Production, purification and analysis of novel peptide antibiotics from terrestrial cyanobacteria

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Abstract

Cyanobacteria are a known source for bioactive compounds, of which several also show antibiotic activity. In regard to the growing number of multi-resistant pathogens, the search for novel antibiotic substances is of great importance and unexploited sources should be explored. So, this thesis initially dealt with the identification of productive strains, especially within the group of the terrestrial cyanobacteria, which are less well studied than marine and freshwater strains. Amongst these, *Chroococcidiopsis cubana*, an extremely desiccation and radiation tolerant, unicellular cyanobacterium was found to produce an extracellular antimicrobial metabolite effective against the Gram-positive indicator bacterium *Micrococcus luteus* as well as the pathogenic yeast *Candida auris*. However, as the sole identification of a productive cyanobacterium is not sufficient for further analysis and a future production scale-up, the second part of this thesis targeted the identification of compound synthesis prerequisites. As a result, a limitation of nitrogen was shown to be the production trigger, a finding that was used for the establishment of a continuous production system. The increased compound formation was then used for purification and analysis steps. As a second approach, *in silico* identified bacteriocin gene clusters from *C. cubana* were cloned and heterologously expressed in *Escherichia coli*. By this, the bacteriocin B135CC was identified as a strong bacteriolytic agent, active predominantly against the Gram-positive strains *Staphylococcus aureus* and *Mycobacterium phlei*. The peptide showed no cytotoxic effects against mouse neuroblastoma (N2a-) cells and a high temperature tolerance up to 60 °C. In order to facilitate the whole project, two standard protocols, specifically adapted for the work with cyanobacteria, were established. First, a method for a quick and easy *in vivo* vitality estimation of phototrophic cells and second, an approach for a high throughput determination of nitrate concentrations in microalgal cultures. Both methods greatly helped to proceed the main objectives of this work, the first one by simplifying the development of suitable cryopreservation protocols for individual cyanobacteria strains and the second one by accelerating the determination of the optimal nitrate concentration for the production of the antimicrobial compound from *C. cubana*. In the course of this cultivation optimization, the ability of cyanobacteria to utilize organic carbon sources for an accelerated cell growth was examined in greater detail. It could be shown that *C. cubana* reaches significantly higher growth rates when mixotrophically cultivated with fructose or glucose. Interestingly, this effect was even further enhanced when light intensity was decreased. Under these low-light conditions, phototrophically cultivated *C. cubana* cells showed a clearly decreased cell growth. This effect might be extremely useful for a quick and economic preparation of precultures.

Kurzzusammenfassung

Cyanobakterien sind als Produzenten bioaktiver Verbindungen bekannt, von denen viele auch antibiotische Wirkung zeigen. Im Hinblick auf die wachsende Zahl multiresistenter Krankheitserreger ist die Suche nach neuartigen antibiotischen Substanzen von großer Bedeutung und unerschlossene Quellen sollten erkundet werden. Daher befasste sich diese Arbeit zunächst mit der Identifizierung produktiver Stämme, insbesondere aus der Gruppe der terrestrischen Cyanobakterien, die weniger gut untersucht sind als marine und Süßwasser-Stämme. Die Arbeiten zeigen, dass *Chroococcidiopsis cubana*, ein besonders austrocknungs- und strahlungsresistentes, einzelliges Cyanobakterium, eine extrazelluläre antimikrobielle Verbindung produziert, die sowohl gegen das Gram-positive Indikatorbakterium *Micrococcus luteus* als auch gegen den pathogenen Hefepilz *Candida auris* wirkt. Da jedoch die alleinige Identifizierung eines produktiven Cyanobakteriums für weitere Analysen und ein anschließendes *scale-up* nicht ausreichen, beschäftigte sich der zweite Teil dieser Arbeit mit den Synthesevoraussetzungen für die Verbindung. Dabei konnte eine Stickstofflimitierung als Produktionsauslöser nachgewiesen werden, eine Erkenntnis, die für die Etablierung eines kontinuierlichen Produktionssystems genutzt wurde. Die daraus resultierende höhere Substanzausbeute wurde für anschließende Reinigungs- und Analyseschritte genutzt. In einem zweiten Ansatz wurden durch *in silico* Analysen identifizierte Bacteriocin-Gencluster aus *C. cubana* kloniert und heterolog in *Escherichia coli* exprimiert. Auf diese Weise wurde das Bacteriocin B135CC als stark bakteriolytisches Peptid identifiziert, das vor allem gegen die Gram-positiven Stämme *Staphylococcus aureus* und *Mycobacterium phlei* wirkt. B135CC zeigte keine zytotoxische Wirkung gegenüber Maus-Neuroblastom-Zellen (N2a) und eine hohe Temperaturtoleranz bis zu 60 °C. Es wurden außerdem zwei Standardprotokolle entwickelt, die speziell für die Arbeit mit Cyanobakterien angepasst wurden. Zum einen eine Methode zur schnellen und einfachen *in vivo*-Vitalitätsmessung phototropher Zellen, um geeignete Kryokonservierungsprotokolle für Cyanobakterien entwickeln zu können, und zum anderen eine Hochdurchsatzmethode zur Bestimmung von Nitratkonzentration in Mikroalgenkulturen. Diese wurde zur Optimierung der Produktionsbedingungen mit *C. cubana* verwendet. Im Zuge dessen wurde auch die Fähigkeit der Cyanobakterien, organische Kohlenstoffquellen für ein beschleunigtes Zellwachstum zu nutzen, näher untersucht. Es konnte gezeigt werden, dass *C. cubana* deutlich höhere Wachstumsraten erreicht, wenn es mit Fructose oder Glucose kultiviert wird. Interessanterweise wurde dieser Effekt sogar noch verstärkt, wenn die Lichtintensität verringert wurde. Unter diesen lichtarmen Bedingungen zeigten phototroph kultivierte *C. cubana*-Zellen dagegen ein

deutlich verringertes Zellwachstum. Dieser Effekt könnte für eine schnelle und wirtschaftliche Anzucht von Vorkulturen genutzt werden.

List of Publications

Peer-reviewed:

M. Witthohn, A. Schwarz, J. Walther, D. Strieth, R. Ulber, K. Muffler (2020a). Novel method enabling a rapid vitality determination of cyanobacteria. Eng Life Sci, 20:580-584.

M. Witthohn, D. Strieth, S. Eggert, S. Kins, R. Ulber, K. Muffler (2021). Heterologous production of a cyanobacterial bacteriocin with potent antibacterial activity. CRBIOT, 3:281-287.

M Witthohn, D Strieth, J Kollmen, A Schwarz, R Ulber, K Muffler (2022). Process Technologies of Cyanobacteria. In: Cyanobacteria in Biotechnology: Applications and Quantitative Perspectives 303-352.

M. Witthohn, A.-K. Schmidt, D. Strieth, R. Ulber, K. Muffler (2022b). A modified method for a fast and economic determination of nitrate concentrations in microalgal cultures. Algal Res, 69:1-13.

M. Witthohn, D. Strieth, R. Ulber, K. Muffler (2023). Continuous production of an antimicrobial metabolite with the terrestrial cyanobacterium *Chroococcidiopsis cubana*. Algal Res, 103193.

Other:

M. Witthohn, A. Schwarz, D. Strieth, S. Lenz, R. Ulber, K. Muffler (2020b). Terrestrische Cyanobakterien als Quelle für antimikrobielle Wirkstoffe, Biospektrum, 07.20, 794-796.

Table of Contents

List of Abbreviations

aa	Amino acid
ABC	ATP-binding cassette
ABR	Algal biofilm reactor
antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
ASN	Artificial seawater nutrients
AT	Acetyltransferase
ATP	Adenosintriphosphat
ATS	Algal turf scrubber
BAC	Bacterial artificial chromosome
BAGEL	BActeriocin GEnome Mining tooL
BBM	Bold's basal medium
BG	Blue-green algae
BGC	Bioactive gene clusters
BLAST	Basic Local Alignment Search Tool
BMPBR	Biofilm membrane PBR
CBB cycle	Calvin-Benson-Basham cycle
CDM (CDW)	Cell dry mass (Cell dry weight)
CPBR	Capillary-driven PBR
CTC	5- Cyano-2,3-Ditolyl Tetrazolium Chloride
CWW	Cell wet weight
DAPI	4',6-diamidino-2- phenylindole
DMSO	Dimethyl sulfoxide
DMT	Drug metabolite transporter
DNA	Deoxyribonucleic acid
CPA	Cryoprotective agent
ED pathway	Entner-Doudoroff pathway
EMP pathway	Embden-Meyerhof-Parnas pathway
ePBR	Emerse PBR
EPS	Extracellular polymeric substances
E_v	Illuminance
FBR	Fixed-bed biofilm reactor
FDA	Fluorescein diacetate
FPLC	Fast protein liquid chromatography
GABA	γ-aminobutyrate

GAP	Glyceraldehyde-3-phosphate
GBL	γ-butyrolactone
gDNA	Genomic DNA
HDPE	High-density polyethylene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFP	Horizontal flat panel
Hik	Histidine kinase
HTS	High-Throughput-Screening
INT	2-(4-Iodo-phenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
ISE	Ion-selective electrodes
KS	Ketosynthase
LC-MS	Liquid chromatography–mass spectrometry
LDH	Lactate Dehydrogenase
MeOH	Methanol
MIC	Minimal inhibitory concentration
MLPR	Multi-layered photobioreactor
MS	Mass spectrometry
MSSePBR	Multi-skin sheet emerse PBR
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NHLP	Nitrile hydratase leader peptide
NMR spectroscopy	Nuclear magnetic resonance spectroscopy
NRPS	Non-ribosomal peptide synthase
OD	Optical density
OGDC	2-oxoglutarate decarboxylase
OPP pathway	Oxidative Pentose Phosphate pathway
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PAM-fluorometry	Pulse amplitude modulated fluorometry
PBR	Photobioreactor
PHB	Polyhydroxybutyrate
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PK pathway	Phosphoketolase pathway
PKS	Polyketide synthase
PPAL	Parallel plate air lift
PPT	4’-phosphopantetheine
PRPC	Photorotating biological contactor

PSBR	Porous substrate bioreactors
RAB	Revolving algal biofilm
RBC	Rotating biological contactor
RFP	Rotating flat plate
RiPP	Ribosomally synthetized and post-translationally modified peptides
RPC	Reversed phase chromatography
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
sdOCT	Spectral domain optical coherence tomography
SDG	Sustainable Development Goal
TAG	Triacylglycerol
TAR	Transformation assisted recombination
TCA cycle	Tricarboxylic acid cycle
TCP	total cell protein
TE	Thioesterase
TOMM	thiazole/oxazole-modified microcin
TR	Transcriptional regulator
UV	Ultraviolet

Chapter I

General Introduction

1 Cyanobacteria – facing new problems with old microbes

Cyanobacteria are among the oldest organisms on our planet, according to fossil records they developed about 3.5 billion years ago [1]. As oxygenic, phototrophic organisms, they have made a major contribution to the formation of the earth's oxygen-rich atmosphere. Thus, cyanobacteria can be seen as the trigger for this far-reaching evolutionary process. These microorganisms are highly adaptable and robust bacteria, found in almost all inhabitable areas on earth. Strains were isolated from salt and fresh water, deserts, glaciers, and hot springs [2]. As a result of these adaptations, cyanobacteria produce a variety of high-value substances with enormous commercial potential. Cyanobacteria, unlike plants, can also use light at wavelengths of 490-620 nanometers for photosynthesis. Special chromophores, the phycobilins, have evolved for this purpose [3]. Various carotenoids also contribute to the extension of the usable light spectrum in cyanobacteria. Both substance classes were successfully established in food industries for some time [4]. Concomitantly, a diverse array of metabolic pathways developed, resulting in a large abundance of different energy storage compounds. Among these are mainly long-chain unsaturated fatty acids, such as gamma-linolenic acid, arachidonic acid or docosahexaenoic acid, which are a health-promoting food additive, mostly used in form of dried cell mass [5]. Furthermore, cyanobacteria produce a variety of biotechnologically relevant substances, such as lipids (e.g., linoleic and α-linolenic acid [6], polysaccharides (especially extracellular polysaccharides [7]), essential amino acids [8], vitamins (e.g., vitamin B12 [9]) and enzymes as proteases, cellulases and amylases [8]. Among the natural substances produced by cyanobacteria are often toxins, as is known from the annual "cyanobacterial blooms" in local swimming lakes. Anatoxin A, produced by *Anabaena*, and microcystins, produced by *Microcystis*, are among the most potent naturally occurring toxins and can also cause severe health damage in humans [10, 11]. Much more beneficial are the numerous cyanobacterial secondary metabolites, which were shown to have antimicrobial or antitumor effects [12]. The diversity of the different substance classes is particularly interesting. These range from peptides, polyketides, alkaloids, and isoprenoids to antiviral polysaccharides [13]. So far, mainly aquatic cyanobacteria were described as a rich source of bioactive natural compounds. However, terrestrial cyanobacteria also produce many medically and biotechnologically interesting substances. Within the orders *Chroococcales*, *Oscillatoriales*, *Pseudoanabaenales* and *Nostocales*, both aquatic and terrestrial cyanobacteria occur [14]. Besides the *Pseudoanabeanales*, these orders include the main producers of bioactive secondary metabolites in the cyanobacterial kingdom [5]. Among them are mainly toxines, antiviral, antifungal, and antibacterial substances, but also substances with proven activity against cancer cells, higher plants and algae [13]. In addition to a broad spectrum of potentially interesting bioactive secondary metabolites, terrestrial cyanobacteria have

several biotechnological advantages over aquatic species. Despite their natural life on land, most terrestrial cyanobacteria can be cultivated submersely in (photo-) bioreactors. They show a high tolerance towards fluctuating cultivation conditions, such as temperature, light input, and gassing [14]. These organisms can even survive complete desiccation without damage, which opens completely new possibilities in the field of bioreactor technology [15]. Terrestrial cyanobacteria are thus on the one hand a promising source of yet unknown bioactive substances and on the other hand possess unique physiological properties that make them highly interesting for biotechnological processes [14].

1.1 Aim of the thesis

The constant and increasing menace from bacterial pathogens resistant to common antibiotics, caused by excessive prescription of these compounds, as well as misuse in the agricultural area [16], is a serious global concern. Especially the so-called "ESKAPE" strains, consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, are clinically most important pathogens, frequently evolving multidrug-resistances. Since these bacteria tend to spread mobile genetic elements harboring antibiotic resistance conferring genes, the development of novel pharmaceutics with high efficacy is an urgent necessity [17]. The aim of this thesis was to participate in facing the emerging threat of multi-resistant pathogens by further exploring the diversity of cyanobacterial bioactive metabolites. After an initial screening and the identification of an antibiotic-producing cyanobacterium, the focus should be on the implementation of a reliable production process, for which the synthesis mechanism had to be elucidated. The results of this approach were addressed in **chapter V**. In the course of the experiments the establishment of a quick and reliable nitrate determination method became necessary; this topic is covered in **chapter IV**. Next to this, a genetic engineering approach should be tested, which included the *in silico* identification of potential biosynthetic gene clusters in the genomic sequence of a productive cyanobacterium, followed by a heterologous production in a suitable heterotrophic expression strain (**chapter VI**). Because common cryoconservation protocols are not suitable for every cyanobacterial strain, methods for a reliable conservation of the identified producers of interesting compounds should be established. A developed method dealing with this topic is described in **chapter III**.

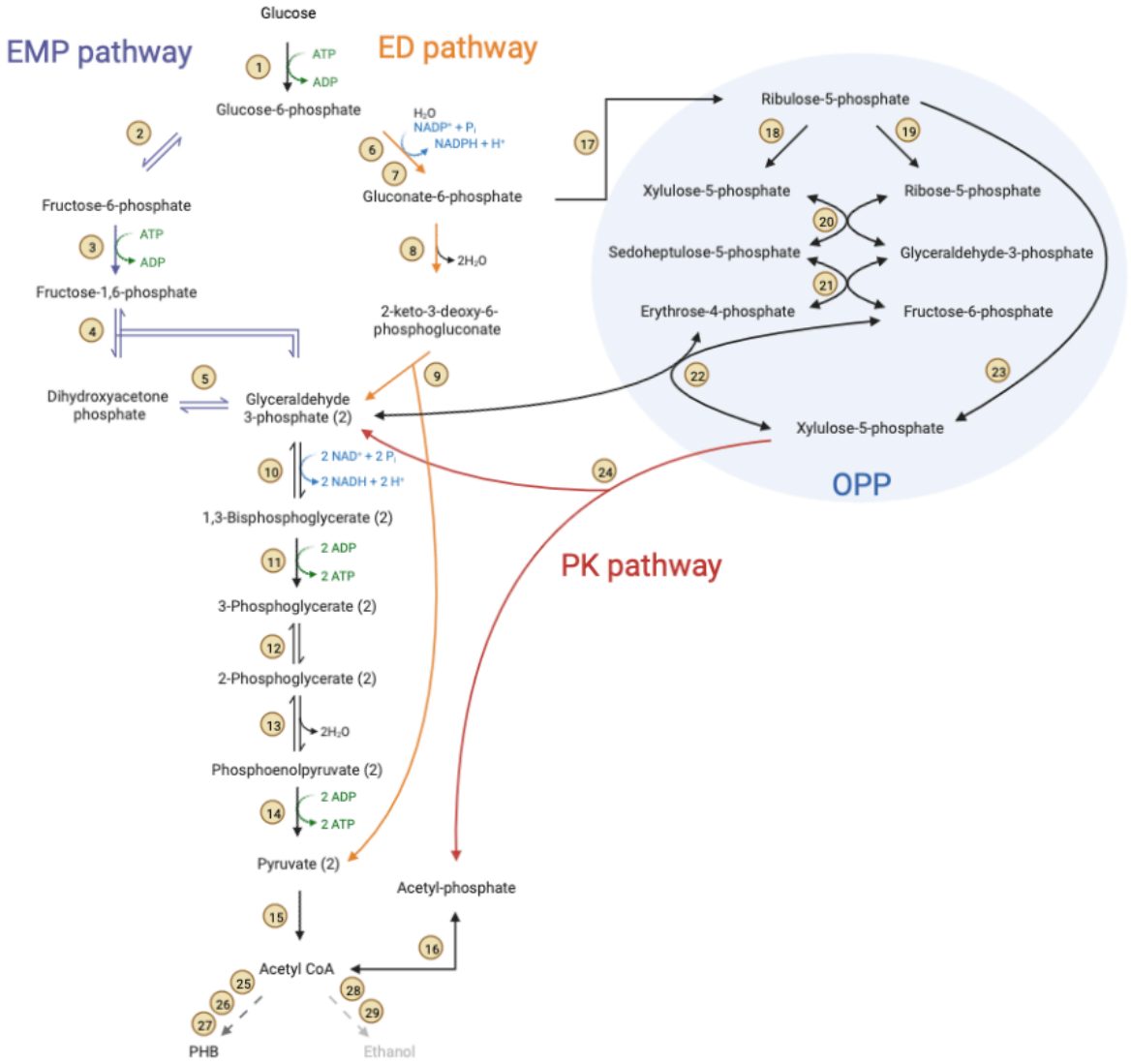
1.2 The versatility of the cyanobacterial metabolism

