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Enzymatic Degradation of Pyridine and Pyridinols

DOCTORAL DISSERTATION

Natural Sciences,
Biochemistry (N 004)

VILNIUS 2025

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Justas Vaitekūnas

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The usefulness of useless knowledge

Abraham Flexner

CONTENTS

THESIS LAYOUT

This thesis is composed of four publications and begins with an Introduction that brings these works under a shared perspective, delineating the primary area of scholarly contribution. Predominantly categorized as exploratory or "blue sky" research, the impetus behind this work is primarily driven by intellectual curiosity. To ensure structured progress, three specific research objectives have been articulated. The Literature review provides a concise summary of xenobiotics, focusing on microbial pathways for their catabolism. This section also discusses methodological advances in the field, highlights the most impactful industrial-scale applications, and discusses potential prospects in xenobiotic research. The results derived from these investigations are thoroughly documented in the respective publications. The Perspective section of this thesis provides a concise overview of the experimental techniques used in the studies and a summary of the key data collected, thus placing the scientific contributions of the research in a broader context. Additional parts include a list of abbreviations, conclusions, summary, reference list, CV, and copies of the publications.

LIST OF PUBLICATIONS

Publication (**I**): **Vaitekūnas, J.**; Gasparavičiūtė, R.; Rutkienė, R.; Tauraitė, D.; Meškys, R. A 2-Hydroxypyridine Catabolism Pathway in *Rhodococcus rhodochrous* Strain PY11. *Applied and Environmental Microbiology* **2016**, *82* (4). https://doi.org/10.1128/AEM.02975-15.

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Publication (**II**): Petkevičius, V.; **Vaitekūnas, J.**; Stankevičiūtė, J.; Gasparavičiūtė, R.; Meškys, R. Catabolism of 2-Hydroxypyridine by *Burkholderia* sp. Strain MAK1: A 2-Hydroxypyridine 5- Monooxygenase Encoded by hpdABCDE Catalyzes the First Step of Biodegradation. *Applied and Environmental Microbiology* **2018**, *84* (11). https://doi.org/10.1128/AEM.00387-18.

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Publication (**III**): **Vaitekūnas, J.**; Gasparavičiūtė, R.; Stankevičiūtė, J.; Urbelis, G.; Meškys, R. Biochemical and Genetic Analysis of 4-Hydroxypyridine Catabolism in *Arthrobacter* p. Strain IN13. *Microorganisms* **2020**, *8* (6), 888. https://doi.org/10.3390/microorganisms8060888.

> *Author Contributions: Conceptualization, **J.V.** and R.M.; methodology, **J.V.**, R.G., J.S., and G.U.; investigation, **J.V.**, R.G., J.S., and G.U.; resources, R.M.; writing—original draft preparation, **J.V.**; writing—review and editing, J.S., **J.V.**, and R.M.; visualization, **J.V.**; supervision, R.M.*

Publication (**IV**): Časaitė, V.; Stanislauskienė, R.; **Vaitekūnas, J.**; Tauraitė, D.; Rutkienė, R.; Gasparavičiūtė, R.; Meškys, R. Microbial Degradation of Pyridine: A Complete Pathway in *Arthrobacter* sp. Strain 68b Deciphered. *Applied and Environmental Microbiology* **2020**, *86* (15), 1–16. https://doi.org/10.1128/AEM.00902-20.

> *Author Contributions: R.M. and V.Č. designed the experiments; R.M., R.S., **J.V.**, D.T., and V.Č. analyzed the data; and R.S., R.R., **J.V.**, D.T., R.G., and V.Č. performed experiments. **J.V.**, R.M., and V.Č. prepared the manuscript*

LIST OF ABBREVIATIONS

2HP	2-hydroxypyridine
4HP	4-hydroxypyridine
THP	2,3,6-trihydroxypyridine
25DHP	2,5-dihydroxypyridine
TF	transcription factor
NAD	nicotinamide adenine dinucleotide
HPLC-MS/MS	high-performance liquid chromatography - mass spectrometry/ or tandem MS
NMR	nuclear magnetic resonance

INTRODUCTION

Proteins are the fundamental constituents vital for sustaining life in all its forms. The comprehension of the roles of enzymes, which are proteins responsible for crucial reactions within cellular structures, forms the foundation of biochemistry, a part of science devoted to elucidating the molecular mechanisms fundamental to life. In the context of this study, proteins are regarded as the functional products of the information encoded within genomic DNA sequences. Consequently, throughout this text the term 'gene function' refers to the function of the protein it encodes.

The number of identified gene sequences is growing exponentially due to enormous advances in DNA sequencing capabilities. However, the comprehensive understanding of their function remains a major bottleneck and lags far behind, forming a significant scientific challenge. As of January 2024, the National Center for Biotechnology Information (NCBI) reports the aggregation of a total number of 2.6×10^{12} nucleobases in their datasets, alongside the further 2.5×10^{13} nucleobases derived from whole-genome shotgun sequencing projects. According to GOLD database[1], more than 550,000 sequencing projects have been carried out, yielding complete or draft genome sequences of 236,493 bacterial species and 92,072 eukaryotic species. Nevertheless, even the most well-characterized organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, have only approximately 80% of their genes annotated in some way. The significance of genes lacking functional characterization is well-illustrated through the example of a minimal synthetic cell. The autonomously functioning bacterium *Mycoplasma mycoides* JCV-syn3.0 had a minimized synthetic genome and was viable, but around one-third of its total genes (149 of 473) had unknown biological functions[2]. Subsequent studies on newer versions of this synthetic organism have significantly enhanced our understanding of the functionalities of these genes[3]. Yet, despite substantial progress, the latest iteration JCVI-syn3A still harbors 16 essential genes with unclear biological functions[4].

The total amount of DNA sequences in NCBI database doubles every 18 months due to the surge in genome and metagenome sequencing initiatives. Given the vast expanse of data, the experimental confirmation of functions for all these proteins is a nearly impossible task. The optimal strategy for functional genome annotation continues to be the computational search for sequence homologues that have been functionally characterized, ideally exhibiting more than 50% sequence identity[5]. The Reference Sequence (RefSeq) collection, as of the release dated January 16, 2024, houses over 300 million distinct entries that were processed via annotation pipelines[6]. The Universal Protein Knowledgebase (UniProt) comprises both the proteins for which functional annotations have been assigned by

homology (TrEMBL; 249,751,891 entries) and those, for which the functions were experimentally confirmed (SwissProt; 570,830 entries)[7]. Additional challenges of identifying substrates for uncharacterized proteins are posed by subtle variations in amino acid composition that can significantly alter the protein-small molecule binding specificity. As a result, many hypothetical enzymes have unknown, uncertain, or incorrect functions. The development of novel computational methodologies, including artificial intelligence (AI), deep neural networks, machine learning, and others, signify substantial advancements in the field of bioinformatics, as recently demonstrated by the highly accurate protein structure prediction algorithms AlphaFold[8] and ESMFold[9]. Experimental confirmation of novel protein functions remains essential, as it not only bolsters the accuracy and reliability of computational predictions but also amplifies the overall effectiveness of advanced big data analytics techniques for protein annotation.

In addition to the fundamental aspect of protein function prediction, there are major translational gains in elucidating unknown enzymatic activities. The novel enzymes have a vast potential for application in synthetic biology and/or metabolic engineering. Moreover, the emerging field of biocatalysis is being recognized as a viable alternative to conventional chemical synthesis. Biocatalysts that possess regio- and stereo-control have immense potential, as they enable the synthesis of complex molecules with exceptional specificity. Furthermore, biocatalysis aligns with the principles of sustainable manufacturing by enabling chemical transformations under benign conditions, thus circumventing the use of toxic metals and solvents. It enhances atomic efficiency by obviating the necessity for the protection and deprotection of functional groups, thereby streamlining the synthesis process. Additionally, biocatalysis leverages renewable and biodegradable resources, contributing to the reduction of environmental impact and fostering the development of green chemistry practices. Through this, biocatalysis not only advances the field of synthetic chemistry but also promotes the adoption of eco-friendly and sustainable methodologies in chemical manufacturing.

A pivotal strategy for identifying useful novel enzymatic activities is studying the metabolism of xenobiotics in microorganisms. It is widely recognized that many bacteria have the capacity to metabolize diverse organic compounds as sources of nutrients for growth, facilitated by an array of enzymes. However, much of the biochemistry involved in these processes is still poorly understood. This lack of information on metabolic pathways can be divided into three categories: the Known Unknowns, the Unknown Knowns, and the Unknown Unknowns. Theoretical proteins derived from DNA sequencing projects, which have hypothetical and/or misannotated functions, and whose roles have not been experimentally elucidated, are classified as Known Unknowns. Likewise, there

exist metabolites that have been identified but do not participate in any documented biochemical reactions (comprising approximately 50% of the KEGG compounds database[10]), or enzymes cataloged in the literature (constituting approximately 20% of the EC database[11]) without any corresponding gene reported—these are termed as Unknown Knowns. The last category, the Unknown Unknowns, represents an uncharted domain of novel proteins, potentially harboring untapped biochemical functionalities. The current challenge in microbiology is that only about 1% of all microorganisms are cultivable under laboratory conditions and do not represent the full phylogenetic diversity[12]. This limitation significantly constrains the scope of gathering comprehensive data through genomic, proteomic and metabolomic resources and integrating such information with functional analyses of enzymes. The exploration of these (Un)Knowns not only underscores the vast *unknown* in microbial biochemistry but also highlights the potential for discovering innovative enzymatic activities that could revolutionize our understanding and application of biocatalysis in various domains, including environmental remediation, synthetic biology, and industrial biotechnology.

