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Gábor Szilveszter Tóth

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“The supernatural seems supernatural.
But the science of tomorrow is the supernatural of today.”

Agatha Christie

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ABSTRACT

Photosynthetic microorganisms, such as cyanobacteria and microalgae play a key role in the ecosystem while also holding great economic potential for a sustainable future. These organisms offer diverse applications, ranging from human and animal consumption to wastewater treatment, and serving as platforms for photosynthetic production of fuels and chemicals. By combining synthetic biology, and metabolic engineering, a wide array of chemicals can be synthesised by these microorganisms, taking advantage of photosynthesis. Through photosynthesis, these microbes convert sunlight and atmospheric carbon dioxide into biomass and/or various chemicals. Immobilising photosynthetic microorganisms in environmentally friendly and biodegradable polymer matrices can transfer the production into a solid-state (hydrogel) system. Immobilised systems emerge as an effective strategy for enhancing production, simplifying operation, and facilitating upscaling.

Key findings include the enhanced production yields of sucrose and ethylene by engineered cyanobacterial *Synechocystis* sp. PCC 6803 strains. Furthermore, *Synechocystis* produced sucrose drives the biotransformation of cyclohexanone to ε-caprolactone in an engineered *Escherichia coli*. The expression of a Baeyer-Villiger monooxygenase in the eukaryotic green alga *Chlamydomonas reinhardtii* is explored as an alternative with photosynthetic co-factor regeneration and O_2 production. The biotransformation is further optimised by the improvement of the strain and coupled with photosynthetic hydrogen production in a stepwise manner. By employing 3D-printing and a photocurable bioink composed of cells, alginate, galactoglucomannan-methacrylate and a photoinitiator, I demonstrate its compatibility with both prokaryotic and eukaryotic photosynthetic microorganisms and ethylene production and biotransformation. The 3D-printed films demonstrate improved stability and present the possibility of creating complex architectures.

The outcomes of this research underscore the versatility of photosynthetic microorganisms for applications in different solid-state chemical production systems. These findings open novel avenues for the utilisation engineered photosynthetic living materials, contributing to the advancement of a more sustainable chemical industry.

KEYWORDS: cyanobacteria, microalgae, immobilisation, 3D-printing, biopolymers, chemical production

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TIIVISTELMÄ

Fotosynteettisillä mikro-organismeilla, kuten syanobakteereilla ja mikrolevillä, on keskeinen tehtävä maapallon ekosysteemeissä. Lisäksi niihin liittyy merkittävää taloudellista potentiaalia. Nämä fotosynteettiset eliöryhmät tarjoavat monipuolisia sovelluksia ihmisten ja eläinten ravinnon tuotannosta jätevesien käsittelyyn, sekä biopolttoaineiden ja muiden kemikaalien tuotannon alustoina. Käyttämällä synteettistä biologiaa ja metabolista muokkausta nämä mikro-organismit saadaan tuottamaan erilaisia kemikaaleja fotosynteesiä hyödyntäen. Fotosynteettiset mikrobit muuttavat auringon valoenergian sekä ilmakehän hiilidioksidin biomassaksi ja kemikaaleiksi. Immobilisoimalla solut ympäristöystävällisiin ja biohajoaviin polymeerimatriiseihin voidaan tehostaa kemikaalien tuotantoa ja yksinkertaistaa solujen käsittelyä sekä helpottaa skaalausta.

Väitöskirjatyössäni havaitsin, että immobilisoimalla muunneltuja syanobakteerikantoja solut tuottavat enemmän sakkaroosia ja etyleeniä kuin solususpensioissa. Lisäksi osoitin, että syanobakteerin fotosynteettisesti tuottama sakkaroosi mahdollistaa sykloheksanonin muuttamisen ε-kaprolaktoniksi geneettisesti muokatussa heterotrofisessa kolibakteerissa. Toisessa lähestymistavassa ε-kaprolaktonia tuottava Baeyer–Villiger-entsyymi siirrettiin *Chlamydomonas reinhardtii* -viherlevään, joka pystyy fotosynteesin avulla tuottamaan reaktiossa tarvittavaa happea ja kofaktoria. ε-kaprolaktonin lisäksi kanta saatiin tuottamaan molekulaarista vetyä vaiheittaisessa reaktiossa. Tein myös 3D-tulostettuja kalvoja alginaatista, galaktoglukomannaani-metakrylaatista, valoinitiaattorista ja soluista koostuvasta biomusteesta, joka kovetettiin valon avulla. Osoitin sen yhteensopivuuden sekä bakteerien, että aitotumallisten mikro-organismien kanssa ja totesin, että kalvossa kiinni olevat solut pystyvät edelleen tuottamaan etyleeniä ja ε-kaprolaktonia. 3D-tulostus tarjoaa mahdollisuuden luoda monimutkaisia rakenteita ja menetelmällä tehdyt kalvot ovat kestäviä.

Tämän tutkimuksen tulokset korostavat fotosynteettisten mikro-organismien monipuolisuutta erilaisissa immobilisaatiosovelluksissa. Väitöskirjani tulokset avaavat uusia mahdollisuuksia fotosynteettisten mikro-organismien käyttöön yhdisteiden tuotannossa, mikä edistää kestävämmän kemianteollisuuden kehitystä.

ASIASANAT: syanobakteerit, mikrolevät, immobilisointi, 3D-tulostus, biopolymeerit, kemian tuotanto

Table of Contents

Abbreviations

2-OG	2-Oxoglutarate
3D	3-Dimensional
AB	Antibiotics
ACC	1-Aminocyclopropane-1-carboxylic acid
ADH	Alcohol dehydrogenase
ATP	Adenosine triphosphate
BVMO	Baeyer-Villiger monooxygenases
Cas9	CRISPR-associated protein 9
CDW	Cell dry weight
Chl	Chlorophyll
CHMO	Cyclohexanone monooxygenase
CNC	Cellulose nanocrystals
CNF	Cellulose nanofibril
CRISPR	Clustered regularly interspaced palindromic repeats
DNA	Deoxyribonucleic acid
EFE	Ethylene forming enzyme
FLV	Flavodiiron proteins
GC	Gas chromatography
GGM	*O*-Acetyl-galactoglucomannan
GGMMA	Galactoglucomannan methacrylate
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid
HIV	Human immunodeficiency virus
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
LAP	Phenyl-2,3,6-trimethylbenzoylphosphinate
LB	Lysogeny broth
LED	Light emitting diode
MIMS	Membrane inlet mass spectrometry
MLG	Mixed linkage glucans
MOPS	3-(*N*-morpholino)propanesulfonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density

Parvi	*Parvibaculum lavamentivorans*
PGR5	proton-gradient-regulation5
PHA	Polyhydroxyalkanoate
PS II	Photosystem II
PS II	Photosystem two
PUFA	Polyunsaturated fatty acids
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
rpm	Round per minute
SAM	*S*-Adenosyl-L-methionine
TAP	Tris-acetate-phosphate
TB	Terrific broth
TMPSM	3-(Trimethoxysilyl)propyl methacrylate
TP	Tris-phosphate
USD	United States dollar
UV	Ultraviolet
Xeno	*Burkholderia xenovorans*
Y (II)	PS II photosynthetic quantum yield

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Tóth, G. S., Siitonen, V., Nikkanen, L., Sovic, L., Kallio, P., Kourist, R., Kosourov, S., Allahverdiyeva, Y. Photosynthetically produced sucrose by immobilized *Synechocystis* sp. PCC 6803 drives biotransformation in *E. coli*. *Biotechnol Biofuels*, 2022; 15:164.

II. Siitonen, V., Probst, A., Tóth, G., Kourist, R., Schroda, M., Kosourov, S., Allahverdiyeva, Y., Engineered green alga *Chlamydomonas reinhardtii* as a whole-cell photosynthetic biocatalyst for stepwise photoproduction of H_2 and ε-caprolactone. *Green Chem*, 2023; 25: 5945-5955.

III. Tóth, G. S., Backman, O., Siivola, T., Xu, W., Kosourov, S., Siitonen, V., Xu, C., Allahverdiyeva, Y. Employing photocurable biopolymers to engineer photosynthetic 3D-printed living materials for chemicals production. *Green Chem*, 2024; 26: 4032-4042.

Publications I, II and III have been published under the terms of Creative Commons Attribution (CC BY) license

Author’s Contribution

Paper I: GST was responsible for the experimental design with input from the co-authors. GST performed the immobilisation, sucrose production and biotransformation experiments. GST contributed to the MIMS measurements. GST analysed the data and drafted the manuscript and contributed to the finalisation of the.

Paper II: GST contributed to culturing of the cells, the biotransformation experiments and the finalisation of the manuscript.

Paper III: GST was responsible for the experimental design with input from the co-authors. GST performed the 3D-printing with the contribution of OB and TS. GST performed the ethylene production experiments and analysed the data. GST drafted the manuscript and contributed to the finalisation of the manuscript.

1 Introduction

Cyanobacteria is a phylum of Gram-negative bacteria inhabiting a wide range of biotopes from hot deserts to arctic snow, but most prevalent in aquatic environments (Stal, 2007). These prokaryotes were the first organisms to evolve oxygenic photosynthesis, a molecular process responsible for life on Earth, as we know it today.

Microalgae are microscopic eukaryotes with a nucleus and chloroplasts, a specialised organelles, responsible for photosynthesis in algae and higher plants. In the field of biotechnology, microalgae and cyanobacteria are often not distinguished from each other, and simply referred to as microalgae, due to their similarities such as microscopic size and the ability to photosynthesise (Heimann & Huerlimann, 2015; Levasseur et al., 2020). Herein the two groups together will be referred to as photosynthetic microorganisms for clarity. While there are other microorganisms, which perform non-oxygenic photosynthesis, these will not be discussed in this work.

Photosynthetic microorganisms are represented in diverse taxonomic groups and exhibit a range of complexities in their morphology. Certain species are unicellular (e.g. *Synechocysti*s sp. PCC 6803, *Chlamydomonas reinhardtii*) while others form multicellular structures such as simple or branching filaments or globular colonies. Many multicellular species possess differentiated cell types for special functions. For example, *Volvox carteri* has both vegetative cells (also called photosynthetic cells) responsible for normal cellular functions, and reproductive cells for the production of offspring. Another example is *Anabaena* sp. PCC7120, which, in addition to vegetative cells, has evolved molecular nitrogen (N_2) fixing heterocyst cells (Meeks & Elhai, 2002; Prochnik et al., 2010). This incredible genetic and physiological variety makes photosynthetic microorganisms prospective candidates for a number of different applications.

1.1 Taxonomy and model organisms

Photosynthetic microorganisms encompass a wide variety of taxa with different and complex evolutionary history. The two main groups are the prokaryotic cyanobacteria and microscopic eukaryotic algae.

The cyanobacteria (formerly blue-green algae) phylum is currently divided into eight orders: *Nostocales, Chroococcidiopsicales, Spirulinales, Pleurocapsales, Chroococcales, Oscillatoriales, Synechococcales, Gloeobacterales* (Komárek, 2014). These prokaryotic organisms developed oxygenic photosynthesis in the early Proterozoic era, filling the atmosphere with molecular oxygen (O_2) and facilitating what is now known as the Great Oxygenation Event (Canfield, 2005; Holland, 2006). Their long evolutionary history and complicated genetic and morphological relations make their taxonomic classification challenging even with the tools of molecular genetics (Whitton, 2012). Many species have different morphologies under different growth conditions and there are species with similar genomes but considerably different morphological characteristics.

At some point during evolution, a heterotrophic eukaryotic cell engulfed an ancient cyanobacterium, which then became the ancestor of the chloroplast in modern algae and plants alike. This phenomenon is known as endosymbiosis and the descendants of the resulting lineage possess the ability to photosynthesise, as well as a nucleus and compartmentalisation of the cellular processes. Photosynthetic algae belong to the *Archaeaplastida*, *Chromista, Rhizaria* or *Excavata* clades. *Archaeaplastida* are characterised by primary endosymbiotic plasmid with a two-layer membrane. This clade contains most of the industrially relevant microalgae and can be divided into *Rhodophyta* or red algae, *Chlorophyta* or green algae, *Glaucophyta* algae and the land plants. *Chromista, Rhizaria* and *Excavata* contain species with chloroplasts with four-layer membranes, a product of secondary and tertiary endosymbiosis events (Burki et al., 2012).

Photosynthetic microorganisms have been extensively studied for a long time leading to the discovery of numerous species with potential in industrial applications as well as genetic and photosynthetic studies. One prominent example is the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) which had its complete genome sequenced in 1996 (Kaneko et al., 1996). This cyanobacterial strain has since become the most extensively studied model organism in the field, resulting in a comprehensive understanding of its genetic makeup and a well-developed molecular and synthetic biology toolkit for biotechnological applications. *Synechocystis* is a unicellular, freshwater cyanobacterium, without N_2 fixing ability (Mills et al., 2020). Its relatively simple genetics and morphology makes this strain desirable for both research and industrial applications. However, it is not a suitable model for more complex, multicellular cyanobacteria. *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) is a filamentous, N_2 fixing, freshwater species with long studied genetics. The genome sequence of A*nabaena* was published in 2001 (Kaneko et al., 2001) and today it serves as a model organism of filamentous cyanobacteria. Its filamentous structure and N_2 fixation capacity make it especially interesting for research focusing on cellular communication, cell differentiation and N_2 fixation.

Among microalgae the most extensively studied model organism is *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*). Its long history dates back to the 19th century when the first morphological descriptions were published followed by the first genetic studies in the early 20th century. The first complete genetic sequence was obtained in 2007 (Harris, 1989; Merchant et al., 2007). *Chlamydomonas* is enveloped by a cell wall and features two flagella, several pyrenoids and an eyespot. It exhibits great versatility, being capable of both sexual reproduction under N_2 starvation and asexual mitosis. Furthermore, while *Chlamydomonas* is capable of photo-autotrophy it is also able to utilise organic carbon sources in mixotrophy, when both light and organic carbon are available or in lack of illumination (Harris, 1989; Merchant et al., 2007; Tran & Kaldenhoff, 2020).

1.2 Significance

1.2.1 Ecological significance

Photosynthetic microorganisms possess the ability to harness light energy for the oxidation of water molecules into molecular O_2 and protons (H^+) through the photosystem II (PS II) complex. The electrons generated through this process travel through the photosynthetic electron transport chain via the plastoquinone pool to the cytochrome b_6/f (Cyt b_6/f) complex and to photosystem I (PSI), eventually leading to the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a necessary co-factor involved in numerous cellular processes. Simultaneously, the proton motive force (*pmf*) generated across photosynthetic membranes is utilised for the synthesis of adenosine triphosphate (ATP). The molecular O_2 produced as a by-product of water oxidation contributes to cellular respiration and the excess is released into the atmosphere (Stirbet et al., 2020). Remarkably, photosynthetic microorganisms are estimated to contribute to over 50% of the O_2 present in the Earth's atmosphere (Pencik et al., 2023).

In aquatic ecosystems, photosynthetic microorganisms are found in two main lifestyles. The free-floating ones are known as the phytoplankton and are found in the open waters of oceans, lakes, and rivers, while benthic photosynthetic microorganisms reside on the ocean floor forming mats or biofilms. The phytoplankton contributes significantly to the primary production of the world and serves as the source of aquatic food webs (Bonachela et al., 2016; Polimene et al., 2017), while benthic species play a crucial role in carbon sequestration by capturing and storing carbon dioxide (CO_2) for extended periods in the deep layers of the oceans. Their ability to sequester CO_2 contributes to the regulation of global carbon cycles and mitigates the accumulation of atmospheric CO_2 (Dunlop et al., 2016; Polimene et al., 2017).

Additionally, the cyanobacterial species capable of atmospheric N_2 fixation are considered the primary nitrogen sources in the oceans, playing a key role in the global nitrogen cycle and consequently fulfil an essential ecological function (Zehr, 2011).

1.2.2 Economic significance

Apart from a crucial ecological role, many photosynthetic microorganisms have considerable economic potential. Due to their vast metabolic diversity, they are capable of producing a number of valuable compounds which find application as biofuel feedstock, pharmaceuticals, additives in food and feed industry and cosmetic products (Camacho et al., 2019; Yap et al., 2021). In 2020 the estimated global market value of the photosynthetic microorganism industry was 3.4 billion USD, projected 4.6 billion $ by 2027 (Research and Markets ltd, 2024). The main applications of photosynthetic microbes are presented in Figure 1.

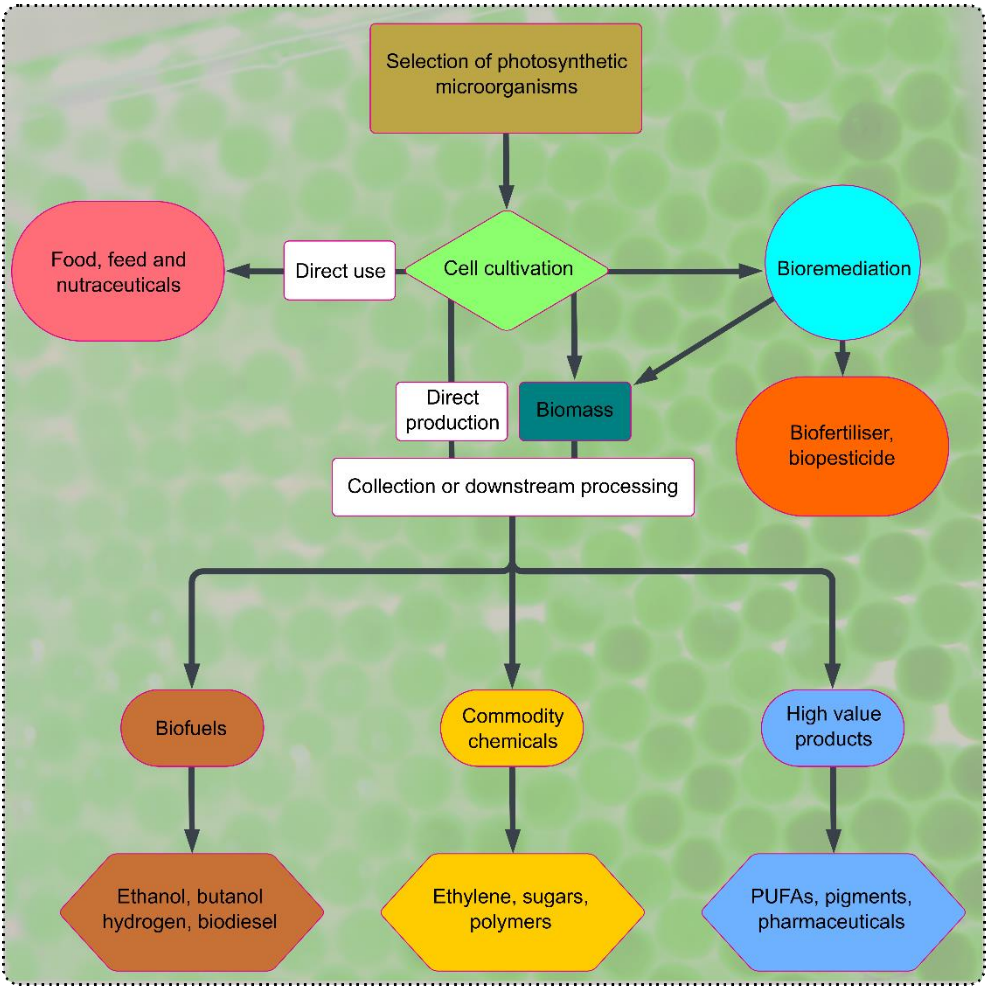

Figure 1. The key routes towards industrial applications of photosynthetic microbes. The photosynthetic cells can be utilised directly for food and feed. Alternatively they can be employed for the production of commodity chemicals, high-value compounds, biofuels or biofertilisers and biopesticides. Photosynthetic microorganisms can be cultivated in mineral medium, or alternatively in wastewater, also performing bioremediation, while also producing valuable biomass for further applications.

Photosynthetic microorganisms grow 5 – 10-fold faster compared to plant crops and approximately 50% of their biomass consists of proteins and carbohydrates, while lipids account for up to 40% of their cellular composition. However, the exact content of the cells is highly dependent on the strain and the culture conditions (Khoo et al., 2020; Singh & Gu, 2010). Lipids synthesised by these cells are an especially rich sources of polyunsaturated fatty acids (PUFAs).