The huge advantage of cyanobacteria over solely heterotrophic microorganisms is their ability to produce a vast array of industrially relevant compounds by using light and CO_2, with only O_2 as "waste material". As the big challenges of our time mainly consist of climate

change, caused by CO_2 and methane release to the atmosphere, and the reckless exploitation of non-renewable resources, this evolutionary achievement may present a key element of the way out. Although the commercial exploitation of microalgae (procaryotic and eucaryotic) is still largely expandable, several industrial branches already count on the versatility of these microorganisms. In case of cyanobacteria, particularly halotolerant *Arthrospira platensis* (often referred to as *Spirulina*) serves as a production strain [18] with an estimated annual global production of about 15,000 t dry mass [19]. The raw, dried biomass is mostly used as food supplement because of its high nutritional value, or as skin care product in cosmetics. Extracted phycocyanin, of which approximately 200 t is produced every year [19], is used as a food colorant or nutraceutical with (suggested) health-promoting effects [20]. Cyanobacteria are also genetically engineered to produce ethanol on an industrial scale; the American company Algenol (Bonita Springs, FL, USA) built a production plant with a proposed productivity of >60,000 L ha^{-1} $year^{-1}$, using different genetically optimized cyanobacterial isolates (mostly *Synechocystis* sp. or *Synechococcus* sp.) [21]. However, today Algenol focusses its main business on the production of microalgae biomass for healthcare concerns. Although the heterologous expression of *Zymomonas mobilis* genes, respectively coding for a pyruvate decarboxylase and an alcohol dehydrogenase, is mainly used to synthesize ethanol with cyanobacteria, some strains are also naturally capable of its production [22]. By exposing *Spirulina maxima* to a NaCl concentration of 1.24 M, during dark anaerobic metabolism, the ethanol yield could be increased 121-fold to 34.5 mg/g dry weight [23]. Although, the involved metabolic pathways and their regulation has not been fully elucidated, it was postulated that the conversion of acetaldehyde to ethanol is catalyzed by two identified bifunctional aldehyde alcohol dehydrogenases [23]. Next to ethanol, which was the first commercialized biofuel product (additive or substitute) [24], also biodiesel can be produced using microalgae. Since cyanobacteria do not naturally accumulate free fatty acids, mostly oleaginous, eucaryotic microalgae are used to produce “third-generation” biofuel, as they accumulate triacylglycerols (TAG) as reserve molecules. In 2020 a global market value of 5,022 billion USD was estimated for algal biofuel, which is predicted to increase to 9.033 billion USD in 2027 [25]. By culture medium adaptations like limitation of the nitrogen source or by exposing the cells to salt stress, the cellular lipid concentration can increase up to 80 % of the total dry weight [20]. However, cyanobacteria possess carbon-concentrating mechanisms and show a high photosynthetic efficiency of up to 10 %, which is two-fold the efficacy of eucaryotic microalgae [26]. Additionally, they possess an effective biosynthetic lipid metabolism, granted by their metabolic plasticity. In combination with the Calvin-Benson-Basham (CBB) cycle, the recently discovered phosphoketolase pathway enables a more efficient acetyl-CoA biosynthesis by reducing the substrate consumption of CO_2 and

ATP [27, 28]. As cyanobacteria are also much more accessible for metabolic engineering than eucaryotic microalgae [29], they are supposed to present the most suitable platform for future biofuel production approaches [30]. By heterologous expression of codon-optimized thioesterase genes and deletion of several cell wall stabilizing genes in the model strain *Synechocystis* sp. PCC 6803, a direct secretion of 197 ± 14 mg L^{-1} free fatty acids could already be achieved [31]. As mentioned before, unlike eucaryotic microalgae, cyanobacteria do not form TAGs as energy storage compounds. However, when stressed by nutrient limitation (*e.g.,* nitrogen, phosphate, or potassium [32]), most studied cyanobacteria produce polyhydroxybutyrate (PHB) from catabolized glycogen storage [33]. Interestingly, this possible substitute for petroleum-based plastics is not formed as an energy and carbon storage [34], which is the case for most heterotrophic bacteria [35]. The precise physiological function is still not elucidated, a possible role as a temporary electron sink was proposed [34]. Outgoing from glucose monomers, which are catabolized mainly via the Embden-Meyerhof-Parnas pathway, PHB is formed by the condensation of two molecules acetyl-CoA, the subsequent reduction of the formed acetoacetyl-CoA to 3-hydroxybutyryl-CoA and a final polymerization step to PHB. The key enzymes involved in this process are the β-ketothiolase PhaA, the acetoacetyl-CoA reductase PhaB [36] and the two-subunit polyhydroxyalkanoate synthase PhaEC [37]. Cyanobacteria have not yet been used for large scale productions of PHB, but they remain the most sustainable solution. However, slow growth rates and, in comparison to heterotrophic producer strains as *Cupriavidus necator*, low cellular PHB contents still are the main drawbacks. While *C. necator* cell dry weight (cdw) can consist of up to 80 % PHB [38], cyanobacteria typically only reach about 10 - 20 % [39]. Consequently, a general metabolic engineering approach comprises the heterologous expression of the PHB synthesis genes *phaABC* from *C. necator* in fast growing cyanobacteria strains as *Synechococcus elongatus* UTEX 2973. By this, the cellular PHB content could be increased 2.4-fold to 16.7 % of cdw, in comparison to the natural producer strain *Synechocystis* PCC 6803 [40]. However, the highest reached specific productivity of 75.2 mg L^{-1} d^{-1} is still comparably low.

The stated examples provide an insight into the remarkable versatility of cyanobacterial carbon and energy metabolism, which enables the direct synthesis of various biotechnologically relevant products and offers diverse entry points for metabolic engineering approaches. Next to the CO_2 fixation process through the Calvin-Benson-Bassham cycle, in combination with the photorespiration pathway, cyanobacteria also possess all known glycolytic pathways to catabolize organic carbon sources. In the conversion process of glucose to the key-intermediate acetyl-CoA, the Embden-Meyerhof-Parnas (EMP) pathway, the Entner-Doudoroff (ED) pathway, the oxidative pentose phosphate (OPP) pathway and the phosphoketolase pathway are involved [28] (**Fig. 1**).



1	Hexokinase	**11**	Phosphoglycerate kinase	**21**	Transaldolase
2	Phosphogluconate isomerase	**12**	Phosphoglycerate mutase	**22**	Transketolase
3	Phosphofructokinase	**13**	Enolase	**23**	Ribulose-5-phosphate epimerase
4	Bisphosphate aldolase	**14**	Pyruvate kinase	**24**	Phosphoketolase
5	Triosephosphate isomerase	**15**	Pyruvate dehydrogenase	**25**	β-ketothiolase (PhaA)
6	Glucose 6-phosphate dehydrogenase	**16**	Phosphotransacetylase	**26**	Acetoacetyl-CoA reductase (PhaB)
7	Phosphogluconolactonase	**17**	6-phosphogluconate dehydrogenase	**27**	Polyhydroxyalkanoate synthase (PhaEC)
8	Phosphogluconate dehydratase	**18**	Ribulose-5-phosphate epimerase	**28**	Bifunctional aldehyde alcohol dehydrogenase (ADHE1)
9	KDPG aldolase	**19**	Ribose-5-phosphate isomerase	**29**	Bifunctional aldehyde alcohol dehydrogenase (ADHE2)
10	Glyceraldehyde phosphate dehydrogenase	**20**	Transketolase		

Fig. 1 Glycolytic pathways in cyanobacteria. Glucose is catabolized via the Embden-Meyerhof-Parnas (EMP) pathway, the Entner-Doudoroff (ED) pathway, the oxidative pentose phosphate (OPP) pathway and the phosphoketolase (PK) pathway. The resulting acetyl-CoA can be further converted to *e.g.* PHB or ethanol (in some strains). Modified according to [28].

The availability of various glycolytic pathways enables cyanobacteria to directly respond to changing environmental conditions, since all pathways differ in effectivity regarding produced ATP and NAD(P)H, ranging from 1 to 2.33 and 0 to 5.33, respectively [28]. So,

for example, under conditions of a limited availability of organic carbon sources, merely the ED pathway is used, as it needs 3.5-fold less catalytic enzymes for the generation of glyceraldehyde-3-phosphate (GAP) from glucose. In this case, the nutrient limitation has a stronger impact than the total ATP outcome, as the EMP pathway is generally more effective than the ED pathway [41]. Eventually all glycolytic routes result into the formation of acetyl-CoA, which is the key substrate for the tricarboxylic acid (TCA) cycle (**Fig. 2**).

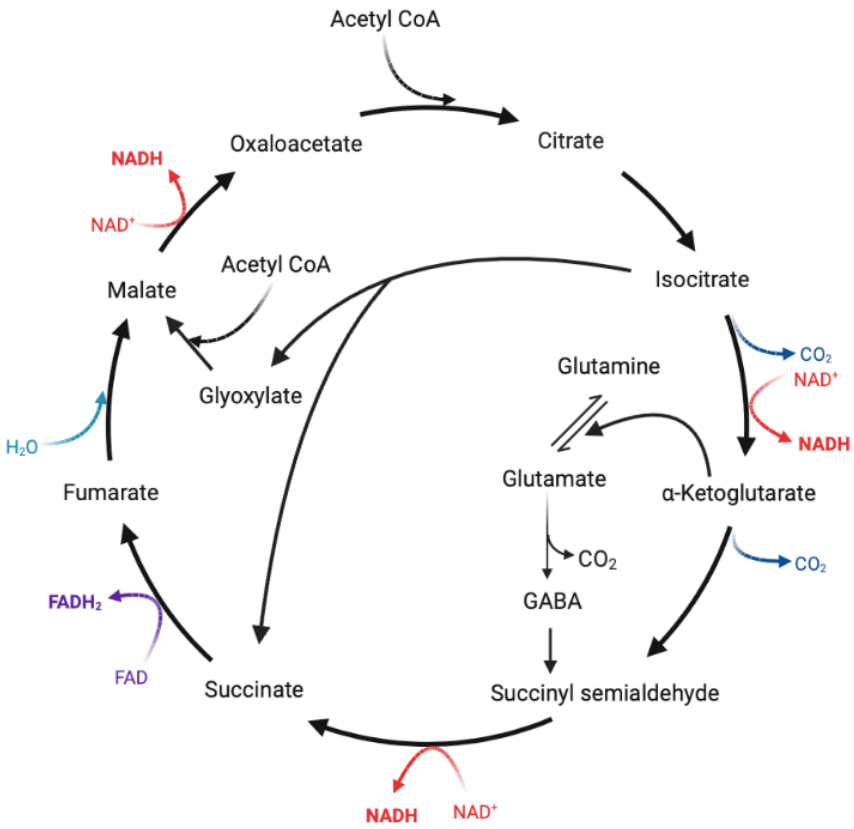


Fig. 2 Tricarboxylic acid (TCA) cycle in cyanobacteria. As it lacks a 2-oxoglutarate dehydrogenase complex, succinate can be generated via the 2-oxoglutarate decarboxylase (OGDC) shunt, the γ-aminobutyrate (GABA) shunt or the glyoxylate shunt.

Because of the lack of a functional 2-oxoglutarate dehydrogenase complex, a long time the TCA cycle was considered to be incomplete in cyanobacteria [42]. However, in these bacteria a much more flexible solution developed. Instead of the conversion of α-ketoglutarate (AKG) to succinyl-CoA, followed by an oxidation to succinate, AKG can either be decarboxylated and dehydrogenated to succinate, with succinyl semialdehyde as an intermediate (2-oxoglutarate decarboxylase (OGDC) shunt [43]), or aminated to glutamate and further decarboxylated to γ-aminobutyrate (GABA), which gets deaminated to succinyl semialdehyde (GABA-shunt) [44]. A third pathway, the glyoxylate shunt, originates from isocitrate which is split to succinate and glyoxylate; by addition of acetyl-CoA, the latter compound is converted to malate and reenters the cycle [45]. These TCA

cycle variants allow several metabolic advantages, such as the maintenance of pH homoeostasis by the proton-consuming decarboxylation reaction of glutamate to GABA [46], the utilization of acetate as an additional source for carbon and energy by the glyoxylate shunt [45] and the strong lead to succinate, which serves as the electron donor for oxidative phosphorylation [47]. Consequently, cyanobacteria can quickly respond to changes in energy provision caused by different light intensities or the availability of organic carbon sources.

The unique adaptability of cyanobacteria is the result of constant evolutionary processes, which started about 3.5 billion years ago. Till then, these organisms were able to survive all climatic changes and to prevail all possible habitats. But for a prolonged existence in such a broad spectrum of life, the development of effective defense mechanisms against many types of competitors seems to be inevitable. And indeed, cyanobacteria are a profound source of structurally very diverse antibiotic substances, of which many are still to be discovered and characterized.

1.3 Cyanobacteria as a source of antimicrobial compounds

As previously mentioned, cyanobacteria are a huge and long-known but and nearly untapped source of bioactive metabolites with antibacterial, antiviral, antifungal and anti-tumoral effects. The group of cyanobacterial antibacterial compounds comprises a huge variety of chemical classes, among these are primarily peptides [48], terpenes [49] and alkaloids [50], but also depsipeptides [51], macrolides/lactones [52], lipopeptides [53], lipids [54] and polyketides [55]. The majority of the identified antibacterial compounds also possesses further bioactive characteristics, especially general cytotoxicity [13]. So, no general rule can be determined linking a specific chemical class to antibiotic activity in cyanobacteria. However, together with depsipeptide and lipopeptide classes, peptides represent 51 % of altogether 260 metabolite families compiled by Demay et al. (2019) [13]. A reason for this may be that cyanobacteria have evolved multiple ways for the synthesis of peptidic biomolecules. Next to the “classic” ribosomal peptide biosynthesis, whose products can additionally be post-translationally modified (RiPPs), also pathways involving non-ribosomal peptide synthases (NRPS) or a combination of NRPS and polyketide synthases (PKS) are used for the generation of bioactive molecules [56].

The precursor peptides of RiPPs are initially ribosomally synthetized and consist of a leader peptide and one or more core peptides, which can be separated by non-functional regions flanking the core peptide(s) [57] (**Fig. 3**). The leader peptide is recognized by maturation enzymes, which perform several post-translational modifications on the core peptide. The leader peptide normally undergoes a proteolytic cleavage in this process. Commonly catalyzed reactions are e.g. dehydration, cyclization, oxidation and methylation [57]. As an

example, cyanobactins first undergo a heterocyclization process, where the amino acids (aa) serine and threonine are converted to their respective oxazoline variant, while cystein is modified to thiazoline [58]. These alterations are followed by a macrocyclization process leading to a cyclic molecule structure. A prominent example for cyanobactins are Patellamide A and C, which were first identified in *Prochloron didemni*, a cyanobacterial symbiont of the ascidian *Lissoclinum patella* [59].

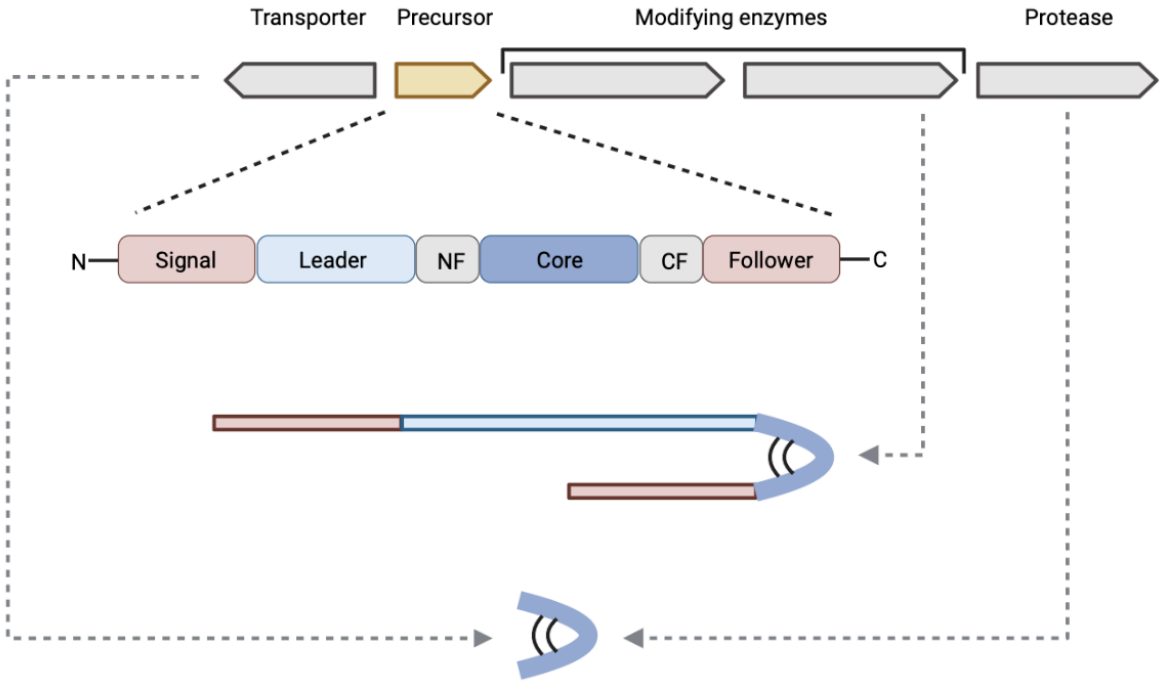


Fig. 3 Typical gene cluster encoding the RiPP precursor protein and maturation/transporter proteins. **Red**, optional aa sequence; **blue**, minimal necessary aa sequence. After the precursor peptide is ribosomally synthesized, the core peptide is surrounded by non-functional regions, followed by an N-terminal leader peptide. In some RiPPs, a C-terminal follower peptide is used instead of a leader peptide. In the maturation process, this part is removed by proteolytic cleavage, and the core peptide is post-translationally modified by several maturation enzymes enabling its bioactivity. Signal regions are used in some RiPPs for the targeted transport to the destination site within the cell, prior to the activation. The protein product of the encoded transporter adjacent to the precursor gene is responsible for the transport of the maturated RiPP over the cell membrane. Modified according to [57, 60].

