2022

PlastoCyan

PROJECT REPORT FOR THE FINAL MEETING

Interreg
EUROPEAN UNION
Austria-Czech Republic
European Regional Development Fund

ÚVOD

Vážení přátelé,

Mikrobiologický ústav AV ČR – Centrum Algatech v Třeboni ve spolupráci s Technickou univerzitou ve Vídni a Univerzitou aplikovaných věd ve Welsu v Horním Rakousku si Vám dovoluje představit projekt Produkce biologicky rozložitelného polymeru polyhydroxybutyrátu (PHB) ze sinic cestou kultivace v odpadních vodách, zkráceně **Plastocyan**, který vznikl v rámci programu INTERREG V-A Rakousko – Česká republika.

Společnost označuje současnost jako dobu plastovou. Plasty jsou univerzální a odolný materiál používaný po celém světě ve všech průmyslových odvětvích. Plasty vyráběné na bázi ropy způsobují vážné ekologické problémy kvůli jejich nerozložitelné povaze. Jakkoli bylo navrženo mnoho strategií pro kontrolu plastového odpadu, mikroplasty, nanoplasty a likvidace plastů stále způsobují obrovskou kontaminaci, která zatěžuje půdu i vodní prostředí. Navzdory vyčerpání neobnovitelných fosilních zdrojů, rostoucímu tempu cen ropy a negativnímu dopadu na životní prostředí je celosvětová poptávka po plastech stále na vzestupu. Tato alarmující situace vede k hledání alternativ. Biologicky odbouratelné plasty vyrobené mikroorganismy, jako jsou polyhydroxyalkanoáty (PHA), se ukazují jako nejlepší řešení, jak nahradit konvenční plasty a chránit tak životní prostředí.

Projekt Plastocyan přichází s novou technologií výroby 100% biodegradovatelného bioplastu - polyhydroxybutyrátu (PHB), přírodní cestou, a to pěstováním sinic v odpadní vodě.

EINFÜHRUNG

Liebe Freunde,

Das Institut für Mikrobiologie des CAS – Algatech Centre in Třeboň, in Zusammenarbeit mit der Technischen Universität Wien und der Fachhochschule Wels, Oberösterreich, möchte das Projekt Produktion von biologisch abbaubarem Polymer Polyhydroxybutyrat (PHB) aus Cyanobakterien durch Kultivierung in Abwasser, kurz Plastocyan, vorstellen, das im Rahmen des INTERREG V-A Programms Österreich - Tschechische Republik eingerichtet wurde.

Das Unternehmen bezeichnet die Gegenwart als das Plastikzeitalter. Kunststoffe sind ein vielseitiges und langlebiges Material, das weltweit in allen Branchen eingesetzt wird. Kunststoffe auf Erdölbasis verursachen ernsthafte Umweltprobleme, da sie nicht abbaubar sind. Obwohl viele Strategien zur Eindämmung von Kunststoffabfällen vorgeschlagen wurden, stellen Mikroplastik, Nanoplastik und die Entsorgung von Kunststoffen nach wie vor eine enorme Belastung für den Boden und die aquatische Umwelt dar. Trotz der Erschöpfung der nicht erneuerbaren fossilen Ressourcen, der steigenden Ölpreise und der negativen Auswirkungen auf die Umwelt steigt die weltweite Nachfrage nach Kunststoffen weiter an. Diese alarmierende Situation führt zu einer Suche nach Alternativen. Biologisch abbaubare Kunststoffe, die von Mikroorganismen hergestellt werden, wie z. B. Polyhydroxyalkanoate (PHA), erweisen sich als die beste Lösung, um herkömmliche Kunststoffe zu ersetzen und so die Umwelt zu schützen.

Im Rahmen des Plastocyan-Projekts wurde eine neue Technologie zur Herstellung von 100 % biologisch abbaubarem Biokunststoff - Polyhydroxybutyrat (PHB) - auf natürliche Weise durch das Wachstum von Cyanobakterien in Abwässern entwickelt.

INTRODUCTION

Dear friends,

The Institute of Microbiology of the CAS – Algatech Centre in Třeboň, in cooperation with the Technical University of Vienna and the University of Applied Sciences in Wels, Upper Austria, would like to introduce the project Production of biodegradable polymer polyhydroxybutyrate (PHB) from cyanobacteria by cultivation in wastewater, abbreviated as Plastocyan, which was established within the INTERREG V-A Austria - Czech Republic program.

The company refers to the present as the plastic age. Plastics are versatile and durable materials used worldwide in all industries. Petroleum-based plastics cause serious environmental problems due to their non-degradable nature. Although many strategies have been proposed to control plastic waste, microplastics, nanoplastics, and plastic disposal still cause enormous contamination, which affects soil as well as aquatic environments. Despite the depletion of non-renewable fossil resources, rising oil prices, and the negative environmental impact, the global demand for plastics is still growing. This alarming situation is leading to a search for alternatives. Biodegradable plastics produced by microorganisms, such as polyhydroxyalkanoates (PHAs), are the best solution to replace conventional plastics and thus protect the environment.

The Plastocyan project has developed a new technology to naturally produce 100% biodegradable bioplastic - polyhydroxybutyrate (PHB) - by growing cyanobacteria in wastewater.