Furthermore, photosynthetic microorganisms are particularly abundant in vitamins and minerals (Camacho et al., 2019; Sousa et al., 2008; Vidanarachchi et al., 2012). *Arthrospira platensis,* commercially known as *Spirulina,* is a cyanobacterium that is highly regarded as a dietary supplement due to its significant nutritional benefits. *Spirulina* is a valuable source of vitamins, proteins, and PUFAs and contains various antioxidant and anti-inflammatory agents contributing to its health-promoting properties (Grosshagauer et al., 2020). *Spirulina* is cultivated in the highest amount in China where it is mainly used as human food and feed in aquaculture (Garrido-Cardenas et al., 2018; Lu et al., 2011). *Chlorella* is another important genus for dietary use. *Chlorella* production world-wide is only about 25% of *Spirulina,* however, the price per ton is higher (Andrade et al., 2018; Garrido-Cardenas et al., 2018).

Beyond dietary supplements, photosynthetic microorganisms are cultivated on an industrial level for pigment production, serving as alternatives to synthetic dyes known for their associated health risks (Dikshit & Tallapragada, 2018). Apart from their bright and appealing colour, pigments derived from photosynthetic microorganisms exhibit additional health-promoting attributes, including antioxidant, anti-cancer, and anti-inflammatory activities. Table 1, modified from Srivastava et al. (2022), outlines the main pigments and pigment-protein complexes found in photosynthetic microorganisms, and their properties.

Table 1. Main photosynthetic microorganism-derived pigments and pigment-protein complexes, their main industrial source organism, colour and application properties (Modified from Srivastava et al., 2022).

Compound	Organism	Colour	Application	References
Phycoerythrin	*Polysiphonia, Batrachospermum*	Red	Antioxidant	Chaubey et al., 2019; Sonani et al., 2018
Phycoyanin	*Oscillatoria, Spirulina, Nostoc, Anabaena*	Blue	Antilipidomic	Tavanandi et al., 2018
Allophycocyanin	*Spirulina*	Bluish-grey	Antioxidant	Rastogi et al., 2015
Chlorophyll *a*	*Chlorella, Spirulina, Nostoc*	Green	Antioxidant	da Silva Ferreira & Sant'Anna, 2017
Fucoxanthin	*Fucus, Phaeodactylum*	Orange	Anti-obesity, anti-diabetic activity	McClure et al., 2018

Compound	Organism	Colour	Application	References
Astaxanthin	*Haematoccoccus pluvialis*	Red	Anti-cancer activity	Ambati et al., 2019; Choi et al., 2008
β-carotene	*Dunaliella, Scenedesmus*	Red-orange	Vitamin A precursor, treatment of night blindness	Xu et al., 2018
Lutein	*Chlorella, Scenedesmus*	Yellow-red	Antioxidant	Dinh et al., 2022
Zeaxanthin	*Scenedesmus, Nannochloropsis*	Yellow	Antioxidant and anti-inflammatory properties	Cha et al., 2008; Ren et al., 2021
Canthaxanthin	*Chlorella, Scenedesmus*	Red-orange	Treatment of blood disorders	Ravaghi et al., 2016
Violaxanthin	*Chlorella*	Orange	Anti-inflammatory activity	Sathasivam & Ki, 2018; Soontornchaiboon et al., 2012
Lycopene	*Chlorella*	Red	Anti-cancer activity	Bhalamurugan et al., 2018; Renju et al., 2014

The main photosynthetic microorganism-derived products used as colorants can be categorised into three main groups: chlorophylls, carotenoids, and phycobiliproteins (Hamed, 2016; Jacob-Lopes et al., 2018). Primarily employed as natural food colorants, as well as constituents in pharmaceutical and cosmetic products (García et al., 2017), photosynthetic microorganism-derived pigments are produced in notable quantities. Currently there is a high demand for β-carotene from *Dunaliella* sp., astaxanthin from *Haematococcus pulvalis,* and phycobiliproteins from *Spirulina* (Berthon et al., 2017; Milledge, 2011; Pagels et al., 2020). Countries with substantial photosynthetic microorganism-derived pigment production include the USA, China, Israel, Australia, India, and Japan (Chakdar & Pabbi, 2017; Maroneze et al., 2020). Global market share of carotenoid production reached 1.7 billion USD in 2017 (this include plant derived carotenoids as well), while of phycobiliproteins it reached 112.3 million in 2013 with exhibiting growth and anticipated continuation of such trends (Farkas et al., 2020; Pagels et al., 2020).

Photosynthetic microorganisms are also a prospective feedstock for biofuel production, however, there are challenges regarding economic feasibility due to the high production costs. Consequently, there is need for the further development of the corresponding technological toolkit (Kim et al., 2022; Maity & Mallick, 2022). Nonetheless, there is a considerable market share of photosynthetic microorganism-based fuels. In this area, Japan is considered a pioneer with a substantial production (Show, 2022) (see more about the biofuel production in section 1.3.1.1).

1.3 Potential industrial application of native and engineered photosynthetic microorganisms

Besides their existing roles as animal feed, dietary supplements, and pigment sources, the use of photosynthetic microorganisms is in emergence for numerous industrial processes. Many species inherently produce high-value compounds, such as anti-cancer or anti-microbial agents, which can be commercialised in the pharmaceutical industry (Prestegard et al., 2009; Saad et al., 2022; Skjånes et al., 2021). Their high lipid and carbohydrate content enables the conversion of the biomass into biofuels such as biodiesel or bioethanol through biorefineries, requiring considerably less land and nutrients compared to conventional feedstocks like rapeseed or sunflower seed cultivation (Kim et al., 2022; Maity & Mallick, 2022).

The utilisation of diverse genetic engineering strategies not only facilitates the optimisation of the production of naturally occurring chemicals but also enables the synthesis of non-native chemicals capitalising on the process of photosynthesis (Wang et al., 2020).

Photosynthetic microorganisms also extend their utility beyond chemical production by the improvement of wastewater treatment systems and bioremediation (Gentili, 2014; Kothari et al., 2012; Salazar et al., 2021). In adherence to the principles of a circular economy the biomass cultivated during wastewater treatment can undergo processing in biorefineries, yielding fuels and other value-added products (Kadir et al., 2018; Rempel et al., 2021). Alternatively, it can be applied as biostimulants or biopesticides in agriculture and horticulture (Costa et al., 2019; González-Pérez et al., 2021). This approach allows for the recovery and utilisation of a substantial quantity of valuable nutrients from waste, while safeguarding the environment and public health.

In the following section the different emerging industrial applications of both naturally occurring and engineered photosynthetic microbes are presented.

1.3.1 Chemical production

The chemical industry plays a crucial role in creating a vast array of products that have become indispensable parts of modern life. However, the manufacturing processes associated with these commodities frequently comes with substantial and, at times, severe environmental damages (Ko et al., 2020). The extraction of petrochemicals, serving as starting materials for a significant proportion of industrial output, causes direct environmental harm (Gani, 2021). Additionally, the storage and neutralisation of waste and hazardous by-products impose significant financial burdens, while accidental spillage poses severe threats to both human health and the environment (Ramiro & Aísa, 2012).

In an attempt to enhance the sustainability of the industry and mitigate its adverse impacts on the environment and consumers, there is a growing exploration of biological solutions as alternatives to conventional chemical production pathways. Two main strategies can be followed for chemical production by photosynthetic microorganisms. The cultivated strains can be naturally occurring or genetically modified. In both cases, the cultivation is often followed by extraction and purification of the desired compound (Di Caprio et al., 2022; Obeid et al., 2022). However, with volatile chemicals (e.g. hydrogen and ethylene) or with the help of genetic engineering it is possible to facilitate the secretion of the product into the growth medium, in which case the extraction can be avoided, but purification or concentration steps are possibly required (Wang et al., 2021). Engineered strains can also be employed as whole-cell biocatalysts in a “substrate in – product out” approach where a chemical is fed to the cells which perform a biotransformation reaction and secrete the product (see more in section 1.4.2).

Commodity chemicals are produced and utilised in large quantities for various purposes from building materials to food products. Consequently, consistent high availability and low prices are necessary to meet global market demands. Key examples of such chemicals include fertilisers, plastic polymers, and sugars (McFarlane & Robinson, 2008; Vaithyanathan et al., 2023). A considerable proportion of these commodities is derived from fossil-based resources, an undesirable and unsustainable practice due to the environmental concerns associated with the petrochemical industry, and the market's inherent volatility and unpredictability (Xiong et al., 2019).

Photosynthetic microorganisms offer a sustainable alternative, utilising photosynthesis to assimilate atmospheric CO_2 into organic monomers or polymers employed in the synthesis of various plastic materials. Poly-hydroxy-alkanoates (PHA), biodegradable polymers with similar characteristics to their petrochemical-based counterparts, have been successfully produced by *Synechococcus subsalsus*, *Synechocystis* sp. PCC 6803, *Anabaena* sp., and

Spirulina sp. under nitrogen-deprived conditions. However, PHA production in photosynthetic microorganisms remains only possible in lab-scale experiments, and the up-scaling requires extensive further research (Costa et al., 2018; Mastropetros et al., 2022; Simonazzi et al., 2021; Sudesh et al., 2002).

ε-caprolactone is another crucial monomer, produced on a large scale to be utilised by the polymer industry for the synthesis of polycaprolactone, an important polymer commonly used in medical implements, cell cultures, 3-dimensional (3D) printing and packaging (Labet & Thielemans, 2009; Srinivasamurthy et al., 2020). ε-caprolactone was successfully produced by the biotransformation of cyclohexanone by engineered *Synechocystis* sp. PCC 6803 and *Chlamydomonas reinhardtii* cells acting as whole-cell biocatalysts (Erdem et al., 2022).

Ethylene is a widely used commodity chemical with multimillion ton annual demand (Haribal et al., 2018). Currently, ethylene is primarily derived from petroleum-based sources with high energy requirement and significant contribution to the overall CO_2 emission of the chemical industry (Amghizar et al., 2020). Ethylene is naturally synthetized by microalga species, however higher yields can be achieved by engineered species as was demonstrated in *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7942 (Carbonell et al., 2019; Thiel et al., 2018a; Vajravel et al., 2020).

Poly-, oligo- and monosaccharides are compounds with versatile applications from polymer industry through bacterial fermentation to food and feed additives. Photosynthetic microorganisms accumulate starch and glycogen as storage compound while sucrose, trehalose and glucosylglycerol act as osmoprotectant against salt stress (Keshari et al., 2019). The biomass with high carbohydrate content can be utilised in multiple ways. The biomass can be used in biorefinery to obtain various products or carbohydrates can be extracted for further use. Alternatively the cells can be engineered to secrete the molecules of interest (Maia et al., 2020; Noguchi et al., 2021; Thiel et al., 2019; Weiss et al., 2017).

Photosynthetic microorganisms have the capacity for organic acid production through extracellular fermentation and photorespiration, however, the fermentation process is transient, only happening in darkness, which renders it economically unfeasible (Kang et al., 2022). Similarly to carbohydrates, organic acids are also employed in polymer and food industry (Lee et al., 2019). As an example, *Chlamydomonas reinhardtii* cells have been used to produce glycolic acid in cell factories (Kang et al., 2022), while the biomass have been demonstrated to be a possible feedstock for fermentation to produce lactic acid (Talukder et al., 2012) and other organic acids (Pendyala et al., 2020).

1.3.1.1 Biofuel production

Currently, our society heavily relies on fossil resources as a primary energy source. The mining and combustion of these materials and the accompanying emission of CO_2 and methane gases have monumental effects on the climate and environment, while their natural deposits are limited (Gani, 2021; Speight, 2015). It is clear, that new, more sustainable energy sources must be found to maintain and improve our societal well-being. The utilisation of photosynthetic microorganisms as raw materials for biofuel production in biorefineries is a prospective way to replace fossil fuels such as coal, mineral oils and natural gas (Alishah Aratboni et al., 2019). Biorefineries, modelled after petroleum refineries, aim to process biomass into biofuels, high-value products, or electricity (Markou & Nerantzis, 2013). Photosynthetic microorganisms are classified as a third-generation feedstock for biorefineries, since their cultivation does not compete with food production and does not require arable land, consequently does not contribute to deforestation. The strains genetically engineered for more desirable properties are termed as 4th generation feedstock (Alishah Aratboni et al., 2019; Aziz et al., 2020; Okeke et al., 2022; S. Wang et al., 2016). Traditionally, the main steps of biofuel production are biomass cultivation, harvesting and dewatering, cell disruption, extraction of valuable compounds, and chemical conversion.

The primary biofuels derived from photosynthetic microorganism biomass are biodiesel, bioethanol and biobutanol followed by biogas. Species with high lipid content are suitable for biodiesel production, whereas those accumulating carbohydrates in high quantities are appropriate for bioethanol synthesis through fermentation (Kim et al., 2022; Maity & Mallick, 2022). For biodiesel production cells are destroyed and the fatty acid extracted followed by transesterification. In this process the fatty acids react with alcohols in the presence of a catalyst resulting in methyl esters and glycerol (Sajid et al., 2016). Economic feasibility remains challenging due to the energy-intensive processes required for cell wall disruption and water removal from wet biomass (Haas & Wagner, 2011; Park et al., 2015). Most commonly *Chlorella*, *Nanochloropsis*, *Chlamydomonas* and *Botryococcus* species are used for biodiesel production (Chen et al., 2015; Cheng et al., 2013; Hidalgo et al., 2015; Im et al., 2015).

Bioethanol can be produced from photosynthetic microorganism species with the high capacity to accumulate carbohydrates such as *Scenedesmus*, *Dunaliella*, *Chlorella*, *Chlamydomonas*, or *Spirulina* (Ho et al., 2013). Similarly to biodiesel production, the process involves the cultivation, harvest and dewatering of the biomass followed by cell disruption. Bioethanol production has also been demonstrated using wet biomass as a starting material. However, in this case the yields were less favourable. Ethanol is obtained by the hydrolysis and fermentation of the carbohydrates content in the biomass followed by distillation (Debnath et al., 2021). Common fermenters include *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Zymomonas mobilis,* potentially aided by engineered photosynthetic microorganisms (Sudhakar et al., 2018). Hydrolysis and fermentation can occur simultaneously or consecutively, with higher yields reported from consecutive reactions (Ho et al., 2013). Butanol can also be produced in a similar way, using different *Clostridium* species as fermenters (Shanmugam et al., 2021). Successful biobutanol production has been demonstrated using Chlorella and Chlamydomonas cells as feedstock (Figueroa-Torres et al., 2020; Onay, 2020). Furthermore, both ethanol and butanol can be produced directly by photosynthetic microbes engineered for this purpose (Liu et al., 2022; Wang et al., 2020).

In a different approach, the biomass of photosynthetic microorganisms has potential as a feedstock for the production of gaseous fuels. Hydrogen and biogas production using these species has demonstrated promising results (Goria et al., 2024; Goyal et al., 2018; Vajravel et al., 2020; Vargas-Estrada et al., 2022). Biomethane is produced through anaerobic fermentation of treated biomass, with enzymatic pre-treatment being the most effective method, albeit limited by high enzyme costs (Rodriguez et al., 2015). Molecular hydrogen (H_2) can be produced from biomass through thermochemical and fermentative methods. With thermochemical methods using high temperatures (900 – 1400 °C) it is possible to achieve high yields. However, CO_2, carbon monoxide (CO) and methane (CH_3) are also released during the process and there is a considerable amount of tar and ash waste formation. Dark fermentative H_2 production involves the use of photosynthetic microorganism biomass with high carbohydrate content as feedstock. With the use of strictly anaerobic fermenters even a small amount of O_2 can hinder H_2 production. For this reason facultative anaerobic bacteria, such as *Clostridium* and *Enterobacter* species are commonly used (Dębowski et al., 2021). Dark fermentation requires expensive biomass pre-treatment, and CO_2 is released as a by-product (Hallenbeck, 2011; Siddiki et al., 2022; Wang & Yin, 2018).

Alternatively, H_2 can be produced directly by photosynthetic microorganisms under special production conditions or by engineered strains (see more about direct H_2 production in section 1.4.1.2).

1.3.1.2 High value compounds

As described above, photosynthetic microorganisms are promising sources of commodity chemicals and biofuels alike. However, the commercialisation of these products remains challenging due to the high costs associated with the biomass cultivation, as well as upstream and downstream processes (Loke Show, 2022; Okeke et al., 2022; Vaithyanathan et al., 2023). To achieve economic viability the market value of the products should be sufficiently high. As described in section 1.2.2 the production of photosynthetic microorganisms as nutraceuticals or pigment sources, already constitutes a lucrative business with established markets. However, photosynthetic microorganisms express a plethora of secondary metabolites besides pigments which have potential in pharmaceutical industry and can be commercialised as high value products.

Many secondary metabolites, such as peptides, pigments, polyunsaturated aldehydes, polysaccharides, PUFAs, vitamins, polyphenols, and amphipathic lipids, possess anti-cancer properties (Andrade et al., 2018). Extracts derived from both marine and freshwater species have demonstrated inhibitory effects on cancer cell growth, with marine diatom extracts proving effective against leukaemia cells, while the Antarctic freshwater species, *Micractinium* sp., and *Chloromonas reticulata* have exhibited the suppression of colon cancer cell growth (Prestegard et al., 2009; Skjånes et al., 2021; Suh et al., 2018). Several *Chlorella*, *Dunaliella*, *Nannochloropsis*, *Isochrysis*, *Tetraselmis* and *Pavlova* species have been identified as having dual anti-cancer and anti-inflammatory properties (Skjånes et al., 2021).

Cryptophycins from the cyanobacterium *Nostoc* sp. GSV224 have demonstrated 100-1000 times higher activity against nasopharyngeal cancer cells and LoVo human colorectal cancer cells than currently available cancer drugs (Patterson et al., 1991), while calothrixins from *Calothrix* sp. exhibit efficacy against human HeLa cancer cells (Rickards et al., 1999).

The advent of antibiotics revolutionised human medicine, yet their excessive use has led to the emergence of multi-resistant bacterium strains necessitating the exploration of new antimicrobial compounds (Vivas et al., 2019). Microalgal metabolites offer potential solutions to this challenge. Antimicrobial compounds like acrylic acids, fatty acids, terpenoids, sterols, carbohydrates, phenols, etc. have been extracted from photosynthetic microorganisms (Saad et al., 2022). Antifungal activity of lyngbyabellins, a class of cyclic depsipeptides from *Lyngbya* and *Moorea* species and microguanidines from *Mycrocistis* sp. has also been reported (Gesner-Apter & Carmeli, 2008; Han et al., 2005). The lipopeptides, majusculamides from *Lyngbya polychroa* and *Lyngbya majuscule* exhibit diverse pharmacological effects, such as antifungal and anti-settlement activity, cytotoxicity and immunosuppressive effects (Tan et al., 2010). Hassalidins, are non-ribosomal cyclic glycosylated lipopeptides from *Hassallina* sp., *Nostoc* sp., *Tolypothrix* sp., *Cylindrospermopsis raciborskii*, and

Aphanizomenon gracile and have been shown to be effective against human pathogenic fungi (Pancrace et al., 2017; Vestola et al., 2014).

Moreover, certain compounds derived from cyanobacteria exhibit activity against parasitic protozoa, although this activity tends to be lower than that of commercially available antibiotics (Vining et al., 2015).

The recent COVID-19 pandemic underscores the urgent need for effective antiviral medications. Calcium spirulan from *Spirulina platensis* exhibits antiviral activity against enveloped viruses such as herpes simplex type-1, human immunodeficiency virus type-1 (HIV-1), mumps, and influenza virus. Additionally, sulfoglycolipids found in multiple photosynthetic microorganisms have demonstrated the ability to inhibit the reverse transcriptase enzyme of HIV (Loya et al., 1998).

1.3.2 Circular bioeconomy concept

The concept of a circular economy strives for the generation of zero waste and pollution throughout a product's lifecycle, from the extraction of raw materials to the final consumer. At the end of its lifespan the waste materials are reintegrated into industrial processes or reintroduced to the environment as organic residue (Nobre & Tavares, 2021). It is clear, that reaching complete circularity while also attaining economic gains is extremely challenging, especially regarding the lucrative valorisation of waste streams.

As described above, photosynthetic microorganism biomass can be utilised for several purposes, while their tolerance for harsh environmental conditions and metabolic versatility makes them capable of prospering on waste materials, such as wastewater from different sources or industrial flue gases (Ferreira et al., 2021; Ranglová et al., 2021; Yadav et al., 2020). While taking up excess nutrients from wastewater and CO_2 from flue gases is a valuable service in itself, the biomass grown on these materials then can be in turn utilised in various ways. Apart from serving as feedstock for biofuel production or a source for high-value compounds, the biomass can be integrated into agriculture or horticulture in the form of biostimulants and biopesticides (Ferreira et al., 2021; Ranglová et al., 2021).