Of the identified Patellamides (A-G), several compounds were shown to possess cytotoxic or multidrug reversing activity [61]. Next to the cyanobactins, also RiPPs that belong to the classes of bacteriocins and microviridins are produced by cyanobacteria [62]. Members of the first one are known for their antimicrobial activity and often contain the non-proteinogenic amino acids lanthionine and methyl-lanthionine and are therefore called lanthipeptides or lantibiotics, in case of possessing an antibiotic activity. Bacteriocins can be identified by a double-glycine motif within the leader peptide sequence, that is

recognized and cleaved by a C39 peptidase domain, being part of an ABC transporter [63]. Although bacteriocins are mostly known to be produced by Gram-positive bacteria, especially by lactic acid bacteria [64], the advances of *in silico* screening methods allowed to also identify members of this family in Gram-negative bacteria, including cyanobacteria [65]. The family of microviridins, N-acetylated tri-and tetra-decapeptides (depsipeptides), often act as (serine) protease inhibitors, and could be used for the treatment of metabolic disorders. Microviridins are relatively large oligopeptides and consist of 12 to 20 amino acids [66]. They were first discovered in and extracted from the cyanobacterium *Microcystis viridis*, followed by successful isolations from several other cyanobacteria. Microviridins can be identified by a conserved N-terminal leader motif within the leader peptide and another conserved amino acid sequence in the core peptide [57].

As already mentioned before, cyanobacterial secondary metabolites can also be synthesized via NRPS-, PKS-, or PKS-NRPS hybrid-pathways, large multienzyme complexes which process and modify precursor peptides to one bioactive molecule [67, 68] (**Fig. 4**).

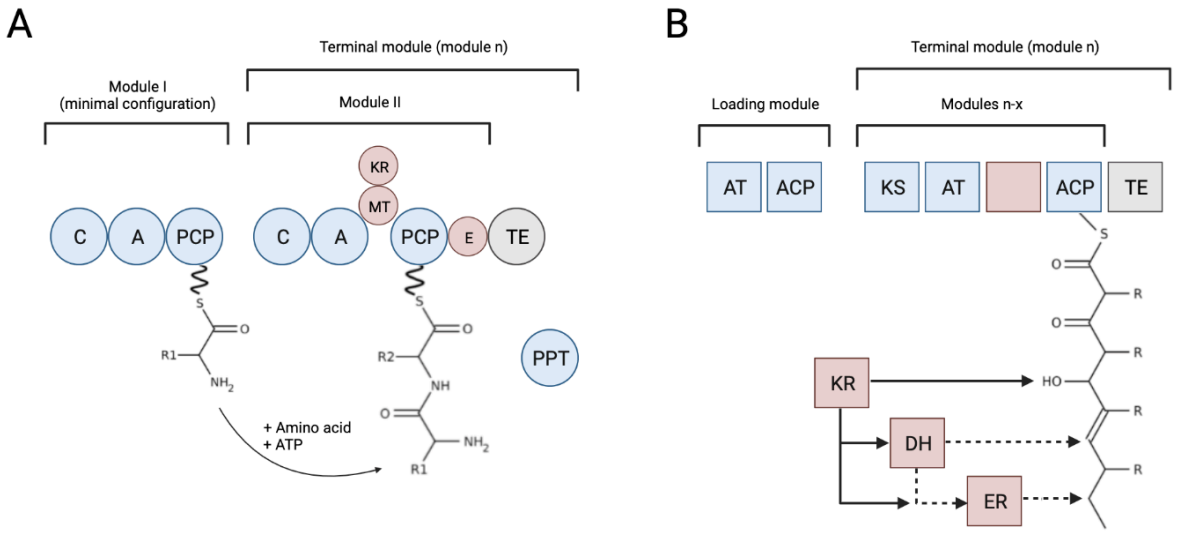


Fig. 4 Enzymatic domains and general process of non-ribosomal peptide and polyketide synthesis by NRPS (**A**) and PKS (**B**) modules. **Red**, optional domains; **blue**, necessary domains. A, Adenylation domain; ACP, Acyl carrier protein; AT, Acyltransferase; C, Condensation domain; DH, Dehydratase; E, Epimerase; ER, Enoyl reductase; KR, Ketoreductase; KS, Ketosynthase; MT, Methyltransferase; PCP, Peptidyl carrier protein; PPT, 4'phosphopantetheinyl transferase; TE, Thioesterase. Modified according to [60].

NRPS consist of different enzymatic modules, each adding an aa to the growing peptide chain. In contrast to ribosomal peptide synthesis, the substrates for chain elongation can either be proteinogenic or non-proteinogenic aas, so huge varieties are possible as about 300 different substrates can be used [69]. Module I, the initiation module, consists of an

adenylation domain (A) that activates the aa substrate by reaction with ATP, a thiolation domain or peptidyl carrier protein domain (PCP) forming an aminoacyl thioester, and a condensation domain (C) which catalyzes the formation of thioester bonds between the thioester intermediates [70]. Before this process, the C domain must be primed by addition of a 4'-phosphopantetheine (PPT) moiety to a conserved serine residue. This essential step is performed by a 4'phosphopantetheinyl transferase [70, 71]. Adjacent elongation modules (M2) add further substrates to the peptide chain, following the same principle. As every module only adds one aa, large NRPS-derived biomolecules require NRPS-cluster with multiple modules. The terminal module possesses a thioesterase domain, which releases the full-length molecule by hydrolysis or cyclisation. Further (optional) modifications can be catalyzed by e.g. ketoreductases, methyltransferases or epimerases [70]. Most cyanobacterial NRPS-pathways also incorporate PKS-modules for product synthesis. One of the molecules which is only NRPS-derived is nostocyclopeptide from terrestrial *Nostoc* sp. strains [72]. A more recently identified variant, nostocyclopeptide M1 from Nostoc sp. XSPORK 13A (**Fig. 5**), acts as a potent antitoxin against the hepatotoxins microcystin and nodularin, by inhibiting drug transporters [73, 74].

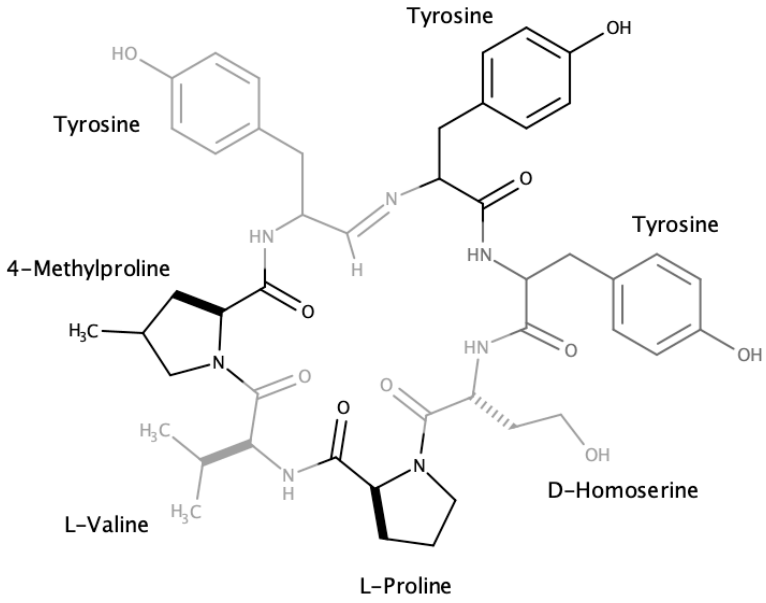


Fig. 5 Molecule structure of the NRPS-derived Nostocyclopeptide M1 from *Nostoc* sp. The pre-mature peptide undergoes a macrocyclization through the formation of an imino bond between the N- and C-terminal tyrosines [60]. The individual aa domains are differently colored for a better distinction. Modified according to [74].

Although nonribosomal peptides and polyketides are completely different molecule families, the biosynthetic process of PKS shows many similarities to the one of NRPS [75] (**Fig. 4**). Just as the PCP-module of NRPS-clusters, the acyl carrier protein of the PKS loading module is activated by transfer of a phosphopantetheinyl-group, catalyzed by a PPTase. A

ketosynthase (KS) catalyzes a condensation reaction to combine the PK-chain with an α-carboxyacyl-extender unit, resulting from an acyltransferase (AT) mediated transacylation of an acyl-CoA-ester (mostly malonyl-CoA) [75, 76]. The polyketide is then elongated by further modules, consisting of the same three domain-components. Additional modifications can be performed by e.g. ketoreductases, dehydratases or enoyl reductases. As in NRPS, the synthesis process is as well completed by hydrolysis or macrocyclization catalyzed by a thioesterase (TE) domain [76]. The alkaloid and potent neurotoxin anatoxin-a from *Oscillatoria* sp. is an example for a solely PKS-derived cyanobacterial bioactive molecule [77], while also NRPS/PKS hybrid-pathways are frequently used for the production of nonribosomal peptides in cyanobacteria. By PKS-mediated addition of polyketides or fatty acid side chains to NRPS-derived peptides, a much greater diversity can be achieved. Examples are the highly toxic compounds nodularin from *Nodularia spumigena*, or microcystin from *Microcystis aeruginosa* [56].

Through the revelation of biosynthetic mechanisms and great advances in genomic and proteomic methods, more and more bioactive secondary metabolites of potential clinical relevance are identified in cyanobacteria (Fig. 6A). However, due to slow growth rates and the need of photobioreactors for cultivation, the huge majority of these substances is not considered for further pharmaceutical use [75]. Moreover, many strain isolates are not yet cultivatable or biosynthetic gene clusters are silent and don't get expressed [78]. An alternative for the direct cultivation of cyanobacteria for secondary metabolite production is the use of synthetic approaches. Until now, several NRPS and PKS cluster, as well as smaller bacteriocin encoding genes, could successfully be expressed heterologously in heterotrophic bacteria or fungi, leading to functional products. The main requirement for this approach is the availability of the respective DNA-material, which is increasingly granted by the growing number of sequenced genomes: in 2021, about 950 different cyanobacterial gDNA sequences were available [79]. As heterologous hosts, primarily standard *E. coli* expression strains were used [59, 80], but also Gram-positive *Streptomyces* strains [81], the yeast *Saccharomyces cerevisiae* [82], and even genetically amenable and fast-growing cyanobacteria as *Anabaena* sp. [83], *Synechocystis* sp. [84] and *Synechococcus* sp. [85] were successfully used. Smaller RiPPs, such as microviridins, can be cloned by direct PCR-amplification and subsequent ligation with a respectively digested expression vector (Fig. 6B). For larger gene clusters, e.g. direct pathway cloning can be applied, which relies on long-amplicon PCR of the biosynthetic genes (whole or partly), followed by HiFi assembly with the target plasmid, harboring complement 5'- and 3'-terminal DNA sequences, from which an exonuclease cleaves the 5' ends and creates annealable 3' overhangs. (Fig. 6D) [78, 86]. By this approach, the first heterologous production of an NRPS-derived anabaenopeptin from *Nostoc punctiforme* was done in *E. coli*, which achieved an >100-fold

product yield, compared to the native synthesis [86]. For large gene clusters, vectors as fosmids, cosmids (about 40-45 kilobase pairs, kb) or bacterial artificial chromosomes (BACs; about 200 kb) can be used [78].

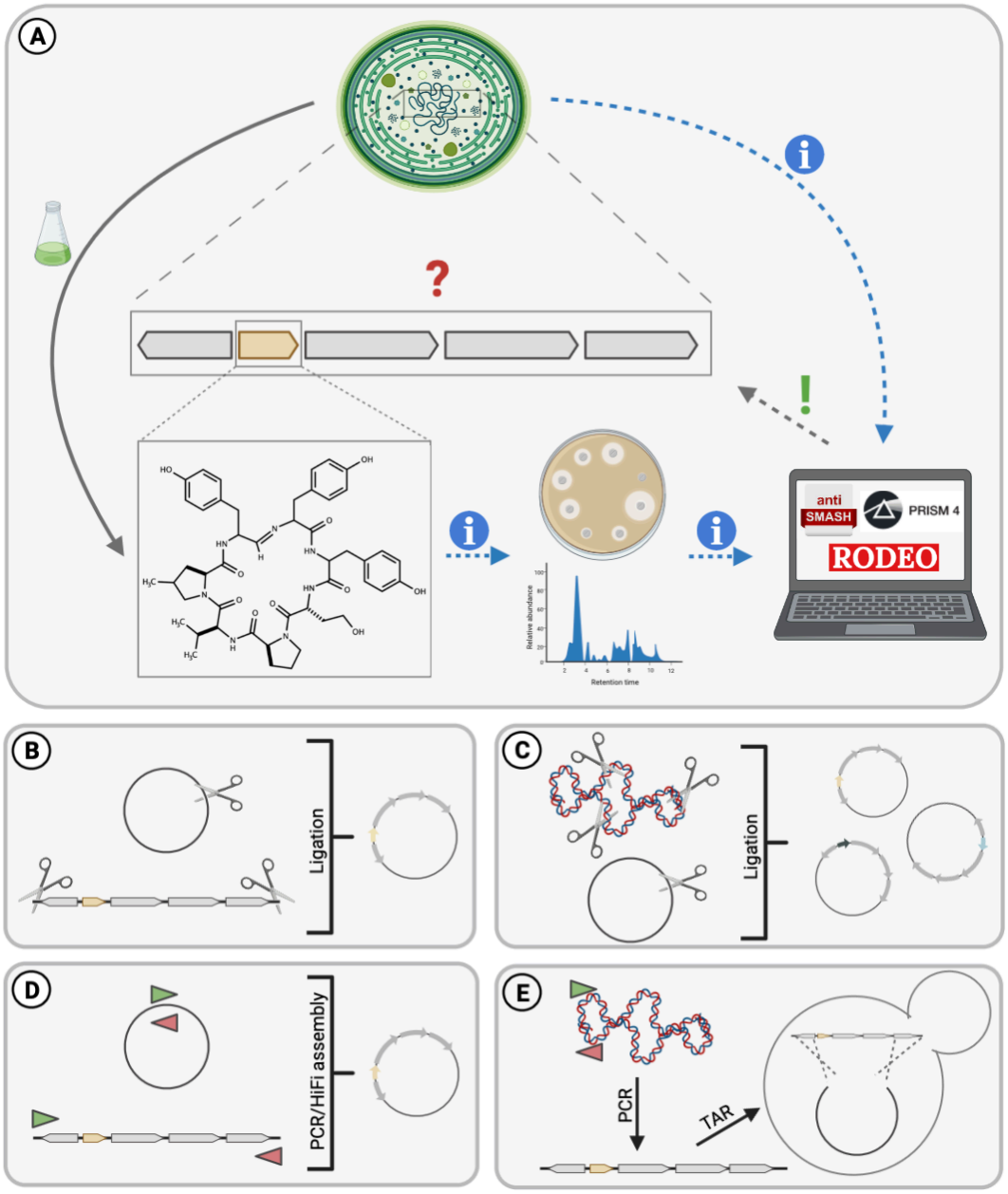


Fig. 6 Bottom-up approach for the identification of biosynthetic gene clusters from cyanobacteria (**A**) and subsequent cloning mechanisms for heterologous product synthesis (**B-E**). **A**, a culturable cyanobacterial strain produces a bioactive substance of unknown identity. For the identification of the associated biosynthetic gene cluster, information gained from bioactivity assays and data from e.g., mass spectrometry (MS)/ nuclear magnetic resonance spectroscopy (NMR) analysis, can be used. Together with strain information and gDNA sequences, *in silico* analysis tools can lead to the identification of biosynthetic gene clusters. **B**, direct ligation of amplified gene cluster and vector. **C**, genomic library cloning approach, digestion of gDNA and ligation with vector. **D**, PCR amplification of gene cluster and vector, followed by HiFi assembly through homologous 5'- and 3'-overhangs. **E**, transformation assisted recombination (TAR) in yeast cells, which possess a strong recombination machinery.

For the previously introduced patellamides from *P. didemni*, a heterologous production was mandatory, as the symbiotic cyanobacterium cannot be cultured under laboratory conditions. Patellamides A, C were cloned by genomic library-based cloning using fosmids as expression vector (Fig 6C) [59], D by shotgun cloning, succeeded by ligation with a BAC [87]. All three molecules were verifiably produced with *E. coli*. Particularly large gene clusters of up to 300 kb can be cloned using the transformation-associated recombination (TAR) approach, which uses the strong recombination machinery of yeast cells [88]. The cells are transformed with a linearized TAR cloning vector and one or several PCR products; sequences which shall be ligated, must possess homologous 5'- and 3'-overhangs. Through homologous recombination, the DNA fragments are assembled with the cloning vector, forming a circular BAC, which can be isolated from the yeast cells and brought into the bacterial target strain [88]. This approach was e.g. used to express the 28.7 kb NRPS/PKS cryptomaldamide gene cluster, natively from *Moorea producens*, heterologously in *S. elongatus* PCC 7942 and *Anabaena* sp. PCC 7120 [89]. With the latter strain, a high-titer production of cryptomalamide was achieved, yielding a concentration of about 26 mg/L [89]. The heterologous production of a cyanobacterial metabolite with antibiotic effects was done in the course of this thesis (see chapter VI), apart from that, an improved production of cyanobacterial substances with antibiotic effects, by using heterologous hosts, does not seem to be in the focus of current research. However, the cloning techniques, even for very large gene clusters, were shown to result in functional, bioactive products. Together with improving *in silico* tools for genome mining approaches, as *e.g.,* antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) [90], and with regard to the menace of multi-resistant pathogens, it will only be a question of time when cyanobacteria and the variety of their synthetized antibiotic metabolites come to the fore. These phototrophic organisms can be a great chance for an emission free and climate friendly production of diverse bioactive compounds, which again leads to the beginning of this chapter and the fascinatingly flexible carbon- and energy metabolisms. The possibilities of mixotrophic cultivation approaches have not yet been fully exploited, but have already shown promising results, not only for the production of various primary, but also bioactive secondary metabolites [91].

2 References

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Chapter II

Process Technologies of Cyanobacteria (Bookchapter)

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Abstract

Although the handling and exploitation of cyanobacteria is associated with some challenges, these phototrophic bacteria offer great opportunities for innovative biotechnological processes. This chapter covers versatile aspects of working with cyanobacteria, starting with up-to-date *in-silico* and *in-vitro* screening methods for bioactive substances. Subsequently, common conservation techniques and vitality/viability estimation methods are compared and supplemented by own data regarding the non-invasive vitality evaluation via pulse amplitude modulated fluorometry. Moreover, novel findings about the influence the state of the precultures have on main cultures are presented. The following sub-chapters deal with different photobioreactor-designs, with special regard to biofilm photobioreactors, as well as with heterotrophic and mixotrophic cultivation modes. The latter topic provides information from literature on successfully enhanced cyanobacterial production processes, augmented by own data.