THE PROJECT'S MISSION

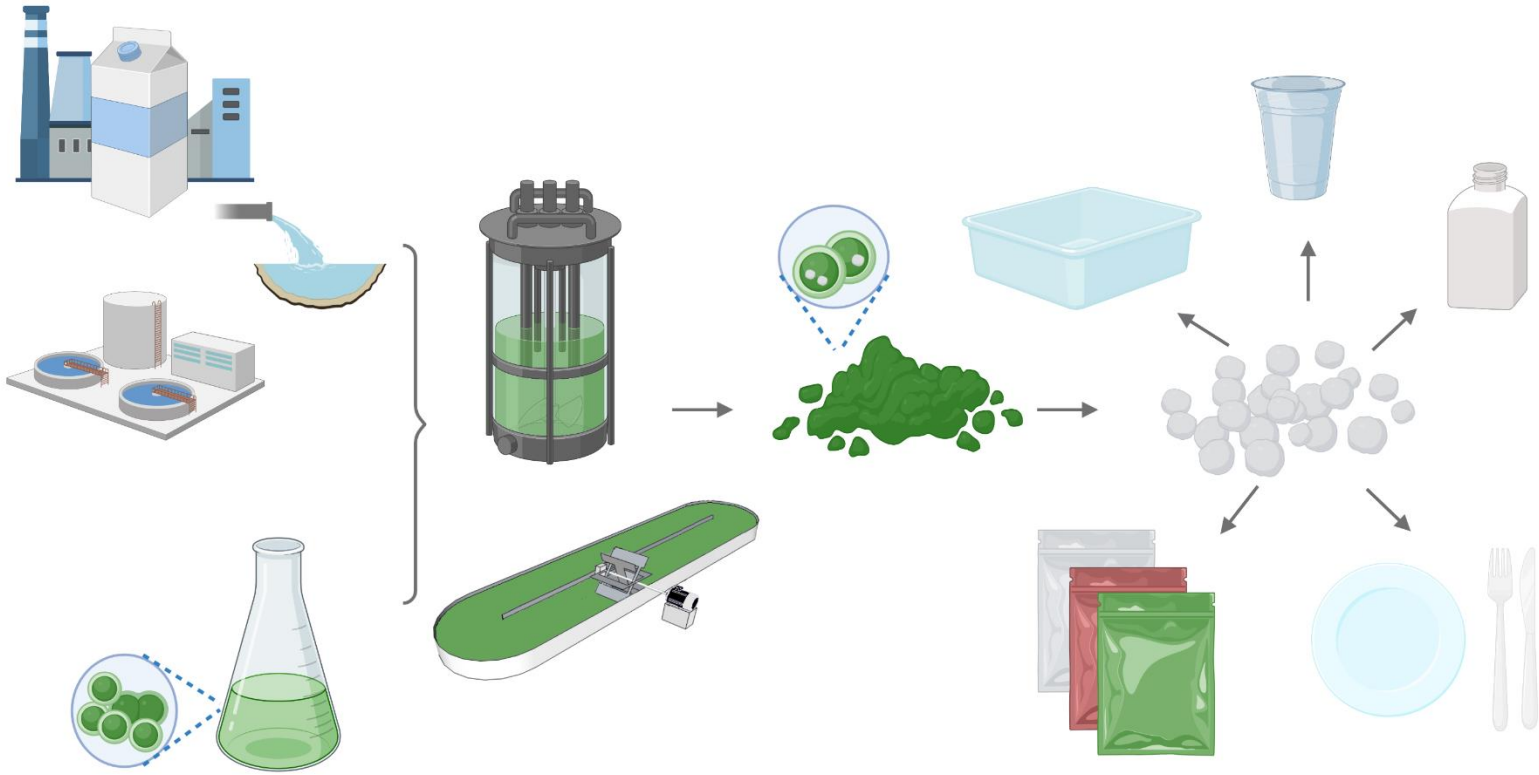
The project’s main objective is to develop ecologically innovative technology for the production of bioplastics from cyanobacteria, which use municipal wastewater and wastewater from the dairy industry as a source of nutrients for their growth.

PHB AND ITS PRODUCTION BY MICROORGANISMS

Polyhydroxybutyrate (PHB) is a thermoplastic, water-repellent, ultraviolet-resistant plastic with properties that replace conventional plastics such as polypropylene or polyethylene.

Under certain conditions, some micro-organisms can form PHB granules which serve as a carbon reservoir. Chemotrophic bacteria produce natural PHB, producing up to 80 % per dry cell weight in several days. Nevertheless, the production cost of PHB is much higher compared to that of conventional plastics. In the production of PHB, up to 50 % cost is precursor substrate material, especially the carbon source. Most cyanobacteria naturally produce 5-20 % PHB of their cell mass. Cyanobacteria cannot compete with chemotrophic bacteria in terms of PHB content or biomass growth rate. However, since cyanobacteria are photosynthesizing organisms, their carbon source is CO_2 obtained from the air. Unlike bacterial PHB producers, photoautotrophic cyanobacteria do not consume sugars and are, therefore, not dependent on crops, making them a green alternative production system.

THE PLASTOCYAN PROJECT



The project has succeeded in developing a cyanobacterial cultivation process where the substrate is wastewater. Two types of wastewater were used for the growth of cyanobacterial biomass (i) municipal wastewater from the treatment process and (ii) wastewater from dairy production. Both substrates are sources of nitrogen (250 mg/L for both substrates) and phosphorus (150 mg/L municipal wastewater and 25 mg/L dairy wastewater), the main components required for cyanobacterial growth.

A strain of the cyanobacterium *Synechocystis* generated by UV mutagenesis with higher PHB production was used for cultivation. A thin-layer outdoor cultivation unit was used for the cultivation in the wastewater of the municipal sewage treatment plant. Cultivation in dairy wastewater was carried out in a closed annular photobioreactor. This was performed to optimize conditions for later testing a genetically engineered strain with higher PHB production and lactose processing ability. Cultivating the strain in both pilot-scale units took 4-6 weeks to achieve the maximum amount of PHB. On the day of biomass harvest, PHB content was 23 % of the dry cell weight in municipal wastewater and 10 % in dairy wastewater. Compared with available data, PHB production in the phototrophic regime (no organic carbon source) of genetically unmodified cyanobacteria is 4-25% under optimal culture medium conditions in pilot-scale units. The strain used in the Plastocyan project achieved 37% PHB cell dry weight under laboratory conditions on a small scale in the optimal growth substrate in the previous study. However, the main benefit of the project is the recycling of wastewater. The results of the project are also above average in this respect. In both types of wastewater, it was possible to grow biomass up to a density of 2-2.5 g/L, while the average values of cyanobacterial growth in wastewater are reported to be 0.6-3.15 g/L.

PHB granules can be extracted from the cells by various extraction methods, mainly using organic solvents such as chloroform, which can be hazardous to the environment. The project also addresses the environmentally and economically friendly alternative of extracting PHB from biomass using so-called ionic liquids belonging to the category of green solvents. In many cases, extracting the product using ionic liquids is more accessible, can be recycled, and therefore reused. The project is also developing a genetically modified strain that can overproduce PHB and use lactose as a carbon source. This could streamline PHB production in closed cultivation units using dairy wastewater. The UV-generated mutant *Synechocystis* sp. PCC 6714 Mt_a24 was genetically improved by eliminating two genes (spsA and glgC) related to competing metabolic pathways. This elimination is supposed to trigger a metabolic shift towards an increased PHB production rate. At the same time, genes enabling lactose utilization (beta-Gal) as a carbon source were cloned and successfully introduced in the cyanobacteria. Moreover, work focusing on increasing PHB production (cloning of plasmids containing phaA and phaB genes) is still ongoing.

<u>The main results achieved in the project include:</u>

a) Optimization of cyanobacteria cultivation in wastewater (urban wastewater, dairy wastewater) for the production of bioplastic - polyhydroxybutyrate (PHB) on a pilot scale.
b) Ecological extraction of PHB from cyanobacterial biomass using ionic liquids.
c) Generating genetically improved strains with the potential for higher PHB production and utilization of lactose for their growth.

