PET Degradation by Microalgae – a Biosynthetic Derivative

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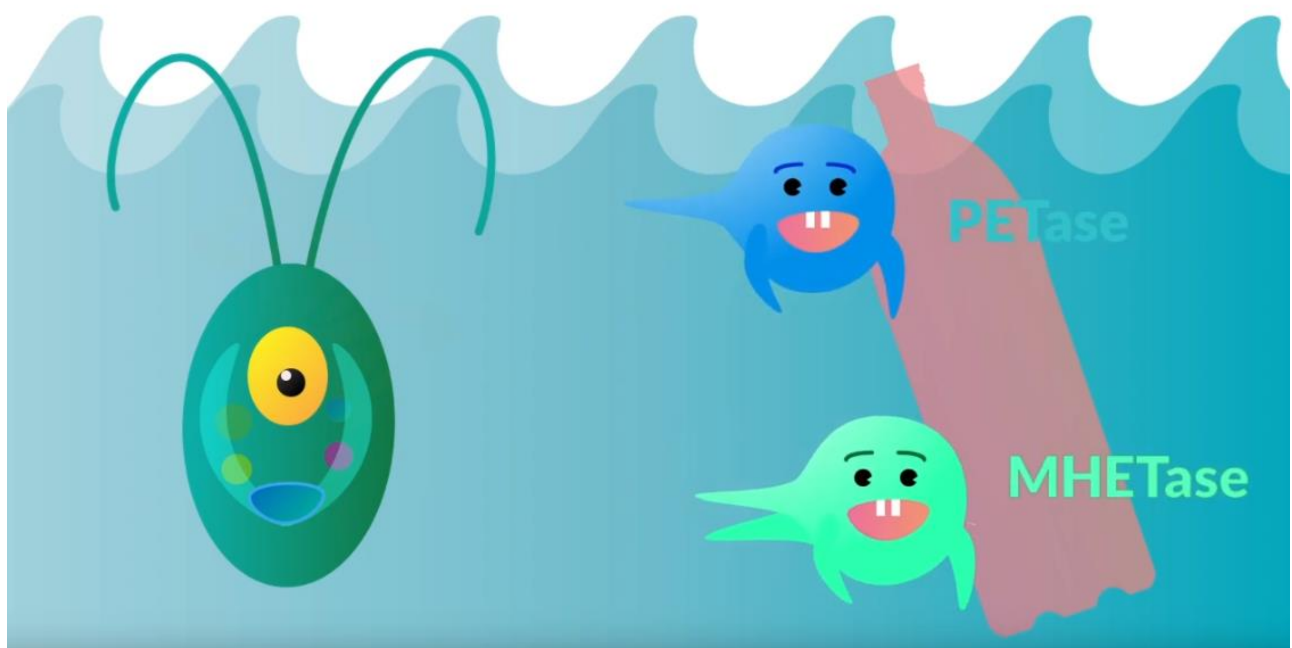

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1 Preface

Looking for an interesting topic for our term paper, we took different points into account. Important to us was to find a topic that we could connect with. During our search through the internet, we came across a video, that introduced a research project that is trying to create microalgae that are able to degrade the common plastic PET.

Given the major problem of plastic pollution in the world and our own interest in an ecological and sustainable future, we were immediately fascinated by this topic. How would this method work? Is it THE solution for the world's plastic problem? A natural organism which is able to digest plastic seemed to be a promising solution, especially for microplastics in the oceans. We wondered how this technique would work and questioned why it is not already in use. Furthermore, we were interested in the process of such a research project and the applied techniques.

We looked at the website of the research team, a group of students at the Humboldt University of Berlin who were working on that problem in the frame of the iGEM, an international competition on synthetic biology. To gain a better insight we started researching and understanding the project. For an interview, we contacted Prof. Dr. Peter Hegemann who was supervising the team during research process. He directed us to his colleague Simon Kelterborn who was willing to help us.

