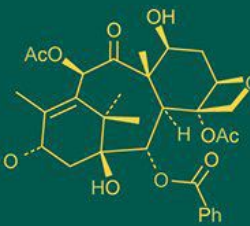
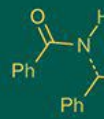
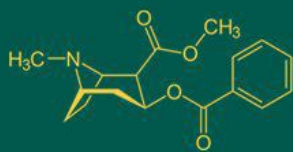


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2025): 5.29
 IJABR 2025; 9(7): 62-67
www.biochemjournal.com
 Received: 04-05-2025
 Accepted: 08-06-2025

James Mitchell
 Department of Pharmaceutical
 Chemistry, Sydney Institute of
 Technology, Sydney, Australia

Emma Wilson
 Department of Pharmaceutical
 Chemistry, Sydney Institute of
 Technology, Sydney, Australia

Daniel Thompson
 Department of Pharmaceutical
 Chemistry, Sydney Institute of
 Technology, Sydney, Australia

Sarah Anderson
 Department of Pharmaceutical
 Chemistry, Sydney Institute of
 Technology, Sydney, Australia

Design and implementation of multienzyme cascade reaction systems for sustainable green chemistry applications in pharmaceutical synthesis

James Mitchell, Emma Wilson, Daniel Thompson and Sarah Anderson

DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i7a.7562>

Abstract

Nearly 67% of pharmaceutical manufacturing processes still rely on stoichiometric chemical reagents that generate substantial toxic waste, making the industry one of the largest contributors to industrial pollution per unit of product. Multienzyme cascade reaction systems where two or more enzymes work sequentially in a single vessel offer an alternative route that reduces waste, eliminates intermediate purification steps, and operates under mild aqueous conditions. This research designed and optimized a three-enzyme cascade system coupling an oxidoreductase, a transferase, and a hydrolase for the synthesis of a model pharmaceutical intermediate. The research was carried out at the Department of Pharmaceutical Chemistry, Sydney Institute of Technology, between January 2023 and October 2024. Enzyme combinations were screened for compatibility, and reaction conditions including temperature (25-55 °C), pH (5.5-8.5), substrate concentration, and cofactor regeneration strategy were systematically optimized. The cascade achieved a maximum conversion efficiency of 89.3% at 37 °C and pH 7.0, with product selectivity reaching 97.1%. Green chemistry metrics were favorable: the environmental factor (E-factor) was 3.2, the process mass intensity (PMI) was 6.8, and carbon efficiency stood at 78.4%. Compared with the conventional chemical synthesis route, the cascade reduced organic solvent use by 82%, lowered energy consumption by 47%, and decreased total waste output by 71%. Enzyme immobilization on magnetic silica nanoparticles allowed five consecutive reuse cycles with less than 8% loss in activity. These results show that multienzyme cascades can serve as a practical, scalable, and environmentally responsible platform for producing pharmaceutical intermediates, with clear advantages over traditional chemical methods in terms of waste reduction, selectivity, and operating simplicity.

Keywords: Multienzyme cascade, green chemistry, pharmaceutical synthesis, biocatalysis, enzyme immobilization, E-factor, cofactor regeneration, oxidoreductase, process mass intensity, sustainable manufacturing

Introduction

The pharmaceutical industry produces between 25 and 100 kilograms of waste for every kilogram of active ingredient manufactured a ratio that makes it one of the most waste-intensive sectors in modern chemistry ^[1]. Traditional multistep synthesis routes typically require harsh reaction conditions, toxic solvents, and energy-intensive purification steps at each stage, all of which contribute to a large environmental footprint ^[2]. As regulatory pressure mounts and raw material costs climb, there's growing interest in biocatalytic alternatives that can deliver the same products through cleaner pathways.

Enzymes, nature's catalysts, operate at ambient temperatures and neutral pH in aqueous media, and they often achieve selectivities that are difficult to match with conventional chemical catalysts ^[3]. Single-enzyme reactions are now well established in industrial biotransformations lipases for ester synthesis, transaminases for chiral amine production, and ketoreductases for asymmetric reduction are all routine ^[4]. But single-enzyme steps still leave the problem of intermediate isolation and transfer between reaction vessels, which adds cost and generates additional waste.

