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Abstract

The rare earth elements (REEs) are metals, which are used in nearly every electronic device. The demand for new REEs is steadily rising. Yearly, millions of tonnes of electronic waste (e-waste) are generated. The e-waste contains valuable metals, but most of them are not recycled.

The recycling of REEs is a challenging process that currently involves the usage of a lot of energy. But also the mining of new REEs is not eco-friendly. It involves the usage of strong acids that harm the environment and mining workers.

In recent years, a newly discovered protein found in a specific bacteria was found to be able to tackle the challenge of REE recycling. The protein can take up the REEs from e-waste, like washing detergents wash dirt out of clothing.

Without any additional preparation of the e-waste other than dust the e-waste, the bacteria is capable of gathering more than 70% of REEs out of the waste.

This could be used in the near future to recycle REEs in an eco-friendly, climate-friendly and energy-saving way.

Introduction

In 2021, around five million tonnes of electronic waste were generated in the EU alone, but less than 40 percent were recycled. This waste often contains valuable metals, but currently most of them are disposed. Some of these disposed metals are the so-called rare earth elements. The rare earths are critical for every electronic device but they are only used in small quantities, so that conventional recycling is not a economically feasible possibility.

For every new smartphone, for example, new rare earths must be mined. This happens mostly in countries where compliance with human and environmental rights are questionable. The following refining of the rare earths is a very energy-consuming, environmentally harmful and climate damaging process. For a single tonne of neodymium, the most used rare earth element, some 75 tonnes of CO₂ are emitted. But the problems do not stop there. There are only a few places on earth where rare earths are mined, because it is mostly not economically viable because of China, who dominates the market.

The largest mine for rare earths is located in China and additionally, China is the largest producer of refined rare earths, which is then used elsewhere to produce electronics. This means that the world's current supply of rare earth elements is largely monopolized by a country, which does not adhere to human and environmental rights.

A solution could be the recycling of rare earths from electronic waste. However, currently established methods are either very expensive, damaging to the environment or use a lot of energy.

A promising alternative could be the usage of bacteria to recover the rare earths. Bacteria have the advantages that they do not need a lot of energy to grow and they only need inexpensive resources to be able to grow.

In this thesis, we tested if, and how rare earth elements can be recycled from electronic waste using bacteria. We outline how this process works and we report our findings from the actual realisation of this process.

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1 Introduction

Rare Earth Elements (REEs) play a critical role in modern-day life. They are used in nearly every device that uses electrical power to operate. A few examples where REEs are essential are: lasers, computer monitors, electric motors, electric generators, high-power magnets, liquid crystal displays (LCDs), solar panels and many more [1].

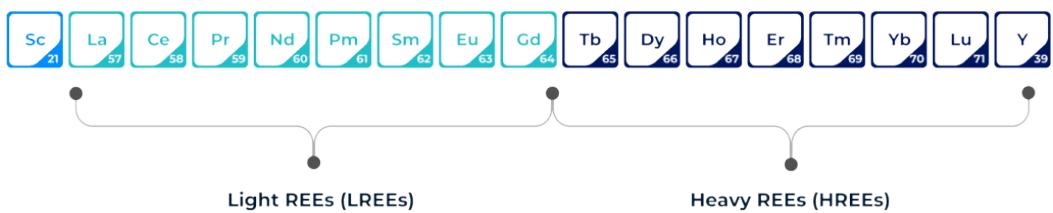


Figure 1.1: List of all rare earth elements. Those 17 elements can be further categorized into the light rare earth elements (LREEs) and the heavy rare earth elements (HREEs). Picture from REIA / Argus Media.

1.1 Problem Setting^{TD}

Given the importance of REEs in the modern world, it is evident that the demand for them is increasing quickly. In the coming years, as the use of electronic devices increases, many of them will become electronic waste. It is vital for the world's future supply of rare earth elements to recycle them from this waste.

Currently used recycling methods for REEs are mostly damaging to the environment and very costly [2]. Therefore, only around one percent of the global REEs supply is from recycled sources [3]. The rest comes from mining, which brings its own challenges. Rare earth ores (REOs) often contain radioactive elements which adds more complexity to the processing of the ores. Also, the extraction of REEs is done by using a process called flotation which produces large amounts of waste water. This waste water is highly problematic, as it often contains radioactive minerals, acids and toxic agents [4].

The processing of REOs does not only damage the environment, but it also contributes to climate change. As an example, 75 tonnes of CO₂— equivalents are emitted for every tonne of newly refined neodymium [5].

There are already thousands of tonnes of electronic waste that contain significant amounts of REEs. Recycling them would reduce the need of mining new REOs and therefore reduce the environmental impact of new electronic devices. Sadly, there is no easy and environmentally friendly process to recycle REEs on an industrial scale.

1.2 Contributions^{MS}

To combat the issues mentioned above, we worked on a way to recycle REEs without the need for large amounts of energy or resources. By using bacteria that produce a special amino acid that allows us to bind the REEs in electronic waste, we achieved just that. Due to the bacteria not needing significant amounts of energy, we managed to remain eco-friendly and cost-efficient. The recycling process works by washing shredded electronic waste with our bacteria solution. After changing the pH value of said solution we can get the REEs back in their pure forms. This process works on a scientific level in a laboratory but could also be used on an industrial scale using large bioreactors and washing tanks.

1.3 Structure of this Thesis^{MS}

This thesis is divided into eight main parts, systematically addressing various aspects of the research project. It begins with an introduction outlining the project's aims and importance. The theoretical background section elucidates the methods and principles underpinning the research. Practical guidance for executing the project is provided in the experimental section. The Results and Discussion segment analyzes the project's outcomes and implications. Project Management reflects on the planning process and evaluates its effectiveness, accompanied by a detailed timesheet. Future Work and Related Work suggest avenues for future research and contextualize the project within existing literature. The conclusion summarizes key findings, challenges, and lessons learned. Additional chapters, such as Acknowledgements, List of Figures, Bibliography, and CV, provide further context and resources. Each section contributes to advancing knowledge in the field, fostering scholarly discourse.

2 Theoretical Background

In order to understand the process of the recovery of rare earth elements from electronic waste with bioaccumulation, the key procedures and techniques are described in this chapter.

2.1 System Overview

In the following section, all used methods and the most important concepts are briefly summarized.

2.1.1 Detection and Measurement of REE concentration^{TD}

Precipitation Reactions

A relatively simple proof if a probe contains REEs is a precipitation reaction. The precipitation reactions work because the rare earths form greater complexes with other molecules which have a different color than the surrounding solution [6]. As an example, a Ce precipitation reaction is shown in figure 2.1 with an orange-red precipitate.

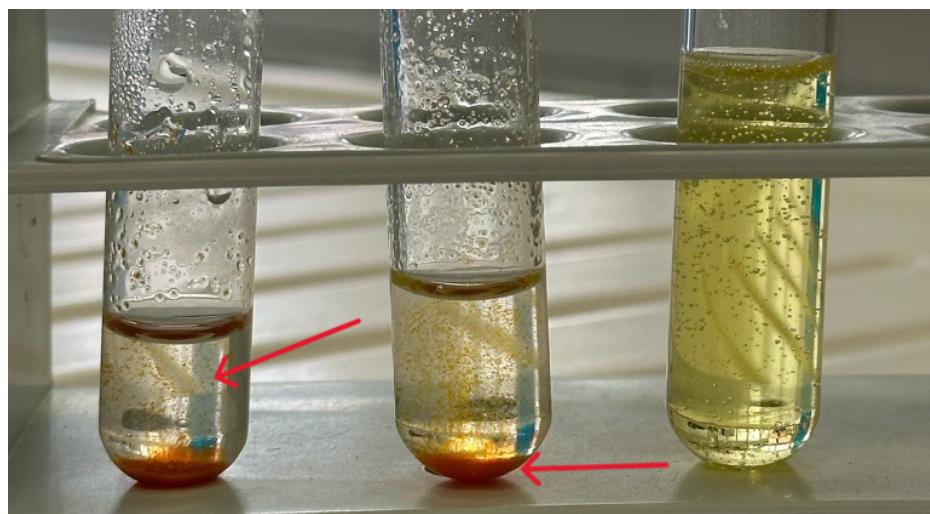


Figure 2.1: Precipitation of a successful REE detection reaction. The red arrows point to the orange-red precipitation.

However, you must be careful because of the REEs chemical similarity, the detection of

a specific REE is not always possible with these precipitation methods. A precipitation reaction might also not be sensitive enough for your use case. So it could be possible that your probe contains rare earths, but you were not able to detect them.

Arsenazo III Assay

A better and more versatile method to detect rare earths in a probe is the so-called arsenazo III assay. With this assay, it is not only possible to detect if rare earths are present, but it is also possible to determine the concentration of REEs [7].

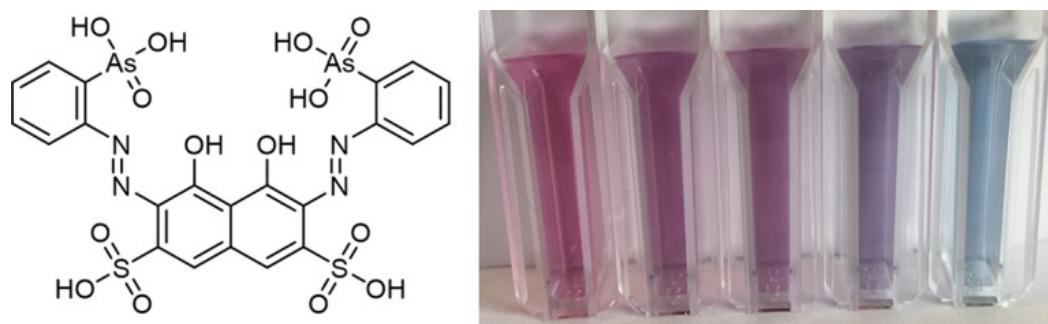


Figure 2.2: Structure of arsenazo III. The color change of the dye depending on the REE concentration is shown on the righthandside. Picture from "Facile Arsenazo III-based assay for monitoring rare earth element depletion from cultivation media for methanotrophic and methylotrophic bacteria" Hoogendorn et al. [7].

Arsenazo III is a metallochromic dye. This means that the dye changes its color depending on the presence of metal ions (for example: 2.2). A second crucial characteristic is that the color of an arsenazo III solution is also dependent on the concentration of some metal ions. The metal ions and the arsenazo III molecule form complexes which block some certain frequencies of light. This property can be used to determine the concentration of rare earths in a probe.

2.1.2 *Methylorum extorquens*

General information^{MS}

Utilizing a special strain of bacteria called *Methylorum extorquens*, we can extract these REEs from electronic waste. This works because the aforementioned bacteria produce an amino acid called lanmodulin which has the unique property of binding to REEs [8]. This technique allows us to wash REEs out of electronic waste in a similar way that surfactants wash the dirt out of laundry.



Figure 2.3: *Methylorum extorquens* in a petri dish.

These bacteria reside in common soil, plant leaves, and dust and can also form symbiotic relationships with some plants. The bacteria appear orange or pink when cultivated on a solid or in a liquid medium. *Methylorum extorquens* utilizes methanol as an energy and carbon source, which is why we had to put methanol in our nutrient media.

Lanmodulin^{TD}

Lanmodulin (LanM) is a protein produced by *M. extorquens*, a lanthanide-utilizing bacteria [8]. LanM is not essential for the growth or survival of *M. extorquens*, and it is only

produced when the bacteria are in a medium with presence of Ln^{III} or Ce^{III} ions [9]. However, the mechanisms that include LanM are not understood as a whole to this day.

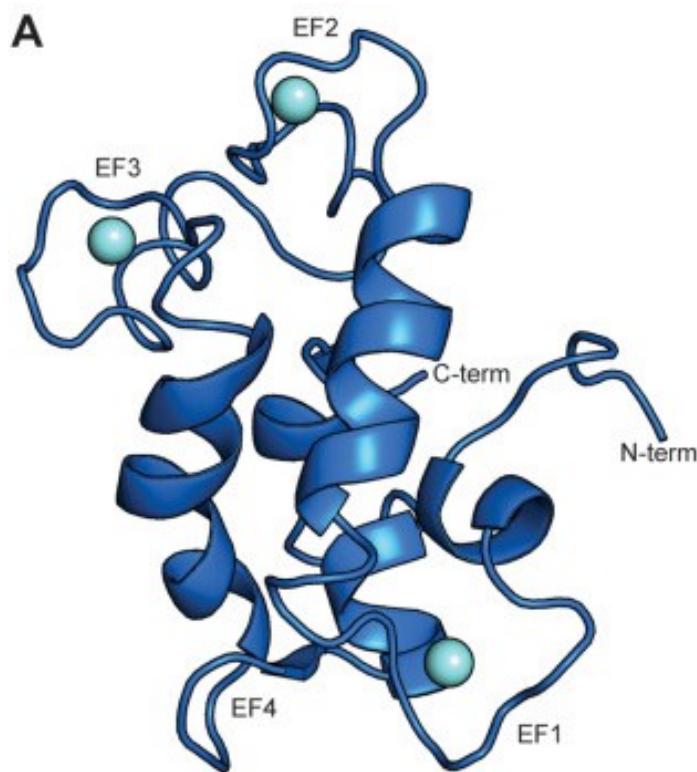


Figure 2.4: Graphical visualization of Lanmodulin structure. EF indicates the EF-hands, this is where the REEs can bind to the protein. In this visualization, the turquoise-colored spheres are Y^{III} ions which are bound to the EF-hands. Picture from "The biochemistry of lanthanide acquisition, trafficking and utilization", Emily R. Featherston and Joseph A. Cotruvo [9].

The most important characteristic of LanM is that the molecule is able to bind lanthanide ions, primarily light REEs (LREEs). When LanM does this, it undergoes a transformation from a disordered state to a compact form of itself. The REEs are hereby bound to the so-called EF-hands which favor to bind to Ln^{III} and other lanthanoids over Ca^{II} which is usually associated with these EF-hands [10].

Protein Extraction^{MS}

To extract lanmodulin, a rare earth element (REE)-binding protein from bacterial cells, shock frosting and sonication methods are employed. Shock frosting involves rapid freezing and thawing, disrupting the cell membrane, while sonication uses high-frequency sound waves to break cell walls. Cooling the bacteria containing lanmodulin prevents amino acid degradation by proteases. These methods ensure efficient extraction of lanmodulin from *Methylorum extorquens* bacteria for study.

Cell Lysis^{MS}

In order to obtain Lanmodulin and REEs, it is necessary to break open the cell walls of the bacteria. This process, known as lysis, can be accomplished using a variety of techniques—either mechanical or enzymatic. Mechanical methods include bead beating, French press lysis, and shock freezing. In contrast, enzymatic lysis can be achieved through lysozyme treatment, which utilizes an enzyme that specifically breaks down bacterial cell walls. For this project, a combination of shock freezing and cell wall disruption using an ultrasonic bath was selected.