Three institutions, the Institute of Microbiology of the CAS - Algatech Centre in Třeboň (MBU), the University of Applied Sciences in Wels, Upper Austria (FH OOE), and the Technical University of Vienna (TU Wien,) are collaborating on the project. Each partner coordinates a specific work package taking into account their expertise.

The following activities describe the results and outputs achieved by the project.

Activity A.T1.1 Cultivation of PHB-producing cyanobacteria in media containing wastewater

Coordinator: MBÚ

In this activity, wastewater from two different sources, (i) wastewater from the municipal wastewater treatment plant in Třeboň and (ii) wastewater from dairy production provided by Madeta a.s., were tested. This activity aimed to create a suitable substrate from the wastewater to obtain cyanobacterial biomass that also forms a biodegradable plastic - polyhydroxybutyrate (PHB).
It was possible to adjust the cultivation parameters to eliminate the presence of cyanobacterial predators that can occur during large-scale cultivations. The cyanobacterium *Synechocystis* sp. was cultured in wastewater on a pilot scale. The growth of *Synechocystis* sp. PCC6714 Mut_a24 was optimized in a pilot-scale cultivation unit called TL-RWP (thin-layer raceway pond) in the medium with wastewater from the municipal wastewater treatment plant in Třeboň. The cultivation was carried out also in a closed annular photobioreactor with dairy wastewater as a substrate, which is the basis for the successful implementation of the technology. The biomass obtained from these cultivations (110 g TL-RWP; 18 g PBR) contained the biodegradable polymer – PHB. On the day of harvesting, PHB comprised 23% of the dry weight of biomass in the municipal treatment plant effluent and 10% in the dairy effluent. The biomass was provided to the partner TU Wien for further analysis.

Partial output A.T1.1.1 Report on the selection and analysis of various wastewaters as a source of nutrients for cyanobacterial cultivation
The so-called centrate from the wastewater treatment plant was centrifuged to obtain wastewater suitable for cultivation. Wastewater was analyzed externally by Povodí Vltavy, a state enterprise and water management laboratory in České Budějovice (Tab. 1). As part of this activity, a test was carried out to determine the effects of the wastewater on growth and the most suitable treatment of the wastewater was selected for further cultivations (Fig. 1). The impact of wastewater treatment (heat, UV treatment, dilution, etc.) was examined to suppress the growth inhibitors and presence of microorganisms (Tab. 2). During the validation, the physiological state of the culture was determined by measuring photosynthesis, the presence of contaminants, and biomass growth, nutrient degradation in the wastewater during cyanobacterial growth, and the PHB content of selected samples (analysis as part of a staff trip to the project partner). Based on the results, UV sterilization was chosen as the most appropriate wastewater treatment for pilot-scale cultivation. Also, four types of dairy wastewater provided by Madeta Inc. were analyzed (labeled M1, M2, M3, and M4 in Table 1). Based on initial tests, the most suitable dairy wastewater (M1) with sufficient nutrients and minimum inhibitors for cyanobacterial growth was selected for PHB production. This wastewater was used for pilot cultivation in a photobioreactor.

Tab 1 Chemical analysis of wastewater, CWW (clean wastewater) wastewater after complete treatment at WWTP, WW (wastewater) centrate taken from activated sludge, HT WW (heat treatment) centrate treated with heat, UV WW centrate treated with UV, M1 sewage from the dairy, M2 wastewater from cheese production, M3 wastewater from cream tanks, M4 wastewater from butter production.

Wastewater analysis [mg L^{-1}]	**CWW**	**WW**	**HT WW**	**UV WW**	**M1**	**M2**	**M3**	**M4**	**BG-11media**
BOD*	6.5	150	190	170	1400	4000	15000	450	-
COD**	34	740	720	730	2700	4700	32000	1900	-
TOC***	15	310	300	300	1100	1800	8200	520	-
Nitrates	-	-	-	-	320	22	8.7	6.2	-
$N\text{-}NO_3$	7.5	<0,15	<0,15	<0,15	72	5.0	2.0	1.4	250
$N\text{-}NO_2$	0.006	0.005	0,005	0.003	0,07	0.01	0.002	0.004	-
$N\text{-}NH_4$	0.02	180	170	170	3.0	1.7	0.47	0.2	-
Total N	**8.7**	**250**	**240**	**250**	**260**	**120**	**120**	**16**	**250**
$P\text{-}PO_4$	2.3	150	130	140	12	21	8.0	1.1	7
Total P	**2.8**	**160**	**140**	**150**	**24**	**30**	**17**	**2.0**	**7**

*Biological oxygen demand; **Chemical oxygen demand; ***Total organic carbon

Tab 2 Determination of bacterial colony units after different wastewater treatments.

treatment	**Colony forming units [CFU/ml]**
CWW	6800
WW	18000
50% ředená WW	9000
HT WW	260
UV WW	0

Fig 1 Cultivation in glass cylinders for the analysis of different types of wastewater.

Partial output A.T1.1.2 Standard Operating Procedure (SOP) for growing cyanobacteria on a pilot scale in media containing wastewater

Standard Operating Procedure for Cultivation Unit

A) thin-layer raceway pond (TL-RWP)

Thin-layer raceway pond placed in a greenhouse with a cultivation area of 5 m^2 and a working volume of 100-600 L, a cultivation layer thickness between 15 and 60 mm, and a flow rate of about 0.2 m/s. Mixing is provided by a paddle wheel.

B) annular photobioreactor (PBR)

A 30 L annular photobioreactor with a total height of 100 cm, outer diameter of 30 cm, and inner diameter of 18 cm, with adjustable internal LED lighting. The culture is mixed with air through tubes located at the bottom of the photobioreactor. The optimum thickness of the cultivation layer (light path) is 5,5 cm.

Standard operational protocol:

Cyanobacterial strain: *Synechocystis* sp. PCC6714 Mt_a24

1. Pre-cultivation of a strain of the cyanobacterium *Synechocystis* from a petri dish into a 5-15 L culture bottle.
2. Seed culture cultivation conditions: sterile inorganic medium -BG-11; pH 8.0; temperature 26-30 °C; continuous illumination with light intensity 100 μmol photons m^{-2} s^{-1}; mixing with air + 1% CO_2 (v/v. Cultivation time - to late exponential growth phase (10-14 days).

3.a Cultivation in an outdoor thin film recirculating tank (TL-RWP) using wastewater from a municipal treatment plant. Approximately 90 L of centrifuged activated sludge, called centrate, is required for cultivation in the TL-RWP. The sediment is 10-20 %, centrifuged at 4000 g for 5 min. Optimum conditions: greenhouse temperature 15-34 °C. Maximum daily solar radiation ≤ 1000 μmol photons m^{-2} s^{-1}.

