatibility	Requires engineered enzymes, e.g., oxidoreductases	Naturally compatible with dehydrogenases, sirtuins	Used by flavoproteins; requires specific binding sites
Production Costs	Lower due to simpler synthesis processes	Higher due to complex biosynthetic processes	Moderate; established industrial pathways
Biodegradability	High; environmentally sustainable	High; extensively recycled in biological systems	High; stable under various conditions
Engineering Flexibility	Flexible in engineered systems with tailored pathways	Limited to naturally evolved enzyme systems	Moderate; depends on flavoprotein presence
Industrial Applications	Therapeutic use, metabolic engineering, biocatalysis	Core to metabolism and pharmaceuticals	Industrial oxidative reactions, detoxification systems

- **Thermodynamic Stability**: NMN is more resistant to degradation under industrial conditions, providing a significant advantage in large-scale reactions requiring prolonged cofactor activity [72].
- **Catalytic Efficiency**: While NMN's catalytic efficiency is often lower in unmodified enzymes, engineered enzymes have achieved activity levels that rival or exceed those using NAD^+. This has been particularly demonstrated in alcohol dehydrogenase and oxidoreductase systems [73].
- **Unique Reaction Mechanisms**: NMN enables reaction pathways that are not easily accessible with natural cofactors. For example, its simpler structure facilitates the design of biocatalytic systems with altered redox potentials, enabling novel synthetic transformations [74].

5.4. NMN in Reaction Mechanisms Beyond Natural Cofactors

NMN has demonstrated unique applications in reactions that require non-standard redox capabilities or altered substrate specificity [75]:

- **Biocatalytic Systems**: NMN-dependent enzymes have been used to drive asymmetric reductions, enhancing enantioselectivity in pharmaceutical intermediate synthesis [76].
- **Synthetic Pathways**: NMN has enabled the design of engineered metabolic pathways with lower energy demands, improving the yield of target compounds in microbial production systems [77].

6. Applications of NMN in Synthetic Biology and Biotechnology: Metabolic Pathway Integration

NMN, a precursor of nicotinamide adenine dinucleotide (NAD^+), has garnered significant interest as a non-natural cofactor in synthetic biology and metabolic engineering [78]. The integration of NMN into metabolic pathways is enabling enhanced yields of valuable bioproducts and the synthesis of novel compounds [62]. This section examines how NMN is used in synthetic biology, with a focus on case studies that highlight how useful it is for metabolic engineering.

6.1. Enhancing Yield Through NMN-Driven Metabolic Pathway Optimization

For the improvement in biobased chemical production, NMN has been utilized in the engineered metabolic pathway. Like, for the utilization of the molecule's compatibility and stability with the engineered enzymes, the introduction of the NMN-dependent oxidoreductases has been carried out. By keeping off the endogenous regulatory bottlenecks and reducing the metabolic burden, these systems frequently outcompete NAD^+-dependent counterparts [61].

- **Case Study**: It was observed in the study that the production of lactic acid by *Escherichia coli* was improved by 25% with the use of NMN-dependent enzymes which decreased the competition for the native NAD^+ pools. Through the desired pathway, more efficient substrate fluxes and energy utilization were enabled by this optimization [79].

6.1.1. Synthesis of Novel Products Using NMN

NMN has facilitated the biosynthesis of molecules that were previously challenging due to NAD^+ dependency. NMN's modified redox potential and ability to act as a bioorthogonal cofactor allow for enzymatic reactions outside the scope of natural systems [62].

- **Case Study**: Researchers engineered a pathway in *Saccharomyces cerevisiae* to synthesize unnatural alcohol derivatives. By integrating NMN as a cofactor for alcohol dehydrogenases, they achieved the production of high-value compounds such as

branched-chain alcohols, which are valuable in the pharmaceutical and fragrance industries [80].

6.1.2. Case Study: NMN in Multi-Enzyme Cascades

NMN is increasingly used in multi-enzyme cascades to streamline complex bioconversions (Figure 3). In one prominent example, a cascade involving NMN-dependent reductases enabled the biosynthesis of chiral amines from prochiral ketones with high enantioselectivity [81]. The use of NMN reduced the need for external NAD^+ regeneration systems, simplifying the reaction design and increasing overall efficiency [1].