1.3.2.1 Wastewater treatment

The vast amount of wastewater generated by our modern society pose a significant environmental threat, containing elevated levels of nitrogen, phosphorus, harmful pharmaceuticals, heavy metals, and other hazardous compounds (de-Bashan & Bashan, 2010; Rawat et al., 2011). The high concentration of nitrogen and phosphorus leads to the eutrophication of natural waters, while pharmaceuticals have adverse effects on the fauna. Heavy metals, accumulating through the food chain, contribute to various health issues in both animals and humans. Effective wastewater treatment, involving the removal or degradation of harmful components, is imperative for preserving human health and environmental sustainability (Godos et al., 2009; Nieto-Juárez et al., 2021; Zhao et al., 2016).

Wastewater treatment is a complex process involving a combination of mechanical and biological steps. Initially, solids with varying sizes are removed through filtering and sedimentation and the resulting wastewater sludge is removed. Subsequently, an aerobic digestion step follows, where ammonia is converted into nitrate and eventually N_2 gas by a bacterial consortium while other digestible organic compounds are also utilised by the growing bacteria. In this step maintaining the proper O_2 levels while removing excess CO_2 is crucial for the effective removal of nitrogen. This is achieved with pumping and vigorous agitation of the wastewater. Additionally, depending on wastewater type and treatment plant specifications, anaerobic digestion and chemical treatment may be incorporated (Grady et al., 2011). However, conventional wastewater treatment methods have their limitations. Certain heavy metals and persistent chemicals cannot be removed, it has high energy requirement and produces substantial amount of greenhouse gases, while the handling of the remaining wastewater sludge is challenging (Mohsenpour et al., 2021).

The utilisation of photosynthetic microorganisms for wastewater treatment offers an environmentally friendly and sustainable alternative. Many species exhibit mixotrophic growth, simultaneously utilising organic carbon and fixing CO_2 through photosynthesis, while the generated O_2 can be employed for the degradation of carbon compounds (Almomani et al., 2019; Singh et al., 2015). However, while many species are capable of utilising organic carbon for growth the complex nature of organic carbon compounds found in wastewater restricts the availability of these compounds for the metabolism of photosynthetic microbes (Mohsenpour et al., 2021). Extensive reports highlight the efficacy of photosynthetic microorganisms for removing nitrogen and phosphorous from horticultural, municipal and industrial wastewater while producing useful biomass (Gentili, 2014; Kothari et al., 2012; Salazar et al., 2021). Furthermore, they demonstrate a high resilience to harsh environmental conditions, and possess the capability to capture heavy metals and degrade various harmful compounds, including painkillers, antibiotics and hormones (Escapa et al., 2017; Parladé et al., 2018; Tripathi et al., 2019; Xiong et al., 2017).

The selection of the species utilised for wastewater treatments is highly dependent on the type of wastewater and the location of the treatment plants. For example, wastewater from paper mills contains high amounts of carbon, however, contains only low amounts of nitrogen and phosphorous, which are highly abundant in municipal and agricultural wastewaters (Gentili, 2014).

Nordic *Scenedesmus* strains have been reported to be effective to remove phosphorous and nitrogen from mixed paper mill, municipal and dairy wastewater (Gentili, 2014), while *Scenedesmus, Nostoc, Synechococcus, Microcystis, Chlorococcum* and *Tetradesmus* species provided a promising solution for the removal of nutrients from greenhouse wastewater (Salazar et al., 2021). Additionally *Chlorella pyrenoidosa* have been utilised for nutrient removal, while the produced biomass holds potential as feedstock for biofuel production (Kothari et al., 2012). Other genera used for wastewater treatment include *Anabaena, Arthrospira, Auxenochlorella, Botryococcus, Chlamydomonas, Desmodesmus, Dunaliella, Haematococcus, Hindakia, Nannochloropsis,* etc. (Li et al., 2019).

Despite the demonstrated efficacy of wastewater treatment using photosynthetic microorganisms, financial challenges persist. The treatment cost of 1 m^3 wastewater with algae was estimated to be 95 € based on a pilot scale system (Gouveia et al., 2016), while with conventional methods it is 0.1-0.2 € (Cashman et al., 2014). The primary cost involved is the energy consumption associated with the adequate mixing of the cultures. This can be reduced by using open pounds with paddlewheels instead of photobioreactors, since pumping has the highest energy cost (Acién et al., 2012; Gouveia et al., 2016). Furthermore, treatment by photosynthetic microorganisms is frequently applied in an additional third step after the energy intensive secondary step, so it does not decrease energy consumption. Instead, the integration of photosynthetic microorganisms into the secondary step, the treatment of settled wastewater (Mohsenpour et al., 2021), and consecutive valorisation of the acquired biomass could contribute to enhanced economic viability (Gouveia et al., 2016; Kothari et al., 2012).

1.3.2.2 Biostimulants and biopesticides

In response to the ever-increasing global population, a proportionate rise in the quantity of available food is necessary, which is only possible to achieve with improved crop yields. This challenge is compounded by diminishing arable land and escalating climate unpredictability. To meet production demands, modern agriculture relies heavily on pesticides and fertilisers, albeit at the cost of negative effects on consumers and the environment. The overuse of conventional chemicals also exacerbates resistance development in target organisms (Isman, 2020). The risk of pathogens developing resistance to biopesticides is lower, due to their multiple modes of action affecting growth, development, reproduction and nutrition (Dar et al., 2021). Photosynthetic microorganisms have potential both as biostimulants and biopesticides to increase crop yield and to protect it from pathogens (Costa et al., 2019; González-Pérez et al., 2021).

Photosynthetic microorganisms, with special regard to cyanobacteria, synthesise a diverse array of secondary metabolites with antifungal, antibiotic, and nematicide properties (El-Mougy & Abdel-Kader, 2013; Rastogi & Sinha, 2009). These effects are attributed to various compounds, including phenolic compounds, tocopherols, carbohydrates, proteins, oils, saponins, sesquiterpenes (Costa et al., 2019).

Cyanobacteria grown in piggery wastewater were shown to be effective against the plant pathogen *Fusarium oxysporum* (Ferreira et al., 2021) while *Chlorella vulgaris* grown in mineral medium or wastewater in outdoor raceway ponds exhibited antimicrobial and antifungal activity against *Clavibacter, Fusarium, Rhizoctonia* and *Phytophtora* strains (Ranglová et al., 2021). Extracts from *Chlorella sorokiniana* were shown to inhibit the growth of *Phytophthora cactorum*, a significant pathogenic oomycote affecting strawberry leaves and fruits post-harvest (Jokel et al., 2023). Various other species display activity against fungal pathogens affecting crops both pre- and post-harvest, including *Botrytis cinerea, Fusarium graminearum, Fusarium oxysporum, Alternalia solani* and *Verticillium albo-atrum* (Jokel et al., 2023).

In agricultural and horticultural practices, the simultaneous use of fertilisers and pesticides is indispensable for sustaining and augmenting production output. However, mineral fertilisers, primarily obtained through mining, have finite deposits and inflict severe environmental damage during extraction (Cao et al., 2023). This necessitates a shift toward more sustainable biological fertilisers and growth stimulants. Biofertilisers, comprising live microorganisms or their derivatives, stimulate plant growth, improve soil quality, and enhance fertility (Pirttilä et al., 2021). Photosynthetic microorganisms grown on wastewater are capable of the accumulation of nitrogen and phosphorous, the primary nutrients provided by commercial fertilisers. Subsequently, the nutrient rich biomass can be re-introduced into the production as fertiliser. Additionally, the cells synthesise phytohormones, compounds capable of enhancing plant growth. Photosynthetic microorganism-based fertilisers are capable of enhancing crop yields by improving nutrient uptake, soil quality, and tolerance to stress and pathogens (Bumandalai & Tserennadmid, 2019; Colla & Rouphael, 2020; Coppens et al., 2016; Guo et al., 2020).

Evidence suggests a significant increase in the sugar and carotenoid content of tomatoes when grown with fertilisers derived from the marine microalga *Nanochloropsis oculata* and the microalgal consortium MaB-Floc, predominantly composed of *Ulothrix* sp. and *Klebsormidium* sp. (Coppens et al., 2016). Furthermore, *Chlorella vulgaris* has been reported to improve shoot and root growth in cucumber plants and the morphology of sugar beets (Barone et al., 2018; Bumandalai & Tserennadmid, 2019). Various *Senedesmus* species have been reported to improve growth of petunia and sugar beets (Barone et al., 2018; Plaza et al., 2018).

1.4 Genetic engineering strategies for chemical production by photosynthetic microorganisms

Engineered photosynthetic microorganisms possess considerable potential for application as cell factories for the production of different chemicals (Sproles et al., 2021). However, despite the recent advances in the metabolic engineering of photosynthetic microorganisms, it still lags behind more extensively utilised heterotrophic microorganisms like *E. coli* or *Saccharomyces* sp. (Fu et al., 2019; Hitchcock et al., 2020; Sproles et al., 2021). By using metabolic engineering, we aim to modify genetic and regulatory processes to increase the production of desired compounds by the cells. The main approaches in metabolic engineering include gene knock-out or overexpression, as well as the introduction of foreign genes into the cells (Naghshbandi et al., 2020; Sproles et al., 2021; Yang, 1998).

Key elements of genetic engineering are the expression vectors, constructed from the genetic elements necessary for expressing the proteins of interest in a microbial cell. The optimal genetic sequences for achieving the best results vary considerably among different photosynthetic microorganism species. Model organisms, such as *Chlamydomonas, Nanochlroposis* and *Synechocystis* already have available modular cloning toolkits comprising of promoters, untranslated regions, introns, terminator regions, reporter genes, tags, and antibiotic resistance genes (Crozet et al., 2018; Poliner et al., 2020; Vasudevan et al., 2019). To maximise gene expression in each organism, codon usage should often be optimised as well. There are many databases and bioinformatics tools available for metabolic engineering such as Chlamy Sequence Optimizer, KEGG pathways or BioBricks (Ogata et al., 1998; Shetty et al., 2008; Weiner et al., 2018, 2020).

Following vector construction, the target sequence needs to be introduced into the genome of the desired organism, a process possible through various methods. In cyanobacteria, heterologous genes can be integrated into the nuclear genome via heterologous recombination or expressed from a self-replicating plasmid (Koksharova & Wolk, 2002). In green algae, the genes can be integrated into either the nuclear or plastid genome (Gan et al., 2018; Scranton et al., 2016). The optimal delivery of the plasmid into the cell largely depends on the target species. For green algae the most frequently used technique is electroporation, but particle bombardment, glass bead agitation, silicon carbide whiskers, nanoparticles and agrobacterium mediated transformation are also possible (Jinkerson & Jonikas, 2015). For cyanobacteria, natural transformation and conjugation, as well as electroporation, are viable options (Hitchcock et al., 2020; Vioque, 2007).

The clustered regularly interspaced palindromic repeats (CRISPR/Cas) gene editing system, derived from bacteria's immune response to viral infection, has recently become available for photosynthetic microorganisms. This system, utilising a single guide RNA to guide the CRISPR-associated protein 9 (Cas9) to degrade exogenous DNA, facilitates precise gene editing with the potential exclusion of selection markers like antibiotic resistance, which is advantageous for industrial applications (Poliner et al., 2018; Y.-T. Zhang et al., 2019). The CRISPR/Cas system is especially advantageous in polyploidy cyanobacteria, since with the traditional homologous recombination transformation the segregation step is often challenging and time consuming. The CRIPR/Cas system improves transformation efficiency and thus accelerates the segregation (Behler et al., 2018; Wendt et al., 2016).

Genetic modifications serves various purposes, including high-value compound production (Chen et al., 2017; Formighieri & Melis, 2016), biofuel production directly or as fourth-generation biofuel feedstock (Xue et al., 2020) or to increase photosynthetic capacity for carbon products (Liang et al., 2018; Santana-Sánchez et al., 2022). The subsequent sections present specific examples of genetic engineering strategies of photosynthetic microorganisms for application in chemical production platforms.

1.4.1 Examples of genetically engineered microorganisms in photosynthetic cell factories for the production of chemicals

In the conceptual framework of the cell factories, microorganisms are considered as production facilities (Villaverde, 2010). The desired chemicals are produced by microbial strains, often engineered for high production yields. These organisms often operate under physiological conditions characterised by lower temperature and pressure and without toxic solvents and catalysts contrary to traditional chemical synthesis methods (Lee et al., 2019). The biosynthesis pathways in microbial cell factories can be divided into three categories: i) native-existing pathways ii) non-native-existing pathways and iii) non-native-created pathways (Cho et al., 2022).

Utilising native-existing pathways means taking advantage of species capable of synthesising valuable chemicals through natural pathways, which can be further optimised through genetic engineering. For instance, *Corynebacterium glutamicum* is capable of the synthesis of high amount of L-glutamate an L-lysine, while *Bacillus* and *Lactobacillus* species can be utilised for L-lactate production (Abedi & Hashemi, 2020; Wendisch et al., 2016). The photosynthetic microorganisms *Haematococcus pluvials* and *Dunaliella sp.* are excellent for the production of astaxanthin and β-carotene respectively, while *Spirulina* is a great source of PUFAs (Grosshagauer et al., 2020; Srivastava et al., 2022).

Non-native existing pathways involve expressing partial or entire biosynthetic pathways from other species in a host cell. This strategy capitalises on the advantageous traits of the host organism, such as rapid growth, high stress tolerance, or photosynthetic activity, to yield products not naturally produced by the cells (Cho et al., 2022). There are a great variety of products synthetized this way in heterotrophic bacteria, fungi and photosynthetic microorganisms and each case requires special considerations regarding the metabolic compatibility of the host and the desired pathway (Yang et al., 2020; Yang et al., 2024).

If we aim to create products, which do not have known naturally occurring biosynthesis pathways, such as many petrochemical based products, we have to utilise non-native-created pathways. For the creation of these pathways enzymes capable of performing the missing reaction are needed. Promiscuous enzymes are capable of acting on various substrates, catalysing multiple reactions and thus, can be utilised to create novel biosynthesis pathways. As an example, aldehyde dehydrogenase enzymes were used to produce 1,5- pentanediol, a building block of polyesters and polyurethanes, from glucose, in *E. coli.* Additionally, rational, computer aided enzyme design can contribute to the creation of biocatalysts capable of performing the desired synthesis steps (Cen et al., 2021; Cho et al., 2022).

The production of industrially relevant chemicals by heterotrophic microorganisms like *E. coli* and *S. cerevisiae* is a well-established and widely used technology (Paul et al., 2019), however, the high costs of raw materials decreases economic gain. In contrast, photosynthetic microorganisms have remarkably low nutrient requirement and no need for organic carbon when sufficient light and CO_2 are available. These factors lower the cost of raw material input and make photosynthetic microorganisms an attractive alternative to heterotrophic microorganism (Nandru et al., 2022).

1.4.1.1 Ethylene producing photosynthetic cells

Renewable energy sources like solar or wind energy are already widely used to replace fossil-based fuels, however, they do not substitute for different petrochemical derived materials, such as solvents, and plastic polymers (Levi & Cullen, 2018). Ethylene is one of the most often used petrochemical with an annual demand reaching millions of tones. This hydrocarbon is used for the synthesis of polyethylene, a ubiquitous component in plastic packaging (Haribal et al., 2018). Currently, ethylene production requires high energy input and results in very high CO_2 emissions (Amghizar et al., 2020).

One possible solution to satisfy the high demand for ethylene while mitigation the negative environmental impact is to introduce enzymatic pathways found in plants, algae and certain bacteria into photosynthetic microorganisms to take advantage of photosynthetic CO_2 fixation. Ethylene, serving as a hormone in plants and algae, is synthesised in a two-step process. First, *S*-adenosyl-L-methionine (SAM) is transformed into 1-aminocyclopropane-1-carboxylic acid (ACC), which is in turn converted to ethylene. The enzymes responsible for these reactions are ACC synthase and ACC oxidase, respectively (Binder, 2020; Bleecker & Kende, 2000; Houben & Van de Poel, 2019). Although this pathway can be utilised for heterologous ethylene production by introducing the enzymes into *Synechococcus elongatus* PCC 7942, the reaction is constrained by the availability of SAM, and cyanide is released as a side product (Jindou et al., 2014).

An alternative enzyme for ethylene production is the ethylene forming enzyme (EFE), found in plant pathogenic bacteria, such as *Pseudomonas syringae*. The ethylene produced by the bacterium interferes with the hormonal signalling of the plant making it more vulnerable for infestation (Weingart et al., 2001). EFE uses 2-oxoglutarate (2-OG) as substrate and has successfully been expressed heterologously in *E. coli* and *S. cerevisiae*. However, in heterotrophic microorganisms, carbohydrates need to be supplied to achieve ethylene production. This limitation can be overcome by introducing the enzyme into photosynthetic organisms, which are capable of incorporating atmospheric CO_2 into the ethylene molecules through photosynthetic pathways (Eckert et al., 2014; Johansson et al., 2017; Lynch et al., 2016).

EFE catalyses two reactions: the ethylene forming reaction using 2-OG as a substrate and the C5-hydroxylation of L-Arg into 5-hydroxy-L-Arg, accompanied by oxidative decarboxylation of 2-OG into succinate (Wang et al., 2000). The ratio between the two reactions is 2:1 however, it depends on the reaction conditions (Fukuda et al., 1992; Kallio et al., 2021; Martinez & Hausinger, 2016). It is worth considering that 2-OG plays an important role in nitrogen assimilation and the maintenance of C/N balance in the cells and thus the depletion of 2-OG pool by EFE has considerable effect on cell metabolism (Fokina et al., 2010; Kallio et al., 2021).

EFE expression has been reported in various photosynthetic hosts, with various molecular engineering strategies proposed to enhance production yields. Common expression hosts include *Synechococcus elongatus* PCC 7942, *Synechococcus elongatus* PCC 11801 and *Synechocystis* sp. PCC 6803 (Kallio et al., 2021).

Ensuring stable expression and a sufficiently high plasmid copy number is essential for sustained enzyme concentration. In earlier works ethylene production stopped after a short period due the instability of the sequence coding the *efe* gene however, codon optimisation and the use of self-replicating plasmids have proven effective in achieving long-term production (Guerrero et al., 2012; Zhu et al., 2015). For further optimisation of *efe* expression alternative ribosome binding sites and 5' untranslated region elements were tested with favourable results (Thiel et al., 2018; Xiong et al., 2015).

In cases where enzyme concentration is not limiting, strategies to increase substrate availability should be employed. EFE uses 2-OG as substrate, which also performs important regulatory and housekeeping tasks in the cell and thus cannot be entirely used for ethylene production. To increase the concentration of available 2-OG, there are several possible strategies: i) The deactivation of 2-OG decarboxylase and succinic semialdehyde dehydrogenase enzymes, which also use 2-OG as a substrate results in decreased consumption (Jindou et al., 2014; Ungerer et al., 2012; W. Xiong et al., 2015; Zhu et al., 2015). ii) The overexpression of phosphoenolpyruvate carboxylase increases the transformation of phosphoenolpyruvate to oxaloacetate allowing the cell to fix more carbon without the involvement of pyruvate and acetyl-CoA. This allows precursors of oxaloacetate, such as 2-OG to accumulate and be utilised for ethylene production (Durall et al., 2020). iii) 2-OG can be supplemented to the cells exogenously. However, in this case the expression of a heterologous 2-OG permease is beneficial, because of the insufficient penetration of the cell membrane by the substrate (Zhu et al., 2015).

1.4.1.2 Hydrogen production

Many species of cyanobacteria and microalgae have the native capacity to produce H_2. The main enzymes responsible for photosynthetic production of H_2 are hydrogenases, which catalyse the reversible reduction of protons to H_2 utilizing electrons received from photosynthetically-reduced ferredoxin/flavodoxin (Gutekunst et al., 2014; Kim & Kim, 2011; Tóth & Yacoby, 2019). Besides hydrogenases, in N_2 fixing cyanobacteria, such as *Anabaena*, photosynthetic production of H_2 is primarily driven by nitrogenase enzymes. Nitrogenases catalyse the ATP-dependent reduction of N_2 to ammonia where H_2 is formed as a by-product. In the absence of N_2 as a substrate, nitrogenase exclusively catalyses the formation of H_2 (Barney, 2020).