Pyridine and its derivatives are ubiquitous environmental contaminants originating from both natural and anthropogenic sources. The pyridine ring is found in alkaloids, coenzymes, and man-made solvents, pesticides, and herbicides. Pyridinols are common intermediate metabolites produced during microbial biodegradation of various *N*-heterocyclic compounds. It is generally recognized that they can be degraded by various bacteria in the environment, however the degradation mechanism of various pyridines has not yet been fully elucidated. Amidst the (un)Knowns, this thesis aims to identify novel enzymes by studying the catabolic pathways of pyridine and pyridinols. To achieve this goal, four objectives were set:

1. To elucidate the degradation pathway of 2-hydroxypyridine in *Rhodococcus rhodochrous* PY11;
2. To decipher the pathway for the catabolism of 2-hydroxypyridine in *Burkholderia* sp. MAK1;
3. To identify 4-hydroxypyridine catabolism genes and proteins from *Arthrobacter* sp. IN13;
4. To determine the principles of pyridine catabolism in *Arthrobacter* sp. 68b.

Scientific novelty

All four bacterial strains analyzed in this study were previously isolated from soil due to their capacity to utilize pyridine or pyridinol as a single carbon source for growth. The investigation into their catabolic pathways began with the identification of degradation genes, followed by their cloning, heterologous expression, and analysis of the resulting reaction products. The novel enzymes obtained were subsequently assessed (either *in vivo* or *in vitro*) for substrate promiscuity to explore their potential applications as biocatalysts.

The first paper "A 2-Hydroxypyridine Catabolism Pathway in *Rhodococcus rhodochrous* Strain PY11" explores how the bacterium *Rhodococcus rhodochrous* PY11 can utilize 2-hydroxypyridine (2HP) as a sole carbon and energy source. The study identifies and characterizes a gene cluster (*hpo*) responsible for the degradation of 2HP, detailing the roles of specific genes and enzymes in this process. The metabolites involved in intermediate steps have also been identified. We have shown that the multicomponent HpoBCDF dioxygenase is responsible for the initial step of 2HP biodegradation. We have been able to solve the long-standing mystery of the ring opening reaction of 2,3,6-trihydroxypyridine (THP) by identifying the culprit—hypothetical cyclase HpoH. Further catabolic step leads to the final degradation products: ammonium ion and α-ketoglutarate. The novelty of this work lies in the experimental discovery of the function of seven new proteins.

The second paper "Catabolism of 2-Hydroxypyridine by *Burkholderia* sp. Strain MAK1: A 2-Hydroxypyridine 5-Monooxygenase Encoded by *hpdABCDE* Catalyzes the First Step of Biodegradation" explores different degradation pathway of 2HP by the *Burkholderia* sp. MAK1. This strain degrades 2HP without producing the blue pigment typically associated with microbial 2HP degradation. The study identifies a gene cluster, *hpd*, responsible for this process, revealing the enzyme 2-hydroxypyridine 5-monooxygenase, encoded by *hpdABCDE*, as pivotal in the initial step of converting 2HP to 2,5-dihydroxypyridine (25DHP). The next step in the pathway is ring cleavage. In contrast to the hydrolytic cleavage of the pyridine ring in *Rhodococcus rhodochrous* PY11, the 25DHP was split oxidatively by HpdF dioxygenase in *Burkholderia* sp. MAK1. Further degradation was proposed to take place via the so-called maleamate pathway, as all genes associated with this pathway have homologues in the *hpd* cluster.

The third paper "Microbial Degradation of Pyridine: a Complete Pathway in *Arthrobacter* sp. 68b Deciphered" presents the full catabolic pathway of pyridine degradation by *Arthrobacter* sp. strain 68b. The study identified a gene cluster responsible for pyridine degradation on a plasmid and detailed the enzymatic steps leading to the conversion of pyridine into succinic acid. The initial step involves a flavin-dependent monooxygenase system for direct ring cleavage, without typical activation steps such as reduction or hydroxylation of

the heterocycle. The subsequent steps of the pathway have been experimentally confirmed. They are catalysed by (*Z*)-*N*-(4-oxobut-1-enyl)formamide dehydrogenase PyrB, amidohydrolase PyrC, and succinate semialdehyde dehydrogenase PyrD.

The final paper, "Biochemical and Genetic Analysis of 4-Hydroxypyridine Catabolism in *Arthrobacter* sp. IN13", focuses on the degradation process of 4HP, identifying a gene cluster responsible for biodegradation and detailing the roles of specific enzymes in the pathway. The study elucidates the biochemical functions of three new proteins, the regiospecific flavin-dependent monooxygenase KpiA, an extradiol dioxygenase KpiC and the 3-(*N*-formyl)-formiminopyruvate hydrolase KpiB.

In summary, these four newly identified catabolic pathways provide fundamental insights into the microbial metabolism of *N*-heterocyclic aromatic compounds, contribute to the understanding of microbial biodegradation capabilities for environmental detoxification. Additionally, a diversity of enzymes from different classes and families were uncovered during the quest to elucidate the microbial degradation pathways of pyridine and its derivatives. Each of these enzymes plays a critical role in the conversion of these targeted organic molecules into simpler, assimilable forms. This enzymatic arsenal has evolved as a consequence of bacterial need to adapt their metabolic pathways to the myriads of chemical compounds encountered across their diverse habitats. As such, these enzymes are not merely biological curiosities; they represent a rich repository of potential biocatalysts that hold immense promise for applications in bioremediation, green chemistry, and synthetic biology. The discovery and characterization of microbial enzymes opens the door to innovative approaches for the detoxification of polluted environments and the sustainable synthesis of valuable chemical products. By leveraging the natural catalytic capabilities of microbial enzymes, scientists and engineers can devise eco-friendly solutions to some of the most pressing environmental challenges of our time, including the breakdown of persistent pollutants and the efficient conversion of renewable resources into biofuels and biodegradable materials.

In this context, the study of bacterial catabolism of xenobiotics transcends basic scientific inquiry, paving the way for transformative advances in environmentally responsible practices and sustainable industrial processes. The exploration of microbial enzymatic diversity is akin to mining a biochemical treasure trove, with the potential to unearth a new generation of biocatalysts that can catalyze reactions with unparalleled efficiency and specificity.

1. Xenobiotics in nature

A chemical substance that is not naturally produced or expected to be present in an organism can be called a xenobiotic. It can also include natural compounds that are present in much higher concentrations than usual, or that are ingested by another organism. However, in the anthropocentric perspective, the term xenobiotic is often reduced to the context of man-made pollutants, e.g. polychlorinated biphenyls, synthetic organochlorines and other pesticides, pharmaceuticals and plastics. It becomes more comprehensible in light of recent estimates that global anthropogenic mass surpasses all living biomass[13].

Other distinct classes of xenobiotics are biologically active secondary metabolites that organisms have evolved to secrete in order to evade, manipulate or kill their enemies. The general structural classes include alkaloids, phenylpropanoids, polyketides and terpenoids. In the search for biologically active molecules that could be used as pharmaceuticals, nutraceuticals, or pesticides, many secondary metabolites have been discovered. Nicotine, salicylic acid and tetracycline are just a few of the compounds used by organisms to mediate ecological interactions that may confer a selective advantage to the organism by increasing its survivability or fecundity. As this arms race evolved, the chemical arsenal expanded, but at the same time the defensive mechanisms of detoxification became more numerous. The prokaryotes even adapted to use these compounds as a source of carbon, nitrogen and energy.

A separate class of xenobiotics consists of non-canonical or damaged metabolites. Spontaneous chemical reactions occur in cells because many metabolites are chemically reactive or unstable. Most commonly, cellular components undergo unwanted modifications due to reactive oxygen species, UV radiation and spontaneous decay. The intricate relationship between organisms and their chemical environment extends to the domain of enzyme function, where the versatility of enzymes in catalyzing diverse chemical reactions reflects the ongoing molecular dialogue between organisms and their surroundings. The paradigm of absolute specificity of enzymes is shifting as numerous examples of enzyme promiscuity are being discovered[14]. There is a growing body of evidence that enzymes can convert a wide range of substrates, albeit with varying efficiencies, a phenomenon known as substrate promiscuity. There is also ample documentation of catalytic promiscuity, based on the ability of a single enzyme active site to catalyze multiple chemical transformations. This promiscuous activity is a double-edged sword. On the one hand, it can be a starting point for the evolution of new enzymes, both for cells and for protein engineers. On the other hand, it consumes valuable resources and can be toxic to organisms. Recent

work suggests that damage control enzymes, which either repair or inactivate the offending metabolites, make up a quarter of the genome and are a plentiful reservoir for new classes of enzymes that use alternative mechanisms or catalyze new reactions[15].

Pyridine and its derivatives constitute a pervasive class of xenobiotics, permeating the environment via both natural and anthropogenic pathways. Unsubstituted pyridine is rarely found in living organisms, but it can be formed through the thermal decomposition of certain amino acids. Predominantly, pyridine's presence in the environment is attributed to anthropogenic activities, given its extensive application as a solvent and a precursor in the synthesis of herbicides, pesticides, and pharmaceutical compounds. Pyridine derivatives are found in a wide variety of natural substances, ranging from universally recognized cofactors such as nicotinamide adenine dinucleotide (NAD) and pyridoxal phosphate (PLP) to specific phytoalkaloids like nicotine, actinidine, and mimosine, in addition to naturally occurring antibiotics such as caerulomycin and piericidin. In addition, synthetic pyridine compounds are widely used as dyes, explosives, pharmaceuticals, and pesticides/herbicides (Fig. 1). Substituting the CH group with a nitrogen atom in aromatic or heteroaromatic rings significantly alters their molecular and physicochemical characteristics, affecting both intra- and intermolecular interactions (Fig. 2). These substitutions can modify various interactions, including lone pairs, dipole-dipole, hydrogen bonding, metal coordination, van der Waals forces, and π-system interactions, potentially resulting in improved pharmacological attributes like binding affinity, functional activity, and target selectivity[16]. Furthermore, incorporating a pyridine ring into pharmaceutical compounds can increase their biological effectiveness, penetration, and stability. A review of US FDA-approved drugs shows that 59% of unique small molecule drugs have a nitrogen heterocycle (62 of which have a pyridine ring)[17]. Nearly 10% of the global agrochemical market consists of 8 crop protection products containing a pyridine moiety[18].