1 Introduction

Bioeconomy describes the transformation from a petroleum-based market economy to a sustainable, post-fossil market economy. The focus is on securing nutrition of a growing population (Sustainable Development Goal “SDG” 2: Zero Hunger), the sustainable use of resources (SDG 12: Responsible Consumption and Production) and climate change (SDG 13: Climate Action). The vision of a post-fossil market economy replaces fossil resources with renewable raw materials, which are used both energetically and materially. However, the production of renewable resources requires intensive cultivation of agricultural land, which leads to a loss of biodiversity. In addition, the availability of agricultural land is limited (see **Fig. 1**), which creates a conflict between tank, trough and plate.

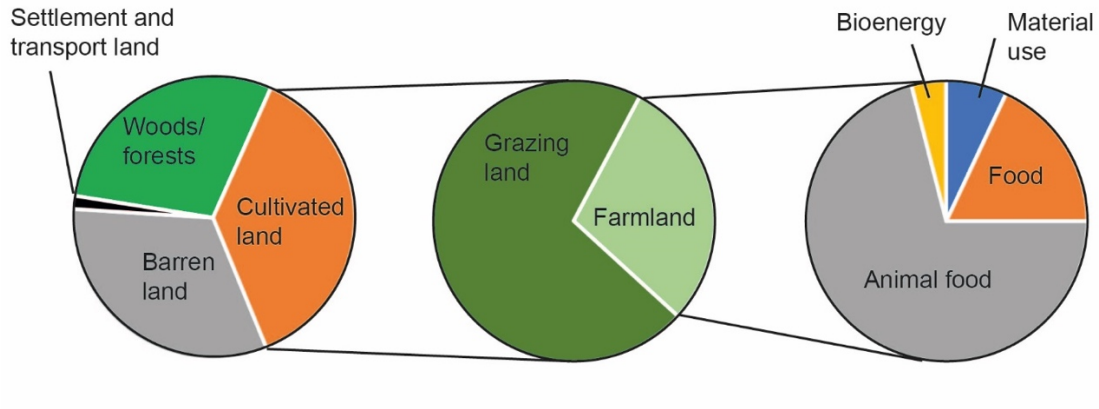

Fig. 1 Current global land use. 37 % are used for agricultural cultivation, 29.1 % are woods and forests, 32.1 % are barren (mountains, desert etc.), and 1.5 % are used for settlement and transportation [1]. The share of cultivated agriculture land is further divided into 71 % used for grazing and 29 % farmland, which in turn is divided into land used for the production of raw materials (6.9 %), food (17.9 %), feed (71 %), and bioenergy (3.8 %). Figure created after [1].

However, renewable resources are most often coupled to the intensive use of fertilizer. This adds substantially to the climate issue, as in most cases the Haber Bosch process is used for ammonia production. It comes along with a significant climate footprint and it is estimated that 1-2 % of the global energy consumption and 1.44 % of global CO_2 emissions are attributed to this process [2]. Further, the use of artificial fertilizer releases high amounts of nitrous oxide (greenhouse gas) [3]. Innovative and new concepts are needed here to meet the challenges in agricultural research such as yield security, climate friendliness, environmental protection and biodiversity conservation. Microalgae can be part of the solution. Photo-autotrophy is a form of life in which, simplified, organisms use light as an energy source to produce oxygen and carbohydrates (sugars) from water and CO_2 and thereby contribute to SGDs 13 and 2. According to current projections, they bind around 30 % of atmospheric CO_2 via the Calvin Benson Bassham cycle (CBB cycle). Cyanobacteria, on which we will focus in this chapter, are prokaryotes, performing an oxygenic photosynthesis, although they are commonly referred to as microalgae. Some cyanobacterial strains form biofilms, meaning they live embedded in a self-produced matrix of extracellular polymeric substances (EPS) that protectively surround them [4]. Depending on their origin, aquatic and terrestrial cyanobacteria can be distinguished. In addition to CO_2, some cyanobacteria also bind atmospheric nitrogen and convert it into bioavailable nitrogen [5], making them interesting to be used as a natural living fertilizer in agriculture as a co-culture with plants [6, 7]. Furthermore, they are also able to bind particulate matter and can be cultivated under flue gas [8]. They live in the most extreme habitats (deserts, hot springs, Antarctica) and are tolerant to fluctuating biotic and abiotic conditions. This makes them easy cultivation organisms, since the common monitoring and controlling of critical cultivation parameters, as temperature and pH, is often not necessary. On the other hand, light is one of the most important substrates which is not dispersible. The intensity and quality of the light can change during cultivation due to *e.g.* mutual shading and thus influences cell physiology and in a consequence growth rate as well as physiology [9]. Each cyanobacterium has organism-specific requirements based on its origin and - depending on the desired target product – in terms of light intensity and light composition as well as temperature [10]. In addition, cyanobacteria can be used to produce a variety of interesting products such as natural dyes, biopolymers, polysaccharides and fatty acids. The biomass itself can be used as a food (supplement), animal feed or fertilizer. Moreover, many cyanobacterial strains are known as producers of various bioactive substances of different chemical classes with, amongst others, antibacterial [11–13], antifungal [14], antiviral [15] and anti-tumoral [16] activity. Several reviews summarize the potential of cyanobacteria as well as potential biotechnological products [17–19].

In this chapter, an overview of modern genome-based screening methods is given. In addition, different ways to preserve and cultivate cyanobacteria are discussed as well as the application of state-of-the-art photobioreactors. Furthermore, alternative ways to increase productivity by heterotrophic and mixotrophic cultivation, and methods for determining cell-vitality and biofilm growth are presented.

2 Genome-based Screening for Potential New Bioactive Substances

The potential of cyanobacteria, especially regarding secondary metabolites, has not yet been exhausted due to the fact that only a small fraction of all existing cyanobacteria have been described up to now [20, 21]. Especially the discovery of bioactive cyanobacterial metabolites is still a hot topic in research, which is indicated by the still increasing number of publications in this research field [22]. The structure of secondary metabolites is as diverse as their field of application. They fulfill various roles including defense against other organisms (antimicrobial substances), metal transporting agents, facilitators for symbiosis, antioxidants, photoprotectors and others [23, 24]. Over the last decades the excessive use of antibiotics has led to the rise of multi-drug resistant bacteria. Thus, the identification of new antibacterial compounds, as well as antifungal and anticancer substances is of high interest [25, 26].

Standard *in-vitro* screening is based on the phenotypic properties of cyanobacteria: *i.e.*, cyanobacterial biomass is collected or grown and usually an extract is prepared, which is then tested in bioactivity assays for its anticancer, antifungal, or antibacterial properties. Commonly used bioactivity tests in lab-scale are summarized by Strieth *et al*. [27]. However, this type of screening can be very laborious and time-consuming, especially in view of the preceding slow growth of the cyanobacteria. This disadvantage is only partially overcome by High-Throughput-Screening (HTS) methods. HTS allows to screen automatically and robot supported of up to 100,000 or more samples per day. This method is mainly used to identify compounds (so called hits) with pharmacological or biological activity. Using HTS only a single concentration (commonly 10 µM) of the compound is tested, which may or may not be sufficient to achieve a response. In quantitative HTS (qHTS), multiple concentrations of the respective compound are tested, which allows the determination of the “minimal inhibitory concentration” (MIC) [28].

It is known that the synthesis pathways leading to such active molecules are mostly encoded by large gene clusters [29]. Due to the ever-increasing number of sequenced genomes and the fact that availability and accessibility of genome data has highly improved, there has been an expansion of genomic screening in the last decade [30–32]. Two

examples of genome data collections are the GEO DataSets from National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) that collects and provides, for example, biomedical and genomic information and the CyanoMetDB. The latter was developed by Jones *et al*. [22] and is an openly-accessible database of cyanobacterial secondary metabolites summarized from 850 peer-reviewed articles published between 1967 and 2020.

Genomic-based screening methods can be divided into *in-vivo* and *in-silico* methods. While *in-vivo* screening methods are mainly based on the detection of the gene(s) of interest using polymerase chain reaction (PCR) *in-silico* methods rely on genome mining approaches. Thereby, a lot of information about an organism can be obtained [33]. Many secondary metabolites have the advantage of being synthesized from so called bioactive gene clusters (BGC) whose structure is similar within their molecular class. It is advantageous that the corresponding nucleotide sequences are very similar across different species [34]. Thus, within a substance family, highly conserved sequences are often found within these gene clusters. For example, LanC is involved in the modification of lantibiotics [35]. Different tools are available for identifying respective BGCs and potential cyanobacterial producers in the genome data space, *e.g.* antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) [36].

In some cases, these analysis tools even can predict the possible structural formula of a molecule without an existing extract and/or structure [33]. If a strain has the LanC it has the potential to produce lantibiotics. Weber & Uk give an overview of existing tools like BAGEL (BActeriocin GEnome Mining tool), Evo Mining and RODEO that can be used to investigate secondary (bioactive) compounds and their corresponding gene clusters (http://secondarymetabolites.org/) [37]. Additional, Basic Local Alignment Search Tool (BLAST) by NCBI can be used to compare unknown DNA or protein sequences to already annotated sequences in various data bases. Methods like blastn (for nucleotide sequences), or blastx (for amino acid sequences) are readily available. Although the number of known cyanobacterial genomes is slowly increasing only a small part of the existing genome space is covered up to date. To gain more information about potential strains, it is important that as many cyanobacteria as possible get sequenced and included in existing genome databases. It is known that a majority of bioactive compounds reported from cyanobacteria are cyclic or linear peptides [38]. Peptide derived compounds can be synthesized in different ways, either through non-ribosomal peptide synthetases (NRPS) or via ribosomal synthesized and post-translationally modified peptides (RiPPs). Additional structure diversity is afforded by mixing NRPS and polyketide synthases (PKS) [39, 40]. Vestola *et al*. characterized the biosynthetic pathway of numerous novel glycosylated

lipopeptides in *Anabaena sp.* SYKE748A [41]. The glycolipopeptide were similar to antimycotic hassalidins A and B found in *Hassallia* sp. Further analysis showed that 20 strains of the genus *Anabaena* carry the hassalidin (*has*) synthetase genes, whereby the structure of antimycotic compound differs, but all inhibit the growth of *Candida albicans*. Finally, they could find the hassalidin synthetase genes in four other cyanobacterial genii [41].

In most cases the genome of a cyanobacterium is not sequenced. In this case analysis can be performed *in-vivo* using PCR. For example, the hassalidin NRPS genes indicate the presence of the *has* gene cluster. Thus, this sequence can be used to screen for potential hassalidin producers [41]. Often a mixture of similar primers is used, so called degenerated primers, to amplify homologous (in different species) or paralogous (within a species) genes. Degenerated primers have a high but not complete sequences similarity to their target, which allows them binding to similar sequences and enables *in-vivo* detection of conserved regions of biosynthetic gene clusters [34]. Thereby, Vestola *et al*. detected the *has* NRPS gene in 31 cyanobacteria. It could be shown that although all investigated strains carried the *has* NRPS gene, five strains were not synthesizing the antimycotic compound [41]. In a further study Neilan *et al*. reported that 70 % of the investigated cyanobacteria contain NRPS gene clusters, which does not necessarily mean that an antimicrobial compound is synthesized by the organisms [42]. Genomic screening for synthesis pathways of bioactive compounds facilitates the identification of cyanobacterial workhorses, but verification of the actual product is crucial. For this reason, genome mining should be coupled to a subsequent bioactivity assay as well as isolation, purification and characterization of the product [43, 44]. Up to now, mainly phenotypic screening methods are used due to the small number of available genomes of cyanobacteria.

3 Strain Conservation of Cyanobacteria

The phylum ‘cyanobacteria’ includes 323 genera with approximately 2,698 known species [45] and represents one of the largest sub-groups of Gram-negative prokaryotes [46]. They occur on almost every place on earth, from oceans over freshwaters to deserts, rocks and hot springs. Due to the many different habitats cyanobacteria live in, they possess a great variety in form, metabolism and growth demands. Therefore, it is a challenging task to implement a strain conservation technique that fits every single strain. However, techniques suiting a wide range of different cyanobacteria were already described.

3.1 Cryopreservation of Cyanobacteria

In the area of biosciences, cryopreservation is the predominantly used method for strain conservation. Since it was (accidently) discovered in 1948 that glycerol can prevent fowl

sperm cells from freezing injuries, this discovery significantly promoted the field of cell conservation [47, 48]. For a long time, it was a common opinion that cryopreservation is not applicable for cyanobacterial strains, or at least only for a very limited number. After thawing of the cells, vitality often decreases dramatically and cells may suffer from freezing damages and die rapidly. A great improvement in cell vitality can be achieved by the two-step cooling method, using a controlled rate temperature decrease and, in most cases, a cell penetrating cryoprotective agent (CPA) [49].

In the process of cryopreservation, there are mainly two critical effects affecting cell viability. The first one being the formation of intracellular ice and the second one being osmotic stress, eventually followed by plasmolysis, caused by cell dehydration. Both effects are directly connected to the cooling rate in the course of cryopreservation. During slow cooling rates, the concentration of extracellular solutes increases, as pure water freezes more rapidly and gets eliminated from the solute-solvent system [50]. Consequently, intracellular water is osmotically driven out of the cells, which have a lower freezing point caused by a higher concentration of dissolved solutes. These physical mechanisms lead to a reduction in cell size and osmotic stress of the cells. However, a too rapid or uncontrolled cooling rate prevents proper dehydration of the cells, which dramatically increases the danger of intracellular ice formation. Cell penetrating CPAs like dimethyl sulfoxide (DMSO), glycerol or methanol (MeOH) are mostly used for the cryopreservation of cyanobacteria. They passively enter the cell over the cytoplasmic membrane, replace the intracellular water, and thus prevent the formation of cell-damaging ice crystals. Moreover, cell permeating CPAs prevent cells from excessive dehydration by equilibrating the osmotic pressure between the intra- and extracellular room. A certain incubation time of the cells after addition of the respective CPA at room temperature and under low light intensity assures the osmotic equilibration between solute concentrations outside- and inside of the cells [51]. However, a prolonged incubation time can also cause cell damages, especially if potentially toxic compounds as DMSO and MeOH are used [52]. In order to overcome the risks of cell damages during cryopreservation, most recent protocols use a two-step freezing method. As a first step, the prepared cryovials are frozen to a sub-zero temperature (usually between -30 and -40 °C), using a defined cooling rate. The cooling rate depends on the used strains, usually a decrease of -1 °C min^{-1} is used. In the course of cooling the cells get slowly dehydrated, which prevents the formation of intracellular ice crystals. The second step implies a rapid cooling to -196 °C with liquid nitrogen. After the freezing process, samples should be stored at ≤ -139 °C, as recrystallization processes can occur at higher temperatures [53]. However, not every laboratory has access to liquid nitrogen, so also higher storage temperatures (mostly -80 °C) are frequently used for cyanobacteria. The success of cryopreservation highly depends on the used strains. Cell viabilities can vary a

lot by application of different CPAs and cooling protocols. In **Table 1**, several cryopreservation approaches are listed, sorted by the respective cyanobacterial order. It shall provide a quick overview about successful cryopreservation methods (with and without usage of liquid nitrogen) and can give hints on appropriate approaches for not yet cryopreserved strains.

Table 1 Overview of different (successful) cryopreservation approaches sorted by cyanobacterial orders. **1S** = one-step freezing with controlled rate; **2S** = two-step freezing with controlled rate, **2S LN** = two-step freezing with controlled rate, involves Liquid Nitrogen; **SF** = one-step freezing without controlled rate. +, generally viable.

Cyanobacterial Order	*No. of strains*	*Vitality (vt), Viability (vb) [%]*	*(Best) Cryoprotectant*	*Cooling Protocol*	*Reference*
Chroococcales	9	> 90 (8/9) (vt)	DMSO 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
	1	> 75 (vb)	DMSO 5 % (v/v)	RT → -80 °C **SF**	[55]
	74	> 60 (62/74) (vb)	DMSO 3 % (v/v)	RT → -30 °C (-1 °C min^{-1}) → -196 °C **2S LN**	[56]
	6	100 (vb)	none	RT → -60 °C **SF**	[57]
	9	+ (8/9) (vb)	DMSO 15 % (v/v)	RT → -40 °C (-3 °C min^{-1}) → -196 °C **2S LN**	[57]
Chroococcidiopsidales	3	> 90 (vt)	DMSO/MeOH 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
Nostocales	34	> 90 (32/34) (vt)	DMSO 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
	2	78 - 97 (vb)	Glycerol 10 % (v/v)	RT → -80 °C **SF**	[55]
	28	> 60 (11/28) (vb)	DMSO 3 % (v/v)	RT → -30 °C (-1 °C min^{-1}) → -196 °C **2S LN**	[56]
	2	0-80 (vb)	none	RT → -60 °C **SF**	[57]

	8	+ (7/8) (vb)	DMSO 15 % (v/v)	RT → -40 °C (-3 °C min^{-1}) → -196 °C **2S LN**	[58]
	10	50-80 (9/10) (vt)	DMSO 5 % (v/v)	RT → - 80 °C (- 1 °C min^{-1}) **1S**	This work
Oscillatoriales	13	> 90 (12/13) (vt)	DMSO 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
	1	> 90 (vb)	Glycerol 10 % (v/v)	RT → -80 °C **SF**	[55]
	39	> 60 (24/39) (vb)	DMSO 3 % (v/v)	RT → -30 °C (-1 °C min^{-1}) → -196 °C **2S LN**	[56]
	1	100 (vb)	none	RT → -60 °C **SF**	[57]
	3	+ (7/8) (vb)	DMSO 15 % (v/v)	RT → -40 °C (-3 °C min^{-1}) → -196 °C **2S LN**	[58]
	2	> 50 (vt)	DMSO 5 % (v/v)	RT → - 80 °C (-1 °C min^{-1}) **1S**	This work
Pleurocapsales	2	> 90 (vt)	DMSO 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
	2	> 60 (1/2) (vb)	DMSO 3 % (v/v)	RT → -30 °C (-1 °C min^{-1}) → -196 °C **2S LN**	[56]
Synechococcales	29	> 90 (27/29) (vt)	DMSO 3 - 5 % (v/v)	RT → -40 °C (-4 °C min^{-1}) → -80 °C (-4 °C min^{-1}) **2S**	[54]
	1	> 80 (vb)	DMSO 1 % (v/v)	RT → - 80 °C (-1 °C min^{-1}) **1S**	[59]
	1	80 (vt)	DMSO 5 % (v/v)	RT → - 80 °C (-1 °C min^{-1}) **1S**	This work
Stigonematales	1	87 (vb)	Glycerol 10 % (v/v)	RT → -80 °C **SF**	[55]
	1	> 60 (vb)	DMSO 3 % (v/v)	RT → -30 °C (-1 °C min^{-1}) → -196 °C **2S LN**	[56]

Usually, 3 – 5 % DMSO (v/v) is the most appropriate CPA for the cryopreservation of cyanobacteria. Only few strains maintain a high viability with MeOH as CPA.