There are three main bottlenecks to overcome for achieving the sustained and effective production of H_2. (i) The O_2 sensitivity of the enzymes involved in H_2 generation, both hydrogenases and nitrogenases; (ii) recycling of photosynthetically-produced H_2 for energy conservation and (iii) the presence of metabolic pathways competing with hydrogenases/nitrogenases for photosynthetic reductants (Nagarajan et al., 2021). Apart from some N_2 fixing species that form heterocysts supporting microoxic environment around the nitrogenase, photosynthetic H_2 production is typically a transient phenomenon, which occurs on the shift from dark anaerobic conditions to the light (Barney, 2020; Tóth & Yacoby, 2019). This is mainly due to the competition of hydrogenase with CO_2 fixation in the CBB cycle, which is activated soon after the onset of illumination, and accumulation of O_2 co-evolved in photosynthesis, which inhibits the activity of hydrogenase enzymes. Sustained photosynthetic H_2 production necessitates specific conditions, such as anaerobic cultivation, special light regimes, the addition of O_2 absorbents or nutrient (sulphur or phosphate) deprivation, leading to the partial inhibition of PS II O_2 evolving complex (Khosravitabar & Hippler, 2019; Miura et al., 1982; Philipps et al., 2012; Tsygankov et al., 2006; Vajravel et al., 2023). Alternatively, hydrogenase enzymes can be engineered for increased O_2 tolerance and direct acceptance of electrons from PSI, thus bypassing the competition with CO_2 fixation and other metabolic pathways (Kanygin et al., 2022; Weyman et al., 2011).

H_2 production in photosynthetic microbes is often affected by H_2 recycling, which is important for energy re-generation. For example, in heterocystous cyanobacteria H_2 produced during N_2 fixation is recycled by uptake hydrogenases, leading to a near absence of H_2 release from cells (Singh et al., 2015). Otherwise, the photosynthetic energy used for H_2 production could be potentially utilised in countless other cellular processes, which leads to a competition. Consequently, common genetic engineering strategies for improving H_2 production involve the downregulation of those competing pathways as well as the H_2 uptake (Kosourov et al., 2014, 2021; Santana-Sánchez et al., 2022; Zhang et al., 2014). The knock-out of uptake hydrogenase enzymes has been shown to be effective to increase H_2 production in *Anabaena*, *Nostoc* and *Synechocystis* strains (Boison et al., 1996; Lindberg et al., 2002; Sadvakasova et al., 2020). For the downregulation of competing pathways, the knock-out of Flavodiiron protein (Flv) 3A enzyme, responsible for photoprotection, has been reported to be effective in *Anabaena*, while in *Chlamydomonas*, proton-gradient-regulation5 (PGR5) mutants with impaired cyclic electron transfer showed increased production (Kanygin et al., 2022; Nagy et al., 2024; Santana-Sánchez et al., 2022). There are additional methods to increase H_2 production, such as the increase the number of heterocysts in N_2 fixing species with genetic or chemical tools or the decrease the ammonium sensitivity of the nitrogenase enzyme for sustained expression (Sadvakasova et al., 2020). Genetic engineering and technological advances make direct photo-H_2 production by cyanobacteria and microalgae a promising approach to obtain green H_2 nevertheless, further optimisations are needed for economic viability.

1.4.1.3 Sucrose secreting photosynthetic cells

In fermentative cell factories heterotrophic microorganisms require sugars or other organic carbon sources which increases the production costs and makes the process competitive with the food industry (Blombach et al., 2022; Lee et al., 2022). In recent years photosynthetic microorganisms garnered interest as a new carbon supplier for industrial processes. Eukaryotic algae typically store the fixed carbon in lipids or starch, which are less readily utilised by heterotrophic microorganisms commonly used in industrial fermentation (Scott et al., 2010). Cyanobacteria also accumulate polysaccharides as storage compounds, however, they synthesise simple sugars as compatible solutes under osmotic or other abiotic stress conditions (Kirsch et al., 2019; Klähn & Hagemann, 2011; Sanz Smachetti et al., 2020).

Salinity is one of the main stress factors in both aquatic and terrestrial habitats. A sudden increase in salt concentration decreases the osmotic potential of the surrounding medium, and results in alterations in the internal ion homeostasis. (Ladas & Papageorgiou, 2000). In response, cyanobacteria actively transport Na^+ and Cl^- ions out of the cells while accumulating osmoprotectant compatible solutes (Hagemann, 2011). These low molecular mass compounds help to balance the osmotic pressure between the interior of the cell and the environment without interfering with other cellular processes (Brown, 1976). Sucrose, alongside trehalose serves as a primary compatible solute in freshwater cyanobacterium species, such as *Synechococcus elongatus* or *Anabaena viriabilis* while in moderately salt tolerant species like *Synechocystis* sp., it is a secondary compatible solute (Lunn, 2002; Salerno & Curatti, 2003). While *S. cerevisiae* readily metabolises sucrose, some *E. coli* strains exhibit low affinity, yet it is possible to engineer strains for optimised sucrose utilisation (Bruschi et al., 2012).

Cyanobacteria can be engineered to accumulate increased amounts of sucrose under salt stress conditions. Introducing sucrose exporter genes, such as *cscB* from *E. coli*, facilitates the removal and secretion of sucrose into the growth medium, simplifying downstream processes and counteracting end-product inhibition. The secreted sucrose can then serve as a carbon source for heterotrophic microorganisms, either grown separately or in consortia with the sucrose producers (Kirsch et al., 2019; P.-C. Lin et al., 2020; Santos-Merino et al., 2021; Weiss et al., 2017).

Most commonly *Synechocystis* and *Synechococcus* strains are used as a chassis for sucrose production, however, the reported yields vary greatly depending on the host strain and engineering strategy. To enhance sucrose production, deactivating enzymes in competitive pathways is a common approach (Santos-Merino et al., 2021). Deactivation of glucosylglycerolphosphate synthase, the final enzyme in the pathway leading to the synthesis of glucosylglycerolphosphate, has been reported to significantly boost sucrose production (Thiel et al., 2019). Upregulating enzymes in the sucrose synthesis pathway, such as sucrose phosphate synthase, a rate-limiting step enzyme, can also increase sucrose production by facilitating the synthesis of sucrose-6-phosphate, the immediate precursor of sucrose. Sucrose-phosphate phosphatase, the subsequent enzyme in the pathway, is a prospective target for upregulation as well (Du et al., 2013; Duan et al., 2016; Lin et al., 2020; Thiel et al., 2018, 2019).

In natural settings, in photosynthetic microorganisms sucrose is synthesised as a response to transient salt stress. With the removal of the stressor, the recycling of compatible solutes becomes essential to maintain osmotic balance and conserve resources. Consequently, there are various metabolic pathways present to break down these sugars (Baran et al., 2017). Therefore, it is important to evaluate sucrose degradation as well as synthesis routes to optimise overall yield.

Among the main sucrose degraders in cyanobacteria are invertases, which irreversibly hydrolyse sucrose to glucose and fructose. Deleting invertases has reportedly increased sucrose yield. Other enzymes capable of sucrose cleavage, such as amylosucrases and sucrose synthases, are less widely distributed in cyanobacteria, with limited studies regarding their functions in industrial sucrose production applications (Kirsch et al., 2019; Liang et al., 2018; Santos-Merino et al., 2021).

Efficient removal of accumulated sucrose, achieved through the expression of the sucrose symporter *cscB,* proves beneficial by acting as a sink to prevent end-product inhibition. Exporting produced sucrose from the cell into the medium facilitates harvest and downstream processing, while keeping the cells intact for additional production cycles (Kirsch et al., 2019; Santos-Merino et al., 2021; Thiel et al., 2019; Weiss et al., 2017).

An alternative approach involves increasing the availability of reducing equivalents. Flavodiiron proteins (Flv) play a key role in redirecting excess electrons to O_2, safeguarding the electron transport chain from damage under fluctuating light conditions (Allahverdiyeva et al., 2015). By deactivating Flv1 and Flv3, electrons can be redirected towards sucrose production and export, which act as electron sinks and compensate for the loss of Flvs under fluctuating light conditions (Santos-Merino et al., 2021; Thiel et al., 2018).

1.4.2 Biocatalytic production of ε-caprolactone by whole-cell biotransformation

The chemical industry produces many products through artificial means, without analogues natural biosynthesis pathways. However, the creation of artificial pathways *de novo* is feasible by using natural biocatalysts. Naturally occurring enzymes are capable of the catalysis of a variety of industrially relevant chemical reactions, functioning under physiological conditions with high reaction rates and substrate specificity (Bell et al., 2021). By utilising the tools of genetic engineering, numerous enzymes can be expressed in microorganisms tailored for industrial production and optimised to meet specific requirements (Lee et al., 2023).

Biotransformation, the biochemical modification of chemicals by whole cells, cell lysates, or purified enzymes, is a prime strategy to synthesise artificial chemicals in a sustainable way (Liese et al., 2006). Employing whole cells instead of purified enzymes has the advantage of a continuously renewing catalyst source without the need of enzyme purification and stability issues regarding the purified enzyme (Tufvesson et al., 2011). Moreover, the co-factors generated by the cells can be harnessed for the reaction. In this regard photosynthetic microorganisms provide a considerable advantage, since they require no additional substrate for co-factor regeneration (Erdem et al., 2022; Schmidt et al., 2015). In this whole-cell biocatalysis approach, the substrate for the biotransformation reactions is different from the carbon source used for cell growth (B. Lin & Tao, 2017). The schematic overview of the biotransformation process is presented in Figure 2.

The whole-cell biotransformation process may involve a single recombinant enzyme catalysing the reaction, or cells expressing entire enzyme cascades to facilitate the multi-step transformation of a substrate (Madavi et al., 2022). When designing a process utilising whole-cell biocatalysts, it is important to first identify the best host cells for the desired pathways. Creating an efficient biocatalyst requires a comprehensive consideration of the metabolism of the cells. Addressing significant bottlenecks, downregulating potential competing pathways, enhancing precursor availability, and resolving redox balance issues are essential steps (Lin & Tao, 2017; Sudheer et al., 2017, 2018).

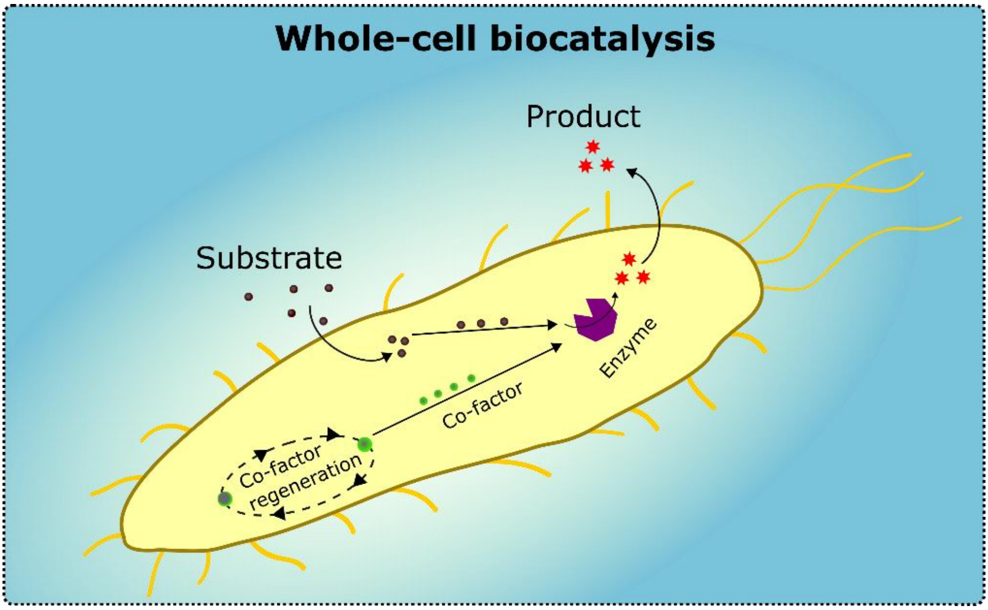

Figure 2. Schematic representation of whole-cell biocatalysis.

ε-caprolactone is an industrially relevant monomer used for the synthesis of polyacprolactone, a biodegradable material used in packaging and employed in 3D-printing, cell cultures and medical applications (Labet & Thielemans, 2009; Lee et al., 2014; Weissermel & Arpe, 2008; Williams et al., 2005). Currently ε-caprolactone is produced on an industrial scale by the Baeyer-Villiger oxidation reaction from cyclohexanone precursor in the presence of peracids, the synthesis of which comes with excessive environmental burden (Sever & Root, 2003).

As for increasing numbers of chemical processes, enzymatic catalysis has been proposed as a more sustainable alternative to the traditional synthesis methods (Bornscheuer, 2017). The group of enzymes capable of catalysing this reaction is called Baeyer-Villiger monooxygenases (BVMOs), found in multiple organisms. These enzymes incorporate O_2 into the molecule in an oxygenation event with NADH or NADPH as a cofactor. However, in heterotrophic organisms, such as *E. coli* NADPH is generated through the metabolism of sugars. This significantly increases the production costs, since glucose or other carbon sources has to be fed to the microorganisms (Erdem et al., 2022; Willetts et al., 2012). Moreover, O_2 availability in heterotrophic cultures is a rate-limiting factor, necessitating intensive agitation and incurring additional expenses. These challenges can be effectively addressed by expressing BVMOs in photosynthetic microorganisms, as they are capable of producing NADPH and release molecular O_2 through photosynthesis. However agitation is still necessary to facilitate effective mass exchange.

Cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* and BVMOs from *Parvibaculum lavamentivorans* ($\text{BVMO}_{\textit{Parvi}}$) and *Burkholderia xenovorans* ($\text{BVMO}_{\textit{Xeno}}$) are among the extensively studied BVMOs. CHMO is capable of catalysing transformation of cyclohexanone to ε-caprolactone in both cell-free environment (Schmidt et al., 2015) and in whole-cell biotransformation (Doig et al., 2003; Silva et al., 2020; Srinivasamurthy et al., 2020). However, in some cases it demonstrates substrate and product inhibition and stability issues (Srinivasamurthy et al., 2020). $\text{BVMO}_{\textit{Parvi}}$ and $\text{BVMO}_{\textit{Xeno}}$ have considerably higher reaction rates compared to CHMO in whole-cell biotransformation when expressed in *Synechocystis* (Erdem et al., 2022).

In the eukaryotic green alga, *Chlamydomonas reinhardtii* the different physiological processes are compartmentalised in the cells, photosynthesis taking place in the chloroplasts. This also means that the heterologously expressed enzyme in the cytosol and photosynthesis are spatially separated. Consequently, a fusion of CHMO and a chloroplast transit peptide, facilitating its translocation to the chloroplast, where it could more efficiently leverage the NADPH produced through photosynthesis was proposed. This approach has been reported to enhance the biotransformation process in *Chlamydomonas reinhardtii* (Paper II).

Both $\text{BVMO}_{\textit{Parvi}}$ and $\text{BVMO}_{\textit{Xeno}}$ have been reported to have high reaction rate and specificity in biotransformation of cyclohexanone to ε-caprolactone when expressed in *E. coli* (Erdem et al., 2022). Furthermore, $\text{BVMO}_{\textit{Parvi}}$ and $\text{BVMO}_{\textit{Xeno}}$ have been successfully expressed in *Synechocystis* cells and biotransformation was conducted. Both enzymes were capable of catalysing the reaction in *Synechocystis* cells, however, $\text{BVMO}_{\textit{Xeno}}$ was proven to have higher reaction rate and more complete conversion of substrate (Erdem et al., 2022).

1.5 Immobilisation as a way to increase bioproduction by photosynthetic microorganisms

Presently, industrially relevant photosynthetic microorganisms are commonly cultivated in either open ponds or enclosed photo-bioreactors, employing suspension cultures to maintain a planktonic state (Chisti, 2007; Noguchi et al., 2021). Although these production systems are adequate for growing a high amount of biomass and offer simple maintenance, the optimal light utilisation in them is impaired by the high culture density (Caldwell et al., 2021; Richardson et al., 2012). Therefore, when the aim shifts from biomass growth to the efficient conversion of light energy into value-added compounds, more focused production strategies became necessary.

An emerging technology to enhance photosynthetic microorganism chemical production efficiency is immobilisation. This approach limits cellular proliferation by physically restricting the space available for the cells, thereby redirecting energy and substrates from biomass accumulation toward chemical synthesis, resulting in higher yields and prolonged production time (Seibert et al., 2018). Moreover, immobilisation offers protection against unfavourable environmental conditions and toxic substrates (Kadimpati et al., 2013). Additionally, it simplifies medium-cell separation, reducing downstream processing expenses, enhancing reusability, and facilitating upscaling (Caldwell et al., 2021; Trelles & Rivero, 2020). Although immobilisation is accepted to be an effective method to increase the production of certain chemicals, the underlying physiological changes and possible negative effects on the cells are not well understood.

When employing immobilised microorganisms for biotechnological processes, it is important to consider that the choice of immobilisation materials and the utilised methods should align with the required physical parameters of the cultivation and production system. Various cultivation modes have been reported concerning the relationship between the immobilisation scaffold and the growth medium (Berner et al., 2015). The immobilised cells can be permanently submerged in the medium, partially submerged or alternating between the medium and the headspace. In situations where excessive contact between the medium and the immobilisation matrix may be detrimental e.g., when a gaseous product has high solubility in the medium or the continuous exposure can facilitate the deterioration of the matrix, it may be beneficial to limit contact (Berner et al., 2015). In such cases, the liquid medium can be provided through an additional substrate, such as melamine foam (Caldwell et al., 2021; Jämsä et al., 2018). The architecture, i.e. physical form of the immobilisation scaffold should in turn adhere to the cultivation mode at the same time providing adequate environment for the cells.

1.5.1 Different immobilisation techniques

In recent years, a plethora of immobilisation techniques has become available (Caldwell et al., 2021). Designing production systems involving immobilised cells requires consideration of several factors, with the relationship between the cells and the immobilisation matrices being foremost. Immobilisation can be classified into passive and active methods. Passive methods take advantage of the natural affinity of cells to adhere to certain surfaces, forming natural biofilms. In contrast, active methods employ chemical or physical means to strongly attach the cells to surfaces or confine them within matrices (Moreno-Garrido, 2008).

Passive methods, such as affinity binding and cell adsorption, are characterised by mild and reversible attachment. These techniques offer advantages such as low impact on cells, cost-effectiveness, and minimal technical demands. On the other hand, these methods do not provide permanent attachment and the cell escape from the surface is considerable. In addition, not every species is readily able to attach to surfaces. Many industrially important organisms prefer planktonic lifestyle and thus the choice of species suitable for this type of immobilisation is restricted (Moreno-Garrido, 2008).

While active immobilisation methods are stronger and more permanent, they require more advanced technical background and affect the cells more profoundly. However, in some cases the physiological effects induced by active immobilisation can be advantageous for specific biotechnological processes. Techniques like confinement behind semi-permeable membranes and entrapment in liquid-liquid emulsion or polymer matrices fall into the category of active immobilisation (Moreno-Garrido, 2008). Membrane confinement involves entrapping cells between a solid surface and a membrane, allowing for medium and product diffusion, while liquid-liquid emulsion entrapment positions cells between two liquid layers (Blanch et al., 1984; Mattiasson, 2018). These techniques do not restrict cell growth very effectively and although membrane confinement has been reportedly used for biosensors, the accumulating biomass often damages the membrane, rendering the system non-functional (Mallick, 2002). Currently polymer matrix entrapment stands as the predominant immobilisation technique (Caldwell et al., 2021; Moreno-Garrido, 2008), extensively employed due to the broad spectrum of available materials with tailorable characteristics. Polymer entrapment is applicable throughout a variety of areas ranging from wastewater treatment to chemical synthesis (Han et al., 2023).

The polymer entrapment technique is notably versatile, however, there are certain important limitations warranting consideration when choosing the employed materials. Key bottlenecks include ensuring sufficient diffusion of substrates and products through the matrix and assessing the potential negative impact of the immobilisation process on cells (Lapponi et al., 2022). Therefore, selecting parameters such as matrix polymer, crosslinking method, and matrix architecture is crucial in accordance with the desired reaction and microbial host (Lapponi et al., 2022; Moreno-Garrido, 2008).

The two major polymer immobilisation approaches are the entrapment of cells within thin films and beads. The thin films formed by the polymer matrix can be supported by diverse materials, including glass (Paper III) blotting paper (Vajravel et al., 2020) or insect screen on Scotch tape support (Kosourov & Seibert, 2009). Films are easy to manufacture manually (Das et al., 2015; Kosourov & Seibert, 2009) or by different coating instruments (Gosse et al., 2008) and can be made from various materials, such as latex, alginate or nanocellulose (Kosourov & Seibert, 2009; Rissanen et al., 2021; Vajravel et al., 2020).