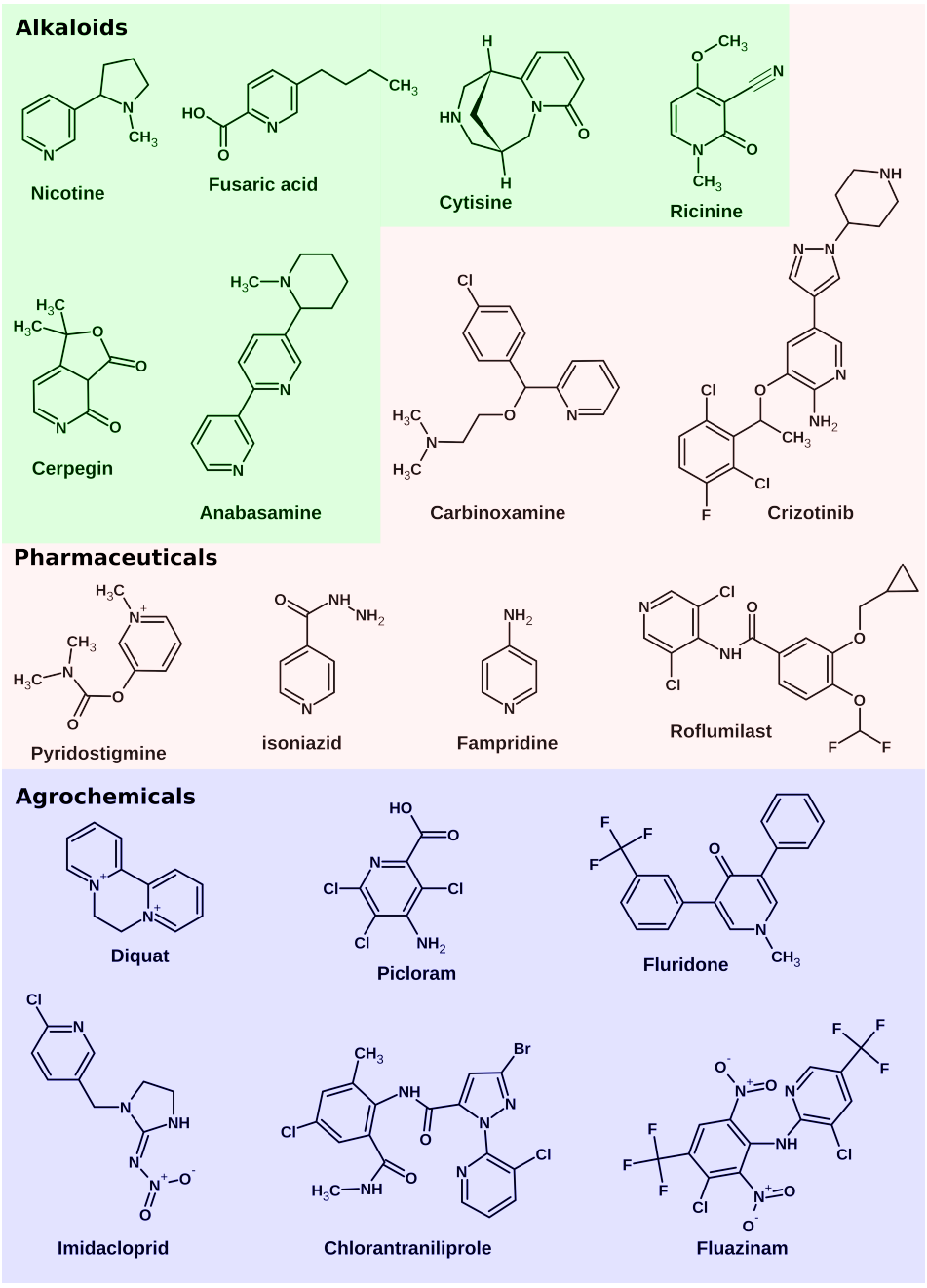
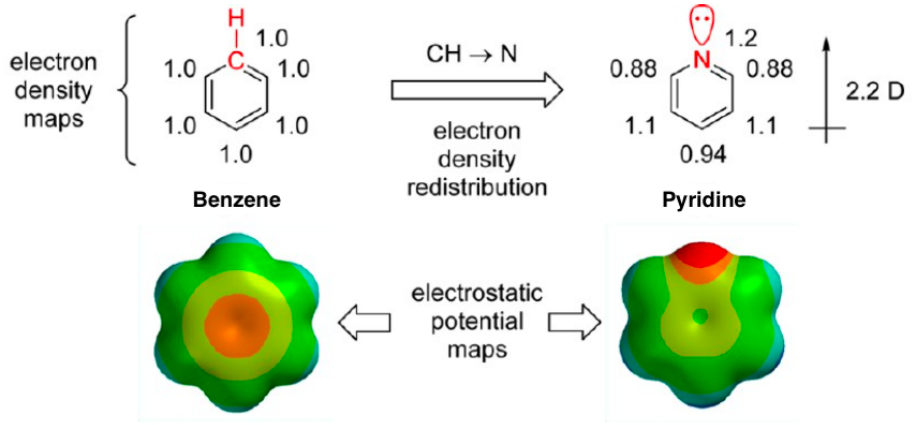


Figure 1. Variety of the xenobiotics containing the ring of pyridine.

Pyridinols, which serve as common intermediary metabolites in the microbial biodegradation of various *N*-heterocycles (pyridine, nicotine, picoline, 2,6-dipicolinic acid), have also been identified as non-specific detoxification by-products in laboratory animals and as an atypical metabolite of NAD—4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate. The heterocyclic

composition of pyridine derivatives augments their solubility, facilitating their transport through soil matrices and the subsequent contamination of groundwater resources. Nonetheless, the presence of these compounds does not tend to result in significant accumulation within natural settings, suggesting their eventual decomposition by abiotic processes and microbial entities. The ability of various soil bacteria to degrade *N*-heterocyclic compounds not only underlines the potential for bioremediation of these toxic xenobiotics, but also highlights the prospective application of such microbial processes in biocatalytic strategies.



Property or Interaction	Benzene	Pyridine
resonance stabilization energy (E_{res})	36 kcal/mol	32 kcal/mol
molecular weight (MW)	78 Da	79 Da
polar surface area (tPSA)	–	12.9 Å^2
lipophilicity (CLogP)	2.142	0.645
basicity (pK_a)	–	5.20
HBA strength (pK_{BHX})	–	1.86
C/N electronegativity (χ)	2.55	3.04
dipole moment (μ)	–	2.2 D
CH/N van der Waals radius (r_w)	1.77 Å	1.55 Å
aqueous solubility (S_{H2O})	1.8 g/L	miscible
ionization potential (IP)	9.23 eV	9.34 eV
3–**3**/**3**–**4** π-stacking energy (ΔE)	−2.71 kcal/mol	−3.23 kcal/mol
Na^+/π-system binding energy (BE)	−27.1 kcal/mol	−20.0 kcal/mol
H2 HBD interaction energy (IE)	−1.47 kcal/mol	−2.23 kcal/mol
C2/C4 carbanion charge demand (CD)	0.29	0.41

Figure 2. Molecular and physicochemical properties of benzene and pyridine (according to Pennington, L. D.; Moustakas, D. T.[16])

2. Isolating bacteria of interest

Bacteria, representing the second most populous domain of life on the planet, are eclipsed only by the sheer abundance of viruses. Throughout the eons, bacteria have exhibited remarkable adaptability, colonizing every conceivable niche on Earth—from terrestrial soils and aquatic environments to extreme locales such as acidic hot springs and the frigid realms of the Arctic biosphere. This ubiquitous presence is a testament to their metabolic versatility, enabling them to extract requisite elements from a vast array of sources to sustain their existence and proliferation.

Classical microbiologists have been using solid culture media since R. Koch's accidental discovery that a single clone of microorganisms could be isolated on potato slices. Since then, by varying the growth conditions, scientists have been able to isolate a wide variety of bacteria with different biodegradation potential. The traditional concept of the experiment is to limit one of the necessary elements such as carbon, nitrogen, sulfur etc. by using the substrate of interest. Progress in bacterial culture methods comes from several directions: from autoclaving growth medium components separately[19], to systematic literature reviews to create a platform that predicts media given an organism's 16S rDNA sequence[20]. However, these technologies have some drawbacks: they are time and resource consuming, not high throughput, and have a low success rate.

Despite the enormous amount of time and effort spent on culturing organisms over the last century and a half, approximately 99% of microorganisms in nature have not been cultured by traditional techniques[21]. Advances in next-generation DNA sequencing capabilities have revealed missing information about uncultured bacteria and provided new tactics for isolating the microorganism. Genome-scale metabolic networks generated *in silico* using genetic information successfully proposed growth medium requirements for *Neisseria meningitidis* and '*Candidatus Pelagibacter ubique*' strain HTCC1062[22,23]. Using reverse genomics, M. Podar's group cultivated three different species-level lineages of human oral *Saccharibacteria*[24]. They obtained polyclonal antibodies against predicted cell surface proteins, labeled them with fluorescent tags and used cell sorting flow cytometry to enrich these bacteria from human oral samples.