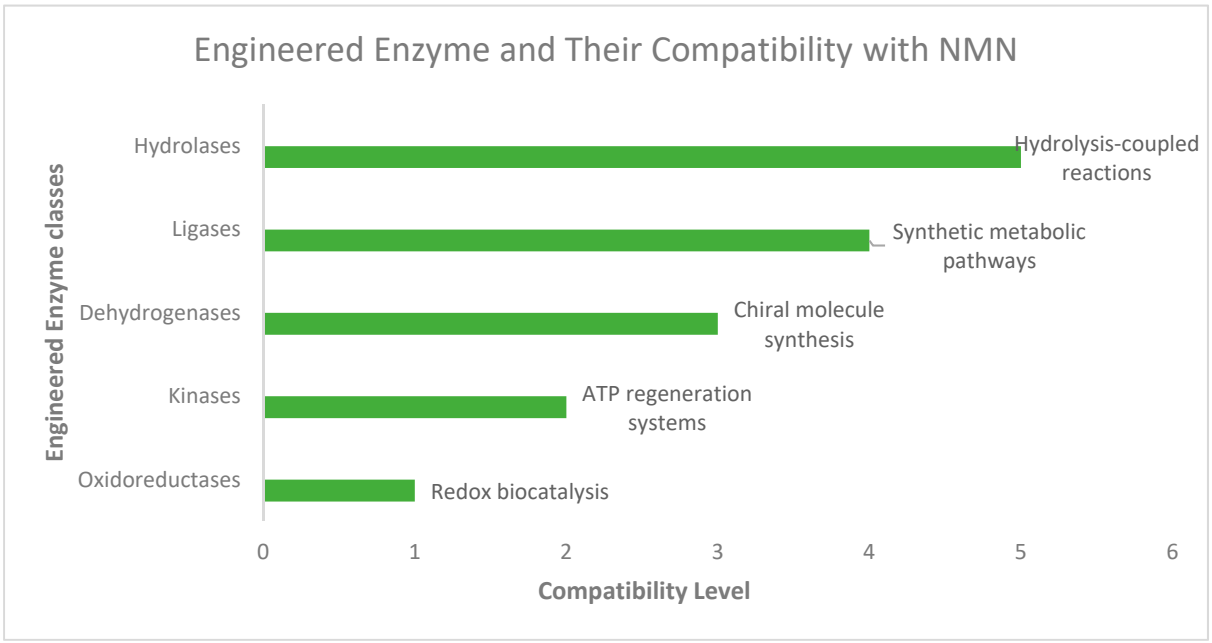

Figure 3. Various enzyme classes and their applications in NMN-based systems, such as ATP regeneration, redox biocatalysis, and synthetic metabolic pathways.

6.2. Biocatalysis Using NMN

Biocatalysis leverages enzymes to facilitate chemical reactions, often with the help of cofactors such as NMN. Recent advancements highlight its potential in chemoenzymatic processes, particularly in areas like pharmaceuticals and biofuels [82]. Below is a high-level overview of NMN applications, with supporting references to recent research [27].

6.2.1. Applications in Pharmaceuticals

- **Chiral Drug Synthesis:** NMN-dependent enzymatic systems have been explored for synthesizing optically pure compounds critical in drug production [83]. For instance, NMN was used in multienzyme cascade systems to efficiently produce biologically active molecules through regioselective and stereoselective transformations. Such processes are particularly beneficial in producing anti-aging compounds and intermediates for NAD^+-boosting drugs [60]. Research has demonstrated the utility of NMN in designing enzyme cascades that improve yields and reduce by-products, critical in pharmaceutical manufacturing [84].
- **Therapeutic Applications:** NMN plays a role in the development of therapies targeting NAD^+ metabolism, especially in diseases where NAD^+ levels are disrupted, such as neurodegenerative disorders and metabolic syndromes. Enzymatic biocatalysis using NMN as a cofactor enables the generation of therapeutic compounds with higher specificity and efficiency [72].

6.2.2. Applications in Biofuels

- **Enhancing Biomass Conversion:** For the optimization of biomass conversion into biofuels, an NMN-driven enzymatic system is being used [85]. This kind of method usually focuses on the enhancement of the enzymatic pathway's efficiency for the production of fermentable sugars from the breakdown of the lignocellulosic materials [86]. NMN's function in recycling and regenerating NAD^+, an essential cofactor in metabolic engineering for the creation of biofuel, is advantageous to this process [86].
- **Sustainable Production Methods:** More sustainable biofuel generation has been made possible by the use of NMN-based biocatalysis in microbial systems. By allowing enzymes to function in milder environments, these systems increase the pace of bioethanol or biodiesel synthesis while lowering energy input and enhancing environmental friendliness [87].

6.2.3. Improved Selectivity

- **Non-natural Amino Acid Synthesis**: With remarkable precision, the synthesis of the synthetic amino acids has been carried out using successfully engineered NMN-dependent enzymes. The selective interaction with the substrate is achieved by the enhanced specificity, which results in the efficient target compound production with fewer side products. This strategy thereby produces extremely selective pharmaceutical and fine chemical production, frequently adapted to have desired qualities for specific purposes [48].
- **Sugar Modifications in Glycosylation Pathways**: To enable the sugar's selective modification, specially for the rare glycosylated product synthesis, the utilization of the NMN is carried out in enzymatic glycosylation pathways [63]. The facilitation of the glycosylated product at specific sites is due to its unique structural characteristics, which is very challenging with natural cofactors. This capacity has resulted in notable progress in the manufacturing of specialty sugars and therapeutic glycoproteins, which are crucial for a range of industrial and pharmaceutical uses [27].