Immobilisation within thin films is particularly appealing when cultivating photosynthetic microorganisms, since these provide optimal light utilisation by mitigating the self-shading effect (Seibert et al., 2018). Photosynthetic microorganisms immobilised in different films have proven effective in various applications, including the production of H_2 and ethylene (Gosse et al., 2008; Kosourov & Seibert, 2009; Rissanen et al., 2021; Vajravel et al., 2020), CO_2 capture (Ekins-Coward et al., 2019) and the development of cell-based sensor arrays (Pannier et al., 2014). A drawback of thin films lies in their mechanical strength, which may be unsatisfactory, particularly when vigorous agitation is required or when handling larger films (Jämsä et al., 2018; Leino et al., 2012). Furthermore, if ionic crosslinking is employed, the high ionic concentration in the medium can lead to rapid degradation of the films (Mallick & Rai, 1994; Rissanen et al., 2021; Vajravel et al., 2020).

Hydrogel beads offer better stability and are more suitable for cultivation requiring strong agitation due to their geometry. Hydrogel beads are most often made from alginate crosslinked with divalent ions in a simple and easily up-scalable process involving dripping the polymer solution containing the cells into the crosslinking reagent (Contreras-Cortés et al., 2019; Damayanti et al., 2021; Teryek et al., 2023). This method has been demonstrated to be effective in various applications, including sucrose, H_2, biodiesel, and succinic acid production (Ercole et al., 2021; Kumoro et al., 2021; Weiss et al., 2017) and wastewater treatment (Lee et al., 2020). However due to the larger volume of the immobilisation matrix, mass transfer limitations often pose a challenge, and in the case of photosynthetic microorganisms, it is essential to consider that light utilisation toward the centre of the bead may be suboptimal (Das et al., 2015; Sheldon et al., 2021; Tong & Derek, 2023; Žnidaršič-Plazl, 2021).

The utility and effectiveness of simple architectures, such as films or beads is extensively reported on, however they come with limitations such as mechanical stability issues or insufficient penetration of light, gases, and nutrients (Trelles & Rivero, 2020; Vajravel et al., 2020; Weiss et al., 2017). Complex structures created by 3D-printing have the potential to overcome some of these challenges. The emerging 3D-bioprinting technologies provide a possibility to achieve increased complexity and versatility in a scalable, user-friendly manner (Lee et al., 2022; Olmos & Kargul, 2015).

3D-printing is already a well-established technique for creating diverse three-dimensional objects from various materials in industrial, medical, and household applications. The layer-by-layer deposition of biological material, known as bioprinting, has gained prominence, particularly in the medical field, where it is widely used for creating models, cell and tissue cultures, and therapeutics (Fu et al., 2022). In tissue engineering applications, the hydrogel matrix surrounding the cells mimics the natural extracellular matrix components of the living tissue and through physical and chemical signalling regulates the activity of the embedded cells (Agarwal et al., 2020; Fu et al., 2022). This method allows for more precise and time-efficient manufacturing of tissue cultures and organoids compared to traditional techniques, such as culturing in liquid media. Specimens produced through 3D-bioprinting more closely resemble *in vivo* samples compared to tissue cultures grown in liquid media on the surface of culture vessels, which makes them better subjects for disease or therapeutic modelling and even transplantation to living hosts for regenerative purposes. Various human tissue types, including skin, cardiac, cartilage, and bone tissue, have been successfully fabricated using 3D-bioprinting (Agarwal et al., 2020; Bodhak et al., 2018; Lim et al., 2019; Phelan & May, 2015; Tomov et al., 2019; Varkey et al., 2019).

In the context of photosynthetic microorganisms, the implementation of 3D-printing technology is in emergence, with proof-of-concept studies demonstrating its practicality in chemical production, bioremediation, wastewater treatment, and even food production (Datta et al., 2023; Lee et al., 2022; Olmos & Kargul, 2015; Uribe-Wandurraga et al., 2020).

Fabricating intricate shapes such as grid patterns or embedded channels from hydrogels, is challenging using manual methods alone. However, 3D-printing allows the precise creation of complex hierarchical structures, offering advanced control over every dimension of the resulting matrix. Maximising the surface area to volume ratio is crucial for adequate gas and nutrient exchange, while it also enables improved light utilisation. Engineered *Synechococcus elongatus* cells immobilised in 3D-printed grid structures composed of Ca^{2+}-alginate have been reported to be effective in the oxidation of artificial dyes (Datta et al., 2023). In another approach *Chlorella pyrenoidosa* cells were added to a polylactide/poly(butylene adipate-coterephthalate) plastic material and printed using fused deposition modelling for the purpose of dye removal by adsorption. Although, in the later approach the cells do not survive the printing process due to the high temperatures involved, nonetheless the composite plastic material containing dead cells successfully absorbed methylene blue dye (Xia et al., 2020).

An interesting application of 3D-printing is the manufacturing of personalised food products for individuals with special dietary requirements. *Chlorella vulgaris* and *Arthrospira pratense* are well known for their beneficial properties as dietary supplements, however, their application in traditional products like bread and pastry is limited due to their strong green colour, which is not commonly associated with these products. 3D-printing using coaxial nozzles enables simultaneous extrusion of materials in a manner where the inner portion of the cylindrical print contains the batter loaded with photosynthetic microorganisms, while the outer layer has the traditional appearance (Uribe-Wandurraga et al., 2020).

A prevalent concept for decreasing atmospheric CO_2 levels to mitigate global climate change, is the development of artificial leaves. 3D-printed leaves were proposed for CO_2 photoreduction using both chemical agents (Chen et al., 2018) and photosynthetic microorganism cells (Liu & Choi, 2019).

Despite its remarkable versatility, 3D-printing is not yet widely employed on a large scale in the biotechnology industry. However, there is intensive research and rapidly growing interest in 3D-bioprinting, with the promise of widespread applications across different areas, offering high throughput and customisation possibilities.

1.5.2 Immobilisation matrices

Synthetic polymers are ubiquitous in daily life in packaging, clothing, and building materials. For immobilisation purposes, some of the frequently applied synthetic polymers are acrylamide, polyurethane, polyvinyl and latexes (Chen et al., 2019; Gosse et al., 2012; Travieso et al., 1996). However, biocompatibility and biodegradation of these materials is often problematic, and their price is comparatively high, which encourages the use of readily available biopolymers like agarose, carrageenan, cellulose, alginate or gelatine (Caldwell et al., 2021; Moreno-Garrido, 2008).

Presently, the preeminent biopolymer utilised for cell immobilisation is alginate, sourced from brown algae (Caldwell et al., 2021; Lapponi et al., 2022; Rissanen et al., 2021). Alginate is a hydrophilic polysaccharide co-polymer, comprised of β-D-mannuronate and α-L-guluronate building blocks. In aqueous environments, alginate forms a viscose solution and undergoes crosslinking facilitated by divalent ions such as Ca^{2+} or Ba^{2+}, in agreement with the "eggbox model", where cations sit in the cavities formed by the arrangement of the hydroxyl groups, leading to the formation of hydrogels (Grant et al., 1973). Dispersing the cells in an alginate solution and subsequently exposing them to ionic crosslinkers in the form of films or droplets is a cost effective and simple way of polymer matrix entrapment immobilisation (Andersen et al., 2015).

Cellulose and hemicellulose are emerging biopolymers sourced from nature, offering alternatives for immobilisation. These polymers are abundant in the cell wall of plants and can be procured from residual streams arising from the forestry sector and agricultural industry. Cellulose, a polysaccharide comprised of lengthy chains of β(1→4) D-glucose building blocks, is of particular interest being the single most abundant biopolymer. While insoluble in aqueous media, cellulose is capable of forming hydrogels due to its hydrophilic character. Cellulose can be obtained in diverse forms with distinct chemical characteristics depending on its source and the applied processing techniques (Curvello et al., 2019; Liu et al., 2020). For immobilisation purposes, TEMPO-oxidized cellulose nanofibrils (CNF) are commonly utilised. TEMPO oxidation facilitates the transformation of the primary hydroxyl groups of cellulose into anionic sodium C6-carboxylate groups. Consequently, cellulose strands can be released by mild mechanical treatment, due to electrostatic repulsion (Jämsä et al., 2018; Klemm et al., 2011). Depending on the length of the resultant cellulose fibres, cellulose nanofibrils (CNF) or nanocrystals (CNC) can be obtained.

Hemicelluloses are a group of branched polysaccharides consisting of various different building blocks such as xylose, arabinose, glucose mannose, galactose, rhamnose. The most important members of this group are xylans, mannans, mixed linkage β-glucans (MLG) and xyloglucans (Ebringerová et al., 2005; Scheller & Ulvskov, 2010). Hydrogels formed by hemicelluloses have been reported as scaffolds for human and animal cell and tissue cultures and other medical purposes (Roos et al., 2008; Wang et al., 2022). *O*-acetyl-galactoglucomannan (GGM) derived from spruce (*Picea abies*) trees, is a cheap material with excellent biocompatibility and susceptibility for chemical modifications, such as methacrylation, which makes it another perspective candidate for immobilisation applications. Spruce is a commonly cultivated crop in the forestry industry and the waste generated during the harvest serves as a valuable feedstock for the synthesis of GGM (Markstedt et al., 2017; Wang et al., 2022).

Composite materials, combining alginate and CNF in different ratios or introducing supplementary polymers like polyvinyl alcohol (PVA) or (MLG), provide versatile options for tailored immobilisation matrices with enhanced mechanical properties and crosslinking efficiency (Leppiniemi et al., 2017; Levä et al., 2023; Rissanen et al., 2021; Virkkala et al., 2023). The addition of chemically modified biopolymers like GGM methacrylate (GGMMA) or gelatine methacrylate conveys novel properties, such as enabling crosslinking by photocuring, as well as improving the mechanical properties of alginate and CNF matrices (Wang et al., 2021).

The vast array of immobilisation matrices with unique chemical and mechanical characteristics emphasises the importance of selecting the right material tailored to the desired application and specific microbial strain.

1.5.3 Crosslinking strategies

Cross-linking imparts a 3D network structure to hydrogels, influencing their viscoelastic properties, solubility, melting point, and other physical attributes (Maitra & Shukla, 2014). Achieving the mechanical stability and rigidity necessary for cell entrapment in various biopolymers necessitates some form of crosslinking. However, compared to the variety of available polymers, the selection of possible crosslinking methods suitable for cell immobilisation is rather limited.

Ionic crosslinking, a frequently employed physical crosslinking strategy, involves the attraction and crosslinking of charged moieties by ions of opposite charge. For immobilisation in alginate, CNF and hemicelluloses divalent cations such as Ca^{2+} or Ba^{2+} are often used for crosslinking (Bialik-Wąs et al., 2021; Vinchhi et al., 2021). While cost-effective and reliable, hydrogels produced through ionic crosslinking cannot withstand environments with high ionic strength, such as wastewater or media with high sodium chloride or sodium bicarbonate concentration or the presence of chelating agents, since these weaken the ionic interactions inside the matrix leading to degradation (Kube et al., 2021; Rissanen et al., 2021).

An alternative approach is the gradual removal of water from the polymer matrix. This process brings the individual polymer strands into close proximity to each other resulting in hydrogen bounding and physical entanglement between the strands. The addition of secondary polymers such as PVA or MLG conveys additional strength and stability to the matrix prepared this way. This process results in a stable scaffold capable of withstanding harsh production conditions. While the characteristics of the resulting matrices are remarkable, the process is time-consuming, and places additional stress on the cells (Jämsä et al., 2018; Rissanen et al., 2021; Virkkala et al., 2023). To ease the stress caused by the loss of water, osmotic dehydration offers a more gentle and controlled alternative to air drying. This method uses an osmolyte separated from the sample by a semi-permeable membrane to decrease the water content. However, the osmotic dehydration process is more time consuming and complicated compared to air drying (Guccini et al., 2022).

Photocuring is an advanced strategy without the drawbacks of drying and ionic cross-linking. During photocuring functional groups, often acrylates or methacrylates, polymerise in the presence of a photoinitiator upon exposure to specific wavelengths of light (Li et al., 2012). The light initiates the decompositions of the photoinitiator resulting in the formation of activated species, which in turn initiate the polymerisation of the functional groups on the polymer strands resulting in crosslinking (Singha & Schlaad, 2012). Commonly used biopolymers, such as alginate, cellulose, GGM and gelatine have available hydroxyl groups, which renders them susceptible for functionalisation with functional groups required for photocuring (Chiulan et al., 2021; Markstedt et al., 2017; Wang et al., 2021). Photocuring requires the careful consideration of the photoinitiator selection, since many of them have been reported to show toxicity towards different cell types (Fedorovich et al., 2009). Moreover, each photoinitiator is associated with a specific wavelength, which often falls within the UV range. Employing UV irradiation for crosslinking initiation ensures controlled reaction, since the standard indoor lighting does not emit UV light in the necessary intensity, however, the required time of the UV treatment should be carefully calibrated, since UV light is damaging to the DNA of the cells (Chiulan et al., 2021; Williams et al., 2005). When employing photosynthetic cells, careful considerations are needed, since the activated species and the UV light both cause damage to the photosynthetic machinery, consequently it is important to find the precise concentration of photoinitiator where of the crosslinking strength and dynamics are satisfactory while preventing irreversible damage to the photosynthetic machinery of the cells (Kumar et al., 2008).

Similarly to matrix materials, the combination of crosslinking methods, such as the supplementation of ionic crosslinking with water removal, provides opportunities for improved results (Jämsä et al., 2018; Virkkala et al., 2023).

However, the choice of the ideal method depends greatly on the characteristics of the polymers used and the requirements of the production system, including agitation, medium composition, temperature, and pH.

2 Aims of the study

Cyanobacteria and eukaryotic microalgae play a key role in the current ecosystem as primary producers and possess substantial economic potential as sources for food, feed, chemicals, and sustainable energy. These photosynthetic microorganisms also provide an opportunity to capitalise on the unique process of photosynthesis as sustainable chemical production platforms.

The application of genetic engineering tools enables the introduction of novel biosynthetic pathways to photosynthetic microorganisms, as well as the optimisation of existing pathways to enhance the production of target chemicals. The immobilisation of cells within polymer matrices further facilitates the increase of production outputs. In my research, I aim for the development of distinct photosynthetic chemical production platforms, utilising *Chlamydomonas reinhardtii* and *Synechocystis* PCC 6803 immobilised in advanced matrix materials.

The specific aims of my thesis were the following:

1. To demonstrate the effect of the immobilisation on the sucrose production and physiological processes of *Synechocystis* cells.
2. To demonstrate the capability of the photosynthetically produced sucrose to drive a biotransformation reaction in an engineered *E. coli* strain.
3. To evaluate and optimise the biotransformation reaction in the engineered eukaryotic photosynthetic alga, *Chlamydomonas reinhardtii* and couple it with photosynthetic H_2 production.
4. To utilise additive manufacturing for 3D-bioprinting an advanced photocurable living photosynthetic material and demonstrate its applicability for ethylene production by *Synechocystis* and biotransformation by *Chlamydomonas* strains.

3 Materials and methods

3.1 Microbial strains and culture conditions

The microbial strains employed in the course of our research project are detailed in Table 2, while the growth conditions for the various experiments conducted are presented in Table 3.

Table 2. List of used microbial strains. The abbreviations, the application of the strain and the corresponding media and antibiotics are also presented. Furthermore, the original publications are provided.

Strain	Abbreviation	Application	AB (µg ml^{-1})	Media	Ref.
Synechocystis PCC 6803 S02	*Synechocystis* S02	Sucrose production	Spectinomycin: 20 Chloramphenicol: 8 Kanamycin: 20	BG11	Thiel et al., 2019
Synechocystis PCC 6803 efe	*Synechocystis efe*	Ethylene production	Spectinomycin: 25 Chloramphenicol: 10	BG11	Thiel et al., 2018
Escherichia coli WΔcscR Inv:Parvi	*E. coli Parvi*	Biotransformation	Ampicillin: 100	LB	Paper I
Chlamydomonas reinhardtii UVM4/CHMO	*Chlamydomonas* UVM4/CHMO	Biotransformation, H_2 production	Spectinomycin:100	TAP or TP	Paper II
Chlamydomonas reinhardtii UVM11-CW/CHMO	*Chlamydomonas* UVM11-CW/CHMO	Biotransformation	Spectinomycin:100	TAP	Paper II
Chlamydomonas reinhardtii UVM11-CW/CHMO_PSAD	*Chlamydomonas* UVM11-CW/CHMO_PSAD	Biotransformation	Spectinomycin:100	TAP	Paper II; Paper III

Table 3. Growth conditions employed with the different microbial strains in the different experiments.

	Sucrose production	Ethylene production	*E. coli* bio-transformation	*Chlamydomonas* biotransformation
Strain name	*Synechocystis* S02	*Synechocystis efe*	*Escherichia coli* WΔcscR Inv:*Parvi*	*Chlamydomonas* UVM4/CHMO; UVM11-CW/CHMO; UVM11-CW/CHMO_PSAD
Temp. (°C)	30	30	30	25
Illumination (µmol photons m^{-2} s^{-1})	200	70 & 35	70	0, 26 & 165
Shaking (rpm)	100	100	100	90
CO_2 (%)	1	Ambient	1	Ambient or 1
Culture type	Suspension, immobilisation	3D-printed film	Suspension	Suspension and 3D-printed film

Synechocystis stock cultures were maintained in 25 ml BG11 medium containing 20 mM HEPES buffer, pH adjusted to 7.5 and supplemented with the respective antibiotics (see Table 2). The cultures were cultivated in 100 ml Erlenmeyer flasks, at a constant temperature of 30 °C, under ~50 µmol photon m^{-2} s^{-1} illumination, and 1% CO_2 concentration while being agitated at 100 rpm, using an orbital shaker.

Chlamydomonas stock cultures were maintained in 40 ml of tris-acetate-phosphate (TAP) medium at a pH of 7.2 supplemented with antibiotics (see Table 2). *Chlamydomonas* cultures were cultivated in 100 ml Erlenmeyer flasks at 25 °C. Light conditions varied between 30 to 60 µmol m^{-2} s^{-1}, and CO_2 concentration was maintained at ambient levels. The cultures were agitated at 120 rpm using orbital shaking.

All photosynthetic stock cultures were cultivated in MLR-351 growth chambers (Sanyo, Japan) under fluorescent light.

E. coli cultures were maintained on lysogeny broth (LB) 1.5% agar plates supplemented with antibiotics (see Table 2), and stored at 4 °C.

Experimental pre-cultures of *Synechocystis* cells were cultivated in 100 ml BG11 medium without antibiotics in 250 ml Erlenmeyer flasks. Pre-cultures were incubated in growth chambers (AlgaeTron AG 130-ECO, PSI, Czech Republic) equipped with white LED lights. The growth conditions were 30 °C temperature, 200 µmol photon m^{-2} s^{-1} light intensity, 1% CO_2 concentration, and continuous agitation at 100 rpm using orbital shaking.

Chlamydomonas pre-cultures were grown in MLR-351 growth chambers (Sanyo, Japan) supplemented with antibiotics (see Table 3). These cultures were maintained at a temperature of 25 °C, under the illumination range of 30-60 µmol m^{-2} s^{-1} and agitated at 120 rpm using orbital shaking. Depending on the used medium (TAP or TP), the CO_2 concentration was either maintained at ambient levels or set at 1% when acetate was not included in the medium as a carbon source.

For *E. coli* pre-cultures, overnight cultivation was carried out in 10 ml culture tubes containing 2-3 ml of LB medium supplemented with antibiotics.

3.2 Chemical production by microorganisms in suspension cultures and immobilised in polymer matrices

3.2.1 Sucrose production and determination

Experimental cultures for sucrose production by suspension cultures were initiated when the pre-cultures reached optical density at 750 nm (OD_{750}) 1.0. At this point *Synechocystis* S02 cells were harvested by centrifugation and resuspended in 100 ml BG11 medium supplemented with 400 mM sodium chloride (BG11+NaCl). After overnight acclimation to the high salt concentration, cultures were pelleted and resuspended in fresh BG11+NaCl medium with OD_{750} adjusted to 0.5, subsequently 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of *cscB* and consequently the export of sucrose into the growth medium. The cultures were incubated under 200 µmol photon m^{-2} s^{-1} illumination at 30 °C temperature, 1% CO_2 with orbital shaking in growth chambers equipped with LED lights (AlgaeTron AG 130-ECO, PSI, Czech Republic). Samples were taken daily for 7 days by pipetting 500 µl from the cultures to an Eppendorf tube, centrifuging the samples and preserving the supernatant at –80°C until analysis.

