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DOCTORAL DISSERTATION

Cyanobacterial biomass in biocatalysis and bioaccumulation processes

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List of abbreviations

ABC-type – ATP-Binding Cassette transporter type
ADHs – Alcohol Dehydrogenases
ATCC – American Type Culture Collection
BG-11 – Blue-Green Medium 11
CALB – *Candida antarctica* Lipase B
CCALA – Culture Collection of Autotrophic Organisms
CFU – Colony Forming Units
COSY – Correlation Spectroscopy
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
DEVP – Diethyl Vinylphosphonate
DKR – Dynamic Kinetic Resolution
DMF – Dimethylformamide
DMHP – Dimethyl (1E)-3-hydroxyprop-1-enylphosphonate
DMSO – Dimethyl Sulfoxide
DMVP – Dimethyl Vinylphosphonate
DNA – Deoxyribonucleic Acid
EDTA – Ethylenediaminetetraacetic Acid
EMDP – Epoxymethyl Dimethyl Phosphonate
EPS – Exopolysaccharide
ERs – Ene-Reductases
ESI – Electrospray Ionization
FAD – Flavin Adenine Dinucleotide
FADH₂ – Flavin Adenine Dinucleotide (reduced form)
FDH – Formate Dehydrogenase
FID – Flame Ionization Detector
GDH – Glucose Dehydrogenase
GMP – Good Manufacturing Practice
HMBC – Heteronuclear Multiple Bond Correlation
HMQC – Heteronuclear Multiple Quantum Coherence
HRMS – High-Resolution Mass Spectrometry

ICP-MS – Inductively Coupled Plasma Mass Spectrometry
ICP-OES – Inductively Coupled Plasma Optical Emission Spectrometry
IREDs – Imine Reductases
KREDs – Ketoreductases
LC-MS/MS – Liquid Chromatography Tandem Mass Spectrometry
LOD – Limit of Detection
LOQ – Limit of Quantification
MDR – Multi-Drug Resistant
MEP – Methylerythritol 4-Phosphate
MIC – Minimum Inhibitory Concentration
NADH – Nicotinamide Adenine Dinucleotide (reduced)
NADPH – Nicotinamide Adenine Dinucleotide Phosphate (reduced)
N.b – Nutrient broth
NSAIDs – Non-Steroidal Anti-Inflammatory Drugs
OYE – Old Yellow Enzyme
PBS – Phosphate-Buffered Saline
PCC – Pasteur Culture Collection
PhnCDE – Phosphonate Transport System (ABC-type)
PTDH – Phosphite Dehydrogenase
REE – Rare Earth Elements
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
SM – Spirulina Medium
TAs – Transaminases
TFA – Trifluoroacetic Acid
TPP – Triphenyl Phosphate
UDP-galactose – Uridine Diphosphate Galactose
UDP-glucose – Uridine Diphosphate Glucose
VOCs – Volatile Organic Compounds
WUST – Wrocław University of Science and Technology

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Scientific achievements

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2. **Samson, S.O.**, Serafin-Lewańczuk, M., Brzezińska-Rodak, M., Klimek-Ochab, M., Żymańczyk-Duda, E. (2024). Complete epoxy phosphonate conversion to dimethyl (1E)-3-hydroxyprop-1-enylphosphonate with photobiocatalysts' assistance. *Symmetry* 16, 1676. <https://doi.org/10.3390/sym16121676>
3. **Samson, S.O.**, Serafin-Lewańczuk, M., Żymańczyk-Duda, E., Brzezińska-Rodak, M., Klimek-Ochab, M., Vítová, M., Čížková, M., Eghbalinejad, M. (2025). Photobiotransformation of xenobiotics by cyanobacterial biomass and its further valorization as bioaccumulator of rare earth elements. Under preparation.

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Conference presentations

1. **Samson, Sunday Ochoi**, Serafin-Lewańczuk, Monika, Żymańczyk-Duda, Ewa. "Cyanobacterial biomass in biocatalysis processes." Poster presented at the *Third Key to Sustainability Symposium*, Wrocław University of Science and Technology, 29–30 April 2025, Wrocław, Poland. <https://keytosustainability.com/>
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<https://www.dbc.wroc.pl/dlibra/publication/154989/edition/115413/content>

6. Brzezińska-Rodak, Małgorzata, Serafin-Lewańczuk, Monika A., Raczyńska, Agnieszka, **Samson, Sunday Ochoi**, Lubiak, Katarzyna D., Piela, Aleksandra, Śliżewska, Agnieszka, Klimek-Ochab, Magdalena, Żymańczyk-Duda, Ewa. “Biocatalysis in the synthesis of high-added-value compounds.” Presented at the *5th Symposium on Biotransformations for Pharmaceutical and Cosmetic Industry*, 13–15 June 2022, Kraków-Częstochowa Upland, Poland.

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Patent applications

1. Biokatalityczny sposób otrzymywania (1*E*)-3-hydroksyprop-1-enylofosfonianu dimetylu z wykorzystaniem szczepu *Synechococcus bigranulatus* CCALA 187. Sunday Ochoi Samson, Monika Serafin-Lewańczuk, Ewa Żymańczyk-Duda, Małgorzata Brzezińska-Rodak, Magdalena Klimek-Ochab.
2. Biokatalityczny sposób otrzymywania (1*E*)-3-hydroksyprop-1-enylofosfonianu dimetylu z wykorzystaniem szczepu *Nodularia moravica* CCALA 797. Monika Serafin-Lewańczuk, Ewa Żymańczyk-Duda, Sunday Ochoi Samson, Małgorzata Brzezińska-Rodak, Magdalena Klimek-Ochab.
3. Biokatalityczny sposób otrzymywania (1*E*)-3-hydroksyprop-1-enylofosfonianu dimetylu z wykorzystaniem szczepu *Leptolyngbya foveolarum* CCALA 76. Monika Serafin-Lewańczuk, Ewa Żymańczyk-Duda, Sunday Ochoi Samson, Małgorzata Brzezińska-Rodak, Magdalena Klimek-Ochab.

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- **Increased Doctoral Scholarship (2022/2023)** – Awarded based on academic merit and research contributions.

Abstract (English)

The growing demand for solutions in the economy that align with the principles of sustainable development is an important driver for the advancement of biotechnology, both in the field of chemical synthesis using biological tools and in environmental bioremediation. Cyanobacteria are useful organisms in this regard, as they can be employed both as photobiocatalysts and as tools for improving environmental quality. This dissertation addresses the potential of selected cyanobacterial strains, *Synechococcus bigranulatus* CCALA 187, *Nostoc cf-muscorum* CCALA 129, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Leptolyngbya foveolarum* CCALA 76, *Kamptomonas animale* CCALA 138, and *Nodularia moravica* CCALA 114 as whole-cell biocatalysts and bioaccumulators that could serve as elements in bioremediation and resource recovery strategies.

A series of photobiocatalytic tests were conducted under controlled conditions to assess the efficiency of transforming acetic acid esters and organophosphonates such as epoxymethyl dimethylphosphonate (EMDPP). Analytical techniques including GC, HPLC, NMR, IR and ICP-MS were used for quantitative analysis of biotransformation, evaluation of stereoselectivity, and determination of rare earth element (REE) accumulation, particularly gadolinium (Gd) and europium (Eu).

The study demonstrated significant catalytic activity of the cyanobacteria. In the case of *Synechococcus bigranulatus* CCALA 187 and *Limnospira* sp. (PCC 8005 and CCALA 27), almost complete bioconversion of EMDPP was achieved within 72 and 24 hours, respectively, resulting in the formation of the product, dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate (DMHPP). Enantioselective hydrolysis of 1-phenylethyl acetate was also observed, with the use of *Leptolyngbya foveolarum* CCALA 76 yielding an enantiomeric excess exceeding 70%. Bioaccumulation experiments showed that cyanobacterial biomass, especially after bioconversion, exhibits a high affinity for REEs, confirming its applicability in bioremediation and resource recovery strategies.

This work demonstrates that cyanobacterial biomass can be utilized in the design of processes aligned with the development of a circular economy, where microalgae act as photobiocatalysts enabling the synthesis of valuable products, and the recovered biomass is valorized for its bioaccumulation capabilities, which in turn enables, for example, the recovery of critical elements. It provides a foundation for future applications in synthetic biology, pharmaceutical synthesis, and environmentally sustainable REE extraction.

Streszczenie (Polski)

Rosnące zapotrzebowanie na stosowanie w gospodarce rozwiązań zgodnych z zasadami zrównoważonego rozwoju jest ważną przesłanką dla rozwoju biotechnologii w zakresie syntezy chemicznej z wykorzystaniem narzędzi biologicznych oraz w zakresie bioremediacji środowiskowej. Cyjanobakterie są w tym przypadku użytecznymi organizmami, które można wykorzystać zarówno jako fotobiokatalizatory jak i narzędzie poprawiające jakość środowiska. Niniejsza rozprawa odpowiada na pytanie jaki jest potencjał wybranych szczepów cyjanobakterii, *Synechococcus bigranulatus* CCALA 187, *Nostoc cf-muscorum* CCALA 129, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Leptolyngbya foveolarum* CCALA 76, *Kamptonema animale* CCALA 138, oraz *Nodularia moravica* CCALA 114, jako całokomórkowych biokatalizatorów oraz bioakumulatorów, które mogą być elementem strategii bioremediacji i odzysku zasobów. Przeprowadzono serię testów fotobiokatalitycznych w kontrolowanych warunkach, aby ocenić efektywność transformacji estrów kwasu octowego oraz organofosfonianów, takich jak epoksymetylo-dimetylofosfonian (EMDPP). Do ilościowej analizy biotransformacji, oceny stereoselektywności i oznaczenia akumulacji pierwiastków ziem rzadkich (REE), szczególnie gadolinu (Gd) i europu (Eu), zastosowano techniki analityczne takie jak GC, HPLC, NMR, IR i ICP-MS.

Badania wykazały istotną aktywność katalityczną sinic. W przypadku *Synechococcus bigranulatus* CCALA 187 and *Limnospira* sp. (PCC 8005 and CCALA 27) uzyskano niemal całkowitą biokonwersję EMDPP odpowiednio w ciągu 72 i 24 godzin i otrzymano produkt, dimetylowy (1E)-3-hydroksypropeno-1-ylowy fosfonian (DMHPP). Zaobserwowano także enantioselektywną hydrolizę octanu 1-fenyletanolu, przy czym użycie szczepu *Leptolyngbya foveolarum* CCALA 76 pozwoliło na uzyskanie nadmiaru enancjomerycznego przekraczającego 70%. Równoległe eksperymenty bioakumulacyjne wykazały, że biomasa cyjanobakterii,

zwłaszcza po biokonwersji, wykazuje wysokie powinowactwo do REE, co potwierdza jej przydatność w bioremediacji i strategiach odzysku zasobów.

Praca ta wskazuje, że biomasa cyjanobakterii może być wykorzystana do planowania procesów wpisujących się w rozwój gospodarki o obiegu zamkniętym, gdzie mikroalgi są fotokatalizatorami pozwalającymi na syntezę wartościowych produktów, a następnie, odzyskana biomasa jest waloryzowana pod kątem zdolności do bioakumulacji, co z kolei umożliwia np. odzysk pierwiastków krytycznych. Wyniki badań stanowią mogą podstawę dla przyszłych zastosowań w biologii syntetycznej, syntezie farmaceutycznej oraz zrównoważonym środowiskowo pozyskiwaniu REE.

CHAPTER ONE

Introduction

1.1. Background

The connection between biotechnology and environmental chemistry has witnessed rapid advances in recent decades, particularly in the development of microbial systems for sustainable catalysis and metal recovery. Cyanobacteria, a group of oxygenic photoautotrophic prokaryotes, have emerged as potent biological agents capable of not only driving complex enzymatic transformations but also accumulating metals (such as rare earth elements) through biosorption, intracellular sequestration, and biomineralization. Their unique physiological and metabolic attributes, including photosynthetic capability, rapid growth, and rich cell wall chemistry, render them highly attractive for applications in green biocatalysis and bioaccumulation.

Biocatalysis refers to the use of biological catalysts, enzymes or whole cells, to mediate chemical reactions under environmentally benign conditions. Microbial biocatalysis has been dominated by heterotrophic organisms, particularly bacteria and fungi. However, cyanobacteria offer the added advantage of phototrophic growth, enabling the direct harnessing of solar energy to drive metabolic processes. This feature has fostered growing interest in their deployment for photobiocatalysis, light-driven biotransformations involving complex substrates such as esters and organophosphonates.

Simultaneously, increasing concerns over the environmental and geopolitical constraints of rare earth element (REE) extraction have catalyzed the search for sustainable recovery technologies. Rare earths such as gadolinium (Gd) and europium (Eu) are critical to diverse technologies including medical imaging, clean energy, and electronics, yet conventional mining practices are often associated with ecological degradation. Cyanobacteria have been increasingly recognized for their metal-binding

potential, attributed to the abundance of functional groups (e.g., hydroxyl, carboxyl, phosphate) on their extracellular polymeric substances (EPS) and cell surfaces, as well as their capacity for intracellular uptake under regulated metabolic states.

1.2. Rationale for the study

While several studies have documented enzymatic activities and heavy metal tolerance in cyanobacteria, few have comprehensively explored their dual functionality in both biocatalytic conversions and metal bioaccumulation under controlled photoautotrophic conditions. Moreover, there is a paucity of research addressing the integration of these functions within a single experimental framework, especially under scalable, reproducible conditions.

The use of cyanobacterial strains such as *Synechococcus bigranulatus* CCALA 187, *Nostoc cf-muscorum* CCALA 129, *Kamptonema animale* CCALA 138, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Nodularia moravica* CCALA 114 and *Leptolyngbya foveolarum* CCALA 76 in the current study provides a valuable opportunity to examine a diverse set of physiological and enzymatic traits. These strains were evaluated not only for their ability to convert organic compounds into value-added products, but also for their REE uptake potential in pre- and post-biotransformation states (Figure 1).

1.3. Research Objectives

The main objectives of this doctoral research were to:

- **Assess** the capacity of selected cyanobacterial strains to catalyze photo-biotransformation reactions involving ester substrates, including compounds containing a P–C bond.
- **Evaluate** the stability of the substrates and optimize the growth medium composition to achieve a balance between biomass productivity and substrate integrity.

- **Determine** the selectivity and conversion efficiency of the selected cyanobacterial strains during biotransformation processes.
- **Conduct** a preliminary investigation of the antimicrobial activity of the obtained bioconversion products.
- **Identify** the most promising cyanobacterial strains for laboratory-scale studies based on their catalytic performance, tolerance to elevated substrate concentrations, and rare earth element (REE) bioaccumulation efficiency.
- **Investigate** the bioaccumulation potential of cyanobacterial biomass, before and after biotransformation, for the recovery of rare earth elements (Gd^{3+} and Eu^{3+}) using inductively coupled plasma mass spectrometry (ICP-MS).

1.4. Significance of the study

This research represents a novel convergence of two biotechnologically important processes, biocatalysis and bioaccumulation, within a single phototrophic microbial platform. By integrating substrate transformation and elemental uptake, it opens pathways for simultaneous bioproduct synthesis and environmental remediation. Additionally, the project aligns with global goals of sustainable chemistry and circular bioeconomy, wherein biological systems are harnessed for both value generation and waste minimization.

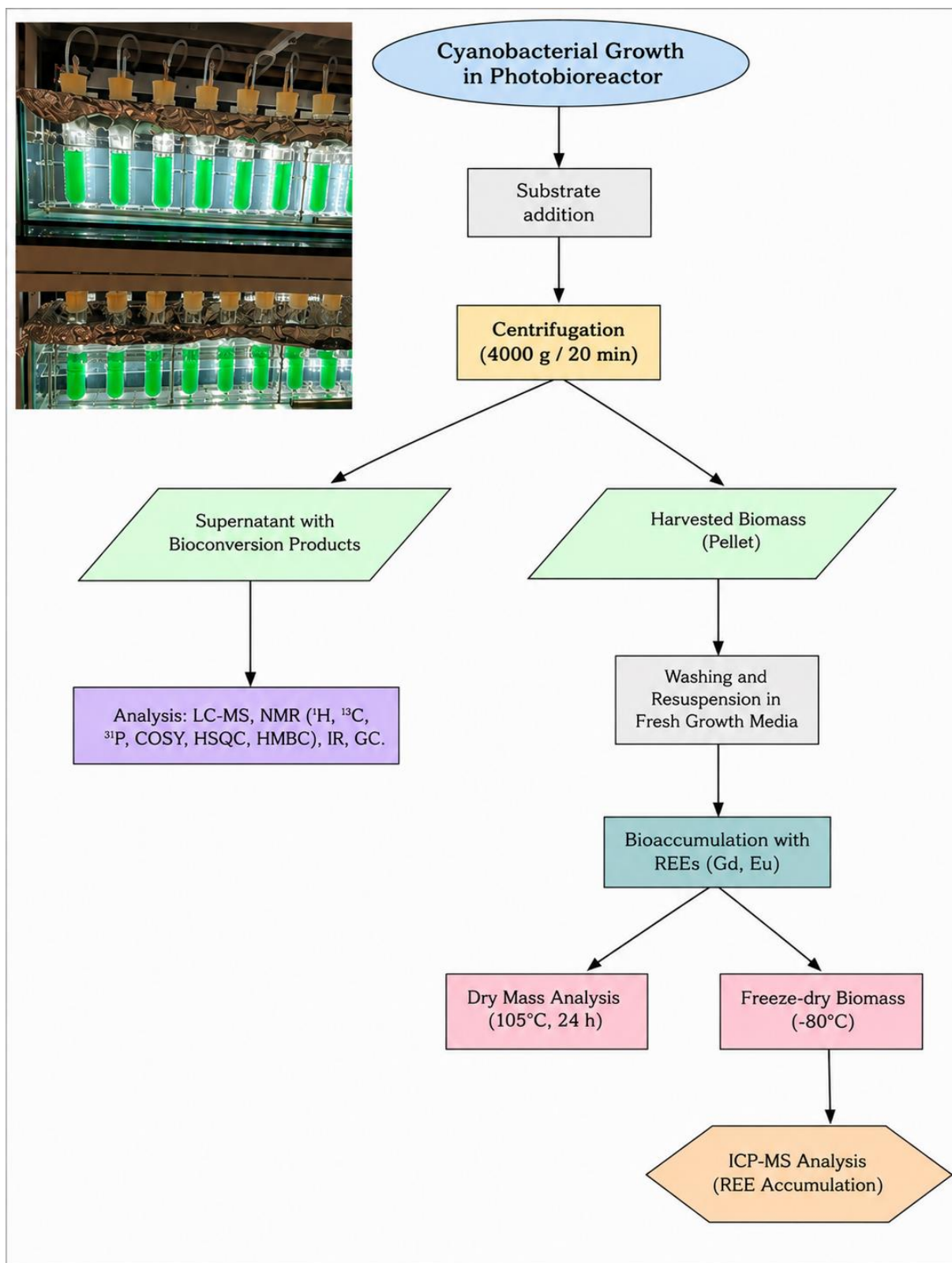


Figure 1. Schematic representation of a combined bioconversion and bioaccumulation processes (sketched by the author).

The biotransformation of organophosphonates into hydroxyphosphonates, particularly the synthesis of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate (DMHPP), illustrates the potential for cyanobacteria in selective reactions with biomedical implications, since phosphonates derivatives are common motives in the drug structures. Furthermore, the use of common medium engineering allowed for tuning substrate stability while maintaining organism viability and activity.

Finally, the application of cyanobacterial biomass for rare earth element recovery not only mitigates environmental pollution but also offers a bioresource pathway for strategic material supply. The capacity to recover REEs using a biological chassis that previously facilitated catalysis offers efficiency, modularity, and sustainability not achievable via conventional processes.

1.5. Limitations and future perspective

This study focuses on a limited number of organic substrates and cyanobacterial strains cultured under laboratory conditions. While the selected strains represent diverse morphophysiological types, the findings may not fully capture the performance spectrum of naturally occurring or genetically modified cyanobacteria. Additionally, while the analytical framework employed ensures high sensitivity and specificity, limitations in yield scalability, product purification, and long-term bioreactor stability remain to be addressed in future studies. Future research will also focus on optimizing the bioaccumulation processes for these strains by extending the acclimation period for post-bioconversion biomass, which could enhance their resilience and bioaccumulation efficiency. Exploring a broader range of REEs and cyanobacterial species will as well deepen understanding of these processes and their potential applications.

CHAPTER TWO

Literature Review

2.1. Conceptual basis of biocatalysis and biotransformation

Biocatalysis refers to the use of biological molecules, primarily enzymes or whole microbial cells, to catalyze chemical reactions. It offers high specificity, efficiency under mild conditions, and reduced environmental impact compared to traditional chemical catalysis [1]. Through selective transformation of substrates, biocatalysis plays a central role in biotransformation processes, enabling the synthesis of enantiomerically pure compounds, complex natural products, and industrial intermediates [2,3], as demonstrated in Figure 2. Biotransformations yield in structurally distinct products under environmentally benign and mild operational conditions and allowed conducting transformations that are often infeasible or inefficient under conventional chemical catalysis [2,4]. Unlike chemical synthesis, which typically relies on extreme pH, high temperatures, and toxic solvents, biotransformations proceed under aqueous, near-physiological conditions, aligning with the principles of green chemistry [5].

The foundational scope of biotransformations encompasses a diverse array of reaction types. These include oxidation and reduction, hydrolysis, esterification, amino-transfer, C–C and C–X bond formations, and even more complex cleavages such as C–P and C–N bonds [5,6]. Such versatility is not merely theoretical, numerous studies have demonstrated the feasibility and efficiency of these transformations across various industrial settings. For instance, Sheldon and Brady [6] critically reviewed the boundaries of biocatalysis and emphasized its application in challenging bond-forming and -breaking processes. Their work expands on the catalytic flexibility of evolved enzymes, demonstrating that with the right engineering, enzymes can be pushed beyond their native substrate and reaction scope.

The biological systems used for biotransformations (as expatiated in section 2.2) are generally categorized into three: purified enzymes, cell-free lysates (crude enzyme mixtures), and whole-cell systems. Each has unique advantages and operational contexts. Purified enzymes provide high specificity, kinetic simplicity, and reduced side-product formation, but often suffer from cofactor dependency and limited robustness [7,8]. In contrast, whole-cell systems offer metabolic self-sufficiency, including in situ regeneration of expensive cofactors like NAD(P)H or ATP [9]. Zhao and van der Donk [9] reviewed various cofactor regeneration strategies and concluded that intracellular pathways provide a cost-effective advantage in large-scale operations, especially in redox chemistry.

Recent studies also showed the importance of choosing the right biocatalyst system based on the complexity of the desired transformation. For example, Liese *et al.* [5] and Singh *et al.* [7] both advocate for whole-cell systems in multistep cascade reactions, where endogenous enzymes and transport mechanisms facilitate product conversion through intermediate stages. These observations are particularly pertinent in industrial biotechnology, where reaction efficiency and scalability dictate process viability.

The sustainability credentials of biotransformations further reinforces their industrial relevance. Enzymes and microbial systems are derived from renewable resources, function in aqueous or non-toxic solvents, and often eliminate the need for hazardous reagents [10,11]. These aspects translate directly to improved E-factors (mass of waste per mass of product) and process mass intensity (PMI), both critical metrics in sustainable process engineering. Sheldon [10] emphasized the role of biocatalysis in advancing resource efficiency, especially in pharmaceutical manufacturing where process waste is tightly regulated.

Integration with synthetic biology further supports the diversification and scalability of biotransformations. Cravens *et al.* [12] outlined microbial chassis engineering

strategies for the overproduction of plant-derived natural products, showing how gene editing and pathway balancing can be used to drive efficient biosynthetic transformations. This development synergizes well with the push toward decentralized, bio-based chemical manufacturing platforms.

Of particular interest in modern biocatalysis is the emergence of photobiocatalysis, where phototrophic organisms or photoactivated enzymes facilitate light-driven chemical conversions. Cyanobacteria have attracted attention in this regard due to their oxygenic photosynthetic machinery and metabolic versatility. These organisms possess the unique capacity to couple solar energy capture with enzymatic redox reactions, thereby offering a sustainable platform for solar-to-chemical energy transduction [13]. Cyanobacteria also harbor numerous oxidoreductases, hydrolases, and transferases that can be exploited in situ for value-added compound production without external cofactors, a significant advantage in bioprocess economics [14].

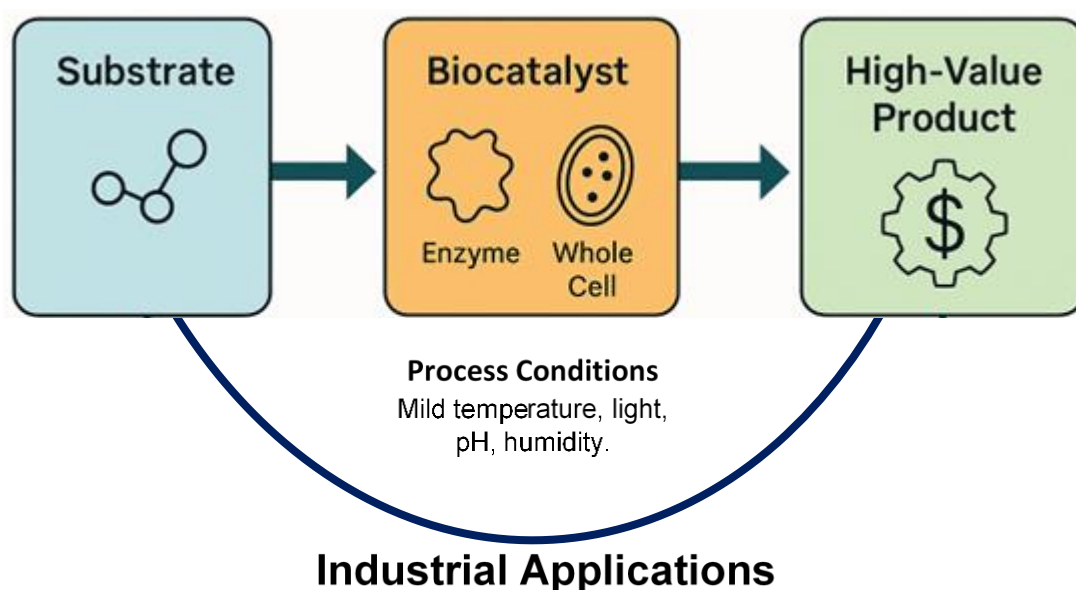


Figure 2. Schematic overview of biocatalysis (*designed by the author*).

Finally, the translation of biotransformations from lab to industrial scale has been facilitated by innovations in reactor design and process intensification. Continuous flow systems and immobilized enzyme technologies now enable long-term operational stability and enzyme reusability, making biocatalytic production

economically viable [15]. These systems allow for seamless integration of multistep reactions in a single setup, reducing residence time, solvent usage, and operational footprint.

2.2. Classes and modes of operation of biocatalysts

Biocatalysts, as it was mentioned, can be operationally categorized into three main systems: (1) purified enzymes, (2) cell-free extracts or lysates, and (3) whole-cell systems. Each of these systems offers distinct catalytic characteristics, operational advantages, and limitations, depending on the complexity of the reaction, substrate specificity, cofactor requirements, economic viability, and scalability [5,7,8].

2.2.1. Purified enzymes

Purified enzymes represent the most refined form of biocatalysts. They are typically isolated from microbial, plant, or recombinant sources and exhibit high catalytic efficiency and substrate specificity. These enzymes provide excellent stereo-, chemo-, and regioselectivity, which is critical in fine chemical and pharmaceutical synthesis [1,4,16]. Their defined kinetic behavior allows detailed mechanistic studies and straightforward reaction modeling.

One of the primary benefits of using purified enzymes is the elimination of cellular barriers and transport limitations that may impede substrate access in whole-cell systems. For example, ketoreductases, lipases, transaminases, and esterases have been widely applied in enantioselective reductions, hydrolyses, and amination reactions due to their broad substrate scope and catalytic precision [4]. The immobilization of purified enzymes on solid supports further enhances their industrial utility. For example, immobilized *Candida antarctica* lipase B (CALB), often marketed as Novozym 435, has become a benchmark biocatalyst for esterification, transesterification, and kinetic resolution of racemic alcohols and amines [17]. Lish *et al.* [17] recently demonstrated that the immobilization protocol significantly influences catalytic

performance in biodiesel production, emphasizing the critical role of matrix selection and enzyme orientation.

Despite their advantages, purified enzymes suffer from several operational constraints. Some enzymes utilize redox cofactors such as the oxidized forms NAD⁺ and FAD, or their reduced counterparts NADH and FADH₂, depending on the specific redox requirement for catalytic turnover, which are prohibitively expensive for large-scale use unless an effective cofactor regeneration system is integrated [9,18]. In addition, they are often sensitive to industrial process conditions such as temperature extremes, pH variation, and organic solvents [19]. To mitigate this, enzyme engineering strategies, such as mutagenesis or fusion to stabilizing protein partners, have been used to enhance solvent tolerance and thermal stability [19].

2.2.2. Cell-free lysates (crude extracts)

Cell-free lysates are typically derived from disrupted microbial cells and contain a complex mixture of enzymes, cofactors, metabolites, and structural proteins. These lysates retain the endogenous cofactors and metabolite pools necessary for catalysis and offer a cost-effective alternative for multienzyme reactions where in situ cofactor regeneration is required [4,20,21]. Alissandratos [21] demonstrated the potential of recombinant *E. coli* lysates in assembling in vitro enzymatic cascades. These systems allow simultaneous expression of multiple enzymatic activities, facilitating multi-step transformations in a single pot. Such systems are particularly advantageous when substrate channeling and intermediate stabilization are needed, as is often the case in redox transformations or chiral amine synthesis.

However, the main limitation of cell-free lysates lies in their compositional complexity and variability. The presence of proteases, side reactions catalyzed by contaminant enzymes, and loss of activity during storage or freeze-thaw cycles are recurring challenges [22]. Moreover, due to the lack of cellular membrane barriers, sensitive

enzymes or unstable intermediates may be quickly degraded, affecting overall yield and reproducibility. Nonetheless, these systems remain a valuable tool for pilot-scale biotransformations, enzyme screening, and academic applications, especially when process economy is a concern and substrate tolerance is moderate [23].

2.2.3. Whole-cell biological systems in biocatalysis

Whole-cell biocatalysts are living or non-viable microbial systems (e.g., bacteria, fungi, yeasts, cyanobacteria) used to catalyze reactions via their native or engineered metabolic pathways. These systems are self-sufficient in cofactor recycling and are ideal for complex biotransformations involving multiple enzymatic steps or high-energy cofactor dependency [7,24,25]. The whole-cell catalysis has several intrinsic advantages. First, cofactors such as NADH, NADPH, and ATP are regenerated in situ via primary metabolism, eliminating the need for costly external supplementation. Second, enzymes are naturally compartmentalized and protected by the cellular environment, which often increases stability and tolerance toward mildly toxic substrates or solvents [15,24].

Biocatalysis has demonstrated the value of bacteria, fungi, yeasts, plant-derived systems, and cyanobacteria in mediating complex biotransformations under mild, sustainable conditions, offering robust enzyme systems, increased solvent tolerance, and the ability to process structurally diverse or recalcitrant substrates.

Fungal biocatalysts, particularly those derived from filamentous species such as *Aspergillus* sp. and *Penicillium* sp. are notable for their ability to produce extracellular oxidative and hydrolytic enzymes. These include laccases, peroxidases, and monooxygenases, which catalyze the oxidation of phenols, aromatic amines, lignin derivatives, and industrial pollutants. Filamentous fungi have shown remarkable utility in transforming hydrophobic or high-molecular-weight substrates, often under biphasic or solvent-rich conditions. [26,27]. Whole cells biocatalysis strongly

dependent on environmental factors such as nutrition deficiency, which is a common tool allowed directing the reactions, as in case e.g. of *Aspergillus niger* cultures applied for stereoselective resolution of heterocyclic phosphonates, including thienyl-, imidazolyl-, and pyrazolyl-substituted derivatives, into optically pure (S)-enantiomers with enantiomeric excess exceeding 98% and yields approaching 100% [28,29]. These results suggested that specific enzymatic pathways involved in stereoselective redox transformations are activated in response to nutrient limitation, likely through secondary metabolic mechanisms that are not constitutively expressed under standard growth conditions.