6.2.4. Expanded Substrate Range

- **Redox Reactions in Synthetic Pathways**: NAD^+ cannot support redox reactions because of steric hindrance or binding constraints, while NMN can because of its structural compatibility with a variety of enzymes [52]. The expansion of the substrate range of the engineered dehydrogenases has enabled the employment of NMN, resulting in the catalytic activity with non-native substrates. As a result, synthetic approaches for producing new chemicals have been developed, including sophisticated materials with specialized features and complicated analogs of natural products [88].

6.2.5. Stabilized Enzyme Activity Under Industrial Conditions

- **High-Value Chemical Production**: When compared with the NAD^+, the NMN has shown higher stability under extreme conditions like high temperatures and pH levels. Because of its improved stability, a greater variety of substrates can be used in synthetic biology applications, resulting in higher reaction rates and consistent enzymatic activity [27]. Consequently, NMN enables dependable and effective biocatalytic processes, especially in the biofuel and pharmaceutical sectors where accuracy and resilience are essential [89]. Modern analytical approaches have been developed to help the development of NMN-based systems. Important new information about the biochemical interactions, structural characteristics, and compatibility of NMN with designed enzyme systems has been made possible by these technologies. Table 3 lists important techniques and how they advance NMN research. Mass spectrometry is

one of these that has been very helpful in measuring NMN turnover and tracking how it integrates into multi-enzyme cascades [90]. Likewise, molecular docking has made it possible to rationally design enzymes that are compatible with NMN by forecasting binding affinities and enzyme interactions [91]. Together, these methods tackle important issues in NMN-based biocatalysis, hastening its use in synthetic biology.

Table 3. Analytical Tools for Studying NMN and Related Systems.

Analytical Tool	Purpose	Key Findings Enabled	Example Study
NMR Spectroscopy	Structural analysis of NMN and intermediates	Confirmation of NMN binding modes in enzymes	Structural studies of NMN-glucose oxidase
Mass Spectrometry (MS)	Quantification in complex biological systems	Detection of NMN turnover in cascade reactions	Analysis of NMN in metabolic engineering
Molecular Docking	Predicting NMN-enzyme binding dynamics	Identification of high-affinity enzyme mutants	Enzyme redesign for NMN compatibility
Isothermal Titration Calorimetry	Measuring NMN binding affinities	Thermodynamic insights into NMN interactions	Binding studies of NMN-dependent oxidoreductases

6.3. Therapeutic and Industrial Potential of NMN

6.3.1. Therapeutic Applications

For targeting metabolic disorder, neurodegeneration, and aging, NMN plays a crucial role [92]. The metabolic functions and the cellular repair mechanism are supported by the replenishing of NAD^+ levels [93]. It has also been shown in studies that NMN regulates the circadian rhythms, enhances the functions of mitochondria, and mitigates age-related decline. NMN is also found to be a promising option for reducing inflammation, improving cognitive function, and as a preclinical model for the neurodegenerative diseases like Alzheimer's [94]. Furthermore, NMN is also found to be beneficial in combating obesity-related complications and improving the insulin sensitivity [87].

Being a promising option in drug development, NMN plays a crucial role in combating aging and metabolic dysfunction [95]. It has found that NMN has also restored the declining level of NAD^+ because of stress or aging, resulting in improvement of overall cellular health and DNA repair [78]. Through the restoration of the NAD^+ levels, neuroinflammation and oxidative stress in neurodegenerative disorders is shielded with the help of the NMN [96]. These are found be a crucial factor in Parkinson's disease and Alzheimer's. In animal models using studies, NMN therapy has been demonstrated to lessen neurodegeneration and improve cognitive functions [97].

6.3.2. Industrial Applications

Greeb synthesis of polymers and fine chemicals is made possible in industries with the contribution of NMN in green chemistry [98]. The biocatalysis is supported by NMN's enhanced stability in the harsh conditions. NMN is said to be valuable for the production of the sustainable and green in biocatalysis because of its compatibility with engineered enzymes, redox properties, and its stability [99]. Through an efficient enzymatic pathway, NMN helps in the designing of glycoproteins and advanced polymers, like high value products. It aligns with the goal of global sustainability because of its part in sustainable manufacturing, which makes it integral to industrial biotechnology [100]. Redox balance being the key factor in the butanol and ethanol production, its optimization is achieved by

using the NMN as an alternative cofactor. As a result, the dependance on fossil fuel reduces with an increase in yield [62]. For the production of rare sugars and glycosylated product types, food additives, and pharmaceuticals and fine chemicals, NMN supplements the enzymatic reactions of the synthetic pathways. When compared with the traditional methods of chemical synthesis, this approach minimizes the environmental effects, enhances the selectivity and decreases the energy inputs [83]. With the utilization of carbon-fixed pathways, the NMN-based engineered enzymes are used in the degradation of the pollutant like phenols and hydrocarbons and synthetic carbon-capture systems. These inventions have led the way for the development of sustainable methods for the carbon reduction and waste treatment [101].