Pre-cultures for bead immobilised sucrose producing cells were prepared as described above and grown until OD_{750} reached 1.5. Subsequently, the cells were collected by centrifugation and resuspended in 1/24 of the original volume in 20 mM HEPES–NaOH pH 7.5 buffer. The cell suspension was mixed with 3% alginate solution (Sigma-Aldrich, US) in 1:12 ratio and mixed homogenously using pipette tip and vortex.

A 10 ml syringe was loaded with the alginate formulation containing the cells, which was in turn dripped into 50 mM $CaCl_2$ solution through a 25 G (0.5*25 mm) hypodermic needle. The cell density in resulting beads corresponded to OD_{750} = 3 (double of the original suspension culture), while the final alginate concentration was 2.75%. The beads were left to solidify for 10 minutes, then the $CaCl_2$ solution was discarded, and the beads were washed twice in sterile MQ water.

The alginate beads were placed in 160 mL filter capped culture flasks (NunC EasYFlask, Thermo Scientific, US) in BG11+NaCl medium supplemented with 1 mM IPTG. Each flask contained 40 ml medium with 10 ml beads. For sucrose production the cultures were incubated as described above for 7 or 30 days. During the 7–day production samples were taken daily, while during the 30 days period samples were taken every 3 – 4 days, and stored at –80 °C until analysis. The medium was removed after every sampling and replaced with fresh during the 30 day cultivation, in a semi-continuous manner.

Sucrose samples analysed using commercially available sucrose/D-glucose Assay Kit (Megazyme, USA) according to the manufacturer's instructions.

3.2.2 Biotransformation by *E. coli*

Sucrose produced over 7 days by immobilised *Synechocystis* S02 cells in BG11+NaCl medium was used to drive the biotransformation reaction in *E. coli Parvi* strain engineered for optimal sucrose utilisation and biotransformation of cyclohexanone to ε-caprolactone (Paper I). Overnight cultures from *E. coli Parvi* were prepared as described above. The overnight cultures were used to inoculate 200 ml terrific broth (TB) medium. The cell culture was incubated at 200 rpm orbital shaking at 37 °C until OD_{600} reached 2 - 3. Subsequently expression of $BVMO_{Parvi}$ was induced by the addition 1 mM IPTG. The protein was expressed overnight at 30 °C. Next, the cells were collected by centrifugation and washed twice with 5 mM HEPES pH 7.5 buffer, then resuspended in the sucrose enriched BG11+NaCl medium taken from sucrose producer bead immobilised *Synechocystis* S02 cultures with OD_{600} adjusted to 8. To ensure continuous $BVMO_{Parvi}$ expression 1 mM IPTG was added. The cultures were incubated in 30 ml volume in baffled Erlenmeyer flasks at 30 °C with 1% CO_2 and 180 rpm orbital shaking. The reaction was initiated by the addition of 5 mM cyclohexanone substrate. Samples were taken 0, 1, 2, 3 and 24 hours after initiation and stored at –80 °C until analysis. Samples were analysed by gas chromatography (GC).

3.2.3 Biotransformation and molecular hydrogen production by *Chlamydomonas* suspension cultures

Chlamydomonas UVM4/CHMO, UVM11-CW/CHMO or UVM11-CW/CHMO_PSAD cells were collected by centrifugation and resuspended in fresh TAP medium. A reaction mix was prepared, consisting of TAP or TP medium, 25 mM MOPS-buffer, 5-10 mM cyclohexanone and 0-1.7% (v/v) ethanol or 0-50 mM fomepizole to inhibit the side reaction. Ethanol and fomepizole concentrations were selected to minimise their growth inhibitory effects. The cells were introduced into the reaction mix with a total chlorophyll (Chl) concentration adjusted to 100 µg ml^{-1}. The reaction flasks were closed with gas-tight rubber stoppers. When TP medium was used, 1% CO_2 was supplemented to the headspace to provide inorganic carbon source. Flasks were incubated with orbital shaking at 90 rpm at 25 °C under light conditions varying between darkness, 26 and 165 µmol photon m^{-2} s^{-1} depending on the experiment. Samples were taken with pipette (beginning and end) or a syringe and needle through the rubber cap.

For H_2 production *Chlamydomonas* UVM4/CHMO cells were used with the air in the headspace of the vials was replaced by argon. This was necessary for the proper functioning of the O_2 sensitive [Fe-Fe]-hydrogenase enzyme catalysing H_2 production. The vials were incubated under 26 µmol photon m^{-2} s^{-1} and H_2, O_2 and ε-caprolactone concentrations were followed for 48 h. Initially samples were taken every 2 h, followed by sampling after 24 and 48 h. Samples were analysed by GC.

3.2.4 3D-printing and rheological measurements

The photocurable 3D-printing bioink was prepared by dissolving 3% Na-alginate (Sigma Aldrich, USA), 4% galactoglucomannan-methacrylate (GGMMA) and 0.05% phenyl-2,3,6-trimethylbenzoylphosphinate (LAP) (Sigma-Aldrich, USA) in MQ water and adding *Synechocystis efe* cells corresponding to 10 µg ml^{-1} Chl a or *Chlamydomonas* UVM11-CW/CHMO_PSAD cells corresponding to 100 µg ml^{-1} Chl a. The ink was loaded into a syringe with piston (Nordson, USA) and printed on glass slides previously treated with 3-(trimethoxysilyl)propyl methacrylate (TMPSM) (Sigma Aldrich, USA) according to the manufacturer's instructions. The thin film prints were created by extrusion printing with a Brinter ONE Multitool 3D bioprinter (Brinter, Finland). The printed films were crosslinked by exposure to 405 nm light with 60 mW cm^{-2} intensity for 10 minutes. The areas of the resulting films were 11.21 cm^{2} for ethylene producer *Synechocystis efe* and 3 cm^{2} for *Chlamydomonas* UVM11-CW/CHMO_PSAD cells performing biotransformation.

Rheology measurements were performed using an MCR 702 MultiDrive rheometer (Anton Paar GmbH, Germany) at room temperature with a PP25 parallel-plate (25 mm diameter, 0.2 mm gap).

For the investigation of photocuring dynamics a constant strain of 0.5% and 5 Hz frequency was used. The samples were exposed to 60 mW cm^{-2} 405 nm light (bluepoint 4 ecocure UV lamp, The Hönle Group, Germany) 20 s after the beginning of the measurement. To investigate the sheer stress tolerance of the films, amplitude sweep measurements were performed with amplitudes of 0.01 - 200% and 5 Hz frequency with 20 points recorded per decade. Before stress sweeps, the samples were crosslinked on the glass plate of the instrument as described above. Storage modulus (G’, describing elasticity), loss modulus (G’’, describing viscosity) and loss factor tangent (tan δ) were recorded.

3.2.5 Ethylene production with 3D-printed biofilms

After 3D-printing, the films were left to recover overnight in a Petri dish filled with BG11 medium under low light (25 µmol photon m^{-2} s^{-1}). After recovery the films were placed in 126 ml vials in 10 ml BG11 medium supplemented with 200 mM sodium bicarbonate (BG11+$NaHCO_3$) and 1 mM IPTG. The vials were sealed with gas-tight rubber stoppers. The vials were incubated at 30 °C at 60 rpm orbital shaking, under 35 or 70 µmol photons m^{-2} s^{-1} illumination. Ethylene was produced under these conditions continuously for 7 days or in a semi-continuous manner for 20 days with the vials opened and the medium changed to fresh every 5 days. Ethylene samples were measured by GC.

3.2.6 Biotransformation with 3D-printed *Chlamydomonas reinhardtii* films

After 3D-printing, the films containing *Chlamydomonas* UVM11-CW/CHMO_PSAD cells were left to recover for 72 h in TAP medium under low light (20 µmol photon m^{-2} s^{-1}) at 25 °C and ambient CO_2 concentration. Subsequently, the films were placed in 3.25 ml biotransformation reaction mix as described in section 3.2.3. The biotransformation reaction was performed in 37 ml infusion bottles sealed by a gas-tight rubber stopper. The bottles were incubated at 90 rpm orbital shaking under 26 µmol photon m^{-2} s^{-1} illumination. 250 µl samples were taken 0, 1, 3, 6, 19, 24, 30, 43, 48 and 72 h after the start of the reaction. Samples were stored at –80 °C until analysis. Concentrations of cyclohexanone cyclohexanol, and ε-caprolactone were determined using GC.

3.3 Product quantification by gas chromatography

For the determination of cyclohexanone, cyclohexanol and ε-caprolactone concentrations, the compounds were extracted using ethyl acetate containing 2 mM acetophenone (Sigma-Aldrich, USA) as internal standard. First 0.06% hydrochloric acid was added to 300 µl sample, then 150 µl ethyl acetate with the internal standard was used for extraction twice, consecutively. The extracted samples were dried using anhydrous magnesium sulphate. After centrifugation the ethyl acetate was collected and analysed by GC-2010 Pro gas chromatograph (Shimadzu, Japan) equipped with HP-5MS 30 m x 0.25 mm (5%-Phenyl)-methylpolysiloxane column (19091S-133, Agilent) with N_2 carrier gas and splitless injection mode. Compound separation happened at 35 °C (hold 3 min), 200 °C (hold 3 min, 10 °C min^{-1}) and 300 °C (hold 3 min, 25 °C min^{-1}). Calibration was done using known amounts of each chemical of interest.

For ethylene concentration determination, 40 µl sample was taken with a gas-tight syringe (Hamilton, US) and injected manually into Clarus 580 gas chromatograph (PerkinElmer, USA) equipped with Carboxen 1010 PLOT 30 m × 0.53 mm capillary column operating at 140 °C and a flame ionisation detector (FID) operating at 350 °C. The air and H_2 flow were 300 ml min^{-1} and 30 ml min^{-1} respectively. The flow rate of the argon carrier gas was 9.8 ml min^{-1}. Total ethylene concentration in the liquid phase and headspace was calculated based on the partial pressure and solubility of ethylene at 30 °C. For calibration, known amounts of ethylene were used.

For the determination of H_2 and O_2 concentration, 150 µl sample was withdrawn from the headspace of the vial and the missing volume was replaced with argon. Samples were analysed with Clarus 500 GC equipped with a thermal conductivity detector and a molecular sieve 5 Å column (60/80 mesh) (PerkinElmer, USA) with argon carrier gas. Air was used as a standard for O_2 and 3% H_2 gas for H_2.

3.4 Chlorophyll determination

The Chl a content of *Synechocystis* cells in suspension cultures was determined by collecting cells from 1 ml suspension by centrifugation followed by resuspension in 90 v/v % methanol and incubation for 5 min in darkness at room temperature. After incubation the samples were centrifuged, and the absorbance of the supernatant was measured at 665 nm with UV-1800 spectrophotometer (Shimadzu, Japan). Values of 730 nm were subtracted from the values at 665 to disregard residual cell particles. Chl a concentration was calculated according to Lichtentaler (1987). In case of Ca^{2+}-alginate bead immobilised cells, beads of 1 ml volume were collected and Chl a was extracted for 48 h with 9 ml 90 v/v % methanol at +4 °C, while from photocured films Chl a was extracted by scraping the films into a 2 ml Eppendorf tube and incubating with 1 ml 90 v/v % methanol for 1 h at 65 °C. Concentration determination was performed identically to suspension cultures.

From *Chlamydomonas* suspension cultures total Chl (a + b) was extracted from 1 ml culture by resuspension in 96 v/v % ethanol following pelleting by centrifugation. After 5 min incubation in darkness, samples were centrifuged again and the supernatant was measured at 649, 665 and 720 nm. Absorbance values at 720 nm were subtracted from the values at 649 and 665 to disregard residual cell particles. Total Chl concentration was calculated according to Harris et al. (1989).

3.5 Real-time gas exchange analyses by membrane inlet mass spectrometry

Membrane inlet mass spectrometry (MIMS) was used to assess carbon fixation and O_2 evolution in sucrose producer *Synechocystis* S02 cells. Both suspension and bead immobilised cultures were investigated in sucrose producing (+NaCl) and non-sucrose producing (-NaCl) conditions. Prima PRO membrane inlet mass spectrometer (Thermo Fisher, USA) was used to monitor gas exchange. Samples were loaded into a modified DW1 O_2 electrode chamber kept at 30 °C by a water jacket. From suspension cultures 2 ml was loaded in the chamber with 10 $\mu g\ ml^{-1}$ Chl a concentration, adjusted in fresh BG11 or BG11+NaCl medium. From bead immobilised cultures, 20 beads were loaded in the medium in which they were incubated during the production period. This medium was used to assess the actual gas exchange in identical conditions to the production state. Samples were supplemented with 1.5 mM sodium bicarbonate and $^{18}O_2$ isotope was in equimolar ratios to $^{16}O_2$. A magnetic stirrer bar was used to continuously agitate the samples in the chamber, which was separated from the vacuum line by a Teflon membrane. The presence of O_2 (*m/z* 32 and 36) and CO_2 (*m/z* 44) were monitored in real time. The $^{18}O_2$ isotope was used to distinguish between respiration and O_2 evolution and to calculate the total and net O_2 production. During the measurement 5 min dark adaptation was followed by 5 min illumination with 500 $\mu mol\ m^{-2}\ s^{-1}$ intensity light from a halogenic light source. Gas exchange rates were determined according to Beckmann et al. (2009).

3.6 Determination of PS II yield

Y (II) was measured by PAM 2000 Portable Chlorophyll Fluorometer (Walz Mess und Regeltechnik, Germany). The suspension cultures, beads or films were placed in a Petri dish under the detector and *Y (II)* was determined under a series of saturation light pulses of 3000 µmol photons m^{-2} s^{-1} for 0.8 s applied on top of 200 µmol photons m^{-2} s^{-1} actinic light.

4 Results

4.1 Increasing sucrose production yields of Synechocystis S02 strain by immobilisation in Ca^{2+}-alginate beads

Immobilisation has been extensively reported to be effective for enhancing chemical production yields of different microbial strains. To evaluate the effect of immobilisation on the sucrose yield of *Synechocystis* S02, the cells were entrapped in alginate beads crosslinked by Ca^{2+} ions. *Synechocystis* cells accumulate sucrose as osmoproectant under salt stress. The S02 strain was engineered for increased sucrose accumulation and secretion into the growth medium, when incubated with NaCl in the growth medium (400 mM). Sucrose production was monitored in both suspension and bead-immobilised cultures, and the yields were normalised to Chl concentration for reliable comparison.

In suspension cultures Chl accumulation progressed in a quasi-linear manner until the 6th day when it reached plateau phase. In bead immobilised cultures Chl concentration was increasing until the 5th day and started to slightly decline thereafter. This drop can be attributed to accumulation of the sucrose in the growth medium leading to mixotrophic metabolic activity or the stress endured due to the prolonged salt exposure. Total sucrose concentration in suspension cultures reached 1910 mg l^{-1} after 7 days, with the specific productivity of ~700 mg mg_{Chl}^{-1} (Figure 3A). In contrast, the maximum sucrose concentration in the medium of bead-immobilised cells was 1150 mg l^{-1} corresponding to ~1200 mg $mg_{Chl\ a}^{-1}$ (Figure 3B – C). This represents an 86% increase in specific sucrose productivity in the immobilised cells based on initial Chl concentration Sucrose concentration in the medium reached a plateau after 7 days, signalling the termination of production.

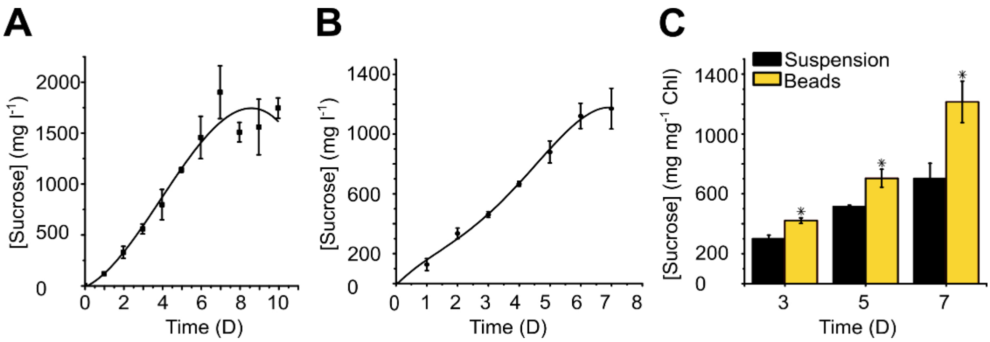

Figure 3. Characterization of sucrose production suspension and alginate-immobilised cultures under salt stress (400 mM NaCl). (A) Total sucrose concentration in suspension cultures; (B) total sucrose concentration in bead-immobilised cultures; (C) specific sucrose production yields in both culture types. Error bars represent the standard deviation of three independent biological replicates (Modified from Paper I).

To explore the potential for long-term production using immobilised *Synechocystis* S02 cells, sucrose accumulation was monitored over 27 days in a semi-continuous system. Samples were collected, and the medium was replenished with fresh BG11+NaCl supplemented with 1 mM IPTG every 3–4 days to prevent end-product inhibition and maintain adequate nutrient levels. The semi-continuous cultivation method significantly prolonged the production period. Sucrose yields increased in each cycle until day 10, reaching a cumulative sucrose concentration of 2200 mg l^{-1}. Subsequently, production gradually declined, but residual sucrose production persisted even after 17 days, reaching a cumulative yield of ~3000 mg l^{-1} by the 27th day (Paper I, Figure 6). Throughout the cultivation period, a gradual accumulation of outgrowth in the culture medium was observed, indicating a degree of cell escape from the immobilisation matrix. The outgrowth in the culture medium reached OD_{750} ~1.5 during the last 4 cycles of production.

4.1.1 Effects of immobilisation and sucrose production on fitness and physiology of *Synechocystis* S02 cells

As previously discussed, sucrose production halts after 7 days in continuous culture conditions. To investigate the correlation between the cessation of production and the photosynthetic CO_2 fixation and respiration of the cells, real-time gas exchange was monitored by membrane inlet mass spectrometry (MIMS) in suspension and immobilised cultures. Gas exchange was studied under sucrose-producing conditions and without active sucrose production, with measurements taken 3 and 7 days after establishing the cultures.

After 3 days, net photosynthetic O_2 evolution was significantly higher ($P = 0.02 - 0.04$) under sucrose producing conditions (+NaCl) both in immobilised and suspension cultures (Figure 4A) compared to non-producing conditions (-NaCl). Similarly, CO_2 fixation rates were higher in producing cultures, likely due to the sucrose production acting as a carbon sink (Figure 4B). However, the difference is only significant in suspension ($P = 0.006$). These observations point to the role of sucrose production in the presence of high NaCl concentration as a strong carbon sink, increasing photosynthetic activity and carbon fixation (Figure 4A – B). The higher gas exchange rates detected in suspension cultures in comparison to immobilised cells are likely attributed to the low gas permeability of the alginate matrix and potential effects of immobilisation on cell metabolism.

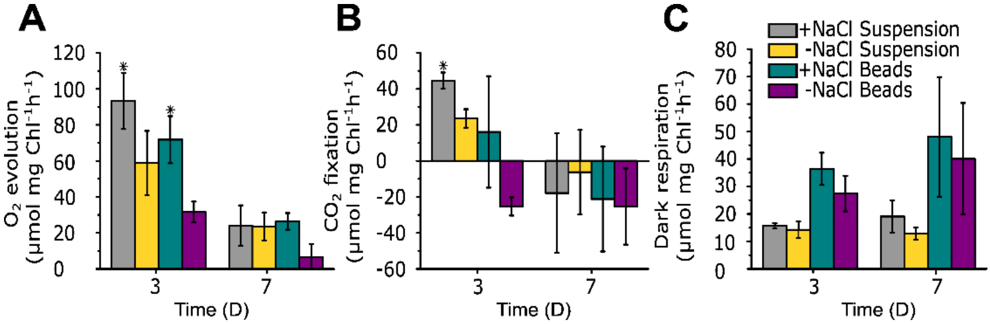

Figure 4. Real-time gas exchange rates in suspension and immobilised cells. (A) Average steady state net oxygen evolution; (B) net CO_2 yield; (C) dark oxygen consumption. The cells were incubated in BG11 supplemented with sodium chloride and 1 mM IPTG (+ NaCl) to induce sucrose production. As a negative control the cells were incubated in BG11 medium supplemented with 1 mM IPTG, but not sodium chloride (-NaCl). Error bars represent the standard deviation of three independent biological replicates (Modified from Paper I).