4.a The centrifuged wastewater is partially sterilized with an immersion UV lamp. Sterilization of the entire volume for one hour is ensured by bubbling air.

5.a The substrate, i.e., wastewater, is loaded into the TL-RWP, and the culture inoculum is added to a final optical density of OD750 = 0.4. (The volume is calculated by calculating the mixing equation: OD_{750}inoculum x X liters = 0.4 x 100 liters).

3.b Cultivation in a closed anaerobic photobioreactor using wastewater (M1 Sub-output A.T1.1.1) from dairy production. For cultivation in a 30 L PBR, 25-27 L of dairy wastewater is required.

4.b The wastewater is partially sterilized with a UV lamp. Sterilization of the entire volume for one hour is ensured by bubbling air.

5.b The substrate, i.e., wastewater, is loaded into the TL-RWP, and the culture inoculum is added to a final optical density of OD_{750} = 0.4. (The volume is calculated by calculating the mixing equation: OD_{750}inoculum x X liters = 0.4 x 30 liters). Culture conditions: temperature 26-30 °C; illumination with 12/12 h light cycle and a light path of 5.5 cm; air mixing.

6. The culture sample is monitored daily for predators (mostly flagellates), which only appear when the culture has grown (2-4 days).
7. Immediately, when the first predators are observed, the pH is adjusted to ≈ 10.5 with 1M sodium hydroxide.
8. A pH ≈ 10.5 is maintained throughout the cultivation period, i.e., 26-30 days.
9. The culture is centrifuged at 17 000g 7 min., and the biomass is lyophilized for subsequent PHB extraction.

Activity A.T1.2 Generating and selection of transformants with increased PHB production

Coordinator: FH OOE

Partial output A.T1.2.1 List of transformants with increased PHB production

The cellular components that serve as primary starting materials for PHB can be stored as glycogen. To make more starting material available for PHB production and improve the metabolic pathway for PHB production in the cyanobacterial strain *Synechocystis* PCC6714 Mt_a24 (further MT_a24) mutant, the glycogen production pathway was inhibited by genetic manipulation. Thereby pushing the metabolic flow more in favor of PHB production. To inhibit glycogen production, one of the major responsible genes, glgC, has been knocked out by molecular biological methods. To do so, plasmids that will integrate a copy of the antibiotic resistance gene Spectinomycin instead of the glgC gene has been generated (Fig. 2). This plasmid has been sequenced and transformed into the strain MT_a24. The presence of the gene has been verified by isolation of the genomic DNA and PCR.

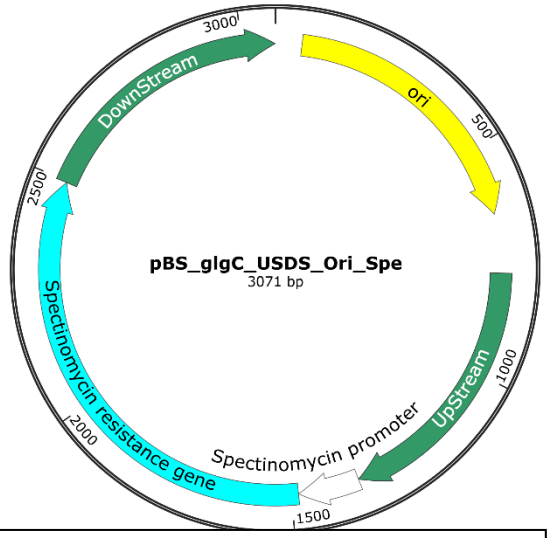


Fig 2 Plasmid map of the generated glgC knock-out plasmid pBS_k.o.glgC.

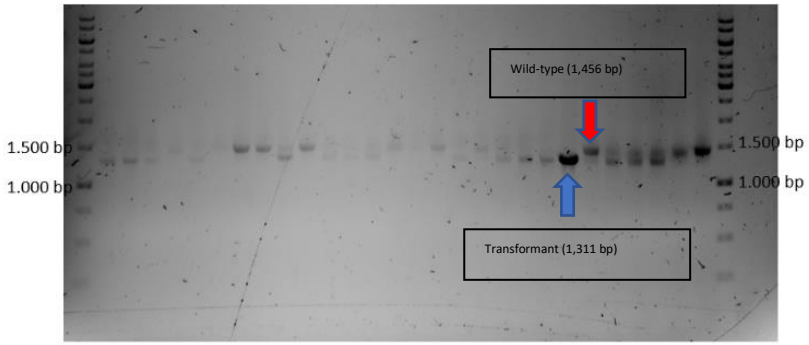


Fig 3 Segregation of *Synechocystis* PCC6714 Mt_a24 transformed with the glgC knock-out plasmid pBS_k.o.glgC.

Since cyanobacteria can have multiple genomic copies per cell, they need to be cultivated for several weeks under antibiotic selection pressure to fully segregate into stable and pure clones that contain only the introduced gene and no copy of the original gene that shall be knocked out (deleted). The transformed cultures have been segregated under increasing antibiotic selection pressure for several weeks. The segregation of the cultures has been analyzed regularly; the knock-out could be confirmed in many clones by PCR (Fig 3). Also, the cultures were segregated to a certain extent over time but did not fully segregate. This could hint that the knocked-out gene is essential for the cyanobacteria and ,they need at least a few copies to survive.

Overproduction of PHB is achieved by increasing the copy number of two genes responsible for PHB production (PhaA and PhaB). The two genes have been isolated and amplified by PCR from the genome of the cyanobacterial strain *Synechocystis* sp. PCC6714. The genes were then cloned via the state-of-the-art – Gibson assembly method into the previously described glgC knock-out plasmid pBS_k.o.glgC (Fig 4). Several slightly different DNA fragments have been generated using various Gibson primers, and then they have been used for this cloning step. However, sequencing the generated plasmids revealed that the PhaA and PhaB genes were never incorporated within the plasmid. Therefore, the two genes fused to a spectinomycin resistance gene have been recently generated synthetically to reach the final goal of overexpression in the cyanobacterial strain *Synechocystis* sp. PCC6714 Mt_a24. This DNA fragment has been cloned into the glgC knock-out plasmid pBS_k.o.glgC via the Gibson assembly. Preliminary sequence analysis showed that the cloning of the PhaAB-overexpression plasmid finally worked. The subsequent step to overexpress them, the transformation in the cyanobacteria, is still ongoing, and it is expected that transformants will be generated by the end of the project period.