IR-Spectroscopy^{MS}

IR spectroscopy utilizes infrared radiation to analyze molecular structure through vibrational motion. Unlike UV-Vis spectroscopy, infrared radiation doesn't induce electronic transitions but affects covalent bond vibrations. Vibrational modes correlate bond type with frequency, forming the basis of IR analysis. Samples intercept infrared radiation, generating spectra displaying absorption intensity against frequency. Peaks denote specific vibrational modes, revealing bond types and strengths. Interpretation involves reference databases and peak intensities, offering insights into functional groups and molecular environments. Challenges arise from extraneous peaks, like alcohol and water, affecting analysis precision.

SDS-PAGE^{MS}

Protein separation techniques are fundamental in biochemical research, facilitating the elucidation of protein structure and function. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stands as a pivotal method for analyzing proteins based on their molecular weight. This technique relies on the denaturation and linearization of proteins through the action of SDS, followed by their separation in a polyacrylamide gel matrix via electrophoresis. The denaturing effect of SDS disrupts protein tertiary structures, rendering them linear and uniformly charged. Subsequent migration through the gel matrix, under the influence of voltage on the gel, allows for separation based on size.

2.2 Detection and Measurement of REE concentration^{TD}

The detection of rare earth elements in a sample is a crucial step in our work. It allows us to quantify the effectiveness of our process.

In modern chemistry, qualitative and quantitative analysis of elements in a sample is usually done with inductively coupled plasma mass spectroscopy (ICP-MS) or atom absorption spectroscopy (AAS). However, as the ICP-MS and AAS use machines that are very, very expensive, these methods were not an option as they exceeded our limited financial resources by far. Instead, we had to search for other methods to detect and quantify rare earths.

In our work, we used two precipitation reactions and one method to quantify the concentration of REEs.

2.2.1 Precipitation Reactions

Cer Precipitation Reaction

The precipitation reaction for cer works by utilizing the oxidation states +III and +IV [11, 6].

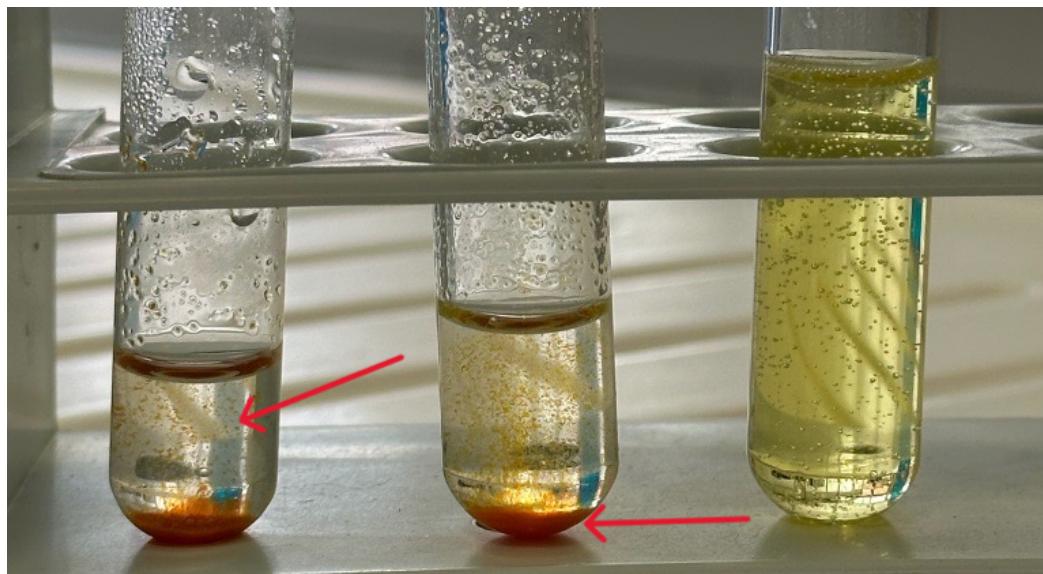


Figure 2.5: Precipitation of a successful cer detection reaction. The test tube on the righthandside does not show any precipitation because the sample was deionized water.

Cer in the aforementioned states forms complexes together with H_2O_2 . The complexes are called cer peroxide hydrates. Their chemical formulas are $\text{Ce}(\text{OH})_2(\text{OOH})$ and $\text{Ce}(\text{OH})_3(\text{OOH})$. These complexes fall out of the solution as a red-brown colored precipitate.

Neodymium Precipitation Reaction

The reaction to detect neodymium is a bit more complicated. It also uses the +III oxidation state of neodymium. The neodymium reacts with acetic acid to form neodymium acetate. As the last step, iodide is given to the solution which forms a blue-colored complex together with the neodymium acetate [6].

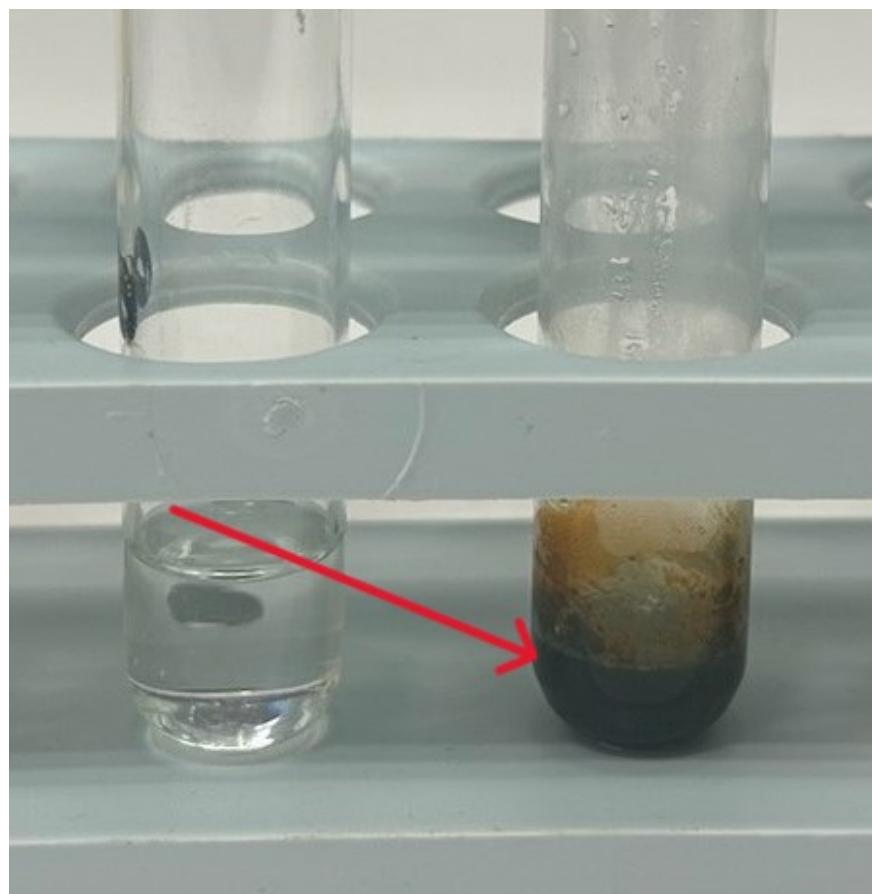


Figure 2.6: Neodymium detection reaction. Neodymium is contained in the right sample. The blue precipitate is clearly visible.

2.2.2 Arsenazo III Assay

Arsenazo III

The arsenazo III assay is based on the dye arsenazo III or ASIII [7]. It is often used to detect calcium, uranium and a lot of other metals, including rare earth elements [12, 13].

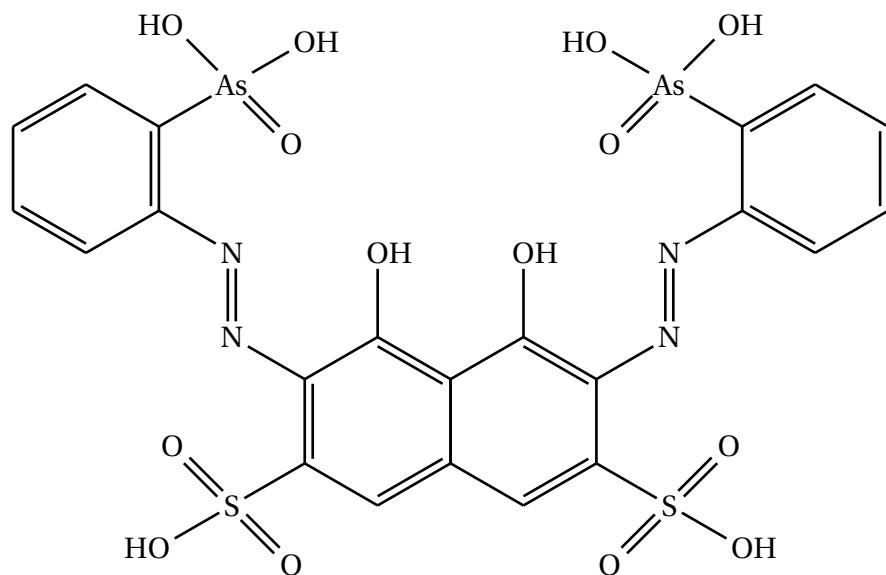


Figure 2.7: Structure of 2,7-bis(2-arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid. Or, in its abbreviated form, arsenazo III.

Arsenazo III was first synthesized in 1959 [14]. In comparison to arsenazo I and II, it possesses two functional arsено groups (see figure 2.7). The arsenazo III dye has the property to change its color based on the pH and the presence of some elements. Normally, the dye has a pinkish-crimson color, but when, for example, thorium is present, the color changes to green. For other elements, other colors have been reported, such as blue for calcium or violet-blue and also green for rare earth elements (see figure 2.8).

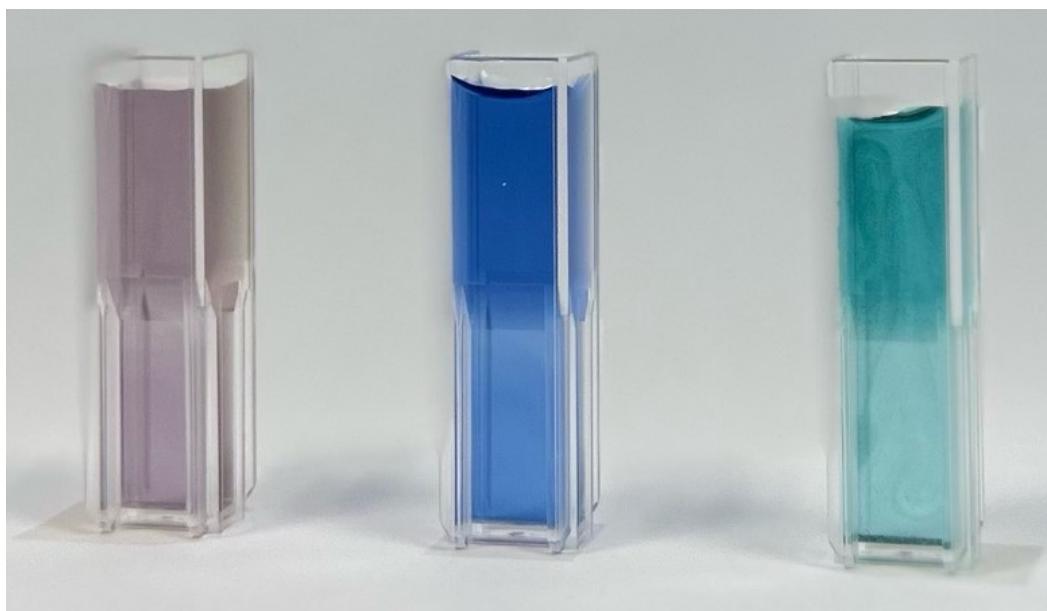


Figure 2.8: Example for different colors of arsenazo III with different samples. The contents of the cuvettes are (from left to right): FeCl_3 , CuSO_4 , NdCl_3 . All are mixed with $10\mu\text{L}$ of 10mM arsenazo III.

The color change happens, because the arsenazo III forms complexes with certain elements. Arsenazo III and rare earths and some other metals form 1:1 complexes [15, 16]. This means that for every molecule of arsenazo III, one rare earth element atom was bound (see figure 2.9). The other arsено group is most likely not used to form these stable complexes.

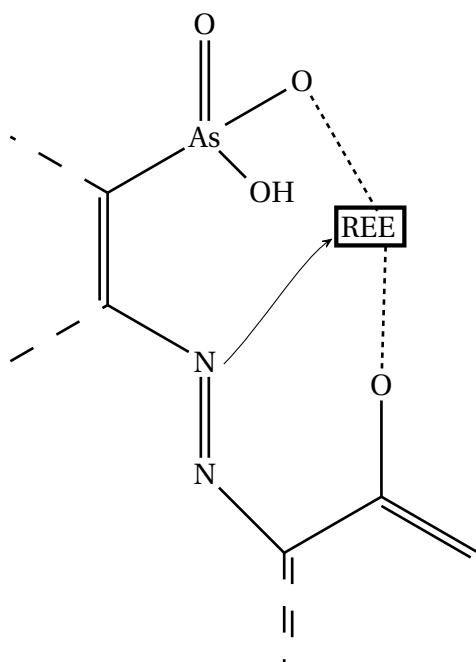


Figure 2.9: An arsenazo III complex with an atom of a rare earth element.

Probe Preparation

To get reliable and correct results, the sample must be prepared beforehand. This happens by adjusting the pH level of the sample solution to around 2.7 to 2.8. This ensures that only rare earth ions interact with the arsenazo III dye. Another advantage of this acidic level is that the ions of the rare earths dissolve better from the sample.

Measuring REE Concentration

The measuring of the concentration of the rare earths works with a UV-Vis-spectrometer. This is a device, that can produce light with a single wavelength. The light goes through the sample and the light intensity is measured. When the intensity of the outgoing light I is set in relation to the intensity of the ingoing light I_0 , the emerging result is the transmittance T [17].

$$T = \frac{I}{I_0}$$

The transmittance is then used to calculate the absorbance A using the following formula [18].

$$A = \log T^{-1} = \log \frac{I_0}{I}$$

The absorbance is the output of the UV-Vis-spectrometer. It is possible to measure just the absorbance at one single wavelength with the device. However, it can also measure the absorbance from a series of wavelengths and plot the result to a spectrum. For the Arsenazo III assay, the absorbance at the wavelength of around 650 nm is important (see figure 2.10).