By day 7, both O_2 evolution and CO_2 fixation rates decreased, indicating a decrease in photosynthetic activity, which was corroborated by PS II effective yield (*Y (II)*) measurements. *Y (II)* dropped from ~0.37 to 0.08 in both immobilised and suspension cultures under sucrose producing conditions. The decline in photosynthetic activity coincided with the termination of sucrose production. Simultaneously, an increase in dark respiration was observed in all conditions except suspension cultures under non-producing conditions (Figure 4C).

Based on these results, immobilisation and sucrose accumulation in the growth medium has considerable impact on cell metabolism, and the periodical removal of the sucrose saturated medium in a semi-continuous cultivation mode is advantageous for long-term productivity.

4.2 Whole-cell biotransformation of cyclohexanone to ε-caprolactone

4.2.1 Biotransformation by *E. coli* using photosynthetically produced sucrose

Sucrose production by photosynthetic microorganisms is primarily seen as an opportunity to supply organic carbon to heterotrophic microorganisms, circumventing agricultural sugar production. Subsequently, we evaluated the capability of sucrose, generated by immobilised *Synechocystis* S02 cells to drive the biotransformation of cyclohexanone to ε-caprolactone without downstream modifications. For this purpose, we employed the heterotrophic *E. coli Parvi* strain expressing the BVMO$_{Parvi}$ enzyme, which was engineered for optimised sucrose utilisation (Paper I). The BG11+NaCl medium enriched with sucrose by the immobilised *Synechocystis* S02 cells was first removed from the producing beads, then inoculated with *E. coli Parvi* cells. The reaction was started by the addition of 5 mM cyclohexanone. Within 2 hours, the majority of the substrate underwent transformation into the product without any undesired side product formation by native alcohol dehydrogenases (ADHs) (Figure 5A). The average product formation rate was 0.9 mM h^{-1}.

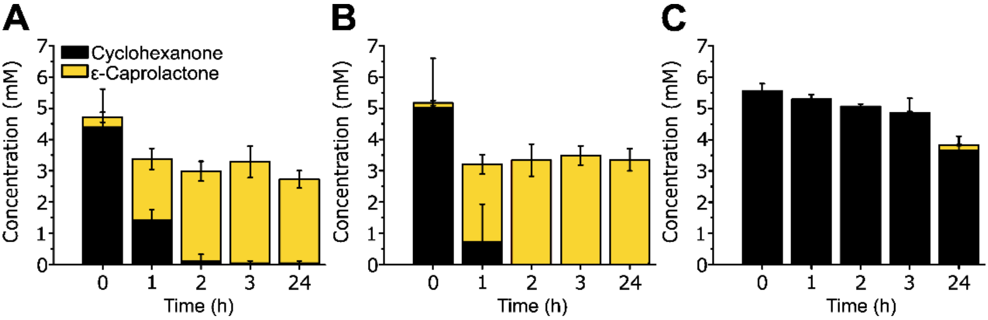

Figure 5. Progression of *E. coli* driven biotransformation of 5 mM cyclohexanone substrate to ε-caprolactone in different media. (A) BG11 + NaCl medium enriched with sucrose by alginate immobilised *Synechocystis* S02 cells; (B) M9 medium supplemented with 10 mM sucrose as positive control and (C) BG11 + NaCl medium without sucrose as negative control (Modified from Paper I).

As a positive control, M9 medium, typically used for the cultivation of *E. coli*, was supplemented with 10 mM sucrose. As anticipated, owing to the higher sucrose concentration, the reaction proceeded more rapidly, with complete transformation of the substrate occurring after 1 hour with average product formation rate of 2.3 mM h^{-1} (Figure 5B). Conversely, in the negative control with only BG11+NaCl medium without any carbon source, no product formation was observed (Figure 5C). These findings underscore the sucrose dependency of the biotransformation reaction, while demonstrating the applicability of photosynthetically produced sucrose to drive the production of the industrially relevant ε-caprolactone in a coupled photosynthetic-heterotrophic production system.

4.2.2 Biotransformation and photohydrogen production by *Chlamydomonas reinhardtii*

While the concept of utilising photosynthetically produced sucrose as a carbon source for heterotrophic microorganisms is promising, it requires the upkeep of multiple microbial strains, while the direct coupling of the systems can be challenging due to the different growth conditions required by the different species and the different reaction times of the sucrose production and biotransformation. The expression of the desired biosynthetic pathways, such as the BVMO, cyclohexanone monooxygenase (CHMO) directly in photosynthetic microorganisms offers the potential to obtain the NADPH and molecular O_2 required by BVMOs directly through photosynthesis eliminating the need of employing heterotrophic organisms. For this purpose, *Chlamydomonas reinhardtii* UVM4/CHMO (*Chlamydomonas* UVM4/CHMO) was engineered and used to drive biotransformation of cyclohexanone to ε-caprolactone.

During the reaction facilitated by this strain, substrate consumption was complete after 48 hours, but only 15% was transformed into the target product, ε-caprolactone, while 85% was converted into the undesired side product, cyclohexanol, by native ADHs (Figure 6A – C HL). The maximum formation rate of ε-caprolactone and cyclohexanol was 0.008 U mg_{Chl}^{-1} and 0.031 U mg_{Chl}^{-1} respectively (Figure 6A HL). To stir the reaction towards product formation, it was necessary to counteract the action of ADHs. After the addition of 30 mM fomepizole, a known inhibitor of ADH, the formation rate of cyclohexanol decreased to 0.013, U mg_{Chl}^{-1}. However, the formation of ε-caprolactone has also slowed down to 0.006 U mg_{Chl}^{-1} (Figure 6A – B HLF). This suggests that fomepizole also inhibits the activity of CHMO. To curb the action of ADHs, while not hindering CHMO, ethanol, a natural product of ADHs, was introduced as a competitive inhibitor, resulting in 0.009 U mg_{Chl}^{-1} specific CHMO activity, a 15% acceleration (Figure 6A – B HLE). However, the ratio of the side product formation still exceeded that of the product. Notably, the addition of ethanol led to complete substrate consumption within the investigated timeframe, unlike fomepizole.

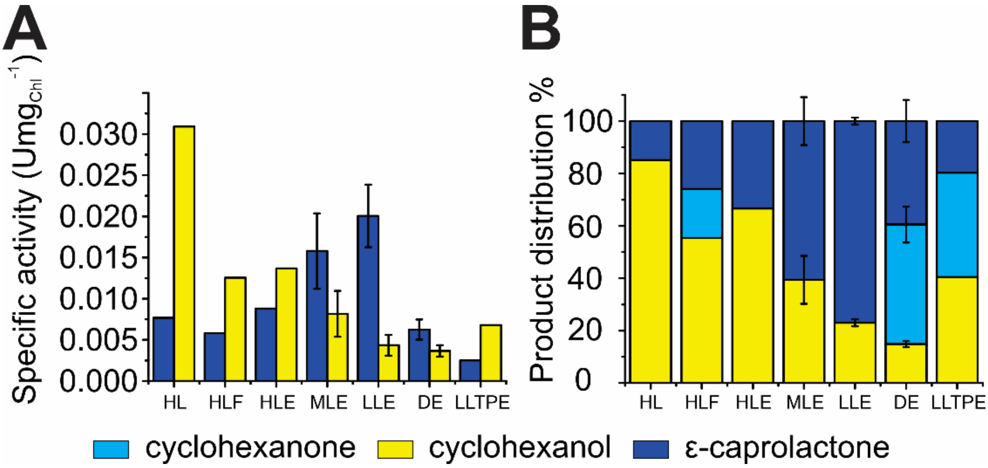

Figure 6. Evaluation of the biotransformation reaction of *Chlamydomonas* UVM4/CHMO. (A) Specific activity of product formation (blue) and side product formation (yellow) normalised to initial Chl. The reaction was performed under high light (165 µmol photons m^{-2} s^{-1}) without additives (HL), with 30 mM fomepizole (HLF), with 1.7% (vol/vol) ethanol (HLE), under medium light (55 µmol photons m^{-2} s^{-1}) with 1.7% (v/v) ethanol (MLE), under low light (26 µmol photons m^{-2} s^{-1}) with 1.7% (v/v) ethanol (LLE), in darkness with 1.7% (v/v) ethanol (DE), under low light (26 µmol photons m^{-2} s^{-1}) with 1.7% (v/v) ethanol and photoautotrophic conditions with 1% CO_2 supplementation (LLTPE). (B) Product distribution 48 h after the reaction was started, substrate (cyan), side product (yellow), and product (blue). The standard deviation are from 2–3 independent replications (Modified from Paper II).

In an effort to optimise ε-caprolactone production, various light intensities were tested. Apart from the initial high light (HL, 165 µmol photons m^{-2} s^{-1}), the reaction was also conducted under moderate (ML, 55 µmol photons m^{-2} s^{-1}), and low light (LL, 26 µmol photons m^{-2} s^{-1}) conditions. Under moderate light, ε-caprolactone formation rate was accelerated by 80%, the product making up 61% of the compounds after termination of the reaction (Figure 6A – B MLE). The best results were achieved under low light, with 77% ε-caprolactone production after 48 hours with the specific product formation activity of 0.02 ± 0.004 U mg$_{Chl}$$^{-1}$ (Figure 6A – C LLE). To ascertain light dependency, the reaction was also performed in darkness. Without light the specific activity was 3 times slower compared to LL, while the side product formation rate was reduced by 18% (Figure 6B DE). This also meant that the reaction did not go to completion in 48 h, with 46% of the substrate remaining. This underlines the need for illumination for the effective biotransformation reaction by *Chlamydomonas* UVM4/CHMO.

It is important to note that all the above-mentioned experiments were performed in TAP medium under mixotrophic conditions, with acetate as a carbon source. In this state light is not essential for their survival but offers additional energy (Puzanskiy et al., 2021). In photoautotrophic cultivation using TP medium without acetate and 1% CO_2 in the headspace, side product formation increased, and reaction kinetics slowed, as the reaction did not go to completion in 48 h. Without acetate, the use of ML or the exclusion of ethanol did not affect the specific product formation activity substantially, it being 0.003 ± 0.0005 U mg_{Chl}^{-1}, however, the side product formation was accelerated twofold, to an average of 0.015 ± 0.001 U mg_{Chl}^{-1} in ML with or without ethanol and in LL without ethanol (Figure 6B LLTPE). It is clear from the results that the presence of acetate in the medium and LL have advantageous effects on the biotransformation reaction.

Chlamydomonas is a eukaryotic microalga, consequently its photosynthesis takes place in the chloroplast, a compartment separated from the cytosol of the cell, where the CHMO enzyme was localised in the *Chlamydomonas* UVM4/CHMO strain. To further enhance the reaction, CHMO was fused with the chloroplast transit peptide, PSAD (subunit of PS I), to translocate CHMO into the chloroplast. This way the enzyme is in close proximity of the photosynthetically produced NADPH, and O_2 generated by water oxidation, which are necessary for its function. For this purpose, a different host strain, *Chlamydomonas reinhardtii* UVM11-CW (*Chlamydomonas* UVM11-CW) was used as a background, resulting from the crossing of UVM11 and CC-124 strains. Using this robust, cell wall-protected strain suitable for large-scale production, improved biotransformation was achieved even without the transit peptide (Neupert et al., 2020; Paper II). The *Chlamydomonas* UVM11-CW/CHMO strain exhibited significantly enhanced biotransformation compared to *Chlamydomonas* UVM4/CHMO ($P = 0.03$) even without the transit peptide, (specific activity: 0.034 ± 0.006 U mg_{Chl}^{-1}) (Figure 7A). Furthermore, the share of ε-caprolactone in the final product distribution has increased from 77% to 85% (Figure 7B). With the addition of chloroplast transit peptide, the specific activity of product formation was further improved in *Chlamydomonas* UVM11-CW/CHMO_PSAD to 0.053 ± 0.01 U mg_{Chl}^{-1}, which is 2-7 times higher than *Chlamydomonas* UVM4/CHMO and 1.6 times higher than *Chlamydomonas* UVM11-CW/CHMO. The reaction also went to completion faster, under 19 h, with 87% ε-caprolactone and 13% cyclohexanol formed (Figure 7B).

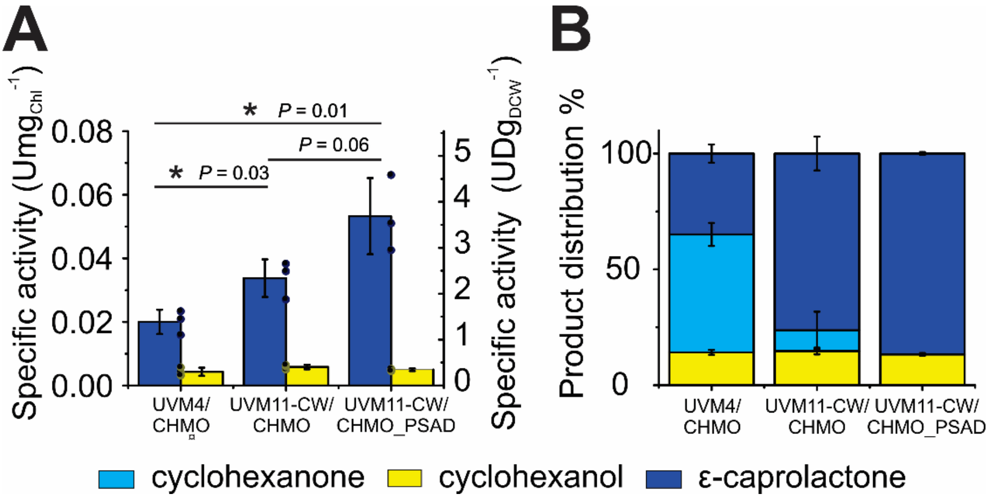

Figure 7. Comparison of biotransformation in *Chlamydomonas* UVM4/CHMO, UVM11-CW/CHMO and UVM11-CW/CHMO_PSAD. (A) The specific activity normalised to initial Chl and DCW derived from the correlation curve. (B) Ratios of the compounds present after 19 h biotransformation. Standard deviations are from three biological replications. * Indicates a significant difference $P < 0.05$. *P*-values were calculated using a two-tailed type II Student's *t*-test (Modified from Paper II).

Chlamydomonas reinhardtii is recognised as a potential green fuel source due to its capacity to generate molecular photohydrogen under anaerobic conditions catalysed by the O_2 -sensitive [Fe-Fe]-hydrogenase enzyme. However, sustaining this process for an extended period in typical culture conditions is challenging, due to the accumulation of O_2 co-produced during photosynthesis. Given that CHMO consumes O_2 for the biotransformation reaction, a protocol was established for H_2 and ε-caprolactone production in a stepwise manner. The reaction commenced under anaerobic conditions, leading to H_2 production by *Chlamydomonas* UVM4/CHMO for approximately 6 hours, yielding a final concentration of 3 mmol l^{-1} (Paper II, Figure 5A). As anticipated, under microoxic conditions, no ε-caprolactone production was observed. However, after 6 h, when O_2 began to accumulate as a result of photosynthesis in the second step, the biotransformation process also initiated, while H_2 production ceased. Without CHMO expression, no discernible difference in H_2 yield was observed. This outcome aligns with expectations, as the two processes are temporally separated and occur under distinct O_2 levels.

4.3 3D-printing and photo crosslinking for the immobilisation of photosynthetic microorganisms

3D-printing is a precise and versatile way to manufacture immobilisation customisable properties and architectures. To create an improved photosynthetic bioproduction platform, this technology was coupled with a plant-based, photocurable bioink, which is impervious to high ionic concentrations and chelating agents in the growth medium, such as high sodium chloride or sodium bicarbonate. To illustrate the compatibility of this approach with both eukaryotic and prokaryotic photosynthetic microorganisms, and chemical production, *Chlamydomonas* UVM11-CW/CHMO_PSAD or *Synechocystis efe* cells were blended with the photocurable ink comprising 3% alginate, 4% GGMMA, and 0.05% LAP (Alg+GGMMA+LAP). This ink, laden with cells, was utilised to 3D-print thin biofilms on glass slides pre-treated for improved attachment.

A homogenous layer was successfully printed, forming an elastic film after photocuring. Throughout this process, the cells encounter various stress factors, including shear stress during extrusion printing, high light intensity during photocuring, and the presence of free radicals derived from the photoinitiator, which are particularly damaging to the photosynthetic machinery. To assess the photoinitiator concentration sufficient for curing, but not detrimental for cell growth, *Synechocystis efe* cells were grown with increasing concentrations of LAP and Irgacure 2959 photoinitiators in a preliminary experiment. A LAP concentration of 0.05% was chosen, resulting in slowed cell growth but not cell death, while still ensuring the formation of stable films.

Y(II) was measured to evaluate the fitness of the cells after printing and curing. Directly after printing and curing *Y(II)* of both cell types were low, 0.2 and 0.4 for *Synechocystis efe* and *Chlamydomonas* UVM11-CW/CHMO_PSDA respectively. To aid cell recovery, the films were incubated in their respective growth medium under low light (25 µmol photons m^{-2} s^{-1}) overnight in case of *Synechocystis efe* and for 72 h in case of *Chlamydomonas* UVM11-CW/CHMO_PSDA cells. Following recovery, *Y(II)* of both cell types increased to a normal level observed in suspension cultures (0.4 and 0.7 respectively) and remained at this level for 6 days under growth conditions.

Curing dynamics of the 3D-printed films were evaluated by photo-rheology measurements, where the change in rheological properties, such as storage modulus (G’) and loss modulus (G’’) were monitored upon exposure to 405 nm light. Samples without cells and with either *Synechocystis efe* or *Chlamydomonas* UVM11-CW/CHMO_PSDA cells were investigated in a thickness of 200 µm. In all samples, the sudden increase of G’ and G’’ was observed after 20 s of exposure to 405 nm light (Figure 8A – C), indicating the start of gel formation from the viscous ink. The gel point, where G’ exceeds G’’ and the sample starts to behave like an elastic gel instead of a fluid, was reached after 30 s. The crosslinking process neared completion after ~150 s in all samples. While the dynamics of the curing process did not significantly differ between inks with or without cells, the final G’ value was twice as high (~20 kPa) in the cell-free samples, indicating that cells decrease film elasticity. At the end of the curing process G’ is approximately one order of magnitude higher than G’’, which signifies the suitability of the Alg+GGMMA+LAP ink to create solid-like, elastic films without ionic crosslinking.

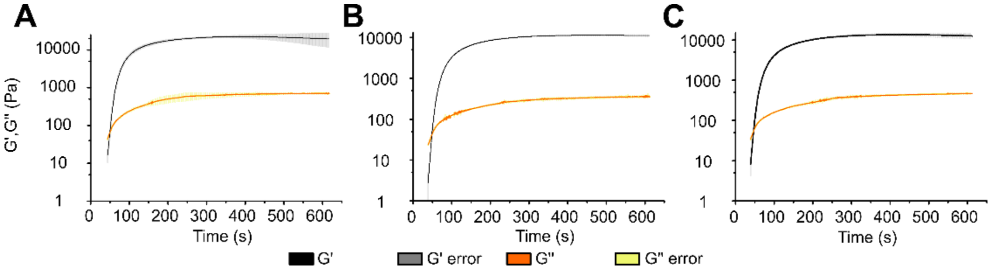

Figure 8. Photo-rheology measurements describing the photocuring process of Alg+GGMMA+LAP ink. (A) Cell-free ink and inks loaded with (B) *Synechocystis efe* and (C) *Chlamydomonas* UVM11-CW/CHMO_PSDA cells. Changes of storage modulus (G’) and loss modulus (G’’) are shown after 20 s exposure to 405 nm light. Shading represents standard deviation (Modified from Paper III).

Subsequently the stress tolerance of the 3D-printed and photocured films was investigated. The overall dynamics of the deformation were not significantly different with or without the cells in the oscillatory stress sweep measurements. However, the onset of deformation occurred significantly earlier in cell containing samples. Films without cells broke down at ~6% shear strain and ~1000 Pa shear stress, while films with *Synechocystis efe* and *Chlamydomonas* UVM11-CW/CHMO_PSDA broke down at 5% stress and ~390 Pa and 7% strain and 800 Pa respectively (Figure 9A). Films with cells were less durable and were destroyed more easily. Interestingly, the samples containing *Chlamydomonas* UVM11-CW/CHMO_PSDA cells were able to endure significantly more stress than the ones with *Synechocystis efe* cells ($P = 0.0035$ with Kruskal-Wallis test). Similarly, the stiffness of the films, as indicated by their shear moduli, was significantly higher in samples without cells ($P < 0.006$), while there was no significant difference between the two cell types (Figure 9B).

