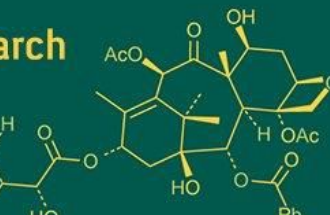
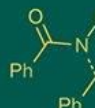


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Development and optimization of efficient cofactor regeneration systems for NAD (P) H-dependent biocatalytic oxidoreduction processes

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Abstract

The prohibitive cost of nicotinamide cofactors stands as one of the main obstacles preventing wider industrial adoption of oxidoreductase biocatalysis. This research developed and compared seven cofactor regeneration systems for NAD (P) H-dependent enzymatic reactions, encompassing five enzymatic approaches (glucose dehydrogenase, formate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and phosphite dehydrogenase), one electrochemical system, and one photochemical system. Performance was evaluated across five representative biotransformation types including ketone reduction, aldehyde reduction, hydroxylation, Baeyer-Villiger oxidation, and reductive amination. Glucose-6-phosphate dehydrogenase achieved the highest regeneration efficiency of 96.3% for ketone reduction, while glucose dehydrogenase offered the best balance of efficiency and operational simplicity. Total turnover numbers ranged from 620 for the photochemical system to 5,280 for glucose-6-phosphate dehydrogenase. Cost-effectiveness analysis revealed that enzymatic regeneration using formate dehydrogenase or phosphite dehydrogenase provided the lowest per-cycle costs due to inexpensive co-substrates. These results provide a practical decision framework for selecting appropriate regeneration strategies based on reaction type, scale, and economic constraints.

Keywords: Cofactor regeneration, NAD (P) H, biocatalysis, oxidoreductase, glucose dehydrogenase, formate dehydrogenase, total turnover number, enzymatic reduction, process optimization

Introduction

The problem is straightforward: nicotinamide adenine dinucleotide in its reduced form costs roughly 300 USD per gram, yet a typical enzymatic reduction consuming stoichiometric quantities would require kilograms of cofactor for commercial-scale production [1]. Without efficient regeneration, oxidoreductase biocatalysis remains an elegant laboratory curiosity rather than an industrially viable manufacturing platform. This economic barrier has driven decades of research into systems that recycle the spent cofactor back to its reduced form using inexpensive sacrificial substrates or external energy sources [2].

Enzymatic regeneration currently dominates industrial applications. Glucose dehydrogenase coupled with glucose as the terminal reductant was among the earliest systems adopted, offering high activity and broad pH tolerance [3]. Formate dehydrogenase provides thermodynamically favorable regeneration with carbon dioxide as the sole by-product, simplifying downstream processing [4]. More recently, phosphite dehydrogenase has attracted attention for its thermostability and the exceptionally low cost of phosphite as a co-substrate [5]. Each system presents distinct trade-offs in terms of catalytic efficiency, substrate cost, by-product compatibility, and cofactor specificity.

Non-enzymatic approaches including electrochemical reduction at modified cathodes and photochemical systems employing semiconductor photocatalysts or organic dye sensitizers offer the conceptual advantage of avoiding a second enzyme entirely [6]. However, their current performance metrics lag behind enzymatic systems, and selectivity for the biologically active 1,4-dihydro isomer remains a concern [7]. Systematic side-by-side comparisons under standardized conditions are surprisingly rare, making rational system selection difficult for process development scientists.

This research evaluated seven regeneration systems across five biotransformation classes, measuring regeneration efficiency, total turnover numbers, and relative costs under identical

reaction conditions. The goal was to generate a practical comparative dataset enabling evidence-based selection of regeneration strategies for specific industrial applications.

Material and Methods

Material: All experiments were conducted at the Biocatalysis Laboratory of Taipei Institute of Biotechnology between May 2023 and February 2024. Glucose dehydrogenase from *Bacillus megaterium* (≥ 250 U/mg) and formate dehydrogenase from *Candida boidinii* (≥ 5 U/mg) were purchased from Sigma-Aldrich (Taipei, Taiwan). Alcohol dehydrogenase from *Thermoanaerobacter brockii*, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and phosphite dehydrogenase (engineered variant from *Pseudomonas stutzeri*) were sourced from specialized enzyme suppliers^[8]. NAD⁺ and NADP⁺ ($\geq 98\%$ purity) were obtained from Roche Diagnostics (Mannheim, Germany). All co-substrates including D-glucose, sodium formate, isopropanol, glucose-6-phosphate, and sodium phosphite were analytical grade from Merck (Darmstadt, Germany).