Yeasts also represent a versatile group of biocatalysts. Species such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica* have been extensively used in whole cell biocatalysis, largely due to their eukaryotic expression systems, capacity for recombinant enzyme production, and intrinsic redox capabilities. There are variety of examples that shows the ability of baker's yeast to reduce aromatic ketones, β -keto esters, diketones and α -haloketones with excellent stereoselectivity, frequently affording the corresponding alcohols with enantiomeric excesses exceeding 95%. These transformations have been extensively applied in the synthesis of pharmaceutical intermediates, flavours, fragrances and fine chemicals, making *S. cerevisiae* one of the crucial whole-cell catalysts in asymmetric synthesis. Comprehensive reviews by Nakamura *et al.* [30], Faber [2], and de Carvalho [31] describe yeast-mediated asymmetric reduction as one of the most advanced and industrially relevant applications of whole-cell biocatalysis. Despite these advantages, yeast-based systems may also exhibit competing metabolic pathways and side reactions due to the presence of multiple endogenous oxidoreductases, prompting the development of recombinant microbial hosts and alternative photosynthetic whole-cell platforms [2,30,31]. These advances have resulted in the investigation of cyanobacteria as sustainable photobiocatalysts, in which reducing equivalents are generated directly from photosynthetic electron transport rather than heterotrophic

metabolism. Żymańczyk-Duda *et al.* showed that baker's yeast, *S. cerevisiae*, can be applied in the stereoselective reduction of diethyl 2-oxoalkylphosphonates, leading to the production of optically pure (*S*)-alcohols with high selectivity. [32]. Similarly, *Y. lipolytica* has been genetically engineered to express surface-bound lipases for biodiesel production from hydrophobic triglyceride substrates, enabling catalysis in non-aqueous media with superior reusability and operational stability [33]. These cases underline the potential of yeast strains not only as hosts for metabolic engineering but also as biocatalysts in their native form for specialty chemical and flavor production.

Plant-derived biocatalysis, though less industrialized, offers unique functionalities. Plant enzymes such as peroxidases, polyphenol oxidases, and glycosyltransferases are capable of catalyzing regioselective oxidation, hydroxylation, and glycosylation reactions relevant to pharmaceutical and nutraceutical chemistry. A representative example is the use of horseradish peroxidase (HRP) from *Armoracia rusticana*, which catalyses the oxidation of phenolic compounds and aromatic amines in the presence of hydrogen peroxide. HRP has been extensively employed in the synthesis of phenolic dimers, lignin-derived polymers, and pharmaceutical intermediates, as well as in environmental bioremediation through the degradation of phenolic pollutants [34,35]. Similarly, suspension cultures from *Catharanthus roseus* and *Nicotiana tabacum* have been applied in the biosynthesis of alkaloids and hydroxylated flavonoids, though scalability and culture stability remain challenges. These systems are particularly valuable for mimicking native plant biosynthetic routes and for functionalizing complex natural product scaffolds [36–38].

Cyanobacteria present another emerging platform for biocatalysis. These phototrophic organisms can perform both primary and secondary biotransformations under carbon-neutral conditions due to their photosynthetic metabolism. Photobiocatalysts have been investigated for different biotransformations such as the asymmetric reduction oxophosphonate esters, with high conversion efficiencies and enantiomeric excess exceeding 95% [39], as well as stereoselective reduction of prochiral ketones and

aldehydes to optically active alcohols through photosynthetically regenerated NADPH [40], cytochrome P450-mediated hydroxylation of natural products and xenobiotics [41], and the light-driven biosynthesis of industrial platform chemicals, including ethanol, isobutanol and 2,3-butanediol, from CO₂ via metabolically engineered cyanobacterial strains [42,43].

In contrast to heterotrophic systems, cyanobacteria provide a model for sustainable, light-driven biocatalysis. Furthermore, cyanobacteria like *Synechococcus elongatus* and *Synechocystis sp.* have been engineered as photoautotrophic whole-cell biocatalysts capable of converting CO₂ and light into industrial platform chemicals and chiral alcohols, offering an unparalleled model of carbon-neutral chemical manufacturing [44,45]. The success of these whole-cell biocatalysts relies not only on their inherent catalytic potential but also on the application of innovative engineering strategies. Immobilization techniques, which have been widely applied allowed to enhance reusability, maintain stability in organic solvents, and facilitate integration into continuous flow reactors. [28].

Despite the strengths, whole-cell systems present their own set of challenges. These include transport barriers across membranes, side reactions from competing metabolic activities, limited tolerance to high substrate/product concentrations, and difficulties in downstream product isolation [46]. However, as the field evolves, the combination of metabolic engineering, immobilization, and bioprocess optimization will be key to unlocking the full potential of these alternative biological systems in industrial biocatalysis [25,46].

Table 1. Comparison of biocatalyst classes and their characteristics

Feature	Purified enzyme	Cell-free lysate	Whole-cell catalyst	Reference
Substrate Specificity	Very High	Moderate to High	Moderate	[20,22,47]
Cofactor Requirement	High (external)	Moderate (endogenous)	Low (internal recycling)	[4,18,22]
Operational Stability	Moderate	Moderate	High	[4,47,48]
Tolerance to Harsh Conditions	Low	Moderate	High	[15,47,48]
Cost of Implementation	High	Moderate	Low	[4,20,22]
Genetic Modifiability	Not Applicable	Not Applicable	High	[4,46]
Multi-Step Conversion	Low	Moderate	Very High	[4,21,49]
Industrial Examples	CALB, KREDs	<i>E. coli</i> transaminase lysate	Recombinant <i>E. coli</i> , <i>S. elongatus</i>	[21,44,45,47,50]

The decision to use a particular biocatalyst system should be guided by the balance of cost, operational feasibility, reaction complexity, cofactor dependency, and environmental constraints as illustrated in Table 1.

2.3. Advantages of biocatalysis over conventional catalytic methods

Biocatalysis has emerged as a transformative methodology in chemical synthesis, providing clear advantages over traditional chemical catalysis in terms of selectivity, sustainability, safety, and process integration. Unlike conventional catalysis, which often relies on high temperatures, extreme pH, and toxic reagents, biocatalysis offers a platform for performing complex chemical transformations under mild, aqueous, and physiologically relevant conditions. This improves the sustainability of the process and influences the reaction efficiency and selectivity [1]. Biocatalysis practical

advantages are process-dependent. Factors such as culturing of particular whole-cells biocatalyst, enzyme production and purification costs, limited enzyme stability under non-optimal conditions, and complex downstream processing requirements can offset these benefits and influence overall process feasibility and scalability [51–54].

From a safety perspective, biocatalysts are non-toxic, non-flammable, and non-corrosive. Their use in water-based systems reduces the risk of fires, explosions, and exposure to volatile organic compounds (VOCs), aligning well with occupational safety standards [23]. This also lowers the infrastructure and insurance costs associated with hazardous chemical storage, transportation, and reaction monitoring. In contrast, chemical catalysis often involves pressurized gases (e.g., hydrogen in metal hydrogenations), strong acids or bases, and reactive organometallic intermediates, all of which require complex containment, waste management, and environmental risk assessments [6]. Moreover, biocatalysis facilitates regulatory compliance, especially in pharmaceutical manufacturing where residual metal contamination is tightly controlled. Biocatalysts leave no metal traces and are thus preferable in late-stage functionalization or API synthesis where purity standards are stringent [55,56].

Table 2. Selected comparative parameters of biocatalytic and non-biological catalytic systems

Feature	Biocatalysis (enzymes / whole cells)	Homogeneous catalysis	Heterogeneous catalysis	Key references
Catalyst type	Enzymes or living cells acting as biological catalysts (a subset of chemical catalysis)	Soluble molecular catalysts (metal complexes, organocatalysts)	Solid catalysts in a separate phase (metals, oxides, supported systems)	[1,3]
Operating conditions	Usually mild temperature, neutral pH, aqueous media;	Broad temperature/solve	Broad temperature/pressur	[1,16,57]

	sensitive to process conditions	nt range; often organic solvents	e range; tolerant to harsh conditions	
Selectivity	Often high chemo-, regio- and enantioselectivity; substrate scope may be narrow	Tunable selectivity via ligand/catalyst design	Often good chemoselectivity; asymmetric variants require special catalysts	[3,55,56,58]
Environmental impact	Potentially low, but strongly process-dependent; catalyst production and downstream processing can increase footprint	Variable; may involve toxic reagents/solvents	Often favorable due to catalyst reuse, but preparation cost/impact may be high	[1,10,59,60]
Catalyst stability	Often limited thermal and operational stability; prone to inhibition/denaturation	Variable; some highly stable, others air/moisture sensitive	Usually high thermal and mechanical stability	[57]
Catalyst reuse	Possible via immobilization or biomass recycling; activity loss may occur	Often difficult separation	Usually easy separation and reuse	[8,61]
Reactor productivity	Often lower volumetric productivity; limited by enzyme activity, mass transfer, cell density	Often high turnover frequency and loading	Often high space-time yields	[1,57]
Cofactor requirements	Frequently required (NAD(P)H, ATP); regeneration systems needed	Not required	Not required	[3,62]

API / pharma constraints	High selectivity advantageous, but requires removal of proteins, endotoxins, and biogenic impurities	Established regulatory frameworks	Established regulatory frameworks	[1,63]
Key limitations	Narrow substrate scope, cofactor dependence, inhibition, regulatory and scale-up challenges	Separation and recovery challenges	Diffusion limits, deactivation, metal leaching	[64][65][51,58]

The characteristics summarized in Table 2 are general trends and may vary significantly depending on catalyst design, reaction class, substrate loading, and downstream processing requirements.

2.4. Limitations of biocatalysis and mitigation strategies

To provide a balanced perspective, the major practical and technological limitations of biocatalytic systems are summarized below, as these factors are essential when comparing biocatalytic and non-biological catalytic approaches.

While biocatalysis offers compelling advantages in modern chemical synthesis, it is not without limitations. These constraints, ranging from substrate specificity and enzyme instability to cofactor dependency, product inhibition, mass transfer issues, and scalability, can impact the broader industrial adoption of enzyme-based systems. As summarized in Table 3, understanding these limitations is essential for overcoming them through enzyme engineering, process optimization, and integration with synthetic biology tools.

Table 3. Major limitations of biocatalysis and representative mitigation strategies

Limitation	Description	Representative examples	Mitigation strategies	Ref.
Narrow substrate scope and enzyme specificity	Native enzymes frequently exhibit high substrate specificity and limited activity toward non-natural or structurally complex compounds.	Alcohol dehydrogenases display reduced activity toward bulky ketones and heterocyclic substrates used in pharmaceutical synthesis.	Directed evolution, rational protein design, semi-rational engineering, enzyme discovery through genome mining.	[16,58, 66,67]
Enzyme instability under industrial conditions	Enzyme activity may decrease under elevated temperatures, extreme pH, organic solvents or mechanical stress.	Lipases and oxidoreductases often exhibit reduced stability in organic media used for poorly soluble substrates.	Enzyme immobilization, cross-linking, protein engineering, extremophile-derived enzymes, reaction engineering.	[1,8,19, 48,68]
Cofactor dependence	Oxidoreductases require costly cofactors (NADH, NADPH, ATP or FAD), making stoichiometric use economically impractical.	ADH- and KRED-catalysed reductions require continuous NAD(P)H regeneration.	Whole-cell catalysis, glucose dehydrogenase, formate dehydrogenase, phosphite dehydrogenase, electrochemical and photochemical regeneration.	[9,18,6 2,69]
Product inhibition and substrate toxicity	High substrate or product concentrations inhibit enzyme activity and reduce productivity.	Hydrolases and oxidoreductases may exhibit inhibition at high substrate loadings used in industrial reactors.	Fed-batch substrate feeding, in situ product removal, biphasic reaction systems, enzyme engineering.	[46,70, 71]
Mass transfer limitations in whole-cell systems	Cell membranes restrict transport of substrates and products, particularly hydrophobic compounds, while endogenous metabolism may consume intermediates.	Intracellular accumulation of aldehydes and limited uptake of bulky substrates in bacterial and yeast hosts.	Cell permeabilization, transporter engineering, metabolic engineering, cell-free biocatalysis, process optimization.	[72,73]

2.5. Biocatalytic approaches to isomer synthesis – selected examples

Isomer synthesis, particularly the preparation of structural, geometric, and stereoisomers, is a critical step in the production of fine chemicals, agrochemicals, and especially pharmaceuticals. Many bioactive compounds exist in chiral or stereospecific forms, with only one isomer exhibiting the desired biological function, while others may be inactive or toxic. Traditional chemical methods for isomer synthesis often involve multistep procedures, chiral auxiliaries, and metal-based catalysts, which are resource-intensive and environmentally problematic. Biocatalysis offers an efficient, selective, and sustainable alternative for synthesizing specific isomeric forms with high yield and purity [1,4,16,56].

2.5.1. Enzymatic racemization and deracemization

Enzymatic racemization refers to the interconversion of enantiomers, which is often used in dynamic kinetic resolution (DKR) processes (Figure 3). This strategy allows one enantiomer of a racemic mixture to be selectively converted into a desired product while the undesired enantiomer is continuously racemized, thus enabling full conversion of racemic substrates, so it refers to the conversion of a racemic mixture into a single, optically pure enantiomer [74]. Unlike classical resolution, which discards one enantiomer, deracemization theoretically allows full conversion of both enantiomers into the desired form, making it more atom-economical. In DKR, catalysts operate simultaneously. The racemase converts the unwanted enantiomer to the desired configuration, while the enantioselective catalyst selectively transforms one enantiomer into a product. Illustrative examples are as follows - the use of racemases and epimerases in conjunction with stereoselective hydrolases or transaminases applied in the synthesis of optically pure amino acids and secondary alcohols. Kroutil *et al.* [75] described a DKR process where mandelate racemase was combined with a stereoselective oxidoreductase to obtain enantiopure (S)-mandelic acid in high yield

and optical purity. This method avoids the 50% yield limitation of conventional kinetic resolution and aligns with green chemistry principles by minimizing waste.

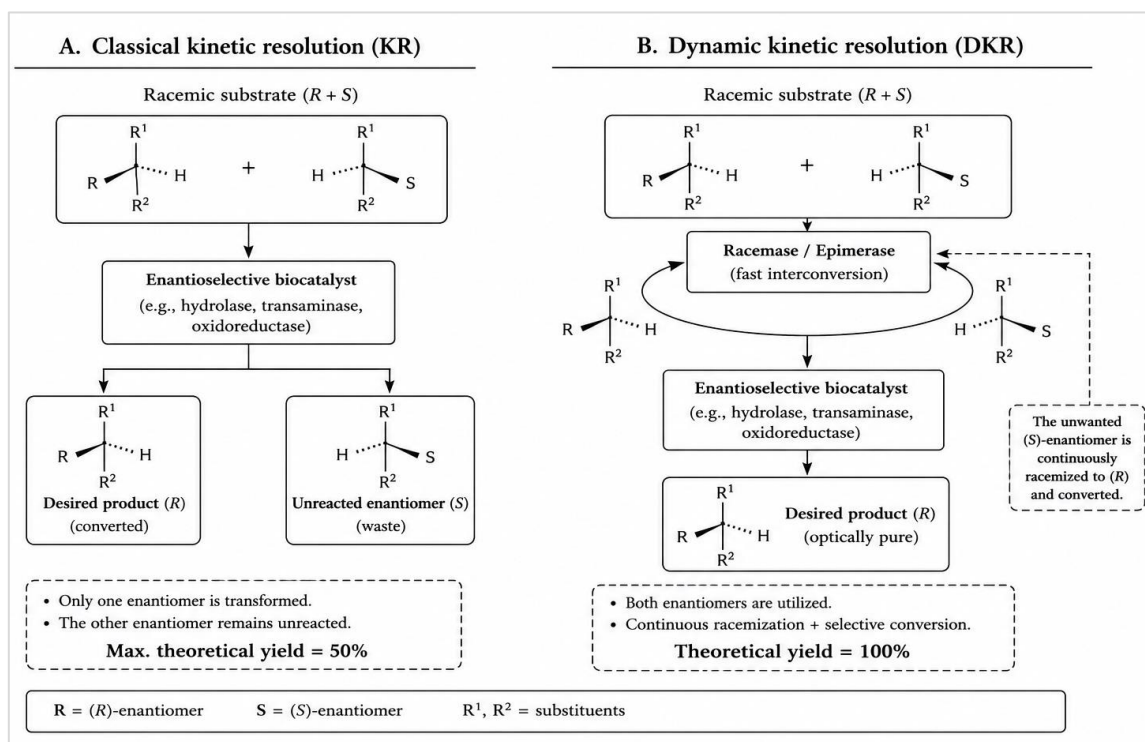


Figure 3. Schematic illustration comparing classical kinetic resolution (KR) and dynamic kinetic resolution (DKR). (designed by the author based on Faber [2], Kroutil *et al.* [76], and Turner [77])

Monoamine oxidase (MAO-N) has been used for oxidative deracemization of racemic amines, followed by stereoselective reduction to yield enantiopure products [78]. These cascade processes are efficient, environmentally benign, and amenable to scaling. Also another combinations have been applied such as amino acid racemases with lipases or transaminases [76,79]. For example, the resolution of racemic 1-phenylethanol can be achieved through lipase-mediated acylation of the (R)-enantiomer while a racemase converts the (S)-enantiomer into (R), thus enabling complete transformation to optically active (R)-acetate [80,81]. Stereoconversion involve the selective conversion of one enantiomer into its mirror image, typically via a two-step oxidation-reduction sequence like in case of alcohols, where the (S)-enantiomer can be oxidized to a ketone intermediate and then reduced back to the (R)-enantiomer

using a stereoselective reductase [82,83]. Enzymes such as alcohol dehydrogenases (ADHs) and Baeyer–Villiger monooxygenases have been engineered or combined in tandem for this purpose. This process is particularly attractive for deracemizing substrates with labile stereocenters or those difficult to resolve via classical methods. While cyclic deracemization involves enzymatic cycles that convert both enantiomers of a racemic mixture into a single product through parallel pathways. An example is the use of oxidase and reductase pairs in which one enantiomer is oxidized and then reduced stereoselectively to the desired isomer. Whole-cell systems are especially suited for such processes due to the presence of multiple cofactor-recycling enzymes and redox mediators that enhance turnover and maintain reaction balance [77,84].

Figure 4 summarizes the principal biocatalytic strategies employed for enzymatic racemization and deracemization of chiral amines and alcohols, integrating oxidative, reductive, and cofactor-coupled processes described in recent literature. Panel (a) illustrates the general principle of enzyme-mediated deracemization via dynamic kinetic resolution or stereoinversion, where an enantioselective oxidase (e.g., monoamine oxidase MAO-N) selectively oxidizes one enantiomer of a racemic amine to the corresponding imine intermediate, which is subsequently reduced stereoselectively to afford a single enantiomer in high optical purity [85,86]. Panel (b) depicts an oxidative–reductive cascade combining alcohol dehydrogenase (ADH) and amine dehydrogenase (AmDH), operating with NAD(H) recycling to enable full redox deracemization of secondary alcohols and amines, a strategy widely applied in industrial asymmetric synthesis [86,87]. Panel (c) represents MAO-N–mediated oxidative stereoinversion coupled with chemical or enzymatic reduction, showing how selective oxidation followed by non-selective or complementary reduction can shift enantiomeric composition toward a single configuration [77,85]. Finally, panel (d) shows cofactor-linked oxidoreductase cycles in which NAD(P)H-dependent enzymes operate within whole-cell systems to maintain redox balance and drive equilibrium toward the desired stereoisomer, indicating the importance of intracellular cofactor

regeneration systems such as glucose dehydrogenase-mediated NAD(P)H recycling [78,87].

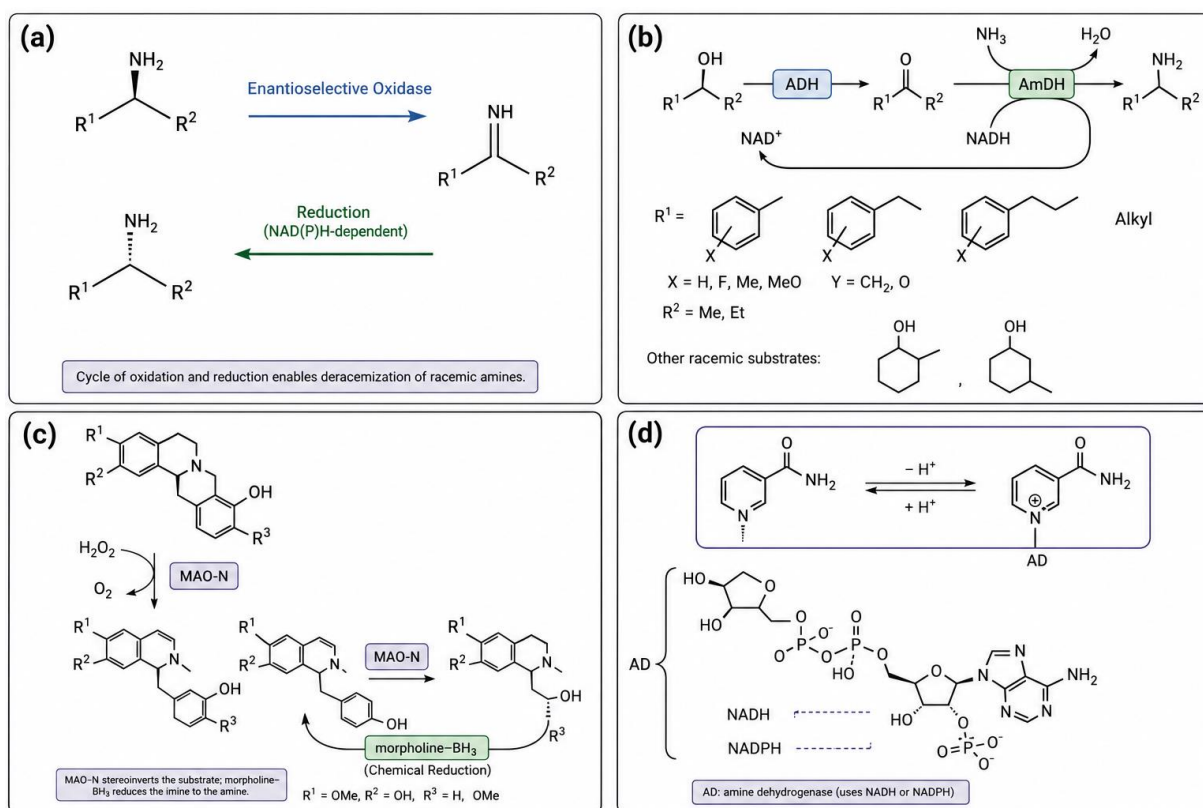


Figure 4. Representative biocatalytic strategies for enzymatic racemization and deracemization of chiral amines and alcohols. **(a)** General enzyme-mediated deracemization via enantioselective oxidation and reduction [85]. **(b)** Coupled alcohol dehydrogenase (ADH) and amine dehydrogenase (AmDH) cascade with NAD(H) recycling [86]. **(c)** Monoamine oxidase (MAO-N)-mediated stereoinversion coupled with reduction [86]. **(d)** Cofactor-dependent oxidoreductase cycles in whole-cell systems [87]. Adapted from Duan et al. (2019), Musa et al. (2019), and de Miranda et al. (2022), distributed under Creative Commons Attribution licenses (CC BY).

2.5.2. Asymmetric isomerization: alkene and ketone systems

Enzymes also catalyze geometric isomerization reactions, such as cis–trans conversion in alkenes or keto–enol tautomerism. Isomerases like maleate isomerase have been

explored for the synthesis of defined geometric isomers of unsaturated acids, which serve as precursors for polymers and surfactants [88]. Of relevance is the biocatalytic transformation of α , β -unsaturated ketones into optically enriched allylic alcohols or saturated ketones. These reactions are commonly mediated by ene-reductases (ERs), which catalyze the stereoselective hydrogenation of activated double bonds [89]. The Old Yellow Enzyme (OYE) family of flavin-dependent ERs has been extensively characterized and engineered for this purpose. These enzymes operate under mild conditions, require only NAD(P)H, as a reductant, and display exquisite stereoselectivity. For example, Müller *et al.* [89] demonstrated that ERs from *Thermus scotoductus* and *Lactobacillus kefir* could selectively reduce chalcone derivatives to (*S*)- or (*R*)-configured dihydrochalcones, depending on enzyme variant. These products are key intermediates in flavor and pharmaceutical industries.

The mechanistic basis of ER-mediated asymmetric hydrogenation has been elucidated through crystallographic, kinetic, and mutagenesis studies. Figure 5 provides a mechanistic and structural overview of flavin-dependent ene-reductases (ERs) belonging to the OYE family, illustrating both their catalytic cycle and substrate scope. As shown in Figure 5a, ER catalysis proceeds via a two-step reductive–oxidative cycle involving a tightly bound flavin mononucleotide (FMN) cofactor. In the reductive half-reaction, NAD(P)H transfers a hydride to FMN, generating the reduced flavin (FMNH₂). In the oxidative half-reaction, the reduced flavin donates a hydride to the β -carbon of an activated α,β -unsaturated substrate bearing an electron-withdrawing group (EWG), while a conserved tyrosine residue delivers a proton to the α -carbon, resulting in stereoselective trans-hydrogenation of the C=C bond [90]. The precise orientation of the substrate within the active site determines whether hydride delivery occurs to the re- or si-face, thereby controlling formation of (*R*)- or (*S*)-configured products. Figure 5b further shows the broad substrate tolerance of OYE-family enzymes, including cyclic enones, aromatic enals, and chalcone derivatives, as well as cases of reversed stereopreference depending on substrate geometry and enzyme

variant [91]. Importantly, enzymes from *Thermus scotoductus* and *Lactobacillus kefir* demonstrate distinct stereochemical outcomes in chalcone reduction due to subtle differences in active-site architecture, indicating the tunability of ERs through protein engineering [90,91].

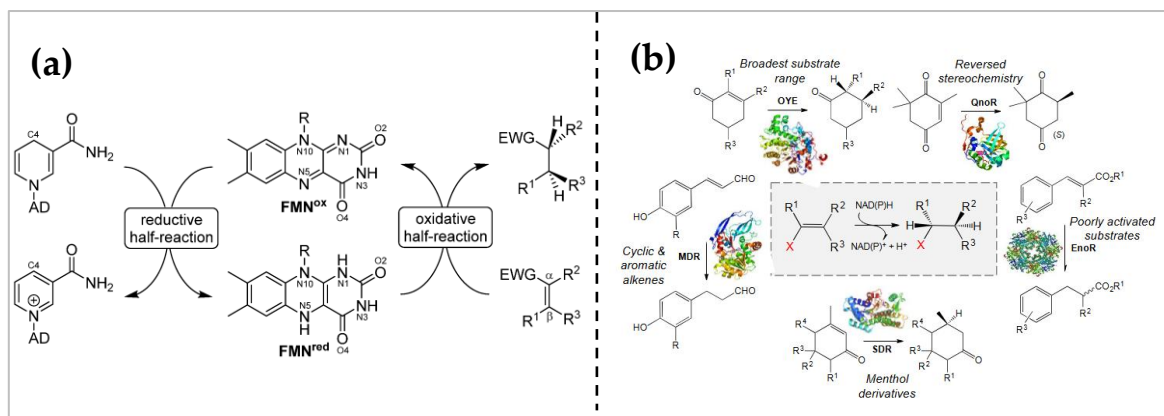


Figure 5. Mechanistic overview and substrate scope of flavin-dependent ene-reductases (Old Yellow Enzyme family). **(a)** Catalytic cycle showing NAD(P)H-dependent reduction of flavin mononucleotide (FMN) and subsequent stereoselective hydrogenation of activated C=C bonds [90]. **(b)** Representative substrate classes and stereochemical outcomes of ene-reductase-catalyzed transformations [91]. *Adapted from multiple sources including Scholtissek et al. (2017) and Toogood and Scrutton (2018), with permission from the American Chemical Society. License number: 6234290248185.*

2.5.3. Epimerization and diastereomer interconversion

Epimerases and racemases are stereoinverting enzymes that catalyze configurational inversion at specific stereogenic centers without altering the molecular backbone of the substrate. These enzymes typically operate via reversible proton abstraction–reprotonation mechanisms or transient oxidation–reduction steps that proceed through planar intermediates, thereby enabling stereochemical inversion under thermodynamic control. Such transformations are fundamental in carbohydrate metabolism, amino acid biosynthesis, and the production of pharmaceutically relevant stereoisomers [66]. A prototypical example is UDP-glucose 4-epimerase (GALE),

which catalyzes the reversible C4 epimerization of UDP- α -D-glucose to UDP- α -D-galactose in the Leloir pathway of galactose metabolism. The reaction proceeds via a tightly bound NAD⁺ cofactor that transiently oxidizes the C4 hydroxyl group to form a 4-keto-UDP-hexose intermediate. Subsequent stereospecific hydride return from the opposite face of the planar carbonyl intermediate results in inversion of configuration at C4. Notably, the NAD⁺/NADH pair remains enzyme-bound during the catalytic cycle, rendering the overall process redox-neutral despite proceeding through an oxidative intermediate [92,93].

Likewise, epimerases have been employed in the transformation of hydroxy acids and α -amino acids into their less common isomers. These isomers often possess distinct pharmacokinetic properties or serve as precursors for semi-synthetic antibiotics and enzyme inhibitors. However, most epimerases are substrate-specific and reversible, posing challenges in achieving high conversions without equilibrium shifting strategies [94]. Recent work has focused on coupling epimerases with selective downstream enzymes or in situ trapping of products to drive the equilibrium toward desired isomers. For example, combining *D*-tagatose epimerase with a selective aldolase has allowed the stereoselective synthesis of rare C7 sugars from glyceraldehyde and *D*-fructose, opening routes to high-value specialty carbohydrates [95].

Figure 6 illustrates the mechanistic approach of stereoinversion catalyzed by mandelate racemase from *Pseudomonas putida*, a paradigmatic Mg²⁺-dependent enzyme operating through a 1,1-proton transfer mechanism. As shown in Figure 6, racemization proceeds via abstraction of the α -proton adjacent to the carboxylate group by a catalytic base (typically Lys166 or His297, depending on orientation), generating a planar enolate intermediate stabilized by coordination to an active-site Mg²⁺ ion. This metal ion plays a critical role in lowering the pK_a of the α -proton and stabilizing negative charge development on the oxygen atoms of the enolate, thereby facilitating reversible proton transfer [95,96]. Subsequent reprotonation from the

opposite face of the enolate intermediate yields the inverted enantiomer, completing the racemization cycle without alteration of the carbon skeleton. The same enolate intermediate can also undergo competing β -elimination under certain conditions, although this pathway is minimized in the native enzyme due to precise geometric constraints within the active site [96].

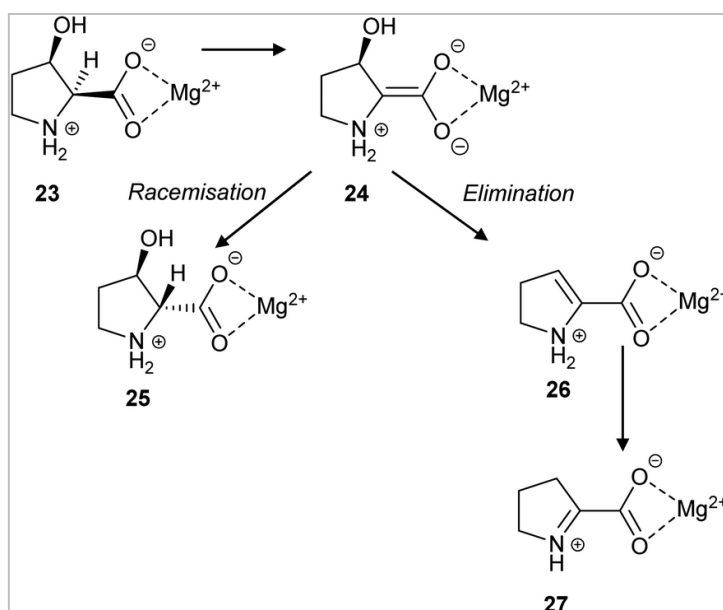


Figure 6. Mechanism of stereoinversion catalyzed by mandelate racemase from *Pseudomonas putida*, illustrating Mg²⁺-stabilized enolate intermediate formation and reprotonation leading to racemization. Adapted from Lloyd et al. (2021) under the Creative Commons Attribution License (CC BY) [96].

2.5.4. Alcohol dehydrogenases and ketoreductases

Alcohol dehydrogenases and ketoreductases are pivotal biocatalysts in asymmetric reduction of ketones and aldehydes to their corresponding alcohols. An important aspect governing the industrial implementation of alcohol dehydrogenases and ketoreductases is the development of efficient hydrogen-transfer systems coupled with in situ cofactor regeneration. Among the available approaches, substrate-coupled regeneration employing 2-propanol has become one of the most widely adopted strategies because it enables simultaneous oxidation of isopropanol to acetone and regeneration of NADH or NADPH without the need for an additional auxiliary

enzyme. Studies by Prof. Szaleniec and co. on (*S*)-1-phenylethanol dehydrogenase from *Aromatoleum aromaticum* showed that this hydrogen-transfer approach enables highly stereoselective reduction of ketones and β -keto esters to optically pure alcohols while maintaining efficient cofactor recycling. Their work further revealed the influence of substrate electronic properties and enzyme-substrate interactions on catalytic activity and stereoselectivity, providing valuable insights for the coherent design and optimisation of industrial alcohol dehydrogenases [97,98]. These findings show how mechanistic understanding, computational modelling and protein engineering can be integrated to improve oxidoreductase performance for asymmetric synthesis.

