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Purification, biomass production and cryo-preservation of aero-terrestrial microalgae and cyanobacteria

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This thesis was carried out in the laboratory of the Department of Biotechnology at the Management Center Innsbruck (MCI) in Austria. It was part of a research project with the aim to develop an economical process to produce valuable bioactive compounds from terrestrial microalgae and cyanobacteria. Algae culture collection MCI ASIB 505, originally from Institute of Botany of the University of Innsbruck, has been used as the basis of this research project.

Microalgae and cyanobacteria have been already proved suitable for a wide range of applications and products. Due their high biodiversity, microalgae and cyanobacteria have attracted researcher's attention already for a few decades.

This thesis mainly focused on MCI ASIB 505 culture collection maintenance, culture purification, test strain cultivation, and on cryopreservation. The main aim was to develop a viable protocol for cryopreservation to replace the costly and time-consuming actively metabolizing maintenance process. In addition, in terms of cultivation and quality assurance, cryopreservation was a far more cost-efficient and faster method. The second objective was to find out the most productive method for microalgae and cyanobacteria biomass cultivation. The growth test was carried out comparing four different techniques for producing biomass: Schott bottle, tubular photobioreactor, conical flask treated with CO_2 and without CO_2. In addition, the culture collection MCI ASIB 505 was purified with antibiotics, antimycotics and with mechanical separation.

Thus, two cryopreservation protocols were developed and examined for strains that grow preferentially in liquid media and for strains that grow preferentially on agar slant. Both methods showed an excellent success and high viability.

Keywords	purification, microalgae, cyanobacteria, cryopreservation, cultivation, culture collection

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Tämä insinöörityö tehtiin Management Center Innsbruck (MCI) -yliopiston bioteknologian osastolla, Itävallassa. Työ suoritettiin osana tutkimusta, jonka tavoitteena on kehittää taloudellisesti kannattava menetelmä tuottaa bioaktiivisia yhdisteitä maanpäällisistä mikrolevistä ja syanobakteereista. Tutkimuksessa on käytetty MCI ASIB 505 – kantakokoelmaa, joka on peräisin ASIB 505 Botany of the University of Innsbruckin kantakokoelmasta.

Mikrolevät ja syanobakteerit ovat jo osoittautuneet monipuolisesti käytettäväksi monenlaisissa sovelluksissa ja tuotteissa. Johtuen korkeasta biologisesta monimuotoisuudesta-, mikrolevät ja syanobakteerit ovat olleet tutkijoiden huomion kohteena jo vuosikymmeniä.

Insinöörityössä keskityttiin MCI ASIB 505 -kantakokoelman ylläpitoon, -kantakokoelman puhdistukseen mahdollisista kontaminaatioista, viljely-menetelmien optimointiin testi-kannoilla sekä kylmäsäilytysmenetelmän kehittämiseen. Tavoitteena oli kehittää ja optimoida toteutuskelpoinen työohje kantakokoelman kylmäsäilytykseen nestemäisessä typessä, jotta korvattasiin käytössä oleva aktiivisesti metaboloituva ylläpito menetelmä. Pitkäaikaissäilytys syväpakastamalla takaa kantakokoelman kantojen geneettisen pysyvyyteen ja on huomattavasti kustannustehokkaampi ja nopeampi tapa, kuin perinteinen jatkuvan ylläpidon menetelmä. Tämän lisäksi tavoitteena oli määrittää tuottavin menetelmä mikrolevän ja syanobakteerin biomassan tuottoon. Kasvu-testi suoritettiin vertaamalla menetelmää, jossa käytettiin Schott-pulloa, Fotobioreaktoria ja-erlenmeyer-pulloa. Lisäksi kantakokoelma puhdistettiin mahdollisilta kontaminaatioilta ja siirrostettiin uuteen elatusaineeseen. Puhdistukseen käytettiin antibioottia, antimykoottia ja mekaanista siirrostusta.

Tehtyjen selvityksien ja kokeellisten kokeilujen perusteella kehitettiin kaksi syväpakastus menetelmää nestemäisessä typessa, kannoille jotka kasvavat valikoivasti nestemäisessä elätusaineessa ja kannoille mitkä kasvaa valikoivasti kiinteällä elätusaineella. Molemmat menetelmät osoittautuivat toimivaksi, toisaalta lisätutkimuksia vaaditaan tulosten toistettavuuden todistamiseksi.

Avainsanat	mikrolevä, syanobakteeria, kylmäsäilytys, viljeily, kantakokoelma

Contents

Nomenclature

ASIB	Algensammlung Innsbruck, registered as no. 505 in the World Direction of Collection of Cultures of Microorganisms
ASIB 505	Die Sammlung von Algenkulturen am Institut fur Botanik der Universität Innsbruck (Österreich)
BBM	Bold's Basal Medium
BBRM	Bold's Basal Rich Medium
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
LN_2	Liquid nitrogen
M_2SO	Dimethyl sulfoxide (DMSO)
MAA	Mycosporine-like aminoacid
MeOH	Methanol
RGR	Relative growth rate
RT	Room temperature
TPBR	Tubular photobioreactor
UTEX	The Culture Collection of Algae, Department of Botany, The University of Texas at Austin, Austin, USA
SAG	Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen, Göttinger, F.R.G

1 Introduction

1.1 Microalgae and cyanobacteria

Algae are mainly oxygen-releasing photosynthetic organisms, with simple body plans, without roots, leaves and stems. Microalgae use carbon dioxide efficiently from atmosphere and convert it to oxygen (O_2), for breathing. With this ability, algae are responsible for more than 75 % of the total global carbon assimilation (carbon fixation) for animal and human use. Furthermore, microalgae and cyanobacteria have already been demonstrated to have potential for a wide range of applications and products, showed in Figure 1.

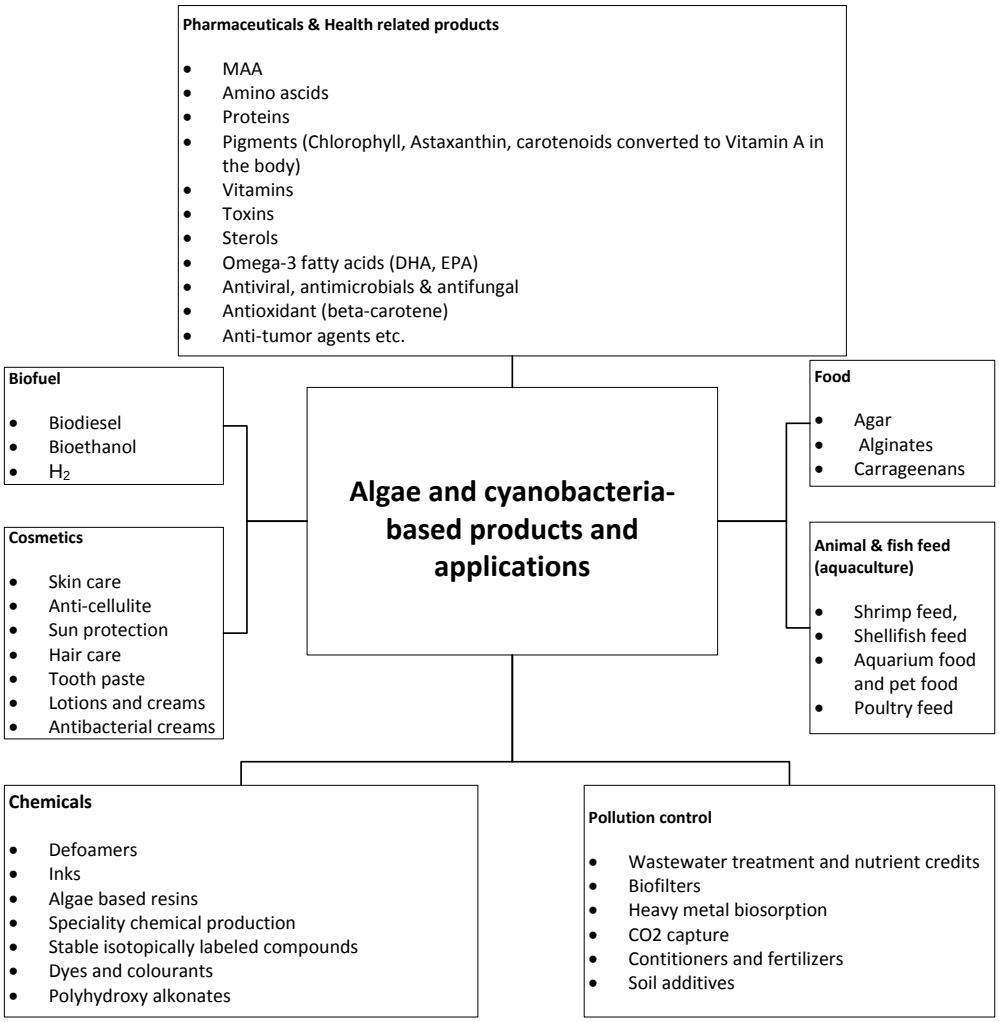


Figure 1. Algae and cyanobacteria based products and applications (adapted from [1]).

Mostly algae are aquatic organisms and do not form an individual monophyletic group, consequently they cannot be easily characterized. Thus, algae as a group are ubiquitous; one species occupy specific habitats. Some algae are simply suspended in water, some grow loosely on soil, trees, and animals, some form symbiotic relationship with other organism (e.g. lichens and corals), and some algae are linked to a substrate like plants and some motile like animals. Above all, the internal cell structure differs greatly, from eukaryotic (e.g. green algae) to prokaryotic (green-blue algae known as cyanobacteria). It is difficult to provide a clear definition for algae. Therefore, thousands of scientific journals and books are dedicated solely to compiling our knowledge of algae [1].

Green algae

Green algae are one of the largest groups of algae. Interestingly, only green algae store their photosynthates within the chloroplast. Hence, green algae have starch grains inside of the plastid; as in concurrently for all other algae, lipids or carbohydrates are located outside of the plastid. From the biochemical point of view, green algae contain proteins, carbohydrates, and lipids, starch being the main product from carbohydrate storage. As seen in Figure 2, green microalgae cell structure/shape may be different, some strains of cells are bigger than others, some strains have different shape, or they contain different amounts of proteins or fats.

The diversity of green algae lipids is extensive, and the composition of cellular lipid can be manipulated increasing the amount of nitrogen. These lipids are mostly polyunsaturated fatty acids (PUFA) that contain more than one double bond, and they are classified as methylene-interrupted polyenes such as omega-3 (EPA, DHA), omega-6 (linoleic acide) and omega-9 (mead acid) and as conjugated fatty acids (rumenic acide). Microalgae are also able to produce mycosporine-like amino acids (MAAs), since they live in an environment with high volumes of sunlight (e.g. *Prasiola sp.*). Green algae (e.g. *Chlorella vulgaris*) proteins are also under interest; they are diverse and can be manipulated by environmental changes, like nitrogen stress [1].

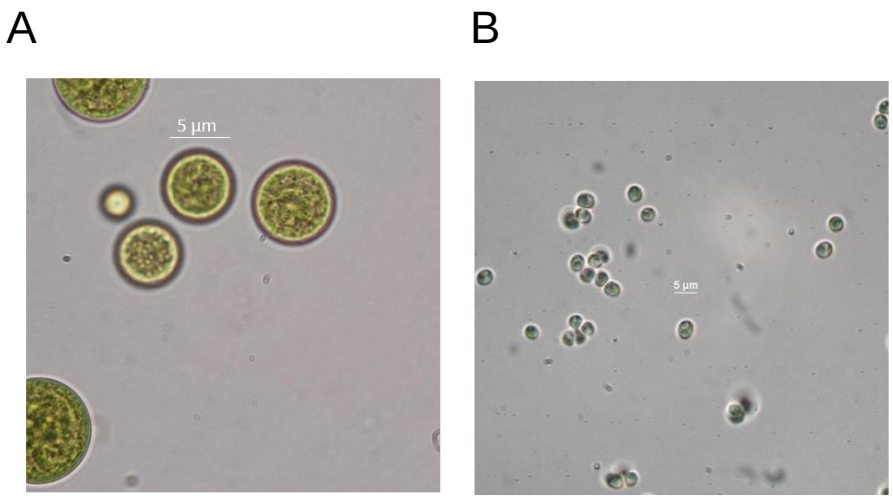


Figure 2. Microalgae *Diplosphaera chordatii* strain (MCI-27) (**A**) and *Prasiola sp.* strain (MCI-28) (**B**) viewed under a light microscope.

Cyanobacteria

Cyanobacteria, also known as blue-green algae, are organisms which historically belong to algae even though they have a prokaryotic cell structure. Cyanobacteria are photosynthetic and can produce their own food. Therefore, cyanobacteria have been named as algae, because of the term of “algae” refers to an aquatic organism that is capable for photosynthesis. Again, due to the blush phycocyanin pigment, cyanobacteria have been named as blue-green algae. [2] Overall, cytologically and biochemically, the cyanobacteria are more like bacteria than algae. Due to the peptidoglycan cell wall, cyanobacteria are an excellent source of protein; even 40-60 % of the dry mass is protein (e.g. *Spiruline*) [1]. Like algae, also cyanobacteria species are not equal among themselves. Some species are toxic and, in high concentrations, can significantly affect the water quality, while some species are commercially very valuable, but mainly inoffensive organisms and priceless for our ecosystem. [1] One way to identify cyanobacteria is to observe the characteristic chain structure as seen in Figure 3 picture A.

A

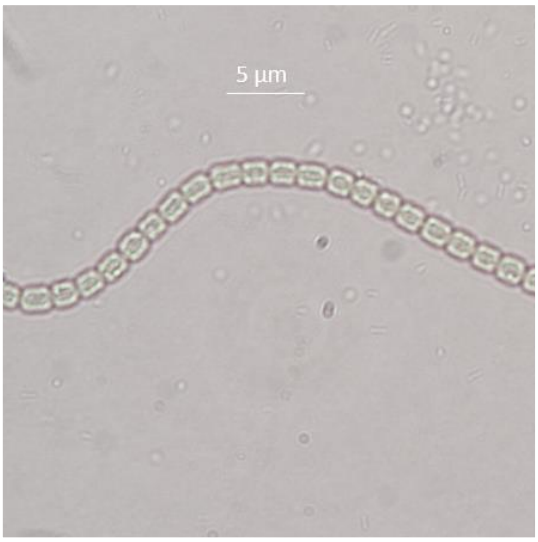


Figure 3. Cyanobacteria *Schizothrix sp.* strain (**A**) viewed under a light microscope.

The principle of microalgae cultivation is simple, but on the other hand,easily manipulated by environmental changes. For cultivation, the following factors were needed: sunlight, carbon dioxide, water, nutrients, optimal temperature, pH, aeration, mixing, light and dark cycles. By changing one of the factors, the product can change significantly or even kill the algae. Light and temperature are the most important factors that affect microalgae biomass productivity, due to the significant effect in the metabolism, enzyme activities and cell structure of algae. There are few different cultivation methods, which are used to produce biomass. Cultivation in open pond and cultivation in photobioreactor are also the most used methods, but fermentation tanks for heterotrophic cultivation and hybrid systems for mixotrophic strains are good alternatives.