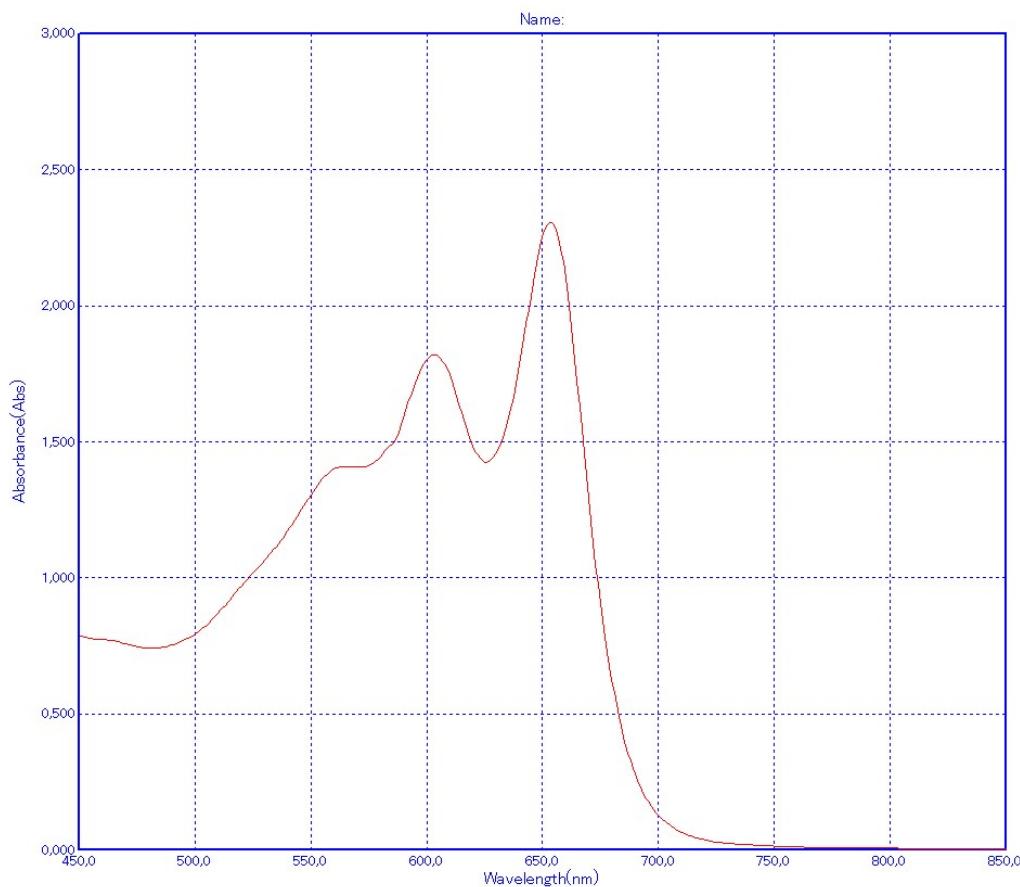


Figure 2.10: Example of a spectrum of an arsenazo III assay. The peak at around 650nm is the product of a complex formed by one rare earth atom and one arsено group.

The final measurement is done with a 1 mL cuvette. Half of it is filled with a phosphate-citrate buffer to ensure a correct pH level. Afterwards, 490 μ L of the sample and 10 μ L of the Arsenazo III dye are added to the cuvette. The solutions in the cuvette have to be mixed, and then a spectrum from 500 nm to 800 nm is recorded. The absorbance at 650 nm is noted. This is later used for calculation of the concentration. Then, 20 μ L of Arsenazo III are again added and mixed into the cuvette. The spectrum and the value at the wavelength of 650 nm are again recorded. The dual measurement is necessary for rare earth concentrations of more than 2 μ mol/L, because it was found that these values suit better for higher concentrations.

These measurements are not only done with the samples but also with solutions that contain a known concentration of rare earths. The values can then be used to calculate a calibration line which in turn gives us the concentration of the samples.

2.3 *Methylorubrum extorquens*

This chapter serves as a detailed explanation of *M. Extorquens*, more specifically its biological origin, its metabolism, and its growth in nutrient solutions in laboratory settings.

2.3.1 Taxonomy^{MS}

Phylum Pseudomonia

Pseudomonadota is a major phylum of gram-negative bacteria (information about gram-negative bacteria will follow further down). They are incredibly diverse, encompassing pathogens, free-living species, nitrogen-fixing bacteria, and many more. *Pseudomonadota* exhibit a large range of shapes and sizes as well as metabolisms and habitats which will also be discussed further down. The diversity of *Pseudomonadota* makes them play a major role in the world's nutrient cycling ranging from crucial ecological relationships with humans to simple things such as nitrogen fixation [19]. *Pseudomonadota* includes five classes but only the class *Alphaproteobacteria* is of importance for us.

Class Alphaproteobacteria

Alphaproteobacteria is a highly diverse class of bacteria belonging to the phylum *Pseudomonadota*. They are named after the first letter of the Greek alphabet (alpha) due to being one of the first major lineages to diverge within the *Proteobacteria* phylum.

This class is incredibly varied, encompassing bacteria with a range of lifestyles including phototrophs (light-using), methanotrophs (methane-utilizing), symbionts (mutually beneficial relationships with other organisms), and pathogens (disease-causing).

Soil, water including cold deep-sea vents, hot springs, and symbiotic relationships even with humans are natural habitats of *Proteobacteria* [20].

Rhizobium: These bacteria form a symbiotic partnership with legumes, such as peas and soybeans. *Rhizobium* colonizes the legume's root nodules and fixes atmospheric nitrogen into a usable form that is essential for plant growth.

Wolbachia: This widespread genus of bacteria lives symbiotically within insects and other arthropods. *Wolbachia* can manipulate the host's reproduction in various ways, sometimes even influencing sex ratios or protecting the host from viruses.

Rickettsia: This genus includes several species that are obligate intracellular pathogens, meaning they can only live and reproduce inside the cells of a host organism. *Rickettsiae* causes various human diseases, including typhus fever and Rocky Mountain spotted fever.

Magnetococcus: These magnetotactic bacteria contain magnetosomes, specialized organelles that allow them to align and move along magnetic fields.

Order Hypomicrobiales

Hypomicrobiales can utilize single-carbon compounds like methanol as an energy source, the bacterium *Methylorum extorquens* does this, for example.

Hypomicrobiales produce carotenoid pigments and therefore appear pink or orange in colonies. These colonies are aerobic, which means they require oxygen for growth. They inhabit a large variety of environments including soils, plant surfaces, root structures, water and dust.

They also play important ecological roles in their habitats, like plant-microbe interactions when metabolizing methanol on plant leaves or carbon and nitrogen cycling in various environments [21].

Genus *Methylorum*

They use specialized pathways to break down methanol for energy and to create biomass. This metabolic capability has potential applications in Bioremediation, which means that this bacteria can clean up methanol-contaminated areas. This family of bacteria is also able to produce valuable chemicals from methanol [22].

Bacteria of the genus *Methylorum* are rod-shaped or slightly bent and show pink or orange pigmentation like every genus that belongs to the order *Methylobacterium*.

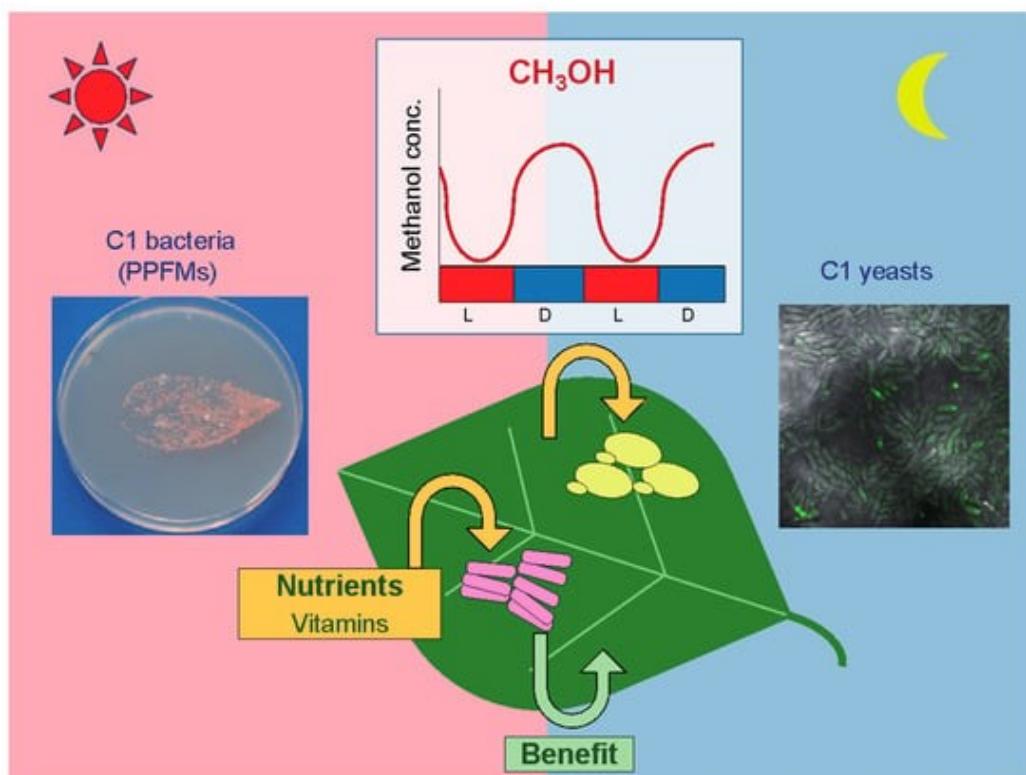


Figure 2.11: Pink *Methylorum extorquens* on a leaf utilizing the plant's nutrients.

Species *Extorquens*

In our thesis, the *extorquens* bacterium species holds immense significance as it displays all the key characteristics of the aforementioned groups to which it belongs. The *Methylorum extorquens* strain is unique in its ability to utilize methanol or methane as its sole source of carbon and energy. Additionally, this bacterium has the capability to metabolize various compounds such as acetate, pyruvate, and succinate, which are converted to energy. This makes the *Methylorum extorquens* strain a particularly fascinating subject for further research and analysis.



Figure 2.12: *M. extorquens* in a sealed petri dish.

2.3.2 Methanol Metabolism^{MS}

Methylorum extorquens exhibits the ability to utilize the simple alcohol methanol CH₃OH as its only source of carbon and energy. This metabolism is explained in three steps:

1. Initiation: Oxidation of Methanol

- Location: Periplasm (the space between the inner and outer cell membranes)

- Enzymes:
 - Methanol dehydrogenase (MposX):
 - * XoxF1: Requires lanthanides for activity, oxidizing methanol to formaldehyde (HCHO) and releasing H⁺.
 - * XoxF2: Less dependent on lanthanides, potentially involved in regulating methanol uptake.
- Importance: Formaldehyde is a toxic intermediate, requiring rapid conversion for *M. extorquens*' survival [23].

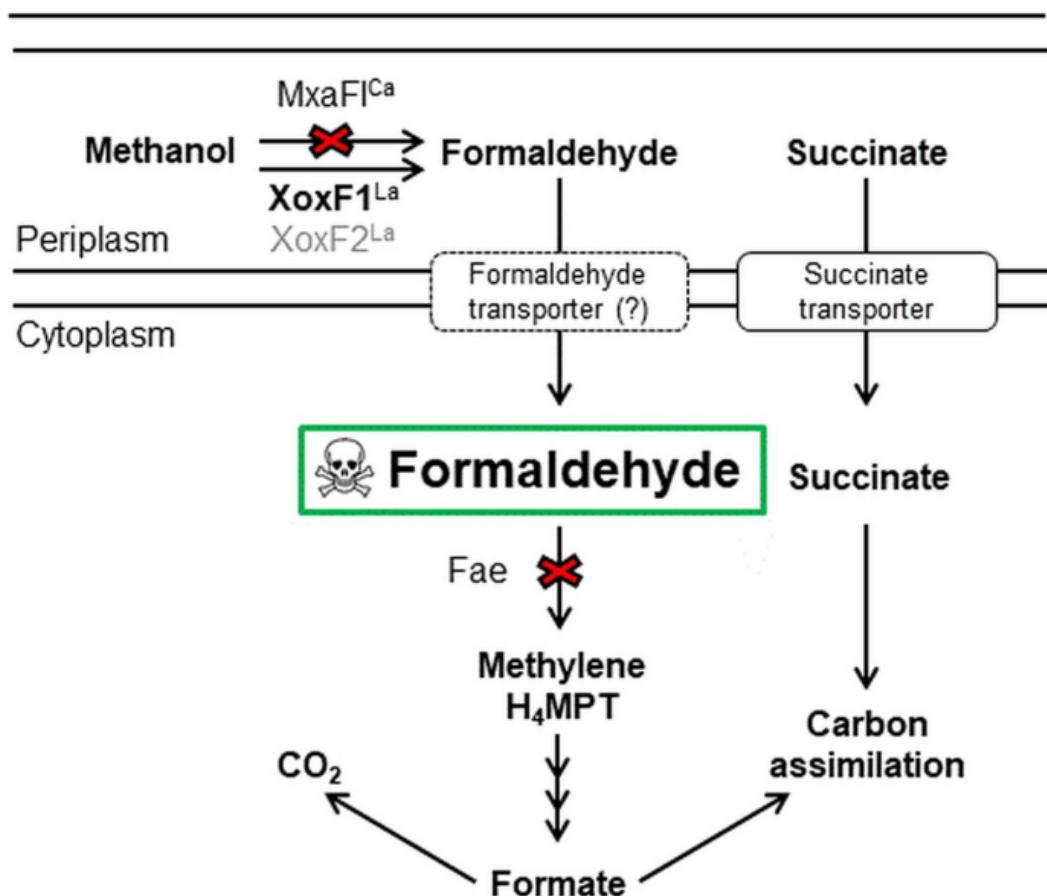


Figure 2.13: Schematic of the metabolic processes to oxidizing methanol to formaldehyde which is reduced or eliminated and used for growth by cells.

2. Capturing the Essence: Fixation of Formaldehyde

- Molecule: Dephosphotetrahydromethanopterin (dH₄MPT) acts as a one-carbon carrier.
- Enzyme: Formaldehyde-activating enzyme (Fae) catalyzes the reaction, attaching formaldehyde to dH₄MPT.

- Significance: Enables the transport of formaldehyde into the cytoplasm for further metabolism [23].

3. Carbon Assimilation: The Serine Cycle Takes Over

- Location: Cytoplasm
- Pathway:
 - Formate dehydrogenase: Oxidizes the formaldehyde-dH₄MPT complex, generating formate (HCOO).
 - Formate acetyltransferase: Condenses formate with acetyl-CoA, forming S-acetyl-CoA.
 - Serine hydroxymethyltransferase: Transfers the one-carbon unit from S-acetyl-CoA to glycine, forming serine.
 - Serine transaminase: Converts serine to pyruvate, a key metabolic intermediate.
- Importance: The serine cycle efficiently converts the one-carbon unit from methanol into usable cellular building blocks [23].