7. Challenges and Limitations

For the application of the NMN as a non-natural cofactor, its stability is a key factor under diverse physiochemical conditions [1]. Significant stability is shown by NMN over a wide pH range, with neutral to slightly acidic conditions (pH 6–7.5) showing the best resilience [102]. Due to the hydrolysis of the glycosidic bonds under alkaline (pH > 9) or acidic (pH < 4) environments, there is an acceleration in the degradation of NMN, resulting in the formation of the ribose derivatives and nicotinamide [28]. Therefore, careful selection of the pH is very important for the utilization of NMN in industrial processes or enzymatic reactions [28].

The NMN was found to be minimally degrading up to 37 °C over extended periods of time and relatively stable at moderate temperature in thermal stability studies [103]. However, above 50 °C, the rapid decomposition of the compound takes place, resulting in the limitation for thermophilic system applications [103]. Its operational durability in biocatalytic applications at high temperature can be improved by adding stabilizing agents, such as buffer systems or divalent metal ions, which have been demonstrated to reduce heat degradation [104].

NMN is also found to be compatible with a variety of organic solvents that retain their structural integrity in acetonitrile or dimethyl sulfoxide (DMSO) or other polar aprotic solvents [105]. However, extended exposure to solvent mixes with low dielectric constants or very hydrophobic solvents can cause destabilization, most likely as a result of changes in solvation dynamics and hydrogen bonding. NMN's utilization increases in non-aqueous enzymatic systems by the careful solvent composition optimization, as it is very important for the maintenance of functional integrity [106].

For the therapeutic potential application of NMN as a cofactor in an enzymatic system, its bioavailability is an important factor [107]. It has shown higher solubility in the aqueous solutions, facilitating rapid distribution and adsorption in the biological system [108]. It has been confirmed in vivo studies that via specific mechanisms of transport, including the Slc12a8 transporter in mammalian models and nicotinamide riboside kinases, NMN is readily taken up by the cells [109].

However, the extracellular enzymatic degradation affects the NMN's bioavailability. The formation of nicotinamide or nicotinamide riboside is catalyzed by the CD157 and CD38 ectoenzymes, resulting in a reduction of its target sites' effective concentrations [107]. Methods include NMN chemical modification for the enhancement of encapsulation within protective delivery systems (e.g., nanoparticles or liposomes) or the enzymatic degradation resistance [110]. As shown in Table 4, the compatibility of the enzymes remains a significant problem. The approaches like directed evolution and protein engineering are actively being explored to tackle this problem. In order to maintain effective concentrations, NMN's pharmacokinetic profile also indicates rapid metabolism and clearance, which may call for the use of sustained-release formulations or periodic administration [50]. Additional

research on NMN formulation modification, including prodrugs and coadministration with enzyme inhibitors, could greatly increase the drug's suitability for use in both therapeutic and industrial applications.

Table 4. The following table summarizes the key challenges associated with NMN utilization in biocatalysis and synthetic biology, along with proposed strategies to address these issues.

Challenge	Cause	Proposed Solution
Enzyme Compatibility	Limited natural enzyme affinity	Protein engineering directed evolution
Stability	Sensitive to pH and temperature changes	Encapsulation, chemical modifications
Cost of Production	Complex synthesis processes	Microbial biosynthesis, pathway optimization
Scalability	Limited large-scale synthesis methods	High-throughput enzymatic production

7.1. Approaches to Enhance the Stability of NMN

The inherent sensitivity of NMN to environmental factors such as pH, temperature, and enzymatic degradation poses challenges for its sustained functionality in biotechnological and therapeutic applications. Novel strategies have been explored to improve NMN's stability in order to overcome these drawbacks and assure its efficient use as a non-natural cofactor. The most promising tactics, such as chemical modification, encapsulation, and co-formulation, are addressed here [111].