Worldwide research on algae is increasing and several large companies and governments invest large amounts of money in research programmes. The aim would be to develop a more efficient process to produce bio-fuel and at the same time decrease production costs. Additions, taxonomists try to classify more unknown algae strains, and researchers try to find more useful/valuable substances for use. [3]

1.2 Cryopreservation

The aim of cryopreservation is to storage a living organism at a very low temperature to maintain their viability, without change in their morphological, biochemical, physiological, and genetic properties [4]. At the low temperature (typically colder than -130 °C) culture cells are an inactive metabolically but able to be revived and restored to normal health during the recovery phase. Cryopreservation is a very common method for retaining microalgae ability and genetic integrity over a long period. [1]

Biological mechanisms of cell damage during cooling and thawing are still not fully understood. Figure 4 shows a graphic illustration of a cryopreservation process with possible stress factors and with mechanisms which cause contribute injury for cells [5]. Even though, cryopreservation is still complicated, due to cooling and thawing steps, hundreds of strains of microalgae have been successfully cryopreserved.

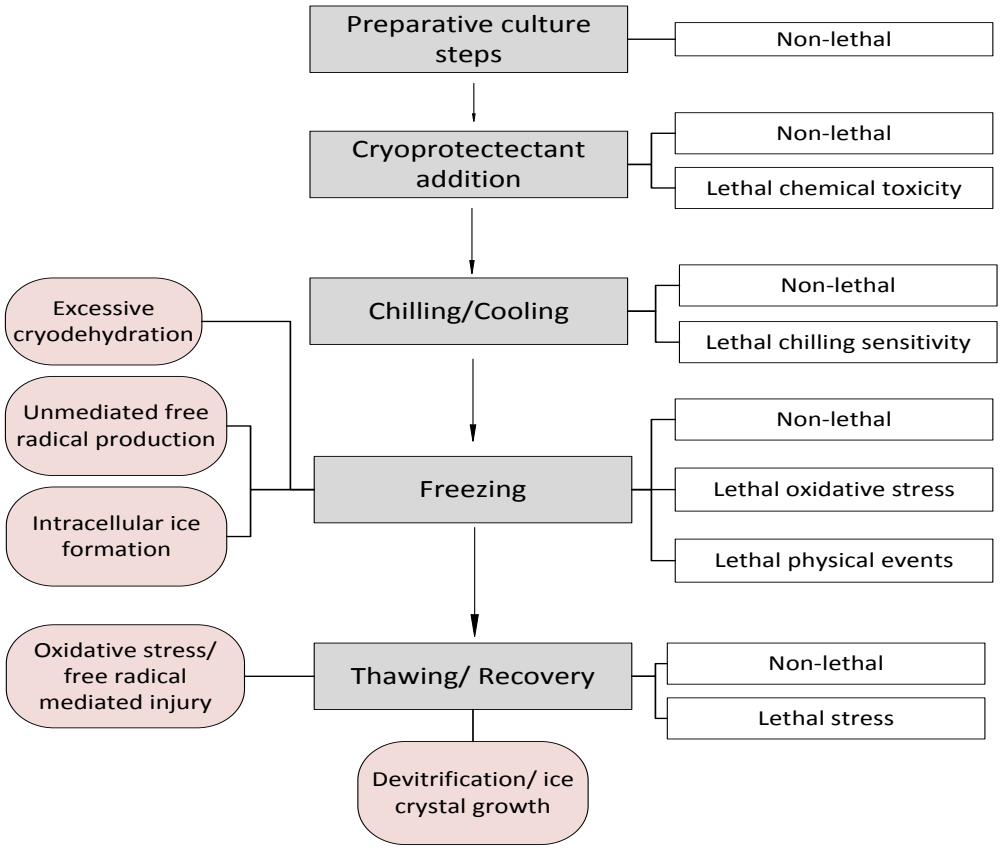


Figure 4. Graphic illustration of a cryopreservation protocol and the stresses which may be associated with each step are described in the white box. In addition, mechanisms which contribute to injury/ stress are highlighted by red for the freezing and thawing/ recovery stages of the protocol (adapted from [6]).

Workload and costs associated with maintaining large actively-metabolizing strain collection, in addition to finite storage capacity, cause a significant limitation. Due to that, cryopreservation as a long-term method would be an excellent solution to eliminate the need for continuous culturing and allows strains storage for theoretically indefinite periods in cryopreservation. In addition, cryopreserved cultures demand very little space and allow storing hundreds of cryovials in one cryotank (Cryodiffusion). Culture storage under liquid nitrogen also protects cultures against of microbial contamination, catastrophic loss and for possible human errors. Therefore, cells do not change in genetic structure during storage at an ultra-low temperature. [5;4]

1.3 MCI project: (co)-Operation SKD (screening cultivation downstreaming)

The aim of Management Center Innsbruck's (MCI) algae project is to achieve an economic process to produce valuable bioactive compounds from microalgae and cyanobacteria. The purpose is not to focus on one product, but to establish processes to be a competent partner in further microalgae-projects and to be able to provide comprehensive customer hyphen-oriented service. Continuous research and development are conducted on algal/cyanobacteria culture condition optimization, analytic and extraction testing of promising substances, fermentation-parameters, downstream processing, and strain characterization based on molecular-genetic techniques. MCI project has several competent partners such as University of Innsbruck, Göttinger University (SAG), FH Oberösterreich (FH OÖ) and the Austrian Drug Screening Institute (ADSI). The collection strategy for the future would be resort of unique culture collection of soil, air, and lichen algae as well as the cyanobacteria of the algae collection ASIB 505. The latest addition is the aim to develop functional and viable method to cryopreserve the whole collection to prevent possible genetic changes in structure and decrease collection maintenance costs.

1.4 Aim of the thesis

The present thesis was part of a research project with the aim to develop an economical process to produce valuable bioactive compounds from terrestrial microalgae. The focus was on algae collection maintenance, culture purification, test strain cultivation, and cryopreservation. In the present thesis project, strains of algae collection ASIB 505 (Table 2) strains were used as test strains. The experimental part of the thesis was carried out in the laboratory of the Department of Biotechnology at the Management Center Innsbruck (MCI).

The aims of this thesis are listed in more detail below:

- Algae collection management, including collection recovery and purification, test strain cultivation, microscope pictures and database update.
- Cultivation of biomass for extractions and assays, using four different methods: tubular photobioreactor, Schott bottle, conical flask in a shaker with and without carbon dioxide and determining the most efficient method for producing biomass with microalgae.
- Development of functional and viable cryopreservation method.

2 Culture collection

2.1 Culture collection and collection strategies

Microalgae culture collections are divided into two different groups: privet collections that are maintained for a specific purpose (not public) and large service collections for public use. More than 11 000 microalgae species are stored in multiple culture collections globally, e.g. in USA, Austria, Australia and in Scotland, most of these require constant sub-culturing to maintain cell viability. To establish microalgae and cyanobacteria culture collection, strains are isolated, purified, cultivated, and screened for nutritional characteristics like high value product, lipid, bioactive compound, and biomass. [1;7].

MCI ASIB 505 collection strains are derived from the ASIB 505 collection from Botanical Institute of the University of Innsbruck (Austria). The ASIB 505 collection of algae started in early 1960s by H. Pitschmann and H. Reisigl. Today the collection comprises 1570 strains [8] with a focus on air, soil, and lichen algae. Collection strains are mostly from alpine areas of a Central Europe (Austria, South Tirol, and Switzerland) and mainly used for education, medicine, biotechnology, or environmental and ecological studies. Original ASIB 505 original cultures are maintained on Bold's Basal Medium (BBM) agar slants (10 g L-1) in glass test tube, which are kept at the optimal temperature of 13 °C and at a light intensity of a 300-3000 lux under cool-white fluorescent lamps on a 12 h day-night rhythm. Strains were transferred every six months to new fresh media, to avoid contamination overgrowth by bacteria and/or fungus. [9;10].

Today’s MCI ASIB 505 culture collection includes both, cyanobacteria (blue-green algae) and eukaryotic microalgae (green algae). The collection maintains 6 cyanobacteria and 48 algae strains. All collection strains are defined as unialgal or unicyanobacte-

rial; in other words, they were classified as single-strains cultures of microalgae. All collection strains are not equal: some strains are very difficult to cultivate, some strains have never been in a culture before, some strains are badly contaminated and some strains have more value than others. [5]

2.2 Maintenance of actively growing cultures

MCI ASIB 505 culture collection backups were storaged for long-term on BBM (Table 3) agar slant with 15 g L-1 agar, in Falcon tubes (50 mL), which are equipped with a cap. The cap has been left loose and wrapped with parafilm to prevent the introduction of contaminants and kept at a high internal relative humidity, but enough loose to allow gas exchange [1]. Falcon tubes were stored, under low temperature and under low light intensity (18 °C, under natural light of a north-facing window) and were transferred to a new fresh media, approximately every six months to prevent contaminations over growth and verify desirable growth environment. To transfer the collection to a new fresh media is very time-consuming, and the risk to contaminate the original culture is high, due to human error. Therefore, the intervals of transfers are precisely regulated and culturing conditions are optimized to retard the growth rate. [1]

For daily use, microalgae cultures are sub-cultured onto nutritionally replete BBM or 3NBBM agar and liquid media such as BBM or 3NBBM. Plates with nutrient culture were used as backups and as a source of biomass for later biomass production using liquid media. These sub-culturing techniques are simple and handy and enable for short term sub-culturing. The following graph, Figure 5, shows the process behind of maintenance of actively growing culture. Cultures are maintained under laboratory growth conditions in a climate-controlled room (20 °C) under room illumination (85 $\mu E/m^2S$) to enable extended viability.

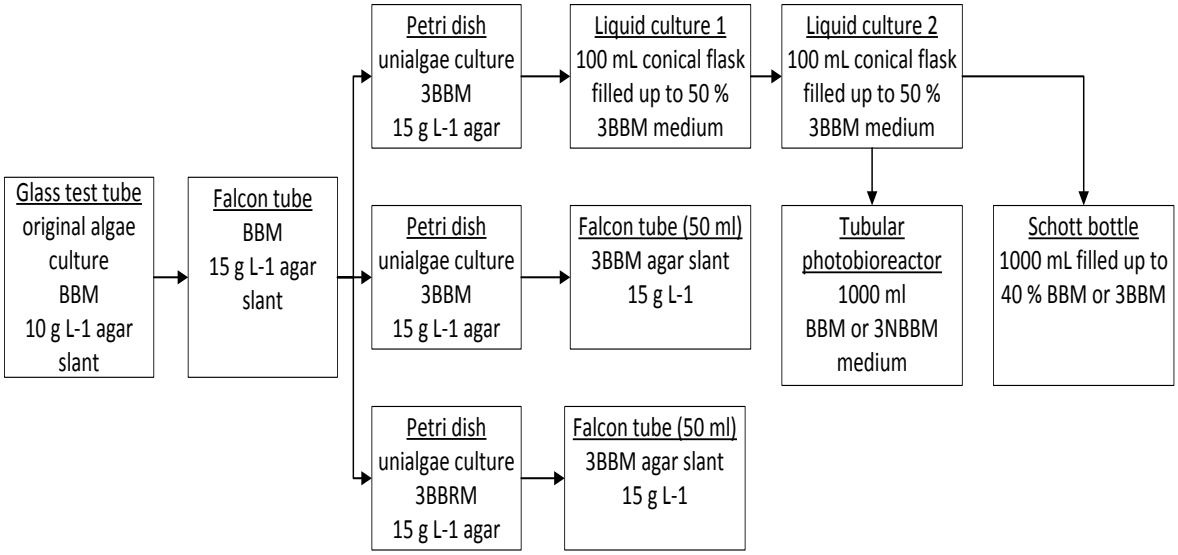


Figure 5. Back-up and pre-culture maintenance process behind of actively metabolized culture. Biomass is produced, for future assays with a tubular photobioreactor (TPBR) and with a Schott bottle.

Maintenance of a large culture collection is laborious and costly, and there is always a risk of dramatic culture loss due to human error or to phenotypic loss. For example, the Aquatic Species Program had over 3 000 microalgae species, and due to the logistical challenge of sub-culturing, only 300 viable species remained at the end of the program. Hence, a viable method for stable long-term storage is vital and provides the basis for any successful culture collection. [7]

2.3 Purity of cultures and database

The aim of culture collection is to obtain a viable culture of a single strain and keep it pure for cultivation. For collection management, MCI ASIB 505 collection database is used and updated during the thesis. The aim of the database is sharing as much information of every strain as possible between researchers and colleagues.

The collection database includes the following data:

- strain id. and collections-number
- original characterization

- information (growth conditions, parameters, features, growth speed and metabolites)
- number of backups and pre-cultures
- cryopreserved culture
- amount of freeze dried mass for each strain.
- microscopic pictures of every strain
- purity (unialgal, axenic, contaminated)
- classification (obtained sequences and used primers)

2.4 Quality control and financial consideration

Actively growing culture collection maintenance is time-consuming, arduous, and also very costly. High costs are based on maintaining qualified personnel and on the interval (weeks to six months) of manual inoculations of strains to a new fresh media. For a large collection, it means easily hundreds of inoculations per month, including needed purification steps. With every transfer, there is potential risk to contaminating the back-up culture or losing the strain. Due to that, quality control management is developed. After every third transfer from original backup, microscopic inspection should be carried out. To reduce risk, of losing strain, several backups and pre-cultures have been made. [1]

3 Materials

3.1 Chemicals

Table 1 lists the chemicals used in the thesis project.

Table 1. Used chemicals

Chemical	Company
Sodium nitrate	Carl Roth GmbH
Magnesium sulfate heptahydrate	Carl Roth GmbH
Sodium chloride	Carl Roth GmbH
Potassium phosphate dibasic	Carl Roth GmbH
Potassium dihydrogen phosphate	Carl Roth GmbH
Calcium chloride dihydrate	Carl Roth GmbH
Zinc Sulfate Heptahydrate	Carl Roth GmbH
Manganese(II) Chloride Tetrahydrate	Carl Roth GmbH
Molybdenum(VI) oxide	Carl Roth GmbH
Copper(II) Sulfate Pentahydrate	Carl Roth GmbH
Cobalt(II) nitrate hexahydrate	Carl Roth GmbH
Boric acid	Carl Roth GmbH
Ethylenediaminetetraacetic acid disodium salt solution	Carl Roth GmbH
Potassium hydroxide	Carl Roth GmbH
Ferrous sulfate	Carl Roth GmbH
Glucose	Carl Roth GmbH
Peptone	Carl Roth GmbH
Agar-Agar	Carl Roth GmbH
Ampicillin	Carl Roth GmbH
Kanamycin	Carl Roth GmbH
Carbendazim	Sigma-Aldrich
Caffeine	Carl Roth GmbH

3.2 MCI ASIB 505 Collection

All algae and cyanobacteria strains were originally provided from the microalgae collection ASIB 505 of the botanical institute of the University of Innsbruck and from the University of Göttingen (SAG). Strains in Table 2 are presently cultured in Management Center Innsbruck (MCI). To avoid nomenclature problems, strains were label with new identification name (strain id.) in MCI.