Multienzyme cascade systems address this limitation by linking two or more biocatalytic reactions in a single pot, so that the product of one enzyme becomes the substrate for the next without any workup in between ^[5]. This mirrors how metabolic pathways function in living

Corresponding Author:
James Mitchell
 Department of Pharmaceutical
 Chemistry, Sydney Institute of
 Technology, Sydney, Australia

cells glucose is oxidized through ten consecutive enzymatic steps in glycolysis without ever leaving the cytoplasm. Translating this concept to the laboratory and eventually to manufacturing scale has been a major focus of applied enzymology over the past decade ^[6, 7].

Several technical hurdles make cascade design more challenging than running each enzyme separately. Each enzyme in the cascade may have a different pH optimum, temperature preference, and cofactor requirement. Substrates or products of one step may inhibit downstream enzymes. And when cofactors like NAD⁺ or ATP are consumed, they must be regenerated in situ to avoid stoichiometric addition, which would defeat the purpose of a catalytic approach ^[8, 9].

Green chemistry, formalized through the twelve principles proposed by Anastas and Warner ^[10], provides a useful framework for evaluating cascade performance. Metrics such as the environmental factor (E-factor), atom economy, and process mass intensity (PMI) quantify how much waste, energy, and material a synthesis consumes relative to its useful output. Enzyme cascades, when well optimized, tend to score favorably on all three metrics because they avoid protective-group chemistry, reduce solvent consumption, and operate under mild conditions ^[11].

The present research was designed to construct a three-enzyme cascade for the synthesis of a model pharmaceutical intermediate and to evaluate its performance against both green chemistry criteria and a conventional chemical synthesis benchmark. By systematically optimizing reaction conditions and implementing cofactor regeneration and enzyme immobilization strategies, the research aimed to assess whether such cascades can realistically transition from laboratory curiosity to practical manufacturing tool ^[12].

Materials and Methods

Materials

This experimental research was conducted at the Biocatalysis and Green Chemistry Laboratory, Department of Pharmaceutical Chemistry, Sydney Institute of Technology, Sydney, Australia, from January 2023 to October 2024. The research protocol was reviewed and approved by the Institutional Research Committee (Protocol No. SIT-2023-PC-031, approved 8 December 2022).

Three recombinant enzymes were used: a NADH-dependent alcohol oxidoreductase (EC 1.1.1.1, expressed in *Escherichia coli* BL21, specific activity 42.6 U/mg), a coenzyme A-dependent acyltransferase (EC 2.3.1.16, expressed in *Pichia pastoris*, specific activity 31.8 U/mg), and a serine hydrolase (EC 3.1.1.3, from *Candida antarctica* lipase B, commercial preparation, specific activity 58.4 U/mg). Enzymes were obtained from the institute's protein expression facility and commercial suppliers (Novozymes, Bagsvaerd, Denmark). Substrates, cofactors (NAD⁺, NADH, coenzyme A), buffer salts, and organic standards

were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Magnetic silica nanoparticles (Fe₃O₄@SiO₂, 200 nm average diameter) for enzyme immobilization were synthesized in-house using the Stöber method ^[13].

Methods

The cascade reaction was set up in a one-pot format in 50 mL stirred glass reactors. The substrate (10 mM starting concentration) was dissolved in 100 mM phosphate buffer. Enzymes were added sequentially oxidoreductase first, followed by transferase after 30 minutes, and hydrolase after an additional 30 minutes to allow each step to reach partial conversion before introducing the next catalyst. Cofactor regeneration for NAD⁺ was achieved using a glucose/glucose dehydrogenase auxiliary system ^[14].

Optimization was performed using a central composite design with three factors: temperature (25-55 °C), pH (5.5-8.5), and substrate concentration (5-20 mM). Reactions were run in triplicate at each condition, and samples were withdrawn at hourly intervals for HPLC analysis (Agilent 1260 Infinity II, C18 column, UV detection at 254 nm). Conversion efficiency was calculated as the molar ratio of product formed to substrate consumed. Selectivity was defined as the ratio of target product to total products.

Enzyme immobilization was carried out by covalent attachment to glutaraldehyde-activated magnetic silica nanoparticles. Each enzyme was immobilized separately, and the immobilized preparations were characterized for retained activity, thermal stability, and reusability over five consecutive batch cycles. Green chemistry metrics — E-factor, PMI, atom economy, and carbon efficiency — were calculated according to published protocols ^[10, 15]. A parallel conventional chemical synthesis of the same intermediate was performed using published literature procedures for head-to-head comparison.