Alcohol dehydrogenases and ketoreductases ability to catalyze the conversion of prochiral ketones into chiral alcohols (Figure 7) has been widely exploited in the manufacture of active pharmaceutical ingredients (APIs), including intermediates for β -blockers, statins, and anti-HIV agents. Yamanaka *et al.* demonstrated that cyanobacterial strains such as *Synechocystis* sp. PCC 6803 can reduce cinnamaldehyde selectively to cinnamyl alcohol with >98% yield under illumination, while the same reaction proceeds only minimally in the dark, confirming that light is directly coupled to cofactor regeneration and reaction efficiency. Thus, the role of light in these systems is not to activate the substrate directly, but to sustain intracellular reducing power via photosynthesis, distinguishing photobiocatalysis from conventional ADH/KRED processes while maintaining the same fundamental hydride-transfer chemistry at the enzyme level [99].

One of the most cited applications is the production of (*S*)-1-phenylethanol and (*R*)-pantolactone using engineered ADHs and KREDs with enantiomeric excesses exceeding 99% [1,16,100]. The advantage of these enzymes lies in their ability to produce single enantiomers with high yield and minimal side reactions, eliminating the need for chiral resolution steps and reducing solvent and reagent use. Engineered libraries of KREDs have been developed to expand substrate scope, increase

thermostability, and allow operation in organic cosolvents. Savile *et al.* [55] described the successful deployment of an evolved transaminase–KRED system for sitagliptin synthesis, replacing a six-step metal-catalyzed process with a one-step biotransformation, thereby significantly improving environmental performance and cost-efficiency.

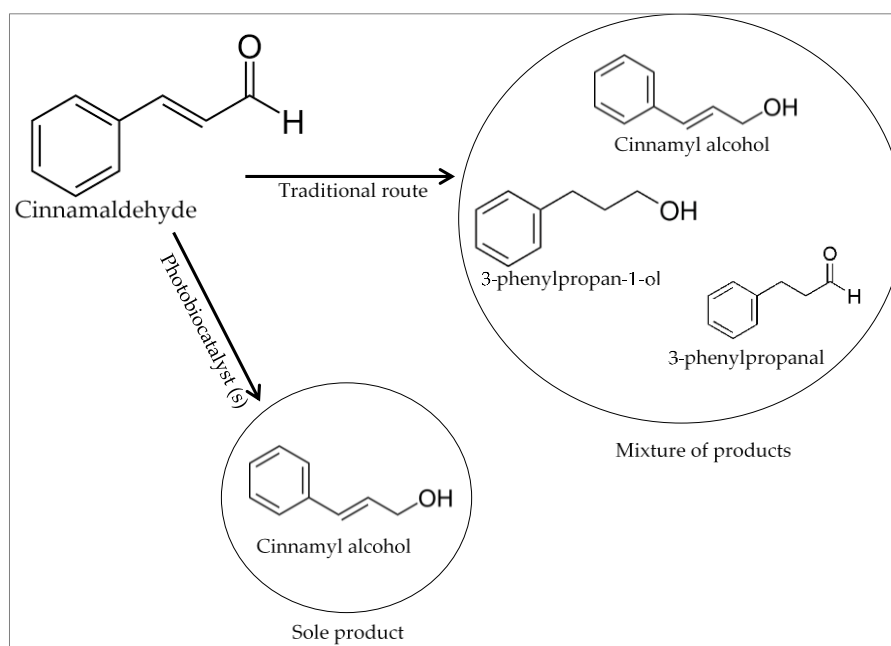


Figure 7. Chemoselective reduction of cinnamaldehyde to cinnamyl alcohol by *Synechocystis* sp. PCC 6803 under light-driven conditions with photosynthetic NADPH regeneration. Scheme designed and drawn by the author based on experimental observations reported by Yamanaka *et al.* [99].

In the traditional catalytic route involving chemical synthesis (Fig. 7), reduction is typically performed using heterogeneous metal catalysts (e.g., Pt-, Pd-, or Ni-based systems under hydrogen), which lead to multiple parallel reactions, including C=O reduction, C=C hydrogenation, and, in some systems, additional side reactions such as ring hydrogenation or dehydration, depending on catalyst composition and process conditions. In contrast, alcohol dehydrogenases (ADHs) and ketoreductases (KREDs) catalyze chemoselective carbonyl reduction via NAD(P)H-dependent hydride transfer, typically preserving adjacent double bonds and aromatic systems. In

photobiocatalytic variants using cyanobacteria, the reduction step is still enzymatic (ADH/KRED-type), while illumination serves to drive photosynthetic cofactor regeneration (NADPH supply) rather than direct substrate activation. This light-driven cofactor recycling mechanism has been experimentally demonstrated in cyanobacterial reductions of cinnamaldehyde and related substrates under illuminated conditions [66,99,101].

In addition to coupled multi-enzyme cofactor recycling systems, a practically important and industrially established strategy for NAD(P)H regeneration involves alcohol dehydrogenases operating with sacrificial alcohol substrates, most commonly isopropanol or ethanol. In such systems, the cofactor is regenerated in situ through oxidation of a low-cost alcohol donor to a volatile carbonyl compound (e.g., acetone), which can be readily removed and thereby shifts the reaction equilibrium toward product formation. This method is widely applied in preparative and industrial ADH/KRED reductions because of its simplicity, scalability, and compatibility with API intermediate synthesis [63,66,101]. Beyond their classical use in ester hydrolysis and transesterification, lipases have also demonstrated catalytic promiscuity and have been applied in non-traditional bond-forming reactions. These include multicomponent reactions such as the Ugi reaction (four-component coupling of an amine, carbonyl compound, isocyanide, and carboxylic acid to form α -acylamino amides), the Passerini reaction (three-component coupling of a carbonyl compound, isocyanide, and carboxylic acid to form α -acyloxy amides), and Knoevenagel condensations (carbon-carbon bond formation between aldehydes/ketones and activated methylene compounds to give α,β -unsaturated products). Enzyme-mediated and lipase-assisted variants of these transformations have been reported by Ostaszewski, Koszelewski and co-workers, showing that biocatalysts can support selective multicomponent and C-C bond-forming processes beyond their native hydrolytic roles (Figure 8) [102,103].

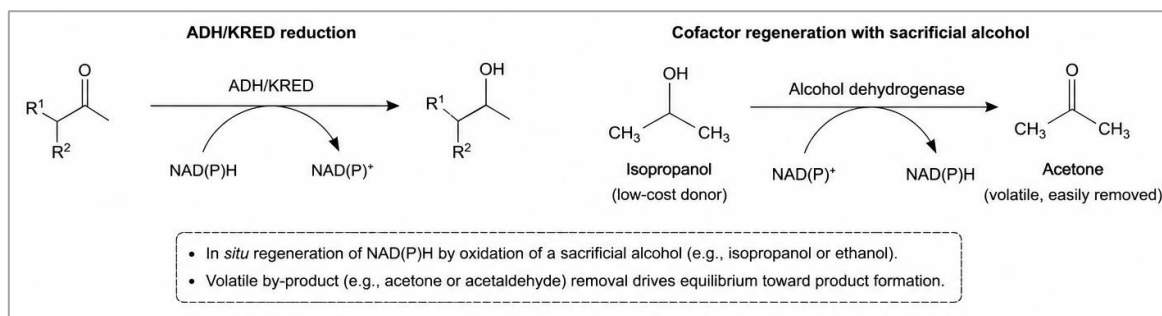


Figure 8. Schematic illustration of cofactor regeneration by alcohol dehydrogenases using sacrificial alcohols and representative examples of lipase catalytic promiscuity. (designed by the author based on Dudzik *et al.* [98]; Koszelewski & Ostaszewski [103]; Kowalczyk *et al.* [104]).

2.5.5. Lipases and esterases

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) catalyze the hydrolysis of ester bonds, producing alcohol and acids. They are among the most robust and widely used enzymes in industrial catalysis, owing to their broad substrate range, high stability, and functionality in organic solvents and biphasic systems [17,48]. A classic industrial example is the resolution of racemic esters using lipases to obtain enantiopure alcohols or acids. CALB (*Candida antarctica* lipase B), immobilized on acrylic resin (e.g., Novozym 435), is a gold standard in such kinetic resolutions [17]. Lish *et al.* [17] reported the application of CALB in biodiesel synthesis and in resolving racemic ibuprofen esters under mild, environmentally friendly conditions [105]. Moreover, lipases are commonly used in non-hydrolytic reactions such as esterification and transesterification in water-restricted systems, further expanding their industrial role in biolubricants, surfactants, and pharmaceuticals.

Beyond their well-established hydrolytic activity, lipases have attracted considerable attention because of their catalytic promiscuity, defined as the ability to catalyze reactions that differ from their native physiological function. This property has substantially broadened the scope of lipase-mediated synthesis, enabling the formation of carbon–carbon and carbon–heteroatom bonds under environmentally

benign conditions. In particular, the research group of Prof. Ryszard Ostaszewski has demonstrated that lipases can efficiently catalyse non-conventional transformations, including Knoevenagel condensations and related tandem chemoenzymatic processes. These studies showed that lipase-catalysed Knoevenagel reactions proceed through genuine promiscuous catalysis rather than secondary hydrolytic mechanisms, thereby expanding the synthetic utility of hydrolases beyond classical esterification and hydrolysis [103,104]. In addition, the same research group reported the successful application of lipase promiscuity in the Markovnikov addition of H-phosphites to vinyl esters for the synthesis of biologically active α -acyloxy phosphonates, further showing the versatility of lipases in sustainable chemoenzymatic synthesis.

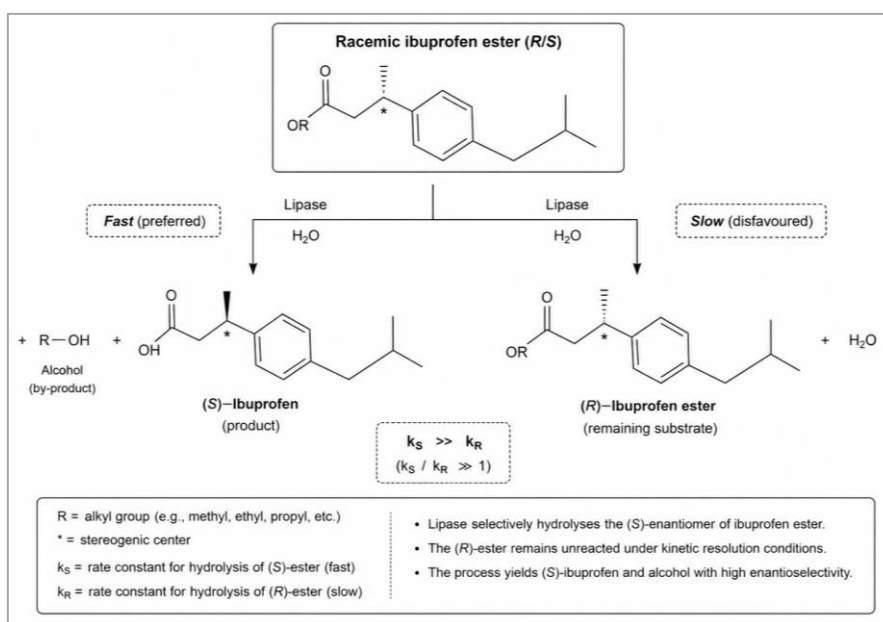


Figure 9. Enantioselective kinetic resolution of racemic ibuprofen esters catalyzed by lipase, showing preferential hydrolysis of the (S)-enantiomer. *Adapted from Ahmed and Thomas (2025), Scientific Reports, under the Creative Commons Attribution License (CC BY) [105].*

A schematic illustration presented in Figure 9 shows the principle of lipase-catalysed enantioselective kinetic resolution of racemic ibuprofen esters, in which the high enantioselectivity enables preferential hydrolysis of the (S)-enantiomer over the (R)-

enantiomer. In the presented reaction sequence, the racemic ibuprofen ester mixture containing both (*R*)- and (*S*)-enantiomers is exposed to lipase under controlled conditions. The (*S*)-ibuprofen ester is preferentially recognized and converted, undergoing rapid hydrolysis to yield the corresponding (*S*)-ibuprofen acid, while the (*R*)-ester reacts much more slowly and remains largely unconverted. This rate difference forms the basis of kinetic resolution, where two products are obtained simultaneously: an enantiomerically enriched acid and the unreacted opposite ester enantiomer. As discussed by Ahmed and Thomas (2025), such systems can be further integrated into dynamic kinetic resolution (DKR) configurations when combined with in situ racemization, enabling theoretical yields above the classical 50% limit of simple kinetic resolution. Their reactor-scale modeling in a membrane bioreactor perspective shows how enzyme selectivity, diffusion, and phase behavior jointly determine conversion profiles and enantiomeric excess over time [105]. In enzymatic kinetic resolution, the efficiency of enantiomer discrimination is quantitatively expressed by the enantioselectivity factor (*E*), which represents the ratio of the specificity constants (or apparent reaction rates) for the fast- and slow-reacting enantiomers. Unlike the general selectivity factor (*S*) commonly used in chemical catalysis, biocatalytic kinetic resolutions conventionally employ the *E* value as the principal measure of stereoselectivity.

2.5.6. Amidases and proteases

Amidases catalyze the hydrolysis of amide bonds to yield carboxylic acids and amines. They are widely employed in the synthesis of β -lactam antibiotics, chiral amides, and agrochemicals. For instance, amidase-mediated hydrolysis of mandelic acid amides provides enantiomerically pure mandelic acid, a key intermediate in semi-synthetic penicillins and cephalosporins [106]. The reaction pathway for penicillin G acylase-mediated β -lactam processing is presented in Figure 10.

Figure 10 shows the penicillin G acylase (PGA)-catalyzed hydrolytic cleavage of penicillin G to yield 6-aminopenicillanic acid (6-APA) and phenylacetic acid, which represents a cornerstone step in the industrial production of semi-synthetic β -lactam antibiotics. In this reaction sequence, the enzyme selectively recognizes the side-chain amide bond of penicillin G and catalyzes its hydrolysis under mild aqueous conditions. Mechanistically, the process proceeds through substrate binding in the active site followed by nucleophilic attack on the amide carbonyl by the catalytic serine residue, forming an acyl-enzyme intermediate. Subsequent hydrolysis of this intermediate releases phenylacetic acid and regenerates the free enzyme, while the conserved β -lactam nucleus is preserved as 6-APA. This intermediate serves as a universal scaffold for the synthesis of numerous clinically important β -lactam antibiotics through subsequent acylation with alternative side chains. As discussed in recent process and engineering studies, improvements in PGA performance have been achieved through enzyme immobilization, protein engineering, and reaction system optimization, enabling higher substrate loading, enhanced operational stability, and improved space-time yields in industrial bioreactors. These developments highlight amidase-class enzymes not only as hydrolytic tools but also as key enabling catalysts in antibiotic manufacturing and β -lactam diversification strategies [107].

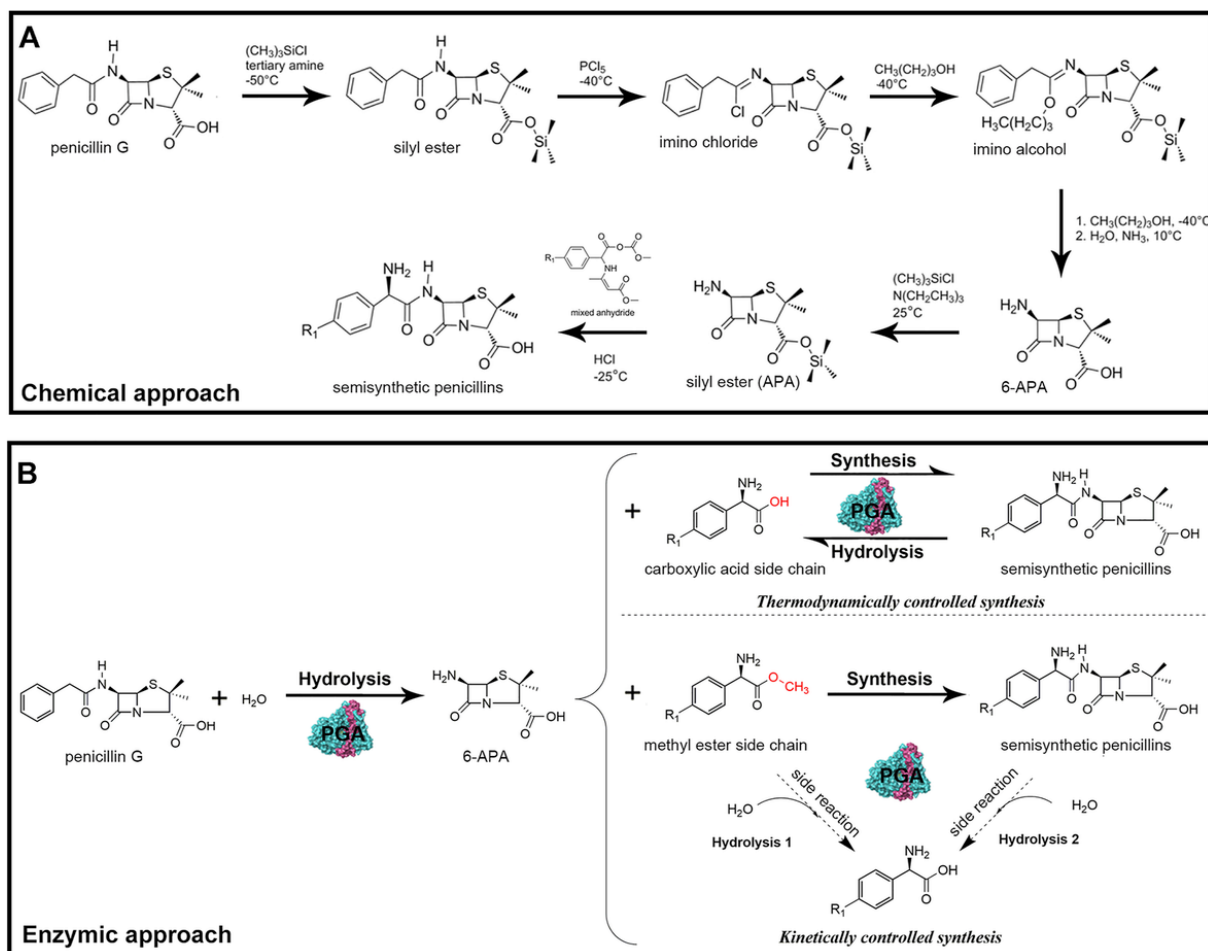


Figure 10. Penicillin G acylase-catalyzed hydrolysis of penicillin G to 6-aminopenicillanic acid (6-APA), a key intermediate in β -lactam antibiotic synthesis. Adapted from Pan et al. *Frontiers in Bioengineering and Biotechnology*, under the Creative Commons Attribution License (CC BY) [107].

Proteases, on the other hand, are extensively used in peptide synthesis and modification. While typically associated with protein degradation, engineered proteases can perform selective hydrolysis of synthetic peptides or catalyze transpeptidation in peptide drug development [108]. These enzymes operate with exquisite sequence and bond specificity, making them ideal for modification of biologically active peptides under mild conditions. Biocatalytic hydrolysis is attractive for its low energy requirements, operational simplicity, and ability to avoid the use of corrosive acids or bases typically employed in chemical hydrolysis. Furthermore,

hydrolases are generally non-toxic, biodegradable, and readily available from microbial sources. In detergent manufacturing, food processing, and pharmaceutical industries, hydrolases have largely replaced conventional catalysts. The enzymatic production of aspartame, synthesis of L-carnitine, and resolution of non-steroidal anti-inflammatory drugs (NSAIDs) are all examples where hydrolases have demonstrated superior yield and selectivity [1,109].

Generally, biocatalytic reduction and hydrolysis reactions offer powerful, green, and highly selective tools for modern chemical synthesis. Through continuous advances in enzyme discovery, engineering, and cofactor management, these catalytic processes now rival or surpass traditional chemical methods in both efficiency and sustainability. As industries continue to adopt biocatalysis for large-scale synthesis, particularly in the context of green manufacturing mandates, reductive and hydrolytic enzymes will remain indispensable tools.

Table 4. Summary of selected enzymatic reaction types for stereoselective isomer synthesis and applications.

Reaction type	Enzyme class	Representative product	Application
Asymmetric reduction	Alcohol dehydrogenases (ADHs), Ketoreductases (KREDs), Imine reductases (IREDs)	Chiral alcohols, chiral amines	- Engineered transaminase for sitagliptin precursor amination, replacing rhodium catalysis - KRED-catalyzed reduction of acetophenone to (S)-1-phenylethanol with NADH regeneration
Hydrolytic resolution	Lipases (e.g., CALB), Esterases, Epoxide hydrolases	Enantiopure esters, alcohols, diols	CALB-mediated resolution of β-amino acid esters with >99% ee

			• <i>A. radiobacter</i> epoxide hydrolase desymmetrizing racemic styrene oxides
Racemization / Epimerization	Racemases, Epimerases	α -Amino acids, secondary alcohols	• Mandelate racemase enabling DKR of phenylethylamine via transaminase coupling • DKR of N-acylated amino acids using racemase/acylase system
Isomerization	Isomerases (e.g., glucose isomerase), Prenyltransferases	Fructose, lactulose, geometric olefins	• Glucose to fructose conversion in high-fructose corn syrup (HFCS) production • Stereoselective olefin rearrangement by engineered double bond isomerases

These enzymatic strategies offer scalable, efficient, and stereoselective routes to isomers that are otherwise challenging to access using classical synthetic chemistry. The diversity of reaction types and enzyme classes (Table 4) shows the flexibility of biocatalysis in asymmetric synthesis, with ongoing innovations in enzyme engineering and high-throughput screening further expanding this biochemical toolkit.

2.6. Microbial cell factories and their applications in biocatalysis

Microbial cell factories refer to genetically optimized microorganisms capable of producing target metabolites, enzymes, or value-added chemicals via biocatalytic pathways. These biological systems have become a foundational platform in modern biotechnology, enabling the sustainable synthesis of pharmaceuticals, agrochemicals, biofuels, and specialty chemicals through metabolic engineering and synthetic biology tools [1,2,4,46]. Unlike isolated enzyme systems, microbial cell factories provide whole cell biocatalysis where enzymatic transformations occur within a living or non-growing but metabolically active microbial host. These systems offer advantages such

as cofactor self-sufficiency, robustness under operational conditions, tolerance to toxic intermediates, and the ability to catalyze complex multistep reactions [18,23,46].

The concept of microbial cell factories involves redirecting and optimizing the native metabolic network of a microorganism to produce desired products efficiently and selectively. This often requires heterologous expression of biosynthetic genes, deletion of competing pathways, optimization of precursor supply, and control of redox balance, energy flux, and metabolite export [46,72]. *Escherichia coli* and *Saccharomyces cerevisiae* are the most commonly used chassis organisms due to their well-characterized genetics, ease of transformation, and scalable cultivation. However, non-conventional hosts such as *Bacillus subtilis*, *Corynebacterium glutamicum*, and *Pseudomonas putida* are increasingly favored for their stress resistance, native metabolic traits, and secretion capabilities [46,72,110].

One of the core advantages of using whole cells as biocatalysts is their ability to regenerate cofactors in situ. Redox reactions catalyzed by dehydrogenases, oxygenases, or reductases require cofactors such as NADH, NADPH, or FAD, which can be continuously regenerated within the microbial metabolism [18,69]. This eliminates the need for costly exogenous cofactor supplementation and simplifies reactor operation. Moreover, whole cells protect sensitive enzymes from degradation by encapsulating them in a natural environment, while maintaining high local concentrations of cofactors and stabilizing multienzyme complexes [48]. This cellular context also enables multistep reactions via intracellular cascades,

Microbial cell factories have been successfully applied in a wide range of industrial and pharmaceutical contexts. In the synthesis of chiral amines, engineered strains of *E. coli* and *Pseudomonas putida* expressing ω -transaminases have demonstrated superior yields and stereoselectivity compared to isolated enzymes, owing to cofactor recycling and better enzyme stability [46,55]. In the field of statin production, microbial strains producing the polyketide compactin (a precursor to simvastatin) have been optimized

for enhanced productivity through modular pathway engineering and precursor supply enhancement [110]. Similarly, recombinant *Corynebacterium glutamicum* strains have been developed to overproduce amino acids such as L-lysine, L-tryptophan, and L-valine, which serve as key precursors for pharmaceuticals and supplements [72,111].

Cyanobacterial cell factories have also shown increasing promises in light-driven biotransformations. For instance, *Synechococcus elongatus* and *Synechocystis* sp. have been engineered to produce fatty acids, isoprenoids, and even stereochemically pure alcohols by harnessing CO₂ and light through photosynthetic electron transport [44,45]. These systems align with sustainable development goals by providing carbon-neutral alternatives for chemical production. Furthermore, microbial platforms are now being employed to produce complex natural products such as artemisinin, taxol precursors, and erythromycin through complete reconstruction of plant or bacterial biosynthetic pathways [111,112].

In contrast to conventional heterotrophic microbial cell factories such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum*, which rely on organic carbon sources and well-established fermentation infrastructure, cyanobacterial systems operate as photoautotrophic or photomixotrophic platforms that derive reducing power and energy directly from light-driven photosynthetic electron transport (Table 5). This fundamental metabolic difference leads to distinct advantages and constraints. Standard bacterial hosts typically provide faster growth rates, higher cell densities, broader genetic toolkits, and more predictable scale-up behavior in dark fermentation processes. Cyanobacteria, by contrast, enable direct coupling of solar energy and CO₂ assimilation to biocatalytic transformations, which is attractive from a sustainability perspective, but often associated with lower volumetric productivities, stronger sensitivity to cultivation conditions, and more complex physiological regulation. As a result, while heterotrophic microbial factories currently dominate industrial biocatalysis, cyanobacterial systems are increasingly explored as complementary

platforms for light-driven redox biotransformations and carbon-neutral synthesis routes [113,114].

Deployment of light-driven whole-cell biocatalysis also introduces engineering and process challenges that are not present in conventional non-phototrophic systems (Table 5). Photobiocatalytic processes require controlled light delivery, management of self-shading effects at higher biomass densities, and reactor geometries that ensure homogeneous photon flux distribution. Light intensity and spectral composition directly influence photosynthetic redox balance and therefore catalytic performance, making process control more complex than in dark bioreactors. In addition, photoinhibition, oxygen evolution, and diurnal metabolic regulation can affect enzyme expression and reaction rates. These factors complicate scale-up and reproducibility compared to standard heterotrophic whole-cell catalysis, where energy supply and redox balance are typically managed through substrate feeding and aeration strategies. Consequently, while cyanobacteria offer unique opportunities for light-powered biotransformations, their practical implementation requires specialized photobioreactor design and tailored process optimization [31,113,115].

Table 5. Comparative summary of heterotrophic whole-cell biocatalysts and cyanobacterial photobiocatalytic systems

Feature	Standard heterotrophic whole-cell systems (e.g., <i>E. coli</i> , yeast)	Cyanobacterial whole-cell systems
Primary energy source	Organic carbon substrates (glucose, glycerol, etc.)	Light-driven photosynthesis + CO ₂ fixation
Reducing power	Generated from metabolic oxidation of carbon sources	Generated directly via photosynthetic electron transport
Typical reactor mode	Dark stirred-tank bioreactors	Photobioreactors requiring controlled illumination
Growth rate & cell density	Typically, fast growth; high cell densities achievable	Slower growth; lower biomass density due to light limitation

Process control	Substrate feed and aeration control redox and energy balance	Light intensity, spectrum, and photoperiod affect metabolism and catalysis
Scale-up maturity	Highly mature industrial scale-up	Scale-up more complex due to photon transfer limits
Typical industrial use	API intermediates, chiral alcohols/amines, fine chemicals	Emerging use in photobiocatalysis and light-driven redox reactions
Representative examples	Recombinant <i>E. coli</i> ADH reductions for chiral alcohol production, industrially applied	Light-driven P450 and ene- reductase reactions in cyanobacteria, proof-of-concept photobiocatalysis
Key limitations	Cofactor cost (requires recycling), substrate toxicity	Light penetration limits, photoinhibition, lower volumetric productivity

2.7. Physiological diversity and biotechnological potential of cyanobacterial metabolites

The exceptional metabolic diversity of cyanobacteria is a direct significance of their long evolutionary history and extraordinary physiological adaptability. Cyanobacteria were among the first organisms to perform oxygenic photosynthesis and have subsequently colonized virtually every illuminated habitat on Earth, including freshwater and marine ecosystems, hypersaline environments, hot springs, polar regions, deserts, acidic and alkaline waters, as well as endolithic and symbiotic niches [116,117]. Their successful ecological radiation has been facilitated by remarkable metabolic plasticity, allowing them to adjust photosynthetic electron transport, carbon and nitrogen metabolism, osmotic regulation, and cellular redox balance in response to changing environmental conditions. Consequently, cyanobacteria possess an extensive repertoire of biosynthetic pathways, including non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and hybrid PKS–NRPS systems, which generate structurally diverse natural products involved in cellular protection, chemical defence, interspecies communication and ecological competition [118,119]. This

biochemical versatility not only reinforces the ecological success of cyanobacteria but also provides an exceptional reservoir of biologically active metabolites with considerable potential for pharmaceutical, agricultural and industrial biotechnology applications [120,121].

Cyanobacteria have been explored not only as photobiocatalysts but they are also meaningful for other fields of biotechnology. The metabolic plasticity of cyanobacteria enables the biosynthesis of a wide array of primary and secondary metabolites with applications spanning healthcare, agriculture, and environmental remediation. Cyanobacteria are prolific producers of bioactive compounds, including cyanotoxins, alkaloids, carotenoids, and polysaccharides [122]. Welker and von Döhren [123] reviewed the metabolic diversity and biosynthetic pathways of cyanobacterial peptides such as microcystins and anatoxins, which exhibit cytotoxic and neurotoxic activities, respectively, and are under investigation for pharmacological applications.

These metabolites (Table 6) are products of core processes such as photosynthesis and carotenogenesis, and their production can be modulated by environmental factors. Primary metabolites are crucial and life supporting, while secondary metabolites are generally not essential for survival but confer ecological advantages, particularly under stress conditions. Primary metabolites produced by cyanobacteria include essential lipids, proteins, and antioxidant molecules, which can be harnessed industrially to produce food additives, bioplastics, natural dyes, and biofertilizers [124–129]. Secondary metabolites include a wide array of bioactive compounds such as alkaloids, phenolic compounds, steroids, essential oils, antibiotics, and cyanotoxins [130–137].

Cyanobacteria's relatively simple genetic architecture facilitates genetic manipulation and biosynthetic optimization, enabling the tailored production of valuable metabolites. Such manipulations are often directed by the cellular response to environmental stressors like nutrient limitation and altered light regimes, which can

significantly modulate photosynthetic and metabolic pathways [138,139]. For example, a study by Chen *et al.* [140] demonstrated that growth under nutrient-deficient and high-light conditions induced substantial production of biopolymers, lipids, essential oils, and starches, in contrast to growth in nutrient-rich media. This two-phase cultivation strategy clearly delineates the stress-induced enhancement of secondary metabolism in cyanobacteria [140,141]. Further evidence by Singh *et al.* [142] described how the Methylerythritol 4-phosphate (MEP) pathway is exploited in cyanobacteria for the synthesis of chlorophylls, fatty acids, and hormonal metabolites, many of which possess potent antimicrobial, anticancer, and antibiotic activities that support their relevance in pharmaceutical applications [142,143].

Table 6. Cyanobacterial metabolites and their applications

Metabolite class	Representative compounds	Functions in cyanobacteria	Biotechnological/pharmaceutical applications	Reference
Phenolic Acids	Gallic acid, Vanillic acid, Chlorogenic acid, Caffeic acid, Ferulic acid, Syringic acid, Gentisic acid	Antioxidant defense against ROS; UV and metal stress tolerance	Anti-aging (gallic, ferulic), skin care (ferulic), cardiac health (syringic, gentisic), antimicrobial	[144–147]
Vitamins	B-complex (B12), C, A, D, E, K	Growth, differentiation, cofactor in enzymatic reactions; stress-induced synthesis	Dietary supplements; Spirulina as a rich source of B12, β -carotene, and E; vitamin D via UV stress	[148–150]
Peptides	Microviridins, Cyanobactins, NRPs, PKs	Secondary metabolism; defense and competition	Antitumor, antimicrobial, antiviral drug leads; structural diversity for drug design	[123,151,152]
Terpenoids	β -carotene, Zeaxanthin, Myxoxanthophyll,	Photoprotection, oxidative stress response, signaling	Anti-aging (carotenoids), antimicrobial (sesterterpenes),	[153–155]

Canthaxanthin, Geosmin, 2-MeBHPs	environmental biomarkers (2-MeBHPs, geosmin)
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2.8. Rare earth elements (REE) and their bioaccumulation mechanisms in cyanobacteria

REEs are essential components in a wide array of high-tech applications, including electric vehicles, wind turbines, smartphones, and medical imaging equipment, yet their extraction from ores and waste streams is environmentally damaging and geopolitically sensitive. Conventional mining and hydrometallurgical techniques employed in REE production are associated with extensive ecological degradation. These processes often involve open-pit mining, acid leaching, and solvent extraction, resulting in considerable greenhouse gas emissions, toxic wastewater discharge, and the generation of radioactive by-products, especially in deposits co-located with thorium or uranium. A comprehensive review by Haque *et al.* [156] published in *Resources* outlines these impacts, noting that current REE extraction techniques are energy-intensive and environmentally burdensome [156]. Further life cycle assessment analyses, such as those conducted by Schulze [157], reinforce these findings by quantifying the carbon and toxicity footprints associated with conventional REE supply chains. Additionally, the U.S. Geological Survey (USGS) has emphasized that REE mining activities pose considerable risks to aquatic systems and soil stability due to the leaching of heavy metals and acidic compounds during ore processing [158].