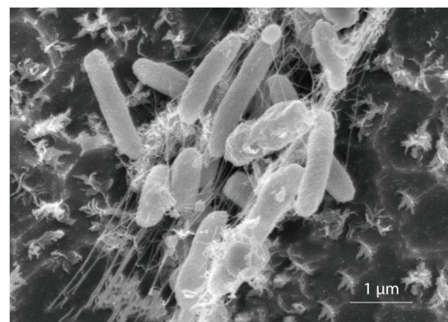
Now, we are going to present the research project of a biosynthetic derivative of two enzymes that are potentially able to degrade polyethylene terephthalate by inserting them into a microalga.

2 Introduction

Plastics are everywhere on Earth. From huge piles near the streets to tiny little microplastic fragments at the bottom of our oceans. Even in our drinking water and growing vegetables microplastics have been found. The plastics will not disappear as for example eggshells, even not after a long period of time. The reason is that the long chains of polymers, which plastics are built of, are very stable and that no natural organism is able to degrade them.

The hope that plastic can be broken down by nature and maybe even recycled afterwards, is a hope that exists ever since humans are aware of the plastic problem. Until recently, no real solution has been found to this problem.

Then in 2016, rays of hope arose: The Japanese scientists Shosuke Yoshida, Kenji Miyamoto and their team of the Kyoto Institute of Technology and the Keio University discovered the two enzymes PETase and MHETase in the microbe *Ideonella sakaiensis.* These enzymes degrade the plastic polyethylene terephthalate, known as PET, by using its carbon as the main energy source for the microbe. More precisely, the enzyme PETase breaks down the polymers into monomers, whereas the MHETase digests these monomers even further. The researchers were surprised about the fact that this enzyme must have evolved over the last 60 years, the timespan plastics became in use. Two points of criticism regarding the discovery by S. Yoshida of the enzyme were, that it took around six weeks for a piece of plastic with the size of a thumbnail to be completely degraded. Furthermore, they have been using a thin PET-film with quite a simple structure which is not comparable with commonly used PET in plastic bottles for example.

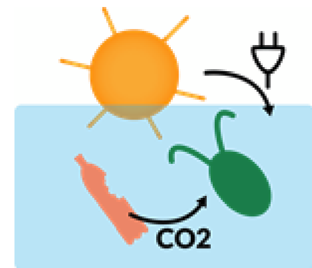


[Figure 1] Microbe Ideonella sakaiensis

With the discovery of these enzymes, a broad field of research opened up and multiple teams started to work on these enzymes. So did John E. McGeehan of the University of Portsmouth, Gregg T. Beckham of the National Renewable Energy Laboratory and H. Lee Woodcock of the University of South Florida. They accidentally built a mutant enzyme that could erode more-highly-crystalline PET, as it is used in plastic bottles. Richard A. Gross of the Rensselaer Polytechnic Institute tried to make the enzyme more stable at higher temperatures. Carbios, a French chemistry company, is currently developing an application of the enzyme at an industrial level with the aim to recycle the monomers that emerge from the first degradation step. They proclaimed, that they succeeded in optimizing the enzyme PETase so well, that it can depolymerize 97% of PET within 24 hours now.

Before and after the discovery of the enzymes in *Ideonella sakaiensis,* investigations for natural degrading processes of plastics were already in progress. In 2017, the biologist Federica Bertocchini successfully found a wax worm caterpillar that could break down polyethylene (PE, mainly used for plastic bags). Also, in 2015, Jun Yang from the Beihang University and his team found a gut bacterium from mealworms consuming polystyrene (PS, harder than PET, used for CD-cases or petri dishes in laboratory equipment) and a bacterium within the Indian meal larvae that degrades polyethylene (PE). The remaining problem in all three cases was that the exact products of the degradation process could not be detected completely.

The biosynthetic technique that we discuss here is also an application and derivative of the PET degrading enzymes found in the microbe *Ideonella sakaiensis*. The research group in Berlin tried to incorporate the enzyme PETase combined with the enzyme MHETase into the freshwater microalgae *Chlamydomonas reinhardtii* with the aim that the algae would digest plastic. This could be a possible solution to reduce the plastic pollution.