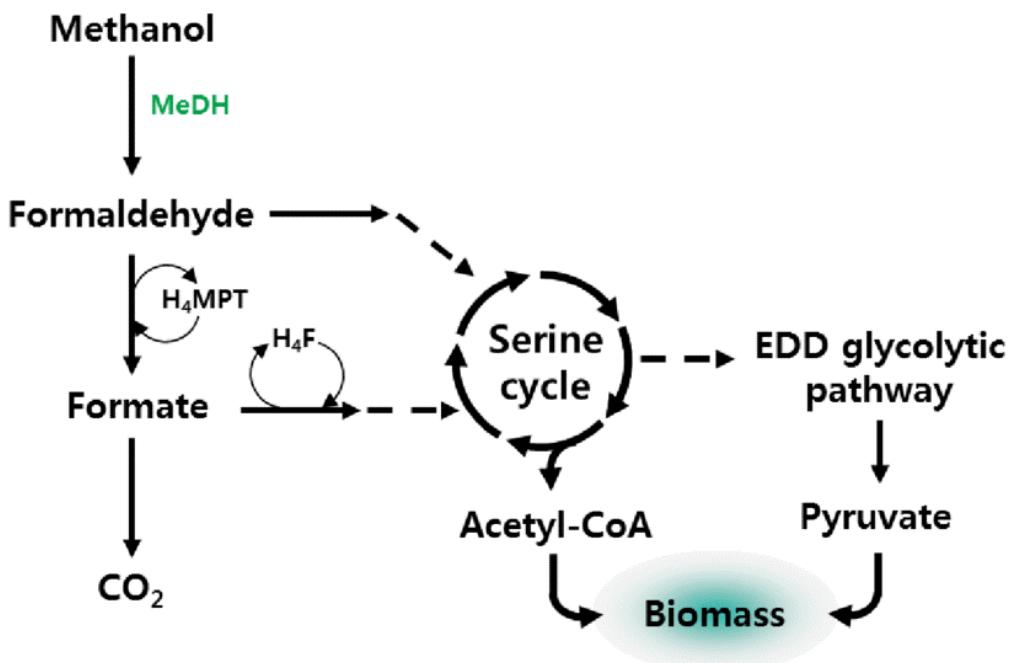


Figure 2.14: *Methylophilus extorquens* metabolizing methanol

2.3.3 Lanmodulin^{TD}

Lanmodulin (LanM) is a protein that was discovered in 2018 in the bacteria *Methylophilus extorquens* [8]. The molecule is around 12kDa in size, and it possesses unique properties, even when compared to other similar proteins. Lanmodulin contains four of the

so-called EF-hands. These hands are normally used to sense Ca^{II} ions. Lanmodulin, however, is able to bind Ln^{III} and other lanthanide ions (which most of the rare earth elements belong to) to this EF-Hands, not only Ca^{II} [10].

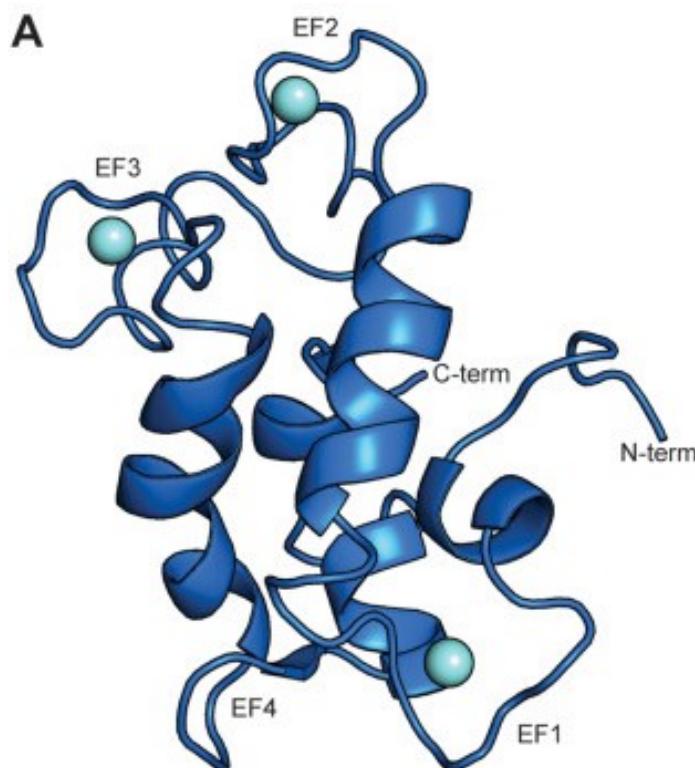


Figure 2.15: Graphical visualization of Lanmodulins structure. EF indicates the EF-hands, this is where the REEs can bind to the protein. In this visualization, the turquoise-colored spheres are Y^{III} ions which are bound to the EF-hands. Picture from "The biochemistry of lanthanide acquisition, trafficking and utilization", Emily R. Featherston and Joseph A. Cotruvo [9].

The second interesting property originates from the ability to bind lanthanide ions. It was found that LanM does not only bind lanthanide ions, but it even favors them to bind to its EF-hands. The affinity for the lanthanides is around 10^8 times higher than for Ca^{II} . This means that, in a solution with, for example, Ln^{III} and Ca^{II} ions, only very few calcium ions will bind to the LanM.

When LanM binds the lanthanide ions, something interesting happens: it undergoes a transformation. It changes its shape and morphs into a sphere-like structure, which contains the ions inside. However, how and why exactly lanmodulin does this, is the subject of ongoing research [24].

2.3.4 Growth^{MS}

Methylorum extorquens thrives at temperatures between 30°C and 35°C, making it a mesophilic bacteria. To promote its optimal growth, the bacteria was placed in a swivel incubator set to this temperature. Additionally, the nutrient solution needs to be slightly acidic to neutral, with a pH range of 6.5-7.5, to further enhance growth. Because *M. extorquens* is an aerobic bacteria, the solution in which it is cultivated must be able to exchange gas and absorb oxygen. This is achieved by sealing the Erlenmeyer flask with a piece of sterile cotton that allows oxygen to pass through while keeping other bacteria and fungi out.



Figure 2.16: Swivel incubator with temperature control used for cultivating *M. extorquens*.

Under optimal conditions, the exponential growth phase of *M. extorquens* typically lasts six to eight hours, during which the number of cells increases rapidly. However, this growth phase comes to a halt due to a lack of nutrients or waste product accumulation, resulting in the stationary phase. After this point, the viability and number of cells gradually decrease, known as the death phase.

2.3.5 Lysis^{MS}

In order to obtain Lanmodulin and REEs, it is necessary to break open the cell walls of the bacteria. This process, known as lysis, can be accomplished using a variety of techniques - either mechanical or enzymatic. Mechanical methods include bead beating, French

press lysis, and shock freezing, while enzymatic lysis can be achieved through lysozyme treatment, which utilizes an enzyme that specifically breaks down bacterial cell walls. For this project, a combination of shock freezing and cell wall disruption using an ultrasonic bath was selected.

2.4 Protein Extraction and Analysis^{MS}

2.4.1 Protein Extraction

To control the binding of REEs, lanmodulin has to be extracted from bacteria cells. This is done with the following steps:

Lysis: Breaking the Cellular Wall

As already discussed in 2.3.5 lysis involves breaking open the bacterial cells to access the intracellular proteins. This is achieved through our chosen methods:

- **Shock frosting**

Shock frosting, also known as freeze-thaw lysis, is a physical method used to disrupt bacterial cells by subjecting them to rapid temperature changes. The process involves freezing the bacterial culture rapidly at low temperatures like -80°C in our case, followed by thawing them at medium temperatures about 38°C to not coagulate the protein. The rapid freezing causes the formation of ice crystals, which physically disrupt the cell membrane. Upon thawing, the ice crystals melt, leading to further mechanical stress on the cellular structures, eventually resulting in cell lysis and the release of intracellular components.

In the case of *Methylophilus extorquens*, shock frosting was an effective way to disrupt cellular integrity, releasing intracellular proteins like lanmodulin.



Figure 2.17: Enterprise freezer that reaches -80°C for shock frosting bacteria.

- **Sonication:**

Sonication, or ultrasonication, is a mechanical method used to disrupt bacterial cell walls through the application of high-frequency sound waves. In this method, the bacterial culture is subjected to ultrasonic waves generated by a sonicator probe or bath. The high-frequency waves create alternating cycles of compression and rarefaction within the culture medium, generating cavitation bubbles. These bubbles undergo rapid expansion and collapse, generating intense shear forces and microstreaming, which disrupt the cell membrane and cell wall.

Sonication is particularly effective for lysing tough bacterial cell walls, including those of gram-negative bacteria like *Methylophilus extorquens*. However, it is essential to optimize sonication parameters such as amplitude, duration, and temperature to avoid excessive heating and denaturation of sensitive intracellular components. Sonication was the final step for effective lysis and disruption of *M. extorquens* cell walls.



Figure 2.18: Ultrasonic bath used to disrupt cell walls.

Cell Debris Removal

After cell disruption, the lysate (cell extract) contains a mixture of cellular components, including proteins, membranes, and nucleic acids. To isolate the proteins, unwanted components were removed by centrifugation. The lysate is spun at high speeds in a centrifuge. This separates the heavier cell debris (pellet) from the soluble components (supernatant) containing the proteins.

Throughout this process, protein concentration and purity were monitored using techniques like SDS-PAGE and IR-Spectrometry respectively, more on these methods and their effectiveness later. To keep the amino acids intact, the epi containing the bacteria needed to be cooled all the time. This prevented protease from consuming and therefore destroying the amino acids of our interest.



Figure 2.19: Centrifuge with cooling function for inhibiting protease.

2.4.2 IR-Spectroscopy

Infrared (IR) spectroscopy has established itself as a cornerstone analytical technique across diverse scientific fields. Its ability to elucidate the structural characteristics of molecules stems from the fundamental interaction between atoms and infrared radiation, providing invaluable insights into the presence of functional groups and the unique vibrational fingerprint of each molecule.

Theory:

The theoretical foundation of IR spectroscopy is based on the well-established principle that molecules can undergo vibrational motion when exposed to specific frequencies of infrared radiation, the radiation matching the molecule's frequency is absorbed. This radiation occupies a distinct region within the electromagnetic spectrum, encompassing wavelengths ranging from approximately $2.5 \mu\text{m}$ to $16 \mu\text{m}$. Unlike ultraviolet-visible (UV-Vis) spectroscopy, which excites electrons to higher energy levels, the energy associated with infrared radiation is insufficient for electronic transitions. Instead, it triggers vibrations within the covalent bonds of the molecule.

The concept of vibrational modes is important to understanding IR spectroscopy. These modes represent the various ways in which the atoms within a molecule can vibrate relative to each other. These atoms are connected by bonds. Stronger bonds, characterized by higher bond energies, vibrate at higher frequencies, while weaker bonds exhibit lower

vibrational frequencies. This distinctive correlation between bond type and vibrational frequency forms the cornerstone of IR spectroscopy.

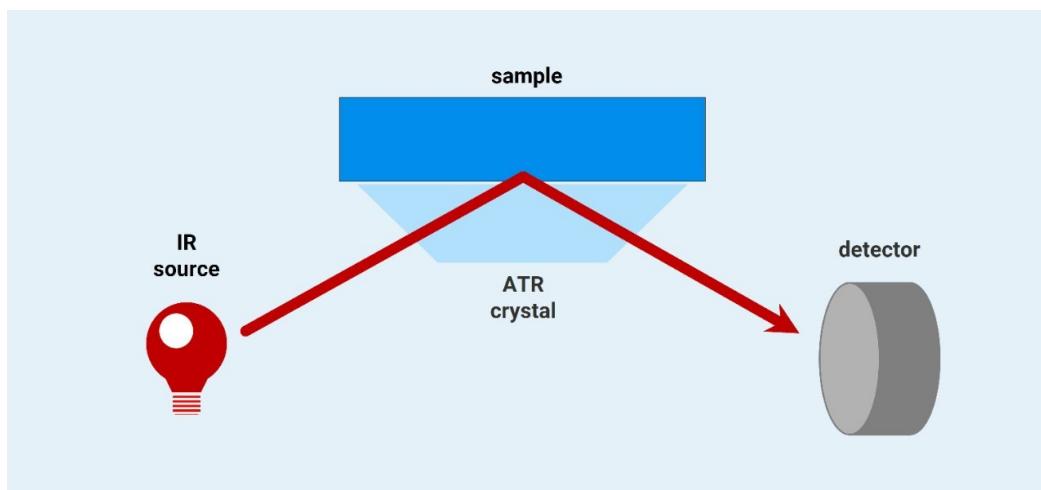


Figure 2.20: Schematic of the reflection of IR light in an ATR (Attenuated Total Reflectance) Spectrometer

The Infrared Spectrometer

This instrument houses a light source that emits a broad spectrum of infrared radiation. The prepared sample, in either solid, liquid, or gas phase, is positioned within the path of this radiation. As the infrared light traverses the sample, specific frequencies corresponding to the molecule's vibrational modes are absorbed. The remaining radiation is subsequently reflected and detected, and a spectrum is generated. This spectrum typically presents the intensity of transmitted or absorbed light (y-axis) versus the frequency or wavelength (x-axis).

Modes:

Transmission Mode: In this mode, the infrared radiation directly transmits through the sample. The resulting spectrum depicts the frequencies absorbed by the molecule, and it is the most commonly employed mode for solid and liquid samples.

Reflection Mode: This mode finds particular utility when analyzing samples that are challenging to prepare as thin films for transmission measurements. In this case, the infrared radiation is directed onto the sample's surface, and the reflected light is captured. The spectrum reflects the molecule's vibrational modes based on the analysis of the reflected frequencies. For the analysis of *Methylococcus extorquens*, this mode was chosen simply because of the available equipment.

Deciphering the Spectrum:

The IR spectrum generated from the experiment bears a resemblance to a unique fingerprint for each molecule. The x-axis typically displays the wavelength. The y-axis represents the intensity of the absorption (or transmittance). The spectrum usually shows diverse peaks, each corresponding to a specific vibrational mode within the molecule. The

location of a peak indicates the type of bond vibration that occurred, while the peak's intensity reflects the strength of the absorption.

By drawing upon extensive reference databases and theoretical calculations, the IR spectrum can be interpreted to glean valuable information about the sample. The presence of specific peaks can confirm the existence of particular functional groups, such as carbonyls ($C=O$), alkenes ($C=C$), and alcohols ($O-H$). The relative intensities of these peaks can even provide insights into the molecule's environment and interactions with neighboring molecules.

2.4.3 SDS-PAGE^{MS}

Outlining the key aspects of SDS-PAGE, a powerful method of protein analysis. Proteins can be separated by size through a process called electrophoresis. This involves applying an electric field to a polyacrylamide gel, which acts as a molecular sieve. The proteins are then forced through the gel based on their size, with smaller proteins moving through faster than larger ones.

Principles of SDS-PAGE

Protein Denaturation and Linearization with SDS

Proteins are biomolecules with three-dimensional structures influenced by various interactions, including hydrogen bonds, disulfide bridges, hydrophobic interactions, and ionic bonds. These interactions dictate a protein's function and overall shape. However, for separation by size in SDS-PAGE, protein conformation becomes irrelevant. This is achieved by sodium dodecyl sulfate (SDS), a strong anionic detergent.

SDS disrupts the noncovalent interactions within proteins, leading to their denaturation. This process unfolds the protein and disrupts its native conformation. Importantly, SDS binds to the unfolded polypeptide chain in a 1:1 ratio along its entire length [25]. This binding is primarily driven by hydrophobic interactions between the hydrocarbon tail of SDS and the hydrophobic amino acid side chains within the protein [26].

A crucial consequence of SDS binding is the masking of a protein's inherent charge. Since proteins possess a diverse array of amino acid side chains with varying pKa values, their net charge at a specific pH can be positive, negative, or neutral. However, with SDS bound along the entire polypeptide chain, the negatively charged sulfate groups of SDS dominate the protein's overall charge. This imparts a negative charge proportional to the protein's mass (due to the constant 1:1 SDS-to-protein ratio).