In response to these environmental concerns, biotechnology offers an emerging, ecologically responsible alternative for REE recovery, particularly through the use of cyanobacteria. As photosynthetic prokaryotes, cyanobacteria possess cell surface structures enriched with functional groups such as carboxyl, hydroxyl, phosphate, and amino moieties, which exhibit strong affinities for metal ions. Recent studies have demonstrated that these organisms are capable of binding and concentrating REEs from dilute aqueous environments. For instance, a 2023 investigation by Paper *et al.* in

Frontiers in Bioengineering and Biotechnology reported that engineered cyanobacterial strains could adsorb lanthanides from solution with high specificity and efficiency, showing the role of extracellular polymeric substances in metal sequestration [159]. Complementary findings by Dell'Aversano *et al.* [160], published in the *Journal of Chromatography A*, demonstrated the analytical characterization of cyanobacterial metabolites involved in metal binding, employing advanced NMR and LC-MS techniques to identify binding interactions and metal-induced changes in metabolite profiles [160]. Furthermore, emerging experimental models show that even low-cost wild-type cyanobacterial consortia can effectively concentrate REEs from mine tailings or e-waste leachates, making this approach particularly attractive for circular economy applications [159,161].

Unlike conventional techniques, cyanobacterial biosorption operates under ambient conditions, requires minimal chemical input, and produces negligible hazardous waste. As the global demand for rare earths continues to rise, the integration of cyanobacterial biotechnology into REE recovery systems could offer a scalable, low-carbon alternative to current practices, contributing to more sustainable and secure critical material supply chains.

Cyanobacteria have garnered significant attention for their ability to biosorb rare earth elements (REEs), a process facilitated by various cellular mechanisms. The primary mode of REE uptake involves surface adsorption, where functional groups on the cyanobacterial cell wall, such as carboxyl, phosphate, and hydroxyl groups, bind metal ions. Research by Gardea-Torresdey *et al.* [162] showed the presence of functional groups such as carboxyl, aldehyde, sulfhydryl, and phosphoryl in the cell walls, which are responsible for the high ion exchange capacity observed in cyanobacteria. These groups provide the cell wall with an overall negative charge, facilitating the attraction and binding of positively charged metal ions. These interactions are predominantly electrostatic or involve ligand exchange mechanisms. For instance, a study by Paper *et al.* [163] showed the role of extracellular polymeric substances (EPS) produced by

cyanobacteria in the biosorption of REEs like cerium (Ce), neodymium (Nd), terbium (Tb), and lanthanum (La), emphasizing the significance of these functional groups in metal binding processes [163].

Beyond surface adsorption, certain cyanobacterial strains, notably those from the genera *Nostoc* and *Anabaena*, have demonstrated the capability to internalize and sequester REEs intracellularly. This bioaccumulation often results in the formation of nanoscale aggregates or intracellular crystallization of lanthanides. Such intracellular processes not only aid in detoxification but also present opportunities for the recovery and refinement of these metals. The presence of specialized cells, like heterocysts in *Anabaena*, which are known for nitrogen fixation, might also play a role in metal sequestration, although further research is needed to elucidate this connection [159]. This insights into the biosorption capabilities of cyanobacteria provided by Fischer *et al.* [164] examined the enrichment of europium, samarium, and neodymium by *Anabaena* species, revealed an almost complete (>99%) biosorption of REE within the first hour of exposure, indicating a highly efficient uptake process. The study also utilized laser-induced breakdown spectroscopy (LIBS) and transmission electron microscopy (TEM) to confirm the presence of electron-dense particles, presumably europium, within the vegetative cells, suggesting active intracellular accumulation of REEs [164].

Following biotransformation, cyanobacterial biomass frequently demonstrates an enhanced capacity for metal ion binding. This improvement is often attributed to cell wall remodeling and increased porosity, both of which facilitate the exposure of additional functional groups capable of interacting with metal ions. Notably, biotransformation-induced stress, such as that triggered by substrate exposure, can stimulate the overproduction of extracellular polymeric substances (EPS). These EPS are enriched with negatively charged functional moieties, including carboxyl, hydroxyl, and phosphate groups, that not only confer cellular protection but also serve as effective metal-binding sites. As emphasized by Paper *et al.* [163], the structural and

chemical complexity of EPS plays a critical role in the sequestration of rare earth elements, with their architecture significantly enhancing biosorption efficiency. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was employed for the quantification of REEs due to its exceptional sensitivity, broad dynamic range, and multi-elemental detection capabilities [165]. This technique is particularly well-suited for trace-level analysis of REEs in complex biological and environmental matrices [166,167].

Non-biological methods for rare earth element (REE) recovery, including sorption by natural clays and engineered adsorbents, ion-exchange leaching, and solvent extraction, remain the dominant approaches in industrial and research contexts due to their scalability and high extraction efficiencies. These physicochemical techniques typically rely on displacement reactions, complexation, or liquid-liquid separation mechanisms to mobilize REEs from ores, industrial wastes, and leachates. For example, ion-exchange leaching using saline electrolytes (e.g., ammonium sulfate) has been widely applied to regolith-hosted clay deposits (“ion-adsorption clays”), where dilute electrolyte solutions displace REE ions adsorbed onto clay minerals, resulting in relatively high extraction yields under controlled conditions [168]. Likewise, engineered sorbents such as activated carbon, silica-based materials, and polymeric adsorbents have demonstrated considerable uptake capacities for REEs from aqueous solutions, offering greater environmental compatibility than solvent extraction while maintaining high affinity and adjustable selectivity through surface functionalization [169].

Despite these advantages, non-biological methods often require significant chemical inputs, energy, and process complexity, which can limit their applicability. Solvent extraction, for instance, is effective for concentrating REEs from high-grade leachates, but its reliance on flammable organic solvents and multi-stage separation increases environmental risk and operational cost, particularly at scale [169]. Sorption techniques, while more environmentally friendly due to the absence of organic

solvents, may still face challenges in selective separation of closely related REE ions without further refining steps, and regeneration of sorbents can generate additional waste streams. As emphasized by Gkika et al, adsorption emerges as a promising alternative to conventional solvent extraction due to its simplicity, versatility, and higher sustainability profile, yet it is generally employed as a complement to other process steps rather than a separate solution in industrial recovery schemes [169].

In contrast, biological methods, particularly biosorption and bioleaching employ natural metabolic or surface binding processes to recover REEs with minimal chemical input and reduced environmental impact. Brown et al. emphasize that biosorption, which involves the passive binding of REE ions to functional groups on microbial cell walls, can achieve significant uptake efficiencies while generating negligible hazardous waste compared to traditional chemical techniques [170]. Moreover, bioleaching using organic acids produced by microorganisms has shown promise for mobilizing REEs from waste materials and ores in a more environmentally friendly manner than aggressive acid leaching commonly used in hydrometallurgical processes [170]. However, biological methods still face challenges in achieving consistent commercial deployment, including slower kinetics, the requirement for optimal microbial growth conditions, and the need for further effective processing of loaded biomass. The comprehensive investigation by Yong Ng et al. reveals that though conventional sorbents typically exhibit adsorption capacities of 10–100 mg g⁻¹, comparable to biological systems, but require acidic regeneration and suffer from gradual performance degradation over multiple cycles [171]. biological systems such as cyanobacteria combine biosorption and bioaccumulation, enabling both surface binding and intracellular sequestration. While biological approaches currently face challenges related to biomass handling, metal recovery from biomass, and long-term operational stability, they offer clear advantages in environmental sustainability, selectivity toward REEs, and compatibility with low-grade streams [170].

Conclusively, Barros *et al.* showed the potential of non-biological sorption for REE removal from wastewater, achieving >90 % lanthanum and cerium removal using zeolite-based sorbents with or without biofilms [161]. Zhang *et al.* reviewed various adsorbent materials, noting that adsorption methods can rival solvent extraction in efficiency while reducing chemical waste [169]. In contrast, biological methods such as microbial biosorption and bioaccumulation offer sustainable alternatives for REE recovery, with promising efficiency and reduced environmental impact [172–174].

2.9. Synthetic biology tools, research gaps and prospects

The integration of synthetic biology with microbial biocatalysis has significantly advanced the design of cell factories. Modular DNA assembly methods, such as Golden Gate, Gibson Assembly, and CRISPR-assisted techniques, now allow rapid prototyping of multigene biosynthetic pathways [110,112]. Systems such as biosensors, feedback loops, and logic gates have been employed to dynamically regulate metabolic flux, ensuring optimal productivity while minimizing cellular stress [111]. For example, metabolite-responsive riboswitches can activate or repress gene expression based on intracellular levels of a key intermediate, maintaining flux balance in long biosynthetic routes.

Microbial cell factories are powerful biocatalytic systems that integrate natural enzymatic selectivity with the robustness and adaptability of engineered metabolism. They represent a cornerstone of green bioprocessing, particularly for complex, multistep chemical synthesis that is impractical or inefficient through chemical catalysis alone. As synthetic biology tools evolve and genome-scale engineering becomes routine, the versatility, productivity, and scalability of microbial cell factories will continue to improve. Despite considerable progress, key knowledge gaps include: The molecular mechanisms underlying substrate specificity and enantioselectivity in biocatalysts, including cyanobacteria; genetic tools and metabolic engineering approaches tailored to enhance biocatalytic and biosorptive capacities; long- term

stability and reuse potential of cyanobacterial biomass in cyclic processes; integration of cyanobacterial systems with downstream chemical recovery and purification technologies.

The present research affirms cyanobacteria's promising dual role in photobiocatalysis and rare earth element bioaccumulation. Their photosynthetic metabolism, versatile enzymatic toolkit, and metal-binding capabilities uniquely position them for sustainable applications in green chemistry and resource recovery.

CHAPTER THREE

Materials and Methods

3.1. Reagents and materials

All chemicals and reagents used in this study were of analytical or chromatographic grade and were used without further purification unless otherwise stated. The substrates (Figure 11) employed for photobiocatalytic transformations include organophosphonates (dimethyl vinylphosphonate, diethyl vinylphosphonate, and epoxymethyl dimethyl phosphonate), and 1-phenylethyl acetate. Rare earth element salts used for bioaccumulation studies include gadolinium chloride (GdCl_3) and europium chloride (EuCl_3). Table 7 provides a comprehensive summary of the chemical reagents used in this research.

Table 7. Chemical reagents

Reagent name	Purpose	Source/manufacturer
Photobiocatalysis substrates		
1-Phenylethyl acetate	Substrate	Sigma-Aldrich (USA)
Dimethyl vinylphosphonate	Substrate	Sigma-Aldrich (USA)
Diethyl vinylphosphonate	Substrate	Sigma-Aldrich (USA)
Epoxymethyl dimethyl phosphonate	Substrate	Specific Polymers (France)
Media macronutrients and buffers		
Sodium bicarbonate (NaHCO_3)	Carbon source	Chempur (Poland)
Sodium carbonate (Na_2CO_3)	Buffer	Chempur (Poland)
Dipotassium hydrogen phosphate (K_2HPO_4)	Phosphate source	Sigma-Aldrich (USA)
Sodium nitrate (NaNO_3)	Nitrogen source	Avantor (Poland)
Potassium sulfate (K_2SO_4)	Sulfate source	Sigma-Aldrich (USA)

Sodium chloride (NaCl)	Osmotic balance	Sigma-Aldrich (USA)
Magnesium sulfate heptahydrate (MgSO ₄ ·7H ₂ O)	Magnesium source	Sigma-Aldrich (USA)
Calcium chloride dihydrate (CaCl ₂ ·2H ₂ O)	Calcium source	Sigma-Aldrich (USA)
Iron-ammonium citrate	Iron source	Sigma-Aldrich (USA)
Chelators & pH stabilizers		
Disodium EDTA dihydrate (Na ₂ EDTA·2H ₂ O)	Chelator	Sigma-Aldrich (USA)
Magnesium sodium EDTA monohydrate	Chelator	Sigma-Aldrich (USA)
Citric acid monohydrate	pH buffer/stop reagent	Sigma-Aldrich (USA)
Micronutrients (trace elements)		
Zinc sulfate tetrahydrate (ZnSO ₄ ·4H ₂ O)	Micronutrient	Sigma-Aldrich (USA)
Manganese sulfate heptahydrate (MnSO ₄ ·7H ₂ O)	Micronutrient	Sigma-Aldrich (USA)
Boric acid (H ₃ BO ₃)	Micronutrient	Sigma-Aldrich (USA)
Cobalt nitrate hexahydrate (Co(NO ₃) ₂ ·6H ₂ O)	Micronutrient	Sigma-Aldrich (USA)
Sodium molybdate dihydrate (Na ₂ MoO ₄ ·2H ₂ O)	Micronutrient	Sigma-Aldrich (USA)
Copper sulfate pentahydrate (CuSO ₄ ·5H ₂ O)	Micronutrient	Sigma-Aldrich (USA)
Ferrous sulfate heptahydrate (FeSO ₄ ·7H ₂ O)	Iron source	Sigma-Aldrich (USA)
Vitamin B ₁₂ (cyanocobalamin)	Growth factor	Sigma-Aldrich (USA)
Analytical & instrumental reagents		
Deuterated chloroform (CDCl ₃)	NMR solvent	Sigma-Aldrich (USA)
Triphenyl phosphate (TPP)	NMR internal standard	Sigma-Aldrich (USA)
Anhydrous magnesium sulfate (MgSO ₄)	Drying agent	Sigma-Aldrich (USA)
Sodium methoxide in methanol	Base catalyst	Sigma-Aldrich (USA)
Dowex® 50W-X8 resin	Ion-exchange catalyst	Sigma-Aldrich (USA)

Acetonitrile	HPLC solvent	Sigma-Aldrich (USA)
Ultrapure water	Universal solvent	In-house (Milli-Q)
Antimicrobial testing reagents		
Dimethyl sulfoxide (DMSO)	Solvent	Sigma-Aldrich (USA)
Cefoperazone	Antibiotic control	Sigma-Aldrich (USA)
Mueller-Hinton agar	Bacterial growth medium	Sigma-Aldrich (USA)
Nutrient broth	Bacterial culture	Sigma-Aldrich (USA)
Sterile paper discs	Antimicrobial diffusion assays	Sigma-Aldrich (USA)

Components used in the preparation of cyanobacterial growth media, including Spirulina medium, BG-11, and BG-11₀, were purchased from Sigma-Aldrich, Chempur (Piekary Śląskie, Poland), and Avantor Performance Materials (Gliwice, Poland). These include macronutrients such as sodium bicarbonate, sodium carbonate, dipotassium hydrogen phosphate, sodium nitrate, potassium sulfate, sodium chloride, magnesium sulfate heptahydrate, calcium chloride dihydrate, and iron-ammonium citrate. Additional reagents involved in chelation and pH stabilization, including disodium EDTA dihydrate, magnesium sodium EDTA monohydrate, and citric acid monohydrate, were incorporated as part of standard media formulations. Vitamin B₁₂ (cyanocobalamin) was prepared as a 5 mg/L stock solution in sterile distilled water, filter-sterilized, and stored at –20 °C in microcentrifuge tubes wrapped in aluminum foil to prevent photodegradation.

Trace element solutions were prepared using zinc sulfate tetrahydrate, manganese sulfate heptahydrate, boric acid, cobalt nitrate hexahydrate, sodium molybdate dihydrate, copper sulfate pentahydrate, and ferrous sulfate heptahydrate. These micronutrients were incorporated in media according to established protocols for

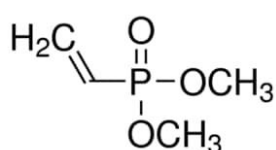
cyanobacterial cultivation and adjusted where necessary to support substrate stability during photobiocatalysis (section 3.3.1 and 3.3.2).

Analytical and instrumental reagents used throughout the study were obtained from Sigma-Aldrich except where otherwise specified. These include high-purity solvents such as acetonitrile for HPLC, ultrapure water, and deuterated chloroform (CDCl_3) for NMR spectroscopy. Triphenyl phosphate was employed as an internal standard in quantitative phosphorus-31 NMR analysis. Anhydrous magnesium sulfate was used for drying organic extracts, and Dowex® 50W-X8 ion-exchange resin (H^+ form, 200–400 mesh) was used during the chemical synthesis of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate. Sodium methoxide in methanol was used as a base catalyst for this chemical synthesis (dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate).

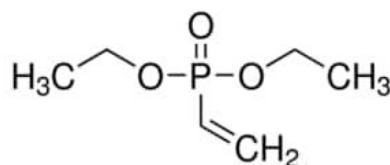
The Malachite Green Assay, used for inorganic phosphate quantification, involved reagents also obtained from Sigma-Aldrich unless otherwise stated. These include malachite green dye prepared at a concentration of 0.045% (w/v) in deionized water, ammonium molybdate tetrahydrate dissolved at 4.2% (w/v) in 4 M hydrochloric acid, and a stop solution consisting of 34% (w/v) citric acid monohydrate. Hydrochloric acid was used to prepare a 4 M stock solution, and phosphate standard solutions were prepared using potassium hydrogen phosphate to establish a standard calibration curve.

Reagents employed in the antimicrobial susceptibility testing of DMHPP were similarly sourced from Sigma-Aldrich unless otherwise stated. Dimethyl sulfoxide (DMSO) was used as the solvent for preparing stock and working solutions of DMHPP. Cefoperazone was used as the positive control antibiotic, while sterile paper discs and nutrient broth were used for bacterial inoculation and testing. Mueller-Hinton agar was prepared according to manufacturer specifications for both disk diffusion and agar well diffusion assays.

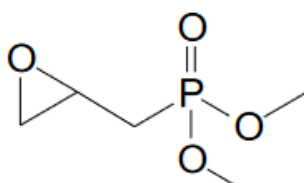
All solution preparations and handling steps were conducted under aseptic or sterile conditions where required.



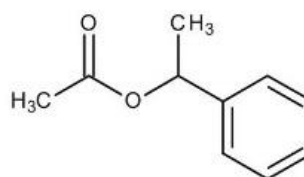
(a) Dimethyl vinylphosphonate



(b) Diethyl vinylphosphonate



(c) Epoxymethyl dimethyl phosphonate



(d) 1-phenylethyl acetate

Figure 11. Selected substrates for photobiocatalysis and bioaccumulation with cyanobacterial strains

3.2. Cyanobacterial strains and microorganisms

The cyanobacterial strains used in this study (Table 8) include *Synechococcus bigranulatus* CCALA 187, *Nostoc cf. muscorum* CCALA 129, *Kamptomena animale*, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 027, *Leptolyngbya foveolarum*, and *Nodularia moravica*. The strains were obtained from recognized culture collections and maintained under sterile photoautotrophic cultivation conditions.

Depending on the strain requirements, cultivation was carried out using Spirulina Medium (SM), BG-11 medium, or nitrogen-deficient BG-11₀ medium. Modified variants of SM and BG-11 media were additionally prepared for experiments involving epoxymethyl dimethyl phosphonate (EMDP) biotransformation.

For antimicrobial susceptibility testing, *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) were used as reference bacterial strains.

Table 8. Microbial strains

Microorganism	Strain code	Collection country	Medium	Function
Cyanobacteria (phototrophic)				
<i>Synechococcus bigranulatus</i>	CCALA 187	Czech Republic	BG-11	Biocatalysis/ bioaccumulation
<i>Nostoc cf-muscorum</i> (cf = confer, i.e., similar to <i>Nostoc muscorum</i> morphologically)	CCALA 129	Czech Republic	BG-11 ₀	Biocatalysis
<i>Limnospira indica</i>	PCC 8005	University of Opole, Poland	SM	Biocatalysis/ bioaccumulation
<i>Limnospira maxima</i>	CCALA 27	Czech Republic	SM	Biocatalysis/ bioaccumulation
<i>Leptolyngbya foveolarum</i>	CCALA 76	Czech Republic	BG-11	Biocatalysis/ bioaccumulation
<i>Kamptonema animale</i>	CCALA 138	Czech Republic	BG-11	Biocatalysis
<i>Nodularia moravica</i>	CCALA 114	Czech Republic	BG-11	Biocatalysis
Heterotrophic bacteria				
<i>Escherichia coli</i>	ATCC8937	WUST	N. b.	Antimicrobial susceptibility testing
<i>Staphylococcus aureus</i>	ATCC 6538	WUST	N. b.	Antimicrobial susceptibility testing

Note: CCALA = Culture Collection of Autotrophic Organisms; PCC = Pasteur Culture Collection; BG = Blue-Green medium; SM = Spirulina medium; N. b. = Nutrient broth; WUST = Wrocław University of Science and Technology, Poland.

3.3. Growth media and cyanobacterial cultivation

Cyanobacterial growth media were prepared according to standard laboratory protocols [175], with particular attention to nutrient composition and sterility (Table 9 and Table 10). Standard and modified media formulations were prepared aseptically from stock solutions and sterilized prior to use. Cyanobacterial strains were cultivated under controlled photoautotrophic conditions using Spirulina Medium (SM), BG-11 medium, or nitrogen-deficient BG-11₀ medium, depending on the physiological requirements of each strain and the experimental objective.

Cultivation parameters, including illumination intensity, temperature, incubation period, and medium composition, were optimized to maintain biomass viability and support subsequent photobiocatalytic and bioaccumulation experiments.

3.3.1. Preparation of Spirulina Medium

The Spirulina medium was constituted by preparing three essential components: Solution 1, Solution 2, and a trace element solution (Table 9 and Table 11).

Table 9. Spirulina medium composition and its modification

Ingredient	Solution 1 (per 0.5 L dH ₂ O)	Solution 2 (per 0.5 L dH ₂ O)	Modified medium (per 1 L dH ₂ O)
NaHCO ₃	13.61 g	—	1.0 g
Na ₂ CO ₃	4.03 g	—	0.2 g
K ₂ HPO ₄	0.50 g	—	1.0 g
Na ₂ EDTA·2H ₂ O	—	0.08 g	0.08 g
NaNO ₃	—	2.5 g	2.5 g
K ₂ SO ₄	—	1.0 g	Removed
NaCl	—	1.0 g	Removed

MgSO ₄ ·7H ₂ O	—	0.2 g	0.2 g
CaCl ₂ ·2H ₂ O	—	0.04 g	0.04 g
FeSO ₄ ·7H ₂ O	—	0.01 g	—
Iron-ammonium citrate	—	—	1 mL (from BG-11 stock)
Trace element solution (SM)	—	1 mL	—
Trace element solution (BG-11)	—	—	1 mL
Vitamin B ₁₂	—	1 mL	1 mL

Note: “—” denotes a non-applicable ingredient.

The trace element solution was formulated in accordance with the specified concentrations and stored under refrigeration to maintain chemical stability. Additionally, the Vitamin B₁₂ stock solution was prepared by dissolving 5 mg of cyanocobalamin in 1 L of sterile distilled water. This solution was subsequently filter-sterilized, aliquoted into 1.5 mL microcentrifuge tubes, wrapped in aluminium foil to prevent photodegradation, and stored frozen until use.

To prepare Solution 1, 500 mL of distilled water was transferred into a beaker containing a magnetic stir bar, and the mixture was gently heated to a temperature range of 50 to 60 °C. The components designated under “Spirulina Medium (Solution 1)” in Table 9 were added sequentially to the warmed water. Each solute was allowed to dissolve completely before the subsequent component was introduced, to prevent precipitation and ensure a homogenous solution.

A similar approach was employed in the preparation of Solution 2. An additional 500 mL of distilled water was dispensed into a separate beaker, equipped with a stir bar, and heated to 50–60 °C. The ingredients listed under “Spirulina Medium (Solution 2)” in Table 9 were added individually, ensuring complete dissolution before adding the next. It was critical at this stage to omit the addition of Vitamin B₁₂, which is heat-labile and was instead incorporated after sterilization.

Solution 1 was prepared in a container with double the required volume capacity to allow for easy combination with Solution 2. Both solutions were sterilized using appropriate sterilization techniques. Upon cooling, the previously prepared and frozen Vitamin B₁₂ solution was aseptically introduced into Solution 2 and mixed gently but thoroughly. Subsequently, Solution 1 and Solution 2 were combined under sterile conditions to yield 1 L of complete *Spirulina* growth medium (Table 9).

3.3.2. BG-11/BG-11₀ medium

The preparation of BG-11₀ medium was identical to that of BG-11, except for the exclusion of sodium nitrate (NaNO₃) as a nitrogen source. This modification was used to support the growth of nitrogen-fixing cyanobacteria such as *Nostoc* sp., which do not require an external nitrogen source due to their ability to fix atmospheric nitrogen [176].

Table 10. BG-11/BG-11₀ medium composition and it's modification

Ingredient	BG-11 Stock Solution (g/10 mL H ₂ O)	BG-11 Medium (per 1 L)	Modified BG-11 Medium (per 1 L)
K ₂ HPO ₄	0.4	1 mL	1 mL
Na ₂ EDTA·2H ₂ O	0.1	1 mL	1 mL
NaNO ₃	—	1.5 g	1.5 g
MgSO ₄ ·7H ₂ O	0.75	1 mL	1 mL
CaCl ₂ ·2H ₂ O	0.36	1 mL	1 mL
Iron-ammonium citrate	0.06	1 mL	1 mL
Citric acid monohydrate	0.06	1 mL	1 mL
MgNa ₂ EDTA·H ₂ O	0.1	1 mL	1 mL
Trace element solution	—	1 mL	1 mL
Vitamin B ₁₂ solution	—	—	1 mL

Note: “—” denotes a non-applicable ingredient.

Stock solutions of the BG-11/BG-11₀ components were prepared by dissolving the relevant quantities of each component (Table 10), in 10 mL of distilled water. These stock solutions were stored under refrigeration to preserve their integrity until required.

For the preparation of 1 L of BG-11 or BG-11₀ medium, 960 mL of distilled water was preheated to 50–60 °C in a sterile beaker containing a magnetic stir bar. Initially, 1 mL of MgNa₂EDTA·H₂O stock solution was added to the preheated water and allowed to dissolve completely. Immediately prior to use, 1.5 g of NaNO₃ was weighed and added to the medium, ensuring full dissolution; this step was omitted in the preparation of BG-11₀ medium. Subsequently, 1 mL each of citric acid, MgSO₄·7H₂O, and CaCl₂·2H₂O stock solutions were added sequentially, with gentle stirring and a wait period of approximately 10 minutes to ensure thorough integration.

Meanwhile, four additional tubes were prepared, each containing 1 mL of the following stock solutions: Na₂CO₃, K₂HPO₄·3H₂O, iron-ammonium citrate, and the trace elements solution (Table 10). Each of these tubes was made up to a total volume of 10 mL using distilled water. The contents of all bottles and tubes were sterilized appropriately. Once sterilized and cooled, the contents of the four tubes were aseptically transferred into the main preparation bottle. The mixture was gently stirred to ensure homogeneity, and the final volume was adjusted to 1 L, thus completing the preparation of sterile BG-11 or BG-11₀ medium suitable for cyanobacterial culture (Table 10).

Table 11. Trace element composition in spirulina and BG-11/ BG-11₀ media

Ingredient	Spirulina Medium (g/L dH ₂ O)	BG-11 Medium (g/L dH ₂ O)
ZnSO ₄ ·4H ₂ O	1.0	0.220
MnSO ₄ ·7H ₂ O	2.0	1.810
H ₃ BO ₃	10.0	2.860
Co(NO ₃) ₂ ·6H ₂ O	1.0	0.0494

Na ₂ MoO ₄ ·2H ₂ O	1.0	0.391
CuSO ₄ ·5H ₂ O	0.005	0.079
Na ₂ EDTA·2H ₂ O	0.8	—
FeSO ₄ ·7H ₂ O	0.7	—

Note: “—” denotes a non-applicable ingredient.

3.3.3. Media modification for *Limnospira* sp.

Modified variants of Spirulina Medium and BG-11 medium were prepared for experiments involving epoxymethyl dimethyl phosphonate (EMDP). In the modified Spirulina Medium, NaHCO₃ was reduced from 27.22 g/L to 1.0 g/L, Na₂CO₃ was reduced to 0.2 g/L, and K₂HPO₄ was adjusted to 1.0 g/L. Iron-ammonium citrate was included at 1 mL/L, while MgSO₄·7H₂O and CaCl₂·2H₂O were maintained at 0.2 g/L and 0.04 g/L, respectively. NaCl and K₂SO₄ were excluded from the formulation, and Vitamin B₁₂ was added at 1 mL/L. In the modified BG-11 medium, the standard macronutrient composition was maintained, while Vitamin B₁₂ was supplemented at 1 mL/L and the trace element composition remained unchanged.

The modifications were introduced with the aim to reduce medium alkalinity and ionic strength while maintaining suitable growth conditions and improving substrate stability during incubation [177–182].

3.3.4. Cultivation conditions

Cyanobacterial strains were aseptically inoculated into 250 mL Erlenmeyer flasks containing 100 mL of the appropriate growth medium. However, modified SM and BG-11 were used in addition to the unmodified media for the culturing of *Limnospira* sp. Cultures were maintained in a controlled photobioreactor system parameters such as, continuous illumination at an intensity of 15 – 22 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, provided by a Power Glo fluorescent bulb (30W, Hagen, QC, Canada); the incubation temperature was maintained at $29 \pm 1^\circ\text{C}$ under sterile conditions, with cultivation

periods ranging from 14 to 21 days, depending on the strain-specific growth requirements.

Cyanobacterial growth was monitored spectrophotometrically by measuring optical density at 720 nm (OD_{720}) in experiments where modified cultivation conditions were applied. Under standard cultivation conditions using unmodified BG-11, BG-11₀, and Spirulina Medium, routine OD measurements were not systematically performed, as these media are well established for the stable growth of the strains investigated and biomass development was evidenced during subsequent experimental procedures, including bioaccumulation and ecotoxicity assays.

3.3.5. Substrate stability analysis

The stability of the substrates in culture media was assessed to ensure their suitability for biotransformation. Each substrate (1 mM) was incubated in 100 mL of growth media (BG-11, BG-11₀, and Spirulina media) for 7 days and analyzed using HPLC and NMR to detect any degradation or transformation.

Importantly, water was not employed as a control medium because it does not represent the physicochemical environment relevant to the biocatalytic systems investigated. Cyanobacterial strains used in biotransformations were conducted exclusively in defined growth media (BG11, BG11₀, and SM), and therefore substrate fate was evaluated under biologically relevant conditions, as commonly recommended for whole-cell biocatalysis studies in literature. This approach enables meaningful comparison between media-only controls and biocatalyst-containing systems.

3.4. Photobiocatalytic transformations method

Photobiocatalytic transformations were performed using actively growing cyanobacterial cultures cultivated under controlled photoautotrophic conditions. Biotransformation reactions were conducted in sterile 250 mL Erlenmeyer flasks containing 100 mL of cyanobacterial biomass suspension. Substrates were added to

achieve a final concentration of 1 mM unless otherwise stated. Depending on the experimental design, incubation periods ranged from several hours up to 7 days. under continuous illumination and controlled temperature conditions. All experiments were performed in triplicate unless otherwise stated.

Following incubation, cultures were centrifuged at 4000 rpm for 20 min to separate biomass from the reaction medium. The supernatants were extracted twice with 50 mL of ethyl acetate. The combined organic phases were dried over anhydrous MgSO₄ for 24 h, filtered through Whatman Grade 1 qualitative filter paper, and concentrated under reduced pressure using a rotary evaporator (IKA® RV 10 digital, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 60 °C. Substrate-specific cyanobacterial strains and experimental conditions are summarized in Table 12.

Table 12. Substrate-specific bioconversion by cyanobacterial strains

Substrate	Amount (mg/100 mL biocatalyst) – 1 mM	Biocatalyst (14 to 21 days culture)	Scalability (mg/ 100 mL biocatalyst) – 4 mM
Dimethyl vinylphosphonate	16.0	<i>Synechococcus</i> sp., <i>Nostoc</i> sp., <i>Kamptanema</i> sp., <i>Limnospira</i> sp.	-
Diethyl vinylphosphonate	16.0	<i>Synechococcus</i> sp., <i>Nostoc</i> sp., <i>Kamptanema</i> sp., <i>Limnospira</i> sp.	-
1-phenylethyl acetate	16.2	<i>Synechococcus</i> sp., <i>Leptolyngbya</i> sp.	-
Epoxymethyl dimethyl phosphonate	16.6	<i>Synechococcus</i> sp., <i>Limnospira</i> sp., <i>Nostoc</i> sp., <i>Nodularia moravica</i>	66.4 [Applied only for <i>Synechococcus</i> sp.]