[Figure 2] Principle of PET-digesting microalgae

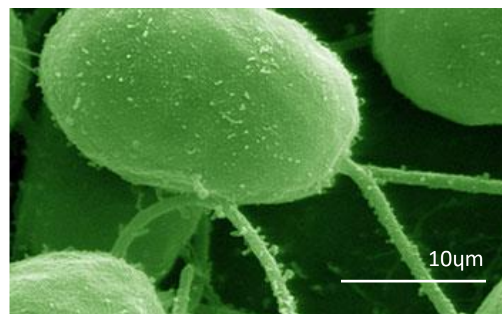
3 Description of Engineering Technique

3.1 Introduction

The aim of the researchers was to develop a new, environmentally friendly way to recycle polyethylene therephtalate (PET). Therefore, the two enzymes PETase and MHETase of the bacterium *Ideonella sakaiensis* got integrated into the genome of a chassis to form a microalga that is able to degrade PET in freshwater and use the carbon as its main energy source.

The enzymes PETase and MHETase are able to break chemical bonds with the help of water, resulting in smaller molecules from their originally larger carbon chains.

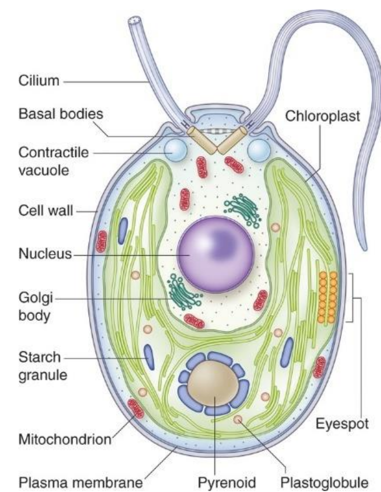
The eucaryotic freshwater microalga *Chlamydomonas reinhardtii (C. reinhardtii)* was used as a chassis since it is a well understood model organism that is very easy to cultivate. Moreover, like plants, it is able to take up carbon dioxide through photosynthesis and use the carbon for its growth. This process is beneficial to reduce the current surplus of carbon dioxide in the atmosphere.



[Figure 3] A false-coloured scanning electron microscope image of C. reinhardtii

3.2 Insertion of the Enzymes into C. Reinhardtii

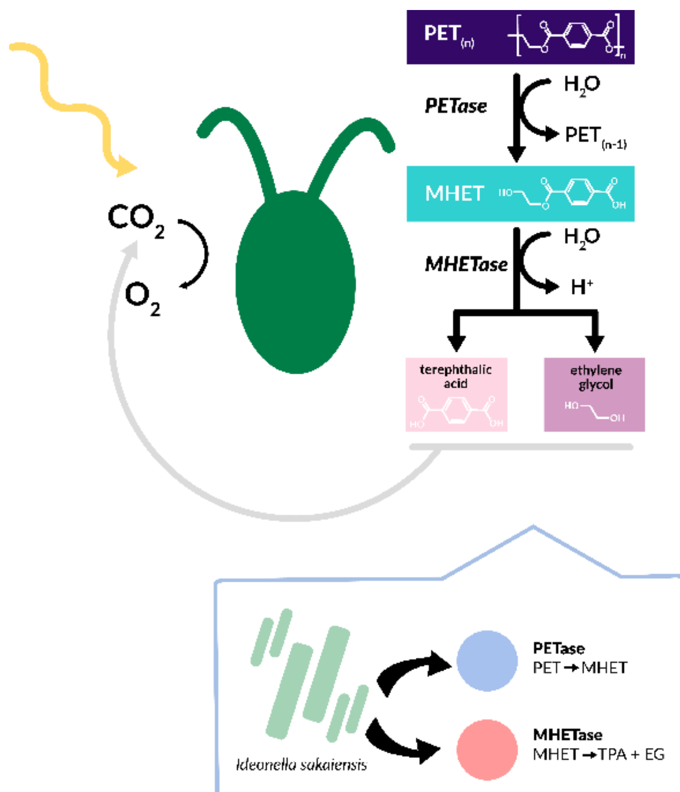
Firstly, DNA encoding for PETase and MHETase were adapted to be expressed in *Chlamydomonas reinhardtii.* The coding sequences of the two enzymes were combined with algae specific promoter (PsaD-Promoter) and terminator sequences. Secondly, the resulting sequences got isolated and inserted into a specific cloning vector. This process is called molecular cloning. Together with an antibiotic selection marker, this genetic construct including PETase, MHETase, PsaD-Promoter and PsaD-Terminator was transformed into the microalgae by electroporation.