Novel, next-generation techniques for the investigation of physiology allow the study of microbiome phenotypes even at the single cell level. Using a fluorescent substrate analogue, flow cytometry or microfluidics-based separation and fluorescence-activated cell sorting (FACS), 1000 cells per second can be sorted. In addition, cells with unique chemical signatures in their Raman spectrum (such as substrate-specific bands or peak shifts due to isotope uptake) can be separated by Raman-activated cell sorting[25]. Cells can also be isolated based on morphological or optical properties using optical microscopy and optical tweezers

or laser microdissection, but these techniques are expensive and have limited throughput. The unlabeled metabolites in the bacterial colony can be detected by nanoscale secondary ion mass spectroscopy with unrivaled sensitivity and spatial resolution[26]. The micro-structured chip systems introduced a new platform for exploring microbial interspecies communication and interactions with the physical environment. Screening of ~100,000 multi-species communities on kChip led to the identification of growth-promoting factors for *Herbaspirillum frisingense*[27]. The custom-designed 'isolation chip' provided a method for culturing novel microorganisms in situ[28].

3. Identification of genotypes associated with biodegradation pathways

Once the bacterial species with the desired metabolic capabilities have been isolated, the next step is to identify the genes that encode the proteins responsible for these processes. Depending on the characteristics of the bacteria (growth rate and yield, available genetic manipulation tools etc.), there are several ways to achieve this goal (Fig. **3**). The most straightforward tactic is to purify native enzymes from the biomass and use mass spectrometry to determine the amino acid sequence of peptides, which easily leads to the genotype (Fig. **3A**). In the era of widely available DNA sequencing capabilities, it is reasonable to assume that the genome sequence of the bacterium of interest is known (even if not 100% complete, it is often sufficient for targeted gene identification). This approach was successfully used to identify the genes responsible for the metabolism of sesamin[29], pendimethalin[30], 6-chloro-2-benzoxazolinone[31], 4-fluorobenzoate[32], acetochlor[33], and 2,4-dinitroanisole[34]. As the metabolic pathways of xenobiotic biodegradation are often inducible in bacteria, they are therefore used as a starting point for gene identification. By growing bacteria on different substrates and analyzing protein expression profiles (1D or 2D gel electrophoresis) or mRNA copy number variations (RNA sequencing), the best gene candidates can be identified without the need for large amounts of biomass (Fig. **3B**). The genotype of metabolism of tetramethylpyrazine[35], sulfoquinovose[36] and toluene, phenol, 4-hydroxybenzoic acid[37] were identified using protein fingerprinting. Prediction of catabolic genes for 3,5-dibromo-4-hydroxybenzoate[38], phenazine-1-carboxylic acid[39], and benzyldimethyldodecylammonium chloride[40] was based on comparative transcriptome analysis.

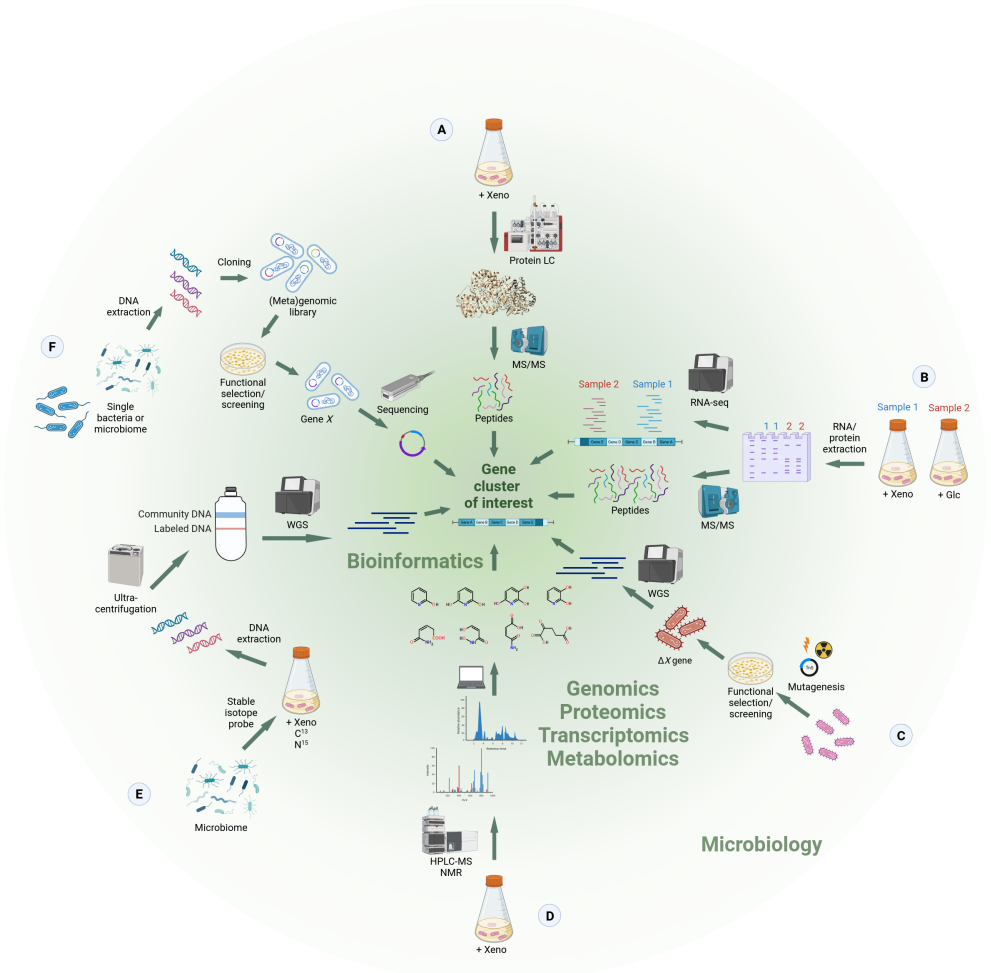


Figure 3. Different (**A**–**F**) approaches for identifying gene clusters of interest. Microbiological techniques facilitate the isolation of target bacteria, exploration of metabolite utilization profiles, mutagenesis (targeted or random), and functional screening. Genomic, proteomic, transcriptomic, and metabolomic methods are employed to gather data on genes and enzymes. The experimental workflows illustrated in **A**–**F** have been widely applied and can be adapted or iterated in user-defined sequences. The resulting datasets are integrated and analyzed with bioinformatics tools to identify genes, promoters, regulatory elements, and proteins of interest. Created with BioRender.com

The alternative approach is to disrupt the phenotype by genetic manipulation. The use of mutagens such as ethyl methanesulfonate, nitrosoguanidine or UV radiation is the simplest method of generating the population of variants and will work with a wide range of host species (Fig. **3C**). The drawbacks of this method are that it requires a high-throughput screening or

selection procedure to identify the phenotype of interest and considerable computational power to determine which mutations are responsible for the phenotype conversion. The genes encoding the enzymes involved in the catabolic pathway of specific compounds are often clustered in operons located in mobile genetic elements such as plasmids and transposons. For this reason, the cultivation of microorganisms under laboratory conditions can, under certain conditions, lead to a spontaneous loss of the biodegradation ability. Comparative genomics of this type of mutant can identify the loss of DNA fragments from the genomes more easily than elucidating which of the point mutations are responsible for the change in phenotype. Analysis of genetic differences in spontaneous mutants led to the determination of the genotypes of thiobencarb[41], buprofezin[42], 2-methyl-6-ethylaniline[43], and 3-phenoxybenzoate[44]. The degradation pathways of BTEX (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene) compounds in *Pseudoxanthomonas spadix* BD-a59 were predicted by genetic comparison with another fully sequenced *Pseudoxanthomonas* strain, which was isolated from different location and later successfully verified[45].

Another genetic technique for generating mutants uses transposon systems as genotype disruptors. The basic concept of this method is to design a specialized minitransposon (Tn5 or others) to integrate stably into a target DNA without its transposase and virtually without preference for a specific DNA sequence (Fig. **3C**). Positive insertion of the transposon is ensured using antibiotic resistance alleles and additional DNA regions are constructed for mapping insertional mutations (by primer walking sequencing, Tn-seq, or plasposons). Not all bacterial species can be manipulated in this way, as they may be resistant to antibiotics and may not have well-developed methods and tools for laboratory genetic manipulation. Nevertheless, there have been many successful adaptations of this technique, such as the identification of the genotypes responsible for degradation of *N*-methylpyrrolidone in *Alicycliphilus* sp. strain BQ1[46], 3-methyldiphenylether by *Hydrogenophaga* atypical strain QY7-2[47], 4,4-dithiodibutyric acid by *Rhodococcus erythropolis* MI2[48], long-chain alkanes in *Alcanivorax dieselolei*[49], and (*S*)-6-hydroxynicotine by *Shinella* sp. strain HZN7[50].

The intermediates of biodegradation pathways can be identified using metabolomics techniques. When sequential metabolites are discovered, the class and/or type of enzyme involved in the reaction can be predicted (Fig. **3D**). The increasing number of protein sequences with experimentally confirmed functions allows more accurate prediction of the genes to be searched for. Predicting the exact gene responsible for a particular reaction from the genomes is still not 100% certain, but the experimental screening required to find the gene of interest is greatly reduced. Metabolomics and protein homology analysis have been used to elucidate the biodegradation genotypes of diphenylether[51], dicamba[52], 2-heptyl-

4(1*H*)-quinolone[53], poly(ethylene terephthalate)[54], mesaconate[55], 2-chloro-4-nitrophenol[56], and others.

Advances in recombinant DNA technologies have expanded the ability to express active proteins in heterologous hosts. Cloning of genomic DNA fragments from bacteria under investigation has become a common technique in molecular biology. However, selecting positive clones from DNA libraries is not straightforward (Fig. **3F**). The transfer of the complete metabolic pathway ensuring exclusive growth on the xenobiotic to other hosts is rarely reported, probably due to improper protein expression profiles and unbalanced quantity of metabolites. The clone of *E. coli* harboring genomic fosmid library constructed from *Cupriavidus* sp. strain ST-14 was selected by growth on 3-nitrobenzoate as sole carbon source[57]. The solid-phase screening methods for the identification of positive clones with degradation pathway genes can be applied if the substrates have certain characteristics, e.g., low solubility in water (e.g. iprodione[58]), can be converted to a colored product (e.g. indole[59 60], 2-hydroxypyridine[61]).