Note: “–” denotes that scalability is not applicable.

The process optimization phase involved a systematic scale-up strategy aimed at enhancing the photobiocatalytic performance of the system using *Synechococcus* species and the target substrate, EMDP. This optimization effort involved a stepwise increase in EMDP concentration from 1 mM to 4 mM (Table 12), while strictly maintaining constant parameters such as the culture volume, light intensity, temperature, aeration, and nutrient compositions.

3.4.1. Biotransformation of 1-phenylethyl acetate process

Photobiocatalytic transformation of 1-phenylethyl acetate was carried out using 7–14-day-old cultures of *Synechococcus* sp. and *Leptolyngbya* sp. under standard cultivation conditions. The substrate was added to cyanobacterial biomass suspensions to achieve a final concentration of 1 mM, followed by incubation under continuous illumination for up to 7 days.

After completion of the reaction, samples were extracted with ethyl acetate, dried over anhydrous MgSO_4 for 24 h, filtered through Whatman Grade 1 qualitative filter paper to remove the drying agent, and concentrated under reduced pressure. Product formation and enantiomeric composition were analysed using chiral gas chromatography (GC).

GC analyses for enantiomeric resolution and quantification of 1-phenylethyl acetate and its chiral bioconversion products were performed using a Shimadzu GC-2010 Plus system (Shimadzu Corporation, Japan) equipped with a flame ionization detector (FID). Separations were carried out on a Chirasil-DEX CB column (25 m $\times$ 0.25 mm, 0.25 μm ; Agilent, Santa Clara, CA, USA) using nitrogen as carrier gas. The initial column temperature of 110 $^{\circ}\text{C}$ was maintained for 15 min and subsequently increased to 200 $^{\circ}\text{C}$ at a rate of 15 $^{\circ}\text{C min}^{-1}$. The total run time was 21 min.

The FID temperature was maintained at 200 $^{\circ}\text{C}$. Nitrogen, air, and hydrogen flow rates were adjusted to 30, 400, and 40 mL min^{-1} , respectively. Analyses were performed in split mode (20:1) with an injector temperature of 200 $^{\circ}\text{C}$, a carrier gas flow rate of 1.2

mL min⁻¹, and an injection volume of 1 µL. Retention times for 1-phenylethyl acetate enantiomers were 10.49 and 12.19 min, while 1-phenylethanol enantiomers were detected at 15.97 and 16.73 min, and acetophenone at 6.62 min respectively.

Conversion percentages and enantiomeric excess (e.e.) values were calculated based on GC peak areas using calibration curves for substrate and products. Data was presented as mean ± standard deviation from duplicate experiments. Conversion was determined as the ratio of total product concentration to the sum of product and substrate concentrations according to Equation 1.

$$\text{Conversion (\%)} = \frac{C_R + C_S}{C_R + C_S + C_{\text{substrate}}} \times 100$$

Enantiomeric excess was calculated according to Equation 2.

$$e.e. (\%) = \frac{C_R - C_S}{C_R + C_S} \times 100$$

The concentrations of substrate and products were determined from calibration curves according to Equations 3–5.

$$C_R = \frac{A_R - b_R}{m_R}$$

$$C_S = \frac{A_S - b_S}{m_S}$$

$$C_{\text{substrate}} = \frac{A_{\text{substrate}} - b_{\text{sub}}}{m_{\text{sub}}}$$

where C_R and C_S represent concentrations (in mol) of R- and S-phenylethanol, respectively, and $C_{\text{substrate}}$ represents the concentration (in mol) of 1-phenylethyl acetate. A , m , and b correspond to chromatographic peak area, slope, and intercept values obtained from calibration curves, respectively.

3.4.2. Organophosphonates biotransformation

Photobiocatalytic transformations of dimethyl vinylphosphonate (DMVP), diethyl vinylphosphonate (DEVP), and epoxymethyl dimethyl phosphonate (EMDP) were carried out using selected cyanobacterial strains cultivated under controlled photoautotrophic conditions. Cyanobacterial cultures aged 14–21 days were used as

biocatalysts. Substrates were added individually to cyanobacterial biomass suspensions to obtain a final concentration of 1 mM unless otherwise stated.

Biotransformation experiments were performed in sterile Erlenmeyer flasks under continuous illumination and controlled temperature conditions for up to 7 days. For scaling-up experiments involving EMDP, substrate concentration was increased to 4 mM using *Synechococcus bigranulatus* CCALA 187 cultures.

Following incubation, reaction mixtures were centrifuged at 4000 rpm for 20 min to separate biomass from the culture medium. Supernatants were extracted twice with ethyl acetate, dried over anhydrous MgSO_4 for 24 h, filtered through Whatman Grade 1 qualitative filter paper, and concentrated under reduced pressure prior to analytical characterization.

Substrate conversion and product formation were monitored using chromatographic and spectroscopic analytical techniques, including HPLC, infrared spectroscopy (IR), high-resolution mass spectrometry (HRMS), and nuclear magnetic resonance (NMR) spectroscopy. Product yield determination was additionally performed using quantitative ^{31}P NMR analysis with triphenylphosphine (TPP) as an internal standard.

3.4.3. Phosphate quantification by Malachite Green assay

The assay was applied to assess phosphate levels in BG-11, BG-110, and Spirulina Medium (SM), as well as in 21-day cyanobacterial cultures before and after substrate addition. In addition, phosphate status was evaluated during bioconversion experiments involving DMVP, including three-day reactions with *Synechococcus bigranulatus* CCALA 187, *Limnospira indica* PCC 8005, and *Limnospira maxima* CCALA 27, and a seven-day reaction with *Nostoc cf. muscorum* CCALA 129. Absorbance measurements were consistently performed at 620 nm for both calibration standards and experimental samples according to established literature protocols [168–170].

The Malachite Green reagent was prepared by dissolving malachite green dye at a concentration of 0.045% (w/v) in deionized water and ammonium molybdate tetrahydrate at 4.2% (w/v) in 4 M hydrochloric acid. These solutions were mixed in a 30:70 (v/v) ratio immediately prior to use. The stop solution consisted of 34% (w/v) citric acid prepared by dissolving citric acid monohydrate in deionized water. A 4 M HCl stock solution was prepared by dilution of concentrated hydrochloric acid (35–38%).

A phosphate stock solution (10 mM) was prepared by dissolving potassium hydrogen phosphate (K_2HPO_4) in deionized water. Calibration standards (0.1–5.0 mM) were obtained by volumetric dilution of the stock solution in deionized water, which also served as the blank. Standards and samples were prepared in identical aqueous matrices to minimize potential matrix effects.

For analysis, 1 mL of standard or sample solution was mixed with 1 mL of freshly prepared Malachite Green reagent and incubated for approximately 20 min at room temperature. The reaction was terminated by addition of 200 μL of the citric acid stop solution. Absorbance was measured at 620 nm using a UV–Vis spectrophotometer (Lambda Bio, PerkinElmer Inc., Waltham, MA, USA) [171]. Absorbance values were plotted against known phosphate concentrations to generate linear calibration curves.

Sample phosphate concentrations were calculated using linear regression according to the equation:

$$[\text{Phosphate}] = \frac{A_{620} - b}{m}$$

where A_{620} is the measured absorbance, m is the slope, and b is the intercept of the calibration curve. This approach was applied to quantify free phosphate released during photobiocatalytic transformation of organophosphonates [183–185].

3.4.4. Purification and structural characterization of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate received as biotransformation product

Purification was carried out by preparative high-performance liquid chromatography (HPLC) using a Teledyne ISCO ACCQPrep HP125 system equipped with a RediSep® Prep C18 reversed-phase column (250 mm × 10 mm, 5 µm). Elution was performed using a water–acetonitrile solvent system with the following gradient: 10% (v/v) acetonitrile isocratic for 10 min, followed by a linear increase to 30% acetonitrile over 10 min, and a final isocratic step at 30% acetonitrile for an additional 10 min. The flow rate was maintained at 5 mL·min⁻¹. Under these conditions, the main biotransformation product (DMHPP) eluted at a retention time of approximately 23 min. Elution was monitored by UV detection at 210 nm [186,187].

After purification with HPLC, the product was analyzed using High-resolution mass spectrometry (HRMS), conducted using a Waters LCT Premier™ XE time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA), equipped with an electrospray ionization (ESI) source operating in positive ion mode. Molecular weights and elemental compositions of products were confirmed using exact mass measurements. Structural elucidation of the purified product was performed by Nuclear Magnetic Resonance (NMR) spectroscopy. Assignments of chemical shifts in the ¹H and ¹³C NMR spectra were confirmed using two-dimensional correlation techniques including ¹H-¹H COSY, ¹H-¹³C HMQC, and ¹H-¹³C HMBC spectra. The spectral data were found to be in agreement with literature values [188].

3.4.5. Antimicrobial susceptibility testing of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate

The antibacterial activity of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate was evaluated using disk diffusion and agar well diffusion methods against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) on Mueller-Hinton agar. DMHPP solutions at varying concentrations were applied to sterile filter discs and

agar wells, with cefoperazone as a positive control and dimethyl sulfoxide (DMSO) as negative controls. After incubation at 37°C for 16–24 hours, inhibition zones were measured to assess antibacterial efficacy.

The DMHPP stock solution was prepared by dissolving 15.8 mg of DMHPP in 1 mL of DMSO to obtain a concentration of 15.8 mg/mL. Serial dilutions were performed to achieve additional concentrations of 7.9 mg/mL, 3.95 mg/mL, and 1.98 mg/mL. The preparation followed standard antimicrobial susceptibility testing procedures as outlined in the Kirby-Bauer disk diffusion susceptibility test protocol [189,190].

Bacterial cultures were inoculated in sterile nutrient broth using aseptic techniques and incubated at 37°C for 12 to 16 hours in a shaking incubator set at 150 to 200 rpm. The bacterial suspension was adjusted to a 0.5 McFarland turbidity standard, corresponding to an optical density at 600 nm of 0.1, which equates to approximately 1.5×10^8 CFU/mL [191].

Mueller-Hinton agar was prepared according to the manufacturer's instructions. A 1 mL aliquot of the bacterial suspension was evenly spread over the agar surface to ensure uniform distribution. Excess culture was removed using a sterile pipette, ensuring consistency in microbial growth assessment [189].

For the disk diffusion method, sterile paper discs were impregnated with 20 µL of each DMHPP concentration and allowed to absorb under a laminar flow hood. The negative control consisted of solvent only, while the positive control used cefoperazone. The discs were placed on the bacterial lawn using sterile forceps, ensuring appropriate spacing to prevent overlapping zones of inhibition. Each experiment was performed in triplicate to ensure reproducibility, in accordance with established antimicrobial susceptibility testing protocols [189,190].

For the agar well diffusion method, a sterile tip borer was used to create 6 mm diameter wells in the agar. The wells were spaced at least 2 to 3 cm apart. Volumes of 70 µL of each DMHPP concentration were added into separate wells. The positive control

involved placing an antibiotic disc (cefoperazone, 30 µg) directly onto the bacterial lawn. The negative control consisted of 70 µL of solvent. Each assay was conducted in triplicate.

Agar plates were incubated at 37°C for 16 to 24 hours. Zones of inhibition were measured using a ruler to assess antibacterial activity. Comparative analyses were conducted based on inhibition zone diameters to evaluate the efficacy of DMHPP against bacterial strains [189].

3.4.6. Chemical synthesis of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate – applied as a structure standard

To complement the biocatalytic route, the target compound, dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate, was also synthesized via a chemical method [192]. In this procedure, sodium methoxide in methanol (0.1 equivalents) was added dropwise to a stirred solution of epoxymethyl dimethyl phosphonate (3.01 mmol, 500 mg) at 0 °C. Following complete addition, the reaction mixture was maintained under stirring at ambient temperature for 1 hour. Subsequently, 2.00 g of Dowex® 50W-X8 (H⁺) ion-exchange resin (200–400 mesh; Sigma-Aldrich, St. Louis, MO, USA), pre-conditioned with methanol, was introduced to quench the reaction. The mixture was stirred for an additional 15 minutes, after which the resin was removed by filtration and rinsed with methanol. The combined filtrates were concentrated under reduced pressure to afford the crude product (490 mg). After synthesis, the products were purified using Flash Chromatography System: Combi Flash® Rf 150 (Teledyne ISCO, Lincoln, NE, USA) and reversed phase column PuriFlash C18-HP, 15 µm, 25 g (Teledyne ISCO, Lincoln, NE, USA). The general procedure of purification included isocratic flow of pure water, flow 10 mL/min, R_f = 2.5 min.

3.4.7. Quantitative determination of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate yield

The product yield of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate, synthesized via the biocatalytic transformation of epoxymethyl dimethyl phosphonate, was determined using quantitative ^{31}P NMR spectroscopy.

In this investigation, a known mass of the standard (10 mg), corresponding to a molar concentration of 32.2 mM, was co-dissolved with the reaction mixture in 1 mL of deuterated chloroform (CDCl_3). The sample thus contained both the biotransformation product (DMHPP) and the standard (TPP) within the same NMR tube. The use of an internal standard allows for quantitative comparison of integrated peak areas in the ^{31}P NMR spectrum, correlating signal intensity with molar concentration under identical experimental conditions [193].

Product yield was evaluated for two substrate concentrations: an initial concentration of 1 mM EMDP in early bench-scale experiments and a scaled-up concentration of 4 mM EMDP in subsequent photobiocatalytic runs. For the 1 mM setup, samples were taken after approximately 3 days, at which point complete bioconversion was observed. For the 4 mM runs, complete biotransformation was achieved between days 5 to 9, with samples taken at day 5, day 7, and day 9 to confirm reproducibility and monitor potential degradation. The molar yield of DMHPP was calculated relative to the known concentration of TPP using integrated NMR peak areas. The formula used was:

$$n_p = \left(\frac{I_p}{I_s} \right) \times n_s$$

Where n_p and n_s represent the amount (mmol) of product (DMHPP) and the amount (mmol) of internal standard (TPP) respectively, while I_p and I_s represent their respective integrated intensities. Yields were subsequently expressed as percentages relative to the amount as:

$$\text{Yield (\%)} = \left(\frac{\text{Amount of product obtained (mol)}}{\text{Theoretical amount of product (mol)}} \right) \times 100$$

This approach provided reproducible quantitative data for product formation, with mean values and standard deviations calculated from the triplicate NMR measurements.

3.5. Bioaccumulation process with cyanobacteria.

This phase focused on how the cyanobacterial strains interact with and retain REEs under controlled conditions. Cultures were exposed to measured amounts of gadolinium and europium, after which the biomass was collected and analyzed to determine the extent of accumulation according to section 4.3.4.

3.5.1. Ecotoxicological screening

Both pre-bioconversion and post-bioconversion cyanobacterial biomasses, obtained as cell pellets after centrifugation ending the photobiocatalysis phase, were assessed for their tolerance to, and bioaccumulation capacity of REEs, specifically Gd and Eu. Stock solutions of each REE were prepared in sterile BG-11 medium and SM at 8 mM by dissolving 0.084 g of GdCl₃ or 0.083 g of EuCl₃ in 40 mL. Working concentrations for preliminary ecotoxicity screening were prepared as nominal exposure levels of 20, 40, and 80 µM. Growth inhibition tests (72 h) were conducted according to OECD Test Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) in sterile 96-well polystyrene microtiter plates to determine sublethal concentrations for each cyanobacterial strain. Cyanobacterial inocula were standardized to 400,000 cells/mL based on absorbance measurements and diluted 1:1 with treatment solutions to yield final test concentrations of 200,000 cells/mL. Each well received 125 µL of REE treatment and 125 µL of the algal suspension, resulting in 250 µL total volume. Columns designated for controls include biomass without REE exposure (post- and pre-bioconversion) and blanks containing only SM and BG-11 medium respectively. Plates were incubated under the same temperature (29 ± 1 °C) and illumination regime

(*Limnospira* sp. and *Leptolyngbya* sp. were exposed to 60–69 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, while *Synechococcus* sp. was exposed to 80–98 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Each treatment was conducted in quintuplicate.

During ecotoxicity assessments, optical densities (OD_{750} and OD_{682}) were measured at 0, 24, 48, and 72 h to monitor growth inhibition, pigment integrity, and overall cell viability.

After ecotoxicological screening, bioaccumulation was conducted on both pre-bioconversion and post-bioconversion biomasses in sterile 100 mL glass test tubes, placed within a controlled photobioreactor aquarium chamber. Incubations were performed in triplicate for each treatment, maintained under constant photoautotrophic conditions at 29 ± 1 °C, with continuous illumination at 20 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. During the 72-h exposure period, OD_{750} measurements were periodically recorded to monitor biomass development. Morphological observations of the cells were performed microscopically using an Olympus BX51 (Olympus, Tokyo, Japan) to detect cellular bleaching, granulation, lysis, or contamination during the bioaccumulation processes.

3.5.2. Dry mass determination

Dry mass determination and quantification of biomass productivity were conducted independently of the elemental analysis. Specifically, 10 mL aliquots of each culture were withdrawn at $t = 0$ and 72 h, and cells were collected by centrifugation at 4800 rpm for 10 min. Pellets were transferred to sterile Eppendorf tubes and dried in an oven at 105 °C for 24 h. Dry biomass was determined gravimetrically.

3.5.3. Preparation of algae samples for REE determination by ICP-MS

After the 72 h bioaccumulation phase, cyanobacterial biomass was harvested for REEs quantification using protocols adapted from Čížková *et al.* [194] and Vítová *et al.* [195]. Cultures were centrifuged at 4800 rpm for 10 minutes, and the resulting cell pellets were subjected to three consecutive washes with their respective media. These

washing steps were essential for removing any residual REEs remaining in the supernatant or loosely adsorbed to extracellular polymeric substances (EPS), ensuring that subsequent elemental analysis would reveal only internalized REEs and not loosely bound surface-associated ions. After the final wash, biomass was immediately frozen at $-80\text{ }^{\circ}\text{C}$ and subsequently freeze-dried. This procedure preserved cellular structure and minimizing any redistribution or loss of accumulated metals during processing.

Sample digestion and ICP-MS measurements were performed by an external analytical facility as follows: Algae samples were prepared by microwave-assisted digestion using a Berghof Speedwave Xpert or ETHOS 1 Milestone system. Approximately 0.10–0.15 g of sample (or the entire available amount) was accurately weighed. Procedural blanks and certified reference materials (CRMs) were digested in parallel under identical conditions.

Each sample was treated with 6 mL of HNO_3 (69%, ROTIPURAN® SUPRA, Carl Roth) and 1 mL of H_2O_2 (30%, trace analysis grade). Microwave digestion was carried out according to the selected program (maximum temperature $200\text{ }^{\circ}\text{C}$, maximum pressure 30 bar), including subsequent evaporation of the reagents. The residue following digestion was reconstituted with deionized water and quantitatively transferred to a final volume of 10 mL in pre-cleaned polypropylene tubes (15 mL).

Prior to ICP-MS analysis (PerkinElmer ELAN DRC-e or Agilent 7900), the samples were further diluted as required with 1% (v/v) HNO_3 containing an internal standard (indium), to a final concentration of $10\text{ }\mu\text{g}\cdot\text{L}^{-1}$. The overall dilution factor ranged from 10 to 1000. Blanks and CRMs were treated identically (typically diluted 10-fold). In addition, post-digestion spiked samples were analysed. Semi-quantitative analysis was performed using multi-element certified reference standards in nitric acid (ASTASOL AN9115MN, Analytika; 18 REEs at $100\text{ mg}\cdot\text{L}^{-1}$ each) or Multi-element Standard XVI (Merck, Supelco) for calibration. The certified reference material INCT-OBTL-5 (tobacco leaves) was used as a matrix CRM.

CHAPTER FOUR

Results and Discussion

4.1. Substrate stability and medium composition effects

The chemical stability of substrates in biological media is critical for the success of biotransformation studies. The NMR results (^{31}P and ^1H) revealed that epoxymethyl dimethyl phosphonate (EMDP) exhibited high stability in BG-11 and BG-11₀ media (section 3.3.2), with no detectable degradation, indicating their suitability for sustained photobiocatalytic applications. In contrast, degradation of EMDP was observed in Spirulina Medium (SM) and its modified variant, as well as in the modified BG-11 medium over a 7-day incubation period (Figure 12).

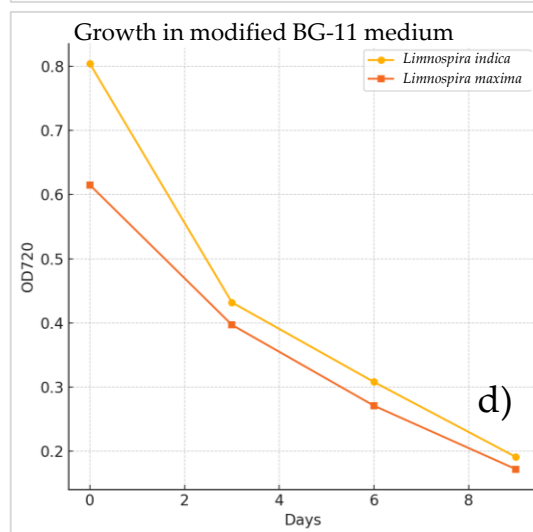
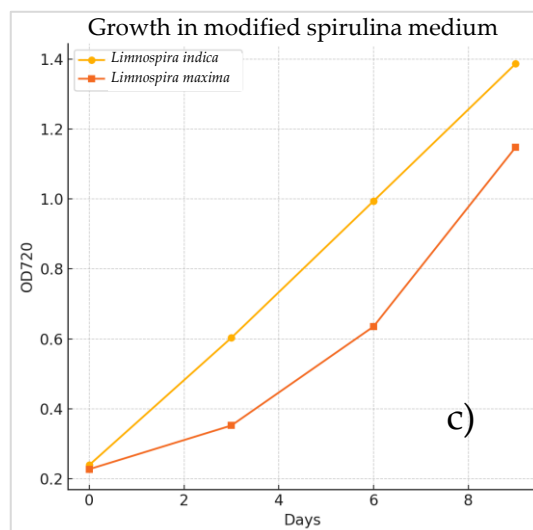
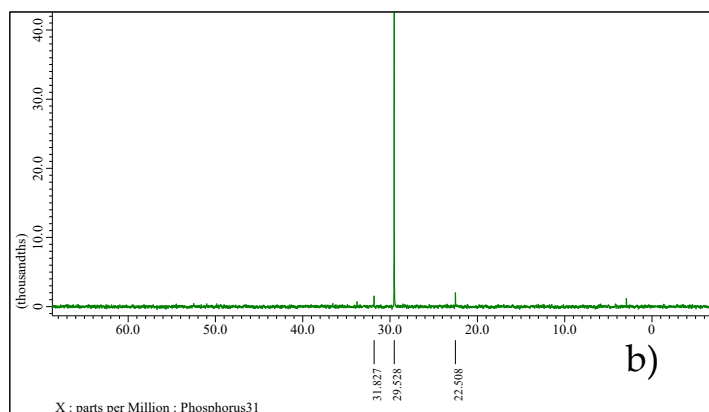
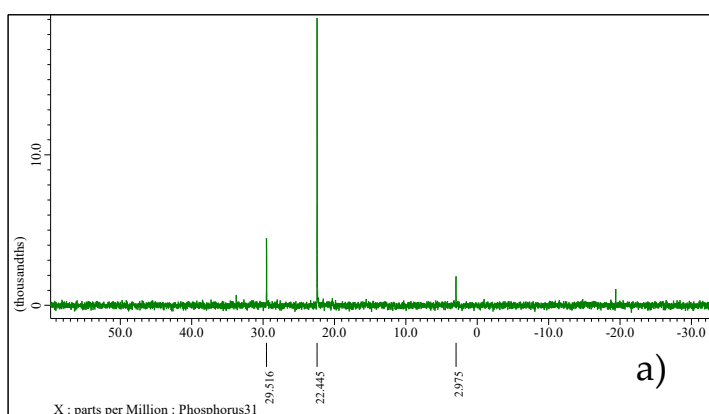


Figure 12. Comparative stability of EMDP and biomass growth of *Limnospira* sp. in modified media (section 3.3.3) over 7 days: (a) ^{31}P NMR spectrum of EMDP in modified spirulina medium; (b) ^{31}P NMR spectrum of EMDP in modified BG-11 medium; (c) growth curve of *L. indica* and *L. maxima* in modified SM; (d) growth curve of both strains in modified BG-11.

The instability of EMDP in modified media (section 3.3.3) is consistent with nucleophilic and coordination-driven transformations of organophosphonates, where elevated concentrations of bicarbonate, phosphate, and metal ions facilitate hydrolysis or structural rearrangement of the epoxide and phosphonate groups Mian *et al.* [196]. Spectroscopic analysis using ^{31}P NMR revealed that EMDP maintained substantial chemical stability in modified BG- 11 medium over the 7- day incubation (Figure 12b), with minimal spectral changes observed. In contrast, modified spirulina medium induced pronounced instability in the substrate (Figure 12a), implicating chemical interaction of the substrate with the medium components.

Similarity to these findings, the growth dynamics of *Limnospira* species displayed a medium-dependent pattern. In modified spirulina medium, both *L. indica* and *L. maxima* exhibited exponential growth (Figure 12c), with *L. indica* achieving higher biomass density. Conversely, modified BG-11 medium supported limited growth (Figure 12d), with both strains experiencing a progressive decline in OD_{720} , indicative of physiological stress or suboptimal nutrient composition despite its compatibility with substrate stability. Figure 12c shows a typical growth response in modified Spirulina medium with a lower initial OD (≈ 0.2), allowing clear observation of biomass increase. In contrast, Figure 12d starts at a higher OD (≈ 0.8) in modified BG-11 medium due to the use of a denser inoculum to ensure measurable optical signals as the experiments were not intended to provide directly comparable growth kinetics but to assess culture viability and biomass response in relation to medium composition and substrate stability. Accordingly, the media were evaluated independently rather than in simultaneous conditions. The progressive decline in cyanobacterial activity

observed in modified medium (Figure 12d) indicates physiological stress and metabolic imbalance due to the alteration of the original medium (BG-11), which adversely affects their enzymatic performance and growth. Under suboptimal medium composition, alterations in nutrient ratios (particularly nitrogen and phosphorus), ionic strength, and metal concentrations can disrupt cellular homeostasis and impair photosynthetic efficiency [197,198].

These dual observations emphasize the inherent adjustment between medium formulations optimized for biomass growth against those favouring substrate stability. Future medium development should focus on achieving a balance between biocompatibility and chemical inertness, particularly when working with organophosphorus compounds such as EMDP. The primary aim behind modifying SM and BG-11 was to optimize cyanobacterial biomass production while concurrently minimizing substrate instability. However, the observed instability of EMDP in the modified media indicates that further refinement is necessary on balancing the nutritional needs of the biomass with the chemical stability of sensitive substrates such as EMDP.

Importantly, the unmodified BG- 11 and BG-11₀ media, which are the principal media used for cyanobacterial cultivation and photobiocatalytic assays, provided a chemically inert environment for EMDP. This compatibility ensures that these media remain the platforms of choice for evaluating cyanobacterial biocatalysis involving epoxyphosphonate substrates. The stability of selected substrates in cyanobacterial media shows the interaction between medium composition, substrate integrity, and experimental reliability.

4.2. Bioconversions with cyanobacteria

The photobiocatalytic activity of selected cyanobacterial strains was investigated using structurally diverse substrates including esters of vinyl phosphonates and epoxy phosphonate and acetate ester. The following sections present the experimental results

and discussions on substrate conversion and product formation and subsequent bioaccumulation studies.

4.2.1. Dimethyl vinylphosphonate (DMVP) and diethyl vinylphosphonate (DEVP) bioconversions

Over a 30- day incubation period, no enzymatic conversion or degradation of either compound by the cyanobacterial strains was observed, as confirmed by ^{31}P NMR spectra (Figure 13 and Figure 14).

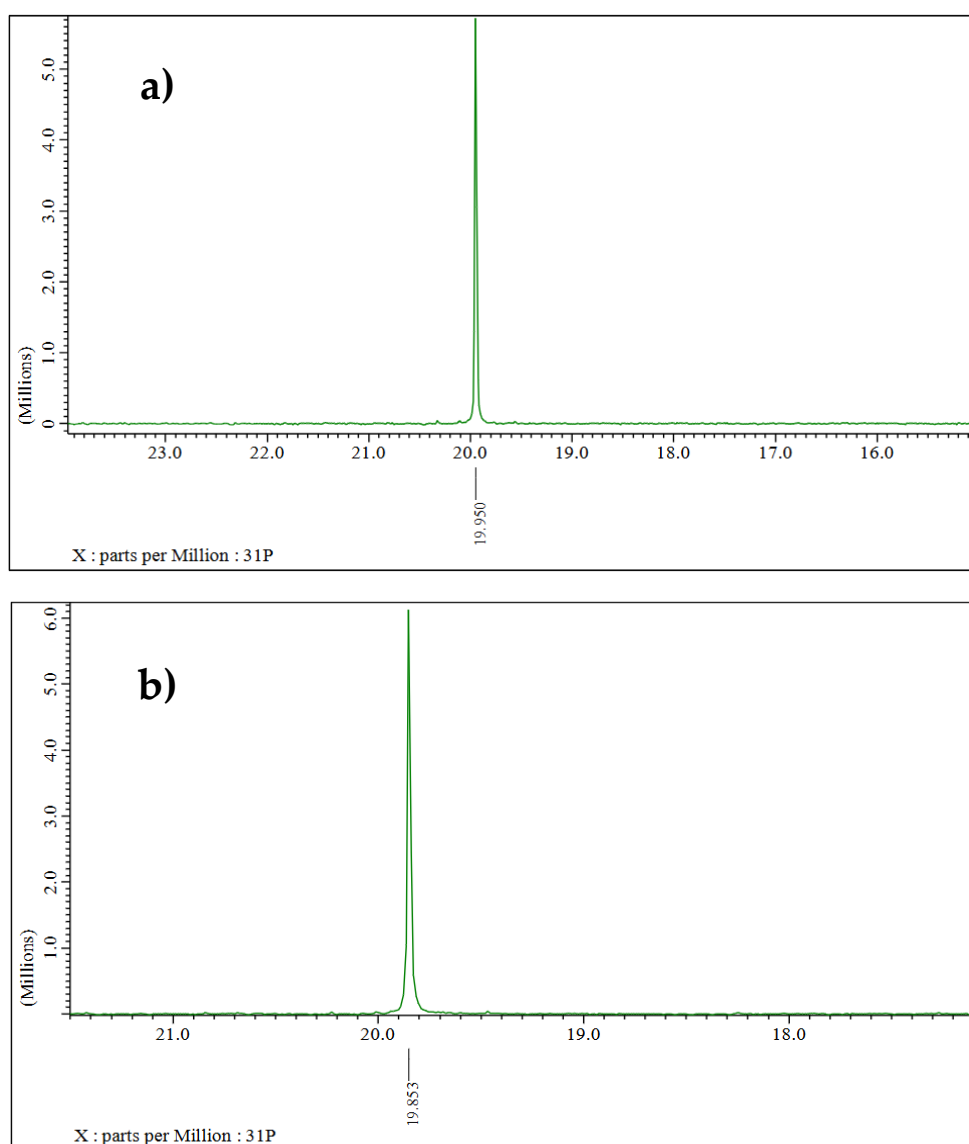


Figure 13. NMR spectra (^{31}P) of dimethyl vinylphosphonate. **a)** substrate sample; **b)** sample after biotransformation (up to 30 days)

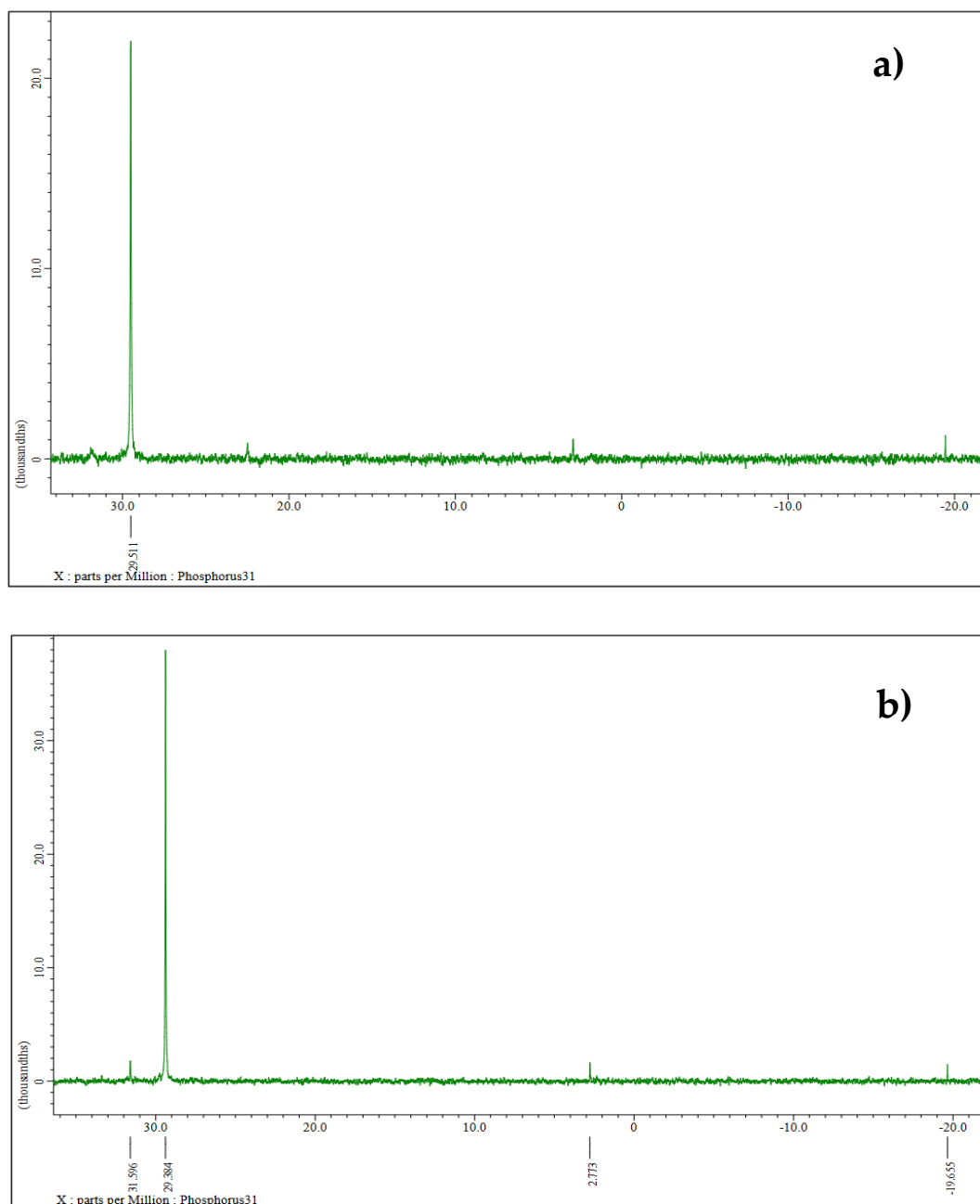


Figure 14. NMR spectra (^{31}P) of diethyl vinylphosphonate. **a)** substrate sample; **b)** sample after biotransformation (up to 30 days)

The inability of cyanobacteria to transform DMVP and DEVP aligns with previous reports showing the limited enzymatic capability of cyanobacteria toward C-P bond cleavage in vinyl phosphonates by Singh and Walker [199]. Despite this lack of bioconversion, inorganic phosphate status was assigned to check if the P-C bond degradation was dependent on the presence of external, easy bioavailable source of phosphorous. It was conducted for following outcomes: photobiocatalytic exposure to

DMVP in the presence of *Nostoc cf-muscorum* CCALA 129, *Synechococcus bigranulatus* CCALA 187, and *Limnospira* spp. (PCC 8005 and CCALA 27), and was confirmed by the Malachite Green assay (Figure 15). This colorimetric assay, recognized for its high sensitivity in phosphate detection [200–202], has been validated in a range of environmental and biochemical contexts with reduced amine interference [203]. This suggests a non- enzymatic, likely hydrolytic process.