[Figure 4] Structure and organelles of C. reinhardtii

In the electroporation method, an electrical pulse was used to create temporary pores in the cell membrane of *C. reinhardtii* through which the expression construct can pass into the cell.

To check whether this nuclear transformation of the genome was successful, the team carried out several investigations. One method they applied is screening with the help of the colony polymerase chain reaction (colony PCR). The results verified that the sequence of the PETase was intact after the insertion into *C. reinhardtii,* but there was no evidence found for the presence of the intact MHETase sequence.



[Figure 5] Degradation of PET by PETase and MHETase providing carbon source to C. reinhardtii

3.3 Degradation Process

Upon expression, the enzyme PETase degrades the long chain polymer of polyethylene therephtalate (PET) with the help of water into single molecules called monomeric mono-2-hydroxyethyl terephthalate (MHET). Subsequently, the enzyme MHETase converts MHET into ethylene glycol (EG) and terephthalic acid (TPA) with the help of water.
Hydrogen cations result as by-products.

The toxity of the end products EG and TPA of the degradation of PET for *C. reinhardtii* has been tested as well. While growth of the microalgae was not limited by TPA, EG was inhibitory, but only above a concentration of 5%.

4 Documentation and Pictures of Research

4.1 The Scientists and the Research Project ChlamyHUB

ChlamyHUB is the name of the research project the student group of the Humboldt University Berlin worked on. They participated at the International Genetically Machine competition (iGEM), that is carried by the iGEM Foundation (Figure 6). With this competition the iGEM Foundation desires to provide a platform for the advancement of synthetic biology.

[Figure 6] Logo of iGEM

"By working together, students […] around the globe are addressing regional and global challenges both inside and outside the lab.", their website explains.

At the competition in 2019, the group of 16 students, self-named Chlamylicious iGEM Team, was quite successful. Nevertheless, we want to focus on the group's research work and not on the competition the team has accomplished.

4.2 What did the group work on?

As shown in their logo (Figure 7) the Chlamylicious iGEM Team worked on different sectors and goals at the same time. The development of the plastic digesting microalgae was only one goal out of five sectors.

[Figure 7] Logo of the research project ChlamyHUB

PET degradation: The implementation of the enzymes found in the microbe *Ideonella sakaiensis* into the microalgae *Chlamydomonas reinhardtii,* and the subsequent aim that the microalgae can degrade polyethylene terephthalate (PET) in sweet water.

Parts: The development of a toolkit of genetic parts, called ChlamyHUB Collection, that should help future iGEM Teams to work with *C. reinhardtii* as a chassis for further research on synthetic biology.

Cultivation: The construction and optimisation of a Do-It-Yourself bioreactor called openPBR. It should become a low-budget alternative to the commonly used, but expensive photobioreactors. The OpenPBR was also designed as habitat for the modified *C. reinhardtii*.

[Figure 8] Bioreactor openPBR

Modelling: The creation of models in which the impact of the enzymatic products on the growth of algae was calculated.

Human Practice: The collection of as much as possible knowledge over algae cultivation, through exchange with other experts and the incorporation of that knowledge into their work. This also included the presentation and visualization of their work to lay people.

4.3 Interview with Simon Kelterborn

Simon Kelterborn

Simon Kelterborn, the Principal Investigator of the Chlamylicious iGEM Team, was willing to give us an interview to gain a better insight into the work of the ChlamyHUB project and the competition. Here is an excerpt from the interview, which can be found in full length in the appendix.