Gain-of-function library screening can also be performed using HPLC-MS/MS, although it is low throughput and more expensive. Using this method, 30 novel microbiome-encoded enzymes were discovered that converted 20 drugs, commonly used in human diseases, into different metabolites[62]. Recombinant DNA techniques and gain-of-function screening or selection methods eliminate the need to cultivate the specialized microorganisms. The DNA library can be constructed from environmental samples. The isolation of metagenomic DNA from contaminated soils led to the discovery of novel petroleum detoxifying enzymes[63] and organohalide destroying reductive dehalogenases[64]. The genotypes of polycyclic aromatic hydrocarbon-degrading bacteria have been elucidated using the DNA-SIP method, based on the incorporation of heavy isotopes ([^{13}C]phenanthrene[65], [^{13}C]anthraquinone[66]) into microbial DNA during growth on labelled substrates (Fig. **3E**). As not all heterologously expressed enzymes can be obtained in active form, other approaches have been developed to isolate novel substrate-induced catabolic genes from environmental metagenomes. In substrate-induced gene expression screening, DNA fragments are ligated into an operon trap vector, the library is subjected to a substrate-dependent gene induction assay, and positive cells are selected by detecting the activity of a co-expressed marker (e.g., GFP) encoded in the vector[67].

4. Translation of catabolic pathways research into practice

Studying catabolic pathways of xenobiotic in microorganisms has provided extensive insights with significant applications in metabolic engineering and synthetic biology. The genes responsible for biodegradation capabilities are often clustered in substrate-inducible operons, allowing elements of the genetic regulatory machinery to be repurposed. This repurposing is central to the

advancement of cell factory engineering, which is currently used to produce valuable chemicals, solvents, monomers, pharmaceuticals, nutraceuticals, antibiotics, etc. Another notable challenge in this field is the need for novel chassis organisms equipped with a comprehensive array of genetic components and regulatory mechanisms, including promoters, ribosomal binding sites, terminators and vectors. Through the investigation of xenobiotic metabolism, several bacterial chassis and their associated genetic toolkits have been identified, notably *Pseudomonas putida* KT2440[68] and *Comamonas testosteroni* CNB-1[69], among others. Furthermore, allosteric transcription factors (TFs) that recognize the structure and report the titer of a given natural product are the basis for genetically encoded *in vivo* biosensors. These biosensors, distinguished by their high specificity for target compounds, are poised to revolutionize high-throughput screening and selection methodologies, facilitating the discovery of novel enzymes and the biosynthesis of value-added chemicals in microbial systems. While bacterial TFs have been acknowledged for decades, only a select few, such as those responsive to lactose or arabinose, have been widely exploited in genetic engineering. A broader repertoire of newly characterized and/or engineered transcription factors has been employed to devise biosensors with tunable sensitivity and specificity, facilitating their application in the monitoring of metabolites and the construction of synthetically robust genetic circuits (as reviewed in[70,71]). An exemplary demonstration of such engineering prowess is the development of an *E. coli* strain “Marionette", featuring 12 highly optimized small molecule sensors[72].

The last two decades have witnessed remarkable advancements in biocatalysis, making it a key technology for sustainable and efficient chemical synthesis. The integration of cutting-edge scientific and technological innovations continues to push the boundaries of what is possible in biocatalysis. The traditional approach to discovering new enzymes typically involves screening natural habitats and employing techniques like enrichment cultures. This method has historically been a foundational tool for identifying microorganisms with specific enzymatic activities, usually metabolism of xenobiotics. This approach has led to the discovery of industrially important enzymes such as nitrile hydratase from *Pseudomonas chlororaphis* B23 that grows on isobutyronitrile containing medium[73]; subtilisin from ovalbumin metabolising *Bacillus subtilis*[74]; (*R*)-enantioselective transaminase deaminating (*R*)-dimethoxyamphetamine from *Arthrobacter* sp. KNK168[75]; P450-BM3 monooxygenase from *Bacillus megaterium*[76] (a phenobarbital inducible enzyme capable of ω-hydroxylation of saturated long chain fatty acids, alcohols, and amides); Δ1-piperideine-2-carboxylate imine reductase from *Pseudomonas putida* ATCC12633[77] (participating in D-lysine metabolism); etc. These biocatalysts have been instrumental in facilitating the multi-ton scale production of chemicals and

pharmaceuticals, including acrylamide, nicotinic acid, and (*R*)-mandelic acid, alongside the synthesis of aspartame via subtilisin and the kilogram-scale synthesis of sitagliptin utilizing an optimized variant of *R*-transaminase[78]. Furthermore, engineered P450-BM3 monooxygenase has been applied for the late-stage functionalization of (*R*)-4-hydroxy isophorone[79], while imine reductases have found application in the synthesis of abrocitinib[80]. These enzymes have been pivotal in biocatalysis, demonstrating their utility in the production of various pharmaceuticals and chemicals at industrial scales.

The adaptation of microorganisms to degrade xenobiotic compounds, substances foreign to biological systems, showcases the rapid evolutionary capabilities of these organisms to exploit novel substrates introduced into the environment by anthropogenic activities. This rapid adaptation is facilitated by specialized enzyme systems and metabolic pathways that have evolved to degrade a wide range of man-made compounds, such as polychlorinated biphenyls, phthalates and other endocrine disruptors. The prevailing focus on reporting positive outcomes in the scientific literature regarding the degradation of xenobiotics by microorganisms presents challenges in accurately discerning the true recalcitrance of pollutants that resist microbial catabolism. This bias towards favorable results skews our understanding of the biodegradability spectrum of xenobiotics, complicating the identification of compounds that are genuinely resistant to microbial degradation and thereby undermining efforts to address environmental contamination comprehensively. Despite the substantial advancements in the discovery of novel biodegradation pathways and the genetic engineering of microorganisms for enhanced bioremediation capabilities, the translation of these laboratory successes to effective, large-scale environmental applications remains limited.

In conclusion, the existence of enzyme systems capable of degrading xenobiotic compounds underscores the remarkable adaptability of microbial life to anthropogenically altered environments. The study of these systems not only provides insights into microbial evolution but also opens avenues for bioremediation technologies aimed at mitigating the environmental impact of xenobiotic pollutants.

PERSPECTIVE

This section summarizes the four research papers (**I—IV**) that make up this thesis.

Isolating bacteria of interest.
In light of the ability of various soil bacteria to degrade *N*-heterocyclic compounds, an investigation was conducted to identify novel bacterial strains capable of metabolizing pyridine and pyridinols. A cohort of soil microorganisms was isolated using solid-state agar plates with a minimal medium supplemented with targeted derivatives of pyridine to restrict the availability of carbon source – a straightforward approach that requires minimal experimental exertion. Through this methodology, a diverse array of bacterial species was isolated, including the four strains described in this work, indicating that the degradation of pyridine and its derivatives is a relatively prevalent phenomenon in environmental contexts. *Rhodococcus rhodochrous* PY11 and *Burkholderia* sp. MAK1 were isolated as capable of utilizing 2HP as a sole carbon source, as documented in the first and second studies, respectively. Concurrently, *Arthrobacter* sp. strain 68b demonstrated proficiency in degrading pyridine. Moreover, this bacterium exhibited versatility in metabolizing 2-methylpyridine, nicotine, and phthalic acid, as elucidated in the third publication. Additionally, *Arthrobacter* sp. IN13 was identified, showcasing the capability to metabolize 4HP and 2HP as sources of carbon and energy, as detailed in the fourth study. Utilizing a straightforward methodology and minimal experimental exertion, a diverse array of bacterial species was isolated, indicating that the degradation of pyridine and its derivatives is a relatively prevalent phenomenon in environmental contexts.

Identification of genotypes associated with biodegradation pathways.
The subsequent phase of the research aimed to elucidate the precise bacterial genotypes instrumental in the bioremediation of the specified xenobiotics. A facilitating factor for this endeavor was the discovery that catabolic processes in all four isolated bacterial strains were inducible by relevant substrates. Cultivation of the bacterial cells in the presence of targeted compounds or succinate ensued, followed by the assessment of *in vivo* degradation activity variances. Additionally, the profiles of inducible proteins were examined using SDS-PAGE and/or mRNA differential display techniques. A particular RNA fragment induced in *R. rhodochrous* PY11 (**I**) exhibited homology to the hypothetical gene gpORF106, previously identified within a gene cluster implicated in nicotine biodegradation in *Arthrobacter nicotinovorans*. This DNA fragment served as a probe for chromosome walking, aiming to pinpoint a genomic locus presumably encoding the biodegradation pathway for 2HP. Consequently, a DNA segment spanning

60,152 base pairs from the *R. rhodochrous* PY11 genome was cloned and sequenced across several plasmids. The purification of two 2HP-inducible proteins and the determination of their N-terminal amino acid sequences, allowed the localization of their corresponding genes within the 60-kb genomic fragment. Integration of these findings led to the identification of a 12-gene cluster (*hpo*) potentially involved in 2HP biodegradation in *R. rhodochrous* PY11. Parallel investigations in *Arthrobacter* sp. 68B (**III**) and *Arthrobacter* sp. IN13 (**IV**) utilizing peptide mass fingerprinting via MS/MS unveiled the substrate-inducible proteins. After partial genome sequencing, a disorganized gene cluster (*pyr*, encompassing 13 genes) accountable for pyridine degradation in *A*. sp 68b and the *kpi* gene cluster, comprising 16 open reading frames responsible for 4HP degradation in *A*. sp IN13, were located. *Burkholderia* sp. MAK1 also exhibited substrate-inducible degradation of 2HP (**II**), as validated by *in vivo* metabolic assays. Nonetheless, the differential display of 2HP-inducible proteins in *Burkholderia* sp. MAK1 did not yield identifiable targets for subsequent analysis. The indigo dye formation assay (indole oxidation) with 2HP-induced *Burkholderia* sp. MAK1 cells produced positive results. The random mutants generated using plasposon (pTnMod-Okm) were screened for the white colony forming phenotype on the 2HP inducible solid medium. The positive clones were tested for their incapacity to utilize 2HP as the sole carbon and energy source. The transposon insertion site within the genome of a selected mutant, *Burkholderia* sp. MAK1 ΔP5, was determined, leading to the discovery of a gene cluster (designated *hpd*) encompassing 12 open reading frames responsible for 2HP degradation in *Burkholderia* sp. MAK1.