Table 2. MCI ASIB 505 algae and cyanobacteria collection

Strain id.	Collectionsnumber	Original characterisation
MCI-1	T1	*Tolypothrix*
MCI-2	T3	*Nostoc punctiforme*
MCI-3	T4	*Nostoc verrucosum*
MCI-4	T6	*Schizothrix sp.*
MCI-5	T7	*Schizothrix sp.*
MCI-6	V152	*Schizothrix sp.*
MCI-7	BS319	*Coccomyxa brevis*
MCI-8	BS775	*Pseudochlorella subsphaerica*
MCI-9	IB142	*Unknown*
MCI-10	IB149	*Unknown*
MCI-11	IB256	*Unknown*
MCI-12	IB273	*Chloroidium sp.*
MCI-13	IB410	*Unknown*
MCI-14	IB459	*Scotiellopsis sp.*
MCI-15	IB472	*Chlamydomonas octigama*
MCI-16	SAG 79.80	*Ettlia texensis*
MCI-17	SAG 213-2a	*Macrochloris radiosa*
MCI-18	T61	*Spongiochloris sp.*
MCI-19	V103	*Klebsormidium dissectum*
MCI-20	V111	*Heterococcus versiculosus*
MCI-21	V142	*Ettila bilobata*
MCI-22	V168	*Tetracystis cf. Tetraspora*
MCI-23	V195	*Scotiellopsis rubescens*
MCI-24	V199	*Hormidiospora verrucosa*
MCI-25	V204	*Chloromonas rosae*
MCI-26	V21	*Unknow*
MCI-27	V219	*Diplosphaera chordatii*
MCI-28	V224	*Prasiola sp.*
MCI-29	V24	*Unknow*
MCI-30	V39	*Muriella terrestris*
MCI-31	V46	*Chlamydomonas culleus*
MCI-32	V50	*Chlamydomonas peterfii*
MCI-33	V6	*Pleurochloris meiringensis*
MCI-34	CV	*Chlorella vulgaris*
MCI-35	CH	*Chlorococcum hypnosporum*
MCI-36	BS110	*Unknown*
MCI-37	BS349	*Unknown*
MCI-38	IB145	*Unknown*
MCI-39	IB505	*Unknown*
MCI-40	-	
MCI-41	SAG379.1C	*Unknown*
MCI-42	IB423	*Unknown*
MCI-43	IB565	*Unknown*
MCI-44	IB514	*Unknown*
MCI-45	E71.10	*Unknown*
MCI-46	-	
MCI-47	IB408	*Unknown*
MCI-48	IB407	*Unknown*
MCI-49	T87	*Unknown*
MCI-50	T90	*Unknown*
MCI-51	T91	*Unknown*
MCI-52	BS351	*Unknown*
MCI-53	BS363	*Unknown*
MCI-54	V143	*Unknown*
MCI-55	V208	*Unknown*
MCI-56	T88	*Unknown*

3.3 Media

The following Bold's Basal Medium (BBM) and Bold's Basal Medium N-free were autoclaved at 121 °C for 20 minutes. For Bold's Basal Rich Medium, peptone and glucose were filter sterilized using a 20 µm syringe filter.

Table 3. Growth media used for the cultivation of algae and cyanobacteria.

Media	**Ingredients**
Bold's Basal Medium (BBM) pH 6.7 (± 0.1)	2.94×10^{-3} M NaNO3 (for 3NBBM 8.82×10^{-3} M) 3.04×10^{-4} M $MgSO_4\times7H_2O$ 4.28×10^{-4} M NaCl 4.31×10^{-4} M K_2HPO_4 1.29×10^{-3} M KH_2PO_4 1.70×10^{-4} M $CaCl_2\times2H_2O$ 3.07×10^{-5} M $ZnSO_4\times7H_2O$ 7.28×10^{-6} M $MnCl_2\times4H_20$ 4.93×10^{-6} M MoO_3 6.29×10^{-6} M $CuSO_4\times5H_2O$ 1.68×10^{-6} M $Co(NO_3)\times6H_20$ 1.85×10^{-4} M H_3BO_3 1.71×10^{-4} M $EDTA\times Na_2$ 5.53×10^{-4} M KOH 1.79×10^{-5} M $FeSO_4\times7H_2O$
Bold's Basal Rich Medium (BBRM) pH 6.7 (± 0.1)	BBM with: 5 g L^{-1} Glucose 5 g L^{-1} Peptone 15 g L^{-1} Agar-Agar
Bold's Basal N-free Medium pH 6.7 (± 0.1)	BBM without 2.94×10^{-3} M $NaNO_3$ 15 g L^{-1} Agar-Agar

3.4 Antibiotics

Table 4 presents the antibiotics used in the thesis project.

Table 4. Used antibiotics.

Antibiotic	**Final-concentration**
Ampicillin ($C_{16}H_{19}N_3O_4S$)	100 mg L^{-1}
Kanamycin ($C_{18}H_{36}N_4O_{11}$)	30 mg L^{-1}

3.5 Antimycotics

Antimycotics are used to prevent fungal contamination. Caffeine (10 mM and 20 mM) has dissolved using distillation water. Carbendazime does not dissolve in water because hydrochloric acid (HCl) has been used as a solvent. For carbendazime, a stock solution with a concentration of 50 mg L^{-1} was prepared, and for caffeine a stock solution with a concentration of 100 mg L^{-1} was prepared. Antimycotics were filter sterilized using a 0.22 µm syringe filter before being added into the medium. Table 5 lists the antimycotics used and their final concentrations.

Table 5. Used antimycotics.

Antimycotics	**Final-concentration**
Carbendazim ($C_9H_9N_3O_2$)	0.05/0.025 mg L^{-1}
Caffeine ($C_8H_{10}N_4O_2$)	1940/3900 mg L^{-1}

3.6 Cryopreservation

A CoolCell (Biocision) alcohol-free cell freezing container was used to pre-freeze the sample under - 80 ℃, to ensure a standardized controlled-rate of -1 °C /minute. Once the desired temperature is obtained, cryovials with samples were directly placed into cryodewar (Cryo Diffusion) and plunged directly into liquid nitrogen, to complete the freezing process (< -130 °C). Methanol and dimethyl sulfoxide (DMSO) were used as cryoprotectants to protect algae cells from freezing damage (Table 6). Stock solutions were autoclaved at 121 °C for 20 min. [11]

Table 6. Cryoprotectants, stock solutions and used concentration.

Cryoprotectant	**Stock solution [%]**	**Final concentration v/v [%]**	**Stock solution [%]**	**Final concentration v/v [%]**
DMSO	10	5	20	10
Methanol	10	5	20	10

4 Methods

4.1 Purification

The aim of purification was to obtain viable cultures of single strains, free of contaminations like bacteria and fungus and pure enough for cultivation in minimal medium [5]. Strains of the ASIB 505 collection were unialgal and unicyanobacterial, but not axenic. Axenic cultures are strains that contain only one strain of organism without any other contaminating organism. Unialgal strains are single-strain cultures that can be cultured granted that heterotrophic bacteria are present. Some bacteria can also produce specific compounds that increase microalgae growth; therefore, axenic cultures are not the target of purification, in this case. [1]

As purification techniques, mechanical separation, antibiotic and antimycotic treatments were carried out. Purification on solid agar medium is most commonly used for growth media. Soil algae grow well on a surface; thus, a solid agar medium was selected for purification instead of a liquid medium.

4.1.1 Mechanical separation

Mechanical separation as purification is based on isolation of single cell colonies to produce a pure culture, making a streak plate. The streak plate method is the way to physically separate a mixed culture into a pure culture including one individual microorganism. Biomass was isolated from solid agar medium by inoculating loop and spread across to a new fresh solid agar plate. Upon incubation (50 µE m^{-2} s^{-1}; 24 h light; 20 °C) over 3-6 days (depending on strain growth rate), colonies will appear and single cells were isolated from a mixed culture to a pure culture. Process was repeated if needed; often this approach has been repeated several times.

It was essential to transfer algae/cyanobacteria biomass before bacteria or fungi showed overgrowth. For some strains, mechanical separation was very successful, but mainly to get pure strains, selective media (antibiotic and antimycotic) were needful. All steps, including agar plate preparation, liquid culture preparation and dilutions were performed in a sterile hood using a laminar flow cabinet, which proved to be very essential to get the desired results.

4.1.2 Antibiotic treatment

Antibiotic treatment was performed to inhibit bacterial growth or eliminate contamination without killing algae. Solid BBM (Table 3) agar containing an antibiotic (Ampicillin and Kanamycin (Table 4)) was used to purify unicellular eukaryotic microalgae [1]. Biomass was spread across of selective antibiotic-containing solid medium, using a loop. After inoculation, plates were placed into an incubator chamber for 3-6 days, depending on the strain growth rate. After that, one algae cell or even a small inoculum, was isolated and inoculated again to fresh BBM agar and incubated for 3-6 days.

It was essential to transfer microalgae biomass before potential contamination showed overgrowth or/and antibiotic became toxic for algae. Generally, microalgae were not affected by the presence of ampicillin, while kanamycin led to reduced growth of algae and even reduced the cell death of some algae [12]. With some strains this approach was repeated several times combined with mechanical separation. [5]

4.1.3 Antimycotic treatment

Antimycotic treatment inhibits the growth of fungi and was therefore used as an inhibitor for fungal contaminations. Carbendazim and caffeine (Table 5) were used as antimycotic agents. For example, carbendazim acts by binding to the fungal microtubules and stopping hyphal growth. In addition, carbendazim binds to the spindle microtubules and blocks nuclear division. In practice, the culture was inoculated on a selective culture dish and placed, into an incubator chamber for 3-6 days. After an antimycotic treatment, the culture was inoculated again to a new fresh solid BBM.

4.2 Proof of purity

To confirm the purity of an algae culture, the culture was cultivated on Bold's Basal Rich Medium (BBRM (Table 3)) including organic substrates such as glucose and peptone. Contamination was determined macroscopically after 7 days arise. Lastly, the strain was classified, according to purity, and several backups and pre-cultures were made. [5]

4.3 Biomass production and upscaling

Algae/cyanobacteria biomass was produced mainly using tubular photobioreactor (TPBR); in addition, a Schott bottle and a conical flask were used. The advantage of a

Schott bottle is the shape, size, price, and handiness. A conical flask is handy for cultivating pre-cultures, due to the small amount of needed biomass.

For biomass production, there are two cases; one is to make a liquid pre-culture from a solid culture, and the other is to up-scaling pre-culture to larger volume, to produce higher amounts of biomass. Figure 6 shows the biomass production process and illustrates the steps behind the up-scale process, behind the pre-culture preparation and downstream. Gradually, increasing the volume of a culture is essential, for cells exponential growth. If the cell density is too low, cell growth starts to decrease. Cultivation to the maximum biomass yield of algae/cyanobacteria is followed by harvesting. During harvesting, desired biomass is removed by centrifugation (4600 rpm, 10 min), followed by two washing steps. Pellets with biomass, are freeze-dried and stored for downstream, in a freezer under – 20 °C.

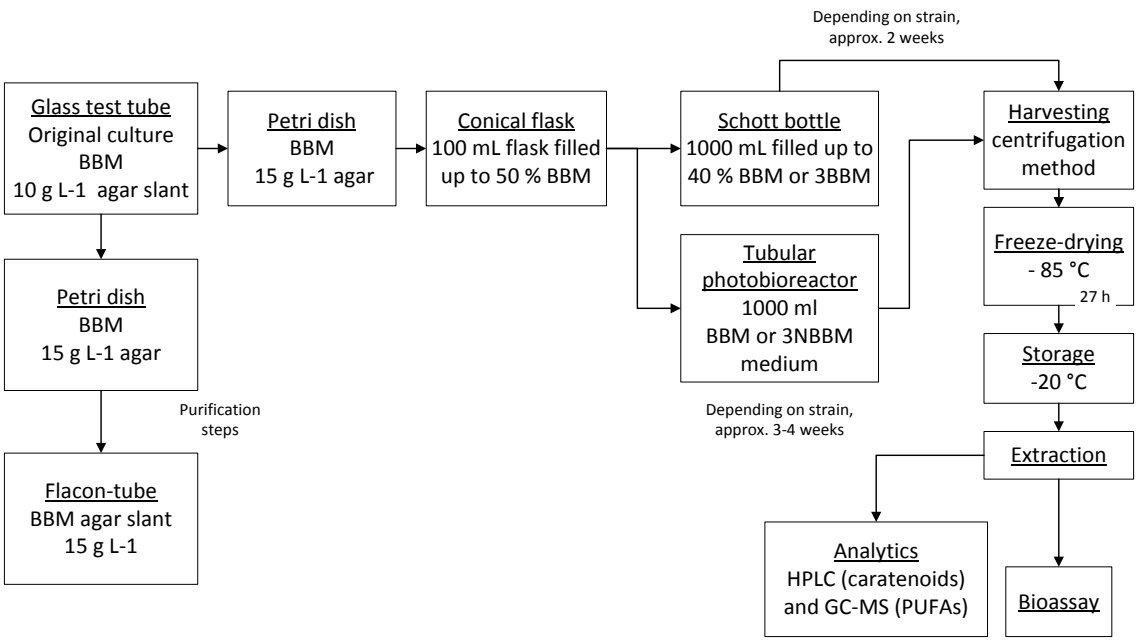


Figure 6. Biomass production process including pre-culture preparation, biomass production and with downstream process have been illustrated in diagram.

As mentioned above, several techniques were used for biomass production. Therefore, biomass growth experiment using different cultivation methods was carried out. The aim of the experiment was to clarify the viability of each method and to compare the results of another. The following four techniques to produce biomass in a liquid culture, with described conditions, were tested:

- Schott bottle of 1000 mL, filled up to 40 % sterile 3NBBM growth medium, illuminated by SANlight M30 LED 200 µE/m^2S, RT 20 °C.
- Conical flask of 100 mL, filled up to 50 % sterile 3NBBM growth medium, under room light 85 µE/m^2S, RT 20 °C.
- Conical flask of 50 mL, filled up to 20 % sterile 3NBBM growth medium. Cultivated with carbon dioxide (CO_2) shaker chamber with 1 % CO_2, under 232 µE/m^2S light, under 20 °C.
- Tubular photobioreactor of 1000 mL, filled up to 80 % sterile 3NBBM growth medium, illuminated by SANlight M30 LED 200 µE/m^2S, RT 20 °C.