I. What was your role within the research group?

I initiated the process for the Humboldt-University to take part at the iGEM competition. Together with Prof. Schmitz-Linneweber, we looked for students who would be interested to take part and also both of us assisted the team all the way to the jamboree: Project finding, lab work, wiki, preparation for the jamboree. Also, I had the privilege of accompanying the team to the Giant jamboree in Boston.*

**The iGEM competition*

II. What was your motivation of choosing a project degrading plastic?

The only condition that I made to the team was to work with the organism Chlamydomonas reinhardtii. *The idea to degrade plastics came solemnly from the students. I guess they felt that's a really big issue for the global environment and currently humanity has no clue how to solve it. Although the use of microorganisms to degrade plastics is not new to iGEM it remains a revolutionary concept to address this issue.*

III. How long did you work on the project?

We started to form the team in summer 2018. The wet lab started in winter 2018/2019 and alternating groups of students kept working on the project until the very last day before leaving Berlin for Boston.

IV. How did you come up with the idea of using the microalgae *Chlamydomonas reinhardtii* as your chassis?

As mentioned above, this condition was given by me as this green algae is the organism I worked with and where my expertise is. Most of the iGEM teams work with bacteria, specifically with E. coli*. So I felt it's important to extend the list of established organisms at iGEM. Being eukaryotic and a phototrophic organism, the green algae* Chlamydomonas reinhardtii *holds great potential for synthetic biology applications. But most importantly, for exactly such a project that aims to degrade a pollutant from the environment you need a chassis that is able to grow there.* E.coli *wouldn't grow in the seawater where the plastic pollution is.*

V. What was your final outcome?

We did not succeed in creating an alga that can degrade plastics. But we achieved to create a toolbox with a set of genetic parts and the openPBR that enables future iGEM teams to work with Chlamydomonas reinhardtii *to foster the "green" synthetic biology.*

We thank you for the interview and your openness!

5 Discussion

The researcher group succeeded in inserting the enzyme PETase into the genome of the DNA of *C. reinhardtii*, whereas this was not the case for MHETase. Unfortunately, it could not be proven whether the algae were able to degrade PET afterwards, or whether the correspondent genome was expressed at all. Nonetheless, the Chlamylicious Team gathered a whole collection of parts, that can be used for further research with *C. reinhardtii* as its chassis. After the iGEM competition, the research group ended their work on the project. They saw it more as a scientific exercise rather than a lifelong research, as S. Kelterborn expressed in the Interview.

In general, the research on organisms that are able to degrade plastic is a broad field that has just opened. Several different organisms which are able to degrade or depolymerise plastics have been recovered. *Ideonella sakaiensis* or the wax worm caterpillar from Federica Bertocchini are only a few examples. Also, the transformation of *C. reinhardtii* into a plastic digesting organism is still a valuable topic. Now it is time to develop these options further as well as search for synthetic applications as the ChlamyHUB tried to do.

The deployment of phototrophic organisms at an industrial level needs further investigation as well. Simon Kelterborn explained that deployment of the modified *C. reinhardtii* on industrial level would be possible, but for now not cost-efficiently enough. Therefore, it is not very likely that this will happen soon.

Another open question is how to deal with the resulting products TPA and EG from PET degradation. According to Simon Kelterborn, one option would be to extract them to make new plastics, although this process seems to be very inefficient. They prefer to enable the algae to use TPA and EG as its main energy source. This is still an unresolved research issue.

In addition, all the recent investigations focused on PET, PE and PS. These are only a small part of all plastic types that exist. It is likely that every plastic type needs an individual approach for its degradation.

An additional discussion is going on whether to use genetically modified organisms in natural environment at all. For example, the European Union is handling this topic very carefully and blocked all attempts of introducing genetically modified organisms freely into the environment. The reason for this is the risk that genetically modified organisms may perturb natural ecosystems. Genetically modified *C. reinhardtii* could therefore be only applied under controlled settings.

Nevertheless, the question whether it is ethically justifiable to genetically change the natural genome for the protection of the environment remains an interesting debate.

Overall, the degradation of plastics by natural organisms will not be the solution to our plastic problem, if we produce and use more and more plastic in our daily life. We must stop or at least reduce the production of new plastic and reuse already existing plastics. Nevertheless, we still need to find solutions for the plastic pollution. One real challenge thereby is the microplastic that we cannot fish out of the water again. Where the ChlamyHUB failed, one day the science will maybe present a solution for that. Simon Kelterborn gave us some hope for this because he confirmed that a similar process as they applied in the freshwater alga *C. reinhardtii* would be possible for a saltwater alga too.