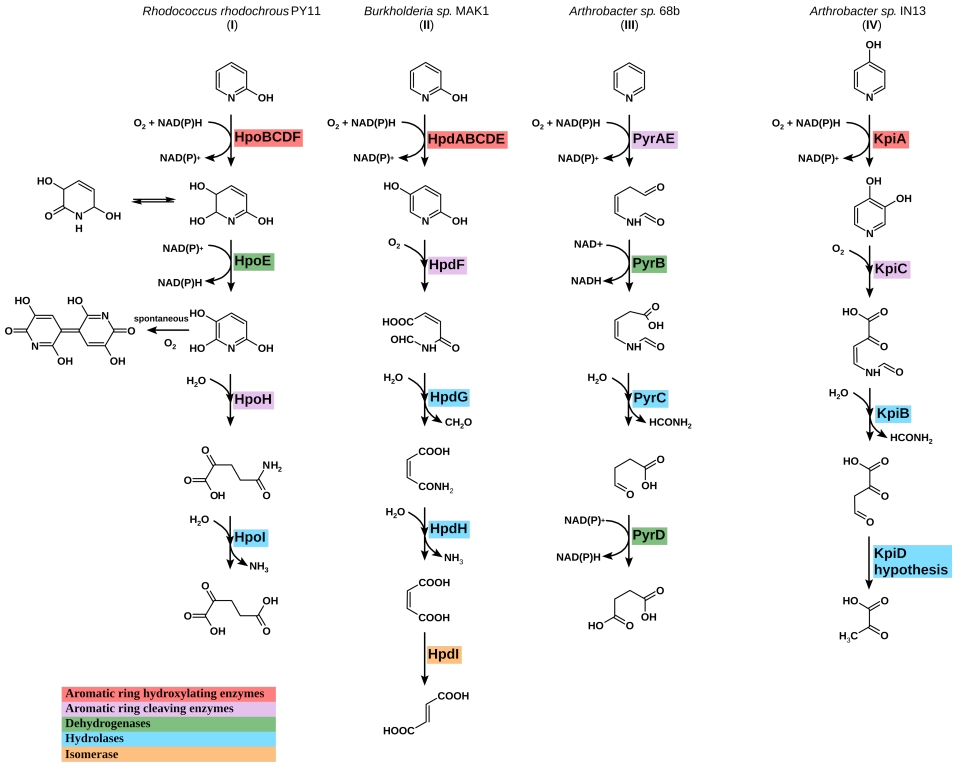
Experimental determination of protein functions.

In the process of elucidating gene clusters pertinent to the study, numerous hypothetical proteins emerged as candidates for further functional analysis. The initial step in the aerobic degradation of aromatic compounds typically involves (di)hydroxylation, catalyzed by a range of mono- or dioxygenases. Preliminary bioinformatics assessments, including superfamily classification and the identification of homologues with experimentally verified activities, facilitated the prediction of enzymes implicated in the initial stages of catabolic pathways. The functional activity of recombinantly expressed proteins was subsequently confirmed *in vivo*, with the hydroxylation reaction products being isolated and characterized through HPLC-MS, ^{13}C NMR, and ^{1}H NMR techniques.

A four-component dioxygenase system (HpoBCDF) from *R. rhodochrous* PY11, categorized within the Rieske non-heme iron-dependent oxygenase family, was found to catalyze the hydroxylation of 2-hydroxypyridine, yielding 3,6-dihydroxy-1,2,3,6-tetrahydropyridin-2-one (**I**). Moreover, the enzymatic activity of 2-hydroxypyridine 5-monooxygenase was attributed to a soluble diiron

monooxygenase within a five-gene cluster (*hpdABCDE*) (**II**) (Fig. **4**). In *Arthrobacter* sp. IN13, the catabolism of 4-hydroxypyridine initiates with hydroxylation mediated by a flavin-dependent monooxygenase (KpiA), producing 3,4-dihydroxypyridine (**IV**). On the contrary, pyridine degradation in *Arthrobacter* sp. 68b involves a direct ring cleavage catalyzed by a two-component flavin-dependent monooxygenase system (PyrAE), consolidating a new family of flavin-dependent enzymes catalysing ring cleavage reactions (**III**). The oxidative cleavage of 25DHP and 34DHP, following their initial hydroxylation, is executed by HpdF and KpiC dioxygenases, respectively. These enzymes do not exhibit notable similarity to established ring-cleaving dioxygenases but are instead homologous to metallo-dependent aminopeptidase and amidohydrolase, respectively. The cyclase HpoH in *R. rhodochrous* PY11, a predicted metal-dependent hydrolase, catalyzes the hydrolytic cleavage of the THP ring.

Furthermore, the functionalities of three dehydrogenases within pyridine(-ol) catabolic pathways were elucidated: HpoE converts 2-pyridone-5,6-dihydro-cis-5,6-diol to THP, while PyrB and PyrD catalyze the oxidation of aldehydes to carboxylates in (Z)-*N*-(4-oxobut-1-enyl)formamide and succinate semialdehyde, accordingly (**III**). Subsequent stages of catabolism involve various hydrolases, with HpdG executing a deformylation reaction, and KpiB and PyrC removing formamide from their respective substrates, though the mechanism—whether it is a single or dual-step process—remains to be clarified. Additionally, two ω-amidases were identified in the metabolic pathways for 2HP degradation: HpoI from *R. rhodochrous* PY11 converts 2-ketoglutaramate to α-ketoglutarate, and HpdH from *Burkholderia* sp. MAK1 transforms maleamate to maleic acid. In summary, the functional activities of 21 proteins across different families were determined, significantly advancing the understanding of the biochemical pathways involved in the degradation of pyridine and its derivatives.



hpo gene cluster of 2HP degradation in *R. rhodochrous* PY11 **(I)**

Gene	Function
hpoR1	Putative transcription regulator
hpoR2	Putative transcription regulator
hpoA	Putative permease
hpoB	Ferredoxin
hpoC	2HP 5,6-dihydroxy-dioxygenase, α-subunit
hpoD	2HP 5,6-dihydroxy-dioxygenase, β-subunit
hpoE	Short-chain dehydrogenase
hpoF	Ferredoxin reductase
hpoG	Hypothetical protein
hpoH	236THP hydrolase
hpoI	2-ketoglutaramate amidase

hpd gene cluster of 2HP degradation in *Burkholderia* sp. MAK 1 **(II)**

Gene	Function
hpdJ	Putative transcription regulator
hpdI	Maleate isomerase
hpdA	2HP 5-monooxygenase subunit
hpdB	2HP 5-monooxygenase regulatory subunit
hpdC	2HP 5-monooxygenase catalytic subunit
hpdD	Ferredoxin
hpdE	NADH-ubiquinone oxidoreductase
hpdK	Putative permease
hpdG	N-Formylmaleamate deformylase
hpdL	Putative chaperone
hpdF	25DHP 5,6-dioxygenase
hpdH	Maleamate amidase

pyr gene cluster of pyridine degradation in *Arthrobacter* sp. 68b **(III)**

Gene	Function
pyrA1	Pyridine monooxygenase
orf43	Hypothetical protein
pyrB	(Z)-N-(4-oxobut-1-enyl)formamide dehydrogenase
pyrC	(Z)-4-formamidobut-3-enoic acid hydrolase
pyrE	Flavin reductase
pyrA2	Pyridine monooxygenase
orf48	Hypothetical protein
orf49	Hypothetical protein
orf50	Hypothetical protein
orf51	Putative transcription regulator
orf52	Hypothetical protein
orf53	Hypothetical protein
pyrD	Succinate-semialdehyde dehydrogenase

kpi gene cluster of 4HP degradation in *Arthrobacter* sp. IN13 **(IV)**

Gene	Function
kpiA	4HP 3-monooxygenase
orf1	Putative transposase
orf2	Hypothetical protein
kpiP	Putative permease
orf3	Pseudogene
orf4	Putative transposase
orf5	Putative transposase
orf6	Hypothetical protein
kpiB	3-(N-formyl)-formiminopyruvate hydrolase
kpiC	34DHP 2,3-dioxygenase
kpiD	Putative hydrolase
orf7	Pseudogene
orf8	Hypothetical protein
kpiR1	Putative transcription regulator
kpiR2	Putative transcription regulator

Figure 4. The identified catabolic pathways of 2HP, 4HP, and pyridine degradation in selected bacteria of this study.

Translation of catabolic pathways research into practice.