During the thawing process, it is essential that a quick warming rate is applied to the cells (*e.g.* water bath, 35 - 45 °C). This is proposed by almost all cryopreservation protocols, as rapid thawing minimizes the risk of intracellular water recrystallization. After thawing, the CPA should be quickly removed, particularly if cytotoxic substances as MeOH and DMSO were used. Glycerol generally won't harm cyanobacterial cells, but it can be used as carbon source by heterotrophic contaminants, if non-axenic cyanobacteria were cryopreserved. During the washing steps, light intensity should be maintained low and centrifugation steps should be avoided or reduced to minimum speed/duration.

3.2 Alternative Conservation Approaches for Cyanobacteria

There are also interesting alternatives to cryopreservation, two of which are shortly summarized in the following chapter.

3.2.1 Lyophilisation

In the process of lyophilisation, frozen samples are dehydrated under vacuum and at low temperatures. Through sublimation, ice crystals are directly converted to a gaseous state, without getting liquid. Lyophilisation is a rather rarely used method for the conservation of cyanobacteria. It has been shown only for a very limited number of strains/genera. Most filamentous, heterocysts-forming strains, like Nostoc or Anabaena, are relatively robust and survive freeze-drying. Cyanobacteria that produce akinetes, thick-walled, cold and desiccation resistant spores, are also described to be insensitive towards lyophilisation [60]. The production of a thick layer of extracellular polymeric substances (EPS) can also lead to a higher viability rate after re-culturing [55]; **Table 2**).

Table 2 Overview of different (successful) lyophilisation approaches with different cyanobacterial strains. +, generally vital.

Strain/s	Viability [%]	Lyoprotectant/ Suspending medium	Reference
Nostoc sp.	< 4	BG-11	[55]
Stigonema sp.	> 60	BG-11	[55]
Synechococcus cedrorum S1C1/J3C1	+	Lamb serum	[60]
Synechococcus elongatus S2C1	+	Human ascites fluid/Beef serum	[60]
Anacystis nidulans Ac1C1	+	Human ascites fluid/Lamb serum	[60]
Microcystis aeruginosa J1/M2/M4	+	Lamb serum/Fetal bovine serum	[60]
Merismopedia elegans Me1	+	Human ascites fluid/Lamb serum	[60]
Gloeocapsa calcarea G1	+	Lamb serum	[60]
Oscillatoria subbrevis Os1	+	Lamb serum	[60]
Anabaena flos-aque Ab1C1	+	Human ascites fluid/Lamb serum	[60]
Anabaena viariabilis Ab2C1	+	Lamb serum	[60]
Nostoc muscorum N1C1	+	Human ascites fluid /Lamb serum	[60]
Tolypothrix tenuis	58 – 92 %	Human serum albumin	[61]
Calothrix brevissima	42 – 96 %	Human serum albumin	[61]
Lyngbya sp. *487/488*	+	Horse serum	[62]
Lyngbya versicolor	+	Horse serum	[62]
Nostoc sp. *387/389*	+	Horse serum	[62]
Nostoc ellipsosporum	+	Horse serum	[62]
Phormidium luridum	+	Horse serum	[62]

When a suitable strain conservation technique needs to be chosen, it could be worth considering lyophilisation. If the concerning strains are amenable to this technique and show high viability rates afterwards, lyophilisation is a useful approach. Freeze-dried cells do not need to be stored at sub-zero temperatures and can be revived more quickly than cryopreserved cells. Moreover, lyophilisation reduces the risk of contaminations and does not promote growth of heterotrophic contaminants [55]. This can be the case after cryopreservation, with CPAs like glucose.

3.2.2 Immobilization

The immobilization of cyanobacterial cells has already been used for a wide range of applications. In photobioreactors, immobilized cyanobacteria can be used for the continuous production of valuable bioproducts [63–65]. Further advantages are the simplification of downstream processes; higher cell densities, combined with improved production effectivity, and the applicability of higher dilution rates [63]. Immobilized cyanobacteria can as well be used for bioremediation processes [66, 67], or the detection of pollutants [68]. However, immobilization can also be applied for the conservation of cyanobacteria. In 1988, Lukavský immobilized six cyanobacterial strains (among several eukaryotic algae) in 2 % agar and stored them under low light intensity at 10 °C. After 32 months, the cells were transferred to fresh cell medium and showed good growth at standard culturing conditions. Overlying the agar tubes with paraffin oil clearly decreased the recovery rate [69]. Also alginate beads (hardened with $CaCl_2$) are suited as shown for the filamentous cyanobacterium *Pseudoanabaena galeata*, which could be preserved for 14 – 18 month at 4 °C in the dark, without a decrease in growth rate or alteration of physiological characteristics [70]. Thereby, cyanobacterial cell metabolism was drastically decreased, or completely stopped. This was indicated by a constant number of cells and no significant alterations in C, H, N and P content [70]. Thus, it is highly likely that genetic changes through mutations or selection of subspecies do not occur. Conservation of cyanobacteria by immobilization with agar or alginate is an interesting alternative to established cryopreservation. It has the advantages of being cheap and accessible for any laboratory. Furthermore, cells can be re-cultured more rapidly than after cryopreservation. Even an encapsulation device for the automated and continuous production of alginate beads has been proposed [71]. However, there is not much data about, which cyanobacterial strains are suited for this kind of conservation method nor is it known, how long cells can be stored this way.

3.3 Commonly used Pre-culture Technologies in Algae Biotechnology

In general, cryopreserved cultures are used to inoculate pre-cultures in lab-scale. To subsequently inoculate the main culture, the cells are harvested in the exponential phase. This procedure minimizes differences in performance, ensures identical starting conditions and minimizes DNA mutations. Different protocols for cryopreservation of microalgae are established and described in the previous chapters, but since microalgae are growing very slow this standard procedure would be very time-consuming. Traditionally, cyanobacteria are preserved as metabolically active serial subcultures, which must constantly be transferred to fresh culture medium in intervals depending on the growth rates of the respective strains. Often, cyanobacteria are immobilized on agar plates and stored at 4 °C and low light intensities resulting in low biomass formation by simultaneously high viability

of the cells (up to 80 %) [72]. In regular intervals, new medium is added to provide the necessary nutrients. This biomass is then used to start a pre-culture. This kind of strain conservation provides the constant availability of vital cyanobacterial cell mass, which is especially helpful for slow-growing strains. However, for medium to large strain collections, this method has the clear disadvantage of being extremely time consuming and labour intensive. Additionally, it was reported that continuous serial subcultures can lead to alterations in the morpho- and genotype due to selection towards subspecies and DNA mutation [73, 74]. Thus, serial subcultures can be useful for a limited period, if fresh cell material is constantly needed. But for long time storage of cyanobacterial strains, cryopreservation or immobilization should be the method of choice.

However, since serial sub-culturing is often used the influence on the main culture needs to be investigated. It is unknown if different growth phases and the fluctuating nutrient concentrations of the pre-culture lead to a different performance in the main culture. To investigate the impact of the age of pre-culture on main cultures, *Nostoc* sp. was cultivated for 56 days in shaking flasks without baffles in a shaking incubator at 24 °C without any medium exchange. After 21, 28, 35, 42, 49 and 56 days of cultivation biomass was harvested and used to start the main cultures that were then cultivated for 14 days in shaking flasks at different light intensities. The typical growth curve of cyanobacteria could be detected including a lag-, an exponential as well as the beginning of the stationary phase (see **Fig. 2** A).

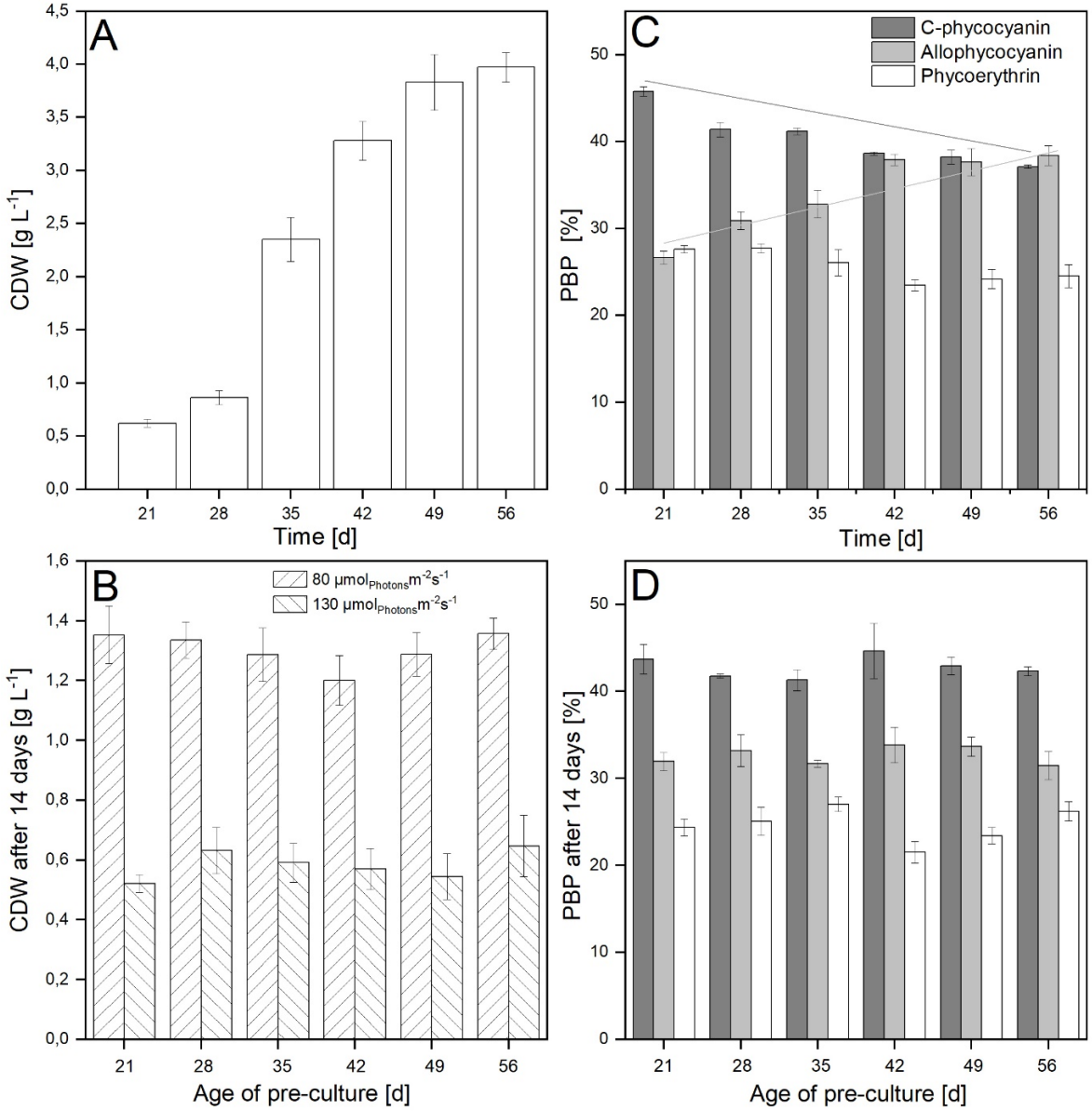

Fig. 2 Influence of different growth phases on the main culture of *Nostoc* sp. A, Cell dry weight (CDW) of the preculture over cultivation time. B, CDW of the respective main cultures inoculated with pre-culture of different age. CDW was determined after 14 days of cultivation at 80 and 130 $\mu mol_{photons}\ m^{-2}s^{-1}$, respectively. C, Influence of the pre-culture cultivation conditions on phycobiliprotein ratio in percent. D, Ratio of phycobiliproteins in the main culture after 14 days of cultivation. Cultivation parameters: 300 mL shaking flasks without baffles, inoculation with 0.1 g cell wet weight (CWW) of *Nostoc* sp. per 50 mL BG-11 medium, 24 °C, 120 rpm (eccentricity 2.5 cm), 130 $\mu mol_{photons}\ m^{-2}s^{-1}$, except the main cultures, n_b (biological replicates) = 3.

In this study, the age of pre-culture had no influence on biomass formation under two different conditions (see **Fig. 2** B). For vitality determination the resazurin assay was used [75]. No influence on vitality could be detected over cultivation time (data not shown). EPS, Pigments and PBP were determined using the method described by Strieth and Stiefelmaier *et al*. [76]. Again, the age of pre-culture had no influence on EPS formation and phycobiliproteins (see **Fig. 2** D) as well as pigment composition (data not shown). Furthermore, the total amount of PBP decreased, whereby also the amount of

C-phycocyanin decreased over time. C-phycocyanin is also used as nitrogen storage is thus degraded due to the decrease of nitrogen in the medium over time. That was interesting, because the ratio of PBP changed in the pre-culture but had no influence on PBP ratio in the main culture.

In conclusion the age of culture had no influence on the productivities of the slow-growing organism of the main culture in terms of biomass formation, but serial subcultures can lead to alterations in the morpho- and genotype due to formation of subpopulations and DNA mutation. Thus strains have to be regularly checked for genomic and morphological integrity and should also be available as original cryopreserves .

4 Characterization of Cyanobacteria

When establishing a suitable conservation method for individual cyanobacterial strains, the evaluation of the cells' condition before and after the conservation process is essential. In contrast to cell vitality tests, viability assays can only superficially differentiate between living and dead cells. Intracellularly impaired or dying cells will still be identified as viable cells. However, such tests are fast and will give first quick indications about cell fitness. For more reliable data, time-consuming cell vitality assays need to be accomplished.

4.1 Cell Vitality

As cyanobacteria tend to have slow to very slow growth rates, a vitality check through this variable can be a time-consuming matter. In addition, the calculation of growth rates by absorption measurement is often not applicable for cyanobacteria. Especially cell aggregates interfere with spectrophotometric methods. In this subchapter, alternative methods for determining cell vitality are introduced.

4.1.1 *In vivo* Growth Fluorometry

A widespread method for the vitality determination of cyanobacteria is the quantification of cell growth by measuring the *in vivo* fluorescence of chlorophyll a. This method is used for diatoms [77], cyanobacteria [78] and green algae [79]. Fluorometric measurements can be performed with a spectrophotometer. A modified variant has been implemented by Karsten *et al*. (2006), in which microalgal cells can be measured in petri dishes [78]. The method's principle remains the same, chlorophyll a gets excited by a light source at 435 - 470 nm [78, 80] and emits light of slightly lower energy. The light intensity is measured by a detector and used for the calculation of growth rates, without need for calibration. An excitation wavelength of 630 nm was proposed for the measurement of phycocyanin and allophycocyanin in cyanobacteria [81]. An advantage of the method is the low amount of needed cell mass. Karsten *et al*. (2006) only used 0.5 µg L^{-1} per run. That minimizes the risk of self-shading and scattering effects [80]. Another benefit is the specificity of the

method, as only living cells are measured and heterotrophic contaminants can be distinguished from cyanobacterial cells [82]. However, although single measurements are only a matter of seconds, the vitality test is still based on the determination of culture growth. Thus, for slowly growing strains several days of culturing are required. Another difficulty of using fluorometry for cyanobacterial growth monitoring is that many strains form cell clusters and aggregates with EPS and are therefore not evenly dispersed in the culture medium. These clusters tend to sediment quickly, which prevents the collection of samples and the quantitative measurement from being reproducible.

4.1.2 Resazurin Assay

The resazurin assay, or Alamar Blue Assay, is based on the reduction of the barely fluorescent, dark purple dye resazurin (redox and pH indicator) to resorufin, which is pink and highly fluorescent. This reaction occurs in a vital cell. If the metabolic activity of the cell is reduced (less vital), less resazurin is converted. By measuring the fluorescence, conclusions can be drawn about the cells vitality. This method is mainly used as bioactivity assay where different amounts of extracts are tested against *e.g. Escherichia coli*. The test strain is resuspended in a buffer solution and placed together with the respective extract and resazurin into a microtiter plate for cultivation. After incubation a colour change indicates no inhibition of the test strain by the used extract [83]. This method cannot only be used as bioactivity assay. Mehring *et al*. showed that the test is also usable to detect vitality of cyanobacteria and is transferable to heterotrophic bacteria and callus cells [75]. In this case, a certain amount of biomass is resuspended in a buffer solution and resazurin is added. The reaction to resorufin only takes place in viable cells and the intensity of the resulting fluorescence can be correlated to metabolic activity. This is a medium to high throughput method for fast and easy determination of cell vitality, which can also be well automated.

4.1.3 Vitality Determination by pO_2 Measurements

The evaluation of cyanobacterial cell vitality should include more classifications than “alive” or “dead”, as important metabolic functions or cell compartments can be damaged without leading to cell death but hampering growth. Therefore, growth experiments are a reliable basis to judge cell viability. However, they have the disadvantage of being very time-consuming in case of cyanobacteria. Since these bacteria perform an oxygenic photosynthesis oxygen is produced during photosynthetic activity. Consequently, oxygen is a good indicator for viable, growing cells. This fact was used to establish an easy vitality test, based on pO_2 measurements [84] (**Fig. 3**).

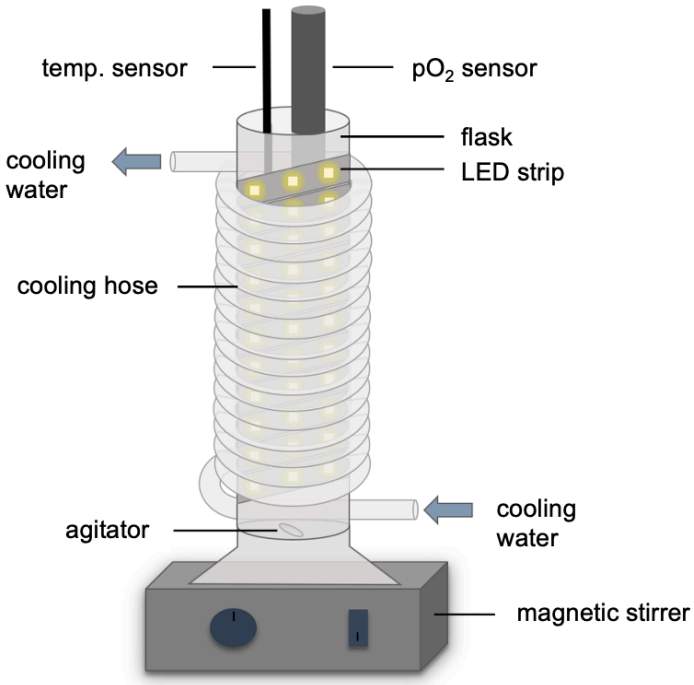

Fig. 3 pO_2 measurement setup for the *in vivo* vitality determination of cyanobacteria. The working volume is 20 - 50 ml. Modified according to Witthohn *et al*., 2020 [84]. Reproduction of this figure is granted by a Creative Commons Attribution License.