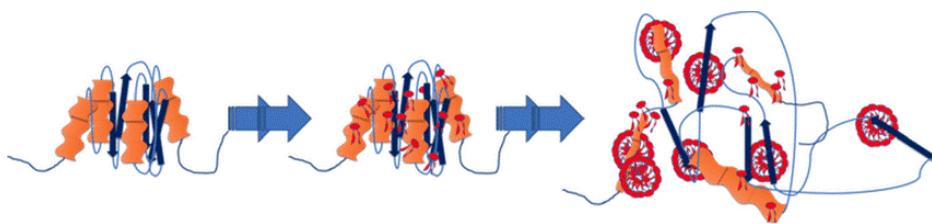


Figure 2.21: Representation of the denaturation effect of a protein promoted by SDS.

Polyacrylamide Gels and Molecular Sieving

The separation of proteins in SDS-PAGE is achieved through electrophoresis, a technique that utilizes voltage to move charged molecules through a gel matrix. In SDS-PAGE, the gel matrix is typically composed of polyacrylamide. This synthetic polymer forms a crosslinked network with pores of varying sizes. The pore size distribution within the gel is determined by the percentage of acrylamide used during gel preparation [27].

During electrophoresis, the negatively charged SDS-protein complexes migrate towards the positive electrode (anode) within the applied voltage. However, their movement is impeded by the polyacrylamide gel matrix. Smaller proteins with minimal hindrance by the gel pores navigate through the network more efficiently, resulting in faster migration. Conversely, larger proteins encounter greater resistance within the tighter pores, leading to slower migration.

This is known as molecular sieving and forms the basis of protein separation by size in SDS-PAGE. Proteins are effectively “sieved” through the gel matrix, with smaller proteins traveling further compared to larger ones. Ultimately, the distance migrated by each protein in the gel reflects its molecular weight, allowing for the creation of a protein profile based on size.

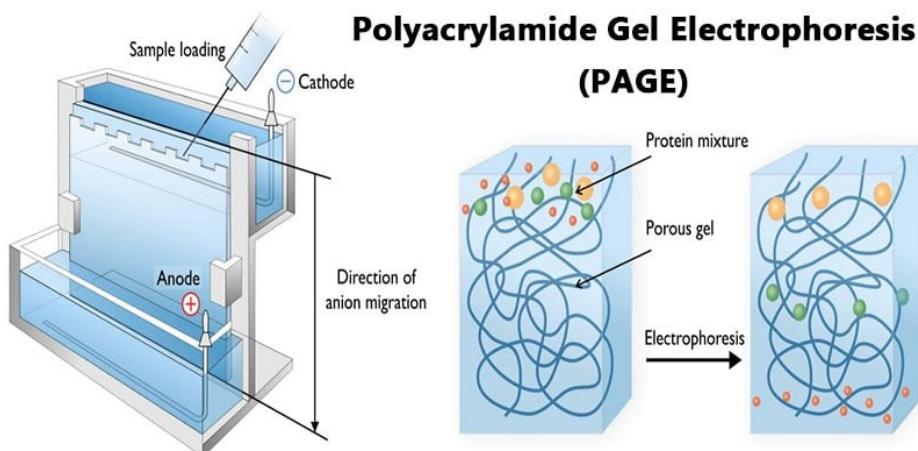


Figure 2.22: SDS-PAGE schematic, (left) SDS-PAGE apparatus, (right) schematic of protein passing through porous gel.

Additional Considerations

While SDS-PAGE is a powerful technique, a few points require consideration:

Disulfide Bridges: SDS does not disrupt disulfide linkages between cysteine residues. Proteins with multiple disulfide bridges may have reduced mobility compared to their predicted molecular weight based on linear sequence.

Glycosylation: The presence of sugar moieties (glycosylation) can slightly affect protein mobility due to increased size and potential interactions with the gel matrix. **Protein-SDS Interactions:** In rare cases, specific protein-SDS interactions may alter the expected migration pattern.

Acrylamide: Usage of Acrylamide requires careful deployment and adequate protection measures. Acrylamide is a toxic neurotoxin that poses a serious health risk when brought in contact with skin, especially mucous membranes.

Curing issues: When implemented into research regarding Lanmodulin and REE recycling, the issue of premature hardening or no hardening occurred. It is not yet clear why the consistency of the gel changed too fast or slow when repeated multiple times with the same amount of chemicals used.

Despite these limitations, SDS-PAGE remains a cornerstone technique in protein analysis due to its simplicity, and reproducibility.

3 Experimental Section

In the following chapter, we describe the work we carried out and why we did it. We begin with methods for cultivating bacteria, which are followed by the extraction of proteins from *M. extorquens*. Afterwards, we describe how we analyzed the proteins. In the end, we show you how we did the arsenazo-III assay.

3.1 Cultivation of Bacteria^{MS}

3.1.1 Solid Media

Preparation of solid nutrient solution for petri dishes:

Materials:

- Peptone 2,5g
- Meat Extract 1,5g
- Agar 7,5g
- H₂O 500mL
- Scale
- Autoclave bottle
- Spatula

Execution:

- Weigh all the necessary ingredients
- Fill the autoclave bottle with around 100ml of water
- Add the dry ingredients to the autoclave bottle
- Mix the dry ingredients with water
- Add the remaining water to the bottle
- Shake until mixed

Finalizing the solid nutrient solution to be poured into petri dishes:

Materials:

- Petri dishes
- Autoclave
- Autoclave indicator tape
- Sterile workbench

Execution:

- Add a piece of indicator tape on the cap of the autoclave bottle
- Autoclave the nutrient solution at 121°C and 1 bar for 15 minutes
- After autoclaving the nutrient solution swiftly pour it into the petri dishes
- Leave the nutrient solution to harden
- Flip the petri dishes carefully on their cover and put them in the fridge

3.1.2 Cultivating Bacteria on Solid Media

Cultivating bacteria from a freeze dried strain, acquired from DSMZ [28].

Materials:

- Freeze dried strain in fused glass vial
- Drigalski spatula
- Petri dish with solid nutrient media
- Blowtorch
- Sterile Workbench
- Pliers
- Liquid nutrient media
- Beaker

Execution:

- Unpack the glass vial and carefully place it in your sterile work environment
- Using a blowtorch, heat the fused tip of the vial whilst holding it with pliers
- Make sure not to heat up too much of the vial, just blast the tip
- When the tip is heated up, carefully hit it on the corner of a beaker to snap it off clean
- Take out the bacteria and mix it with a small amount of liquid media
- Pour about 500ul of the mix into a petri dish, distribute it with a Drigalski spatula
- Close the petri dish and place it in an incubator at 30°C

3.1.3 Liquid Media

Preparation of solid nutrient solution for petri dishes: Materials:

- Peptone 2,5g
- Meat Extract 1,5g
- Methanol 5ml
- H₂O 500ml
- Scale
- Autoclave bottle
- Spatula
- Erlenmeyer Flask 100ml – 250ml
- Volumetric pipette 10ml
- Peleus Ball

Execution:

- Weigh all the necessary ingredients
- Fill the autoclave bottle with around 100ml of water
- Add the dry ingredients to the autoclave bottle
- Add the Methanol to the autoclave bottle using the volumetric pipette and Peleus Ball
- Mix the ingredients with water
- Add the remaining water to the bottle
- Shake until mixed

Finalizing the solid nutrient solution to be poured into Erlenmeyer Flasks and inoculated with bacteria and REEs:

Materials:

- Erlenmeyer Flask 100ml – 250ml
- Autoclave
- Autoclave indicator tape
- Sterile workbench

Execution:

- Add a piece of indicator tape on the cap of the autoclave bottle
- Autoclave the nutrient solution at 121°C and 1 bar for 15 minutes

- After autoclaving the nutrient solution let it cool down to room temperature
- Pour the nutrient solution into the Erlenmeyer Flask to fill it about 25%
- Put the leftover nutrient solution into the fridge, make sure it is sealed properly

3.1.4 Inoculating liquid nutrient media

Adding *M. extorquens* and REEs to the liquid nutrient media.

Materials:

- NdCl₃ 1000µl
- Ce 1000µl
- Erlenmeyer Flask with nutrient solution
- Petri dish with cultivated bacteria
- Microliter pipette 1000µl
- Microliter pipette tips 1ml
- Sterile Workbench
- Cotton

Execution:

- Working in a sterile work environment equip the Microliter Pipette with 1ml capacity tips
- Use the tip to scrape off a tiny bit of the *M. extorquens* culture (Orange to pink) and drop it into the Erlenmeyer flask holding the liquid nutrient solution
- Repeat three times to create a solution for Neodymium, Cerium, and no REE
- Add 1ml of NdCl₃ to the first of three flask
- Add 1ml of CeCl₃ to the second of three flasks
- The third flask stays untouched and serves as a control solution to compare to the REE solutions
- Seal the flasks with a piece of cotton, make sure not to press it in too hard to allow oxygen to pass through

3.2 Protein Extraction^{MS}

The recipe for the protein lysis buffer was given to us by our supervisor Mr. Seeburger [29, p. 52].

Material:

Protein Lysis Buffer:

- NaPO₄ 50mM
- NaCl 300mM
- Imidazole 5mM
- Mix all ingredients

We did not add the imidazole, because the chemical was not available at that time.

- Bacteria in liquid media
- H₂O
- Laemmli Buffer
- Lysis Buffer
- Temperature-controlled centrifuge
- Beaker
- Freezer or Liquid Nitrogen
- Ultrasonic Bath
- 1ml Epi

Execution:

- Fill a 1ml Epi with bacteria in liquid media
- Centrifuge that Epi for 5min at 5000RPM
- After centrifugation decant the liquid media out of the epi, be careful not to pour out the bacteria pellet that has formed on the bottom of the container
- Add an equal amount of water to your bacteria pellet and freeze it with a high-power freezer or liquid nitrogen
- Thaw the bacteria pellet fully in water no warmer than 37°
- Repeat this process 3 times
- Sonicate the Bacteria pellet while still in the epi for 5min in an ultrasonic bath
- Centrifuge the epi at 14000RPM and 4°C for 25min
- Drop the pellet found at the bottom of the epi into a Lysis-Buffer
- Centrifuge it at 2000RPM for 2min
- Pour the liquid component into a Laemmli Buffer
- Heat it at 95°C for 5min or at 70°C for 10min
- Continue with SDS Page or methods that require extracted Protein

3.3 Protein Analysis^{TD}

3.3.1 IR-Spectrometry

The IR-Spectrometry was done using an ATR-IR-Spectrometer. This meant that we could place the sample directly onto the device, without additional sample preparation.

The following table 3.1 gives an overview of the lysed samples of *M. extorquens* we measured, what treatments we gave the bacteria to grow in and when we measured the samples after the treatment.

Type of sample	Treatment	Weeks of growth after treatment
Proteins dissolved in water	no treatment	0, 1 and 2
Cell paste	no treatment	0, 1 and 2
Proteins dissolved in water	0,5mL of Ce-solution	0, 1 and 2
Cell paste	0,5mL of Ce-solution	0, 1 and 2
Proteins dissolved in water	1mL of NdFeB-magnet, dissolved in water	0, 1 and 2
Cell paste	1mL of NdFeB-magnet, dissolved in water	0, 1 and 2

Table 3.1: Types of samples measured with IR-Spectrometry.

We put a drop of a sample onto the ATR-IR-Spectrometer and measured the IR spectrum. Before each measurement, we cleaned the ATR crystal with a drop of ethanol on a soft tissue.

3.3.2 SDS-PAGE

The SDS-PAGE was carried out as it is described in the guide of the manufacturer of our electrophoresis cell, Bio-Rad Laboratories, Inc. [30], with some slight modifications.

Gel Casting:

Materials and Methods:

Ingredient	Volumina for Stacking Gel	Volumina for Resolving Gel
30% Acrylamide	1,485mL	4,5mL
0,5M Tris-HCl pH6,8	3,78mL	-
1,5M Tris-HCl pH8,8	-	3,75mL
10% SDS	150µL	150µL
diH ₂ O	9mL	5,03mL
TEMED	18µL	7,5µL
10% APS	90µL	75µL

Table 3.2: Ingredients for SDS-PAGE gel.

- Mix all ingredients except for TEMED and APS
- Add TEMED and APS to the resolving gel
- Transfer resolving gel into the gel cassette
- Add ethanol on top, to ensure that the stacking gel binds to the resolving gel
- Wait until the resolving gel has polymerized
- When the resolving gel is firm, pour off the ethanol
- Put the gel comb into the gel cassette, so that one end is between the glasses
- Add TEMED and APS to the stacking gel
- Fill the stacking gel into the gel cassette
- Make sure that no air bubble is between the tines of the comb
- Press the comb gently into the fluid gel
- Wait until the stacking gel has polymerized

We increased the amount of TEMED and APS by 20 percent compared to the manufacturer's guide, because oftentimes the resolving gel did not polymerize.

Running Buffer:

Materials and Methods:

- Tris Base 30,30g
- Glycine 144,70g
- SDS 10,00g
- Mix all ingredients with 1000mL of deionized water

Probe Preparation:

Materials and Methods:

Laemmli-Buffer:

- 0,5M Tris-HCl pH6,8 3,75mL
- Glycerol 100% 7,5mL
- 1,0% Bromophenol blue 0,3mL
- 10% SDS 6,0mL
- diH₂O to 30mL
- Mix all ingredients
- When the buffer is yellow, add Tris base until it is blue

Probe Preparation:

- Mix the sample with the same amount of Laemmli-Buffer
- Heat the mixture to 95°C for 5min

We did not add β -mercaptoethanol to the sample/buffer mixture, because the chemical was not available at that time.

Performing Gel Electrophoresis:

Materials and Methods:

- Insert the gel(s) with removed comb(s) into the running module
- Fill the gel box with the running buffer to around three quarters
- Insert 30 μ L of protein marker into one well
- Insert 15 μ L of sample into the remaining wells
- Fill the gel box completely with running buffer
- Put the lid onto the box and plug in the electrodes
- Let the SDS-PAGE run for 60min at 100V

Gel Staining:

- 10% Acetic Acid
- 50% Methanol
- 0,1% Coomassie Blue
- Mix all ingredients
- Remove the glass front plate gently from the gel

- Cut the stacking gel carefully off
- Remove the gel cautiously from the spacer plate
- Put the gel into the staining solution
- Let the gel stain overnight

Gel Destaining:

- 10% Acetic Acid
- 40% Methanol
- Mix all ingredients
- Rinse the stained gel under deionized water
- Put the stained gel into the destaining solution
- Let the gel destain overnight
- After the gel destained, rinse it with deionized water
- The gel can be stored in a fridge in deionized water

3.4 Arsenazo-III Assay^{TD}

The arsenazo-III assay was carried out, as described by Hoogendorn et al. [7], with slight modifications, because of some missing chemicals.