All four isolated bacterial strains demonstrated significant efficacy in metabolizing their respective substrates, rendering them viable candidates for incorporation into bioremediation strategies. Notably, *Arthrobacter* sp. 68b emerged as the most promising organism, attributed to the relatively higher toxicity of pyridine compared to pyridinols, positioning it as a superior bioremediative agent against more hazardous xenobiotics. The potential repurposing of the identified putative transcription regulators within the biodegradation gene clusters for the development of inducible protein expression modules is particularly intriguing, especially for utilization in non-conventional hosts such as *Rhodococcus*, *Burkholderia*, and *Arthrobacter*. Furthermore, the construction of an engineered biosensor specifically for pyridine detection could

significantly enhance monitoring and management strategies for contaminated environments. The paramount application identified in our research is the exploitation of hydroxylating enzymes. The regioselective monooxygenase isolated from *Burkholderia* sp. MAK1 exhibited remarkable substrate flexibility[81], paving the path for subsequent explorations into other soluble diiron monooxygenases and their broad-spectrum hydroxylation capabilities[82]. Additionally, the HpoI ω-amidase, involved in the 2-hydroxypyridine degradation pathway in *R. rhodochrous* PY11, was discovered to possess enzymatic promiscuity, efficiently deaminating D,L-glutamate and D,L-aspartate. This enzyme presents as a viable candidate for novel asparaginase applications, both in pharmaceuticals and food production. The other enzymes involved in the catabolic pathways of the pyridine derivatives hold out the promise of further applications and insights in the near future.

CONCLUSIONS

- The dihydroxylation of 2-hydroxypyridine followed by a hydrolytic cleavage of the pyridine ring in *Rhodococcus rhodochrous* PY11 is an atypical pathway for degradation of aromatic compounds.
- The pyridine ring is oxidatively cleaved by HpdF dioxygenase in *Burkholderia* sp. MAK1 during the catabolism of 2-hydroxypyridine, resembling a common strategy in the biodegradation of aromatic compounds.
- *Arthrobacter* sp. IN13 cells catabolize 4-hydroxypyridine via a conventional pathway consisting of a regiospecific flavin-dependent hydroxylating monooxygenase KpiA, a 3,4-DHP 2,3-dioxygenase KpiC and the 3-(*N*-formyl)-formiminopyruvate hydrolase KpiB.
- An unorthodox pathway of pyridine degradation in *Arthrobacter* sp. 68b involves a direct ring cleavage catalyzed by a two-component flavin-dependent monooxygenase system (PyrAE) without typical activation steps such as reduction or hydroxylation of the heterocycle.

SUMMARY/SANTRAUKA

Baltymai yra būtini visoms gyvybės formoms. Tiriant molekulinius gyvybės mechanizmus, ypač svarbią dalį sudaro kataliziniai baltymai – fermentai. Dėl didžiulės DNR sekoskaitos galimybių pažangos, įvairius fermentus koduojančių hipotetinių genų sekų skaičius auga eksponentiškai. 2024 metų sausio mėnesio duomenimis, Nacionalinis biotechnologijų informacijos centras (NCBI) savo duomenų bazėje jau talpino $2{,}5\times10^{13}$ nukleobazių, gautų iš įvairių DNR sekoskaitos projektų. Buvo atlikta daugiau kaip 550 000 sekoskaitos projektų, kurių metu gauta 236 493 bakterijų rūšių ir 92 072 eukariotų rūšių pilnų arba dalinių genomų sekų[1]. Deja, eksperimentiškai patvirtinti šių baltymų funkcijas ir suprasti veikimo mechanizmus vis dar išlieka dideliu moksliniu iššūkiu, kadangi tai gana lėtas ir sudėtingas procesas. Net geriausiai apibūdintų organizmų, tokių kaip *Escherichia coli* ir *Saccharomyces cerevisiae,* tik apie 80 % genų yra priskirtos kokios nors funkcijos. Genų funkcijų patvirtinimo svarbą gerai iliustruoja minimalios sintetinės bakterijos pavyzdys: autonomiškai veikianti bakterija *Mycoplasma mycoides* JCV-syn3.0 turėjo minimalų sintetinį genomą ir buvo gyvybinga, tačiau maždaug trečdalio visų jos genų (149 iš 473) biologinės funkcijos nebuvo žinomos[2].

Dėl sparčiai augančių genomo ir metagenomo sekoskaitos iniciatyvų, bendras DNR sekų kiekis NCBI duomenų bazėje padvigubėja kas 18 mėnesių. Atsižvelgiant į didžiulę duomenų apimtį, eksperimentinis visų naujų hipotetinių baltymų funkcijų patvirtinimas yra beveik neįveikiamas uždavinys. Genų anotavimui dažniausiai taikoma strategija, kai ieškoma baltymų homologų, kurie buvo funkciškai apibūdinti ir, idealiu atveju, pasižyminčių didesniu nei 50 % tapatumu. Universali baltymų duomenų bazė (UniProt) talpina baltymus, kuriems funkcinės anotacijos buvo priskirtos pagal homologiją (TrEMBL; 249 751 891 įrašas), ir baltymus, kurių funkcijos patvirtintos eksperimentiškai (SwissProt; 570 830 įrašų). Homologija pagrįstas baltymų funkcijos anotavimas gana patikimai prognozuoja fermentų katalizuojamos reakcijos tipą, tačiau negali atskleisti substratinio savitumo. Net ir labai subtilūs aminorūgščių sudėties skirtumai gali nulemti kitokį baltymų ir mažų molekulių prisijungimo specifiškumą. Todėl daugelio hipotetinių fermentų funkcijos nežinomos, neaiškios arba neteisingai priskirtos. Naujų duomenų apdorojimo metodikų, įskaitant dirbtinį intelektą, giliuosius neuroninius tinklus, mašininį mokymąsi ir kitas, taikymas daro didelę pažangą bioinformatikos srityje, kaip neseniai parodė itin tikslūs baltymų tretinių struktūrų prognozavimo algoritmai AlphaFold ir ESMFold. Nepaisant to, eksperimentinis naujų baltymų funkcijų patvirtinimas išlieka labai svarbus, nes jis padidina bendrą pažangių didžiųjų duomenų analizės metodų, skirtų baltymų anotacijai, veiksmingumą.

Be fundamentinio baltymo funkcijos nustatymo aspekto, nežinomų fermentų charakterizavimas turi ir pritaikomumo naudą. Nauji fermentai turi didžiulį pritaikymo potencialą sintetinėje biologijoje ir metabolinių kelių inžinerijoje. Be to, besiformuojanti biokatalizės sritis yra pripažįstama kaip perspektyvi įprastinės cheminės sintezės alternatyva. Biokatalizatoriai, pasižymintys regio- ir stereoselektyviomis savybėmis leidžia lengviau sintetinti sudėtingus enantiomerinius junginius. Be to, biokatalizė atitinka tvarios gamybos principus, nes leidžia atlikti chemines transformacijas mažiau kenksmingomis sąlygomis, todėl nereikia naudoti toksiškų metalų ir tirpiklių. Ji taip pat didina atominį efektyvumą, nes nebereikia naudoti apsauginių funkcinių grupių, taip supaprastinant patį sintezės procesą. Be to, biokatalizėje naudojami atsinaujinantys ir biologiškai skaidūs ištekliai, taip prisidedant prie kenksmingo poveikio aplinkai mažinimo ir skatinant ekologiškos chemijos plėtrą. Dėl to biokatalizė ne tik padeda plėtoti sintetinės chemijos sritį, bet ir skatina ekologiškų ir tvarių metodikų diegimą chemijos pramonėje.

Viena iš naujų fermentų paieškos strategijų yra ksenobiotikų degradacijos mikroorganizmuose tyrimas. Daugelis bakterijų geba metabolizuoti įvairius organinius junginius, kurie į pagrindinį ląstelės metabolizmą yra įjungiami galybės skirtingų fermentų. Tačiau didelė šių procesų biochemijos dalis vis dar menkai suprantama. Šį informacijos apie junginių metabolizmo kelius trūkumą galima suskirstyti į tris kategorijas: žinomi nežinomieji, nežinomi žinomieji ir nežinomi nežinomieji. Teoriniai baltymai, gauti iš DNR sekoskaitos projektų, kurių funkcijos yra hipotetinės ir (arba) neteisingai priskirtos ir kurių vaidmuo nėra eksperimentiškai išaiškintas, priskiriami žinomiems nežinomiesiems. Taip pat yra tarpinių metabolitų, kurie buvo identifikuoti, bet nedalyvauja jokiose aprašytose biocheminėse reakcijose (sudaro apie 50 % KEGG junginių duomenų bazės), arba charakterizuotų fermentų, kurie neturi jokių atitinkamų juos koduojančių genų – šie vadinami nežinomais žinomaisiais. Paskutinioji kategorija – nežinomi nežinomieji – yra neištirta baltymų aibė, kurioje gali slypėti dar nenustatytos biocheminės funkcijos. Šios grupės atstovų paiešką limituoja vienas iš didžiųjų mikrobiologijos iššūkių: tik nedidelę dalį (spėjama iki 1 %) visų mikroorganizmų galima kultivuoti laboratorinėmis sąlygomis. Šis veiksnys gerokai apriboja naujų duomenų rinkimo, naudojant genomikos, proteomikos ir metabolomikos metodus, ir tolimesnės informacijos integravimo su fermentų funkcine analize galimybes. Aukščiau išvardintų (ne)žinomų dalykų tyrimas ne tik pabrėžia, kad mikrobų biochemijoje yra daug nežinomųjų, bet ir išryškina galimybes atrasti naujus fermentus, kurie galėtų pakeisti mūsų supratimą ir biokatalizės taikymą įvairiose srityse, įskaitant aplinkos apsaugą, sintetinę biologiją ir pramoninę biotechnologiją.

Piridinas ir jo dariniai yra dažnai aptinkami gamtoje, atsirandantys tiek iš gamtinių, tiek iš antropogeninių šaltinių. Piridino žiedas aptinkamas alkaloiduose,

kofermentuose, sintetiniuose tirpikliuose, pesticiduose ir herbiciduose. Piridinoliai yra įprasti tarpiniai metabolitai, susidarantys mikrobiologiškai skaidant įvairius *N*-heterociklinius junginius. Visuotinai pripažįstama, kad juos gali skaidyti įvairios aplinkoje esančios bakterijos, tačiau įvairių piridino darinių skaidymo mechanizmai dar nėra iki galo išaiškinti.