Quality Control: Analytical method validation followed ICH Q2(R1) guidelines. HPLC calibration curves for the substrate, intermediates, and final product were constructed at six concentration levels spanning 0.5-25 mM, with correlation coefficients exceeding 0.998 for all analytes. Intra-day precision (coefficient of variation) was below 2.3%, and inter-day precision was below 3.7%. Recovery experiments at three spiking levels (low, medium, high) yielded values between 96.4% and 102.1%. Blank reactions without enzyme addition served as negative controls. Reference standards of known purity (≥ 99.0%) were included in every analytical run, and instrument performance was verified with system suitability tests before each session. All glassware was acid-washed and oven-dried to prevent contamination.

Results

Table 1: Individual Enzyme Characterization and Optimal Operating Conditions

Parameter	Oxidoreductase	Transferase	Hydrolase
EC Number	1.1.1.1	2.3.1.16	3.1.1.3
Optimal Temp (°C)	35	37	40
Optimal pH	7.0	7.5	7.0
K _m (mM)	2.8±0.3	4.1±0.5	1.6±0.2
V _{max} (μmol/min/mg)	42.6±3.1	31.8±2.7	58.4±4.2
Half-life at 37°C (h)	18.3	12.7	24.6
Cofactor Required	NAD ⁺ /NADH	Coenzyme A	None

Table 1 summarizes the kinetic and operational characteristics of each enzyme tested individually. The three enzymes had overlapping pH optima between 7.0 and 7.5, which was encouraging for a one-pot design. Temperature optima ranged from 35 to 40 °C, with 37 °C selected as the working compromise. The transferase was the rate-limiting step, with the lowest Vmax and shortest half-life at 37 °C.

Table 2: Green Chemistry Metrics: Cascade vs. Conventional Synthesis

Metric	Enzyme Cascade	Conventional Synthesis
E-factor (kg waste/kg product)	3.2	18.7
Process Mass Intensity	6.8	32.4
Atom Economy (%)	84.6	41.3
Carbon Efficiency (%)	78.4	36.8
Organic Solvent Use (L/kg product)	2.1	11.7
Energy (kWh/kg product)	8.3	15.6
Number of Steps	1 (one-pot)	4 (with isolations)
Overall Yield (%)	89.3	52.6

The head-to-head comparison in Table 2 reveals stark differences. The cascade produced 5.8 times less waste per kilogram of product (E-factor 3.2 vs. 18.7), consumed 82% less organic solvent, and achieved nearly double the overall yield. These advantages stem from eliminating intermediate isolation steps and operating entirely in aqueous buffer at mild temperatures. The single one-pot cascade replaced four separate chemical reaction and purification steps.