7.1.1. Encapsulation Strategies

For the protection of the NMN from degradation, encapsulation is found to be a robust method by NMN's isolation from destabilizing environmental factors [112]. Numerous methods of encapsulating have been examined:

- **Liposome-based Encapsulation:** A versatile and biocompatible delivery is possible because of liposomes made up of phospholipid bilayers. It not only protects the oxidative and hydrolytic degradation of the NMN but also improves its bioavailability by stimulating target delivery to the cellular systems [113]. NMN's further stability is also enhanced by the functionalization of the specific antibodies or liposomes with ligands such as polyethylene glycol (PEG) [114].
- **Nanoparticle-based Encapsulation:** The stability of the NMN in harsh conditions is also possible with polymer-based nanoparticles, including those derived from poly (lactic-co-glycolic acid) (PLGA). By enabling regulated release profiles, these nanoparticles sustain steady NMN concentrations for a long period of time [115]. Also, the protection against the oxidative stress is enhanced by the incorporation of the stabilizing agents like antioxidants into the nanoparticle matrix [116].
- **Hydrogel Systems:** This approach is a promising option for the NMN's stability maintenance in an aqueous environment by its immobilization in hydrated work. Hydrogels are perfect for both in vitro and in vivo applications because of their adjustable traits, which provide precise monitoring of the release kinetics and environmental responsiveness of NMN [117].

7.1.2. Chemical Modifications

Chemical derivatization of NMN provides a means to enhance its stability by altering its physicochemical properties [27]:

- **Prodrug Development:** Converting NMN into prodrug forms, such as esterified derivatives, increases its resistance to enzymatic degradation [118]. Prodrugs are

designed to remain stable during storage and administration, undergoing conversion to active NMN only upon reaching specific intracellular environments [119].

- **Cyclization Strategies:** Cyclization of NMN's structure to form stable intermediates can reduce its susceptibility to hydrolysis and oxidative degradation [120]. Cyclized analogs maintain the functional properties of NMN while exhibiting enhanced resistance to environmental stressors [121].
- **Conjugation with Stabilizing Agents:** Covalent attachment of NMN to stabilizing molecules, such as polyethylene glycol (PEGylation), has been shown to improve its solubility, reduce enzymatic degradation, and extend its functional half-life. PEGylation also decreases immunogenicity, which is beneficial for therapeutic applications [122].

7.1.3. Co-Formulation Techniques

Co-formulation of NMN with complementary stabilizing agents is another effective strategy to enhance its robustness:

- **Antioxidant Additives:** Incorporating antioxidants such as ascorbic acid or tocopherol into NMN formulations minimizes oxidative degradation, particularly during storage or in oxidative environments [123,124].
- **Enzyme Inhibitors:** Co-formulation with inhibitors of ectoenzymes (e.g., CD38 or CD157) prevents the enzymatic breakdown of NMN into nicotinamide riboside or nicotinamide, preserving its functional concentration for longer durations [87].
- **Buffer Optimization:** Formulating NMN in buffer systems with optimal pH (typically 6.5–7.5) and ionic strength has been shown to significantly reduce hydrolytic degradation. Specialized buffers, such as those containing zwitterionic compounds, provide additional stabilization [62,125].

7.1.4. Emerging Strategies

Recent advances in molecular engineering and material sciences have introduced novel approaches for NMN stabilization:

- **Metal–Organic Frameworks (MOFs):** MOFs are crystalline porous materials that can encapsulate NMN, offering exceptional protection against thermal and oxidative stress while enabling controlled release in desired environments [126].
- **Enzyme Shielding via Protein Engineering:** Engineering NMN-utilizing enzymes with tailored binding pockets that stabilize NMN during catalytic processes represents an innovative approach to indirectly enhance NMN stability [127].

7.2. Integration of NMN into Existing Biochemical Systems

The use of NMN as a non-natural cofactor presents a transformative opportunity to enhance enzymatic processes and metabolic engineering applications. However, integrating NMN into native systems traditionally reliant on NAD^+ involves significant biochemical challenges. These challenges, including enzymatic incompatibility, altered cofactor specificity, and disruptions to metabolic equilibrium, necessitate innovative strategies to facilitate seamless integration [1].

7.2.1. Biochemical Challenges of Replacing NAD^+ with NMN

Replacing NAD^+ with NMN in native biochemical systems is hindered by intrinsic structural and functional differences [128]. NAD^+, as a canonical redox cofactor, is widely utilized by dehydrogenases, oxidoreductases, and other enzymes, which have evolved highly specific binding pockets tailored to its molecular architecture [129]. NMN, lacking the dinucleotide structure and the adenine moiety of NAD^+, often exhibits reduced

or absent binding affinity to these enzymes. This incompatibility limits NMN's direct substitution in NAD^+-dependent pathways [130].

Additionally, the replacement of NAD^+ with NMN can disrupt tightly regulated intracellular cofactor pools. NAD^+ serves not only as a redox cofactor but also as a substrate for non-redox processes, such as ADP-ribosylation and sirtuin-mediated deacetylation [131]. Substituting NAD^+ with NMN risks perturbing these auxiliary pathways, potentially leading to unintended metabolic consequences. The reduced cellular concentrations of NMN compared to NAD^+ further compound these challenges, making effective integration into native systems difficult without substantial modifications [132].