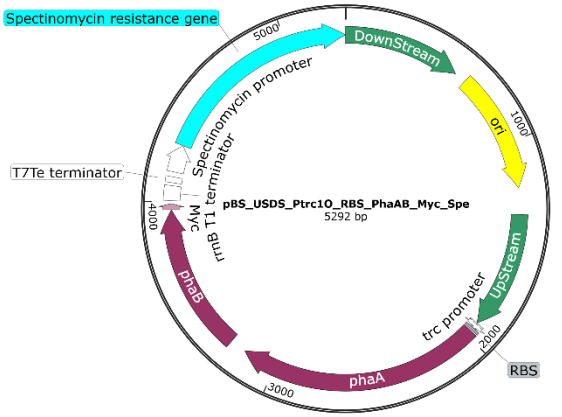


Fig 4 Plasmid map of the designed PhaAB expression plasmid pBS_glgC::PhaAB.

Partial output A.T1.2.2 List of transformants capable of processing lactose

Since cyanobacteria are unable to break down lactose, a component of dairy waste, a gene for ß-galactosidase, which breaks down lactose into glucose and galactose, has been introduced. For this approach, three variations of a ß-galactosidase gene were each cloned in a cyanobacterial plasmid vector. First, the bacteria *Escherichia coli* LacY and LacZ genes, second, the powerful ß-galactosidase genes originating from fungi *Trichoderma reseii* (BGA1) and bacteria *Bacillus circulans* (BgaD-D). The designed plasmids can be seen in Fig. 5. The amino acid sequence of the genes has been codon-optimized for cyanobacterial protein expression, and the respective DNA has been fused to suitable export sequences that allow secretion of the produced enzymes in the surrounding external medium. The synthesized DNA fragments were then cloned into cyanobacterial expression vectors. The plasmids pBS_spsA::bGAl-bact and pBS_spsA::bGAl-fungal were successfully cloned and sequenced. Since two versions of a ß-galactosidase gene were already successfully cloned in the desired plasmid, the efforts to generate the third version (pBS_spsA::lacYZ) were stopped.

Subsequently, the cyanobacterial strain *Synechocystis* sp. PCC6714 Mt_a24 was transformed by both plasmids. The presence of the ß-galactosidase gene has been confirmed by colony PCR (Fig 6).

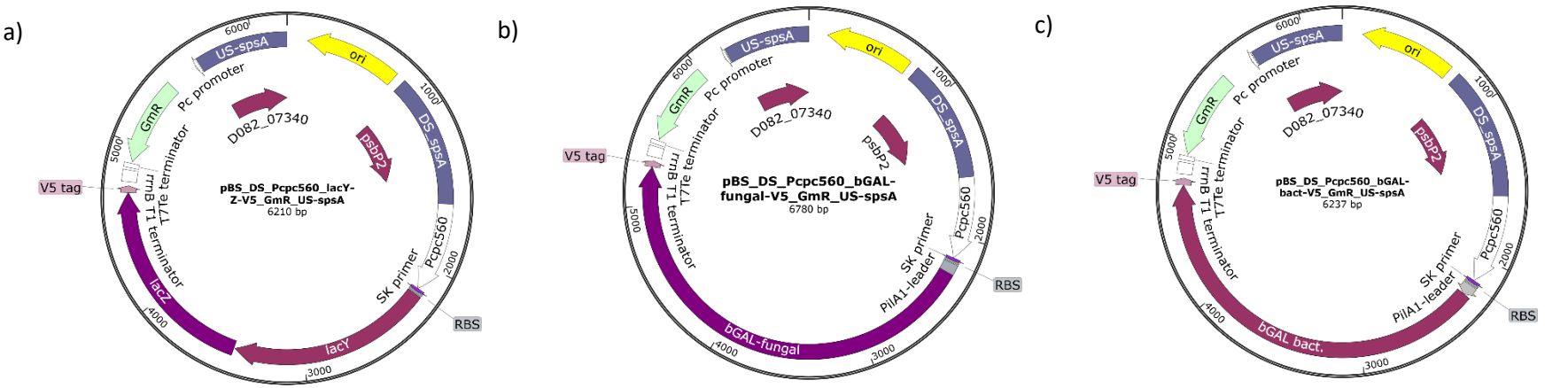


Fig 5 Plasmid map of the generated ß-galactosidase expression plasmids a) pBS_spsA::lacYZ b) pBS_spsA::bGAl-bact c) pBS_spsA::bGAl-fungal.

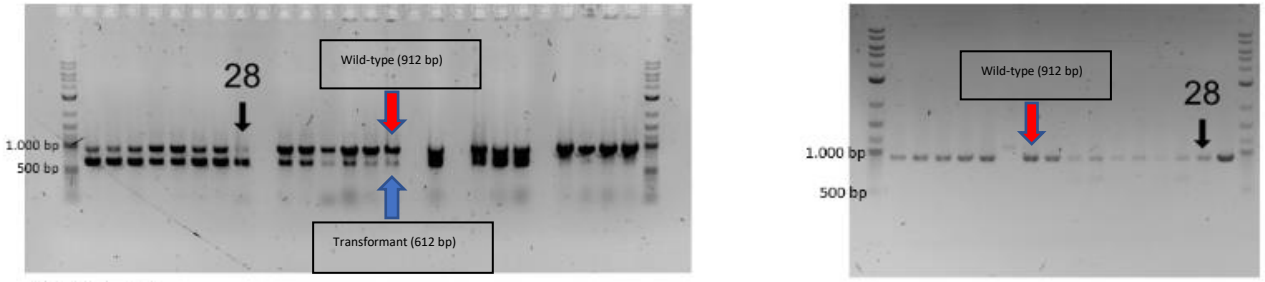


Fig 6 Segregation of the strain *Synechocystis* sp. PCC6714 Mt_a24 transformed with the ß-galactosidase expression plasmids pBS_spsA::bGAl-bact (left) and pBS_spsA::bGAl-fungal (right).

The generated plasmids are designed in such a way that the ß-galactosidase genes integrate at the location of the spsA gene within the genome of the cyanobacterial strain *Synechocystis* PCC6714. Thereby at the same time as the ß-galactosidase is expressed, or respectively integrated, and a competitive metabolic pathway is switched off. Also, in this case, the cyanobacterial cultures need to be cultivated for several weeks under antibiotic selection pressure to fully segregate into stable and pure clones that contain only ß-galactosidase and no copy of the original spsA gene.

This process is still ongoing, and the transformed cultures are regularly analyzed. As can be seen in Fig 6, one clone (#28) was almost fully segregated (a), but a couple of days later, the ratio of the original strain‘s spsA gene vs. the ß-galactosidase gen was inverted again (b). So far, no fully segregated and stable cultures could be obtained. The segregation process is still ongoing.