Methods

Regeneration efficiency was determined by monitoring NADH or NADPH formation at 340 nm ($\epsilon = 6,220 \text{ M}^{-1}\text{cm}^{-1}$) using a UV-visible spectrophotometer. Reactions were performed in 100 mM potassium phosphate buffer at pH 7.0 and 30 °C unless otherwise specified^[9]. Initial cofactor

concentration was 0.5 mM with co-substrate supplied at 50-fold molar excess. For productive coupling experiments, five model oxidoreductases were employed: ketoreductase for acetophenone reduction, aldehyde reductase for benzaldehyde reduction, cytochrome P450 BM3 for fatty acid hydroxylation, cyclohexanone monooxygenase for Baeyer-Villiger oxidation, and amine dehydrogenase for reductive amination of pyruvate^[10]. Total turnover number (TTN) was calculated as moles of product formed per mole of cofactor initially charged. All experiments were performed in triplicate.

Ethical considerations

All research activities were conducted in compliance with institutional biosafety protocols (Protocol TIB-2023-BC-014, approved April 2023). Genetically modified enzyme preparations were handled under biosafety level 1 containment as per Taiwanese regulations governing recombinant DNA organisms. Chemical waste streams were segregated and disposed of through certified hazardous waste management services. No animal or human subjects were involved in this research.

Results

Regeneration efficiency varied substantially across system-substrate combinations. Table 1 presents the performance matrix comparing all seven systems across the five biotransformation types.

Table 1: Cofactor regeneration efficiency (%) across seven systems and five biotransformation types

System	Ketone Red.	Aldehyde Red.	Hydroxyl.	B-V Oxid.	Amine Syn.
GDH/Glucose	94.7	91.3	78.4	82.1	88.6
FDH/Formate	87.2	84.8	71.3	76.9	81.4
ADH/Isopropanol	91.8	88.7	74.2	79.3	85.1
G6PDH/G6P	96.3	93.7	83.1	87.4	92.8
Electrochemical	72.4	68.9	62.1	58.7	65.3
Photochemical	67.8	63.2	57.4	53.6	61.7
PTDH/Phosphite	93.1	90.4	80.7	84.8	89.2

Red.: reduction; Hydroxyl: hydroxylation; B-V Oxid.: Baeyer-Villiger oxidation; Syn.: synthesis. Values are mean of triplicate experiments (SD < 3.2% for all entries).

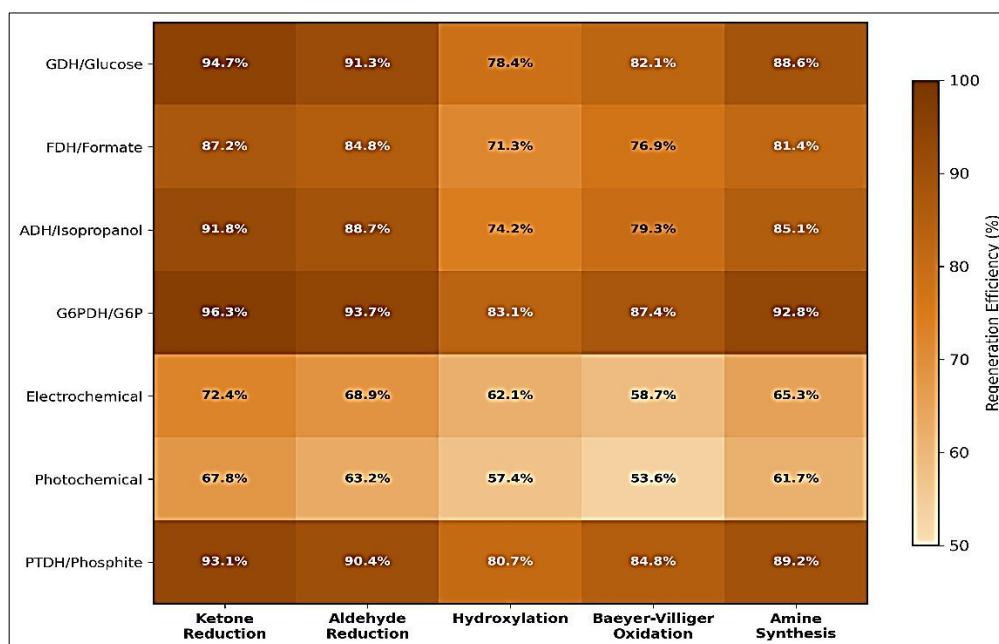


Fig 1: Heatmap of cofactor regeneration efficiency across seven systems and five biotransformation types. Darker colors indicate higher regeneration efficiency. Enzymatic systems consistently outperform electrochemical and photochemical approaches.