7.2.2. Strategies to Overcome Enzymatic Incompatibility

To address these challenges, a range of strategies has been developed, focusing on modifying enzymatic systems or engineering pathways that leverage NMN's unique properties while ensuring compatibility with native biochemistry [133].

Engineering Hybrid Enzymatic Systems

- **Rational Enzyme Engineering:** Directed mutagenesis of NAD^+-dependent enzymes can be employed to enhance their affinity for NMN [134]. Structural analyses of enzyme-cofactor interactions enable targeted modifications to the binding pocket, accommodating NMN's smaller size and altered charge distribution [135]. For example, introducing polar or positively charged residues in proximity to NMN's phosphate group can stabilize binding and improve catalytic efficiency [136].
- **De Novo Enzyme Design:** Computational protein engineering allows for the creation of entirely new enzymes optimized for NMN. These de novo enzymes can be tailored for specific catalytic roles, ensuring efficient utilization of NMN in synthetic pathways [137].

Cofactor Recycling Mechanisms

- **Artificial Cofactor Regeneration Systems:** Cofactor recycling is a critical strategy to maintain NMN availability in reactions requiring continuous redox turnover [138]. Enzyme pairs can be engineered to form closed-loop recycling systems, wherein NMN is oxidized and reduced repeatedly within the reaction cycle [139]. For instance, NMN-specific oxidoreductases can be coupled with auxiliary enzymes to regenerate NMN from its oxidized form (NMN^+) using an external electron donor or acceptor [18].
- **Metabolic Pathway Rewiring:** Endogenous pathways can be reprogrammed to prioritize NMN biosynthesis and recycling over NAD^+ production. This involves redirecting flux through enzymes like nicotinamide phosphoribosyltransferase (NAMPT) and suppressing competing pathways that consume NMN [140].

Synthetic Biology Approaches

- **Synthetic Pathway Construction:** Synthetic pathways incorporating NMN can be designed de novo, bypassing the need to modify native enzymes. These pathways utilize NMN-specific enzymes and minimize cross-talk with NAD^+-dependent systems [141]. For example, NMN can be employed in synthetic cascades for producing fine chemicals or biofuels, where its unique redox properties are advantageous [60].
- **Cofactor Mimics and Chimeras:** Hybrid cofactors combining structural features of NAD^+ and NMN can be synthesized to enhance compatibility with native enzymes. These chimeric molecules preserve the redox functionality of NMN while retaining the binding affinity of NAD^+, providing a transitional solution for integration into existing systems [142].

Dynamic Regulatory Systems

- **Allosteric Modulation:** Enzymes can be engineered with allosteric sites responsive to NMN or NMN analogs. This approach enables fine-tuning of enzymatic activity in response to NMN availability, ensuring efficient flux through NMN-dependent reactions [143].
- **Temporal and Spatial Control:** Utilizing inducible systems or compartmentalized expression of NMN-utilizing enzymes can reduce competition with NAD^+ pathways [144]. For example, NMN-specific reactions can be localized within organelles or encapsulated within synthetic microcompartments to isolate them from NAD^+-dependent processes [3].

7.3. Regulatory and Economic Concerns Surrounding NMN Applications

7.3.1. Regulatory Hurdles in Therapeutic Applications

The translation of NMN from preclinical research to therapeutic applications faces significant regulatory challenges [145]. As a bioactive compound intended for human use, NMN must comply with stringent regulatory frameworks established by authorities such as the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA), and similar entities worldwide [18]. Key regulatory hurdles include:

- **Safety and Toxicology Assessment:** While NMN has demonstrated a favorable safety profile in preclinical studies and early clinical trials, comprehensive long-term toxicity and pharmacokinetic studies are required [23]. Potential concerns include off-target effects, accumulation in non-target tissues, and metabolic perturbations, which must be thoroughly investigated to ensure its safety for chronic use [146].
- **Efficacy Validation:** It is a very critical step to show the therapeutic efficacy across a variety of patient population with the disease. Strong clinical trial data, such as randomized, placebo-controlled studies, are required for regulatory approval in order to demonstrate the efficacy and consistency of NMN [147].
- **Classification as a Drug or Nutraceutical:** The NMN's classification varies globally, which influences the market entry and approval pathway [148]. For instance, while NMN is sold as a dietary supplement in certain places, it may require a full drug approval procedure in others, which would result in significant economic and regulatory challenges [149].
- **Manufacturing Standards and Quality Control:** To ensure the consistent stability, purity, and quality of the products, it is mandatory to adhere to Good Manufacturing Practices (GMP). Therefore, it is a challenge to develop the reproducible and scalable production that can match with these standards, specially the stability of the NMN under harsh conditions [150].