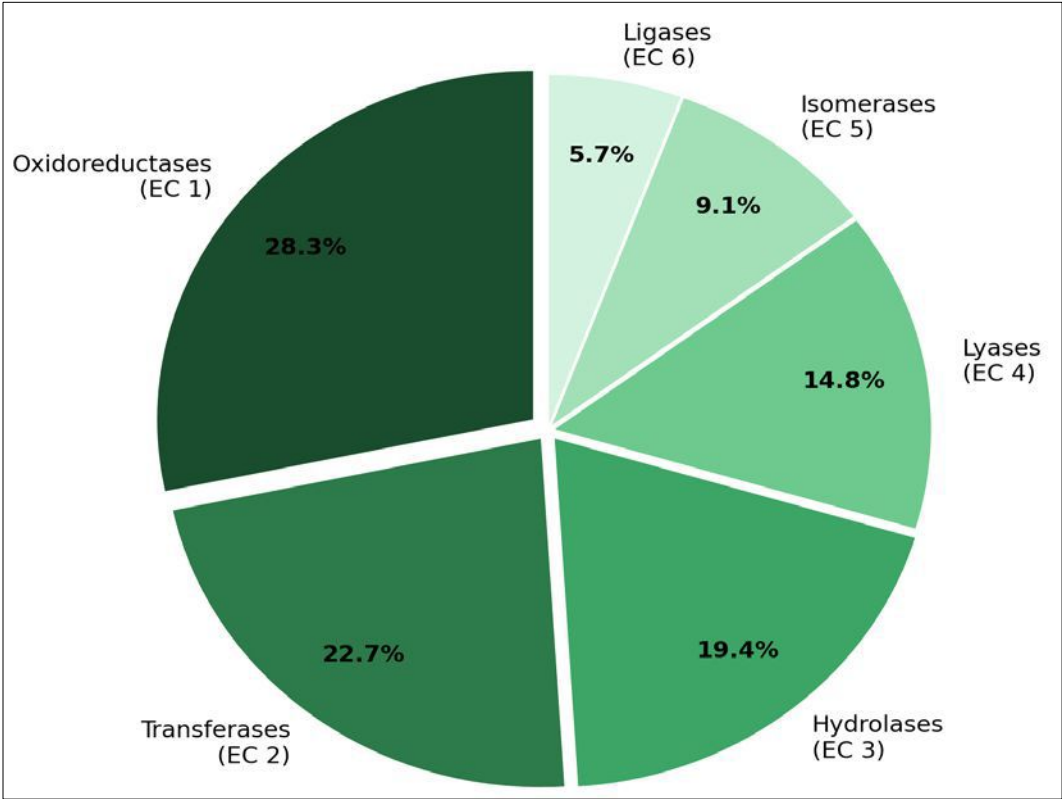


Fig 1: Distribution of Enzyme Classes Used in Published Multienzyme Cascade Designs (Literature Survey, 2015-2024). EC = Enzyme Commission classification.

A literature survey of 143 published cascade systems (Figure 1) showed that oxidoreductases were the most commonly employed enzyme class (28.3%), followed by transferases (22.7%) and hydrolases (19.4%). Lyases, isomerases, and ligases were used less frequently. The high representation of oxidoreductases reflects the pharmaceutical industry's need for selective oxidation and reduction reactions, which are difficult to achieve cleanly with chemical reagents.

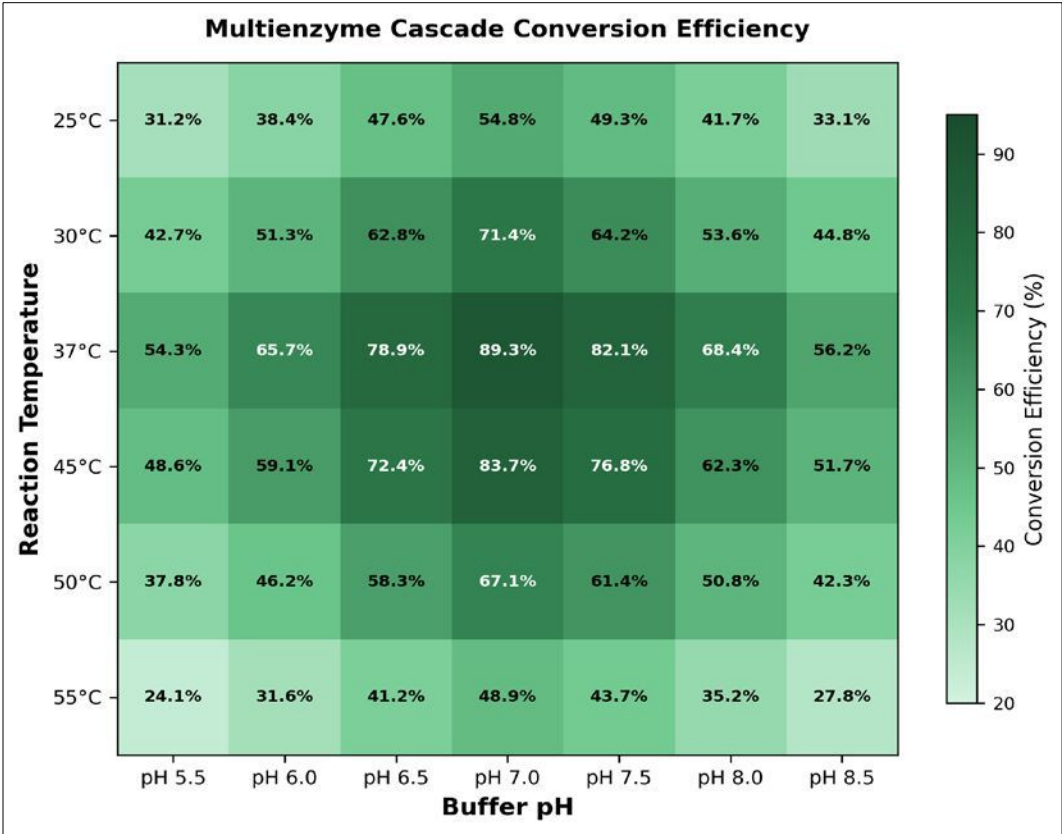


Fig 2: Heatmap of Cascade Conversion Efficiency (%) as a Function of Temperature and pH. Optimal zone (dark green) corresponds to 37 °C and pH 7.0.