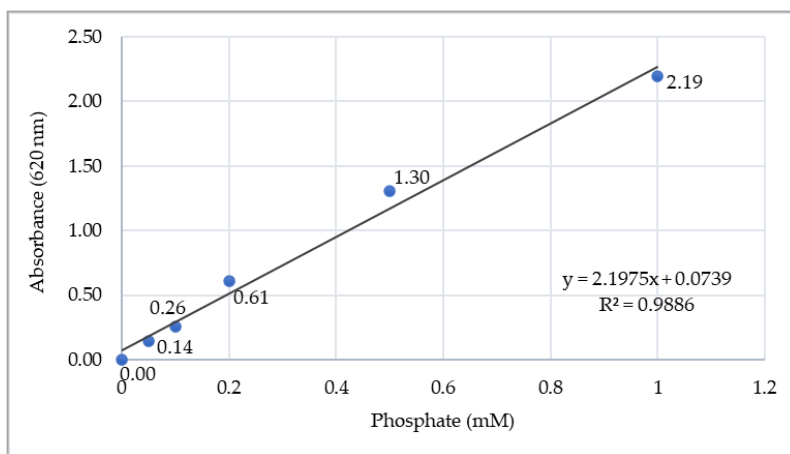


Figure 15. Malachite Green assay standard curve (K_2HPO_4).

Figure 15 showed the standard curve using the Malachite Green Assay, establishing a correlation between phosphate concentration and absorbance at 620 nm. This standard curve was used to quantify the observed inorganic phosphonates (Table 13) during photobiocatalysis of vinyl phosphonates.

Table 13. Concentration (mM) of inorganic phosphonate in the test samples (A_{620})

Sample	0 h		72 h	
	A_{620}	Conc. (mM)	A_{620}	Conc. (mM)
<i>Nostoc</i> sp. + DMVP	0.082	0.007	0.049	0.023
<i>Synechococcus</i> sp. + DMVP	0.044	0.069	0.108	0.006
<i>Limnospira indica</i> + DMVP	0.141	0.023	0.037	0.066
<i>Limnospira maxima</i> + DMVP	0.520	0.212	0.195	0.050
Control:				

<i>Nostoc</i> sp. (21-days culture)	0.599	0.251	0.106	0.005
<i>Synechococcus</i> sp. (21-days culture)	0.572	0.237	0.056	0.020
<i>Limnospira indica</i> (21-days culture)	6.200	3.041	0.287	0.095
<i>Limnospira maxima</i> (21-days culture)	6.200	3.041	0.320	0.112
BG-11 Medium	0.572	0.237	–	–
BG-11 ₀ Medium	0.599	0.251	–	–
SM Medium	6.200	3.041	–	–

Note: “–” indicates that the test was not applicable under the given conditions.

The Malachite Green Assay results in Table 13 indicate that the detected inorganic phosphonate observed in the cultures of *Nostoc* sp., *Synechococcus* sp., and *Limnospira* sp. exposed to DMVP phosphate levels were relatively low. This showed that any phosphate release was minimal and likely due to non-enzymatic hydrolysis rather than a direct biocatalytic process. The presence of background phosphate levels in control samples further supports this observation. For samples such as *Limnospira* sp. (21-day culture) and SM Medium in Table 13, elevated turbidity resulted in apparent absorbance values outside the original calibration range of the malachite green assay. To address this, these samples were appropriately diluted prior to measurement, and the reported values were calculated by multiplying the measured OD by the corresponding dilution factor to place them within the instrument’s valid range. This approach aligned with standard analytical practice when measuring highly turbid samples to ensure the reliability of absorbance data.

The lack of observable enzymatic conversion of DMVP and DEVP by the cyanobacterial strains used in this study could be due to genomic and physiological constraints, including the absence or limited expression of phosphonate transport and C–P bond cleavage genes (*phn*), as well as the tight regulation of photosynthetic

electron transport and intracellular redox metabolism, which prioritize photoautotrophic growth over biotransformation [204–206].

4.2.2. Bioconversion of epoxymethyl dimethyl phosphonate

This section reveals the bioconversion of epoxymethyl dimethyl phosphonate by cyanobacterial strains, with emphasis on substrate specificity, enzymatic activity towards substrate, and structural confirmation of the resulting product.

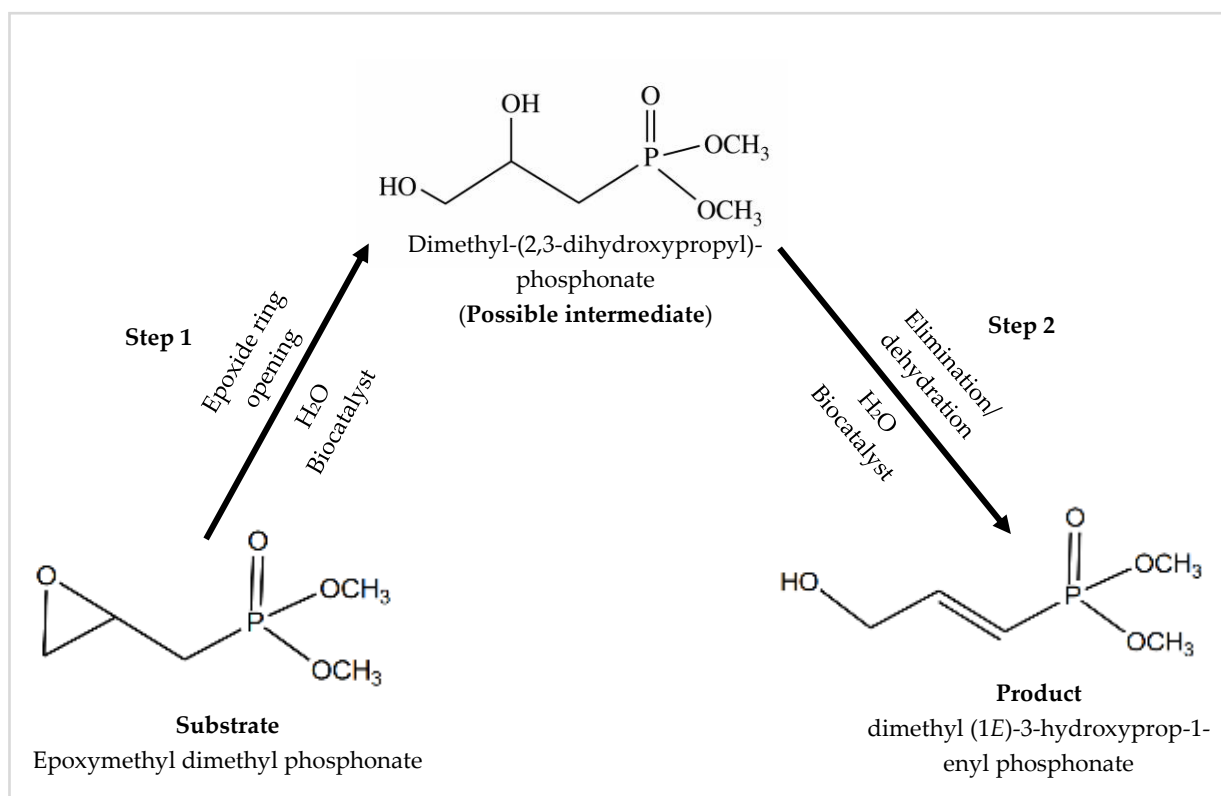


Figure 16. Possible two-step bioconversion of epoxymethyl dimethyl phosphonate by biocatalysts (*Synechococcus* sp. and *Limnospira* sp.).

The EMDP envisioned to undergo enzymatic ring-opening reactions mediated by the biocatalysts (Figure 16) and possibly result in the formation of dimethyl-(2,3-dihydroxypropyl)-phosphonate as the intermediate product (step 1). While Step 2 involves elimination to form the (E)-alkenylphosphonate product, dimethyl (1E)-3-hydroxyprop-1-enylphosphonate. Hydroxyphosphonates possess distinct physicochemical properties and biological activities that are relevant in various

biotechnological applications. For example as key building blocks in the synthesis of nucleoside analogs and other bioactive compounds [207].

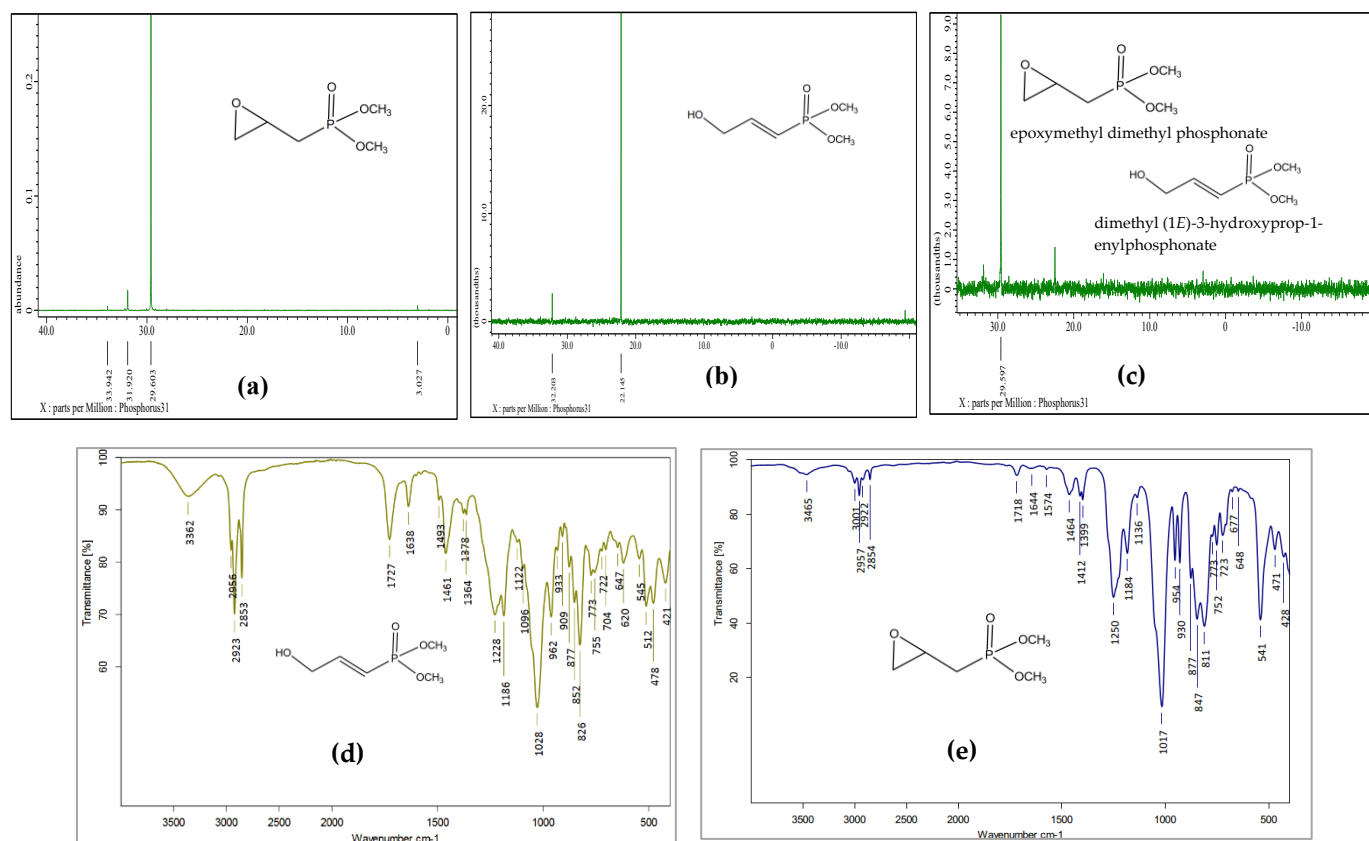


Figure 17. Spectral analysis of the biotransformation process: (a) ^{31}P NMR spectrum of the substrate, EMDP; (b) ^{31}P NMR spectrum of the complete transformed substrate, EMDP to product, DMHPP; (c) ^{31}P NMR spectrum showing partial substrate, EMDP conversion; (d) IR spectrum of the complete transformed substrate, EMDP to product, DMHPP (e) IR spectrum of the substrate, EMDP [186].

Spectroscopic characterization, as shown in Figure 17, substantiates the molecular transformation of EMDP. The ^{31}P NMR spectrum of the unconverted substrate (Figure 17a) exhibited chemical shifts typical of phosphonate groups. Following complete bioconversion (Figure 17b), notable spectral shifts in the phosphorus environment indicated the formation of product. The partially converted intermediate (Figure 17c) displayed mixed spectral features, confirming simultaneous presence of substrate and product, and thereby offering insights into reaction kinetics. The IR spectrum of the fully transformed product (Figure 17d) corroborated these findings by confirming

vibrational bands associated with unsaturated C=C and hydroxyl groups, consistent with the molecular structure of dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate. While Figure 17e shows the IR spectrum of the substrate, enabling full comparison with the final product [208–210].

The obtained product (Figure 17) from the bioconversion of epoxymethyl dimethyl phosphonate subsequent analyses were as follow – MS (TOF MS ES+) Calcd for C₅H₁₁O₄P [M + H]⁺ 167.0473; found: 167.0481; IR: 3362 (OH), 1638 (CH=CH) cm⁻¹; ³¹P NMR (CDCl₃, δ, ppm): 22.64; ¹H NMR (CDCl₃, δ, ppm): 3.69 (d, *J* = 11 Hz, 6H, 2×P(O)OCH₃), 4.26–4.31 (m, 2H, CH₂OH), 5.96 (ddt, *J* = 2, 17, 21 Hz, 1H, P-CH=CH), 6.86 (ddt, *J* = 3, 17, 23 Hz, 1H, P-CH=CH); ¹³C NMR (CDCl₃, δ, ppm): 52.58 (d, *J* = 6 Hz, 1C, POCH₃), 62.36 (d, *J* = 22 Hz, 1C, HOCH₂CH=CH), 113.22 (d, *J* = 191 Hz, 1C, P-CH=CH), 153.69 (d, *J* = 5 Hz, 1C, P-CH=CH) [188]. The assignment of signals on ¹H and ¹³C NMR were further confirmed by ¹H COSY, ¹H-¹³C HMQC, and ¹H-¹³C HMBC spectra.

4.2.3. Conventional transformation of epoxymethyl dimethyl phosphonate

The chemical transformation of epoxymethyl dimethyl phosphonate (EMDP) successfully yielded dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate (DMHPP), providing a reliable reference standard for comparison with the biocatalytically produced compound (Figure 18). DMHPP was isolated in 81.4% yield with a purity of 85%. The isolated yield is comparable to those reported for similar phosphonate transformations under mild basic conditions, showing the suitability of this approach for preparing analytical standards [211].

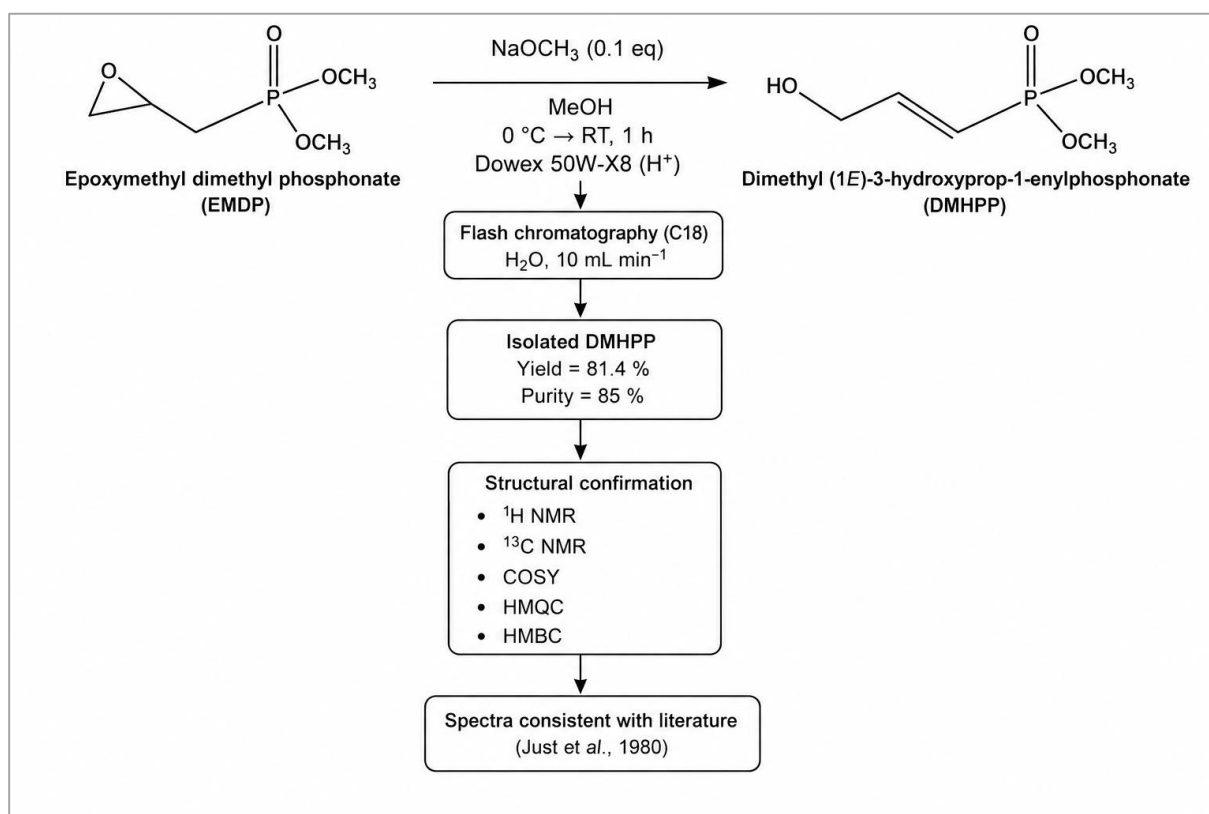


Figure 18. Schematic representation of the chemical synthesis of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate (DMHPP) from epoxymethyl dimethyl phosphonate (EMDP).

The chemically synthesized DMHPP provided a reliable reference for comparison with the product obtained through biocatalysis. The combined use of one- and two-dimensional NMR spectroscopy is well established for the structural elucidation of organophosphorus compounds, as originally reported by Just et al. [188]. Spectroscopic characteristics between the chemically synthesized standard and the enzymatically produced compound provided reliable evidence that both approaches generated the same product, thereby confirming the identity of the biocatalytic product.

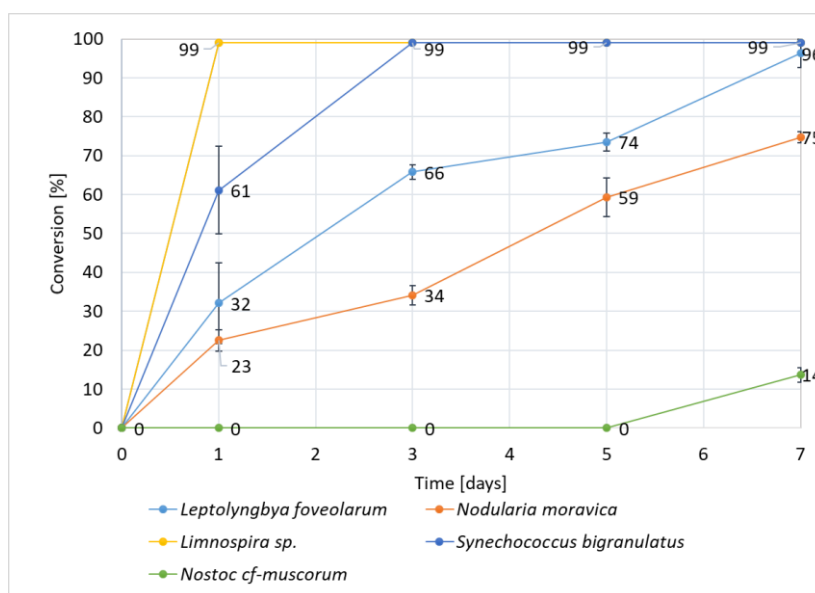


Figure 19. Comparative analysis of the bioconversion percentages by cyanobacterial biocatalysts [186].

Figure 19 presents a comparative assessment of six cyanobacterial strains, *Synechococcus bigranulatus* CCALA 187, *Nostoc cf-muscorum* CCALA 129, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Leptolyngbya foveolarum* CCALA 76, and *Nodularia moravica* CCALA 114, for their catalytic efficacy in converting EMDPP into dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate. All strains showed progressive conversion over a 7-day period, though significant differences in efficiency were observed. *Limnospira* sp. and *Synechococcus* sp. strains exhibited the most rapid and complete bioconversion, achieving 99% conversion degrees within 24 h for *Limnospira* sp., and 72 h for *Synechococcus* sp., and maintaining this level of efficiency throughout the experimental period (Figure 19). This high performance is likely attributable to their advanced photosynthetic apparatus and their compatibility with the experimental photonic conditions, facilitating enhanced photobiocatalytic activity. By contrast, *Leptolyngbya foveolarum* CCALA 76 and *Nodularia moravica* CCALA 114 display moderate percentage conversion efficiencies, attaining 96% and 75% conversion respectively by day seven. *Nostoc cf-muscorum* CCALA 129 was the least effective, with only 14% conversion over the same period, potentially due to intrinsic

physiological constraints such as lower substrate permeability or limited enzymatic repertoire relevant to EMDP metabolism.

Table 14. Bioconversion degree (%) of epoxymethyl dimethyl phosphonate (EMDP) by different cyanobacterial strains and the corresponding yield (%) of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate (DMHPP) obtained using selected biocatalysts at different substrate concentrations.

Bioconversion degree (%)						DMHPP yield (%)		
Day	<i>L. foveolarum</i>	<i>N. moravica</i>	<i>Limnospira</i> sp.	<i>S. bigranulatus</i>	<i>N. cf. muscorum</i>	<i>S. bigranulatus</i> (1 mM)	<i>Limnospira</i> sp. (1 mM)	<i>S. bigranulatus</i> (4 mM)
1	32.0 ± 10.0	23.0 ± 2.0	99.0 ± 0.0	61.0 ± 11.0	0.0	10.0 ± 1.1	8.4 ± 2.5	6.5 ± 1.2
3	66.0 ± 2.0	34.0 ± 2.0	99.0 ± 0.0	99.0 ± 0.0	0.0	7.1 ± 0.7	5.9 ± 0.4	6.5 ± 1.2
5	74.0 ± 2.0	59.0 ± 4.0	99.0 ± 0.0	99.0 ± 0.0	0.0	2.6 ± 0.4	0.7 ± 0.0	3.2 ± 0.5
7	96.00 ± 1.00	75.0 ± 0.0	99.0 ± 0.0	99.0 ± 0.0	14.0 ± 2.0	1.0 ± 0.4	0.0 ± 0.0	2.9 ± 0.2

The data presented in Table (14) showed the percentage conversions and yields of bioconversion product. In terms of yield, *Synechococcus* sp. consistently outperformed *Limnospira* sp. across the time frame. At 1 mM EMDP, *Synechococcus* sp. achieved a peak yield of 9.95 ± 1.08 % of the product on day 1, which steadily declined to 1.00 ± 0.40 % by day 7. In contrast, *Limnospira* sp. began with a comparable initial product yield of 8.42 ± 2.53 % but declined precipitously, reaching zero by day 7. Given these results, *Synechococcus* sp. was selected for optimization under a higher substrate concentration of 4 mM, where a maximum product yield of 6.50 ± 1.20 % was observed by day 3, similar to the day 1 value of 6.46 ± 1.20 %, with a gradual reduction to 2.93 ± 0.20 % by day 7. Wiemer and Wiemer [212] and Hecker and Erion [213] reported that phosphonate esters can insert asymmetrically into lipid bilayers, leading to increased retention and altered distribution. This characteristic provides a compelling rationale for the experimentally observed low yield of DMHPP from the supernatant (Table 14), despite near-complete EMDP conversion (Figure 19). These insights could also justify the bioaccumulation potential of cyanobacterial strains. Also, these bioconversion yields can be in correlation to the increased capacities of cyanobacterial strains to bioaccumulation of REE's what is discussed in section 4.3.4.

The more pronounced decline in DMHPP yield observed for *Limnospira* sp. compared with *Synechococcus* sp. suggests strain-dependent differences in the capacity to sustain EMDP biotransformation. Such variability is consistent with previous studies showing that cyanobacterial species differ in their enzymatic repertoire and metabolic characteristics, resulting in differences in substrate specificity and catalytic efficiency [205,214]. In addition, the catalytic activity of cyanobacteria is closely linked to their physiological state and photosynthetic metabolism, which can influence the availability of cellular resources for biotransformation processes [204]. Therefore, the higher and more sustained product yield observed for *Synechococcus* sp. reveal a greater capacity to maintain catalytic activity towards EMDP under the experimental conditions applied in this study.

EMDP exhibited very good susceptibility to bioconversion under photoautotrophic conditions. The selective bioconversion of EMDP to dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate by *Synechococcus bigranulatus* CCALA 187 and *Limnospira* sp. is likely attributable to the presence of epoxide hydrolases, which catalyze the nucleophilic attack on the strained three-membered epoxide ring [215]. This functional group is absent in the other tested organophosphonates, which could explain their inertness under photoautotrophic conditions. EMDP's physicochemical properties, such as its water solubility, moderate lipophilicity, and electrophilic center, further enhance its accessibility to enzymatic systems. These observations show a structure–activity relationship governed primarily by functional group compatibility with native cyanobacterial enzymatic machinery. However, *Nostoc cf-muscorum* CCALA 129 showed limited capability due to the shift of their large portions of metabolic resources toward nitrogen fixation rather than photobiocatalysis as expatiated by Herrero *et al.* [216,217]. This strain-specific activity suggests a distinct distribution of epoxide hydrolase enzymes across cyanobacterial taxa.

The transformation of epoxymethyl dimethyl phosphonate into dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate by cyanobacterial strains is, as we can presume, a

two-step biochemical process: epoxide ring opening, yielding a vicinal diol intermediate, and a subsequent dehydration/elimination, resulting in the conjugated vinyl phosphonate product. The ability of certain cyanobacterial strains to catalyze this reaction, while others cannot, points toward species-specific differences in enzymatic equipment, gene regulation, and metabolic prioritization.

The first step, epoxide hydrolysis, is most plausibly catalyzed by epoxide hydrolases (EHs), enzymes that catalyze nucleophilic attack on the strained epoxide ring, converting it to a 2,3-diol. The enzymatic basis for this activity in microbes has been extensively reviewed by Hult and Berglund [218], who emphasize that microbial EHs are widespread in both prokaryotic and eukaryotic organisms and can act on a variety of substrates, including terminal epoxides like EMDP. Their work shows that EHs possess a conserved catalytic triad (Asp-His-Asp/Glu) and often function under mild conditions, making them likely candidates for spontaneous or constitutive expression in cyanobacteria. This directly supports the idea that the cyanobacterial conversion of EMDP begins via EH-mediated ring opening, a reaction broadly feasible in microbial hosts [218]. Similarly, Archelas and Furstoss [219] demonstrate that microbial EHs exhibit significant substrate promiscuity and stereoselectivity, suggesting that naturally occurring cyanobacterial EHs (even if not specialized) could process the EMDP substrate, especially due to the electron-withdrawing effect of the phosphonate group, which increases epoxide ring susceptibility. Their work further supports the view that such transformations can proceed in wild-type, non-engineered strains under standard cultivation [219]. The second step of the bioconversion, conversion of the diol to a conjugated hydroxyalkenyl phosphonate, likely proceeds via dehydration of the hydroxyl intermediate, mechanisms previously observed in microbial phosphonate-processing pathways [220].

4.2.4. Antibacterial plate testing of epoxymethyl dimethyl phosphonate (EMDP) and its bioconversion product, dimethyl (1*E*)-3-hydroxyprop-1-enylphosphonate (DMHPP)

This part constitutes a supplementary experiment to our main research. The rationale for testing EMDP and its bioconversion product, DMHPP, for antibacterial activity (Figure 20) lies in the established bioactivity of organophosphorus compounds, particularly those bearing C–P bonds, vinyl, or epoxide moieties. Such structures are frequently found in synthetic phosphonates with reported antimicrobial or inhibitory effects due to their structural mimicry of phosphate esters and their potential to disrupt microbial metabolism [221]. This evaluation aimed to determine whether either compound exhibited inherent antibacterial effects and to assess any biosafety concerns relevant to scale-up or environmental deployment. Although no significant antimicrobial activity was observed, the testing contributes to defining the bioactivity boundaries of these compounds and supports their suitability for biocatalytic applications where non-toxicity is essential.