7.3.2. Economic Feasibility of Industrial-Scale NMN Production

For NMN's widespread employment in industrial and therapeutic cases, the economic viability of NMN production is also important. Still, multiple challenges limit its feasibility [151]. Initially, the enzymatic and chemical synthesis of NMN asks for high production cost, which is a significant barrier. The high cost is because of its stringent reactions conditions, specialized enzymes, and high purity precursors, which lead to considerably higher per gram cost than other cofactors like nicotinamide riboside or NAD^+ [152].

Secondly, even though the enzymatic synthesis of the NMN provides a scalable and sustainable method of production, it also requires extensive optimization to reduce cost and produce higher yields [153]. For the reduction in production cost, advances in enzyme engineering like enhancement of substrate specificity and catalytic efficiency is very important [154].

Then comes the cost and the availability of the raw materials including the ribose phosphate and nicotinamide, which influences the expenses of overall NMN production directly [83]. Using alternate feedstocks or introducing advances in precursor synthesis to meet supply chain constraints could help ease this difficulty [155].

Lastly, NMN's demand in industrial and therapeutic sectors also plays a crucial role in the determination of the cost and production scale. The expansion of its use in pharmaceuticals, biofuel production, and biocatalysis would drive economies of scale, which will ultimately reduce the per unit cost of the NMN [156].

7.3.3. Addressing the Challenges

Several strategies are important to achieve economic feasibility and address regulatory hurdles. For NMN production, efficacy, and safety, the development of the standardized guidelines can be made by the collaborative efforts of the regulatory agencies, industry, and academia. The production costs can be lowered, and the efficiency can be improved by the use biotechnological methods like continuous bioprocessing and metabolic engineering. International trade and NMN distribution can be facilitated and approval procedures streamlined by establishing worldwide harmonization of rules through a development of consistent standards across regions [157]. The economic and ecological sustainability of NMN manufacturing can also be improved by implementing sustainable practices, such as using bio-based production techniques and reusing cofactor intermediates.

8. Future Directions

Despite significant advancements in understanding NMN as a therapeutic agent and biochemical tool, key gaps remain in the research landscape, particularly in the realms of synthesis, application, and safety evaluation. Addressing these gaps is essential for translating NMN-based systems into scalable and clinically viable solutions.

A critical area of deficiency lies in the large-scale synthesis of NMN. While current enzymatic and chemical synthesis methods have demonstrated feasibility, they often face limitations in yield, scalability, and cost-effectiveness [158]. Enzymatic synthesis, though promising for producing NMN with high purity, is often constrained by cofactor requirements and enzyme instability under industrial conditions [159]. Chemical synthesis, on the other hand, can lead to undesirable by-products, complicating downstream purification processes [160]. Developing novel, efficient, and sustainable synthetic methods—potentially integrating biocatalytic and chemical strategies—is vital for meeting the growing demand for NMN in therapeutic and industrial applications [161].

Another major gap is the integration of NMN into multi-enzyme cascades. Although NMN has been recognized as an effective intermediate or cofactor in synthetic pathways, its utilization in complex enzyme cascades remains underexplored [162]. Challenges include optimizing enzyme compatibility, stabilizing NMN under diverse reaction conditions, and managing its dynamic conversion to other metabolites [163]. Advances in enzyme engineering, pathway design, and reaction modeling are needed to fully exploit NMN in multi-enzyme systems, which hold promise for applications ranging from biomanufacturing to carbon sequestration [164].

A pressing concern is the insufficient toxicity profiling of NMN, particularly for its long-term therapeutic use. While preclinical studies suggest NMN is well-tolerated at low to moderate doses, there is limited data on its effects during prolonged high-dose administration [165]. Risks such as cumulative toxicity, metabolic disruptions, or off-target effects have not been comprehensively assessed. Furthermore, NMN's interactions with existing drugs, as well as its effects on vulnerable populations, such as individuals with metabolic disorders or children, require thorough investigation. Robust preclinical and

clinical studies employing diverse models and longitudinal monitoring are essential to establish a comprehensive safety framework.

Emerging Technologies

The significance and the efficiency of NMN can be optimized by the potential of the revolutionary NMN-dependent systems. This includes the utilization of cutting-edge technologies like synthetic biology platforms, CRISPR-based engineering, and artificial intelligence (AI).

For the use of NMN, the design of the AI-driven enzymes shows a transformative way to tackle the challenges. The best enzyme changes that improve thermal stability, substrate selectivity, and catalytic activity can be found and predicted by researchers using machine learning algorithms that have been trained on large amounts of biochemical data [166]. Generative design models and AlphaFold are AI technologies that facilitate the quick designing of enzymes particularly suited for NMN production or use. Additionally, by simulating multi-enzyme cascades, these options can direct pathway optimization and forecast bottlenecks [167]. In order to scale NMN synthesis and incorporate it into artificial biological systems, this expertise is essential.