Materials and Methods:

Phosphate-Citrate-Buffer:

- Citrate monohydrate 1,767g
- Na₂HPO₄ · H₂O 564,2mg
- dH₂O to 100mL
- Mix all ingredients
- Measure pH. It should be 2,8

Arsenazo-III stock solution (10mM):

- Arsenazo-III 77,64mg
- dH₂O to 10mL
- Mix all ingredients

Sample preparation:

- Adjust pH of sample to 2,7 to 2,8 with HCl

Preparation of Calibration Lines:

- NdCl₃ 100mM in water:

NdCl₃ 250,6mg

diH₂O to 10mL

Mix all ingredients

- NdCl₃ 1mM in water:

Dilute NdCl₃ 100mM 1:10

Dilute NdCl₃ 10mM 1:10

- Adjust pH to 2,7 to 2,8

- Prepare solutions with the following concentrations of NdCl₃:

- 0µM, 0,1µM, 0,2µM, 0,5µM, 0,7µM, 1µM, 2µM, 5µM, 7µM, 10µM, 20µM, 50µM, 100µM

- NdCl₃ 50mM in nutrition solution:

Adjust pH of solution to 2,7 to 2,8

Take 50mL

Add NdCl₃ 125,3mg

- NdCl₃ 1mM in nutrition solution:

Dilute NdCl₃ 50mM 1:5

Dilute NdCl₃ 10mM 1:10

- Prepare solutions with the following concentrations of NdCl₃:

- 0µM, 0,1µM, 0,2µM, 0,5µM, 0,7µM, 1µM, 2µM, 5µM, 7µM, 10µM, 20µM, 50µM, 100µM, 150µM, 170µM, 200µM, 250µM

Performing the Assay:

- Dilute arsenazo-III stock solution (10mM) 1:10
- Add 500µL of phosphate-citrate-buffer into a 1mL cuvette
- Add 490µL of sample to the cuvette
- Add 10µL of arsenazo-III 1mM
- Record UV-Vis spectrum from 500nm to 800nm
- Note absorbance at 650nm
- Add 20µL of arsenazo-III 1mM
- Record UV-Vis spectrum from 500nm to 800nm
- Note absorbance at 650nm

- Repeat for every sample

4 Results and Discussion

4.1 Cultivation of Bacteria^{MS}

Upon acquisition of the bacterial strain, it underwent cultivation on agar plates followed by incubation. However, initial observations within the first week of incubation did not yield satisfactory results, as *Methylorubrum Extorquens* (*M. Extorquens*) failed to produce a visibly discernible orange culture indicative of successful growth.

By the third week of cultivation, a distinct orange dot appeared on the surface of the incubated agar plate, signifying the emergence of bacterial growth. The bacteria were meticulously scraped from the solid nutrient media for subsequent cultivation in liquid nutrient media, thereby facilitating a transition from solid to liquid growth conditions.



Figure 4.1: First *M. extorquens* culture developed from an acquired strain.

Subsequent growth in liquid media exhibited a remarkable proliferation of bacterial colonies. To maintain optimal growth conditions and prevent overpopulation-induced stress, a

routine procedure of decanting 75% of the liquid culture from the flasks and replenishing them with fresh liquid media was implemented on a weekly basis. This critical step ensured the sustained viability and productivity of the bacterial population.

Toward the latter stages of the project, flasks were emptied completely, yet bacterial colonies regenerated solely from residual deposits adhering to the inner walls of the flasks.

It was also observed that the addition of Methanol had a significant impact on *M. extorquens'* growth speeding up it's growth by 20%-50%, this is explainable by *M. extorquens'* methanol metabolizing capabilities.

4.2 Protein Analysis^{TD}

With the analysis of the lysed bacteria, we wished to be able to determine if the bacteria are capable of producing LanM. We tried two methods for protein analysis, namely the IR-Spectrometry and the SDS-PAGE. However, both methods did not bring any valuable results.

IR-Spectrometry

The IR spectra of our samples looked all the same, and they did not have any significant differences. In general, all spectra showed the lysis buffer we used to lyse the bacteria (see figures 4.2 and 4.3).

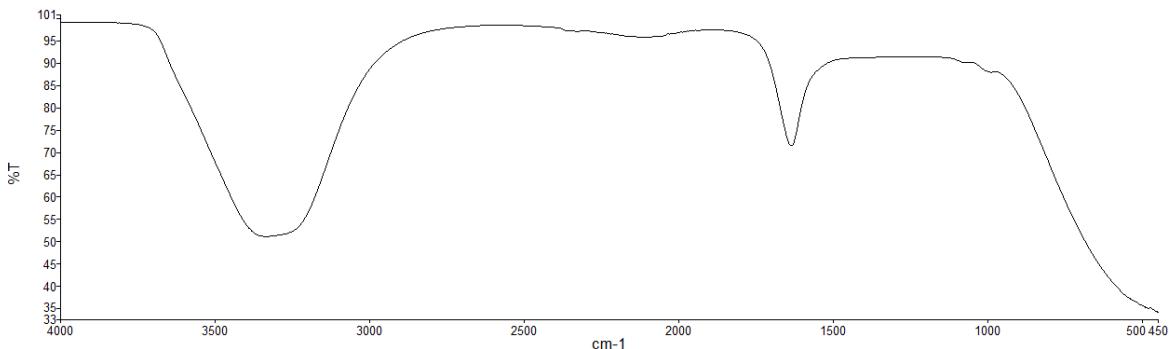


Figure 4.2: IR-Spectrum of proteins solved in water, from a culture of *M. extorquens*, to which no rare earths were given.

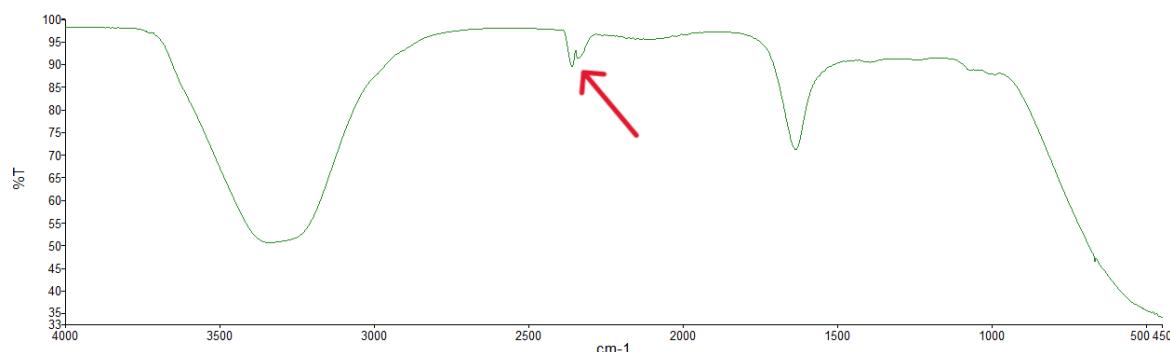


Figure 4.3: IR-Spectrum of proteins solved in water, from a culture of *M. extorquens*, to which the NdFeB-solution was given. The marked valley stems from the wrongly calibrated IR-Spectrometer.

There were no results because maybe we did not have enough amounts of bacteria, which would have more proteins, to make their spectra more visible. It could also be that we did not achieve to break the cell wall of enough bacteria, so that we could analyze the proteins.

SDS-PAGE

The SDS-PAGE was really challenging to perform, because most of the time, the gel did not polymerize. This could be due to old chemicals, or the chemicals did not have the right temperature to link and form the gel. However, we tried the SDS-PAGE multiple times, and we did not find the correct reason why our gel sometimes polymerized and sometimes not.

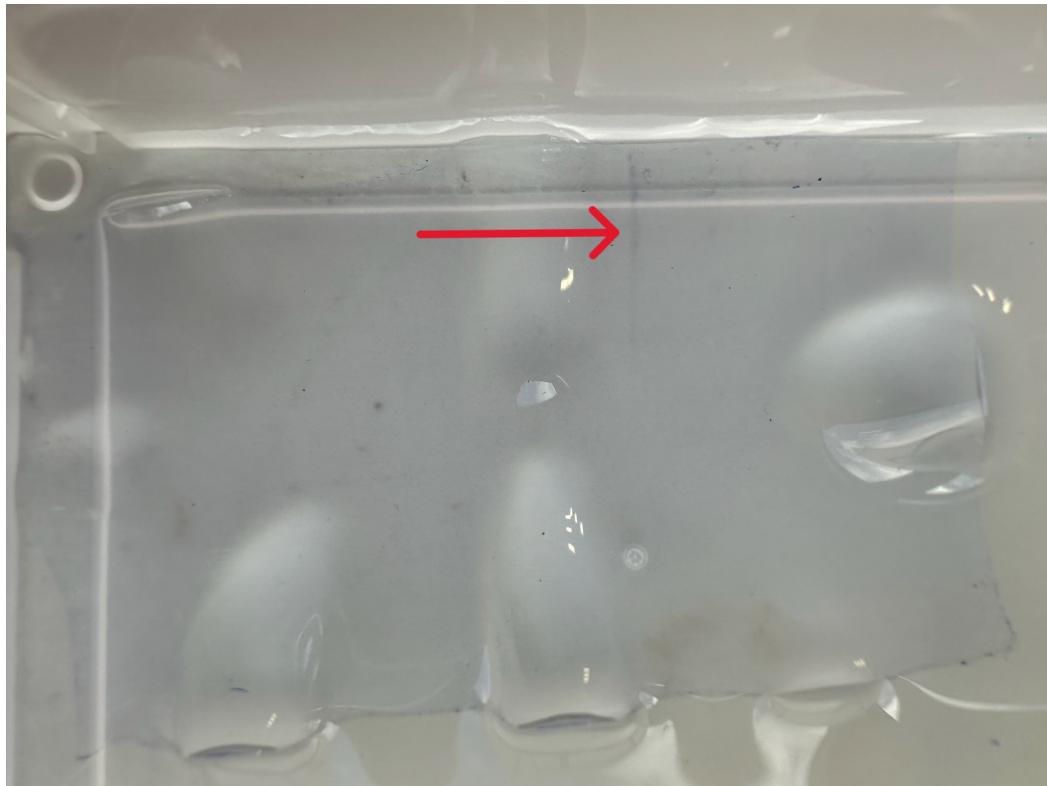


Figure 4.4: Photo of a SDS-PAGE gel. It does not show any bands, except for the one marked stripe.

When we had the gel, the further process was really straightforward. However, this does not mean that any results are achieved. The gels did not show any clear bands. Not even the marker was clearly visible. This could be because the marker was old, or our staining solution was old. But after we made a new staining and destaining solution, the result looked the same as in figure 4.4.

Because the complete process of the SDS-PAGE takes a whole day to carry out, we decided to abandon this method to not waste any more time.

4.3 Arsenazo-III Assay^{TD}

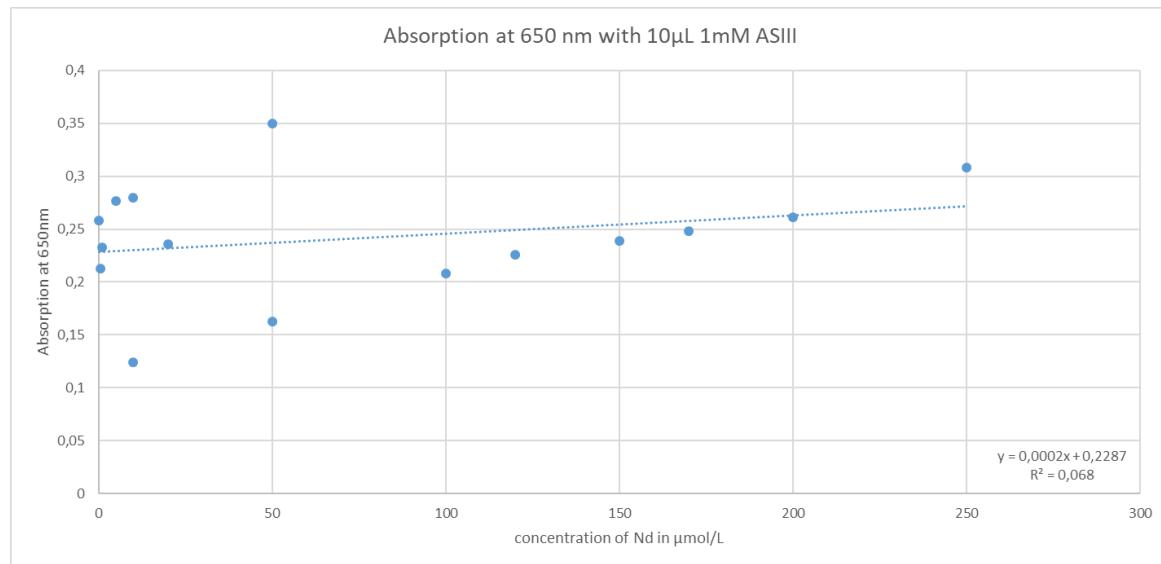


Figure 4.5: Calibration line of absorption at 650nm with 10µL of 1mM arsenazo-III added.

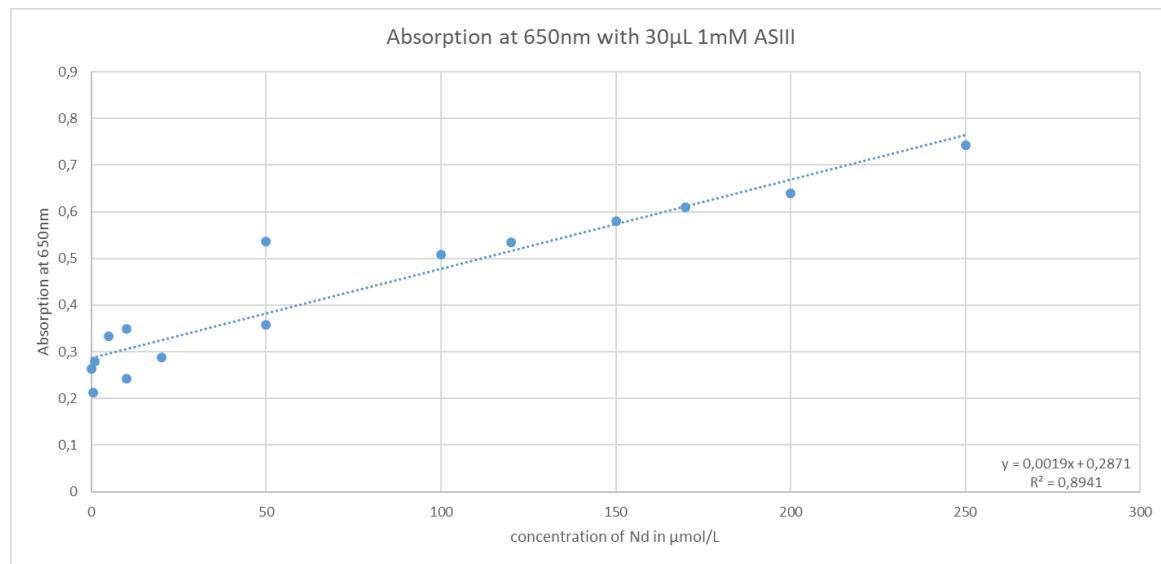


Figure 4.6: Calibration line of absorption at 650nm with 30µL of 1mM arsenazo-III added.