The microalgae strain, *Prasiola sp*., was used as a test strain. All four methods were carried out with using 3NBBM (Table 3), to support algae growth. For Schott bottle and for Tubular photobioreactor, the aeration was done by gas flushing, to ensure constant aeration. For conical flasks, table shaker and shake chamber with 1 % of CO_2, were used. Constant aeration is an essential factor because without mixing the culture, cells became dead and no more growth occurred. Viability of each method was observed and quantified by optical density (OD) detection at a wavelength of 750. Using results of optical density detection, RGR also known as exponential growth rate, was determined. RGR determine the algae maximal growth rate per day, where t1 is time one (in days) and t_2 is time two (in days), N_1 is the optical density (750 nm) at time one, and N_2 is the optical density (750 nm) at time two.

$$RGR\ (\mu) = \ln (N_2 / N_1) / (t_2 - t_1) \quad (1)$$

Furthermore, cell concentration, expressed as a dry cell weight per culture volume, was determined using the dry weight measurement.

4.4 Cryopreservation methods for maintaining microalgae cultures

4.4.1 Cryoprotection

Cryoprotectants are chemicals that protect cells from a cell damages during freezing and thawing. Cryoprotectants are defined into two groups, impermeable and permeable. These groups are ranged in their ability to protect the cells for cryopreservation. For algae, typically permeable cryoprotectants as dimethyl sulfoxide (Me_2SO) and methanol (MeOH) were used. Advantage of permeable cryoprotectant is ability to freely permeate cell membrane, hence to provide protection against freeze damage [7]. Although, that they are protecting cells is essential to note that cryoprotectants were iden-

tified as a toxicity agent and can potentially damage cells. Due to that, optimization of cryoprotectant concentration is a very important aspect of optimization of microalgae cryopreservation protocols. [6]

Methanol with 5 % (v/v) and 10 % (v/v) and dimethyl sulfoxide with 5 % (v/v) and 10 % (v/v) were chosen for this thesis. The above-mentioned concentrations were based on several earlier studies. 10 % methanol and 10 % dimethyl sulfoxide concentration were used as an experimental trial.

4.4.2 Freezing

Two-step freezing process was chosen for this thesis based on success of earlier studies [7]. In two-step freezing processes in the first phase of this process, the temperature is lowered at a controlled rate, -1 °C /minute using a CoolCell freezing container until the desired temperature is attained in this thesis it was -80 °C. Once the desired temperature is obtained, cryovials with sample is plunged directly into liquid nitrogen to complete the freezing process (< -130 °C). It has been reported that, with cryoprotectant and without cryoprotectant, two-step freezing process yielded higher cell viabilities than one-step or rapid freezing [7]. If the cooling process is too fast the chemical potential of the extracellular solution decreases much faster than the rate at which water can diffuse form the cell. However, cooling process can be too slow as well, then the cell will respond by losing water across the semi-permeable plasma membrane. During the freezing, there are few potential stress factors to injure the cell, illustrated in Figure 4. [7]

4.4.3 Thawing and recovery

Like freezing, also thawing has recently shown to be influenced by the cell viability (Figure 2). Several variables, for example, thawing speed, nutritional requirements, temperatures, light levels, cell volume, and the shear forces (centrifugation and pressure from pipetting) can influence the viability of cells. Too slow thawing may cause ice crystal growth what causes a cell damages. It is an essential to remove quickly as possible the freezed media including cryoprotectants and replace it with a new fresh non-toxic media. It is also important to choose optimal warming rate for warming the samples from – 130 °C to the room temperature. Optimal warming rate would be ~70 °C per minute. Hence, water bath with 37 °C is chosen for rapid thawing. In general, optimal warming rate decrease ice crystals reformation and at the same time minimizes the

time when cells are in direct contact with potentially toxic, like cryoprotectant (methanol and/or DMSO). [7; 6]

For a cryopreservation method with a liquid culture, suspension was immediately diluted to the fresh medium (to avoid potential damages) and after that, two washing steps were followed. Suspension was centrifuged gently to pellet cells after every washing step. Old supernatant was replaced with a new fresh medium. However, there is a risk that centrifugation may cause remarkable stress for algal cells. Accordingly, samples were centrifuged as gently as possible, and for an agar slant method, centrifugation step was eliminated. After two washing steps, the re-suspended culture was plated on solid BBM and on solid BBM including 5 g L^{-1} glucose. Glucose as an organic substrate support algae recovery and detect the ability of algae to produce a new fresh biomass. In addition, liquid culture was made using 100 mL Erlenmeyer flask which was filled up to 50 % of 3NBBM medium including 0.8 mL inoculated culture.

For an agar slant method, suspension was directly pipetted out of vial (without harming algal culture on agar slant) and replaced with a fresh medium, two washing steps were followed. For experimental reasons, including storage suspension after every washing step, washing suspension was plated on solid BBM, BBRM, and BBM including 5 g L^{-1} glucose after every washing step to support culture recovery and to observe the potential culture loss.

Microalgae cultures are typically grown under high illumination of light; however, cryoinjury make cells more susceptible to biochemical and physical disturbances including light. Therefore, samples were placed after thawing, into fridge for 24 hours, under very low light level at 8 °C degrees. Afterwards samples were located at room temperature under normal light conditions. After three days of recovery, cultures were placed into incubator and liquid cultures into carbon dioxide shaker (1 % CO_2). [7] More detailed protocols for both cryopreservation methods can be found in Chapter 4.4.5. Assessment of viability

It is difficult to determine, the population of an algal units that survive over long-term storage under liquid nitrogen and can resume normal growth after thawing. For positive results, even one algal unit is enough to define minimum success of a cryopreservation. For successful results, the whole process is an important, starting with preparation of pre-cultures and ending up to the recovery phase. A few methods for determining

the viability after recovery of cryopreservation were carried out. The most direct way to determine viability was to transfer thawed test strains (*Prasiola sp.* (MCI-28), *Chlorococcum hypnosporum* (MCI-35) and *Chloroidium sp.* (MCI-12)) on the nutrient agar plate or in the liquid medium. For nutrient agar plate, BBM with 5 g L-1 glucose were used. Presence of glucose not only support recovery, but also shows potential contaminations in culture. For liquid culture, 3NBBM media was used. After thawing, test strain *Prasiola sp.* (MCI-28) culture growth was observed and quantified by optical density (OD) detection at a wavelength of 750. Furthermore, dry mass was determined using dry weight measurement, at the end of an experiment (Table 8). After thawing living cells they were observed microscopically (Nicon Eclipse 50, Nikon Metrology) and macroscopically.

4.4.4 Used cryopreservation protocols

Cryopreservation was performed for an experimental reason using two different agents; cryopreservation with liquid culture and cryopreservation on agar slant. For both used methods, protocols were loaded. The protocol followed in cryopreservation is based on a guide provided by the Univesity of Texas at Austin [13] and on the interview with Sebastian Perkams [14].

Protocol for strains that grow preferentially in liquid culture.

The following three microalgae strains were used as test strains: *Prasiola sp.* (MCI-28), *Chlorococcum hypnosporum* (MCI-35) *and Chloroidium sp*. (MCI-12). Strains were cultivated in shake flask in fresh Bold's Basal Medium with three times nitrogen (3NBBM) (Table 3) over 5 days under room light (85 µE/m^2S), at 20 °C controlled room temperature. Before cryopreservation algae cultures should be in exponential phase. A liquid culture of the algae of interest is grown in medium that supports active growth. Bold's Basal Medium with three times nitrogen (3NBBM) was chosen.

First, 5 mL of algae culture was placed in a 15-mL sterile Falcon tube and hidden for subdued light because an algal algal culture should be kept in subdued light any time they are directly exposed to a methanolic solution. Falcon tubes were covered with aluminum foil, and placed into a 4 °C refrigerator for 24 h before samples were stored in cryovials.

The following materials were prepared in an advance:

- Sterile cryoprotectants stock solutions (SL):
- 10 % methanol (for 5 % (v/v))
- 20 % methanol (for 10 % (v/v))
- 10 % DMSO (for 5 % (v/v))
- 20 % DMSO (for 10 % (v/v))
- 1.8-mL cryovials
- A CoolCell freezing container, constant 1 °C /minute freeze rate
- Three of a 12-position square storage box designed to hold 1.8-mL cryovials
- 4 sterile 100 mL Erlenmeyer flasks
- Bold's Basal Medium with three times nitrogen (3NBBM), pH 6.7 (± 0.1)
- Bold's Basal Medium (BBM) 15 g L^{-1} Agar-agar plates, pH 6.7 (± 0.1)
- Bold's Basal Medium (BBM) 15 g L^{-1} Agar-agar, 5 g L^{-1} glucose plates, pH 6.7 (± 0.1)

Then, 0.7 mL of algae in liquid culture 3NBBM was placed into 1.8 mL cryovial. After that, 0.7 mL of the cryoprotectant (DMSO or methanol with different/ wanted concentration) solution was added to the vial and contents were mixed quickly, but gently. Used final concentrations (v/v) of cryoprotectant were 5 % methanol, 10 % methanol, 5 % DMSO and 10 % DMSO.

Cryovials were then placed into a CoolCell freezing container and placed into a -80 °C freeze for 2 hours (at least 1.5 hours but not as long as overnight). A CoolCell freezing container cooled the samples down constantly 1 °C/ minute. After 2 hours in the -80 °C freezer, the CoolCell was removed and cryovials are immediately placed into the 12-position square storage box. Storage box was then placed into the rack, which was placed into the liquid nitrogen dewar for short-term or long-term storage. It would be noted that the storage dewar must never run out of liquid nitrogen, even briefly, and the storage box must only be removed from the dewar for brief periods of time (max 3 min.) so that the contents of cryovials do not rise above approximately -130 °C.

Thawing is the most critical point of cryopreservation. Therefore, the cryovials were removed one by one from liquid nitrogen dewar and quickly inserted into the 37 °C water bath. The cryovial was gently agitated during thawing and transferred to 10 mL fresh 3NBBM as soon as crystals start to melt (approximately in 2 min.).

After that, falcon tube with 10 mL of fresh media and with living algae was centrifugated with 2000 rpm (as gentle as possible), 10 min, and 8 °C. During the centrifugation algae were separated from cryoprotectant and from old media. Supernatant was gently decanted. After first washing step Falcon tube was filled with new fresh 5 mL of 3NBBM and left undisturbed for several minutes. Centrifugation was repeated with 2000 rpm, 10 min, 8 °C and supernatant was removed as before and supernatant is gently decanted.

Then, 1mL of fresh 3NBBM was added into Falcon tube to suspend the pellet of algae after two washing steps. Next, 0.8 mL of recovered algae culture was transferred into 100 mL Erlenmeyer flask with 10 mL of fresh 3NBBM. 0.1 mL of recovered algae culture was inoculated on BBM agar plate and 0.1 mL of recovered algae culture on BBM agar plate including 5 % glucose.

Finally, the plates and the Erlenmeyer flask were then placed in dark conditions into an 8 °C refrigerator for 24 h (cryo-injury makes cells more susceptible to physical and biochemical disturbances), afterwards they were located at room temperature (20 °C) under normal light conditions. After 3 days, conical flask with algae culture was placed into carbon dioxide (CO_2) shaker (1 % CO_2; 232 µE/m^2S, 20 °C) and plates were placed into incubator (50 µE/m^2; 24 h light; 20 °C).

Protocol for strains that grow preferentially on agar slant

The following three microalgae strains were used as test strains: *Prasiola sp.* (MCI-28), *Chlorococcum hypnosporum* (MCI-35) *and Chloroidium sp*. (MCI-12). For each strain, two samples were made. One sample was inoculated from BBM agar plate and the other from BBRM agar plate into cryovial including an agar slant (15 g L^{-1}, 3BBM).

A nutrient agar slant of a composition known to support growth (3NBBM) of the alga of interest was prepared inside of a 1.8-ml cryovial. The vial contained approximately 1.0 mL of nutrient agar (15 g L^{-1}). After the slant solidifies, the alga of interest was inoculat-

ed on the agar slant and then placed into an incubator at 20 °C under 24 h light (normal growth conditions). The culture is ready for cryopreservation when a good amount of biomass (lawn) forms on the agar slant. The algal culture should be in exponential phase. After that, cryovials including algae were hidden for subdued light (an algal culture should be kept in subdued light any time they are directly exposed to a methanolic solution). Vials were covered with aluminum foil, and placed into a 4 °C refrigerator for 24 h before samples were stored in cryovials.

The following materials were prepared in advance:

- A CoolCell freezing container, constant 1 °C /minute freeze rate
- 1.8 mL cryovials
- 10 % methanol stock solution
- Bold's Basal Medium with three times nitrogen (3NBBM), pH 6.7 (± 0.1)
- Bold's Basal Medium (BBM) plates with 15 g L^{-1} Agar-agar, pH 6.7 (± 0.1)
- Bold's Basal Medium (BBM) plates with 15 g L^{-1} Agar-agar and 5 g L^{-1} glucose, pH 6.7 (± 0.1)

Then, 10 mL of 5 % methanolic culture medium was prepared using 10 % methanol stock solution and 3NBBM. After that, cryovials with agar slant and algae were gently filled with room temperature 5 % methanolic culture media to 1.5 -1.8 mL under low light conditions. Most of the algal lawn should remain on the agar after the solution has been pipetted into the vial.

Cryovials were then placed into a CoolCell freezing container at -80 °C freeze for 2 hours (at least 1.5 hours but not as long as overnight). A CoolCell freezing container cooled the samples down constantly 1 °C/ minute. After 2 hours in the -80 °C freezer, the CoolCell was removed, and cryovials were immediately placed into the 12-position square storage box. Then storage box was placed into the rack which was placed into the liquid nitrogen dewar, for short-term or long-term storage. It would be noted that the storage dewar must never run out of liquid nitrogen, even briefly, and the storage box must only be removed from the dewar for brief periods of time (max 3 min.) so that the contents of cryovials do not rise above approximately -130 °C.

Thawing is the most critical point of cryopreservation. Therefore, the cryovials were removed one by one from liquid nitrogen dewar and quickly inserted into the 37 °C water bath. The cryovial was gently agitated during thawing and left in the water bath until all ice had melted (approximately in 2 min). When, significant number of algae, were still on agar slant, solution was removed by pipette without disturbing the algae on slant.

After the solution with 5 % methanol was removed, vial was filled up with 1.0 mL of fresh 3NBBM (very slowly) and left undisturbed for several minutes. After a few minutes, media was gently removed using pipette, and the vials was filled up again (very slowly) with 1.0 mL of fresh 3NMMB and again left undisturbed for several minutes. Finally, the media was gently removed by a pipette without harming the algae culture on slant. If the algae did not remain on the agar surface when the solution was first thawed, centrifugation was performed (2000, rpm 5 min) before decanting the liquid in each wash.

Finally, the washing solutions were plated on nutrient agar (15 g L^{-1} BBM and BBM with 5 g L^{-1} glucose). Cryovials with agar slant and algae and plates with washing solution were placed under normal growth conditions (20 °C temperature-controlled room under room light). After 3 days, cryovials and plates were transferred into incubator (50 µE/m^2; 24 h light; 20 °C).

A successfully cryopreserved culture will produce new biomass on the culture surface within few weeks and may be inoculated to a fresh slant when desired.