6 Summary

The paper presents a biosynthetic derivative of the plastic degrading enzymes PETase and MHETase found in the microbe *Ideonella sakaiensis.* A research group of students at the Humboldt University of Berlin tried to insert the open reading frames of the two enzymes into the genome of the microalgea *Chlamydomonas reinhardtii,* with the aim to modify *C. reinhardtii* in a way that it becomes able to degrade plastic in and from its sweet water surrounding. Unfortunately, they failed in developing a clone of the *C. reinhardtii*, that was able to degrade plastic, even though the PETase was successfully inserted into the genome. In any case, what the researchers have started should be continued in some form, as it is a promising idea for solving the plastic problem we are dealing with.

7 References

Main sources for all paragraphs

- https://2019.igem.org/Team:Humboldt_Berlin
- Interview with Simon Kelterborn (see appendix)

Additional sources for different paragraphs

Preface

- Video: https://www.youtube.com/watch?v=eSFRQVzlrrM

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Presentation of research group

- https://igem.org/Main_Page
- https://2019.igem.org/Team:Humboldt_Berlin

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- Figure 4 : https://www.nsf.gov/news/mmg/mmg_disp.jsp?med_id=79712&from=mmg
- Figure 6 : https://igem.org/Main_Page

8 Appendix

- Interview with Simon Kelterborn

Interview ChlamyHUB – Simon Kelterborn

Berlin, 01/08/21

Personal Questions:

- What were your tasks/what was your role in the research group?

I initiated the process for the Humboldt-University to take part at the iGEM competition. So together with Prof. Schmitz-Linneweber, we looked for students who would be interested to take part and also both of us assisted the team all the way to the jamboree: Project finding, lab work, wiki, preparation for the jamboree. Also, I had the privilege of accompanying the team to the Giant jamboree in Boston.

Questions of Process:

- What was your motivation of choosing a project degrading plastic?

The only condition that I made to the team was to work with the organism Chlamydomonas *reinhardtii.* The idea to degrade plastics came solemnly from the students. I guess they felt that's a really big issue for the global environment and currently humanity has no clue how to solve it. Although the use of microorganisms to degrade plastics is not new to iGEM it remains a revolutionary concept to address this issue-

- How long did you work on the project?

We started to form the team in summer 2018. The wet lab started in winter 2018/2019 and alternating groups of students kept working on the project until the very last day before leaving Berlin for Boston.

- How did you come up with the idea of using the microalgae *Chlamydomonas reinhardtii* as your chassis?

As mentioned above, this condition was given by me as this green algae is the organism I worked with and where my expertise is. Most of the iGEM teams work with bacteria, specifically with *E. coli*. So I felt its important to extend the list of established organisms at iGEM. Being eukaryotic and a phototrophic organism, the green algae *Chlamydomonas reinhardtii* holds great potential for synthetic biology applications.

But most importantly, for exactly such a project that aims to degrade a pollutant from the environment you need a chassis that is able to grow there. *E.coli* wouldn't grow in the seawater where the plastic pollution is…

- What made you so sure that it would work?

We were never sure it can work. Actually, we thought the opposite: “To the best of our knowledge, it will not work. But let’s try it anyway and see what’s happening”. No PI would work on such a project because there are probably too many obstacles, so such projects are never worked on in “real science life”.

And I think that’s exactly the motivation of the iGEM competition. To work on “the impossible” and to expand the boundaries of what we think is possible. In the end most of the 300 iGEM projects fail, but as you can see on the finalist, some teams do succeed, which nobody anticipated. And most importantly, from all problems that each of the teams face we all learn where exactly are the missing parts and maybe next year another team comes up with a new idea to solve this exact issue. So the boundary’s what is possible are constantly shifting.

- The topic is quite new; did you have other competitors while researching?