In figure 4.5 it is clearly visible, that the values for the absorption fluctuate heavily. The resulting calibration line should not be used for calculating the concentration. Therefore, we used the graph in figure 4.6 to calculate the remaining concentration of Nd in the nutrient solution.

Initial Nd concentration	Nd concentration after one week of bacteria growth	Absolute Change	Percentage change
0µmol/L	-39µmol/L	-39µmol/L	-%
200µmol/L	49,68µmol/L	-150,32µmol/L	-75,16%
1mmol/L	279,42µmol/L	-720,58µmol/L	-72,58%

Table 4.1: Initial and remaining concentration of Nd in the nutrient solution.

In table 4.1, *M. extorquens* demonstrates its efficiency. In just one week of growth, the bacteria were able to accumulate more than 70% of neodymium in a solution. It was irrelevant, how concentrated the neodymium was.

These results are very promising. We did not only confirm that *M. extorquens* is able to accumulate Nd but without additional changes, it is naturally able to bind more than 70% of the given neodymium.

5 Project Management

5.1 Planning

Nº	Milestone	Date of Achieval
MS_1	Cultivation of Bacteria	09.11.2023
MS_2	Extraction of LanM	07.12.2023
MS_3	Detection of LanM	14.03.2024
MS_4	Binding of LanM to Rare Earth Elements	29.02.2024
MS_5	Separation of Rare Earths from LanM	not confirmed yet

Table 5.1: Planned milestones and their date of achievement.

5.2 Evaluation^{TD}

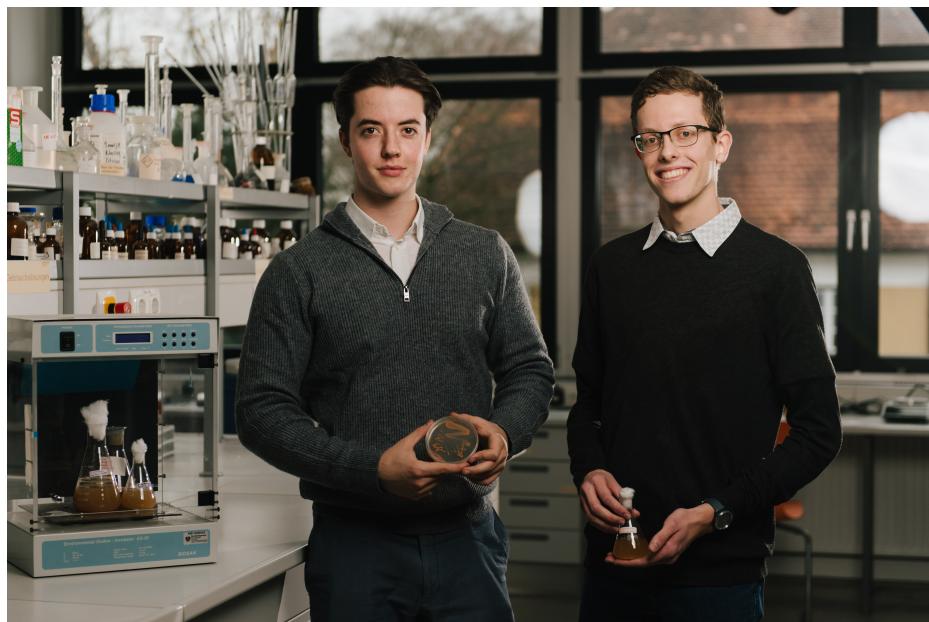


Figure 5.1: The project team

When we started to conduct some research for the project in the summer break, we also simultaneously began to plan the work with agile project management methods. As it turned out, doing the project management this way was really helpful. During our work,

we encountered a lot of obstacles which we had not thought of before, which resulted in a slower progress than we had previously expected.

Another problem that we encountered was that we simply could not do our project the way we had planned at the beginning. Due to limited financial resources and equipment, we could not carry out our planned work. A lot of methods we tried out did not produce the expected or reliable results. When we ran into these problems, we had to change how we want to achieve our planned goals. This also meant that one of our planned milestones (MS_3 Detection of LanM, see table 5.1 in section 5.1) was achieved later and also in a different way than we initially expected.

After we had tried our new approach, we finally achieved promising results. This brought fresh air into the project because we saw that progress was being made. After weeks of repeated failure, we found new motivation to keep going.

Our new approach requires less expensive resources and is simpler to carry out. Overall, this made our project better, and it did not change our main goal. The transformation from our first approach to the other would not have been possible if we had not used agile project management methods.

5.3 Timesheet

5.3.1 Tobias Daxecker

Date	Work	School Time	Free Time
03.08.2023	Research	0,00	3,00
18.08.2023	Research	0,00	2,00
24.08.2023	Research	0,00	2,00
03.09.2023	Research	0,00	1,00
06.09.2023	Research	0,00	2,00
07.09.2023	Research	0,00	4,00
08.09.2023	Preparation of nutrient solution	0,00	6,00
14.09.2023	Cerium detection	10,00	0,00
21.09.2023	Production of solid culture medium	10,00	0,00
22.09.2023	Organization of teams	0,00	1,00
28.09.2023	Profile, milestones	0,00	1,00
02.10.2023	Research	0,00	1,00
05.10.2023	Preparation of nutrient solution, detection of rare earths	10,00	0,00
06.10.2023	Freezing the bacteria	0,00	1,00
09.10.2023	Research	0,00	1,00
10.10.2023	Research, research plan	0,00	2,00
12.10.2023	Cultivation of M. extorquens, preparation of SDS-PAGE	10,00	0,00
15.10.2023	Draft research plan	0,00	3,00
16.10.2023	Draft research plan, research	0,00	1,00
18.10.2023	Research plan draft, research	0,00	2,00
19.10.2023	Prepare SDS-PAGE, design poster	10,00	0,00
24.10.2023	Research, research plan	0,00	1,00
27.10.2023	Research plan Draft, research, newspaper article	0,00	3,00
30.10.2023	Newspaper article, writing the thesis	0,00	3,00
31.10.2023	Writing the thesis	0,00	4,00
01.11.2023	Writing the thesis	0,00	6,00
02.11.2023	Writing the thesis	0,00	5,00
03.11.2023	Writing the thesis	0,00	5,00
06.11.2023	Research, writing the thesis	0,00	2,00
09.11.2023	Perform SDS-PAGE, separate Nd from Fe from magnets	10,00	0,00
10.11.2023	Follow up SDS-PAGE	0,00	2,00
12.11.2023	Newspaper article, writing the diploma thesis	0,00	2,00
14.11.2023	Newspaper article, writing the diploma thesis	0,00	2,00

Date	Work	School Time	Free Time
16.11.2023	Preparing an SDS-PAGE, cultivating the bacteria	10,00	0,00
19.11.2023	Writing the thesis	0,00	6,00
20.11.2023	Cultivating the bacteria, writing the diploma thesis, Jugend Innovativ application	0,00	3,00
23.11.2023	SDS-PAGE performed, bacteria cultivated, new fume cupboard installed	10,00	0,00
24.11.2023	Placing gel in destaining solution, preparing for open day	0,00	1,00
26.11.2023	Writing the diploma thesis	0,00	4,00
27.11.2023	Submission ECO Bonus	0,00	1,00
30.11.2023	Photo for newspaper article, preparation for open day, cultivation of bacteria	10,00	0,00
03.12.2023	Research, writing the diploma thesis	0,00	3,00
07.12.2023	Neutralizing cerium, IR spectroscopy, protein determination according to Bradford assay	10,00	0,00
10.12.2023	Research, writing the diploma thesis	0,00	5,00
11.12.2023	Research, writing the thesis	0,00	1,00
14.12.2023	Prepare new medium, repeat REE detection, add REEs to bacteria, cultivate bacteria	10,00	0,00
21.12.2023	IR spectrometry with lysed bacteria, cultivate bacteria with REEs	10,00	0,00
28.12.2023	Research, writing the thesis, project report Jugend Innovativ	0,00	3,00
29.12.2023	Research, writing the thesis, project report Jugend Innovativ	0,00	2,00
01.01.2024	Research, writing the diploma thesis	0,00	3,00
02.01.2024	Research, writing the thesis	0,00	4,00
03.01.2024	Research, writing the diploma thesis	0,00	4,00
04.01.2024	Research, writing the diploma thesis	0,00	3,00
11.01.2024	Measurement spectrum of NdFe, measurement fluorescence NdFe	10,00	0,00
12.01.2024	Writing the diploma thesis, Jugend Innovativ project report	0,00	1,00
14.01.2024	Writing the diploma thesis, Jugend Innovativ project report	0,00	3,00
15.01.2024	Writing the diploma thesis, Jugend Innovativ project report	0,00	1,00
16.01.2024	Writing the project report	0,00	3,00
17.01.2024	Writing the project report	0,00	2,00

Date	Work	School Time	Free Time
18.01.2024	Nd detection, UV-VIS measurements, cultivation of bacteria	10,00	0,00
22.01.2024	Writing the project report	0,00	1,00
23.01.2024	Writing the project report	0,00	2,00
24.01.2024	Writing the project report, discussing the report so far	0,00	1,00
25.01.2024	Recording some sequences for video, cultivating the bacteria, producing a pH buffer, writing the project report	10,00	0,00
26.01.2024	Writing the project report	0,00	2,00
27.01.2024	Writing the project report	0,00	6,00
27.01.2024	Writing the thesis	0,00	1,00
28.01.2024	Writing the project report	0,00	1,00
30.01.2024	Writing the project report	0,00	1,00
08.02.2024	Video shoot, production of a new culture medium, cultivation of the bacteria	10,00	0,00
16.02.2024	Carrying out an arsenazo III assay	0,00	5,00
20.02.2024	Writing the diploma thesis	0,00	2,00
21.02.2024	Writing the thesis	0,00	5,00
22.02.2024	Writing the thesis	0,00	3,00
23.02.2024	Writing the thesis	0,00	3,00
26.02.2024	Diploma thesis	0,00	1,00
28.02.2024	Writing the diploma thesis	0,00	1,00
29.02.2024	Arsenazo-III assay, cultivation of bacteria	10,00	0,00
03.03.2024	Writing the thesis	0,00	4,00
04.03.2024	Writing the diploma thesis	0,00	5,00
05.03.2024	Writing the thesis	0,00	1,00
06.03.2024	Cost plan	0,00	1,00
07.03.2024	Microscopy, preparation of a new culture medium, cultivation of bacteria	10,00	0,00
08.03.2024	Presentation for job market	0,00	1,00
10.03.2024	Presentation for job fair, writing the diploma thesis	0,00	2,00
11.03.2024	Presentation for job fair, Writing your thesis	0,00	1,00
14.03.2024	Arsenazo-III assay, cultivation of bacteria	10,00	0,00
17.03.2024	Writing the diploma thesis, correcting the diploma thesis	0,00	7,00
18.03.2024	Writing the diploma thesis, correcting the diploma thesis	0,00	2,00
19.03.2024	Writing the diploma thesis	0,00	1,00

Date	Work	School Time	Free Time
20.03.2024	Writing the diploma thesis	0,00	3,00
21.03.2024	Writing the diploma thesis	0,00	2,00
21.03.2024	Arsenazo-III assay, cultivation of bacteria, preparation of new culture medium	10,00	0,00
22.03.2024	Writing the diploma thesis, correcting the diploma thesis	0,00	6,00

Total sum of school time work hours: 200,00 Total sum of free time work hours: 192,00

Braunau/Inn, 22.03.2024

Tobias Daxecker

Ort, Datum

Unterschrift

5.3.2 Mathias Standhartinger

Date	Work	School Time	Free Time
03.08.2023	Research	0,00	3,00
18.08.2023	Research	0,00	2,00
24.08.2023	Research	0,00	2,00
03.09.2023	Research	0,00	1,00
06.09.2023	Research	0,00	2,00
07.09.2023	Research	0,00	4,00
08.09.2023	Preparation of nutrient solution	0,00	6,00
14.09.2023	Cerium detection	10,00	0,00
21.09.2023	Production of solid culture medium	10,00	0,00
05.10.2023	Production of nutrient solution, detection of rare earths	10,00	0,00
06.10.2023	Freezing the bacteria	0,00	1,00
12.10.2023	Cultivation of M. extorquens, preparation of SDS-PAGE	10,00	0,00
18.10.2023	Research plan draft, research	0,00	2,00
19.10.2023	Prepare SDS-PAGE, design poster	10,00	0,00
03.11.2023	Project poster	0,00	2,00
03.11.2023	Registration for competitions	0,00	2,00
09.11.2023	Performing SDS-PAGE, separating Nd from Fe from magnets	10,00	0,00
10.11.2023	Follow up SDS-PAGE	0,00	2,00
13.11.2023	Planning Jugend Innovativ Video	0,00	2,00
14.11.2023	Newspaper article, writing the diploma thesis	0,00	2,00
16.11.2023	Preparing an SDS-PAGE, cultivating the bacteria	10,00	0,00
20.11.2023	Cultivating the bacteria, Writing the thesis	0,00	2,00
23.11.2023	SDS-PAGE performed, bacteria cultivated, new fume cupboard installed	10,00	0,00
24.11.2023	Placing gel in destaining solution, preparing for open day	0,00	1,00
30.11.2023	Photo for newspaper article, preparation for open day	10,00	0,00
07.12.2023	Neutralizing cerium, IR spectroscopy, protein determination according to Bradford assay	10,00	0,00
14.12.2023	Prepare new medium, repeat REE detection, add REEs to bacteria, cultivate bacteria	10,00	0,00

Date	Work	School Time	Free Time
21.12.2023	IR spectrometry with lysed bacteria, cultivate bacteria with REEs	10,00	0,00
26.12.2023	Research, REE, LanM	0,00	8,00
29.12.2023	Research, division of labor and project structure Diploma thesis	0,00	6,00
11.01.2024	Measuring the spectrum of NdFe, measuring the fluorescence of NdFe	10,00	0,00
15.01.2024	Scripts for short video on the project	0,00	2,00
16.01.2024	Writing the project report	0,00	2,00
18.01.2024	Nd detection, UV-VIS measurements, cultivation of bacteria	10,00	0,00
23.01.2024	Writing the project report, preparation for video shoot	0,00	4,00
24.01.2024	Writing the project report	0,00	3,00
24.01.2024	Preparation for video shoot	0,00	3,00
25.01.2024	Follow-up video shoot	0,00	1,00
25.01.2024	Recording some sequences for video, cultivating the bacteria, producing a pH buffer, writing the project report	10,00	0,00
27.01.2024	Designing the project report	0,00	4,00
28.01.2024	Designing the project report	0,00	6,00
29.01.2024	Correction of the project report	0,00	2,00
05.02.2024	Preparation for video shoot	0,00	4,00
07.02.2024	Scripts and preparation for video shoot	0,00	4,00
08.02.2024	Video shoot, production of a new culture medium, cultivation of the bacteria	10,00	0,00
16.02.2024	Carrying out an arsenazo-III assay	0,00	5,00
19.02.2024	Stockfootage and preparation of video editing	0,00	4,00
21.02.2024	Writing the diploma thesis	0,00	4,00
23.02.2024	Writing the thesis	0,00	3,00
26.02.2024	Diploma thesis	0,00	2,00
28.02.2024	Writing the diploma thesis	0,00	3,00
29.02.2024	Arsenazo-III assay, cultivation of bacteria	10,00	0,00
01.03.2024	Writing the diploma thesis	0,00	3,00
02.03.2024	Writing the thesis	0,00	4,00
04.03.2024	Writing the thesis	0,00	3,00
05.03.2024	Writing the thesis	0,00	3,00
06.03.2024	Cost plan	0,00	1,00
06.03.2024	Writing the thesis	0,00	4,00
07.03.2024	Writing the thesis	0,00	5,00
07.03.2024	Video editing	0,00	6,00