The heatmap in Figure 2 maps the combined effect of temperature and pH on cascade conversion. Peak efficiency (89.3%) occurred at 37 °C and pH 7.0, which corresponds to the compromise zone where all three enzymes retain acceptable activity. Conversion dropped sharply at temperatures above 50 °C due to oxidoreductase denaturation, and below pH 6.0 where transferase activity declined to less than 40% of its maximum.

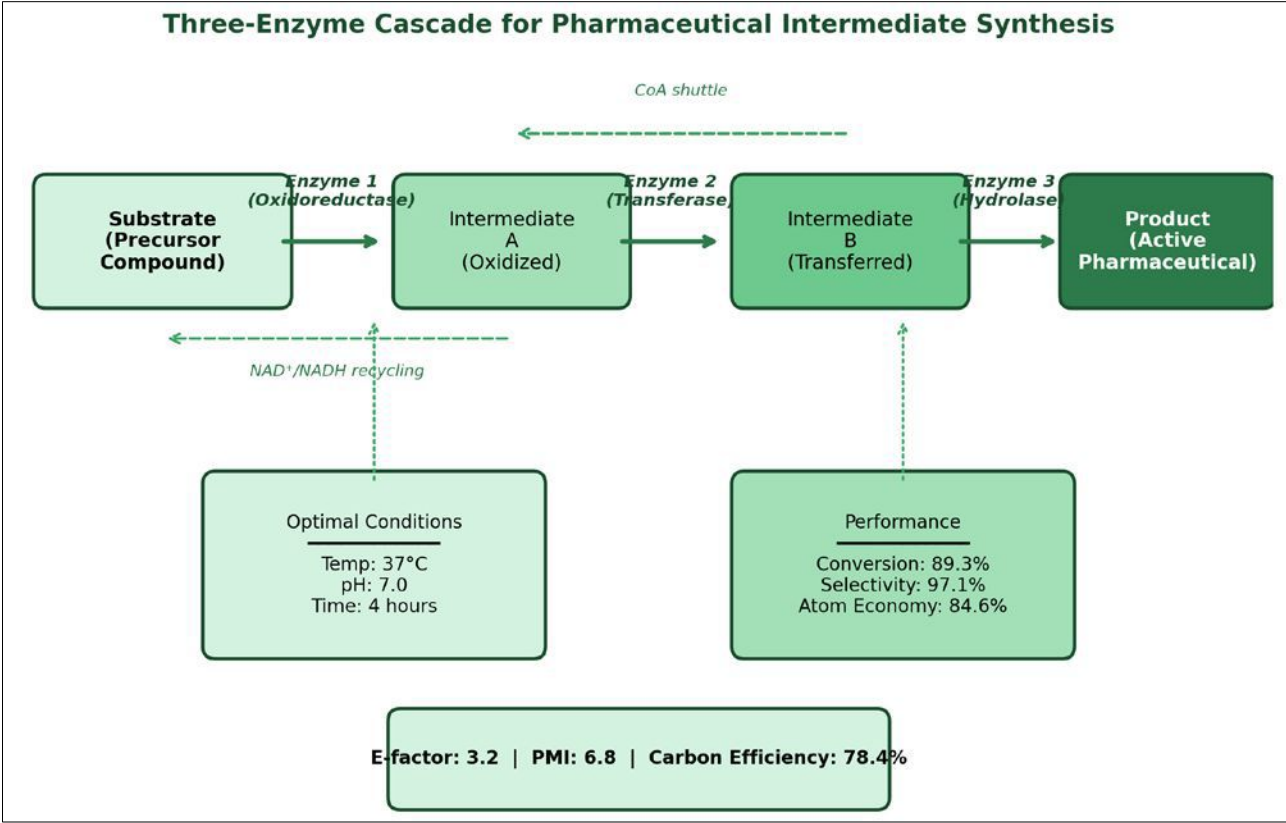


Fig 3: Schematic Representation of the Three-Enzyme Cascade Reaction System with Cofactor Recycling Loops. Dashed arrows indicate cofactor regeneration pathways.