Activity A.T1.3 Selection of PHB-producing transformants and purification of PHB from biomass

Coordinator: TU Wien

Partial output A.T1.3.1 Report about the selection of transformants on a laboratory scale
No transformants for lactose metabolization are applicable at this stage of the project (Activity A.T1.2).

Partial output A.T1.3.2 Standard Operating Procedure (SOP) for purification of PHB from cyanobacteria.
The ionic liquids (IL), EMIM chloride, EMIM acetate & EMIM diethyl phosphate were chosen for the study because they are highly corroding, can dissolve the microalgae biomass, are very polar (PHB is thus not soluble), have good dissolving properties and are partially able to cleave ester bonds (depends on the basicity of the respective groups: Acetates > Diethylphosphates> Chlorides; with the rule: the more alkaline, the more ester bonds are cleaved).
Based on the preliminary results of the first experiments, EMIM diethyl phosphate was chosen as the most suitable IL. Complete dissolution of biomass was observed with the following parameters: 10 g IL completely dissolve 1 g biomass.
PHB should not dissolve due to its high polarity. Accordingly, the IL-biomass mixture can be separated from the resulting PHB via centrifugation/ filtration. However, the biomass-IL mixture's viscosity was very high, so complete separation from the precipitating PHB and liquid phase is impossible.
One can dilute the ILs with water (up to 5% w/w) before the dissolving power decreases. This depends on the free hydrogen bond acceptor donors of IL. The cosolvent must not form a hydrogen bond with IL; however, cosolvents could lower the IL-biomass mixture's viscosity without changing its dissolving properties.
Cosolvents with non-altering Kamlett Taft parameters would be:

- DMSO
- Acetonitrile
- Gamma-valerolactone
- Cyrene – Dihydrolevoglucosenone

Since Cyrene was detected as the most persistent chemical in the range of these previously mentioned cosolvents, the chemical was chosen as the appropriate method to reduce viscosity.
Once the viscosity is reduced, it is necessary to test if 100% of the dissolved biomass can be recovered when precipitating the mixture with water. After adding water, PHB could be well separated from the IL-biomass mixture again. Various precipitation and washing reagents were tried to increase the purity of the precipitated pellet further:

- Repeated washing step of the PHB pellet with pure methanol.
- Repeated washing step of the PHB pellet with pure acetone.
- Repeated washing step of the PHB pellet with pure cyrene.
- Repeated washing step of the PHB pellet with pure IL.
- Repeated washing step of the PHB pellet with pure hexane.
- Repeated washing step of the PHB pellet with pure chloroform.

As soon as this method is finalized, we can look forward to a greener extraction method of PHB compared to the current extraction method (chloroform extraction) in the course of the Plastocyan project, which may further advance bioplastic production in the future.

Activity A.T1.4 Sharing knowledge and improving collaboration.

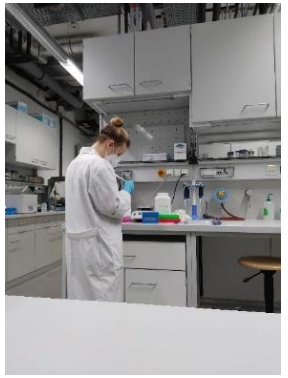
Partial output A.T1.4.1 Staff exchange report

Staff Exchange 1

In the period 21.11.-25.11.2021, the employee of the Institute of Microbiology, Romana Beloš, took part in a staff exchange of employees at project partner 3 (TU Wien), to learn the methodology for the extraction of polyhydroxybutyrate (PHB) from cyanobacteria biomass. As part of this short internship, R. Beloša learned to analyze PHB using high-performance liquid chromatography (HPLC). HPLC is used to separate sample components and determine their presence and concentration. The employee brought samples of lyophilized biomass of cyanobacteria grown in wastewater from the municipal wastewater treatment plant in Třeboň. Mainly samples after different treatments of wastewater (unsterilized wastewater, UV sterilization, and heat sterilization compared with BG-11 growth medium) and samples from pilot cultivation were tested. TU Wien has modern equipment for the analytical determination of the content of various substances, and the decision of the PHB content is one of the standardized methods here. Doctoral students Ricarda Kriechbaum and Julian Kopp introduced to Roman Beloša in detail the processing and analysis of samples. Before the investigation, the samples were hydrolyzed with acid (or base), and then PHB in the form of trans-crotonic acid was detected on HPLC. Subsequent analysis, comparison of values with the standard, and calculation of the PHB concentration were performed using the Chromeleon analytical program. At the same time, the results of these measurements helped to optimize the processing of the substrate (wastewater) from the municipal wastewater treatment plant before its use (Tab. 3). The purpose of this stay was to deepen cooperation, perform necessary analyzes within the project and transfer know-how for determining PHB content using HPLC to Czech workplace. This exchange stay benefited the CZ partner in implementing a new methodology in the workplace. Based on the transfer of know-how, the MBU workplace was subsequently able to purchase the necessary equipment (PHB standard, column for HPLC) so that the analyzes from further tests for the optimization of PHB production by cyanobacteria in wastewater could take place at the MBU workplace. The methodology was successfully introduced at the Institute of Microbiology in Třebon.

Tab 3 PHB analysis of samples of the cyanobacterium *Synechocystis* sp. Mt_a24 was growing for ten days in wastewater after various treatments.

sample	PHB content [% per dry biomass]
Heat treatment	9.2
Non-treated WW	5.8
UV treatment	8.4
BG-11	5.1



Staff Exchange 2

From November 2 to November 4, 2022, Tomáš Grivalský from the Institute of Microbiology of the Academy of Sciences of the Czech Republic, Algatech Center (MBÚ), participated in an exchange stay in the partner organization of the University of Applied Sciences in Wels (FH OO). During a short internship, he worked on the detection of the degree of segregation process in transformants of the *Synechocystis* sp. PCC6714 Mt_a24 strain and also on the optimization of the protocol for determining β-galactosidase activity in an improved strain capable of processing lactose. *Synechocystis* is a single-cell organism with multiple genome copies per cell. After transformation, the foreign DNA must be integrated into all copies of the genome by a homologous recombination process to ensure the integrated DNA's stability. However, the DNA cannot segregate into all copies in certain cases. The PCR determines the degree of segregation and, thus, the presence of foreign DNA. During this staff exchange, the employee mastered the PCR method of detecting the presence of genes for PHB overproduction, and he will be able to apply this method at the Czech workplace (MBÚ). This technique will need to be used prior to pilot-scale cultivation to avoid the loss of improved properties of the transformed strain. In addition, he worked with an employee of project partner 2 - Kevin Trenzinger, on optimizing the protocol for measuring beta-galactosidase activity in transformants capable of using lactose as a substrate. This protocol is essential for selecting the most suitable strain for growth in dairy wastewater. Based on this protocol, strains capable of beta-galactosidase production were screened (Tab. 4), and the selected strains were transferred to the Czech workplace for further testing. Kevin Trenzinger guided the MBÚ employee through the entire FH OO workplace, which has a lot of modern laboratory equipment with well-established operating procedures. This can have a positive effect on building further cooperation. The goal and result of this internship were to deepen cooperation and transfer know-how of detecting the presence of genes for PHB overproduction and lactose processing using PCR protocol.