Date	Work	School Time	Free Time
07.03.2024	Microscopy, preparation of a new culture medium, cultivation of bacteria	5,00	0,00
08.03.2024	Presentation for job fair	0,00	2,00
08.03.2024	Video editing	0,00	6,00
11.03.2024	Presentation for job exchange, JI video	0,00	3,00
13.03.2024	Writing the thesis	0,00	3,00
14.03.2024	Writing the thesis	0,00	4,00
14.03.2024	Arsenazo-III assay, cultivation of bacteria	10,00	0,00
15.03.2024	Writing the diploma thesis	0,00	5,00
16.03.2024	Writing the diploma thesis	0,00	4,00
18.03.2024	Writing the diploma thesis	0,00	3,00
19.03.2024	Writing the diploma thesis	0,00	5,00
20.03.2024	Writing the diploma thesis	0,00	6,00
21.03.2024	Writing the diploma thesis, correcting the diploma thesis	0,00	7,00
21.03.2024	Arsenazo-III assay, cultivation of bacteria, preparation of new culture medium	10,00	0,00
22.03.2024	Writing the diploma thesis, image correction	0,00	6,00

Total sum of school time work hours: 195,00 Total sum of free time work hours: 189,00

Braunau/Inn, 22.03.2024

Mathias Standhartinger

Ort, Datum

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6 Future Work^{MS}

Optimization and Industrialization: To enhance the industrial viability of this initiative, several essential measures must be implemented. Foremost, there is a critical imperative to optimize bacterial proliferation efficiency and subsequent Rare Earth Element (REE) yield. This necessitates the exploration of innovative methodologies aimed at reducing growth duration and optimizing nutrient utilization by *M. extorquens*. Additionally, the substitution of methanol, the primary substrate for bacterial metabolism, with methane, a more rudimentary and economically advantageous precursor, presents a promising avenue, considering its compatibility with *M. extorquens* metabolism.

For the full-scale industrialization of this project, comprehensive testing within large-scale bioreactor systems is paramount. Concurrently, integration of diverse forms of electronic waste (e-waste) is essential. Identifying the most economically viable categories of e-waste requires extensive experimental evaluation. In parallel with these assessments, there is a pressing need for the development of a high-capacity, efficient, and durable shredding apparatus specifically designed to handle various types of E-Waste.

This endeavor poses multifaceted challenges, particularly regarding safety and cost considerations. An industrial-grade E-Waste shredder must be inherently non-combustible and adept at processing metal, plastic, fiberglass, and adhesive materials. Moreover, it must maintain optimal power consumption levels and facilitate straightforward maintenance protocols. Such considerations are integral to the successful implementation of sustainable and efficient recycling practices within industrial contexts.

Rare Earth Element Mining: *Methylorum extorquens* (*M. extorquens*) showcases remarkable Rare Earth Element (REE) binding capabilities, presenting not only an avenue for e-waste recycling but also a potential revolution in the mining of new REEs. Presently, traditional REE mining processes are notorious for their heavy environmental footprint, characterized by significant pollution emissions.



Figure 6.1: Rare earth element mining in China.

Unlike gold or iron, REEs are not typically found in large, concentrated deposits, necessitating extensive excavation of ore-rich regions. Often, these sites contain ores with radioactive components like uranium, adding another layer of environmental concern.

Following excavation, REEs are further concentrated through processes involving sulfuric acid. Unfortunately, the liberation of REEs from minerals during this stage results in the release of sulfur oxides, contributing significantly to acid rain formation. Moreover, the utilization of sulfuric acid, along with hydrochloric and nitric acids for leaching REEs, poses substantial risks to both workers and the environment.

The final critical step involves the separation of REEs from the acidic solution. Organophosphate solvents such as tributyl phosphate (TBP) serve as selective host molecules, facilitating the dissolution of REEs and their separation from aqueous solutions laden with impurities. However, TBP is known to be toxic and persistent in the environment, posing significant safety concerns throughout its lifecycle.

In summary, the conventional REE extraction and refining processes are marred by severe pollution of water and air. Ecosystems suffer disruption, aquatic life is adversely impacted, and human health is jeopardized, underscoring the urgent need for alternative, more environmentally sustainable approaches.

Bioleaching: Utilizing lanmodulin's distinct properties, integral to our planned recycling initiative, we aim to directly extract Rare Earth Elements (REEs) from ores. This methodology holds significant promise in terms of cost reduction and environmental preservation by mitigating pollution. However, before implementation, rigorous experimentation and validation are imperative. Factors such as efficiency, bacterial survival within bioreactors in mining environments, and operational feasibility necessitate thorough research and optimization. This can be achieved through localized trial runs in mines, gradually integrating and refining our methods on a broader scale.

7 Related Work^{TD}

There are some other studies that are somewhat close to our work. Most of them have the same basic idea at their core. That is to use *M. extorquens* or lanmodulin to recycle rare earth elements.

An example for the usage of only lanmodulin would be the work of Dong et al. [31]. Their approach was to take lanmodulin and attach it to a microbead (a small sphere made of agarose, see bottom left of figure 7.1). The product of this procedure is the immobilized lanmodulin. They made a lot of the immobilized lanmodulin and put it into a column. Afterwards, they let a solution which contained ash from a coal power plant, which in turn contained some REEs, flow through the column. The REEs get caught by lanmodulin, and every other metal flows freely through the whole column. After that, they washed their column, and then they began separating the different rare earths. They achieved this by giving solutions with different pH values into their column. Lanmodulin releases only some certain rare earths at a certain pH which is useful for separating them. When every rare earth has been extracted, the column can be cleaned and even be reused for the next recycling process.

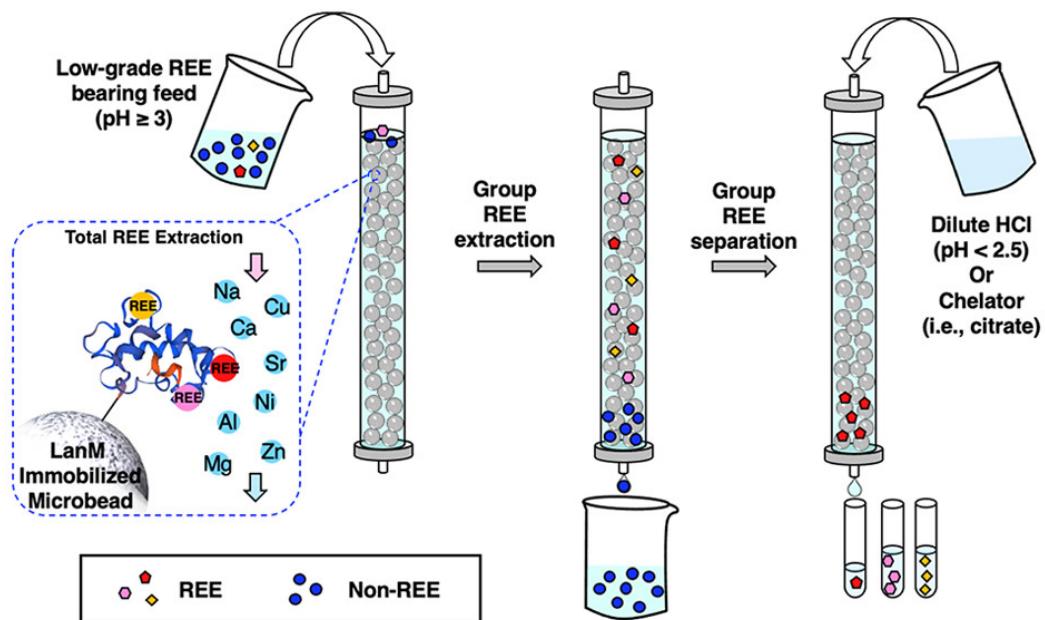


Figure 7.1: Overview of the work which inspired this thesis. Picture from "Bridging Hydrometallurgy and Biochemistry: A Protein-Based Process for Recovery and Separation of Rare Earth Elements", Dong et al. [31].

This is a very clever process that even inspired this thesis. However, this work is not easy to reproduce. It requires costly chemicals and machinery, which only a company or a university can afford. Therefore, it was not feasible at our school. What must also be taken into consideration is that they used a genetically modified bacteria which produced the lanmodulin. This step alone would take too long to achieve for a diploma thesis.

Good et al. took another approach, which is surprisingly similar to our work. Their basic idea was to let *M. extorquens* grow in a solution which contains electronic waste (figure 7.2) and find methods to increase the yield of this recycling method [32]. This approach is fairly similar to our own work. However, this work did not inspire us because the paper was first published on December 27th 2023, when we already had worked three months on our project.

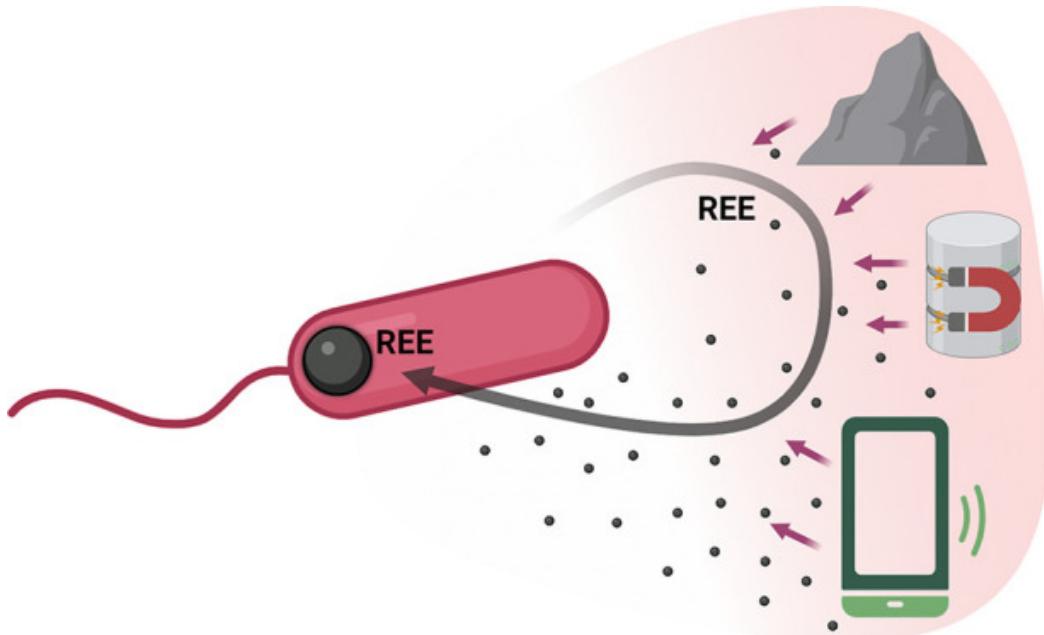


Figure 7.2: Very simplified abstract of the work from Good et al. Picture from "Scalable and Consolidated Microbial Platform for Rare Earth Element Leaching and Recovery from Waste Sources", Good et al. [32].

The main difference to our work is their technological advantage. They used a genetically modified strain of *M. extorquens* AM1, which is called $\Delta mxaF$. They deleted the gene *mxaF* to ensure that the growth of the bacteria is dependent on the uptake of rare earths. This led to a higher rare earth uptake capacity per bacteria.

Another remarkable difference is that they did not only let the bacteria grow with crushed magnets, but also with a crushed smartphone. This did have, interestingly, no significant impact on the growth of *M. extorquens* AM1 $\Delta mxaF$, according to their study.

After that, they improved the yield by adding an organic acid to the bacteria's growth

medium. This helped to extract the rare earths from the crushed magnet (and smartphone). What also boosted their yield was that they genetically engineered *M. extorquens* AM1 $\Delta mxaF$ even further.

We can conclude that our project was done with the minimum of resources you can use to achieve some good results. Compared to the above-mentioned studies, we had very limited resources and only basic laboratory equipment, so we could do only basic work. What the other studies achieved is really great, but we showed that it is possible to be part of the newest developments of science without expensive materials and equipment.

8 Conclusion^{TD}

The process of recycling of rare earths from e-waste using bacteria is a more eco-friendly and energy efficient way than currently established recycling methods. In our project, we achieved to carry out this process and to determine its efficiency. Hereby, it is important to know that we only measured the natural capacity of *M. extorquens* without any additional changes.

In brief, our project can be summarized as follows: We found a way to efficiently recycle rare earth elements from e-waste. This works with the bacteria *Methylorum extorquens*, which has the ability to use rare earth elements in its metabolism. This property of the bacteria is essential because the e-waste is simply given in a crushed form to the culture medium. The rare earths accumulate naturally in the bacteria. The bacteria can then be opened to recover the rare earths.

These results are very promising. We did not only confirm that *M. extorquens* is able to accumulate Nd but without additional changes, it is naturally able to bind more than 70% of the given neodymium.

We learned a lot during the time of this project, because neither of us had previous knowledge in the field of microbiology. This meant that we had to research everything from the ground up. In the beginning, we thought we would do a lot of things differently than we do now. But after three months of work, we came to a dead end because our school lacked the required equipment. This had the consequence that we had to pivot our work in a new direction. Afterward, we finally managed to achieve results.

The key method, which we discovered late in the project was the arsenazo-III assay. This assay is a method to determine the concentration of rare earth elements in a sample. Without this method, we would not have achieved any results at all, because all the other methods we tried did not work well enough.

What is also noteworthy is that we learned that at any given time something unexpected can happen, which ruins the work of a whole day.

Acknowledgements

We would like to express our gratitude for the smooth and conflict-free cooperation within our project team. Without it, our endeavor wouldn't have been possible.

Our supervisor, Benjamin Seeburger M.Sc., deserves a special mention for helping us acquire all the necessary materials and assisting us with research.

We are also thankful to Herbert Ofenmacher and Richard Sommerauer for providing us with e-waste samples and neodymium magnets for our research.

Additionally, we would like to thank Dipl. Ing. Andreas Scherfler and Dipl. Ing. Bernhard Schmeitzl who, despite being supervisors of other teams, were eager to assist us with some of the problems that arose during our project.

We would also like to extend our sincere thanks to the members of other project teams, who were fun and engaging to be around. We would like to give a special shout-out to Robert, Jan, Emily, Lilli, Magda, Samantha, Anna, and Mara.

We would be remiss in not mentioning the 'L' key on Tobias' keyboard, which fell victim to hours of vigorous typing for this thesis and got destroyed.

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