5 Results

5.1 ASIB 505 microalgae collection purification

At the beginning of the culture collection purification, contamination level was observed between pure and contaminated. Mostly culture collection was contaminated with bacteria and/or fungus. In some cases, contamination had already overgrown the strains and almost leads to the strain loss (e.g. E71.10 and IB149). In addition, strain contaminations may cause several problems for an assay or in worst case lead to the algae/cyanobacteria death. Above all, for some strains living symbiosis with bacteria is vital (e.g. *Coccomyxa brevis*).

Purification steps for every strain were specific depending on the strain level of contamination and considering the strain type. For some strains, mechanical separation was enough to separate algae from contamination, concurrently for some strains several purification methods were carried out (Chapter 4.1) without any success. For instance, for strain *Scotiellopsis rubescens* (MCI-23) and for strain *Chloromonas rosae* (MCI-25) one mechanical separation was enough to have a pure culture, meanwhile strain *Clamydomonas noctigama* (MCI-15) and strain named as T90 (MCI-50) were not affected by any treatment. Nonetheless, most effective way of purification for algae turned out the combination of mechanical separation and an antibiotic treatment. However, the cells of *Coccomyxa brevis* (MCI-7) were killed when antibiotic treatment was performed.

For several badly fungus-contaminated cultures antimycotic treatments were carried out. Despite the toxicity of an antimycotic, the treatment only affects the fungus cells and not the algae cells. Antimycotic treatment with caffeine and with carbendazim was carried out for all badly contaminated strains. For some strains, caffeine eliminated fungus cells, but for some strains, caffeine was found ineffective against contamination. For example, the strain named as BS110 was one of them. As seen in Figure 7, after caffeine treatment fungus contamination can be still detected. At the same time, carbendazim treatment proved to be effective in eliminating fungus. As seen in Figure 7, when the culture was transferred from carbendazim to the BBM plate, it appeared fungus free. After last purification with kanamycin, the culture was classified as unialgal.

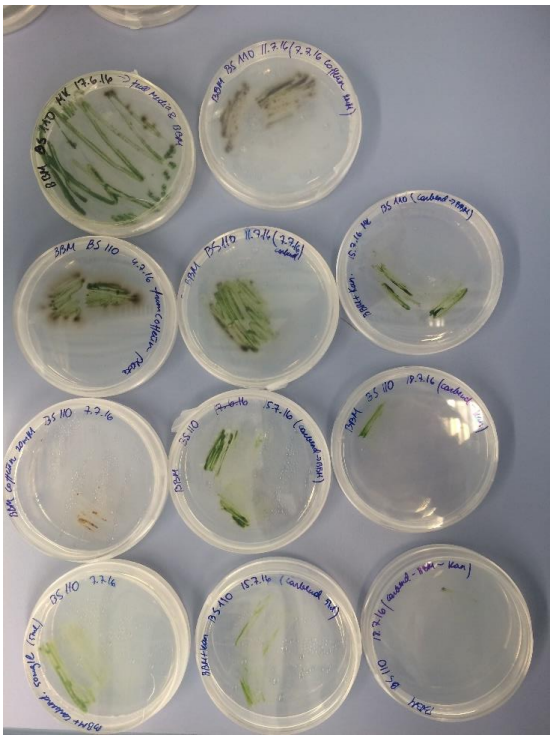


Figure 7. Strain BS110 purification results. Purification steps were carried out for algae strain, named as BS110. Strain was treated with caffeine, carbendazim and kanamycin. After every treatment, the culture was inoculated to the BBM agar to recover. Purification started from the left upper corner. Purification steps were performed as follows: BBM, caffeine, BBM, caffeine and to the carbendazim, BBM, kanamycin,BBM.

To summarise, 20 algae strains and one cyanobacteria out of 54 strains, were classified as pure in the database (Appendix 3). Of the remaining strains, 11 strains were classified as impure, but pure enough for cultivation and for experimental use. Two strains were overgrown by contamination and therefore not capable for a cultivation. Four strains of the collection named as V208, T88, V143, and IB505 are new ones and are now under classification. Overall, there seems to be no one effective way for purification; successful purification requires much effort and several tries using different methods and is extremely time-consuming.

5.2 Biomass production using four different techniques

The aim of experiment was to show which cultivation technique produced the largest amount of fresh weight of biomass, out of four different ways of cultivation. As a test strain, the algae strain named *Prasiola sp.* (MCI-28) was selected. Before cultivation, the test strain was purified and classified as pure and transferred from a solid culture to a liquid culture. Experiments were carried out using a tubular photobioreactor, a Schott bottle, and a conical flask with CO_2 and without CO_2 as cultivation methods. All four

methods were illustrated in Figure 8. The growth of strain *Prasiola sp.* was observed and quantified by optical density (OD) detection at a wavelength of 750 nm. Initial optical density of each sample was between 0.3-0.4. At the end of the cultivation, dry mass was quantified. Cultivation experiment is shown graphically in Figure 9 ja in Figure 10. OD was measured for nine days from conical flask and from Schott bottle. From tubular photobioreactor, samples were taken for nineteen days and from conical flask treated by carbon dioxide (CO_2) for fifteen days.

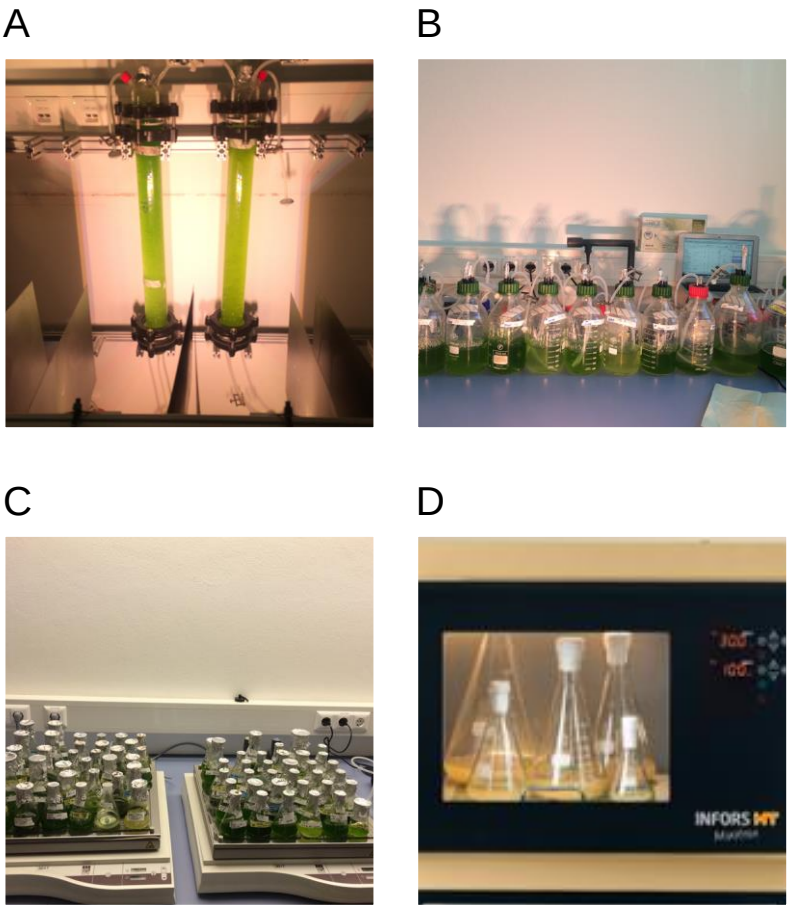


Figure 8. Four different techniques used for biomass production. Cultivation with tubular photobioreactor (TPBR) (**A**), Schott bottle (**B**), conical flask (**C**), and conical flask in the shaker,with CO_2 (1 %) (**D**).

As summarized in Figure 9 and in Figure 10, cultivation in a conical flask, with carbon dioxide (CO_2) showed the highest growth rate of the compared cultivation techniques. All other methods were cultivated without carbon dioxide (CO_2), and the same trend was observed. Respectively, biomass cultivation with tubular photobioreactor and with Schott bottle showed considerable potential and are worth noting. As the growth curves indicate (Figure 9 and Figure 10), the experiment was stopped too early, when cultivat-

ing with a Schott bottle and with a conical flask. If the experiment had lasted ten more days, the results would be more comparable. It was predictable that cultivation with carbon dioxide would be more productive than cultivation without carbon dioxide.

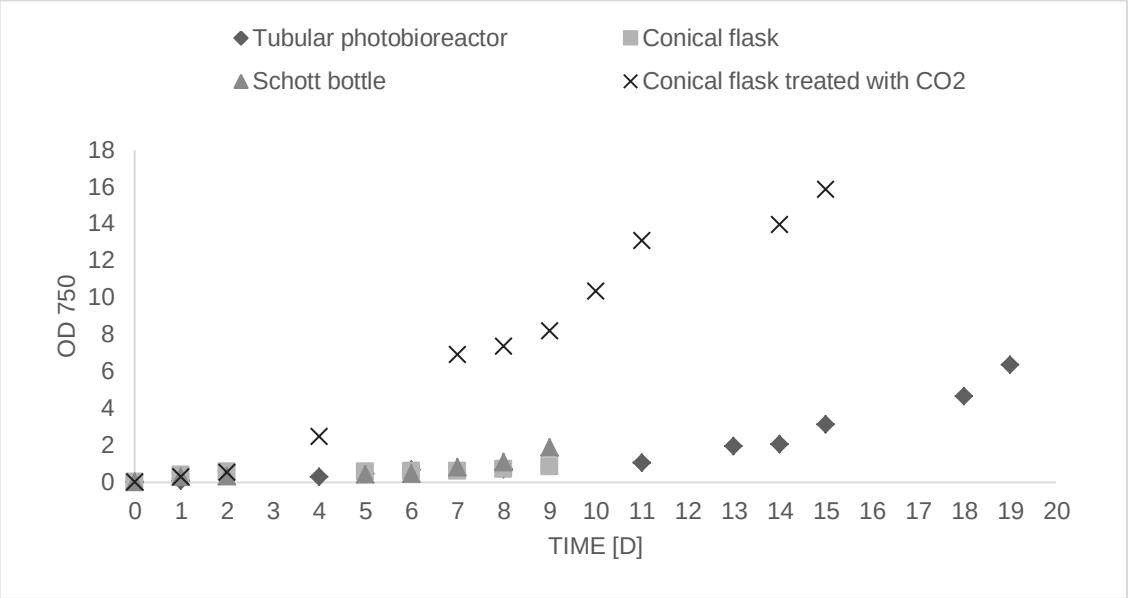


Figure 9. Four different biomass cultivation techniques plotted on linear scale. *Prasiola sp.* was used as a test strain.

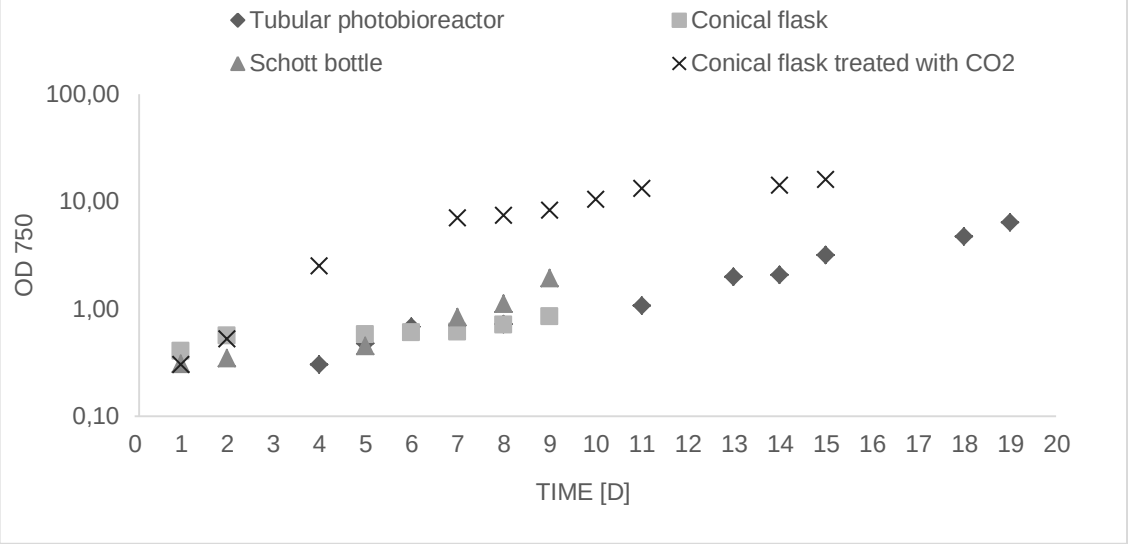


Figure 10. Growth curves for four different cultivation techniques plotted on a logarithmic scale. The linear relation proofed the exponential growth during the experience. *Prasiola sp.* was used as a test strain.

The highest RGR and the highest amount of dry mass were measured to a conical flask with the addition of CO_2. The second-best result was obtained with a Schott bot-

tle, the third best with a tubular photobioreactor, and the lowest amount of dry mass and the lowest RGR was measured with conical flask without the addition of CO_2.

Table 7. Four different cultivation methods compared with each other. Relative growth rate (RGR) and dry mass were determined, and RGR was calculated based on exponential phase of growth. For calculations, Equation (1) was used.

Cultivation method	**Relative growth rate (RGR)**	**Dry mass (g/L)**
Tubular photobioreactor	0.20	2.04
Schott bottle	0.38	0.34
Conical flask	0.17	0.20
Conical flask treated with CO_2	**0.53**	**5.29**

When reviewing the results, it should be considered that cultures were not totally homogenized when samples were taken. Especially, in a tubular photobioreactor biomass also grows on the vessel. In addition, cell size is bigger with a higher amount of illumination and due to CO_2 treatment, which can distort the results of optical density.

5.3 Cryopreservation

In this thesis, the type and concentration of cryoprotectant were tested. Commonly used cryoprotective agents such as methanol (MeOH) and dimethyl sulfoxide (Me_2SO) were used. Also, the method of cryopreservation was examined. In this experiment, cryopreservations with a liquid medium and on an agar slant were tested.

To determine the precise fraction of algae units in a culture that survive freezing/thawing stress and resume on normal growth after procedure is quite difficult. The most common method for assessment of viability is to observe the ability of individual algal units to form colonies on an agar plate. Therefore, the samples were plated on an agar after thawing, and results were assessed macroscopically. In addition, algae's ability to build fresh biomass was examined experimentally by calculating the maximal growth rate per day (Chapter 5.3.3). The growth of *Prasiola sp.* in liquid culture was observed and quantified by optical density (OD) detection at a wavelength of 750 nm. No chlorophyll or other pigments are absorbing the light at wavelength of 750 nm,

therefore, to ensure that desired particles are defined and not components that are not under focus.

The initial optical density of each sample was between 0.3-0.4. Viability was also observed macroscopically and microscopically. At the end of the experiment, dry mass was quantified from each sample and compared to the results of OD. [5]

5.3.1 Cryopreservation with liquid medium

Prasiola sp.