Ši disertacija skirta identifikuoti naujus fermentus, tiriant piridino ir piridinolių katabolinius kelius. Tikslui pasiekti buvo iškelti keturi uždaviniai:

1. Išsiaiškinti 2-hidroksipiridino skaidymo kelią *Rhodococcus rhodochrous* PY11 bakterijose;
2. Nustatyti 2-hidroksipiridino katabolizmo kelią *Burkholderia* sp. MAK1 bakterijose;
3. Identifikuoti 4-hidroksipiridino katabolizmo genus ir baltymus iš *Arthrobacter* sp. IN13 bakterijose;
4. Nustatyti piridino skaidymo principus *Arthrobacter* sp. 68b bakterijose.

Visos keturios šiame tyrime analizuotos bakterijos buvo išskirtos iš dirvožemio dėl jų gebėjimo panaudoti piridiną arba piridinolį kaip anglies šaltinį augimui. Katabolizmo kelių tyrimai prasidėdavo nuo genų, atsakingų už šių junginių skaidymą, nustatymo. Po to šie genai buvo klonuojami, vykdoma jų raiška bakterijose ir ieškoma efektyviausių aktyvių fermentų formų bei analizuojami susidarę reakcijų produktai. Vėliau, atrasti nauji fermentai buvo plačiau charakterizuojami (*in vivo* arba *in vitro*) nustatant substratinį specifiškumą, siekiant ištirti jų, kaip biokatalizatorių, panaudojimo galimybes.

Visi darbo metu gauti rezultatai buvo paskelbti keturiuose straipsniuose. Pirmajame iš jų nagrinėjamas *Rhodococcus rhodochrous* PY11 bakterijų gebėjimas įsisavinti 2-hidroksipiridiną kaip vienintelį anglies ir energijos šaltinį. Buvo nustatyta ir apibūdinta genų sankaupa (*hpo*), atsakinga už 2HP skaidymą, išsamiai aprašytas konkrečių genų ir fermentų vaidmuo šiame procese. Taip pat pavyko nustatyti tarpinius metabolitus. Parodėme, kad daugiakomponentė HpoBCDF dioksigenazė yra atsakinga už pradinį 2HP biologinio skaidymo etapą. Taip pat įrodėme, jog hipotetinė ciklazė HpoH katalizuoja hidrolizinę THP žiedo atidarymo reakciją, kuri ilgus metus neturėjo priskirto fermento. Tolesniame katabolizmo etape susidaro galutiniai skilimo produktai – amonio jonas ir α-ketoglutaratas. Šio darbo naujumas – eksperimentinis septynių naujų baltymų funkcijos nustatymas.

Antrajame darbe nagrinėjamas kitoks 2HP skaidymo būdas. *Burkholderia* sp. MAK1 bakterijos skaido 2HP nesusidarant mėlynam pigmentui, kuris paprastai siejamas su mikrobiniu 2HP skaidymu. Tyrimo metu nustatėme, kad už šį procesą atsakinga *hpd* genų sankaupa. Buvo patvirtinta, kad fermentinis 2-

hidroksipiridino 5-monooksigenazės kompleksas, kurį koduoja *hpdABCDE* genai, yra atsakingas už 2HP konversiją iki 2,5-dihidroksipiridino. Kitas kelio etapas - žiedo skilimas. Skirtingai nei *Rhodococcus rhodochrous* PY11 vykstantis hidrolitinis piridino žiedo skilimas, *Burkholderia* sp. MAK1 bakterijose 25DHP buvo skaidomas oksidaciniu būdu, už kurį atsakinga HpdF dioksigenazė. Buvo pasiūlyta, kad tolesnis skaidymas vyksta vadinamuoju maleamato keliu, nes visi su šiuo keliu susiję genai turi homologus hpd sankaupoje.

Trečiajame straipsnyje "Mikrobinis piridino skaidymas: iššifruotas pilnas *Arthrobacter* sp. 68b katabolinis kelias" analizuojamas piridino skaidymo katabolinis kelias šiose bakterijose. Tyrimo metu buvo nustatyta, kad už piridino skaidymą atsakingų genų sankaupa (*pyr*) yra plazmidėje. Pavyko išsamiai charakterizuoti fermentus, kurie piridiną suskaido iki gintaro rūgšties. Pradiniame etape dalyvauja nuo flavino priklausoma monooksigenazės sistema, skirta tiesioginiam žiedo skėlimui, be tipiškų aromatinį žiedą aktyvuojančių etapų: redukcijos ar hidroksilinimo. Tolimesni skaidymo kelio etapai taip pat buvo patvirtinti eksperimentiškai. Juos katalizuoja (Z)-*N*-(4-oksobut-1-enil)formamido dehidrogenazė PyrB, amidohidrolazė PyrC ir sukcinato semialdehido dehidrogenazė PyrD.

Paskutiniame straipsnyje "Biocheminė ir genetinė 4-hidroksipiridino katabolizmo kelio *Arthrobacter* sp. IN13 bakterijose analizė" dėmesys buvo skirtas 4HP skaidymo procesui. Nustačius genų sankaupą (*kpi*), koduojančią už skaidymą atsakingus baltymus, buvo apibūdinti konkretūs fermentai ir jų vaidmenys šiame kelyje. Tyrime buvo identifikuoti trys nauji baltymai – regiospecifinė nuo flavino priklausoma monooksigenazė KpiA, ekstradiolinė dioksigenazė KpiC ir 3-(*N*-formil)-formiminopiruvato hidrolazė KpiB – bei ištirtos jų biocheminės funkcijos.

Apibendrinant galima teigti, kad šie keturi naujai nustatyti katabolizmo keliai suteikia esminių įžvalgų apie *N*-heterociklinių aromatinių junginių mikrobiologinį metabolizmą. Be to, siekiant išsiaiškinti piridino ir jo darinių bakterinio skaidymo kelius, buvo atskleista įvairių klasių ir šeimų fermentų įvairovė. Kiekvienas iš šių fermentų yra esminis ir būtinas elementas piridino ir jo darinių katabolizmo kelyje skaidant šias organines molekules iki centrinio metabolizmo junginių. Toks fermentų arsenalas išsivystė dėl bakterijų poreikio pritaikyti savo metabolinius kelius prie daugybės cheminių junginių, su kuriais jos susiduria savo aplinkoje. Taigi, šie fermentai nėra tik biologinės įdomybės - jie yra turtinga potencialių biokatalizatorių saugykla, kuri yra labai perspektyvi bioremediacijos, žaliosios chemijos ir sintetinės biologijos srityse. Bakterinių fermentų atradimas ir apibūdinimas atveria duris naujoviškiems užterštos aplinkos detoksikacijos ir tvarios vertingų cheminių produktų sintezės metodams. Pasinaudodami natūraliomis fermentų katalizinėmis savybėmis, mokslininkai ir inžinieriai gali sukurti ekologiškus sprendimus kai kuriems aktualiausiems šių

laikų aplinkosaugos iššūkiams, įskaitant patvariųjų teršalų skaidymą ir veiksmingą atsinaujinančių išteklių pavertimą biodegalais ir kitais naudingais junginiais.

Dėl to, ksenobiotikų katabolizmo bakterijose tyrimai peržengia fundamentinių mokslinių tyrimų ribas ir atveria kelią pažangai aplinkosaugos, atsakingos praktikos ir tvarių pramonės procesų srityse. Bakterinių fermentų įvairovė yra biocheminis lobynas, kuriame galime atrasti naujos kartos biokatalizatorių, galinčių katalizuoti neprilygstamo efektyvumo ir specifiškumo reakcijas.

Išvados

1. *Rhodococcus rhodochrous* PY11 skaido 2-hidroksipiridiną netipiniu aromatinių junginių skaidymo būdu: piridino žiedas pirmiausia yra dihidroksilinimas, po ko seka hidrolizinis žiedo atidarymas.
2. 2-hidroksipiridino katabolizmo iš *Burkholderia* sp. MAK1 kelyje piridino žiedą atidaro dioksigenazė HpdF, o tai yra įprasta aromatinių junginių biodegradacijos strategija prokariotuose.
3. *Arthrobacter* sp. IN13 ląstelės katabolizuoja 4-hidroksipiridiną įprastiniu keliu, kuriame dalyvauja regiospecifinė nuo flavino priklausoma hidroksilinanti monooksigenazė KpiA, 3,4-DHP 2,3-dioksigenazė KpiC ir 3-(*N*-formil)-forminopiravato hidrolazė KpiB.
4. *Arthrobacter* sp. 68b nestandartinis piridino skaidymo kelias prasideda tiesioginiu žiedo atidarymu, kurį katalizuoja dviejų komponentų nuo flavino priklausoma monooksigenazės sistema (PyrAE) be įprastų aktyvacijos etapų, tokių kaip heterociklo redukcija ar hidroksilinimas.

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SOFTWARE USED

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Publication **II**

Petkevičius, V.; **Vaitekūnas, J.**; Stankevičiūtė, J.; Gasparavičiūtė, R.; Meškys, R. Catabolism of 2-Hydroxypyridine by *Burkholderia* Sp. Strain MAK1: A 2-Hydroxypyridine 5- Monooxygenase Encoded by hpdABCDE Catalyzes the First Step of Biodegradation. *Applied and Environmental Microbiology* 2018, *84* (11). https://doi.org/10.1128/AEM.00387-18.

Publication **III**

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Publication **IV**

Časaitė, V.; Stanislauskienė, R.; **Vaitekūnas, J.**; Tauraitė, D.; Rutkienė, R.; Gasparavičiūtė, R.; Meškys, R. Microbial Degradation of Pyridine: A Complete Pathway in *Arthrobacter* Sp. Strain 68b Deciphered. *Applied and Environmental Microbiology* 2020, *86* (15), 1–16. https://doi.org/10.1128/AEM.00902-20.

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