Tab 4 Fluorescence measurements for determining beta-galactosidase activity in selected transformants of *Synechocystis* sp. PCC6714 Mt_a24. Fluorescence was measured from both the pellet and the supernatant. A UV mutant without the β-galactosidase gene is a control (blank).

Strain / Fluorescence values	**#56**	**#67**	**#75**	**#86**	**#79**	**#39**	**Mt_a24 (blank)**
Pellet	13911	17559	109753	16221	72194	9984	261
Supernatant	303	434	697	418	560	392	338

Staff exchange 3

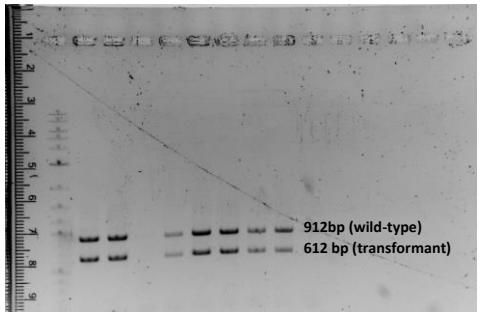
Ricarda Kriechbaum, Vienna Technical University (Working Group: Integrated Bioprocess Development), stayed three days (9.11.-11.11.2022) at the partner organization FH OÖ Wels during an employee exchange programme of the Intereg EU project Plastocyan.. During this exchange, the software Snapgene was discussed, PCR approaches and consecutive agarose-gel electrophoresis were performed, and β-Galactosidase activity was measured with an enzymatic assay and compared to the activity measurements performed at TU Wien.

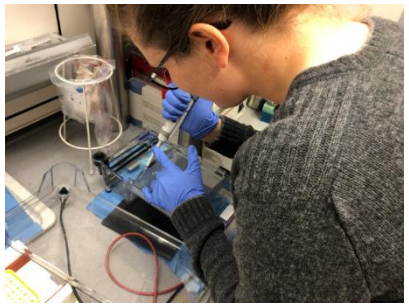
Workflow

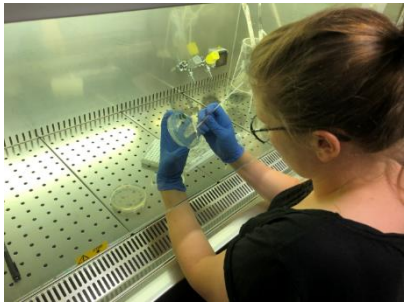
1. **Snapgene introduction + detailed discussion of the cloning strategy and codon optimization in *Synechocystis* sp. PCC6714**

During the exchange, the segregation process of transformants of the *Synechocystis* sp. PCC6714 Mt_a24 strains were observed and visualized via agarose-gel electrophoresis of a conducted PCR approach. *Synechocystis* sp. is a unicellular organism with multiple genome copies per cell. Therefore after transformation, the introduced host-DNA has to be integrated into all copies of the genome through a homologous recombination process. This is later checked with a multiplex PCR approach consisting of 3 different Primers. Ricarda Kriechbaum was introduced to Snapgene, the programme used by FH OÖ to design the plasmids. She is now able to use described PCR method to check the segregation process at TU Wien and reproduce the results.

2. **Screening PCR – Multiplex PCR (using three primers)**







3. **β-galactosidase Activity Assay using Fluorescein Di-β-D-Galactopyranoside (FDG)**

Aim: Comparison of the SOPs of TU Wien and FH OÖ

The β-gal activities of the transformants were compared with the activity of the *Synechocystis* strain Mt_a24 and quantified using a standard calibration curve. This method was compared to the method of TU Wien and optimized.
Regression equation based on calibration curve to link the measured fluorescent intensity (absorption; y) to the produced amount of β-gal activity (x) [ng/mL]: $y = 14{,}57x + 291{,}76$

Tab 5 β-galactosidase activities of screened transformants. 7 Transformants with β-gal gene from the fungal or bacterial origin and Mt_a24 as a control. The absorption was measured in triplicate, and β-gal was calculated via regression equation.

β-gal host	Strain	1. mes	2. mes	3. mes	Mean ± Deviation	ng/mL
no BGAL	Mt 24	22,33	-9,67	-9,67	1,00 ± 15,08	0
fungal	79	10,33	-6,67	-12,67	-3,00 ± 9,74	0
fungal	86	-2,67	-8,67	-7,67	-6,33 ± 2,62	0
fungal	75	-7,67	-8,67	-7,67	-8,00 ± 0,47	0
bacterial	28	-7,67	-8,67	-10,67	-9,00 ± 1,25	0
fungal	67	-8,67	-9,67	-7,67	-8,67 ± 0,82	0
fungal	39	-7,67	-9,67	-7,67	-8,33 ± 0,94	0
fungal	50	-11,67	-8,67	-9,67	-10,00 ± 1,25	0

The strains showed no β-gal activities compared to the previous experiment and need to be further checked and analyzed.

4. Open House FH Wels – project presentation with Roll-Up:

On November 11, 2022, an open day was held at FH OÖ to introduce potential students to the various fields of study that can be studied at FH Wels. Kevin Trenzinger (FH OÖ) and Ricarda Kriechbaum (TU Wien) introduced the project to the students and the possible field of activity after graduation.

Staff exchange 4

From November 28 to December 1., 2022, Kevin Trenzinger (FH OÖ) participates in a staff exchange in the partner organization Institute of Microbiology of the Academy of Sciences of the Czech Republic, Algatech Center (MBÚ) in Třeboň. Cultivation in a 30 L photobioreactor (PBR) of the transformant KO glgc #5 in wastewater from the dairy industry is carried out here. The transformant is based on *Synechocystis* sp. PCC6714 Mt_a24, in which one gene (glgC - glucose-1-phosphate adenylyltransferase) was knocked down. This should lead to an increased yield of PHB. Due to the addition of wastewater from the dairy industry, various parameters such as pH and nutrient supply do change. Accordingly, the cultivation has to be adapted. Cultivation on this pilot scale will also enable new insights to be gained and, above all, new methods to be applied at the workplace at the Upper Austrian University of Applied Sciences (Wels). This is important because, up to now, cultivation has only been done on a small scale in FH OO, and new challenges will arise due to the upscaling. Therefore, adapting the cultivation procedure during this process to the new conditions is essential. The stay in Třeboň also enables the staff in Wels to perform experiments on a pilot scale, thus improving future projects or collaborations.