Freezing algae easily damages cells and results in severe loss of viability. Cells are exposed to many physical and chemical stresses such as, temperature decreasing down to a few degrees below the suspension freezing point can alter overall metabolic patterns, which are caused by differential effects of various enzyme reactions and temperature rates. Also, thawing exposes cells to the potentially lethal effects of changing electrolyte concentration and temperature effects on enzymatic rates [15]. Therefore, the two-step freezing method and the controlled thawing method were selected.

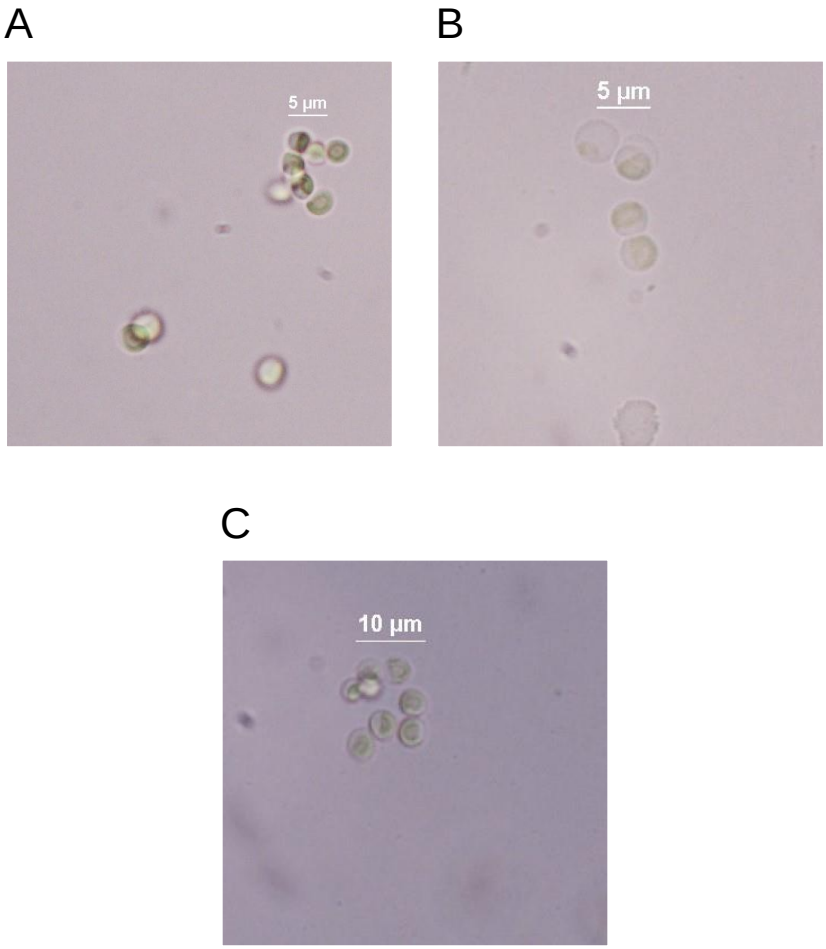


Figure 11. Microscope pictures taken after thawing to observe the viability of cryopreservation. Algae strain *Prasiola sp.* showing successful recovery from cryopreservation. Cryopreserved with liquid medium using 5 % methanol as cryoprotectant (**A**). Cryopreserved with liquid medium using 5 % dimethyl sulfoxide as cryoprotectant (**B**). Cryopreserved with liquid medium using 10 % dimethyl sulfoxide as cryoprotectant (**C**).

Microscope pictures were taken after thawing to detect the number of living cells of algae. As can be observed in Figure 11, regardless of type or concentration of cryoprotectant agent, some living cells are observed. The size or incidences of cells do not exhibit any significant differences between the methods. For satisfactory results, even one living algae unit would show the success of the method.

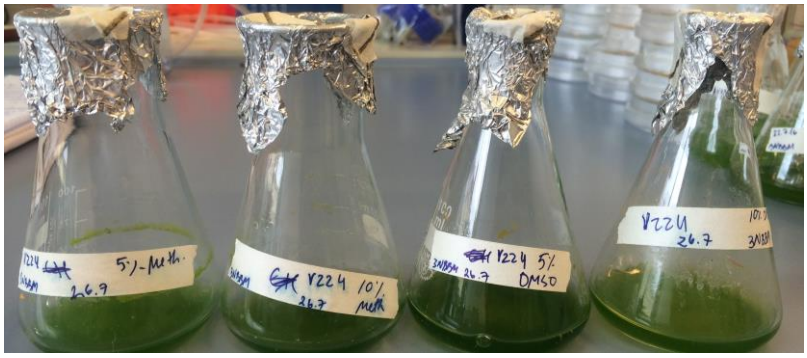


Figure 12. Recovery from cryopreservation. For all four cultures, *Prasiola sp.* was used as test strain. The first sample on the left was cryopreserved using 5 % methanol as cryoprotectant, the second with 10 % methanol, the third with 5 % DMSO and the fourth with 10 % of DMSO. All four samples were cryopreserved using the agar slant method. The picture was taken after 10 days of cultivation in liquid culture (3NBBM) after thawing.

After thawing, the thawed culture was transferred to a liquid 3NBBM to detect the ability of cells to grow actively again. As can be seen in the Figure 12, *Prasiola sp.* shown excellent ability of recover from cryopreservation. Macroscopically observed, the viability of *Prasiola sp*. is higher using DMSO as cryoprotectant, especially 5 % DMSO culture shows highest cell concentration in culture. However, the culture viability does not exhibit any significant differences between the methods; thus it seems that cells grow normally despite which cryoprotectant and concentration has been used.

Comparison of test strains *Chlorococcum hypnosporum, Prasiola sp.* and *Chloridium sp*.

Cryopreservation in a liquid culture, using as test strain *Prasiola sp.* (Chapter 5.3.2) shows high viability, but the method may be unsuitable for other strains of algae. Therefore, *Chloridium sp.* and *Chlorococcum hypnosporum* were also tested. Test strains were cryopreserved in a liquid culture and were treated with 5 % and 10 % methanol, and with 5 % and 10 % dimethyl sulfoxide (DMSO) as cryoprotectant. For comparison, 5 % methanol treated samples were chosen out of each test strain to examine the effect of cryoprotectant. Viability was assessed macroscopically.

As can be seen in Figure 13, cryopreservation was successful. For all three-test strains, the ability of cells to produce new fresh biomass was detected in liquid culture and on agar. Dark green colour in liquid cultures show high cell density and prove the cells' ability to produce biomass after thawing. On solid media, including glucose, bac-

terial contaminations were detected, but most important, no fungus contamination was observed. Earlier studies, with the same technique and with the same strains show frequent fungus contaminations and, in some cases (DMSO 5 % and 10 %) cells did not grow at all in a liquid culture. On the basis of this, the used protocol and working methods show substantial potential for storing algae strains in liquid nitrogen for the long-term maintenance.

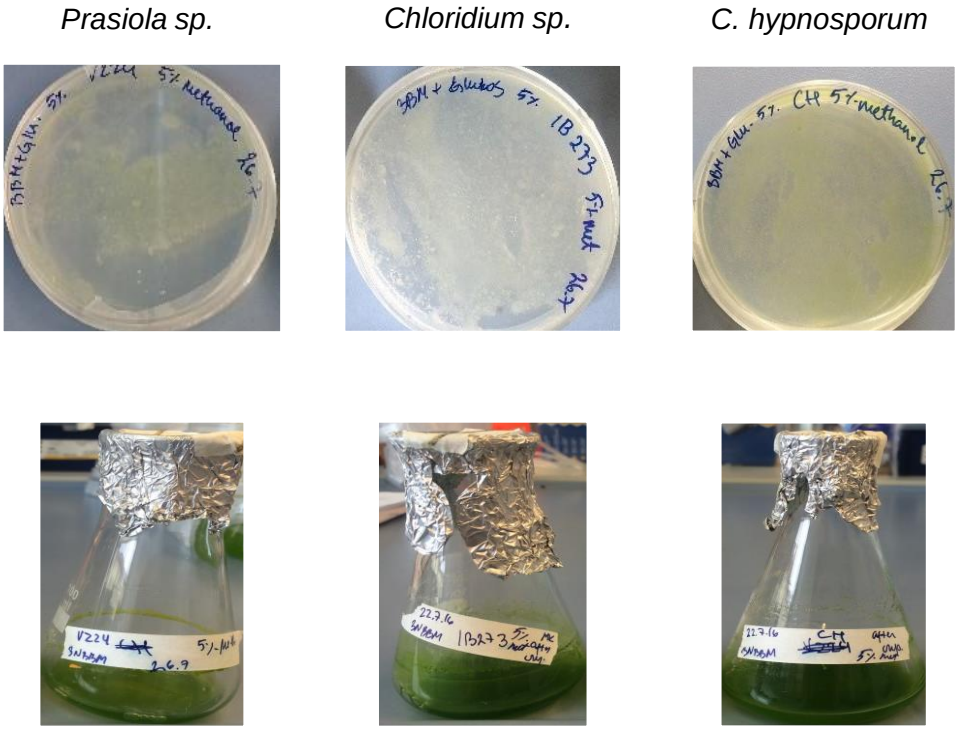


Figure 13. Successful cryopreservation. Test strains were cryopreserved with liquid culture method using 5 % (v/v) methanol as a cryoprotectant. The thawed culture was plated on BBM agar including 5 g L^{-1} glucose to observe the potential contamination. Also, thawed culture was transferred to new fresh 3NBBM to examine the algae potential to produce new biomass.

5.3.2 Cryopreservation on an agar slant

Cryopreservation on an agar slant was tested for the first time with the collection of ASIB 505. The method was selected based on the feature of species in ASIB 505 culture collection. The aero-terrestrial algae grow preferentially on solid agar which provides a desired growth medium for algae. Alternatively, the culture grows directly as a lawn, on a tiny agar slant (volume of 1 mL) which is placed inside of the cryovial, in contrast to the liquid culture method. The difference between these two methods is based on the texture of the nutrient medium and on the thawing process. The thawing

process of agar slant method is more sensitive for recovered cells than the liquid culture method where all samples were centrifuged during the washing. For the experimental reasons, to obtain numbers of loosed viable cells during the washing, washing solution were plated on agar (BBM, BBM including glucose 5 g L^{-1}) after every washing steps the results are illustrated in Figure 14 and in Figure 15.

Prasiola sp.

As seen in Figure 14, after the first wash and after the second wash, viable cell loss is significant. As seen on Sample **(A)** and on Sample **(B)**, cannot be notice that the two cultures are remarkably pure and no contaminations by bacteria or by fungus can be detected. Especially Sample **(B)** culture would be perfect for direct cell counting due to the single cell columns.

A B

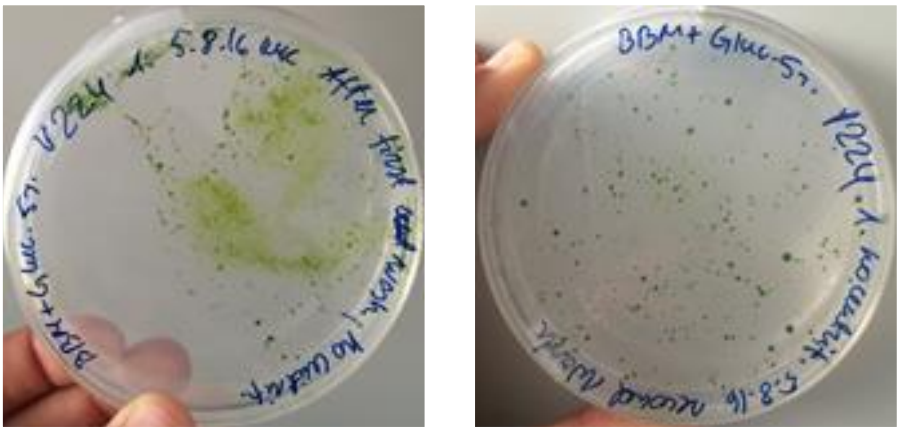


Figure 14. Picture taken after 10 days of recovery from cryopreservation using agar slant method. Sample **A** was pipetted after the first washing step and Sample **B** after the second washing step, during the thawing process. BBM agar including 5 g L^{-1} glucose was used as a growth substrate.

Comparison of *Chlorococcum hypnosporum, Prasiola sp.* and *Chloridium sp.*

Cryopreservation on an agar slant, using as test strain *Prasiola sp.* (Chapter 5.3.2) shows high viability, but the method may be unsuitable for other strains of algae. Therefore, *Chloridium sp.* and *Chlorococcum hypnosporum* were also tested. Test strains were cryopreserved on agar slant, treated with 5 % methanol as cryoprotectant.

For experimental reasons, during the thawing, the washing solution was plated on solid agar to observe the possible wastage of viable cells. As can been seen in Figure 15 (first row), in the second wash solution there was a significant number of viable cells. In addition, these culture plates would be perfect for cell counting due to single cell columns. The second and third row on Figure 15 shows the ability of cells to grow actively after cryopreservation on an agar slant. In these cases, biomass was inoculated after the recovery phase from agar slant. Even though algae were transferred on fresh sterile organic agar after thawing, overgrowth by contaminants was avoided. As seen on Figure 15, the purity of cultures was excellent, the *Chloridium sp.* and *Chlorococcum hypnosporum* (*C. Hypnosporum*) strains showed some bacterial contaminations, but most important, the cultures were fungus free.

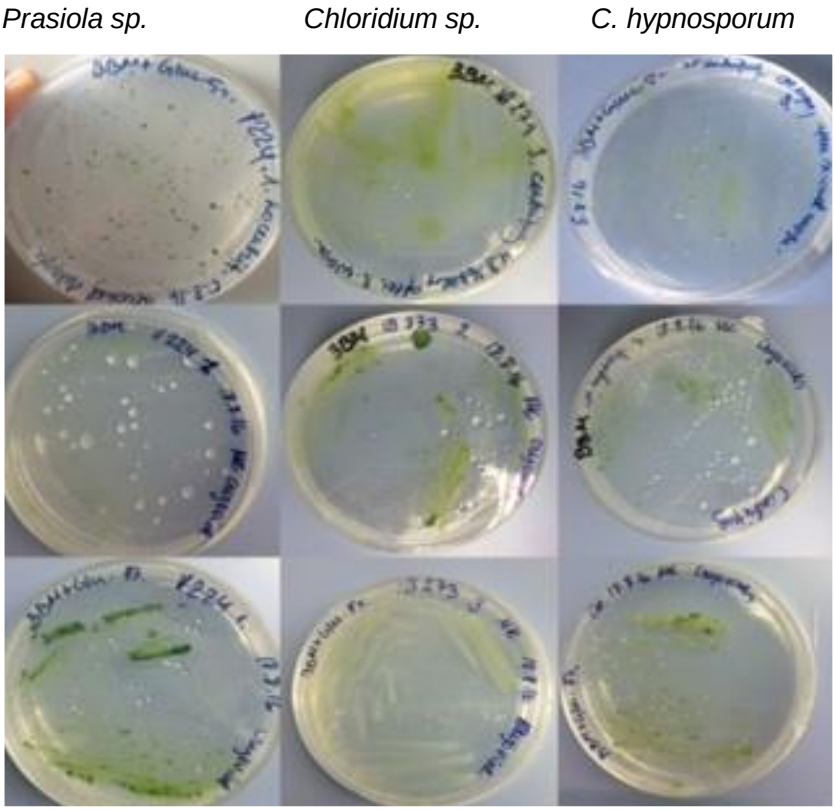


Figure 15. Pictures taken after 10 days of thawing when the cryopreservation method with an agar slant technique was performed. On the first row on top, samples are transferred on BBM agar plate including 5 g L^{-1} glucose, after the second washing step during the thawing process. The second row samples are from cryovial agar slant after sample have had 9 days' recovery after thawing, as a growth substrate BBM agar has used. The third row samples are the same as second row samples but the growth substrate includes 5 g L^{-1} glucose to support the recovery period and to detect possible contamination.