A cell wet mass pellet of 0.5 g cryopreserved and thawed *Nostoc* sp. was resuspended in BG-11 medium and applied to the measuring flask. The culture was stirred at about 500 rpm and heated to 27 °C. The LED strip provides light for photosynthesis; the pO_2 increase was measured by a sensor inside the culture medium. The slope of the resulting graph can be compared to the one obtained by fresh, not cryopreserved cells (**Fig. 4A**) [84]. In this way, different CPAs can be tested in a relatively small amount of time. For example, the results shown in **Fig. 4B** can theoretically be obtained in 5 – 6 hours, as one run takes about 30 min.

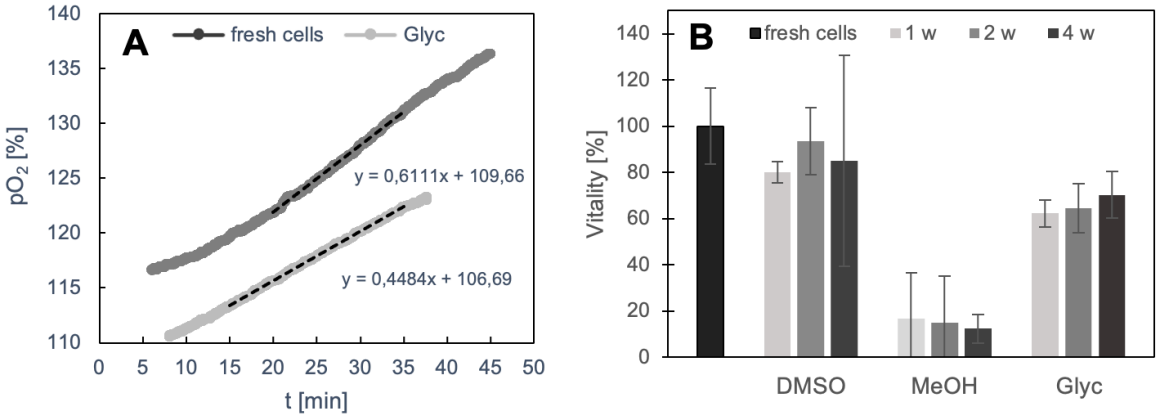

Fig. 4 A, pO_2 increase for vitality determination of *Nostoc* sp. (formerly referred to as *Trichocoleus sociatus*) cells cryopreserved with glycerin as CPA. Measurements have been performed in the device shown in figure 3. B, obtained vitality data of *Nostoc* sp. cells cryopreserved with different CPAs. Modified according to Witthohn *et al*., 2020 [84]. Reproduction of this figure is granted by a Creative Commons Attribution License.

Thereby it could be shown that DMSO is the most appropriate CPA for cryopreservation of *Nostoc* sp. Moreover, the comparison of these results with data from "classic" growth experiments demonstrate the reliability of the pO_2 measurement technique (**Fig. 5**).

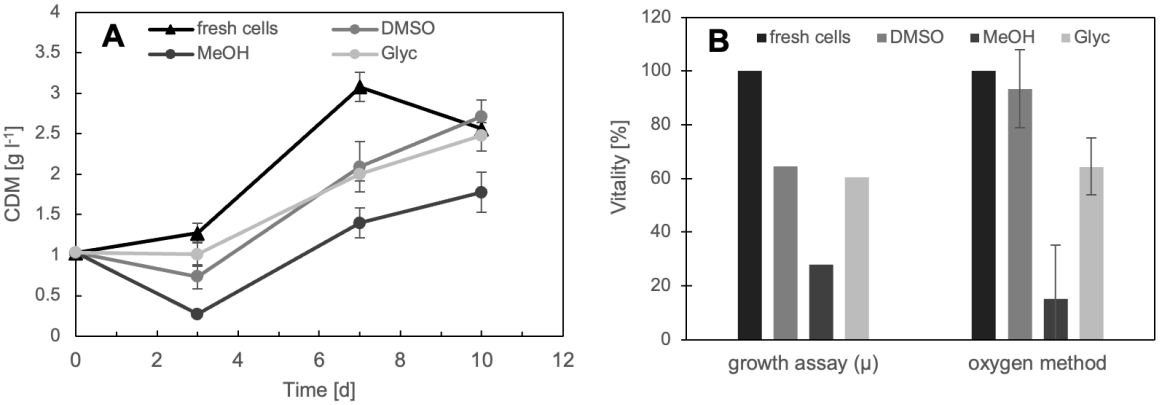

Fig. 5 A, Growth assay with *Nostoc* sp. (formerly referred to as *Trichocoleus sociatus*) by determination of CDM. The cells were cryopreserved for two weeks with different CPAs (DMSO/MeOH 5 %, Glyc 15 % v/v). B, comparison of growth assay and pO2 measurement as vitality determination methods. Modified according to Witthohn *et al*., 2020 [84]. Reproduction of this figure is granted by a Creative Commons Attribution License.

The vitality determination by means of pO_2 increase constitutes an easy and functional approach for the quick evaluation of cyanobacterial cell states. The data shown for *Nostoc* sp. could reflect the growth behaviour in shaking flasks. Although this method can give no hints on specific cell damages as vital staining with different dyes does, it nevertheless can be used to predict the anticipatory cell growth. As the proof of concept was only presented for one strain, more data with different cyanobacteria would be interesting.

4.1.4 Spectral domain Optical Coherence Tomography (sdOCT) and Pulse Amplitude modulated (PAM)-fluorometry

In this chapter, two further non-invasive methods to characterize cyanobacterial growth are presented; the spectral domain optical coherence tomography (sdOCT) and pulse amplitude modulated (PAM)-fluorometry (**Fig. 6**). The growth behaviour under identical cultivation parameters can be seen as indicator for cell vitality. Cyanobacterial growth on surfaces can be measured by means of chlorophyll activity using a PAM-fluorometer (Imaging-PAM). A saturation pulse in the form of red light (620 nm) is given via the PAM fluorometer, which excites the chlorophyll molecules. This raises the electrons in the electron transport chain to a higher energy level. When the electrons fall back to their ground state, energy is released in the form of heat or radiation. This energy can also be used for photochemical processes. Accordingly, radiation is at its maximum, when the energy used for photochemical processes is at its minimum. The reaction centre of photosystem II can be inactivated, or the electron acceptors reduced by a short, strong flash of light, allowing the radiated heat to be measured [85]. The Imaging-Win software (Heinz Walz GmbH, Effeltrich, Germany) can be used to record the emitted radiation and thus determine the spread of the biofilm in two-dimensional space via the activity of the reaction centre (photosystem II). By this, it is possible to describe growth curves of surface-associated growing cyanobacteria in a short time (about 1 minute per plate of 5 colonies). The technique is thus applicable for the evaluation of cyanobacterial cell viability and cell vitality after cryopreservation, for example.

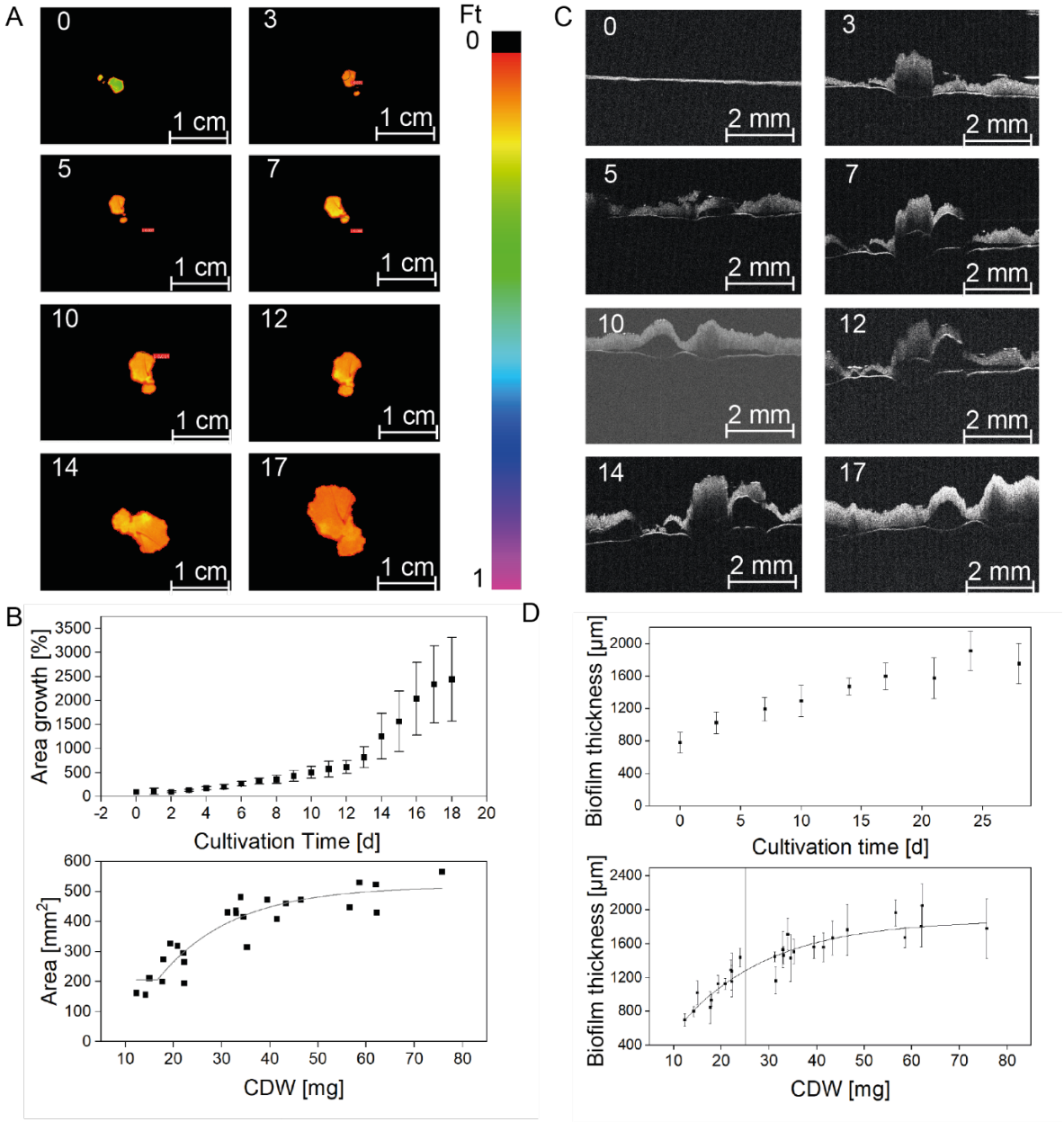

Fig. 6 Non-invasive methods for cell growth characterization as indicator for cell vitality by the example of *Nostoc* sp. (A) Area growth determined by chlorophyll-a fluorescence using PAM fluorometry over a cultivation period of 17 days. n_b (biological replicates) = 3 (B) Area growth in percent over cultivation time and correlation of area with cell dry weight (CDW). (C) Biofilm thickness measured using OCT over a cultivation period of 17 days. n_b = 3, n_t (technical replicates) = 30 (D) Biofilm thickness over cultivation time and correlation of biofilm thickness with cell dry weight (CDW). Cultivation parameters: Solid BG-11 medium, 24 °C, 100 $\mu mol_{photons}\ m^{-2}s^{-1}$, 400 ppm CO_2, 30 days, n_b = 3

In principle, the method relies on measuring the surface covered by cyanobacteria when growing attached to a surface as a biofilm, like on an agar plate. This is accomplished by measuring chlorophyll a emission when excited at 620 nm (**Fig**. **6** A). Both, the fluorescence intensity, and the area from which fluorescence occurs can be measured. Only the absolute area is used to calculate growth. The areas can be reproducibly measured by defining fixed thresholds for fluorescence. The cyanobacterial growth is described as the increase in the

surface covered by the bacteria (**Fig**. **6** B). Typical growth phases of the biofilm area over the cultivation time could be reported [86]. The correlation between area and biomass was linear up to ten days, afterwards deviations between replicates increased (Fig. **6** B). This can be explained by the increasing biofilm thickness that can be determined using non-invasive sdOCT (**Fig**. **6** C). Here, the contrast is achieved by the different light scattering properties of the biofilm and thus provides information about its microstructure and thickness without any use of contrast agents [87]. A problem when using sdOCT to determine the layer thickness is that above a certain biofilm thickness (also depending on the pigment content), mutual shadowing occurs, and the biofilm cannot be completely imaged. In this case, it is no longer possible to distinguish between cavity and shadowing, which makes the evaluation of the data more difficult. Furthermore, it is not possible to distinguish between water deposits and biofilm as well as between cells and EPS [86]. Similar to the spreading of the biofilm, biofilm thickness could also not be linearly correlated with CDW meaning (see **Fig. 6** D), real growth rates cannot be determined. This is probably because the ratio of biomass to EPS changes over the cultivation period (data not shown), which allows the biofilm to become thicker without forming cell mass. Furthermore, the thickness of the biofilm depends on the stored water in the EPS. Therefore, this method can be used to determine re- and dehydration of biofilms (**Fig**. **7**). Biofilm thickness changes and the associated water loss of *Nostoc* sp. biofilms growing on borosilicate glass, PMMA and silicone at 24 °C and a relative humidity of 30 % (typical cultivation conditions in aerosol-based photobioreactors (description of the PBR see chapter 0) without aerosol supply) were documented by sdOCT (**Fig**. **7**). To better compare the influence of different substrates on the dehydration of the biofilm, the respective half-lives were calculated at which the biofilm showed 50 % of its initial thickness. Respective values determined were 30.27 ± 7.26 min (borosilicate glass), 27.60 ± 5.13 min (PMMA) and 18.90 ± 6.70 min (silicone). In comparison, a water film reduced its thickness by 50 % after only 8 min (data not shown).

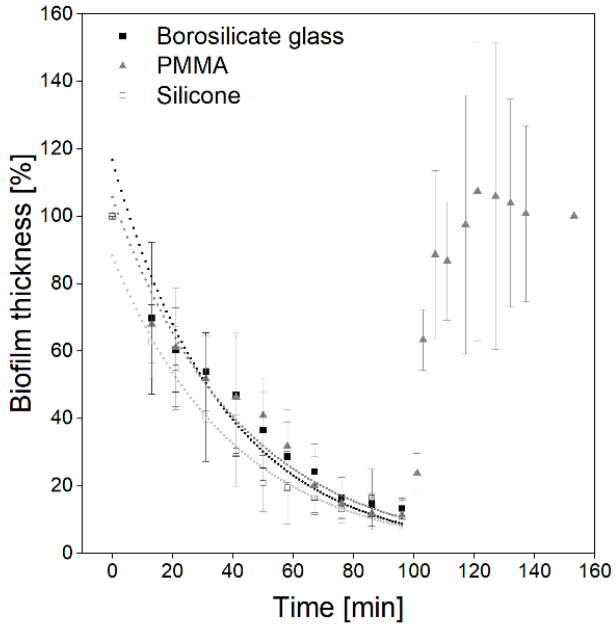

Fig. 7 De- and rehydration of *Nostoc* sp. biofilms. Dehydration was performed on different materials (borosilicate glass, PMMA, and silicone) and rehydration was only performed on silicone in an aerosol-based photobioreactor. The biofilm thickness was recorded by OCT at 24 °C and 30 % relative humidity and plotted as a percentage (n_b = 5).

This shows that EPS protects the biofilm from dehydration and that the change in layer thickness can be determined reliably and with low deviations using sdOCT. Based on the results obtained, sdOCT is suitable for visualizing the surface morphology and for dehydration and rehydration experiments of biofilms, assuming that no cavities are formed. Additional, growth curves can be used as additional information source to gain more information about the state of the cells. Furthermore, characterization of growth behaviour using PAM fluorometry or sdOCT under the same cultivation conditions can be applied to determine cell vitality and viability, since growth only occurs when the cells are viable and biomass formation depends on metabolism activity (cell vitality). Both methods are suitable for a fast und simple characterization of the surface-associated growth of cyanobacteria [86]. It should be mentioned again, that for the calculation of growth rates a linear correlation between horizontal spreading and CDW is essential, which is not possible with this method. However, sdOCT is suited for characterization of surface attached cyanobacteria and allows to obtain qualitative data on biofilm development and cell vitality.

4.2 Cell Viability

In contrast to cell vitality tests, viability assays can only superficially differentiate between living and dead cells. Intracellularly impaired or dying cells will still be identified as viable cells. However, such tests are fast and will give first quick indications about cell fitness. For more reliable data, time-consuming cell vitality assays need to be accomplished.

4.2.1 Staining Methods

In many cases, the number of living cells is used as an indicator for culture viability after cryopreservation. For this purpose, the cells are stained and evaluated by microscopy. In this process either only dead cells are stained, as the colorants can cross their damaged cell membrane, or only living cells are stained because of enzymatic activation of the dye. A staining approach addressing cell vitality by combining different stains was developed by Tashyreva *et al*. (2013). They used a series of different dyes to determine the physiological state of *Phormidium* cells. With SYTOX green, damaged cell membranes could be revealed; by staining with 4',6-diamidino-2- phenylindole (DAPI), degraded DNA was shown and with 2-(4-Iodo-phenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 5- Cyano-2,3-Ditolyl Tetrazolium Chloride (CTC) the respiratory function of the cells could be verified.

There are several staining methods for cell viability testing established for bacteria, however, not all work for cyanobacteria. For example, it was found that propidium iodide, which is meant to stain only non-viable cells, also stained viable cells of filamentous cyanobacteria [88]. It was postulated that this is due to intracellular channels between the cells [89, 90]. In the following, compounds frequently used for viability staining of cyanobacteria are presented. Depending on the used substance, stained cells can be observed either via light, or fluorescence microscopy. The bisazo dye tryptan blue [54, 91] and the triphenylmethan compound erythrosine b [92] are staining agents detectable via light microscopy. Tryptan blue binds to proteins of cells with an impaired cell wall; the staining procedure takes 5 to 10 min [91]. Intact cell walls of viable cells are not permeative for the dye. A prolonged incubation should be avoided since tryptan is cytotoxic and can thus lead to false positive results. Moreover, this substance should be handled with care, as it is teratogenic [93] and carcinogenic [94]. Erythrosine b on the other hand is used as a food colouring and therefore a non-toxic compound. The staining process is as quick as with tryptan blue and it also acts on proteins of cells with damaged cell walls [95].