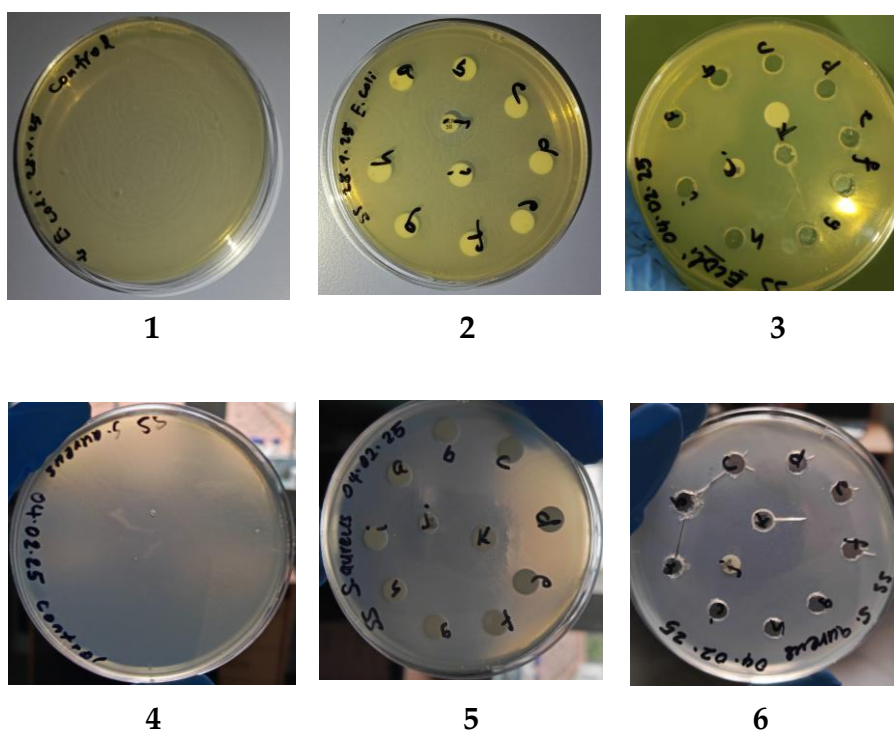


Figure 20. Antimicrobial susceptibility testing using disk diffusion (**Plates 2 and 5**) and agar well diffusion (**Plates 3 and 6**). Plates **1** and **4** are controls with *E. coli* and *S. aureus*.

DMHPP (a – d) and EMDP (e – h) were tested at 15.8, 7.9, 3.95, and 1.98 mg/mL, with controls: i = DMSO, j = cefoperazone 30 µg, k = EMDP (24.56 mg for disk diffusion, 85.96 mg for agar well diffusion).

The disk diffusion method (Table 15) showed no antibacterial activity for both the substrate, epoxymethyl dimethyl phosphonate, and the product, dimethyl (1E)-3-hydroxyprop-1-enylphosphonate, at concentrations ranging from 0.0396 mg to 0.316 mg per disk. Even at an undiluted concentration equivalent to 24.56 mg per disk, EMDP produced a zone of inhibition of 0.7 cm against *Escherichia coli* and 1.4 cm against *Staphylococcus aureus*. However, DMHPP did not exhibit any inhibitory effect, indicating its ineffectiveness at tested concentrations. Cefoperazone, used as the positive control, showed significantly larger inhibition zones of 0.9 cm for *E. coli* and 1.2 cm for *S. aureus*, demonstrating a strong antibacterial activity.

Table 15. Antimicrobial activity of EMDP and DMHPP using the disk diffusion method

Microbial strain (McFarland standard)	Zone of inhibition (cm)										
	Substrate (EMDP)					Product (DMHPP)				Antibiotic (CEP30)	Solvent (DMSO)
	0.0396	0.079	0.158	0.316	24.56	0.0396	0.079	0.158	0.316	30 µg	22 mg
	mg	mg	mg	mg	mg*	mg	mg	mg	mg		
<i>Escherichia coli</i>	0.00	0.00	0.00	0.00	0.70	0.00	0.00	0.00	0.00	0.90	0.00
<i>Staphylococcus aureus</i>	0.00	0.00	0.00	0.00	1.40 ± 1.41E-01	0.00	0.00	0.00	0.00	1.20	0.00

Note: Volume of each sample applied to the disk = 20 µL. Equal concentrations of substrate and product were applied, except where indicated by *.

The agar well diffusion method (Table 16) produced similar results, where EMDP exhibited no inhibition at concentrations up to 1.106 mg per well. However, at 85.96 mg, EMDP produced an inhibition zone of 1.65 cm against *E. coli* and 1.55 cm against *S. aureus*, confirming its antibacterial potential at a high concentration. DMHPP exhibited no antibacterial activity at tested concentrations, confirming its inert nature in antimicrobial contexts. The positive control, cefoperazone, produced inhibition zones of 0.9 cm for *E. coli* and 1.2 cm for *S. aureus*, consistent with the disk diffusion

method. The lack of antibacterial activity in DMHPP, even at high concentrations, suggests the need for structural modifications or its use in combination with other antimicrobial agents to enhance its potential efficacy in future applications.

Table 16. Antimicrobial activity of EMDP and DMHPP using the agar well diffusion method

Microbial strain (McFarland standard)	Zone of inhibition (cm)										
	Substrate (EMDP)					Product (DMHPP)				Antibiotic (CEP30)	Solvent (DMSO)
	0.1386 mg	0.2765 mg	0.553 mg	1.106 mg	85.96 mg*	0.1386 mg	0.2765 mg	0.553 mg	1.106 mg	30 µg	77 mg
<i>Escherichia coli</i>	0.00	0.00	0.00	0.00	1.65 ± 7.07E-02	0.00	0.00	0.00	0.00	0.90	0.00
<i>Staphylococcus aureus</i>	0.00	0.00	0.00	0.00	1.55 ± 7.07E-02	0.00	0.00	0.00	0.00	1.20	0.00

Note: Volume of each sample applied to the well = 70 µL. Equal concentrations of substrate and product were applied, except where indicated by *.

Although phosphonate-containing compounds are known to exhibit antimicrobial activity, their efficacy depends strongly on structural features that enable active transport into cells and inhibition of specific metabolic enzymes, such as those involved in cell wall biosynthesis [221]. In the present case, the tested compounds likely lack the structural motifs required for efficient uptake or target binding, resulting in negligible biological activity at low concentrations. Alternatively, the tested bacterial strains may possess intrinsic resistance mechanisms that reduce their susceptibility to these phosphonate derivatives. However, the absence of significant antibacterial activity does not preclude the chemical and pharmaceutical relevance of the investigated compounds. DMHPP represents a valuable synthetic intermediate, as it can be further oxidized to dimethyl (1*E*)-3-oxoprop-1-enylphosphonate, which is utilized in the synthesis of β -lactam antibiotics and related bioactive molecules in medicinal chemistry; as scaffolds for enzyme inhibitors, antiviral agents, and anticancer compounds due to their ability to mimic phosphate esters and act as transition-state analogues [186,221,222]. In addition, previous studies also demonstrated that certain phosphonate derivatives function as adjuvants and exhibit

enhanced antibacterial effects when combined with conventional antibiotics, leading to reduced inhibitory concentrations and improved efficacy [223].

4.2.5. Bioconversion of 1-phenylethyl acetate

Figure 21 presents GC chromatograms confirming successful enantioselective bioconversion while Figure 22 present the graphical representations of the concentrations of the standards. The emergence of distinct peaks for (*R*)- and (*S*)-1-phenylethanol supports enzymatic activity favouring selective hydrolysis of 1-phenylethyl acetate. The GC chromatograms also confirm the formation of acetophenone (Figure 21 c-e), which arises via oxidation of 1-phenylethanol by endogenous alcohol dehydrogenases (ADHs).

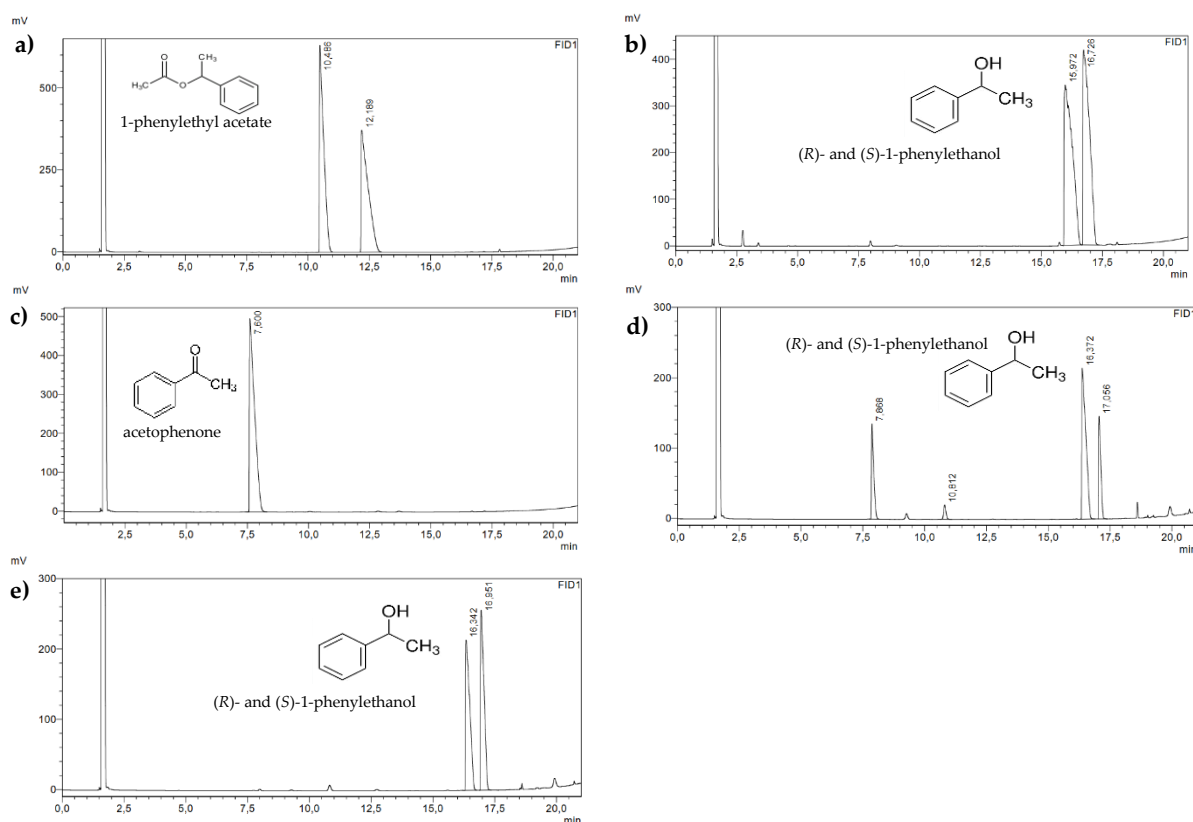


Figure 21. Representative gas chromatograms of 1-phenylethyl acetate bioconversion. (a) Standard 1-phenylethyl acetate; (b) standards of (*R*)- and (*S*)-1-phenylethanol; (c) standard acetophenone; (d) reaction mixture obtained after 24 h biotransformation

with *Leptolyngbya foveolarum* (14-day culture); (e) reaction mixture obtained after 24 h biotransformation with *Synechococcus bigranulatus* (14-day culture).

The chiral alcohol products, particularly (*S*)-1-phenylethanol, are valuable intermediates in the synthesis of pharmaceuticals, including β -blockers and antihistamines, as well as in fragrances and agrochemicals [147,224]. From a green chemistry perspective, achieving this transformation under mild, aqueous, and energy-efficient conditions is both scientifically and industrially significant.

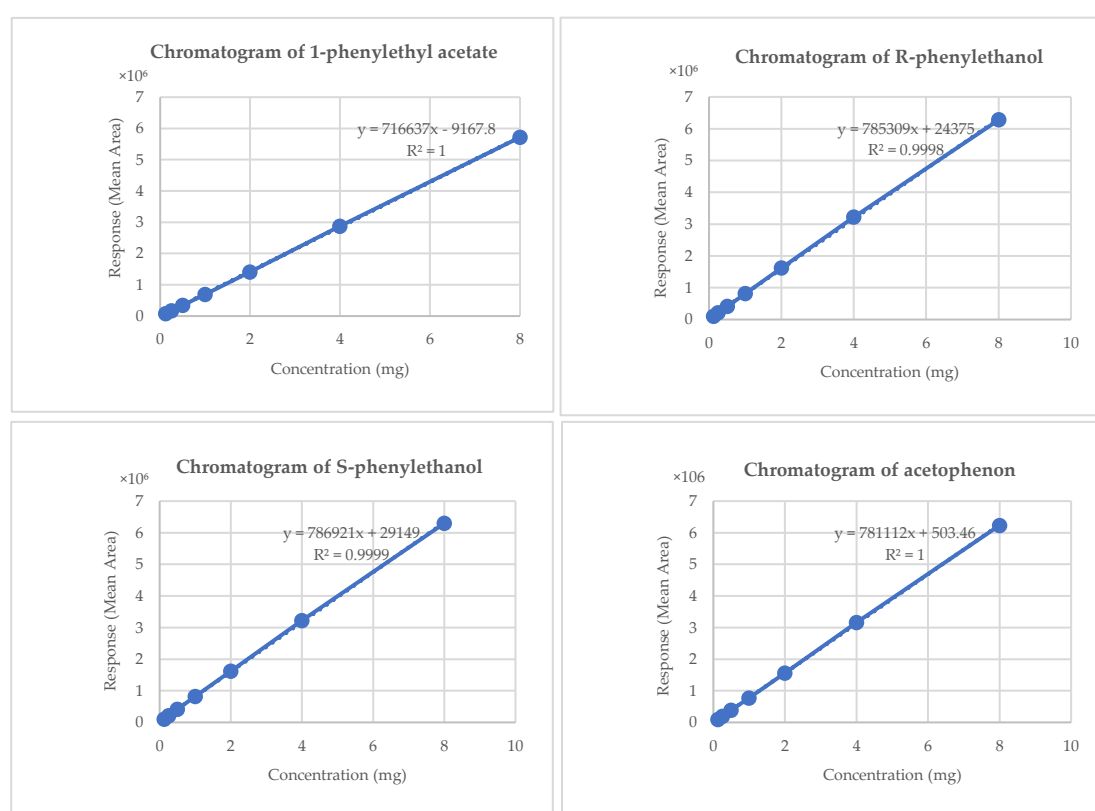


Figure 22. Calibration curves for quantitative GC analysis of 1-phenylethyl acetate, (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, and acetophenone.

The optimization process for the bioconversion of 1-phenylethyl acetate aimed to enhance the enantiomeric excess (e.e.) of the final product, phenylethanol. Key parameters assessed include the comparison between automatic and manual growth chambers, the duration of biomass production, photobiocatalysis time, optimization of light exposure, and the selection of a suitable cyanobacterial strain. Through these

evaluations, *Leptolyngbya foveolarum* CCALA 76 was identified as the most bioactive strain under the phytotron growth chamber. The biomass of this strain was subsequently utilized for the bioaccumulation of rare earth elements (REEs), yielding promising results. In comparison with the research published by Śliżewska *et al.* [187], specifically in terms of reproducibility, the findings aligns with the obtained results.

Table 17. Biocatalytic conversion of 1-phenylethyl acetate by *Synechococcus bigranulatus* and *Leptolyngbya foveolarum* under varying cultivation conditions.

Biocatalyst	Culture age	System	Time (h)	Conversion (%)	ee Product (%)	ee Substrate (%)
<i>Synechococcus</i> sp.	7 days	Phytotron, 47 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	2	35.6 $\pm$ 12.8	5.8 $\pm$ 0.3	4.0 $\pm$ 1.5
			4	50.0 $\pm$ 6.7	5.5 $\pm$ 5.1	5.2 $\pm$ 3.3
			6	67.8 $\pm$ 1.3	3.8 $\pm$ 0.3	7.7 $\pm$ 0.2
			8	75.1 $\pm$ 1.2	10.7 $\pm$ 0.3	29.1 $\pm$ 3.0
			24	99.1 $\pm$ 0.6	0.5 $\pm$ 0.4	28.9 $\pm$ 18.8
<i>Leptolyngbya</i> sp.	7 days	Phytotron, 47 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	2	44.3 $\pm$ 1.1	0.6 $\pm$ 0.2	1.0 $\pm$ 0.0
			4	50.3 $\pm$ 0.4	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1
			6	72.6 $\pm$ 7.4	1.8 $\pm$ 0.6	0.9 $\pm$ 0.2
			8	77.9 $\pm$ 7.2	1.8 $\pm$ 0.1	1.0 $\pm$ 0.1
			24	92.8 $\pm$ 5.7	8.3 $\pm$ 1.8	3.2 $\pm$ 0.2
<i>Synechococcus</i> sp.	14 days	Phytotron, 47 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	2	30.4 $\pm$ 5.6	4.0 $\pm$ 0.6	2.3 $\pm$ 0.1
			4	44.9 $\pm$ 10.8	37.2 $\pm$ 0.0	39.1 $\pm$ 0.0
			6	71.9 $\pm$ 4.4	3.6 $\pm$ 1.7	7.3 $\pm$ 0.7
			8	59.0 $\pm$ 3.4	9.3 $\pm$ 2.5	12.3 $\pm$ 1.7
			24	99.2 $\pm$ 0.4	1.0 $\pm$ 0.3	74.7 $\pm$ 12.0
<i>Leptolyngbya</i> sp.	14 days	Phytotron, 47 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	2	25.7 $\pm$ 10.8	9.4 $\pm$ 9.5	1.9 $\pm$ 1.3
			4	32.8 $\pm$ 9.3	3.7 $\pm$ 0.1	1.0 $\pm$ 0.1
			6	33.6 $\pm$ 0.7	3.4 $\pm$ 0.4	1.1 $\pm$ 0.1
			8	54.5 $\pm$ 37.3	6.2 $\pm$ 1.3	1.4 $\pm$ 0.4
			24	92.0 $\pm$ 6.6	42.7 $\pm$ 9.1	68.1 $\pm$ 41.6
<i>Synechococcus</i> sp.	14 days	Phytotron, 22 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	24	82.2 $\pm$ 14.6	42.3 $\pm$ 29.9	59.9 $\pm$ 0.0
			48	97.3 $\pm$ 3.1	21.9 $\pm$ 14.9	0
			72	99.8 $\pm$ 0.1	20.3 $\pm$ 6.3	0
<i>Leptolyngbya</i> sp.	14 days	Phytotron, 22 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	24	96.9 $\pm$ 0.6	34.3 $\pm$ 7.8	2.1 $\pm$ 1.6
			48	98.2 $\pm$ 1.7	14.8 $\pm$ 6.1	4.2 $\pm$ 2.0
			72	99.7 $\pm$ 0.0	38.6 $\pm$ 4.4	0

			168	99.7 ± 0.1	74.8 ± 1.5	0
<i>Leptolyngbya</i>	14 days	Manual chamber, 22	24	78.2 ± 3.2	7.8 ± 4.5	4.0 ± 0.4
sp.		μmol photons m ⁻² s ⁻¹	72	97.8 ± 1.5	35.1 ± 7.6	19.5 ± 7.0
			168	99.7 ± 0.0	84.8 ± 2.3	0

In Table 17, most biotransformation experiments for 1-phenylethyl acetate were performed using phytotron-grown cultures. Data obtained in the manually controlled chamber are presented only for *Leptolyngbya foveolarum* and serve as a reference for comparison with previously reported experiments conducted under less strictly regulated cultivation conditions. This comparison enabled assessment of the influence of cultivation environment and light regime on the catalytic performance and stereoselectivity of cyanobacterial biomass. The results indicate high conversion degrees and enantiomeric selectivity in both growth chambers.

Table 17 reports results obtained with 7-day and 14-day-old cultures at ~47 μmol photons·m⁻²·s⁻¹ and ~22 μmol photons·m⁻²·s⁻¹ respectively, provided by 30% cool white (CW). Values are expressed as mean ± standard deviation (SD) from duplicate experiments. Reported parameters include the degree of substrate conversion (%) and the enantiomeric excess (e.e.%) of both the product (1-phenylethanol) and the residual substrate (1-PEAc). The data presented in the Table 17 shows the influence of biomass age and light intensity on biotransformation kinetics and stereoselectivity, with older cultures and lower light intensities generally favouring higher enantioselectivity, particularly in *L. foveolarum*. However, *Synechococcus* sp. (Table 17) exhibited a rapid increase in bioconversion efficiency over 24 hours, achieving near-complete transformation by 24 hours. *Leptolyngbya* sp. displayed a slower conversion rate, requiring up to 48 hours to reach comparable conversion efficiencies using two weeks old cultures. Particularly in *Leptolyngbya foveolarum*, the improved enantioselectivity observed in older cultures and under lower light intensity may be attributed to physiological changes associated with culture maturation and photoautotrophic metabolism. As cyanobacterial cultures progress from exponential to later growth

phases, metabolic activity becomes less focused on biomass production and more favourable for secondary enzymatic processes, including hydrolytic transformations. Moreover, reduced light intensity minimizes photoinhibition and helps maintain intracellular redox balance, thereby providing more stable conditions for enzyme-mediated catalysis [204,205].

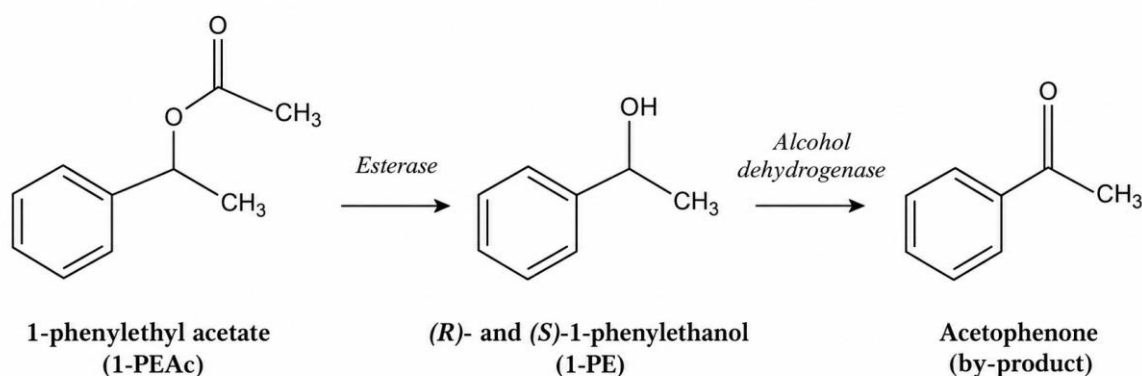


Figure 23. Schematic representation pathway for the biotransformation of 1-phenylethyl acetate by cyanobacteria.

The formation of acetophenone (Figure 23) was observed primarily during extended biotransformation periods, particularly when substrate conversion exceeded 90% (Table 17; Figure 21d, e), indicating subsequent oxidation of the initially formed 1-phenylethanol. This phenomenon became increasingly evident after 24 h of biotransformation, coinciding with near-complete hydrolysis of 1-phenylethyl acetate. Under such conditions, the concentration of the alcohol product increased substantially, providing a larger substrate availability for endogenous alcohol dehydrogenases capable of catalysing the oxidation of secondary alcohols to their corresponding ketones. Similar oxidation pathways have been extensively documented in microorganisms, where alcohol dehydrogenases catalyse the reversible interconversion of 1-phenylethanol and acetophenone as part of aromatic compound metabolism [225,226].

The effect of this secondary reaction on stereoselectivity appears to be particularly relevant when considering the temporal evolution of product enantiomeric excess

values. In several experimental variants, the enantiomeric excess of 1-phenylethanol initially increased but subsequently increased or declined despite continued substrate conversion. For example, *Synechococcus bigranulatus* cultivated for 14 days at 22 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ exhibited a product ee of 42.3% after 24 h, which decreased to 21.9% and 20.3% after 48 h and 72 h, respectively, while conversion simultaneously increased from 82.2% to 99.8% (Table 17). This behaviour shows that secondary transformations occurred after the primary hydrolysis step. A similar phenomenon has been reported in microbial kinetic resolutions where alcohol dehydrogenases selectively oxidise one enantiomer of 1-phenylethanol, thereby modifying the enantiomeric composition of the remaining alcohol pool and reducing the observed enantiomeric excess [225,226].

In contrast, *Leptolyngbya foveolarum* maintained or increased product enantiomeric excess during prolonged incubation, reaching 74.8% ee after 168 h under phytotron conditions and 84.8% ee after 168 h in the manual chamber. This trend shows that hydrolytic activity remained dominant over competing oxidative pathways, allowing progressive enrichment of the preferred alcohol enantiomer. Similar observations have been reported for whole-cell biocatalytic systems in which the balance between esterase-catalysed hydrolysis and alcohol dehydrogenase-mediated oxidation determines both product yield and optical purity [224,227].

The likely origin is the oxidation of 1-phenylethanol by alcohol dehydrogenases (ADHs), which convert secondary alcohols to ketones. A detailed enzymatic basis for this reaction is provided by studies on *Azoarcus sp.* strain EbN1, a denitrifying ethylbenzene-degrading bacterium [225]. Kniemeyer and Heider [225] reported the isolation and characterization of a (S)-1-phenylethanol dehydrogenase (PED) that catalyzes the NAD^+ -dependent oxidation of (S)-1-phenylethanol to acetophenone [225]. The enzyme was shown to be strictly stereoselective for the (S)-enantiomer, and its activity was linked to redox metabolism in anaerobic aromatic hydrocarbon degradation. This mechanistic detail directly supports the notion that acetophenone

formation in this study is not a non-specific oxidative artifact, but rather the outcome of a highly specific enzymatic pathway conserved across multiple bacterial lineages.

Further structural and biochemical analysis by Höffken *et al.* [226] revealed the high-resolution crystal structure of this PED, confirming the positioning of substrate and cofactor in a catalytic triad consistent with other short-chain dehydrogenase/reductase (SDR) family members. This work demonstrated the stereoselective complementarity between (*S*)-1-phenylethanol and the enzyme active site, thereby explaining its enantiospecific oxidation. The authors also observed that PED expression in *Azoarcus* is environmentally regulated and tied to metabolic demand for NADH regeneration, a feature potentially mirrored in cyanobacteria under redox stress or prolonged light exposure.

The setting of accurate and repeatable conditions in this bioconversion was crucial for receiving the biomass for its further prospecting in bioaccumulation processes. Ślizewska *et al.* [187] has previously demonstrated that enantioselective hydrolysis of 1-phenylethyl acetate to (*R*)-1-phenylethanol, with enantiomeric excesses exceeding 86% and selectivity (*E*) values up to 283, achieved using *Leptolyngbya foveolarum* CCALA 76, *Nostoc cf-muscorum* CCALA 129, and *Synechococcus bigranulatus* CCALA 187 cultivated for 21 days under a 16:8 light–dark photoperiod at 27 °C. Building on this approach and setup conditions, the current study employed both 14-day and 7-day cyanobacterial cultures, incubated in a phytotron-based photobioreactor system and a custom manual bioreactor, respectively. The transformation of 1-phenylethyl acetate was explored using *Synechococcus bigranulatus* CCALA 187 and *Leptolyngbya foveolarum* CCALA 76. The efficiency of biotransformation was assessed based on enantiomeric excess (e.e.) of 1-phenyl ethanol and 1-phenyl ethyl acetate, conversion degree, and product formation (Tables 17).

4.3. Bioaccumulation capacity of cyanobacterial biomass prior to and following photobiocatalytic transformation

Following the photobiocatalytic conversion of selected substrates (EMDP and 1-PEAc), the secondary aim of this research was to evaluate the potential of cyanobacterial biomass for rare earth element (REE) uptake, specifically gadolinium (Gd) and europium (Eu). Bioaccumulation of REEs by cyanobacteria offers a feasible approach to metal recovery from aqueous environments, aligning with global efforts in green bioremediation and circular economy strategies [159,172,228]. The integration of biotransformation and bioaccumulation studies presents a complete framework for evaluating the dual functional potential of cyanobacterial strains, both as biocatalysts and biosorbents and aligns with the zero-waste strategy. To comprehensively assess the suitability of the biomass for REE bioaccumulation, the following investigations were performed: microscopic evaluation of biomass morphology, assessment of culture purity and contamination, determination of biomass dry weight, and quantification of Gd and Eu accumulation by ICP–MS.

4.3.1. Microscopic examination of pre- and post- bioconversion biomass

To assess the physiological integrity of cyanobacterial cells prior to and following photobiocatalysis, microscopic evaluations were conducted using light microscopy (Figure 24).

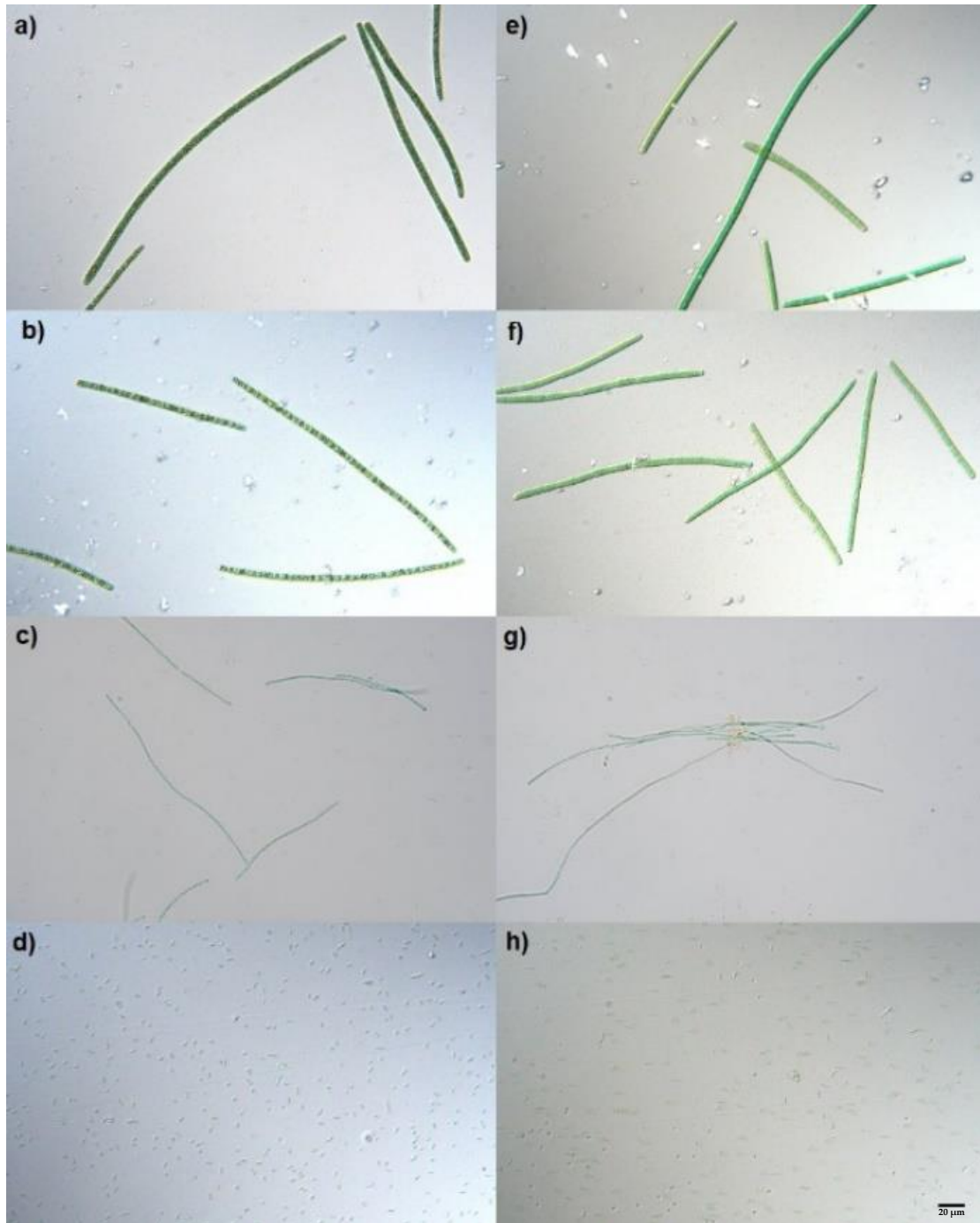


Figure 24. Light microscope images of *Limnospira indica* (a, e), *Limnospira maxima* (b, f), *Leptolyngbya foveolarum* (c, g) and *Synechococcus bigranulatus* (d, h). Pre-bioconversion biomass (a – d) and post-bioconversion biomass (e – h). Images were captured at 60× magnification (Scale bar 20 μm).

The observations confirmed conservation of viable cellular morphology in both pre- and post-bioconversion biomass. Cells retained structural integrity with no visible signs of lysis, aggregation, or abnormal pigmentation, indicating that the

biotransformation processes did not induce significant cytotoxic effects under the applied conditions. No significant amount of contamination was observed, supporting the reliability of biomass for subsequent ecotoxicological and bioaccumulation assessments. The absence of significant contamination is critical to ensure the reliability of downstream ecotoxicological and bioaccumulation assessments, as competing microbial populations can skew uptake profiles or influence the physiological state of the biomass [229].

4.3.2. Growth inhibition assay (ecotoxicology) results

Ecotoxicity results revealed significant strain-specific variations in REE tolerance (Figures 25), supporting previous findings that show the adaptive mechanisms of cyanobacteria in response to heavy metals and other stressors [230]. Growth inhibition presented in Figure 25 was determined in accordance with the Organisation for Economic Co-operation and Development (OECD) Test Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) based on changes in cyanobacterial biomass over the 72-h exposure period relative to untreated controls. Optical density (OD₆₈₀) measured at 0, 24, 48, and 72 h was used as a representative for biomass, and inhibition was calculated from differences in OD increase between treated and control cultures over time. Thus, the reported values show treatment-induced effects on biomass changes rather than a single endpoint measurement.