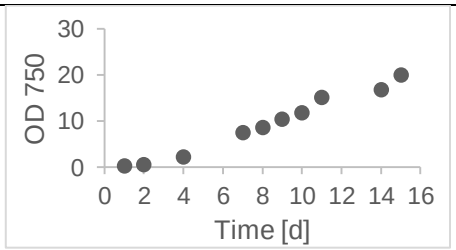
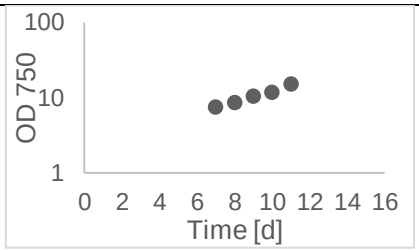
5.3.3 Determine viability by growth rate method

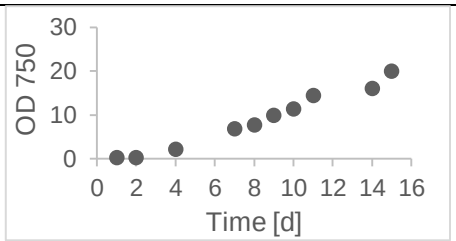
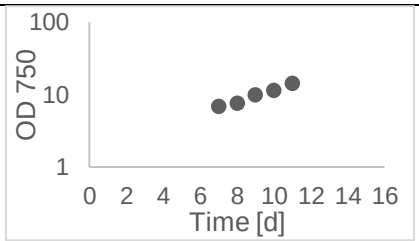
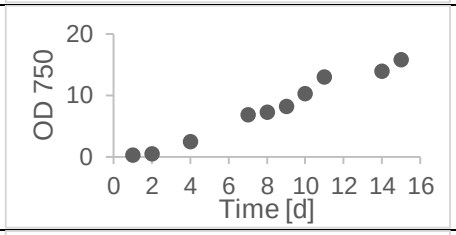
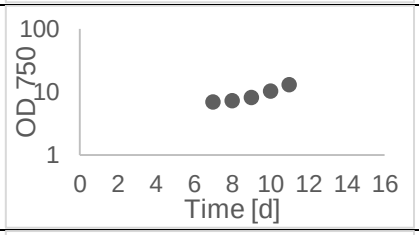
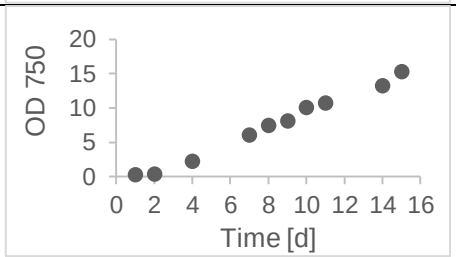
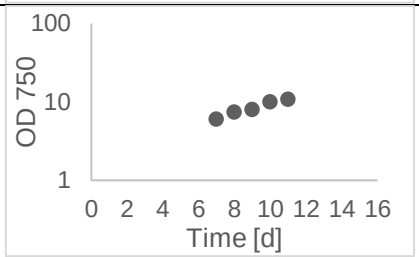
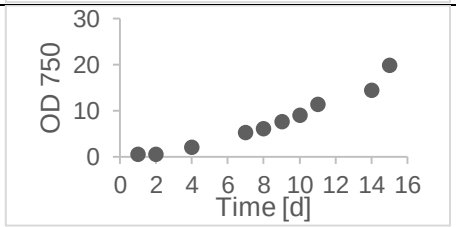
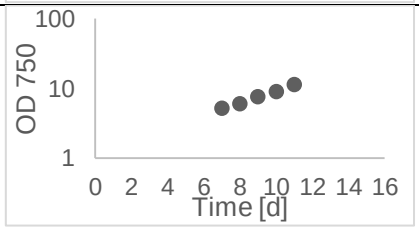
To examine the ability of cells to actively produce a new fresh biomass, after combined stress of freezing/thawing, a recovery growth test was performed. Since all three test strains showed similarly results after thawing, *Prasiola sp.* test strain was chosen for the growth test. Further, for all samples, 5 % methanol was chosen as a cryoprotectant agent.

The viability of the strains was examined by cultivating biomass in a liquid culture over fifteen days, during the cultivation optical density (OD) was measured and relative growth rate (RGR) calculated (1).

As positive control, a non-cryopreserved *Prasiola sp.* strain (from same pre-culture as test strains) was cultivated in the same way as cryopreserved samples after a recovery period (10 mL volume of 3NMMB in CO_2 shaker). Samples were diluted, with minimal medium to increase the optical density to 0.3. After the growth test, dry weight of each sample was quantified and compared to each other as shown in Table 8.

Table 8. Recovery growth test results. Four samples were cryopreserved using two different freezing protocols, cryopreservation with a liquid media (1)(2) and cryopreservation on an agar slant (4)(5). Both methods were compared to the positive control (3). As the cryoprotectant agent, 5 % methanol and 5 % DMSO were used. As a test strain *Prasiola sp.* was used.

	Samples are cryopreserved as follows (except positive control).	**OD measurement results over 15 days were plotted on linear scale.**	**Slope present five consecutive days' results of measurements in logarithmic scale. Based on these measurements, the maximal growth rate per day was calculated.**	**Results: μ [1/d], dry mass = m [g/L]**
1	In liquid culture, treated with 5 % methanol			μ = 0.18 R^2 = 0.98 m = 5.43

2	In liquid culture, treated with 5 % DMSO			μ = 0.19 R^2 = 0.99 m = 5.86
3	Positive control, **non-cryopreserved**, cultivated in liquid BBM			μ = 0.16 R^2 = 0.94 m= 5.29
4	On agar slant, treated with 5 % methanol			μ = 0.14 R^2 = 0.97 m = 5.29
5	On agar slant, treated with 5 % DMSO			μ = **0.20** R^2 = 0.99 m = **6.96**

The aim of the experiment was to detect the ability of cells to actively grow again after cryopreservation and compare the results to those for the positive control. Surprisingly, the results met the target, and the cells' ability to divide remained unchangeable. As can been seen in Table 8, all samples were grown almost identically, regardless of used cryopreserved method and regardless of used cryoprotectant agent with different concentrations. The highest obtained biomass was 6.96 g L^{-1}; it was obtained from Sample 5. Also, the highest growth rate, 0.20 1/d was observed for the same sample. Nevertheless, the differences between Sample 5 and the other samples were too small to prove that the agar slant method, using 5 % DMSO would be the most optimal method for microalgae cryopreservation. Secondly, after cryopreservation, the interest was focused on lag-phase to detect how long it would take for a culture to start grow again. As seen in Table 8, the lag phase of all four cultures was same as it was with the positive control.

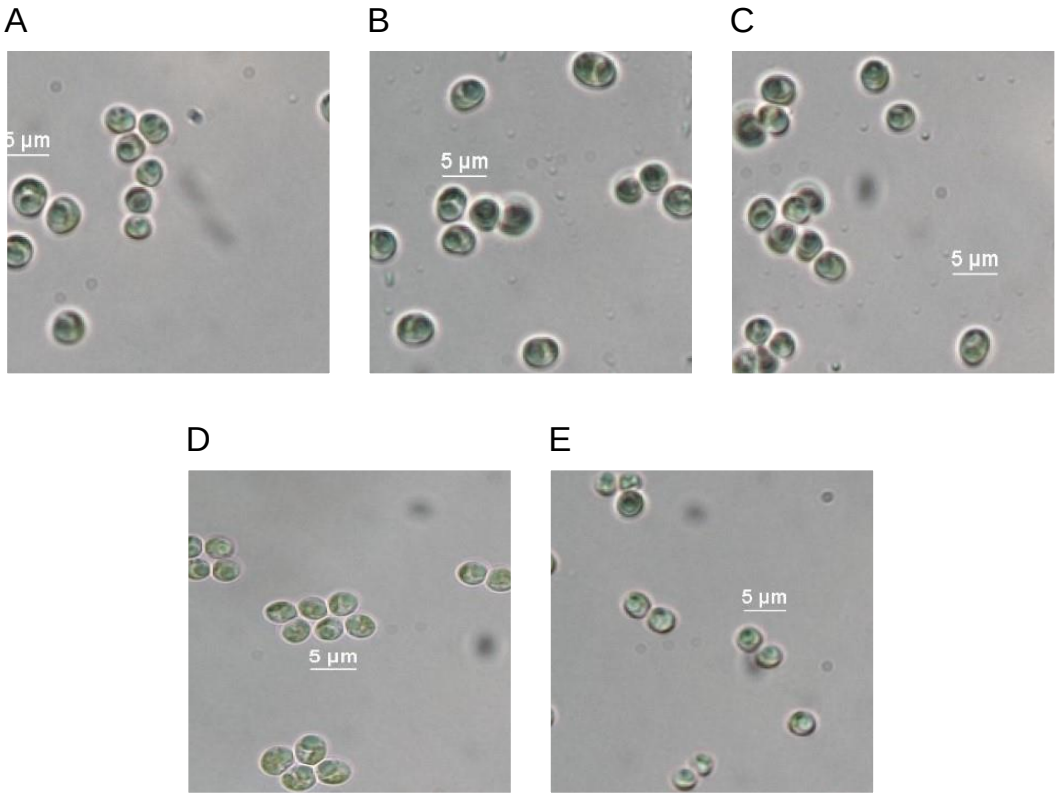


Figure 16.Microscope pictures taken after growth screening test. Samples **A** and **B** are cryopreserved using liquid culture method. Picture **C** was taken from a positive control. Pictures **D** and **E** depict samples that are cryopreserved on an agar slant. For all samples, 5 % methanol was used as a cryoprotectant (except for the positive control).

At the end of the experiment, microscope pictures were taken to observe the cell growth phase and the size of the cells in each sample. As seen on Figure 16, at the end of the experiment, every sample cell was still in an exponential phase (cell division were observed). This means that those cultures would have had high potential to increase cell density (higher OD) and correspondingly produce more biomass, if the experiment had not been stopped. In addition, it was detected that for some reason, cryopreserved cells with agar slant were more “organized” (Figure 16) than cells which were cryopreserved using liquid medium.

6 Discussion

6.1 MCI ASIB 505 collection purification

At the beginning, the aim of purification was to purify collection, as axenic. During the purification, it was noticed that getting axenic cultures was not truly possible. For some strains, bacteria play an important role and for some strains symbiosis relationship with to fungi is vital. Therefore, strains were purified as a unialgal and as a unicyanobacterial. Thus, unialgal and unicyanobacterial became the targets of purification instead of axenic.

Several purification methods were performed (Chapter 4.1) to purify the ASIB 505 collection. First of all, mechanical separation as antibiotic-free approach was carried out for all strains of the collection. As reported by Droop (1967), mechanical separation method was preferred over antibiotic-based treatments due to negative impact of chemicals to microalgae and cyanobacteria. For example, *Chlorococcum hypnosporum*, *Chloroidium sp.*, *Hormidiospora verrucosa* mechanical separation was sufficient to obtain unialgal culture without using chemicals. Antibiotic and antimycotic treatment was carried out only when the mechanical separation method appeared unsuccessful. Within this thesis, antibiotic treatment was used for the most part of collection due to the high contamination rates. However, the aim was to treat species as little as possible with different chemicals in order to maintain the genetic stability.

Brown (1964) reported that caffeine at concentration of 0.01-0.03 M would inhibit fungi and protozoa. Due to that, caffeine (10mM and 20mM) were used in agar medium to repress fungi. Caffeine is a non-synthetic antifungal agent and act as a metabolic inhibitor, as do antibiotics [5]. In addition, some species of bacteria use caffeine as a major source of carbon as nutrient (*Pseudomonas putida*), while others are inhibited in the presence of caffeine [16]. In this thesis, caffeine as antimycotic was a success in inhibiting contamination. However, for some species caffeine inhibits also algae; one of these species was named as BS110. The purification steps were illustrated in figure 7 in Chapter 5.1. As second antifungal agent, carbendazime, was used to purify cultures from contamination. Carnendazime belong to the benzimidazole family, and they have a trait to bind to microtubules and to interfere with cell division and transport. UTEX (University of Texas at Austin) has reported that carbendazime is a successful method to clean up contamination and also an inexpensive method. For heavy contaminated cultures, UTEX researchers repeated the carbendazime treatment two to three times

and used fungicides as a preventative measure [17]. Like caffeine, also carbendazim was discovered to be a successful method to use for ASIB 505 culture collection. Several contaminated cultures were effectively purified. The potential keys for success were fast culture inoculation from selective culture to new fresh agar media to avoid a toxicity hood by antimycotic and sterile working methods. It should also be noted that in earlier trials, using an antimycotic as an antifungus agent was ineffective.

For the cyanobacteria strain, antibiotic-free approach has been carried out to purify cultures without harming the strains morphological and physiological features. As an antibiotic-free method, BBM without a nitrogen source was used as growth medium.

There is no single method applicable for every species. Different algae will probably be contaminated with a wide range of bacteria and fungi, each of which will differ in sensitivity to the various chemical agents. In addition, it is necessary to experiment with different concentrations and types of chemicals; however, it should be noted that they must be selectively toxic. At the same time, it should be noted in mind that every chemical treatment can change the species' morphological and physiological features.

6.2 Cryopreservation as a high potential technique for a long-term storage

Cryopreservation of eukaryotic microalgae and cyanobacteria are already routinely applied into microalgae culture collection management, for example UTEX Culture Collection of Algae in Texas, USA use cryopreservation as long-term storage method [13]. Range of methods were reported such as rapid, one-step, two-step, encapsulation-dehydration, verification, high pressure freezing (novel technique) and mixed methods [7]. Two-step cooling technique which allows successful recovery with a viability of >50 %, was chosen for this thesis. According to G. J. Morris. (1978), the method was unsatisfactory with only 32 strains out of 252 freezed microalgae strains. In addition, this method was adopted because of the simplicity and low concentration of needed cryoprotective agent [18]. In this thesis, the two-step freezing method was successful with all three test strains regardless the used freezing protocol. The results in Table 8 show no difference in recovery of cell concentration of all samples regardless of the used method.