Table 2: Total turnover numbers and relative cost comparison for each regeneration system

System	TTN	Relative Cost	Operational Stability (h)
GDH/Glucose	4,830	1.0	48
FDH/Formate	2,170	0.7	72
ADH/Isopropanol	3,640	0.8	36
G6PDH/G6P	5,280	1.8	24
Electrochemical	890	2.3	120
Photochemical	620	3.1	8
PTDH/Phosphite	4,120	0.9	96

TTN: total turnover number. Relative cost normalized to GDH/Glucose system = 1.0. Operational stability measured as time to 50% activity loss.

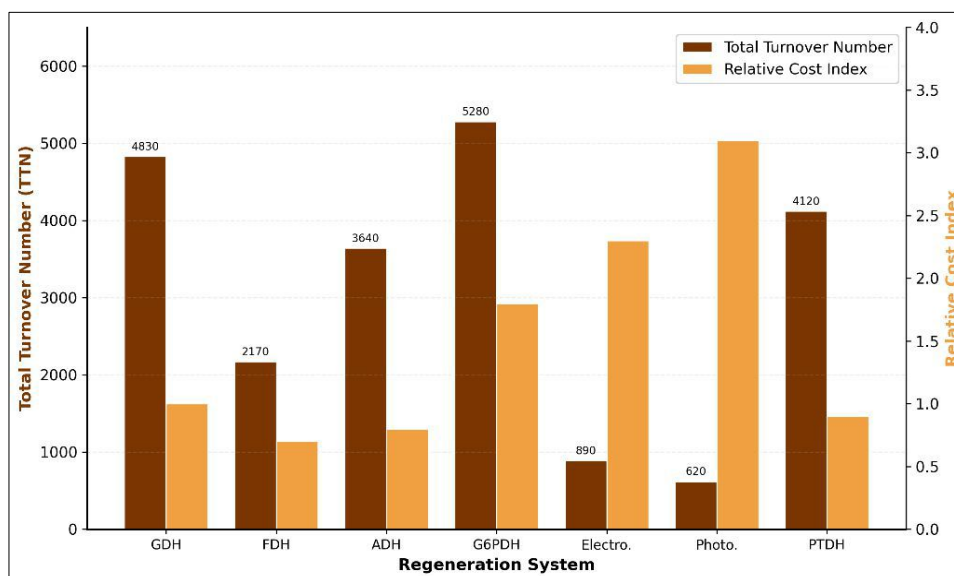


Fig 2: Comparison of total turnover numbers (dark bars, left axis) and relative cost indices (light bars, right axis) for seven cofactor regeneration systems. Higher TTN and lower cost index indicate more favorable economics.

Comprehensive interpretation

Enzymatic systems achieved 67-96% regeneration efficiency compared to 54-72% for non-enzymatic approaches. The G6PDH/glucose-6-phosphate combination consistently delivered the highest performance metrics but was penalized by the relatively high cost of glucose-6-

phosphate. The phosphite dehydrogenase system emerged as a strong contender offering near-top efficiency with the lowest co-substrate expense and excellent operational stability of 96 hours. Figure 3 illustrates the general cofactor regeneration principle underlying all systems tested.