Fluorescein diacetate (FDA) [96–99] and SYTOX green are fluorescent stains frequently used for cyanobacteria [100–102]. FDA is a non-fluorescent molecule which can cross the bacterial cell membrane of living cells. Upon entering the cell the compound is hydrolized to the yellow-green fluorescent compound fluorescein, which can be detected under ultraviolet light [103]. It is often used in combination with propidium iodide, an analogon of ethidium bromide, which stains DNA of dead cells [97]. SYTOX green also acts as nucleic acid stain of cells with damaged plasma membranes [104]. It can be excited at 488 nm and emits light of 523 – 530 nm. However, just as propidium iodide, it was described as non-applicable for filamentous cyanobacteria [89].

5 Photobioreactors

Due to their diversity, cyanobacteria can be cultivated in many different ways. Heterotrophic cultivation in stainless steel fermenters, for example, is possible, although it has some disadvantages compared to phototrophic cultivation [105]. For example, there is a risk of contamination by adherent heterotrophic bacteria, which cannot be completely removed from the cultures even with complex isolation methods [106–108]. Another major advantage of phototrophic and mixotrophic cultivation is that CO_2 can be used as a carbon source. This not only allows the use of cheaper cultivation media, but is also of great interest, especially in the current times when global climate goals depend on a reduction of carbon dioxide emissions. Chapter 6 gives a more detailed view on the advantages of the different cultivation modes of cyanobacteria, with a focus on mixotrophic cultivation. Furthermore, the type of cultivation has an influence on the profile of the synthesised secondary metabolites, making some processes depended on photo-autotrophic growth. The systems for phototrophic cultivation can be divided into open and closed systems based on their design. Open systems in the form of open ponds are still the most commonly used form for large-scale cultivation. This is mainly due to the fact that they are cheap and easy to set up and operate. However, due to the open design, there is a high evaporation rate of water and additionally a risk of contamination. Furthermore, gas exchange is poor, leading to low biomass productivities, which in turn adds to the bad biomass to land ratio of such facilities. Therefore, open pond systems are not covered further here, instead this chapter is focussing on closed photobioreactors (PBR).

5.1 Closed photobioreactors (PBRs)

Closed systems have several advantages over open systems. The risk of contamination is significantly reduced, and the closed design enables more diverse construction options. This allows the surface area to volume ratio to be optimised, which is particularly important with regard to an optimal light supply for phototrophic organisms. As light is not dispersible, systems with a low surface area to volume ratio lead to inhomogeneous light distribution. A good compromise must be found here, as a large surface area to volume ratio automatically results in a large footprint of the reactor. The light can be supplied either as artificial or natural light and also the material of the PBR will heavily impact light intensities and quality due to different refractive indices. Optimized light supply significantly increases biomass productivity compared to open ponds. Furthermore, the use of resources such as water is also improved, as the problem of evaporation is eliminated. In this chapter, only a brief description of the different submerged PBR designs is given, as the focus here will be on biofilm reactors. The interested reader is referred to existing reviews in the literature [109–111].

The simplest form of a submerged PBR is the stirred tank, which due to its geometry offers poor light distribution and is therefore less suitable for phototrophic cultivation. Flat panel PBR are characterised by small depth, improving light supply. Beside these, there are multiple PBR designs based on tubular geometry. Vertical tubular PBR, are operated as a bubble column or airlift reactor and the mixing in these reactors can be controlled by adjusting the gassing rate. Horizontal tubular PBRs consist of tubes that can be arranged in different shapes and lengths. The horizontal arrangement simplifies the illumination by natural light [109]. However, submerged systems are not the optimal cultivation method, especially for biofilm-forming cyanobacteria, as they do not grow submerged by nature but surface-associated.

5.2 Attached (Biofilm) Cultivation of Cyanobacteria

Biofilm formation usually follows the following principle [110]: i) initial adhesion of cyanobacteria to the surface by adsorption (reversible), ii) adhesion by the formation of EPS (irreversible), iii) formation of EPS, iv) biofilm growth by attachment of additional cyanobacteria and organisms, and v) partial detachment of the biofilm due to loss of integrity. The formation of a biofilm and the resulting growth in this form can be utilised with the help of specially designed reactors. Cultivation in the form of biofilms can thus bring a multitude of advantages, like the potential of a reduced water to biomass ratio [112] and a reduction in costs compared to submerged PBRs. The reduced costs are the result of several factors. These include the already mentioned reduced water consumption as well as increased biomass production. Furthermore, the harvesting of cyanobacteria as biofilm is significantly simplified, since the biomass can, for example, simply be scraped off the surface and the separation of the biomass from the process water is much easier than for planktonic cultures. Depending on biofilm thickness, cells benefit from better light availability especially in the outer regions of the biofilm. The lower layers may become light limited if the biofilm is too thick, which can reduce the productivity of the cells in the biofilm [113]. This problem can be avoided by regular harvesting, which in turn is not a major problem if considered in the design of the reactor [114]. Another advantage of regular partial harvesting is a faster re-growth of the biofilm, which in turn can increase productivity [115]. A disadvantage of biofilm cultivation is the unwanted, spontaneous detachment of biofilm, which then continues to grow in the medium, or biofouling and clogging of the complete PBR system.

In the cultivation of biofilms, a distinction can be made between submerged systems, in which cultivation takes place in a liquid medium, and surface-associated systems, in which the biofilms grow air-exposed. These systems will be discussed separately in the following. Table 3 gives an overview on existing systems for biofilm cultivation attached to surfaces.

The table shows that most systems are aimed at either optimising biomass productivity or maximising the production of lipids, which can be used *e.g.* for the production of biofuels. In addition, the most important application is the treatment of wastewater, to remove high concentrations of pollutants, like nitrogen and phosphorus.

Table 3: Comparison of different Photobioreactors for the cultivation of cyanobacteria or microalgae attached to surfaces. (PBR – photobioreactor, NA – not applicable)

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity (g m^{-2} day^{-1})	Reference
Vertical PBR	Air-exposed	*Phormidium*, *Pseudanabaena*, *Nitzschia*, *Scenedesmus*	Removal of nitrogen and phosphorous	-	0.125	6.70 – 7.20	[114]
Multiple plates PBR	Air-exposed	*Botryococcus braunii*	Lipid and hydrocarbon production	-	0.54	49.10	[116]
Multiple layer vertical plate attached PBR	Air-exposed	*Scenedesmus obliquus*	Lipids	-	0.54	50 - 80	[117]
Multi-skin sheet emerse PBR (MSSePBR)	Air-exposed	*Coleofasciculus chtonoplastes*, *Nostoc* sp.	Biomass	-	0.046	1.7	[118]
Capillary-driven PBR (CPBR)	Air-exposed	*Scenedesmus* sp.	Lipid production, removal of nitrogen and phosphorous	-	0.077 – 0.538	10	[119]
Twin-layer PBR	Air-exposed	*Isochrysis* sp., *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Nannochloropsis* sp.	Biomass	-	10.72	0.6 - 1.8	[120]

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity (g m^{-2} day^{-1})	Reference
Biofilm cultivation system	Air-exposed	*Haematococcus pluvialis*	Optimization of water footprint	-	0.001	6.0	[121]
Attached biofilm reactor	Air-exposed	*Pseudochlorococcum*	Biomass	-	0.00025	6 - 8	[122]
Emerse PBR (ePBR)	Air-exposed	*Nostoc* sp.	Biomass and EPS	-	0.0025	2.4	[123]
Twin-layer system	Air-exposed	*Halochlorella rubescens*	Removal of nitrogen and phosphorous	-	NA	6.3	[124]
Attached PBR	Air-exposed	*Scenedesmus* sp.	Removal of NH_4Cl, $CuSO_4$, tertracycline, norfloxacin and sulfadimidine	-	NA	6.2	[125]
Fixed-bed biofilm reactor (FBR)	Air-exposed	*Chlamydomonas* sp.	Biomass, removal of nitrogen, phosphorous and Cu(II)	-	0.8	49.70	[126]
Horizontal flow lanes	Intermittently submerged	*Phormidium*, *Pseudanabaena*, *Nitzschia*, *Scenedesmus*	Removal of nitrogen and phosphorous	-	0.140	4.50 - 9.90	[114]

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity (g m^{-2} day^{-1})	Reference
Revolving algal biofilm (RAB)	Intermittently submerged	*Chlorella vulgaris*	Biomass	-	2.94 or 1.85	18.90	[127]
Rotating algal biofilm reactor (RABR)	Intermittently submerged	Mixed algal culture	Removal of nitrogen and phosphorous	8 | 535 | 8000	0,24 | 2,72 | 4,26	5,50 | 20,00| 31,00	[128]
Rotating biological contactor (RBC)	Intermittently submerged	*Chlorella sorokiniana*	Biomass	11	0.362	20.1	[129]
Photorotating biological contactor (PRBC)	Intermittently submerged	*Klebsormidium* sp.	Biomass	15	1.57	0.45	[130]
Rotating biological contactor (RBC)	Intermittently submerged	Mixed algal culture	Treatment of aqueous effluent containing diesel oil	4	0.83	NA	[131]
Rotating flat plate (RFP) PBR	Intermittently submerged	*Chlorella vulgaris*	Biomass, lipids	8	0.286	2.99	[132]
Rotating algal biofilm reactor (RABR)	Intermittently submerged	Mixed algal culture	Removal of wastewater nutrients, lipid production	8	NA	0.96	[133]

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity ($g\ m^{-2}\ day^{-1}$)	Reference
Parallel plate air lift (PPAL)	Submerged	*S. obliquss*, *C. vulgaris*, *Coccomyxa* sp., *Nannochloris* sp., *Nitschia palea*, *Oocystis* sp., *Oocystis polymorpha*	Lipids	15	0.064	1.10 - 2.08	[134]
Biofilm membrane PBR (BMPBR)	Submerged	*Chlorella vulgaris*	Secondary effluent treatment	280	-	NA	[135]
Attached algal culture system	Submerged	Mixed algal culture	Fatty acids production, removal of nitrogen and phosphorous	0.2	0.0136	0.58 - 2.57	[115]
Attached algal cultivation	Submerged	*Scenedesmus*, *Chlorella*, *Pediastrum*, *Nitzschia*, *Cosmarium*, filamentous microalgae and others	Lipids	8000	33.1	9.10	[136]
Semi-continuous flat plate parallel horizontal PBR	Submerged	*Scenedesmus obliquus*, *Nitzschia palea*	Lipids	0.288	0.072	2.1 – 2.8	[137]
Algae biofilm PBR	Submerged	*Botryococcus braunii*	Lipids	NA	0.275	0.71	[112]

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity (g m^{-2} day^{-1})	Reference
Moving bed PBR	Submerged	*Nostoc* sp.	Biomass	65	11.26	NA	[138]
Roof-installed parallel plate microalgae biofilm reactor	Submerged	*Scenedesmus obliquus*	Wastewater treatment	5	0.5	2.5	[139]
Algal turf scrubber	Submerged	Mixed algal culture	Removal of nitrogen, phosphorous and chemical oxygen demand	200	1	5.0	[140]
Horizontal flat panel (HFP) PBR	Submerged	Mixed algal culture	Removal of phosphorous	NA	2	12.21	[141]
Algal biofilm reactor (ABR)	Submerged	*Chlorella, Phormidium*	Biomass	3	0.063	4.0	[142]
Algal turf scrubber (ATS)	Submerged	Mixed algal culture	Phosphorous removal	-	2.67	33 - 39	[143]
Biofilm capillary reactor	Submerged	*Synechocystis* sp.	Biomass	NA	NA	NA	[144]
Attached PBR	Submerged	Mixed algal culture	Biomass, nutrient removal	15	0.171	1.57 – 1.91	[145]

Reactor	Biofilm placement	Cyanobacteria/Microalgae	Product	Liquid reservoir (l)	Growth surface (m^2)	Biomass productivity ($g\ m^{-2}\ day^{-1}$)	Reference
Tubular biofilm PBR	Submerged	*Chlorella sorokiniana*	Removal of carbon, nitrogen and phosphorous	7,5	1.1	NA	[146]
Algal-based immobilization reactor	Submerged	*Scenedesmus*	Removal of nitrogen and phosphorous	96	NA	NA	[147]

5.3 (Partly-)Submerged Biofilm PBRs

The reactor systems for submerged or partly-submerged cultivation of biofilms can be further divided (see **Fig. 8**). One possible classification is into fixed bed, in which the biofilm grows on a fixed surface, and fluidised bed reactors, in which the support material behaves like a fluid. The fixed bed can be further subdivided into vertical, horizontal and rotating reactors, depending on the orientation of the support material. For fluidised bed reactors, a further distinction can be made between mobilised and immobilised systems [148]. A further possible division of (partly-)submerged biofilm PBRs is the distinction between dynamic and stationary systems, whereby dynamic systems include all reactors in which the substratum is moved [149]. This categorisation of reactors is also applied here.

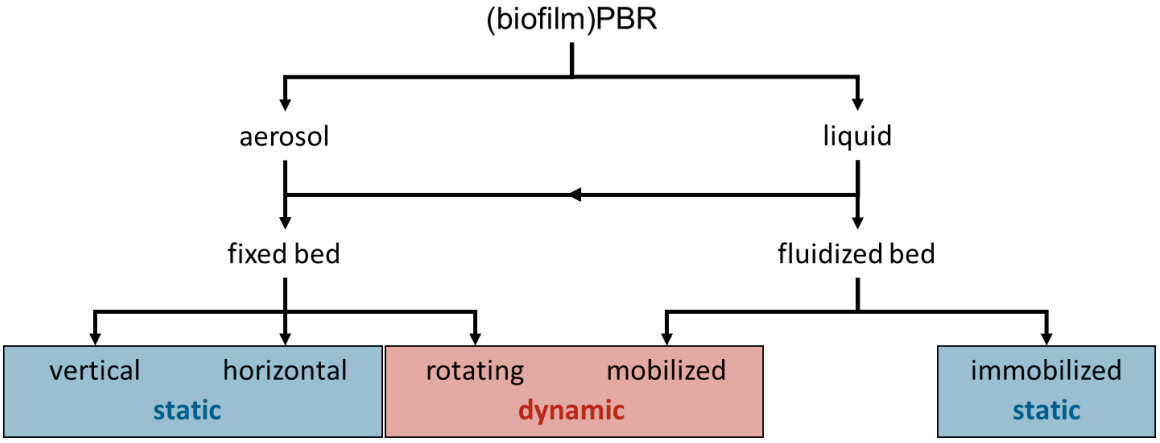

Fig. 8 Classification of (biofilm) photobioreactors

5.4 Dynamic Systems

Dynamic biofilm PBRs are defined by the surface on which the biofilm is cultivated and thus also the biofilm itself is moved in the medium. The movement serves to simulate natural growth conditions, for example by imitating the tide. Furthermore, in systems where the biofilm is not constantly submerged, the gas exchange can be improved. In the attached algal culture system, the supporting material, *e.g.* made from polystyrene foam, is located on the bottom of a growth chamber, which is fixed on a rocking mechanism (see Figure 9 A) [115]. The growth chamber is shaken gently by 15° via the horizontal axis and illumination is continuous with 110 - 120 $\mu mol_{photons}$ m^{-2} s^{-1} from the top. Manure wastewater is used as medium. Harvesting is accomplished by scraping the biofilm off the surface. Another dynamic biofilm PBR is the rotating biological contactor (see **Fig. 9** B) [131]. The applied RBC consist of 27 acrylic discs mounted on a PVC shaft, which serve as growth substratum. 35% of the respective disc surface is submerged in the cultivation medium. Due to the rotation of the discs with the biofilm, it is only temporarily submerged and otherwise exposed to the air. The rotating algal biofilm reactor (RABR) [128] is based on the same principle. Instead of individual disks, it has a rotating tube supporting the growth substratum. Another

design consists of a paddle wheel, in which the paddles are made of the supporting material. In both designs, the respective growth surfaces were 40 % submerged. Compared to suspended cultures, the RABR achieved higher biomass productivity, which was increased from 7,4 g m^{-2} d^{-1} in suspended cultivation to 20 – 31 g m^{-2} d^{-1} in the RABR. Hodges *et al*. [150] also used the RABR described by Christenson and Sims [128] to remove solids from petrochemical wastewater. They observed a significant increase of solids removal and biomass productivity compared to open pond experiments. Another dynamic system for increasing biomass productivity combined with facilitated harvesting is the Revolving Algal Biofilm (RAB) cultivation system [127]. In this reactor, the supporting material is stretched around drive shafts in the form of a flexible mat. Different geometries can be achieved by a triangular or a vertical arrangement of the drive shafts, whereby higher productivity is achieved with the latter. Only the lower drive shaft and thus only a small part of the surface is submerged in the medium. On a pilot-scale, biomass productivity with the RAB was increased by 302 % compared to a classic raceway pond (8.5 m^2). Walther *et al*. [138] developed a submerged biofilm reactor based on a moving bed bioreactor. The carriers were made of high-density polyethylene (HDPE) with a size of 1 - 5 cm. The glass reactor has a volume of 65 litres and mixing is achieved by gassing at the bottom. To avoid dead zones, an inclined plate is installed next to the gassing unit. A cultivation of *Nostoc* sp. was successfully carried out in the reactor.