As a cryoprotectant agent, several cryoprotectants were reported such as impermeable cryoprotectants glycerol and sucrose and permeable cryoprotectants as dimethyl sulfoxide (DMSO) and methanol. Glycerol is often used for bacterial cryopreservation, but

for algae, it has been shown ineffective in many instances [19]. Sucrose, again, works extracellularly by competing for water molecules, in turn, dehydrating the cell. Methanol and dimethyl sulfoxide can freely permeate the cell membrane and provide protection against cryo-injury. When used in combination with intracellular cryoprotectants and extracellular cryoprotectants, increased success in cryopreservation can be achieved. Latest studies have reported that DMSO has been shown to be more successful than methanol; methanol causes algae cells to become more light-sensitive than DMSO [7]. In this thesis methanol and dimethyl sulfoxide were examined and the results showed that both cryoprotective agents were successful with chosen test strains and did not significantly affect cell concentration. In the future, it would be interesting to test sucrose as a cryoprotectant before freezing or adding sucrose to the recovery medium. Previous studies have showed that the colony size was much larger using sucrose, but at the same time no difference was detected in the recovery phase. Although the combination of DMSO (10 %) and sucrose (200 mM) yielded higher cell viabilities for all strains tested, some strains exhibited lower viability compared to others. It has been suggested that the effectiveness of sucrose or other sugar would depend on the ability of microalgae to utilise them as heterotrophic growth substrates. [7]

Two freezing protocols were employed, using two different growth agents: the first protocol was for strains that grow preferentially on an agar slant, and the second was for strains that grow preferentially in a liquid culture. Both methods are routinely used in UTEX Culture Collection of Algae in Texas, USA. The three microalgae species, *Prasiola sp.* (MCI-28), *Chlorococcum hypnosporum* (MCI-35) *and Chloroidium sp*. (MCI-12) were randomly chosen as test strains. For recovery tests *Prasiola sp.* was chosen. Both protocols were described in chapter 4.4.5 and were carried out during this thesis. The ability of recovery was determined based on strain's ability to actively produce new biomass after freezing and thawing.

Results indicated that these three-species survived both freezing protocols and that the cells' ability of building new biomass was excellent. However, the agar slant method, especially when cryopreserved with 5 % DMSO as a cryoprotectant, yielded a higher cell viability and showed that the cells were able to produce biomass with less contamination after thawing (Table 8 and Figure 14). By comparing the results of growth rate tests, cells' activity to build new fresh biomass was the same, regardless of used protocols. Furthermore, when the results were compared to a positive control, it was observed that the ability of build new fresh biomass had remained unchanged. Due to the

remarkable ability of recovery, the culture purity level and the simplicity of the method, cryopreservation using an agar slant protocol is applicable to a board range of microalgae (MCI ASIB 505); nevertheless, it is not perfect and there are still unknown optimisation and variables which may yield further improvements in cell viability. In addition, more species should be tested to be assured of method viability. It is necessary for further studies to notice that all species may not survive from radical growth agent changes. The results of both freezing protocols were significant, comparing results of earlier studies with the same culture collection. Especially, contamination was the biggest issue which affected a culture so much that the microalgae cells lost the activity to grow again [20]. Presumably, the potential key for success was careful and notably sterile working methods. In addition, some changes to earlier used protocols were made.

In addition, the future aim would be to test the possibility of freezing the same culture again after samples were thawed once. As observed in results, at every washing step potential biomass was removed by washing media; therefore, a washing solution, which would allow to freeze the sample again (biomass on agar slant are intact) would be utilized for co-cultures. Using this method, labour costs can be notably minimized and time saved.

6.3 Most productive way to produce biomass

Growth tests were performed using four potential cultivation methods, cultivating with a tubular photobioreactor, with a Schott bottle and with a conical flask without CO_2 and with CO_2. These methods were defined as a closed (or mostly closed) vessel for phototrophic production where energy is supplied via electric light. The aim is to use cultivated biomass to produce commercial product, either an extract or cells. Algae biomass production requires a biomass that is cost effective and consistent in quality from batch to batch. Several conditions affect the yield of biomass such as light, nutrients, circulation, gas exchange, sterilization, temperature, and pH. Light is the most important parameter in the design and construction of a vessel. Light can be supplied continually or in light-darks cycles. In addition, the presence of CO_2 support algae biomass production and is consequently an important factor take into account [5]. As summarized in Figure 9 and in Figure 10, cultivation in conical flask, with carbon dioxide (CO_2), shows the highest growth curve out of four tested cultivation methods. It was predicted that cultivation with carbon dioxide would be more productive that cultivation without carbon dioxide, but such a significant difference as observed in Figure 9 and in Figure 10 was

unexpected. On the basis of the results, it can be inferred that the presence of CO_2 for cultivation is important. Despite the results, it is an important to carry out further growth tests to ensure the reliability of the results. As mentioned in chapter 4.3. cultivation tests were stopped too early to have reliable results.

The Schott flask turned out in cultivation test as a potential vessel for biomass production. As for method simplicity, needed space and low costs, the Schott flask has all these advantages compared to a tubular photobioreactor. For laboratory use, the Schott flask as a cultivation method has substantial; nonetheless, results can be different when lifting the cultivation volume is necessary.

In summary, when the cultivation target is based more on quantity than quality, cultivation with CO_2 will be the best alternative. Rather, if the aim of the cultivation is more to analyze and make experimental stress tests, cultivation with tubular photobioreactor would be a more optimal choice. Cultivation with tubular photobioreactor showed to be more stable based on the standard growth curve, where lag phase, log phase (exponential), stationary phase and death phase are showed. As mentioned earlier, the experiment of algae biomass cultivation was stopped too early to have reliable results, and it would be important to make more growth tests to ensure the reliability of the results.

6.4 Financial saving

Compared to the current method of maintenance and sub-culturing, cryopreservation decreases the laboratory costs and is an effective method for maintenance a large culture collection over a long period with high stability. The MCI ASIB 505 collection with 54 species is definitely not the largest possible collection; nonetheless, the maintenance of the collection needs much effort from personnel and are costly; therefore, cryopreservation would be an excellent alternative for long-term storage. In addition, all species are not equally valuably, which means that without cryopreservation it would not be conceivable to maintain low-value species that are not valuable now but can be in the future.

For instance, it took one month to purify and transfer the MCI ASIB 505 collection to the new minimal nutrient media. The optimal interval of transfer is one time per year or every six months, depending on the collection. That means that for every strain, an average of 6 or 3 workhours are needed. For a collection of 1000 species, it means

3000 workhours per year, or if the transfer interval is every six months, then 6000 workhours are needed. It is clear that the maintenance of an actively metabolizing culture collection is very time-consuming and costly, which causes significant limitations to the maintenance of large collections including low-value strains. In the future, the aim would be to store the MCI ASIB 505 collection for a long term, using cryopreservation as a storage method.

7 Reference

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Appendix 1. Cryopreservation viability test

Table 1. Four samples are cryopreserved using two different freezing protocol, cryopreservation with a liquid media and cryopreservation on an agar slant. Both methods were compared to the positive control. As a cryoprotect agent, 5 % methanol has been used. As a test strain *Prasiola sp.* was used.

Days → Method ↓	**1**	**2**	**4**	**7**	**8**	**9**	**10**	**11**	**14**	**15**
In liquid culture. treated with 5 % methanol	0.34	0.57	2.19	7.53	8.64	10.42	11.74	15.18	16.74	20.00
In liquid culture. treated with 5 % DMSO	0.31	0.36	2.31	6.92	7.75	10.02	11.46	14.44	16.18	20.00
Positive control, non-cryopreserved, cultivated in liquid BBM	0.30	0.52	2.48	6.91	7.34	8.18	10.34	13.06	13.94	15.86
On agar slant, treated with 5 % methanol	0.32	0.37	2.24	6.09	7.51	8.11	10.14	10.78	13.24	15.30
On agar slant, treated with 5 % DMSO	0.52	0.52	2.06	5.24	6.08	7.6	9.06	11.38	14.44	19.88

Table 2. After cryopreservation, produced biomass was determined by dry mass measurement, at the end of the growth test.

Sample	**Dry mass (g) per 7 ml**	**Dry mass (g) per 1000 ml**	**Time (min)**
In liquid culture, treated with 5 % methanol	0.038	5.43	19
In liquid culture, treated with 5 % DMSO	0.041	5.86	19
Positive control. non-cryopreserved, cultivated in liquid BBM	0.037	5.29	20
On an agar slant, treated with 5 % methanol	0.037	5.29	20
On an agar slant, treated with 5 % DMSO	0.049	7.00	21

Appendix 2. Data of biomass growth-test using four different cultivation techniques

Table 1. The growth of *Prasiola sp*. was observed and quantified by optical density (OD) detection at a wavelength of 750 nm. Four different biomass cultivation methods have been examined in period 12.8.16-2.9.16.

Days → Method ↓	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**15**	**18**	**19**
Tubular photobio-reactor	0.1	-	0.3	0.5	0.7	-	0.7	-	-	1.1	-	2	3.1	4.7	6.3
Schott bottle	0.3	0.3	-	0.4	0.5	0.8	1.1	1.9	-	-	-	-	-	-	-
Conical flask	0.4	0.6	-	0.6	0.6	0.6	0.7	0.8	-	-	-	-	-	-	-
Conical flask CO_2	0.3	0.5	2.5	-	-	6.9	7.3	8.2	10.3	13.1	14	16	-	-	-

Table 2. Four different biomass cultivation methods have been examined using test strain *Prasiola sp.* and produced biomass have been determined by dry mass measurement.

Biomass cultivation method	**Dry mass (g) per 1000 ml**	**Time (min)**
Tubular photobioreactor	2.04	11
Schott bottle	0.46	12
Conical flask	0.17	10
Conical flask with CO_2	0.37	12

Appendix 3. Database of MCI ASIB 505 culture collection

Database was created during the purification process and during the biomass cultivation.

Table 1. Database MCI ASIB 505

Strain id.	**Collec-tionsnumber**	**Original characterisation**	**Purity before**	**Purity now**	**Information**
MCI-1	T1	*Tolypothrix*			Cyanobacteria
MCI-2	T3	*Nostoc punctiforme*	pure	pure	Cyanobacteria
MCI-3	T4	*Nostoc verrucosum*			Cyanobacteria
MCI-4	T6	*Schizothrix sp.*		unpure	Cyanobacteria
MCI-5	T7	*Schizothrix sp.*	fungus free	fungus free	Cyanobacteria
MCI-6	V152	*Schizothrix sp.*		pure	Slow to cultivate
MCI-7	BS319	*Coccomyxa brevis*		fungus free	Symbiotic with bacteria?
MCI-8	BS775	*Pseudochlorella subsphaerica*	fungus free	fungus free	Slow to cultivate
MCI-9	IB142	*Unknown*			Purity better in liquid culture
MCI-10	IB149	*Unknown*			Was hardly contaminated, one green ball inside of medium. Treated with antibiotic and with antimycotic mix.
MCI-11	IB256	*Unknown*	fungus free	pure	Fast to cultivate
MCI-12	IB273	*Chloroidium sp.*	pure	pure	Fast to cultivate, **cryopreserved**
MCI-13	IB410	*Unknown*	fungus free	pure	Fast to cultivate, amino acid rich
MCI-14	IB459	*Scotiellopsis sp.*		pure	Fast to cultivate
MCI-15	IB472	*Chlamydomonas octigama*		unpure	Fast to cultivate in liquid and on agar. Antibiotics and antimycotics don't worked with this strain.
MCI-16	SAG 79.80	*Ettlia texensis*		pure	Extremely slow to cultivate
MCI-17	SAG 213-2a	*Macrochloris radiosa*			Extremely slow to cultivate on liquid or/and on agar
MCI-18	T61	*Spongiochloris sp.*	pure	pure	Fast to cultivate
MCI-19	V103	*Klebsormidium dissectum*			Grow very fast in liquid culture, but do not grow on agar
MCI-20	V111	*Heterococcus versiculosus*		fungus free	Was hardly contaminated. Grow much better in liquid culture than on agar.
MCI-21	V142	*Ettila bilobata*		pure	Good for up-scale
MCI-22	V168	*Tetracystis cf. Tetraspora*			Strain was over growth by contami-nation, botanic back-up was used to recover the strain.
MCI-23	V195	*Scotiellopsis rubescens*		fungus free	Fast to cultivate
MCI-24	V199	*Hormidiospora verrucosa*		pure	Fast to cultivate
MCI-25	V204	*Chloromonas rosae*		pure	Fast to cultivate
MCI-26	V21	*Unknow*			Grow fast in liquid culture but slow on agar.

MCI-27	V219	*Diplosphaera chordatii*	pure	pure	Fast to cultivate
MCI-28	V224	*Prasiola sp.*	pure	pure	MAA production, colour change to yellow with stress factor. Fast to cultivate. **Cryopreserved**
MCI-29	V24	*Unknow*			Biomass grow fast
MCI-30	V39	*Muriella terrestris*	pure	pure	
MCI-31	V46	*Chlamydomonas culleus*	pure	pure	Canthaxanthin
MCI-32	V50	*Chlamydomonas peterfii*		unknow	Strain founded in August
MCI-33	V6	*Pleurochloris meiringensis*			Very contaminated, botanic back-up used for new plates.
MCI-34	CV	*Chlorella vulgaris*	pure	pure	Slow to cultivate
MCI-35	CH	*Chlorococcum hypnosporum*	pure	pure	Fast to cultivate, interesting strain, **cryopreserved**
MCI-36	BS110	*Unknown*			Not the slowest but not the fastest to build biomass.
MCI-37	BS349	*Unknown*			lost
MCI-38	IB145	*Unknown*			lost
MCI-39	IB505	*Unknown*			New one
MCI-40	-	-	-	-	-
MCI-41	SAG379.1 C	*Unknown*			Biomass grow very slow
MCI-42	IB423	*Unknown*			Contaminated strain. Nothing worked out to purify this strain.
MCI-43	IB565	*Unknown*			Was overgrowth by contamination. Botanic back-up will be needed.
MCI-44	IB514	*Unknown*		pure	Very good strain, fast to cultivate.
MCI-45	E71.10	*Unknown*			Culture was overgrowth with contamination. Botanic back-up used for new cultures and now plates look very good.
MCI-46	-	-		-	-
MCI-47	IB408	*Unknown*		pure	Very good strain to up-scale, grow fast.
MCI-48	IB407	*Unknown*		pure	Very good strain to up-scale, grow fast.
MCI-49	T87	*Unknown*		pure	Was very contaminated, treated with antibiotic and with antimycotic
MCI-50	T90	*Unknown*		unpure	Any purification method does not work with this strain.
MCI-51	T91	*Unknown*		fungus free	Terrestrial, grow on glass and on bottom of conical flask. Biomass production ok in liquid culture but not on agar.
MCI-52	BS351	*Unknown*		unpure	Biomass grow good, but not pure.
MCI-53	BS363	*Unknown*		fungus free	Cyanobacteria, after cell lysis biomass turned to orange.
MCI-54	V143	*Unknown*		fungus free	Got on August, grow fast, potential for up-scaling
MCI-55	V208	*Unknown*			Got on August, new one
MCI-56	T88	*Unknown*		fungus free	Got on August, carbendazim treatment