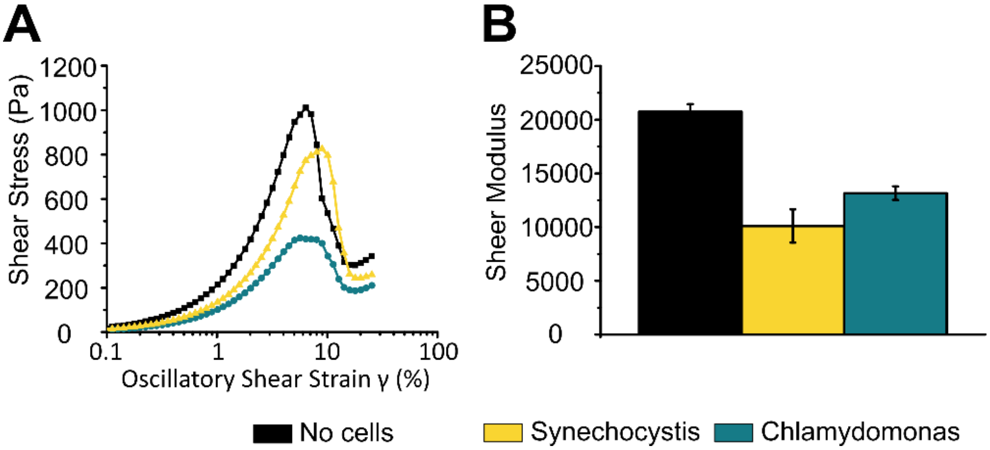

Figure 9. Rheological properties of photocured films. (A) Oscillatory strain sweeps to measure stress tolerance of 3D-printed films. Oscillatory shear stress is shown in relation to shear strain for inks without cells and with *Synechocystis efe* and *Chlamydomonas* UVM11-CW/CHMO_PSAD cells, respectively. Data points represent the average of 2–3 measurements. 20 points per decade were recorded. (B) Average of shear modulus of films without cells, with *Synechocystis efe* and *Chlamydomonas* UVM11-CW/CHMO_PSAD cells. The error bars represent the standard deviation of 2–3 measurements (Modified from paper III).

Y(II) and rheological measurements prove that utilising Alg+GGMMA+LAP inks in conjunction with 3D-printing and photocuring allows for the production of robust films capable of harbouring photosynthetic cells without impeding their photosynthetic activity.

4.3.1 3D-printed biofilms for chemical production

To ascertain the applicability of the 3D-printed biofilms for chemical production, films harbouring *Chlamydomonas* UVM11-CW/CHMO_PSDA cells were used to drive biotransformation of cyclohexanone to ε-caprolactone. In this reaction, the matrix must allow unhindered entry of the substrate into the cells and the exit of the resulting product. The choice of a matrix with sufficient permeability is crucial to prevent the inhibition or slowing down of substrate and product diffusion, which could lead to potential losses through ADHs action, product polymerisation, or evaporation in an open system.

In the 3D-printed Alg+GGMMA+LAP films, the biotransformation reached completion in 30 hours with a final product-to-side product ratio of 87% and 13%, respectively (Figure 10A). The maximum product formation rate was 158 ± 2 µmol h^{-1}, while the side product formation rate was 28.1 ± 4.8 µmol h^{-1} (Figure

10B). This seems considerably slower, than the rates observed in *E. coli,* however it is important to note that in that strain a different, much faster enzyme was expressed. Consequently the two reactions are not comparable. To evaluate the specific activity of the cells, the product formation rate was normalised to Chl concentration and cell dry weight (CDW). The resulting maximum specific activity of the immobilised *Chlamydomonas* UVM11-CW/CHMO_PSDA cells were 0.051 ± 0.033 $\mathrm{Umg_{Chl}}^{-1}$ and 3.46 ± 0.2 $\mathrm{Ug_{CDW}}^{-1}$ (Figure 10B), which is comparable to suspension cultures (Paper II). The ratio of product to side product remained consistent as well. On the other hand, immobilisation offers opportunities for upscaling the process and simplifies the separation of the product from the cells, eliminating the need for centrifugation and harsh extraction.

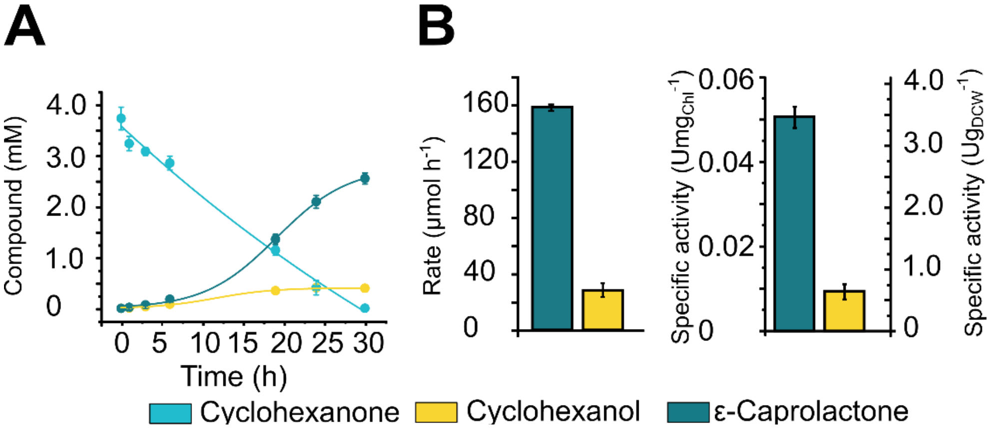

Figure 10. Whole-cell biotransformation by *Chlamydomonas* UVM11-CW/CHMO_PSDA cells entrapped in 3D-printed photocured Alg+GGMMA+LAP films. (A) Time course of the formation of ε-caprolactone and cyclohexanol from cyclohexanone. (B) Maximum formation rate of ε-caprolactone and cyclohexanol and specific activity normalised to Chl and DCW of ε-caprolactone and cyclohexanol (Modified from Paper III).

As a different type of reaction, ethylene production by *Synechocystis efe* cells was also performed in the 3D-printed, photocured Alginate+GGMMA+LAP matrix. After crosslinking the films were submerged in BG11+$NaHCO_3$ medium in gas-tight vials. Contrary to Ca^{2+} crosslinked alginate films, the photocured films did not disintegrate upon immersion in the medium with high sodium bicarbonate concentration. They remained intact and firmly attached to the glass slides for the 7-day cultivation period. During the 20-day long-term production, some films detached and were cut by the moving glass due to agitation. Remarkably, the cut films retained their integrity without further degradation, and the damage did not affect ethylene production.

Ethylene concentration steadily increased until the fifth day, when it reached a plateau phase. The maximum ethylene yield was 700 µmol mg_{Chl}^{-1} after seven days (Figure 11A), with a maximum formation rate of 7.0 mol mg_{Chl}^{-1} h^{-1}. Despite the high stiffness of the matrix, a substantial Chl accumulation was observed, indicating cell growth (Figure 11B).

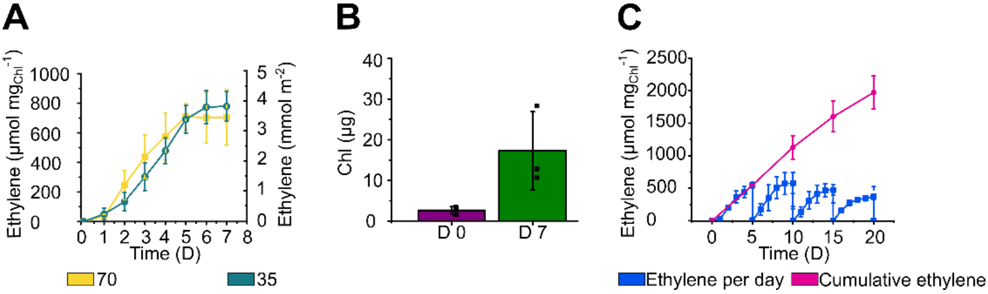

Figure 11. Ethylene production by *Synechocystis efe* cells immobilised within 3D-printed photocured Alg+GGMMA+LAP films. (A) The specific and areal ethylene production yields in a batch production system under 35 (teal) or 70 (yellow) µmol photons m^{-2} s^{-1} illumination. (B) Chl contents after overnight recovery (D 0) and 7 days of ethylene production (D 7). (C) Long-term semi-continuous ethylene production and cumulative total ethylene yield during 20-day semi-continuous production. Every 5 days the medium and the headspace were refreshed. The drop of the ethylene concentration to 0 in the end of each cycle is caused by opening of the vials. Error bars represent the standard deviation of 3 biological (with 2-3 technical) replicates (Modified from Paper III).

Ethylene production was also monitored in a semi-continuous system for 20 days to evaluate the presented method for long-term production. The vials were opened, and the medium exchanged to fresh every 5 days. The highest specific ethylene yield was achieved in the second cycle and thereafter gradually decreased in every cycle. Ethylene production did not cease by the 20th day with more than 350 mg_{Chl}^{-1} yield in the last cycle (Figure 11C), which reinforces the applicability of the concept of Alginate+GGMMA+LAP films 3D-printed on a glass slide for chemical production purposes for an extended period.

5 Discussion

5.1 Immobilisation improves chemical yields of photosynthetic cells and simplifies upscaling endeavours

In our study, immobilisation notably enhanced photosynthetic sucrose and ethylene production yields on the basis of Chl. Although it did not impact the specific rate of cyclohexanone biotransformation to ε-caprolactone, it greatly simplified the separation of cells from the reaction mixture, a valuable aspect for potential scale-up efforts (Paper I; Paper III).

The 86% increase in specific productivity achieved by alginate bead immobilisation of the sucrose producer *Synechocystis* S02 is comparable to similar works (Weiss et al., 2017; Paper I), while the ethylene yield obtained from photocured 3D-printed Alg-GGMMA-LAP films was the highest reported from the *Synechocystis efe* strain (Rissanen et al., 2021; Virkkala et al., 2023; Paper III). Immobilisation simplifies the implementation of semi-continuous cultivation systems, beneficial for both sucrose and ethylene production. As corroborated by the MIMS measurements, the accumulation of sucrose in the medium initiates a decrease in photosynthetic CO_2 fixation and an increase in cell respiration (Paper I; Paper III). This can be attributed to the ability of *Synechocystis* cells to utilise sugars in a mixotrophic metabolism, when they are available in high enough concentration (Muñoz-Marín et al., 2024; Solymosi et al., 2020). This, together with end product inhibition and the depletion of the nutrients in the medium necessitates the periodic exchange of the medium. Similarly, ethylene production requires high amount of available carbon, which is commonly supplied by sodium bicarbonate in the growth medium, which in turn should be refreshed periodically (Paper I; Paper III).

While immobilisation proves effective for chemical production by photosynthetic microorganisms, certain limitations must be considered. The two main bottlenecks are the mechanical stability of the matrix and its permeability. Matrices crosslinked by ionic interactions can be susceptible to the ionic strength of the medium and the presence of chelating agents, especially when the architecture is more vulnerable, for example in thin films (Rissanen et al., 2021). Beads, a more robust immobilisation architecture, overcome some issues but may hinder light utilisation and mass transfer toward their centre, leading to uneven cell performance (Das et al., 2015; Tong & Derek, 2023; Paper I). The stability and permeability of the matrix are strongly connected, since the harder it is for nutrients, products and gases to penetrate the matrix, the more vigorous agitation is needed, as seen in the cease of sucrose production. The MIMS measurements demonstrate the low permeability of the beads, which is even more pronounced in case of bigger molecules, such as sucrose (Paper I). While ethylene is a gaseous product and penetrates the matrix easier compared to sucrose, the continuous high production rate requires high amounts of carbon as substrate, which is most easily supplied in the form of sodium bicarbonate, which results in high ionic strength in the medium and the degradation of the matrices cross-linked by ions (Rissanen et al., 2021; Vajravel et al., 2020). To overcome these issues it is important to select and tailor the immobilisation matrices to the desired processes and continuously improve the permeability and stability of the matrix through chemical modifications and discover new possible matrix materials, such as the photocurable GGMMA presented in Paper III.

5.2 Biotransformation of cyclohexanone to ε-caprolactone is transferable to *Chlamydomonas reinhardtii*

The microbial production of ε-caprolactone could greatly contribute to a more sustainable chemical industry. While the biotransformation of cyclohexanone to ε-caprolactone has been effectively demonstrated in *E. coli*, the reaction is greatly dependent on an organic carbon source, particularly in the form of sugars (Paper I). Numerous photosynthetic microorganisms have shown promise in producing the necessary amount of sucrose for heterotrophs performing chemical production (Hays et al., 2017; Lin et al., 2020; Weiss et al., 2017), and our research has established *Synechocystis* S02 as one of the viable candidates (Paper I). However, when aiming for the co-cultivation of a sucrose producer photosynthetic and a heterotrophic microorganism, the sucrose production process should be further optimised to increase production yield and speed. This is especially important when the “substrate-in-product-out” approach is used, with volatile compounds. In case of cyclohexanone and ε-caprolactone, slower reaction may result in substrate and product losses through evaporation and the action of ADHs. However, if photosynthetic microorganisms are used to supply sugars for heterotrophic cells, there is still a need to maintain two distinct cell cultures with different requirements. To address this challenge, the biotransformation pathway was expressed directly in photosynthetic microorganisms (Erdem et al., 2022; Paper II).

The expression of different BVMO enzymes in the cyanobacterium *Synechocystis* sp. PCC 6803 yielded promising results (Erdem et al., 2022; Malihan-Yap et al., 2022; Tüllinghoff et al., 2022). Notably, in cyanobacteria the respiration and photosynthesis is confined to the same compartment, resulting in the complex regulation of involved processes (Mullineaux, 2014; Nikkanen et al., 2021). In contrast, in eukaryotic photosynthetic microorganisms, such as *Chlamydomonas reinhardtii* the spatial separation of respiration and photosynthesis provides possible novel strategies optimising biotransformation reactions. Indeed, translocating the CHMO enzyme into the chloroplast considerably enhanced the biotransformation reaction compared to cytosolic expression, likely attributed to the close proximity of photosynthetically produced NADPH and O_2 (Paper II).

For further optimisation, another step was the inhibition of ADHs competing with CHMO for the substrate. The addition of ethanol proved preferable over fomepizole, possibly due to the lower affinity of CHMO towards linear compound over cyclic ones. Furthermore, lower light intensities and the inclusion of acetate as a carbon source were also beneficial for the biotransformation reaction (Paper II). A possible explanation for the positive effects of low light is the high NADPH to ATP ratio under low light conditions, linked to the mitochondrial respiration limited by the low photosynthetic O_2 production in dense algal cultures (Scoma et al., 2014; Sheng et al., 2001). Additionally, CO_2 fixation and biomass accumulation, which would otherwise redirect energy from the biotransformation, is slower (Maltsev et al., 2021). This effect is similar to the growth inhibition induced by immobilisation. The advantageous effect of the acetate is due to the activity of respiratory catabolism, besides the photosynthetic pathway, resulting in the additional accumulation of NADPH (Paper II). This also highlights the potential of photomixotrpohic grow of microalgae for industrial applications (Patel et al., 2019).

Additionally, the coupling of CHMO-mediated biotransformation with H_2 production in a stepwise manner highlights the versatility of the producer strain, offering potential avenues for novel production systems. However, challenges associated with industrial-scale application, including factors such as mixing, heat and mass transfer, and associated costs, need careful consideration (Paper II).

5.3 Coupling photocuring and 3D-printing is an effective way for the immobilisation of photosynthetic microorganisms for various applications

The 3D-bioprinting of living photosynthetic microorganisms was so far predominantly utilised for CO_2 capture and wastewater treatment (Lee et al., 2022; Xia et al., 2020; Xue et al., 2023), our study demonstrates its application for the fabrication chemical production platforms. Through the mixing of alginate and GGMMA we successfully implemented photocuring, thereby circumvented the necessity for ionic crosslinking (Paper III).

Employing these techniques, we crafted stable films with favourable rheological properties, however, the presence of the cells in the matrix changed some of its properties (Paper III). While not influencing the dynamics of the photocuring, the addition of either *Synechocystis efe* or *Chlamydomonas* UVM11-CW/CHMO_PSAD cells to the matrix decreased the final G' values by 50%, to ~10 kPa indicating diminished elasticity in films containing cells. Nevertheless, these films exhibited satisfactory physical characteristics, and their stability during cultivation was noteworthy (Paper III). The final G' values surpassed those of a photocurable ink composed of modified alginate and gelatine (~300 Pa; Yuan et al., 2017) and were comparable to the photocurable mixture of GGMMA and TCNF (10-60 kPa; Xu et al., 2019). The observed effect of cells on the matrix is similar to the ability of bovine chondrocytes to interact with matrix monomers, restricting polymerisation and quenching free radicals derived from the photoinitiator (Chu et al., 2020). Additionally, the presence of the cells decreases stress tolerance and rigidity of the films. Interestingly the impact of *Synechocystis efe* cells were more pronounced than that of *Chlamydomonas* UVM11-CW/CHMO_PSAD (Paper III) possibly attributed to distinct characteristics of their cell surface or interactions with matrix materials, or variations in cell size. Notably, the influence of cells on matrix characteristics was more significant compared to reports on Ca^{2+} crosslinked alginate or Ca^{2+} and PVA crosslinked TCNF films (Rissanen et al., 2021), underscoring the need for tailored matrix selection based on the specific cells and processes involved, as different materials and crosslinking methods yield varying effects on matrix stability.

This immobilisation method proved effective for conducting biotransformation in *Chlamydomonas* UVM11_CW/CHMO_PSAD and ethylene production, even over extended durations in *Synechocystis efe* cells. The positive effects were particularly prominent for ethylene production, where the specific ethylene productivity was considerably higher than reported for suspension cultures (Vajravel et al., 2020) and cultures immobilised in Ca^{2+}-alginate, Ca^{2+}-

TCNF and Ca^{2+}-TCNF-MLG films (Rissanen et al., 2021; Virkkala et al., 2023). Notably, there was no significant difference in ethylene production under 35 and 70 µmol photons m^{-2} s^{-1}, suggesting that 35 µmol photons m^{-2} s^{-1} is already sufficient for light saturation of the production system (Paper III). This highlights the effectivity of immobilisation in thin films for optimised light utilisation. Additionally, the photocured films, in contrast to Ca^{2+} crosslinked alginate, remained intact in the medium containing 200 mM sodium bicarbonate, emphasising the heightened chemical resistance of the material (Paper III). This is especially important for applications where the medium has detrimental effects for the matrix, such as in wastewater treatment with immobilised cells, where matrix stability is often an issue (Cruz et al., 2013; Han et al., 2022; Rissanen et al., 2021).

Beyond the aforementioned applications, 3D-printed and photocured alginate+GGMMA+LAP films containing photosynthetic microorganisms hold promise for medical uses. Polymer films comprising alginate and CNF are already extensively employed for wound treatment (Alven & Aderibigbe, 2020; Ouyang et al., 2022; Rana et al., 2024), and the incorporation of photosynthetic microorganisms can introduce additional benefits through the antimicrobial and anti-inflammatory properties of these cells (Choo et al., 2020; Demay et al., 2021; Shaima et al., 2022).

6 Conclusion and future perspectives

In conclusion, this study underscores the versatility of photosynthetic microorganisms as valuable tools for sustainable chemical production, particularly when immobilised in polymer matrices. Our findings demonstrate the effectiveness of Ca^{2+} crosslinked alginate beads in enhancing the sucrose output of *Synechocystis* sp. PCC 6803 S02 cells. The produced sucrose proves to be a viable carbon source, to drive the biotransformation of cyclohexanone to the industrially relevant ε-caprolactone, employing *E. coli* WΔcscR Inv:Parvi as the biocatalyst. Additionally, the successful implementation of the biotransformation process using engineered *Chlamydomonas reinhardtii* cells was achieved.

Furthermore, the integration of 3D-printing and photocuring emerged as a promising method for immobilising photosynthetic microorganisms in films resilient to the challenges posed by high ionic strength in the medium. The outcomes of this research lay the groundwork for the development of an industrially relevant photosynthetic chemical production platform, leveraging immobilised cyanobacteria and microalgae.

In future studies the implementation of 3D-printed and photocured alginate+GGMMA+LAP should be investigated in diverse applications, including wastewater treatment, and the production of high-value-added products. The potential of 3D-printing technology to construct more intricate, multilayer structures, such as artificial leaf configurations for enhanced immobilisation, also presents an exciting avenue for further investigation.

In summation, I firmly believe that this technology holds significant potential to make substantial contributions to advancing a more sustainable chemical industry.

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