Well, over the last year, many iGEM teams worked on finding a biological way to degrade plastics, so we expected that there would be “competitors”. Actually there was even a team working with *Chlamydomonas reinhardtii* on the same topic. However, the original iGEM spirit is not about competing against each other but to compete together for the best solution.

- What was your biggest success/most thrilling moment?

I guess this question would have to be answered from one of the students. For me the most thrilling moments were towards the end when the students came to me full enthusiasm to update me on the newest results.

Questions of Technical Aspects and General Understanding:

- Could you give a short summary of the research topic?

So besides the original plan to build a green alga that is able to degrade plastics, it was the aim to supply the iGEM community with tools that enable future iGEM teams the use of this organism.

- What is the UVM4 strain of *Chlamydomonas reinhardtii* exactly?

UVM4 = UV-mutagenized clone 4. This strain was randomly mutated by UV-radiation and selected for clones that show higher expression of transgenes.

· For what reason did you develop the openPBR exactly?

One of the limiting factors to work with phototrophic organisms like *C. reinhardtii* are the expensive Photobioreactors. So both, for our own experiments and to enable other teams the possibility to build their own low-budget reactor we developed the openPBR.

· At "Variation of the Cultivation Density", which ratio is denser? (1:10 or 1:100)

1:10 is denser. It means 1 part algae in 10 part media (e.g. 1 ml algae + 9 ml fresh media)

· Could you give a brief explanation on how you implemented the enzymes PETase and MHETase into the DNA of *C.reinhardtii* ?

DNA that codes for PETase and MHETase were codon-adapted to be expressed in *C. reinhardtii* and synthesized (= ordered from a company). In our lab we combined this DNA with an algae specific promoter (PsaD-Promoter) and a Terminator sequence. We used a cloning method called "MoClo" to combine these different parts. To amplify and store these genetic sequences it is routine to vectors that are replicated in *E. coli* cells. Together with a antibiotic selection marker this genetic construct (PsaD-Promoter – PETase – PsaD-Terminator) is then transformed into the Chlamydomonas genome by electroporation. We select on the antibiotic containing plates for cells that were transformed and eventually contain the pETase gene.

· Could give a brief explanation on how your Chlamy is living in the bioreactor? How does the process of degrading PET work?

We supply the algae with the nutrients they need. Additionally they got the light from the reactor and air, bubbled through the chamber for CO2 supply. The idea was that the PETase got secreted by the cells to degrade the PET. However we never could prove that the PETase was expressed and secreted to the media.

· What happens with the end products TPA and EG? Are they filtered out of the water? And is it then possible to make plastic out of them again? If yes, did you try this?

So there are different ways how to use TPA and EG. Either they can be extracted from the media and used to make new plastics. As this process seems to be very inefficient, our preferred way would be to give the algae the opportunity to use the TPA and EG for their metabolism, so they can use these chemicals as their main carbon source. However, we left this issue for the next iGEM team. J

· What is your final product/ What are your findings?

We did not succeed in creating an algae that can degrade plastics. But we achieved to create a toolbox with a set of genetic parts and the openPBR that enable future iGEM teams to work with *Chlamydomonas reinhardtii* to foster the "green" synthetic biology.

· Did you take any judicial steps for getting a patent or to licence one of your awards?

No. We believe science should be open source, so everything we did is documented and available from our wiki (https://2019.igem.org/Team:Humboldt_Berlin)

Questions to the Future:

· After the iGEM, how did the team and the project move on? What is your current status?

Two students choose their topic for their bachelor thesis from this iGEM project but in general the project was over with the end of the iGEM competition.

· What are your future plans? Did you make any attempts to implement the Chlamy also on industrial level? If not, how successful would you estimate that to be?

No, we saw this project as a scientific exercise. The use to phototrophic organisms in an industrial level seems very attractive because such organisms can use their "energy" for living from the sunlight and even take up CO_2, which is the biggest challenge for this century. However, to grow and maintain big bioreactors with algae is too expensive to be cost efficient for a commercial setting. Unfortunately, as long as the price for energy and CO_2 are so low there are only very few applications where the use of phototrophic organisms is cost-efficient.

· Would there be a possibility of a similar process for saltwater algae?

Of course!

We thank you for the interview and your openness!