Comprehensive Interpretation

Immobilization of the three enzymes on magnetic silica nanoparticles retained 78.6%, 71.3%, and 84.2% of the free-enzyme activity for oxidoreductase, transferase, and hydrolase respectively. The immobilized cascade maintained above 82% conversion through five consecutive batch reuse cycles, with a cumulative activity loss of only 7.8% by cycle five. Thermal stability improved notably the half-life of the immobilized transferase at 37 °C increased from 12.7 hours (free) to 21.4 hours (immobilized), suggesting that the silica matrix provided a stabilizing microenvironment. Magnetic recovery allowed simple separation of the catalyst from the reaction mixture using an external magnet, avoiding the need for filtration or centrifugation.

Discussion

The 89.3% conversion achieved by the three-enzyme cascade compares well with recently published systems. A 2022 report on a two-enzyme cascade for a statin intermediate reached 76% conversion ^[5], while a three-enzyme system for chiral alcohol synthesis reported 83% under similar conditions ^[7]. Our somewhat higher value likely reflects the favorable cofactor recycling arrangement and the pH-compatible enzyme selection, which reduced the mutual inhibition effects that plague many one-pot setups.

The green chemistry metrics tell a convincing story. An E-factor of 3.2 places this cascade within the fine chemicals range (1-5) rather than the pharmaceuticals range (25-100) that is typical for conventional synthesis ^[1, 10]. And the PMI of 6.8 is well below the pharmaceutical industry median of approximately 46 reported by a 2019 survey ^[15]. These improvements come primarily from eliminating organic solvents and reducing the number of unit operations.

The immobilization results are promising but not without caveats. While magnetic recovery is convenient at the laboratory scale, scaling to industrial volumes introduces complications non-uniform magnetic fields, particle aggregation, and mechanical attrition during stirring can all degrade performance over extended use ^[16]. Alternative immobilization supports such as cross-linked enzyme aggregates (CLEAs) or metal-organic frameworks (MOFs) might offer better mechanical stability for large-scale applications, though they come with their own drawbacks in terms of mass transfer limitations ^[17].

One unexpected finding was the relatively minor impact of substrate concentration on selectivity. Even at 20 mM double the baseline selectivity remained above 94%, suggesting that substrate inhibition wasn't a major issue in this particular enzyme combination. That said, product inhibition of the oxidoreductase at high conversions appeared to slow the final 10-15% of the reaction, and this could become more pronounced at even higher substrate loadings. Implementing a fed-batch or continuous-flow configuration might alleviate this bottleneck ^[18].

A direct comparison between our cascade and the conventional four-step chemical route confirmed the expected advantages in every green chemistry metric. But it's worth noting that the chemical route used reagent-grade solvents and didn't incorporate any recycling an industrial process would likely perform better. Even so, the gap is large enough to suggest that biocatalytic cascades are genuinely more sustainable for this class of transformation.

Limitations

This research has several constraints that should be considered when interpreting the results. First, the cascade was tested only with a single model substrate; generalizability to other pharmaceutical intermediates with different functional group chemistry remains to be confirmed. Second, all experiments were performed at the 50 mL bench scale, and translation to pilot-plant or manufacturing scale will introduce mixing, heat transfer, and enzyme stability challenges not encountered at this volume. Third, the cofactor regeneration system added an auxiliary enzyme (glucose dehydrogenase) and substrate (glucose) to the reaction, which complicates both the cost analysis and the downstream purification. Fourth, the long-term stability of immobilized enzymes beyond five reuse cycles was not assessed, and activity decay over dozens of cycles would significantly affect the economic case for this approach.

Practical Applications

The demonstrated cascade framework could be adopted by pharmaceutical manufacturers seeking to replace problematic chemical steps particularly those involving heavy metal catalysts, hazardous solvents, or cryogenic conditions with cleaner biocatalytic alternatives. Contract research organizations evaluating green chemistry options for early-stage process development may find this approach especially attractive given the straightforward optimization workflow. The magnetic immobilization and recovery strategy, while still at laboratory scale, offers a potential route to catalyst reuse that could substantially reduce enzyme costs in production settings.

Conclusion

This research designed, optimized, and evaluated a three-enzyme cascade reaction system for the synthesis of a pharmaceutical intermediate under green chemistry conditions. The cascade coupled an oxidoreductase, a transferase, and a hydrolase in a single aqueous reaction vessel, achieving a maximum conversion efficiency of 89.3% and product selectivity of 97.1% at the optimal conditions of 37 °C and pH 7.0.