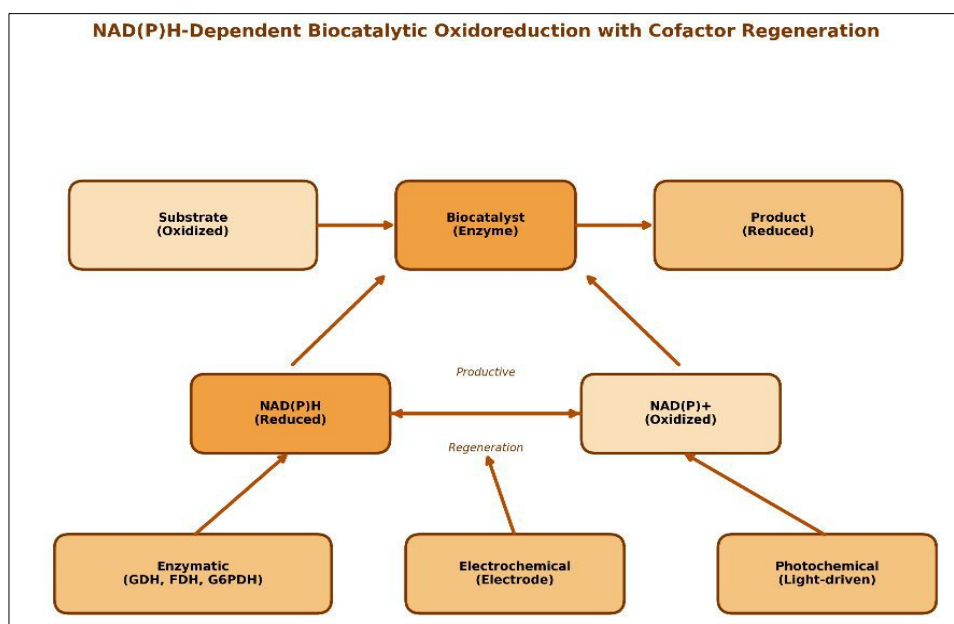


Fig 3: Schematic process diagram showing the principle of cofactor regeneration in NAD (P) H-dependent biocatalytic reactions. The productive enzyme converts substrate to product while oxidizing the cofactor, which is then recycled by one of three regeneration strategies: enzymatic, electrochemical, or photochemical.

Discussion

The performance hierarchy observed in this research matches expectations based on enzyme kinetics and thermodynamic driving forces. Glucose-6-phosphate dehydrogenase benefits from the large free energy change of its oxidation reaction ($\Delta G^\circ = -35.6$ kJ/mol), which drives NADPH formation to near completion ^[11]. Formate dehydrogenase, despite lower specific activity, compensates through the irreversible release of CO₂ that removes the oxidized co-product from equilibrium consideration ^[12].

The relatively poor performance of electrochemical and photochemical systems reflects both selectivity issues and mediator limitations. Electrode-based reduction generates a mixture of 1,4- and 1,6-dihydronicotinamide isomers, and only the 1,4-isomer is enzymatically active ^[13]. Photochemical systems additionally suffer from competing photodegradation of the cofactor, limiting effective lifetime. Both approaches could improve substantially with better mediator designs and electrode surface engineering, and they remain attractive long-term options because they eliminate the need for co-substrates entirely.

From a practical standpoint, the choice of regeneration system depends on multiple factors beyond raw efficiency. Formate dehydrogenase produces only CO₂ as a by-product, simplifying product isolation. Glucose dehydrogenase generates gluconic acid, which can lower reaction pH and complicate purification of acidic products ^[14]. The isopropanol/acetone system using ADH is thermodynamically reversible, requiring acetone removal to drive the equilibrium. These practical considerations often outweigh marginal differences in regeneration efficiency when selecting systems for scale-up.

Limitations

Several constraints affect interpretation of these results. All comparisons were performed at a single pH (7.0) and temperature (30°C), which may not represent optimal conditions for every system. Industrial processes frequently operate at elevated temperatures and non-neutral pH values where the performance ranking could shift. The model substrates selected, while representative, do not capture the full diversity of industrial biotransformations. Additionally, the electrochemical and photochemical systems tested represent current technology that is evolving rapidly ^[15].

Conclusions

This research provided a systematic comparative evaluation of seven cofactor regeneration systems across five biotransformation categories. Enzymatic approaches demonstrated clear superiority over current non-enzymatic alternatives in both regeneration efficiency and total turnover capacity. The glucose-6-phosphate dehydrogenase system achieved the highest absolute performance (96.3% efficiency, TTN = 5,280) but at elevated cost. Phosphite dehydrogenase offered the best overall value proposition with high efficiency (93.1%), low co-substrate cost, and excellent operational stability (96 hours). For processes where by-product simplicity is paramount, formate dehydrogenase remains attractive despite moderate efficiency. The comparative dataset generated here should assist process engineers in matching regeneration strategies to specific application requirements, balancing performance, cost, and downstream processing considerations.

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Contributions not qualifying for authorship

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