Another powerful tool for the development of the NMN-dependent pathway is the CRISPR–Cas genome editing technology [168]. This is used to optimize the metabolic flux for specific uses by fine-tuning the balance between the NAD^+ pools and NMN through NAD^+ biosynthetic enzymes encoding gene modification [77]. Additionally, for the enhancement of the efficiency and the stability of the NMN-related pathways, and for the introduction of synergistic changes, multiplex editing is enabled by the CRISPR. Also, the therapeutic and biotechnological utility of the NMN can be advanced by engineered mammalian cells or microbial strains with improved tolerance to the tailored enzymatic profiles and NMN perturbations [169].

The versatility of the NMN as a cofactor is highlighted by its application in a cell-free synthetic biology system. This system minimizes endogenous pathway interference and enables complete control over NMN-dependent reactions [170]. This method is profitable in the high-value compound synthesis or designing of modular enzymatic cascades. Cell-free systems, when paired with CRISPR-engineered components and AI-guided enzyme design, can facilitate extremely sustainable, scalable, and effective processes [133].

Additional collaborations between NMN and advancements in synthetic biology are anticipated to increase its possible uses. Using NMN-dependent enzymes to construct dynamic regulatory networks, real-time control over metabolic flow could improve production efficiency and flexibility [35]. The opportunities for programmable therapeutics and precise medicine are opened by combining the biosensors with optogenetic systems by allowing the NMN-dependent pathway's external regulation [171]. Additionally, the optimized metabolic circuit development is increased by the NMN-based system's rapid prototyping, which is allowed by the advancement of high-throughput screening platforms and microfluidics [172].

Addressing these research gaps and leveraging emerging technologies will be pivotal in realizing the full potential of NMN-dependent systems. Efforts should focus on conducting comprehensive toxicity studies, optimizing multi-enzyme cascades, and developing scalable synthesis methods to ensure safety and efficacy in therapeutic contexts. The combination of cell-free systems and AI, CRISPR offers unparalleled opportunities for innovation, driving the design of sustainable, adaptable, and efficient NMN-based platforms. By adopting these transformative tools and bridging current knowledge gaps, researchers can unlock new frontiers in biotechnology and therapeutic development, positioning NMN as a cornerstone of future metabolic engineering and synthetic biology applications.

9. Conclusions

The integration of NMN into advanced biotechnological and therapeutic applications underscores its unique role as a non-natural cofactor with transformative potential. Its capacity to modulate NAD^+ metabolism, enhance enzymatic efficiencies, and serve as a versatile intermediate has positioned NMN as a cornerstone in both metabolic research and applied biotechnology. Key findings from this review highlight its pivotal contributions across diverse domains.

In biocatalysis, NMN has demonstrated its utility in enhancing enzymatic reactions, offering novel routes for high-value compound synthesis. However, research gaps in optimizing NMN's integration into multi-enzyme cascades, managing enzyme compatibility, and achieving scalable synthesis remain pressing [62]. Addressing these challenges through advanced enzyme engineering and pathway optimization will be critical for unlocking NMN's full potential in industrial processes.

In synthetic biology, the application of NMN is being revolutionized by emerging technologies. AI-driven enzyme design has enabled the rapid development of NMN-specific enzymes with enhanced stability and efficiency, while CRISPR-based genome engineering has refined metabolic pathways to balance NAD^+ and NMN flux. Cell-free systems, leveraging NMN's unique properties, have facilitated precise, modular, and scalable workflows, paving the way for innovative applications in biomanufacturing and programmable biosystems [173]. These technological synergies provide a strong foundation for further integration of NMN into dynamic and adaptive synthetic platforms.

In the context of therapeutics, NMN's role in mitigating age-related pathologies and enhancing cellular metabolism has shown promise, though critical gaps in toxicity profiling and long-term safety evaluation remain. Comprehensive investigations into dose-dependent and chronic effects, tissue-specific responses, and potential drug interactions are imperative to ensure its clinical viability. Robust preclinical and clinical studies will be essential to establish NMN as a safe and effective therapeutic agent, particularly for metabolic and age-associated disorders [28].

Looking forward, NMN's integration into advanced biotechnology offers exciting opportunities for innovation across fields. By addressing current research limitations and leveraging cutting-edge tools, researchers can unlock its potential to drive advancements in precision medicine, sustainable bioprocessing, and metabolic engineering. NMN's unique properties as a non-natural cofactor, combined with the power of AI, CRISPR, and cell-free platforms, position it as a transformative agent in the future of biotechnology. As the field progresses, NMN is poised to become a linchpin in the development of next-generation therapeutic and biotechnological solutions, redefining the boundaries of what is achievable in synthetic biology and beyond.

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