During the exchange of employees, it is also planned to check the state of segregation of these transformants using PCR. This is essential as it must be ensured that the genes remain knocked-down. This will also allow the method to be confirmed in Trebon and possible optimizations to be carried out.

Activity A.T1.5 Bilateral final meeting with end users

Partial output A.T1.5.1 Bilateral final meeting with end users

YOUR NOTES

PARTNERS AND CONTACT INFO

Institute of Microbiology, Czech Academy of Sciences, Centre Algatech

The Algatech Třeboň Centre is one of the departments of the Institute of Microbiology (MBU), and since its foundation in 1962, it has gained extensive experience in all areas of microbial research. It is currently one of the most competent institutions in the South-West Bohemia region conducting research in algal cultures. MBU has many years of experience in the cultivation of various strains of microalgae and cyanobacteria used for biomass production and isolation and identification of bioactive substances, as well as in the development and testing of different culture units on laboratory and pilot scales. Various physiological, biochemical, and biophysical techniques optimize the cultivation process. The MBU has also developed several analytical methods to detect multiple compounds present in biomass.

Address: Novohradská 237, 379 01 Třeboň, Czech Republic

Web: www.alga.cz

Project coordinator for MBU: Tomáš Grivalský, grivalsky@alga.cz

Fachhochschule Oberösterreich Forschungs- und Entwicklungs GmbH, AG Biosciences

AG Biosciences works closely with the Bio- and Environmental Technology (FH OOE) program and has well-equipped molecular biology laboratories using a range of established techniques for the genetic engineering of cyanobacteria and yeasts. It focuses on the sustainable production of valuable compounds in different microorganisms: the use of waste products by yeasts in the IWB Combined Agro Forest Biorefinery project, the biodiversity of snow algae in FWF-funded projects, and the establishment of cyanobacteria as a sustainable production system. Methods for genetic engineering of cyanobacteria have been established in the INTERREG ATCZ15 project and in the FFG project, and extensive knowledge has been gained on their cultivation and PHB production.

Address: Roseggerstrasse 15, 4600 Wels, Austria

Web: www.fh-ooe.at/campus-wels

Project coordinator for FH OOE: Alexander Zwirzitz, alexander.zwirzitz@fh-wels.at

Technische Universität Wien - Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften, Integrierte Bioprozessentwicklung

The research group Integrierte Bioprozessentwicklung at the Institute of Process Engineering, Environmental Technology and Biological Sciences of the Faculty of Technical Chemistry of TU Wien has more than ten years of experience in the development of microbial bioprocesses of various scales. In this research group, microbial bioprocesses are developed and optimized in an integrated way, i.e., strains are generated, bioreactor cultures are designed and implemented on a scale of 0.1 - 30 L, different products are isolated and purified in complex purification steps, and their applicability is also characterized and tested. Subsequently, these processes are transferred to an industrial scale. Over the last three years, the group has also been working with microalgae and cyanobacteria and developing new technologies for controlled cultivation. Furthermore, methods for the isolation and characterization of valuable products (e.g., carotenoids, glycogen, lipids) from microalgae have been developed.

Address: Getreidemarkt 9, 1060 Wien, Austria

Web: www.vt.tuwien.ac.at/biochemical_engineering/integrierte_bioprozessentwicklung/

Project coordinator for TU Wien: Oliver Spadiut, oliver.spadiut@tuwien.ac.at

SOUHRNNÉ INFORMACE

Název projektu
Produkce biologicky rozložitelného polymeru polyhydroxybutyrátu PHB ze sinic cestou kultivace v odpadních vodách.
Číslo projektu, zkratka
ATCZ260, PlastoCyan
Program
Program Interreg V-A Rakousko-Česká republika, Evropský fond pro regionální rozvoj
Prioritní osa
Životní prostředí a zdroje
Doba trvání projektu
01. 06. 2021 – 31. 12. 2022
Vedoucí partner
Mikrobiologický ústav AV ČR v.v.i.
Partneři projektu
• Fachhochschule Oberösterreich Forschungs- und Entwicklungs GmbH
• Technische Universität Wien - Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften
Alokované prostředky EFRR
€ 335 365.62
WEB:
https://www.at-cz.eu/cz/ibox/po-2-zivotni-prostredi-a-zdroje/atcz260_plastocyan
www.at-cz.eu/cz

BASIS INFORMATIONEN

Projektname
Herstellung von biologisch abbaubarem Polymer Polyhydroxybutyrat PHB aus Cyanobakterien durch Kultivierung in Abwasser.
Projektnummer, Akronym
ATCZ260/ PlastoCyan
Programm
Programm Interreg V-A Österreich – Tschechische Republik, Der Europäische Fonds für regionale Entwicklung
Prioritätsachse
Umwelt und Ressourcen
Projektdauer
01. 06. 2021 – 31. 12. 2022
Lead Partner
Mikrobiologický ústav AV ČR v.v.i.
Projektpartner
• Fachhochschule Oberösterreich Forschungs- und Entwicklungs GmbH
• Technische Universität Wien - Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften
Genehmigte EFRE-Mittel
€ 335 365.62
WEB:
https://www.at-cz.eu/cz/ibox/po-2-zivotni-prostredi-a-zdroje/atcz260_plastocyan
www.at-cz.eu/cz

BASIC INFORMATION

Project name
Production of the biodegradable polymer polyhydroxybutyrate PHB from cyanobacteria via wastewater cultivation.
Project number/Acronym
ATCZ260/PlastoCyan
Program
Interreg V-A program Austria-Czech Republic, European Regional Development Fund
Priority axis
Life environment and sources
Project duration
01. 06. 2021 – 31. 12. 2022
Lead partner
Institute of Microbiology, Czech Academy of Sciences
Project partners
• University of Applied Sciences Upper Austria
• Vienna University of Technology - Institute for Process Engineering, Environmental Engineering and Technical Biosciences
Budget from ERDF
€ 335 365.62
WEB:
https://www.at-cz.eu/cz/ibox/po-2-zivotni-prostredi-a-zdroje/atcz260_plastocyan
www.at-cz.eu/cz