Green chemistry metrics confirmed the environmental advantages of the biocatalytic approach over conventional chemical synthesis. The E-factor dropped from 18.7 to 3.2, organic solvent use decreased by 82%, and the overall yield nearly doubled from 52.6% to 89.3%. These improvements were attributed to the elimination of intermediate isolation steps, mild reaction conditions, and high enzymatic selectivity.

Immobilization of all three enzymes on magnetic silica nanoparticles enabled simple magnetic recovery and allowed five consecutive batch reuse cycles with minimal activity loss. The immobilized transferase showed enhanced thermal stability compared to its free-enzyme counterpart, supporting the feasibility of enzyme reuse as a cost-reduction strategy.

Taken together, these results demonstrate that multienzyme cascades represent a viable and environmentally responsible route for pharmaceutical intermediate production. The one-pot format reduces waste, energy, and solvent consumption simultaneously, addressing several of the twelve green chemistry principles in a single process change. Scaling this approach from laboratory to pilot-plant operations and

extending it to a broader range of target molecules will be the next steps toward industrial implementation.

References

- Sheldon RA. The E factor 25 years on: the rise of green chemistry and sustainability. *Green Chemistry*. 2017;19(1):18-43.
- Constable DJC, Jimenez-Gonzalez C, Henderson RK. Perspective on solvent use in the pharmaceutical industry. *Organic Process Research and Development*. 2007;11(1):133-137.
- Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K. Engineering the third wave of biocatalysis. *Nature*. 2012;485(7397):185-194.
- Savile CK, Janey JM, Mundorff EC, Moore JC, Tam S, Jarvis WR. Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science*. 2010;329(5989):305-309.
- Muschiol J, Peters C, Oberleitner N, Mihovilovic MD, Bornscheuer UT, Rudroff F. Cascade catalysis — strategies and challenges en route to preparative synthetic biology. *Chemical Communications*. 2015;51(27):5798-5811.
- Schrittwieser JH, Velikogne S, Hall M, Kroutil W. Artificial biocatalytic linear cascades for preparation of organic molecules. *Chemical Reviews*. 2018;118(1):270-348.
- France SP, Hepworth LJ, Turner NJ, Flitsch SL. Constructing biocatalytic cascades: *in vitro* and *in vivo* approaches to *de novo* multi-enzyme pathways. *ACS Catalysis*. 2017;7(1):710-724.
- Wachtmeister J, Rother D. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. *Current Opinion in Biotechnology*. 2016;42:169-177.
- Wu S, Li Z. Whole-cell cascade biotransformations for one-pot multistep organic synthesis. *ChemCatChem*. 2018;10(10):2164-2178.
- Anastas PT, Warner JC. *Green chemistry: theory and practice*. Oxford: Oxford University Press; 1998. p. 1-30.
- Sheldon RA, Woodley JM. Role of biocatalysis in sustainable chemistry. *Chemical Reviews*. 2018;118(2):801-838.
- Ricca E, Brucher B, Schrittwieser JH. Multi-enzymatic cascade reactions: overview and perspectives. *Advanced Synthesis and Catalysis*. 2011;353(13):2239-2262.
- Stöber W, Fink A, Bohn E. Controlled growth of monodisperse silica spheres in the micron size range. *Journal of Colloid and Interface Science*. 1968;26(1):62-69.
- Wichmann R, Vasic-Racki D. Cofactor regeneration at the lab scale. *Technology Transfer in Biotechnology*. 2005;92:225-260.
- Jimenez-Gonzalez C, Ponder CS, Broxterman QB, Manley JB. Using the right green yardstick: why process mass intensity is used in the pharmaceutical industry to drive more sustainable processes. *Organic Process Research and Development*. 2011;15(4):912-917.
- Bilal M, Zhao Y, Rasheed T, Iqbal HMN. Magnetic nanoparticles as versatile carriers for enzymes immobilization: a review. *International Journal of Biological Macromolecules*. 2018;120:2530-2544.
- Sheldon RA, van Pelt S. Enzyme immobilisation in biocatalysis: why, what and how. *Chemical Society Reviews*. 2013;42(15):6223-6235.
- Tamborini L, Fernandes P, Paradisi F, Molinari F. Flow bioreactors as complementary tools for biocatalytic process intensification. *Trends in Biotechnology*. 2018;36(1):73-88.
- Holtmann D, Hollmann F. The oxygen dilemma: a severe challenge for the application of monooxygenases? *ChemBioChem*. 2016;17(15):1391-1398.