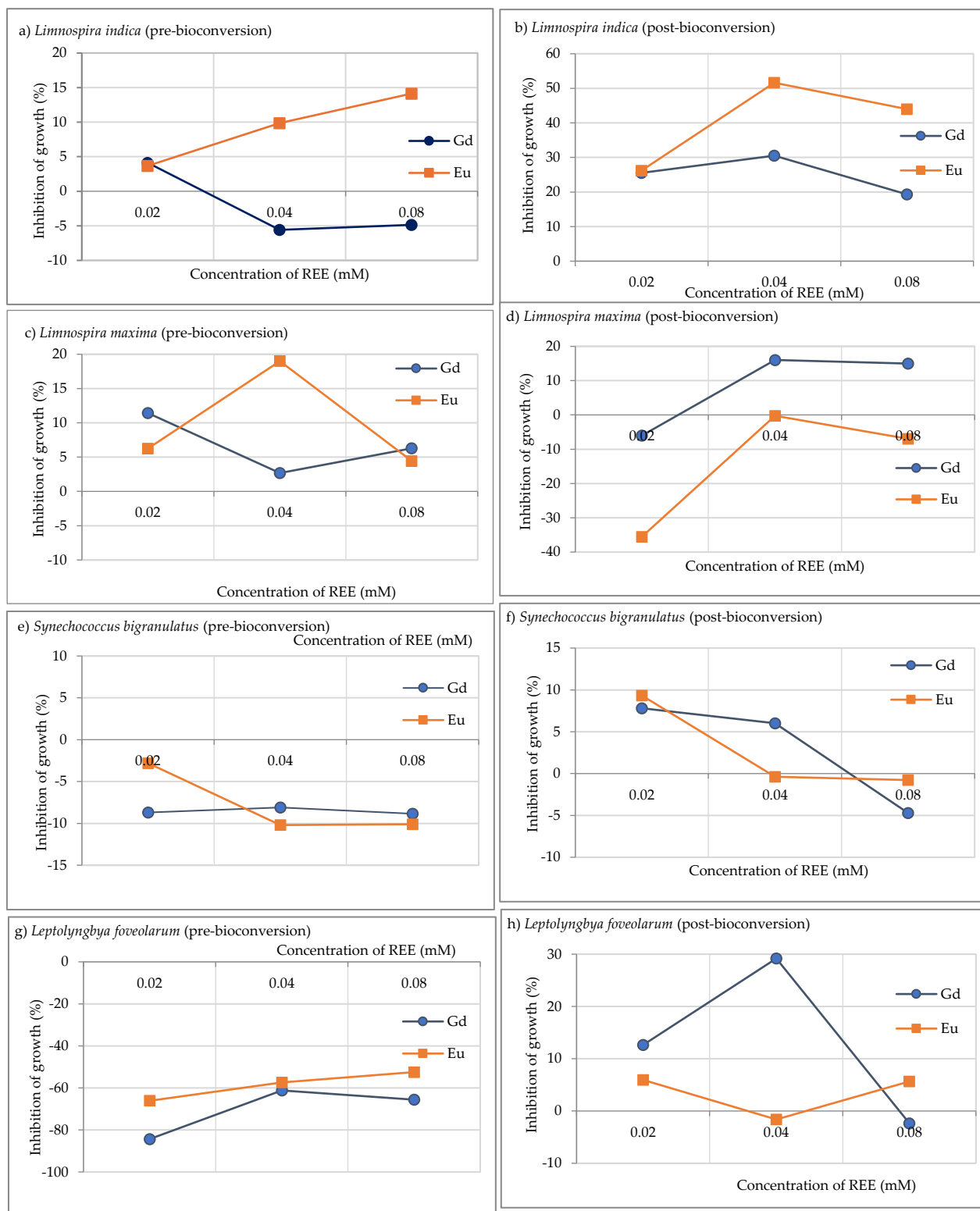


Figure 25. Growth inhibition (%) of four cyanobacterial strains after 72 h exposure to gadolinium (Gd) and europium (Eu) at three concentrations, expressed as mean $\pm$ SD (n = 5) relative to untreated controls.

In this research, hormetic-like responses were most evident in *Limnospira* sp. (Figure 25a-d). In the *Limnospira* sp. pre-bioconversion biomass (Figure 25a, c), Gd at the lowest concentration (0.02 mM) caused stronger inhibition than at higher levels. In the post-bioconversion strains (Figure 25b, d), inhibition was most pronounced at 0.04 mM Gd. However, Eu consistently produced strong inhibitory effects at higher concentrations (0.04 and 0.08 mM) in both pre- and post-bioconversion biomass of *Limnospira* sp. A comparable pattern was observed in *L. foveolarum* (Figure 25h), where post-bioconversion biomass growth was more inhibited at low Gd exposure concentrations (0.02 and 0.04 mM) than at 0.08 mM, and Eu at the lowest concentration, 0.02 mM also exerted stronger inhibition. In Figure 25g (pre-bioconversion biomass of *L. foveolarum*), none of the tested REE concentrations produced growth inhibition, indicating a negative response with no detectable adverse effect. By contrast, *S. bigranulatus* (Figure 25e, f) showed stable growth across exposure levels, indicating higher tolerance.

These hormetic responses to REE treatments as observed in *Limnospira* strains, and increased sensitivity of *Leptolyngbya* sp. post-bioconversion biomass, and the consistent tolerance of *Synechococcus* sp. reveals the existence of clear strain-specific differences in REE response profiles as summarized in Figure 26.

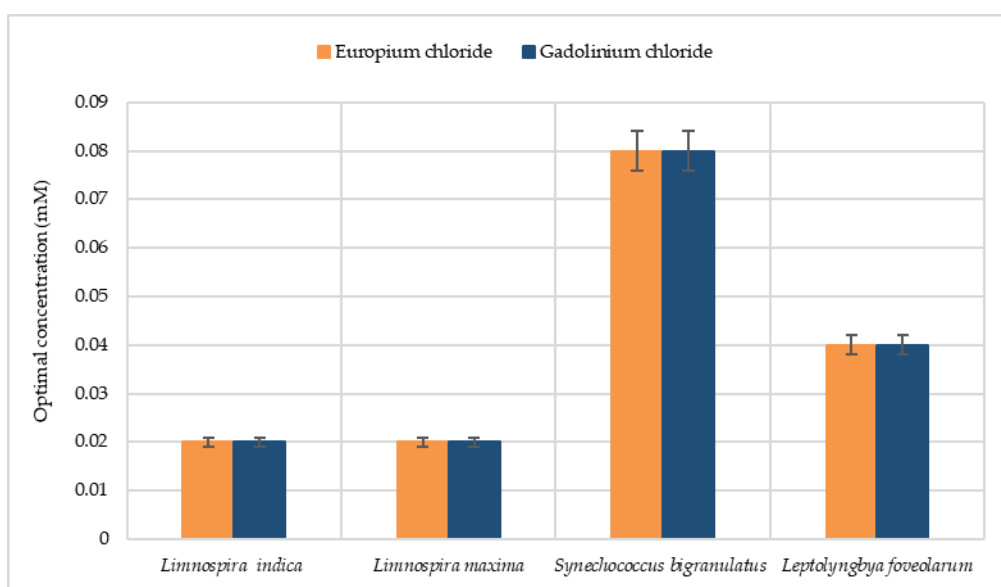


Figure 26. Graphical representation of the optimal concentrations of REEs.

However, these disparities could also be attributed to the role of extracellular polymeric substances (EPS), which bind metal ions and limit their intracellular uptake [231]. Limited EPS production in *Limnospira* sp. and *Leptolyngbya* sp. could be responsible for their increased sensitivity. Furthermore, oxidative stress from REE exposure is known to disrupt photosystem II, lowering chlorophyll content and reducing biomass productivity [232]. Notably, post-bioconversion biomass displayed slightly greater resilience at low REE levels, indicating that metabolic adjustments during the biotransformation phase induce protective responses, such as upregulation of stress proteins or efflux systems. This aligns with reports that *Synechococcus* sp. possess efficient heavy-metal efflux systems and strong surface binding capacity due to negatively charged functional groups such as carboxyl, hydroxyl, and phosphate [233–235]. Their metabolic plasticity under stress [236–238], further enhance their effectiveness as a promising candidate for REE bioremediation applications.

These preliminary assays were conducted at three exposure concentrations (20, 40, and 80 μ M). While this design does not yield a full dose-response curve, it allowed for empirical determination of final concentrations for the bioaccumulation phase (Figure 26). Interestingly, in some cases, the lowest concentrations induced stronger inhibitory responses than higher concentrations, a phenomenon (hormesis) previously reported in toxicity studies where stress responses are triggered at sub-lethal levels, resulting in a non-linear response. This aligns with the findings by Gjata *et al.* [239] who showed that several REEs (Ce, Nd, Gd, Dy, Yb, Ho, Lu) in millimolar concentrations produce toxicity (e.g. growth inhibition, oxidative stress) in *Lemna minor*. Whereas in fungi exposed to La, Ce, and Nd, low doses stimulated growth while higher ones were inhibitory [240].

4.3.3. Dry mass analysis of bioaccumulated REEs

After 72 h of bioaccumulation experiment, pre-bioconversion cyanobacterial samples treated with REEs exhibited higher increase in biomass weight compared to controls (untreated pre-bioconversion cyanobacterial samples). This trend was observed across

all treated samples except for Gd treated *Synechococcus* sp. (Figure 27a). This observation aligns with previous studies that highlight the enhanced resilience and bioaccumulative potential of cyanobacteria when exposed to REEs [172]. In post-bioconversion biomass of *Synechococcus* sp. and *Limnospira maxima*, a decrease in biomass weight was observed, more significant in controls compared to REE treated samples (Figure 27b). This shows that the response to REE treatment vary among different cyanobacterial species and REEs with some beneficial effect on the cyanobacterial growth or induce an accumulation of these specific REEs (Eu and Gd) contributing to increased biomass [164,230,241].

Figure 28 shows the descriptive comparisons between cyanobacterial biomass before and after bioconversion in bioaccumulation studies and aims at assessing general trends in strain-specific responses, rather than formal significance testing.

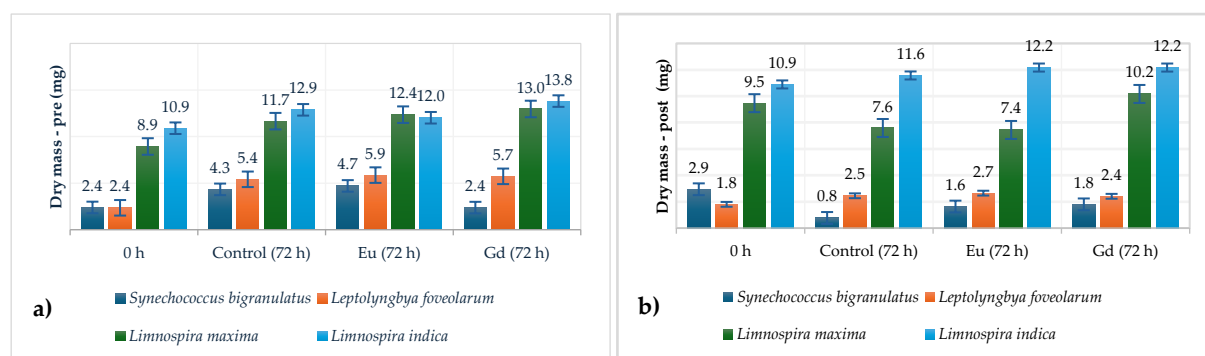


Figure 27. Dry mass assessment of pre- (a) and post- (b) bioconversion cyanobacterial biomasses after 3 days of bioaccumulation. **Control** = pre-bioconversion (a) and post-bioconversion (b) biomass without REE treatment; **Eu** = biomass treated by Europium chloride at defined concentrations; **Gd** = biomass treated by Gadolinium chloride at defined concentrations.

4.3.4. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of bioaccumulated REEs

ICP-MS data (Figure 23) reveals that pre-bioconversion biomass of *L. indica* exhibited Eu bioaccumulation capacity of 7.5 ± 0.6 mg/g and Gd accumulation of 9.1 ± 0.7 mg/g.

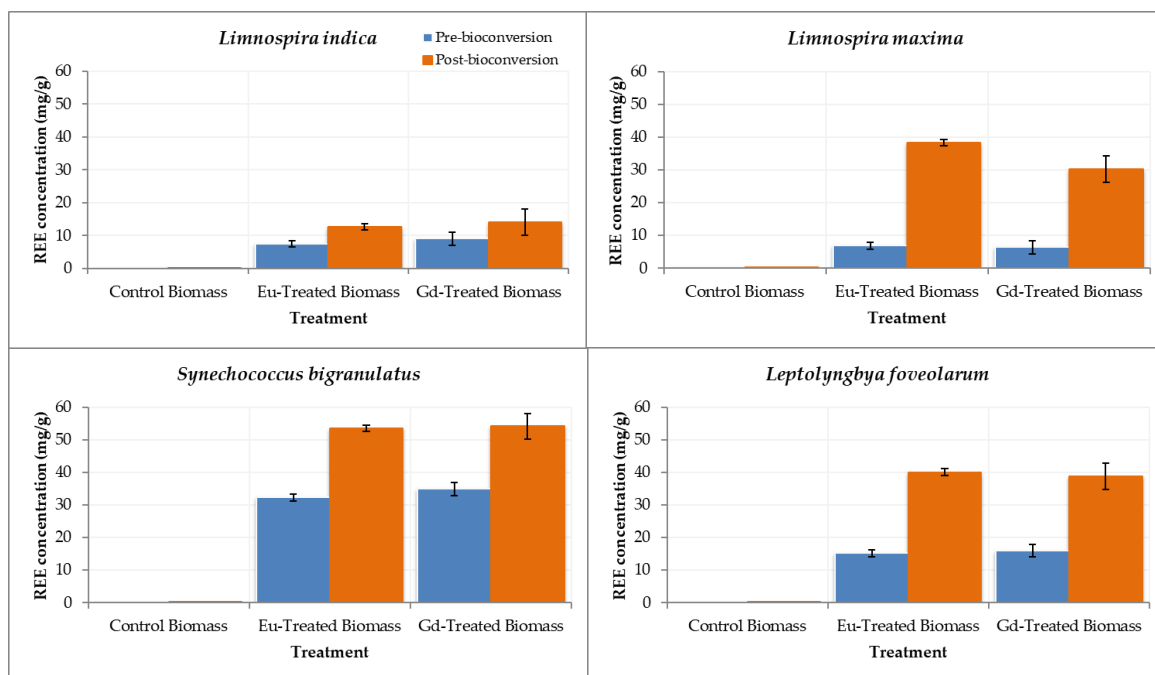


Figure 28. Graphical illustration of ICP-MS analysis of bioaccumulated REEs

After bioconversion (i.e., post-bioconversion biomass), these values increased to 12.7 ± 0.7 mg/g and 14.0 ± 0.2 mg/g respectively. *L. maxima* followed a similar trend, with Eu accumulation increasing from 6.9 ± 0.8 mg/g pre-bioconversion to 38.3 ± 6.0 mg/g post-bioconversion. The corresponding increase for Gd was from 6.4 ± 0.3 mg/g to 30.3 ± 3.6 mg/g. The most pronounced increases were observed in *S. bigranulatus*, where Eu accumulation surged from 32.3 ± 1.1 mg/g to 53.6 ± 1.6 mg/g post-bioconversion. Gd accumulation increased from 35.0 ± 3.3 mg/g to 54.2 ± 2.8 mg/g. *L. foveolarum* also exhibited significant increases, with Eu bioaccumulation rising from 15.2 ± 0.9 mg/g to 40.1 ± 1.4 mg/g. Gd increased from 16.0 ± 1.8 mg/g to 38.8 ± 1.4 mg/g.

Table 18. Analysis of bioaccumulated REEs by *Limnospira indica*

Sample	Pre-bioconversion (mg/g)	Post-bioconversion (mg/g)
Control Biomass	0.009 ± 0.003	0.014 ± 0.011
Eu-Treated Biomass	7.5 ± 0.6	12.7 ± 0.7
Gd-Treated Biomass	9.1 ± 0.7	14.0 ± 0.2

Table 19. Analysis of bioaccumulated REEs by *Limnospira maxima*

Sample	Pre-bioconversion (mg/g)	Post-bioconversion (mg/g)
Control Biomass	0.004 ± 0.002	0.005 ± 0.002
Eu-Treated Biomass	6.9 ± 0.8	38.3 ± 6.0
Gd-Treated Biomass	6.4 ± 0.3	30.3 ± 3.6

Table 20. Analysis of bioaccumulated REEs by *Synechococcus bigranulatus*

Sample	Pre-bioconversion (mg/g)	Post-bioconversion (mg/g)
Control Biomass	0.001 ± 0.001	0.008 ± 0.003
Eu-Treated Biomass	32.3 ± 1.1	53.6 ± 1.6
Gd-Treated Biomass	35.0 ± 3.3	54.2 ± 2.8

Table 21. Analysis of bioaccumulated REEs by *Leptolyngbya foveolarum*

Sample	Pre-bioconversion (mg/g)	Post-bioconversion (mg/g)
Control Biomass	0.002 ± 0.002	0.002 ± 0.001
Eu-Treated Biomass	15.2 ± 0.9	40.1 ± 1.4
Gd-Treated Biomass	16.0 ± 1.8	38.8 ± 1.4

The presented Table 18 – 21 showed the bioaccumulation of rare earth elements (REEs) by cyanobacteria, comparing pre-bioconversion and post-bioconversion biomass in tabular forms. The four cyanobacterial strains examined, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Synechococcus bigranulatus* CCALA 187, and *Leptolyngbya foveolarum* CCALA 76, demonstrated substantial variations in REE uptake, with a consistent trend of increased bioaccumulation following bioconversion. The observed patterns support the findings that cyanobacteria offer biologically selective and efficient platforms for the sequestration of critical elements such as Eu and Gd from aqueous environments, a concept corroborated in recent literature exploring microbe-mediated REE recovery strategies [172,242].

All REE accumulation values reported in Tables 18-21 was calculated per gram of freeze-dried cell mass (mg g⁻¹ DW), also represented in graphical form in Figure 28. Biomass digestion and ICP-MS measurements were carried out by an external

analytical facility using established and validated procedures as elucidated in the materials and methods section.

Notably, the biotransformation product DMHPP, formed from epoxymethyl dimethyl phosphonate, exhibits amphipathic properties that could promote its interaction with or retention in the plasma membrane and change its permeability and special conformation. Its molecular structure, featuring a hydrophobic alkene chain and a negatively charged phosphonate headgroup can be recognized, in part as structural analogue (Figure 29) of undecaprenyl phosphate (C_{55} -P), a lipid carrier known to be incorporated into prokaryotic cell membranes and undergo active translocation via lipase systems. This molecule is of special importance for prokariota, since it is involved in the cell wall building blocks translocation from cytoplasm to the periplasmic space. Notably, C_{55} -P is recycled across the membrane by the UptA–PopT translocase complex as elucidated by Oluwole *et al.* [243], suggesting that structurally analogous compounds such as DMHPP could similarly associate with or engage membrane-related mechanisms.

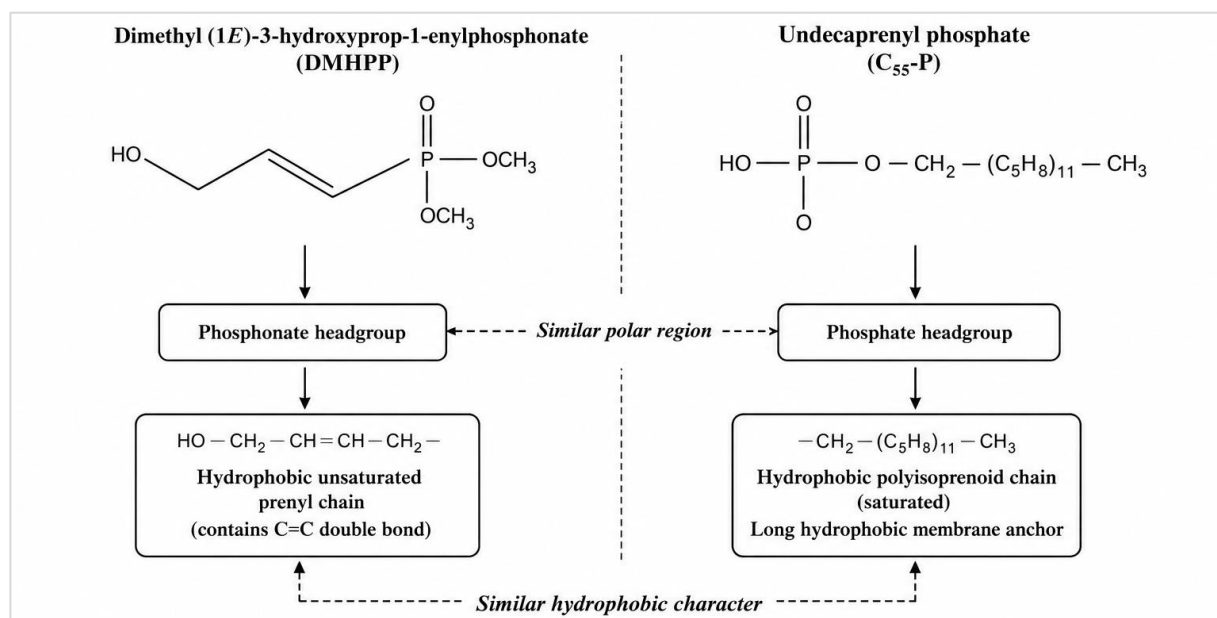


Figure 29. Structural comparison of dimethyl (1E)-3-hydroxyprop-1-enylphosphonate (DMHPP) and undecaprenyl phosphate (C_{55} -P).

DMHPP shares key physicochemical features with undecaprenyl phosphate, including a phosphorus-containing polar headgroup and a hydrophobic carbon chain (Figure 29). Although DMHPP possesses only a short unsaturated allylic chain, in contrast to the long polyisoprenoid membrane anchor of undecaprenyl phosphate, these shared amphiphilic characteristics may contribute to similar recognition by membrane-associated enzymes.

The ICP-MS data shows that bioconversion significantly enhances the ability of cyanobacterial biomass to accumulate REEs, supporting the hypothesis that structural and biochemical modifications in the biomass contribute to improved metal sequestration. For instance, a study on biochar, a form of biomass, highlights that its properties, such as surface area, porosity, pH, surface charge, functional groups, and mineral components, can be tailored to enhance metal sorption. These modifications can significantly improve the biochar's capacity to adsorb various metals from contaminated environments [244]. In another development, research on biomodification in mineral processing demonstrates that microbial-induced changes to mineral surfaces can enhance metal recovery. Microorganisms can alter the surface properties of minerals through biochemical interactions, leading to improved metal sequestration [245].

These values clearly indicate that bioconversion plays a crucial role in improving REE sequestration across all tested strains. The observed enhancements in REE bioaccumulation post-bioconversion are consistent with findings from previous studies. Research by Paper *et al.* [246] on *Nostoc* sp. 20.02 reported maximum adsorption capacities ranging from 84.2 to 91.5 mg/g for cerium, neodymium, terbium, and lanthanum [247]. The post-bioconversion bioaccumulation values observed in the present study, particularly for *L. maxima* and *S. bigranulatus*, approach or exceed these values, which suggest that bioconversion enhances cyanobacterial metal uptake to levels comparable with the most efficient biosorption strains reported in literature. Similarly, in previous investigation by Yushin *et al.* [248], *Arthrospira platensis*

demonstrated a maximum biosorption capacity of 101 mg/g for gadolinium at pH 3.0 and 20°C [248]. The increases observed in *S. bigranulatus* and *L. foveolarum* post-bioconversion align with these findings, reinforcing the efficacy of these strains as viable candidates for large-scale REE biorecovery [247–249].

These strains' high uptake efficiency is consistent with findings by Rastogi and Sinha [121], who identified *Synechococcus* sp. as having robust metal-binding properties due to their dense exopolysaccharide (EPS) layers and high surface area-to-volume ratios. Additionally, it aligns with the work of Calderón *et al.* [250]; Volesky & Holan [251], where enhanced biosorption in microbial biomass was attributed to the abundance of functional groups (e.g., carboxyl, phosphate) on cell surfaces that form stable complexes with trivalent metal ions [250], resulting to maximum biosorption capacities of 200 - 300 mg/g for heavy metals in brown algae [251].

Additionally, prior studies have shown that the biosorption of europium, samarium, and neodymium by *Anabaena cylindrica* is both rapid and nearly complete within minutes of exposure [164]. The substantial increases in europium and gadolinium bioaccumulation post-bioconversion, particularly in *L. maxima*, *S. bigranulatus*, and *L. foveolarum* suggests that modifications made to the biomass, such as biotransformation with organic compounds, could optimize biosorption kinetics, possibly through increased binding site availability or enhanced metabolic activity [252].

The observed increases in europium and gadolinium accumulation align with existing literatures, confirming the efficiency of post-bioconversion biomass in metal sequestration. The results emphasize the potential of bioconversion as a tool to optimize cyanobacterial bioaccumulation capacity, with applications in bioremediation and resource recovery. For instance, a pertinent study by Pandhal *et al.* [253] investigated the harvesting of environmental microalgal blooms for remediation and resource recovery, assessing economic and microbial community impacts. The

research highlights the feasibility of utilizing microalgae for environmental clean-up and the recovery of valuable resources [253].

Table 22. Comparative biosorption capacities of microbial taxa for metal and rare earth element uptake

Biosorbent group	Representative taxa	Target metal(s)	Biosorption capacity (mg/g)	Mechanism	Key ref.
Cyanobacteria	<i>Arthrospira (Spirulina) platensis</i>	La ³⁺ , Ce ³⁺	~80–120 mg/g	Surface adsorption; intracellular accumulation	[254]
	<i>Synechococcus sp.</i>	Eu ³⁺ , Nd ³⁺	~60–100 mg/g	Cell surface binding; biomineralization	[164]
Microalgae	<i>Chlorella vulgaris</i>	Cd ²⁺ , Pb ²⁺	~100–200 mg/g	Metabolism-dependent uptake; adsorption	[255]
	<i>Scenedesmus sp.</i>	REE (mixed)	~50–150 mg/g	Surface binding; intracellular uptake	[256]
Fungi	<i>Aspergillus niger</i>	La ³⁺ , Ce ³⁺	~150–300 mg/g	Passive biosorption (cell wall chitin/glucans)	[257] – [259]
	<i>Penicillium sp.</i>	REE (mixed)	~100–250 mg/g	Cell wall binding	[260]
Bacteria	<i>Bacillus sp.</i>	La ³⁺	~40–80 mg/g	Surface adsorption; limited uptake	[261]
	<i>Pseudomonas sp.</i>	REE (mixed)	~30–70 mg/g	Adsorption via functional groups	[256]

To further emphasize the competitiveness of cyanobacterial biomass in REE bioaccumulation, a comparative evaluation of major biosorbing taxa (cyanobacteria, microalgae, fungi, and bacteria) is presented in Table 22. Fungal systems generally exhibit the highest biosorption capacities, primarily due to the high density of functional groups in their cell walls. However, these systems rely entirely on passive

adsorption mechanisms. In contrast, cyanobacteria show slightly lower but still competitive uptake capacities, while offering the additional advantage of active intracellular accumulation of REEs. This dual mechanism, combined with phototrophic growth and metabolic flexibility, reveals the suitability of cyanobacteria as useful platforms for REE recovery. Notably, the uptake capacities observed in this study are within the range reported for cyanobacterial systems in literatures, showing their competitive performance relative to other microbial biosorbents as shown in Table 21.

CHAPTER FIVE

Conclusions

The present study shows that cyanobacteria can function as multifunctional whole-cell biocatalysts, integrating selective photobiotransformation of organophosphorus compounds and acetate ester with efficient bioaccumulation of rare earth elements. Diverse cyanobacterial strains explored include *Synechococcus bigranulatus* CCALA 187, *Nostoc cf-muscorum* CCALA 129, *Limnospira indica* PCC 8005, *Limnospira maxima* CCALA 27, *Leptolyngbya foveolarum* CCALA 76, and *Kamptenema animale* CCALA 138. the study systematically evaluated their ability to perform photobiocatalytic transformations of industrially relevant substrates (vinylphosphonates, epoxyphosphonate, and acetate compounds) and accumulate critical rare earth elements (REEs), particularly gadolinium (Gd) and europium (Eu). Table 23 shows the major findings in this study.

Table 23. Summary of the major experimental findings obtained in this study

Research objective	Most significant result	Experimental conditions	Best-performing system
Selection of photobiocatalytic medium	Complete EMDP stability	Unmodified BG-11 and BG-11 ₀ media	BG-11/BG-11 ₀
Biotransformation of vinyl phosphonates	No biotransformation observed	DMVP and DEVP; up to 30-day incubation	All investigated strains
Fastest EMDP bioconversion	99% conversion within 24 h	1 mM EMDP	<i>Limnospira</i> spp. PCC 8005 and CCALA 27
Highest stable EMDP bioconversion	99% conversion after 72 h	1 mM EMDP	<i>Synechococcus bigranulatus</i> CCALA 187
Highest DMHPP yield	10.0 ± 1.1%	1 mM EMDP; 24 h	<i>Synechococcus bigranulatus</i> CCALA 187
Highest DMHPP yield at increased substrate concentration	6.5 ± 1.2%	4 mM EMDP; 24–72 h	<i>Synechococcus bigranulatus</i> CCALA 187
Chemical synthesis of DMHPP	81.4% isolated yield	Conventional synthesis	Reference compound (DMHPP)

Highest conversion of 1-phenylethyl acetate	99.7% conversion	14-day-old biomass; 22 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; 168 h	<i>Leptolyngbya foveolarum</i> CCALA 76
Highest enantioselectivity (phenyl alcohol)	84.8% ee	14-day-old biomass; manually controlled growth chamber; 22 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; 168 h	<i>Leptolyngbya foveolarum</i> CCALA 76
Highest europium accumulation	$53.6 \pm 1.6 \text{ mg g}^{-1}$	Biomass after EMDP bioconversion	<i>Synechococcus bigranulatus</i> CCALA 187
Highest gadolinium accumulation	$54.2 \pm 2.8 \text{ mg g}^{-1}$	Biomass after EMDP bioconversion	<i>Synechococcus bigranulatus</i> CCALA 187

The major conclusions are as follows:

- The composition of the cultivation medium was found to be a key factor determining the success of photobiocatalytic processes. Unmodified BG-11 and BG-11₀ media ensured complete stability of epoxymethyl dimethyl phosphonate (EMDP) and were therefore selected as the optimal media for subsequent investigations.
- Dimethyl vinylphosphonate (DMVP) and diethyl vinylphosphonate (DEVP) were resistant to biotransformation by all investigated cyanobacterial strains. The small amounts of inorganic phosphate detected originated from non-enzymatic hydrolysis rather than biological cleavage of the C–P bond, confirming the high stability of vinyl phosphonates under the applied experimental conditions.
- Epoxymethyl dimethyl phosphonate proved to be an excellent substrate for cyanobacterial photobiocatalysis. The highest catalytic activity was exhibited by *Limnospira* spp. PCC 8005 and *Limnospira maxima* CCALA 27, which converted 99% of 1 mM EMDP within 24 h, while *Synechococcus bigranulatus* CCALA 187 achieved 99% conversion after 72 h.
- The biotransformation product was explicitly identified as dimethyl (1E)-3-hydroxyprop-1-enylphosphonate (DMHPP) using NMR spectroscopy, infrared spectroscopy and high-resolution mass spectrometry. Conventional synthesis

yielded an identical compound, confirming the structure of the biotransformation product.

- *Synechococcus bigranulatus* CCALA 187 produced the highest DMHPP yield, reaching $10.0 \pm 1.1\%$ after 24 h using 1 mM EMDP. Increasing the substrate concentration to 4 mM maintained complete substrate conversion but reduced product yield, showing intracellular retention or membrane association of DMHPP.
- Photobiocatalytic conditions for the kinetic resolution of 1-phenylethyl acetate were successfully optimized. *Leptolyngbya foveolarum* CCALA 76 cultivated for 14 days and incubated under $22 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 168 h exhibited the highest substrate conversion (99.7%) and the highest product enantiomeric excess (84.8% ee).
- Cyanobacterial biomass remained morphologically intact after photobiocatalysis and enabled its direct application in subsequent bioaccumulation experiments. Biomass obtained after photobiocatalysis efficiently accumulated rare earth elements. The highest accumulation capacities were achieved by *Synechococcus bigranulatus* CCALA 187, reaching $53.6 \pm 1.6 \text{ mg g}^{-1}$ for europium and $54.2 \pm 2.8 \text{ mg g}^{-1}$ for gadolinium.
- Photobiocatalytic treatment enhanced the biosorption performance of cyanobacterial biomass. Biomass recovered after biotransformation consistently exhibited greater rare earth accumulation than untreated biomass.
- The integration of photobiocatalysis and bioaccumulation into a single microbial system represents a green alternative to conventional chemical and metallurgical processes.

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