5.5 Stationary Systems

In stationary biofilm cultivation systems, the supporting material and thus also the biofilm is fixed in place. The only movement is caused by the flow of the medium over the biofilm. The substratum can be arranged in the form of vertical plates, as is the case in the parallel plate air lift (PPAL) reactor according to Genin *et al*. [134]. The reactor consists of a glass chamber with a volume of 15 l, in which two vertical plates made of acrylic glass are located. Various supporting materials can be attached to these plates. The gas supply is located at the bottom of the reactor and between the two plates. The lighting is provided from the side. The multi-layered photobioreactor (MLPR) consists of several alternating layers of cell suspension layers and transparent medium layers, separated only by membranes [151]. The incident light is diffused by the medium layers and thus evenly distributed in the MLPR, providing illumination over a larger area. A simpler vertical system for attached cultivation was used by Lee *et al*. [136]. In this system, the attaching material was suspended in the form of several rectangular nylon meshes in a raceway pond, so that the flow runs across the length of the mesh. Lee *et al*. compared growth directly with a suspended culture and achieved a 2.8-fold increase in biomass and total lipid productivity in the attached system. In addition to a vertical arrangement, a horizontal one is of course also conceivable, such as in the flow lane biofilm reactor [114]. This consists of horizontal channels of different

depths. The medium flows over the biofilm by itself due to a slight tilt of the reactor. The lighting is also provided from above. Boelee *et al*. simulated a wave effect by pouring the medium at regular intervals from a reservoir. The algae biofilm photobioreactor (see **Fig 9** E) by Ozkan *et al*. [112] also relies on the independent flow of the medium through a slight negative slope. The reactor consists of a concrete plate that serves as a growth surface. The medium is fed at the highest point and collected and recirculated at the lower end. Schnurr *et al*. [137] use a flow forced by pumps in their semi-continuous flat plate parallel horizontal PBR. This allows them to precisely adjust the flow velocity and thus also the shear stress for the biofilm. The reactor consists of 18 small parallel PBR's, which are all operated with the same parameters. A system based neither on vertical nor horizontal flat growth surfaces is described by Gao *et al*. [135] in the form of a biofilm membrane PBR. As substratum, flexible fibre bundles were used. They were completely submerged in the medium. The fibres are submerged in a 0.5 m deep reactor made of plexiglass and the lighting is provided from the outside. In the biofilm capillary reactor (see **Fig. 9** F) [144], the medium is transported by capillary forces through a thin reaction chamber with the biofilm on its inner surface. A segmented flow can be used to alternately supply the biofilm with medium and air.

5.6 Air-exposed Biofilm PBRs

Air-exposed cultivation of biofilms in reactors is not as well studied as submerged cultivation, but in recent years it has become increasingly popular. Especially for terrestrial cyanobacteria, this type of cultivation is advantageous, as their natural habitat is imitated. The supply of media can be carried out primarily in two ways: i) supply via a liquid medium, which is available to the biofilm on one side, while the other side of the biofilm is exposed to air and ii) supply via a nutrient mist (aerosol). The first type includes a multiple layer vertical plate PBR described by Liu *et al*. [117], for example. The supporting material consists of filter paper fixed on glass. The medium is passed through the filter paper, so that the biofilm grows exposed to the air on the outside, which optimises gas exchange and light absorption. The light is diluted between the individual surfaces, which are arranged in an array fashion. The same reactor was also used by Cheng *et al*. [116] and in addition in a horizontal arrangement. The biomass productivity of the vertical multi-layer PBR was about 10 times higher than in the horizontal reactor. A simpler construction of an air-exposed biofilm reactor was shown by Boelee *et al*. [114]. A vertical plate made of different layers of geotextiles serves as substratum. Nutrients were supplied by continuously adding the liquid medium to the biofilm at the upper edge. Cultivation was carried out with continuous illumination. Harvesting can be done in this reactor by simply scraping the biofilm from the surface. Xu *et al*. [119] used a capillary-driven PBR (CPBR) consisting of polyester microfibers that were vertically attached in bundles. The lower end was placed in

the medium, which was distributed over the fibres by capillary forces. The illumination was from above and the biofilm grows completely exposed to air. Porous substrate bioreactors (PSBRs) (see **Fig. 9** C) are another alternative for the emerse cultivation of biofilms [152]. The biofilm grows on a porous substrate, which on the one hand serves as a barrier to the liquid medium and as a growth surface, but on the other hand also allows the transport of water and nutrients. Scherer *et al*. [118] developed a multi-skin sheet PBR for the emerse cultivation of terrestrial cyanobacteria as biofilm. The design was optimised for later application in facades. In this case, nutrients are supplied via an aerosol. The biomass productivity could be increased in comparison to suspended cultures. The emerse photobioreactor (ePBR) (see **Fig. 9** D) developed by Kuhne *et al*. [153] and further improved by Strieth *et al*. [123, 154] is an aerosol-based PBR specifically designed for the cultivation of terrestrial cyanobacteria. The ePBR was fully characterised in terms of aerosol distribution to ensure optimal nutrient supply. Through the optimisation, the biomass formation of *Nostoc* sp. could be almost tripled. An influence of the surface on the growth of the biofilms with regard to biomass productivity could not be observed. Another version of the ePBR is the hexagonal ePBR developed by Stiefelmaier *et al*. [155] which differs in its geometry.

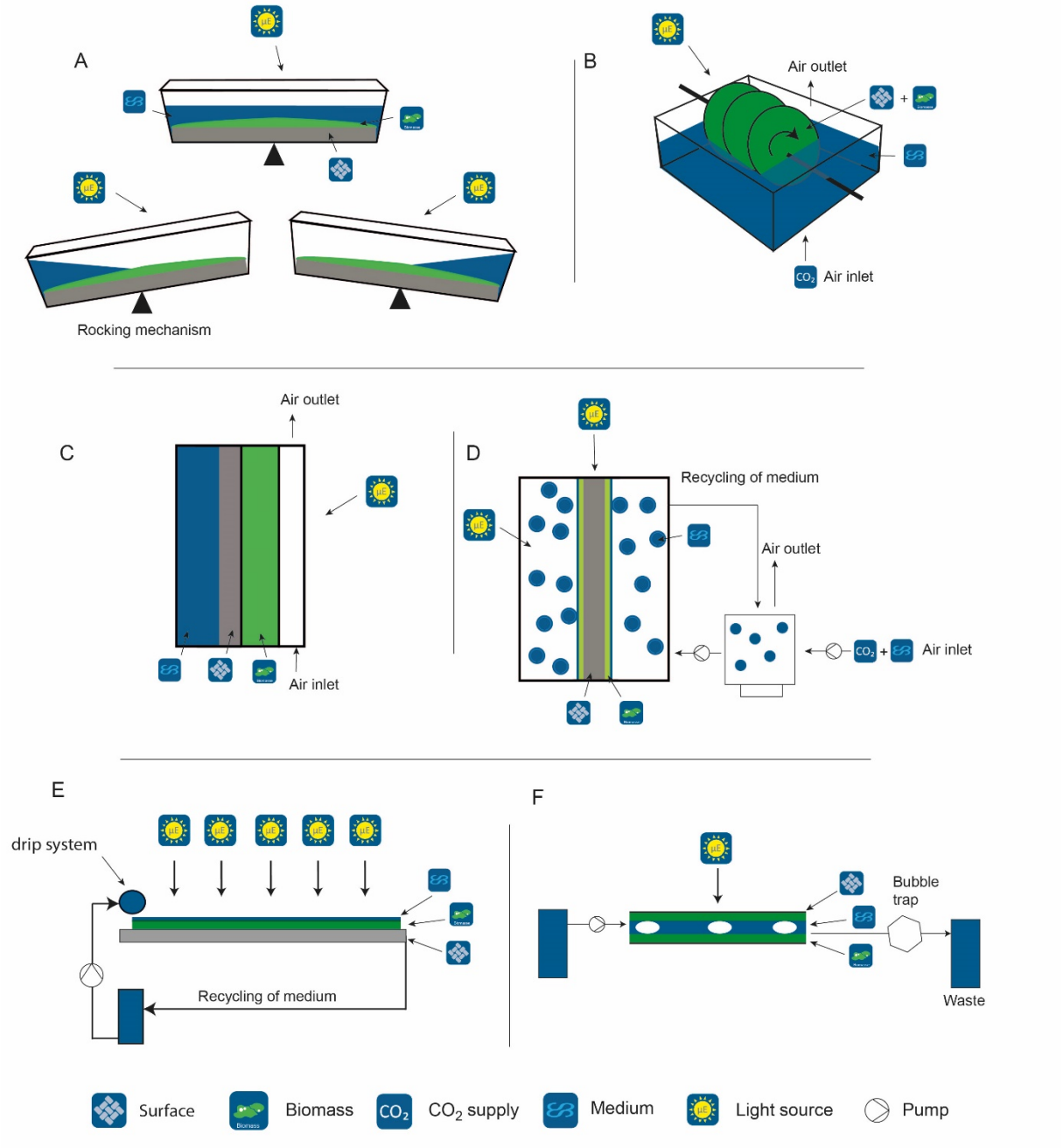

Fig. 9 Overview of Biofilm Photobioreactors. A: Attached algal culture system with a rocking mechanism (modified from Johnson and Wen 2010 [115]), B: Rotating biological contactor (RBC) (modified according to Mukherji and Chavan 2012 [131]), C: Porous substrate bioreactor (PSBR) (modified according to Podola *et al*. 2017 [152]), D: Emerse Photobioreactor (ePBR) (modified according to Strieth *et al*., 2021 [7]), E: Algal biofilm photobioreactor system (modified according to Ozkan *et al*. 2012 [112]), F: Biofilm capillary reactor (modified according to Heuschkel *et al*. 2019 [144]).

6 Cultivation Modes of Cyanobacteria

Among the prokaryotes, cyanobacteria are the only organisms that are capable of oxygenic photosynthesis. Just as algae and higher plants, cyanobacteria also possess photosystems I and II. However, in difference to plants, photosynthesis and cell respiration can be performed simultaneously at the thylakoids [156]. Moreover, the CO_2 fixation efficiency is 10 to 50-fold higher than in plants [157]. Besides using CO_2 as sole source of carbon and energy, many cyanobacteria can also metabolize organic carbon sources, like glucose. In cyanobacteria, all known glycolytic pathways could be identified [158]. Carbohydrates can be metabolized via the oxidative pentose phosphate pathway (OPP), the Entner-Doudoroff (ED) pathway [159], as well as via the phosphoketolase- [160] and the Embden-Meyerhof-Parnas (EMP) pathway. All pathways eventually result in acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle. In the past, it was assumed that cyanobacteria possess an incomplete TCA cycle, missing the α-ketoglutarate-dehydrogenase [161]. However, through synthesis of a 2-oxoglutarate decarboxylase and a succinic semialdehyde dehydrogenase, which were first identified in *Synechococcus* sp. PCC 7002, the cycle is closed. These two enzymes catalyse the conversion of 2-oxoglutarate to succinate, with succinate semialdehyde as intermediate product [162] (**Fig. 10**).

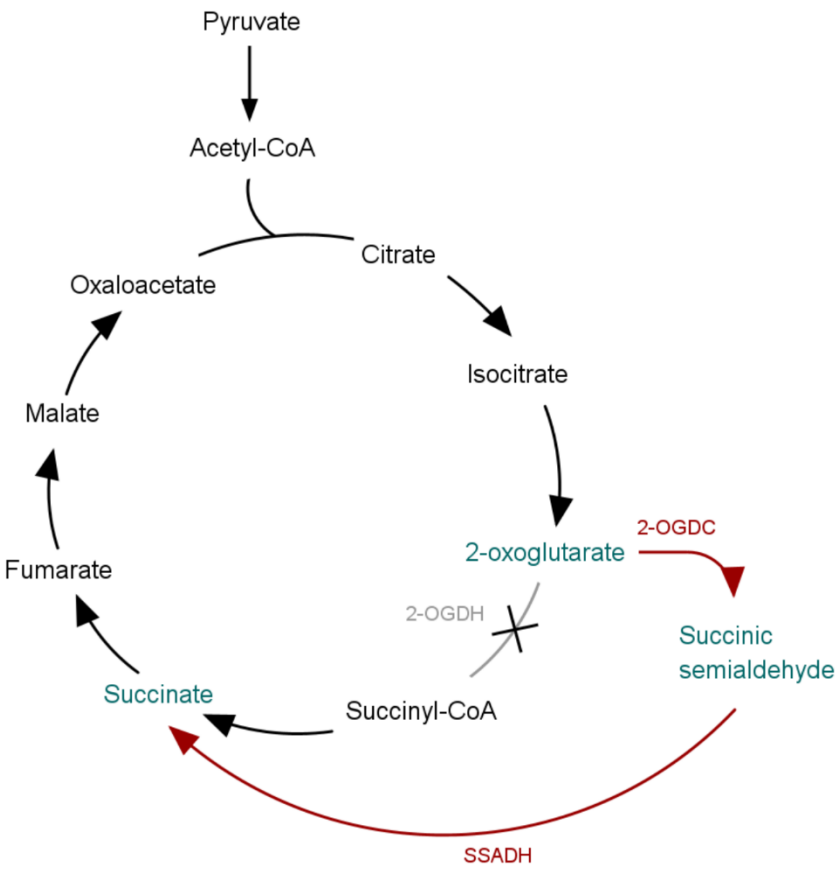

Fig. 10 Schematic diagram of the citric acid cycle in cyanobacteria. SSADH, succinic semialdehyde dehydrogenase; 2-OGDC, 2-oxoglutarate decarboxylase; 2-OGDH, 2-oxoglutarate dehydrogenase. Modified according to Zhang & Bryant, 2011 [161].

Moreover, the γ-aminobutyrate (GABA) shunt, which catalyses the conversion of glutamate to succinate, also contributes to a fully functional TCA cycle [163].

The mixotrophic cultivation of cyanobacteria confers great advantages over phototrophic cultivation. Several studies report that many cyanobacteria show a clearly enhanced growth if an organic carbon source is concomitantly applied with light. However, many cyanobacterial strains are contaminated with heterotrophic bacteria or fungal species. These organisms start growing when an organic carbon source is provided. For an estimation of contaminant share in cyanobacterial cultures, Walther *et al*. developed a qPCR method, which enables a differentiation between cyanobacterial cells and those of heterotrophic bacterial contaminants by means of specific DNA primers [164]. They could show that heterotrophic cultivation of the terrestrial cyanobacterium *Nostoc* sp. (formerly referred to as *Trichocoleus sociatus*) does not lead to a high concentration of contaminant cells. The cdw partition of *Nostoc* sp. shortly dropped to 90 % after two days of cultivation and quickly rose again to about 100 % of total cdw [164]. Similar results were shown for heterotrophic batch, mixotrophic batch and mixotrophic fed-batch cultivations of *Nostoc* sp.

and *Desmonostoc muscorum* (formerly referred to as *Nostoc muscorum*) with different carbon sources [165].

By addition of 0.5 g l^{-1} glucose, *Spirulina* sp. reached growth rates of >0.05 h^{-1}, compared to >0.02 h^{-1} at photoautotrophic conditions [166]. It was simultaneously observed that photoinhibition, which occurred from approximately 30 W m^{-2} (approx. 138 $\mu mol_{photons}$ m^{-2} s^{-1}) in autotrophic cultures, was completely unascertainable at mixotrophic conditions. By addition of glucose to the cultivation medium, light intensities of 50 W m^{-2} (approx. 230 $\mu mol_{photons}$ m^{-2} s^{-1}) could be applied. Above this value, growth could not be further increased, but also no drop in growth rates was noticed [166]. Similar results could be obtained with the terrestrial cyanobacterium *Nostoc* sp. (**Fig. 11**, this work).

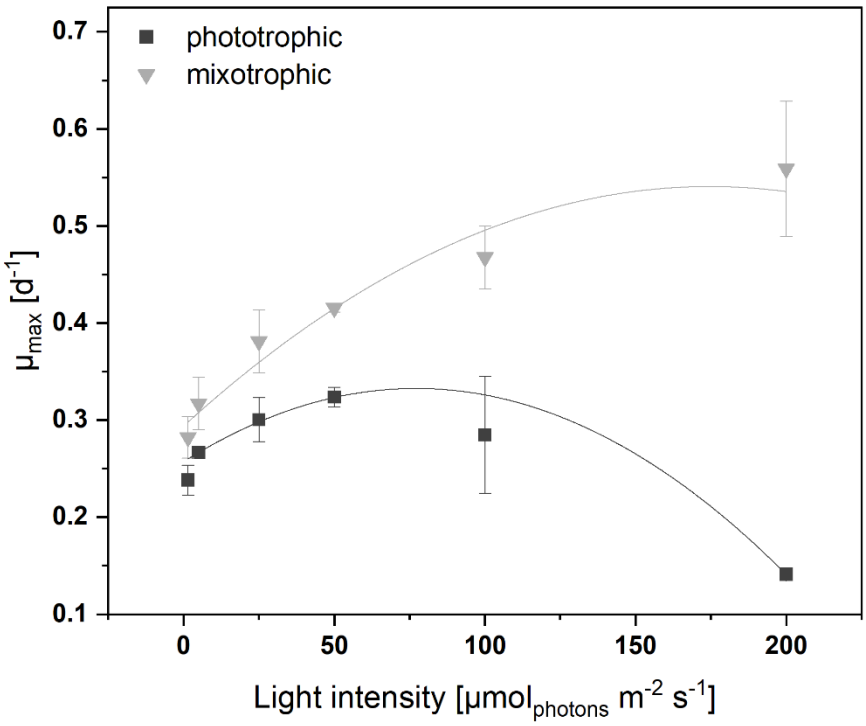

Fig. 11 Growth rates of *Nostoc* sp., phototrophic/mixotrophic cultivation (2.5 g L^{-1} raffinose), as function of light intensity. BG-11 medium, pH 7, t = 2 days, T = 27 °C, n =120 rpm, N= 5.

At mixotrophic cultivation, a linear growth rate increase between 1.5 and 100 $\mu mol_{photons}$ m^{-2} s^{-1} was measured. At higher light intensities, a plateau was reached. In solely phototrophic cultures, photoinhibition at light intensities over 100 $\mu mol_{photons}$ m^{-2} s^{-1} was noticed. This effect is caused by an excessive photon flux in the cell, which cannot be consumed by the Calvin-Benson-Basham (CBB) cycle. These electrons react with water molecules and form cell damaging hydrogen peroxide [166]. It has been suggested that dissolved carbohydrates have a protective impact against photoinhibition [166, 167]. Moreover, addition of carbon sources protects the cells from photoinhibition by significantly diminishing the chlorophyll content [168].

Schwarz *et al*. tested growth of two terrestrial cyanobacteria, *Nostoc* sp. and *Desmonostoc muscorum*, under phototrophic, heterotrophic and mixotrophic conditions [165]. The latter cultivation mode was also tested in combination with fed-batch cultivation. Especially *Nostoc* sp. showed significantly increased growth with fructose, glucose, galactose and raffinose

(0.25 % w/v respectively), when the cells were once again supplied with the respective organic carbon source after five days of cultivation. By addition of raffinose, a cell dry weight (cdw) of 1.32 g l^{-1} at heterotrophic cultivation and 1.49 g l^{-1} at mixotrophic cultivation could be reached after already two days of cultivation. This is 1.9/2.1 times more cdw than by phototrophic cultivation. *Nostoc* sp. showed very promising growth under hetero-/mixotrophic conditions with glucose or raffinose, further experiments with different concentrations of organic C-source and de-/increased light intensity were done for this subchapter (**Fig. 12**).

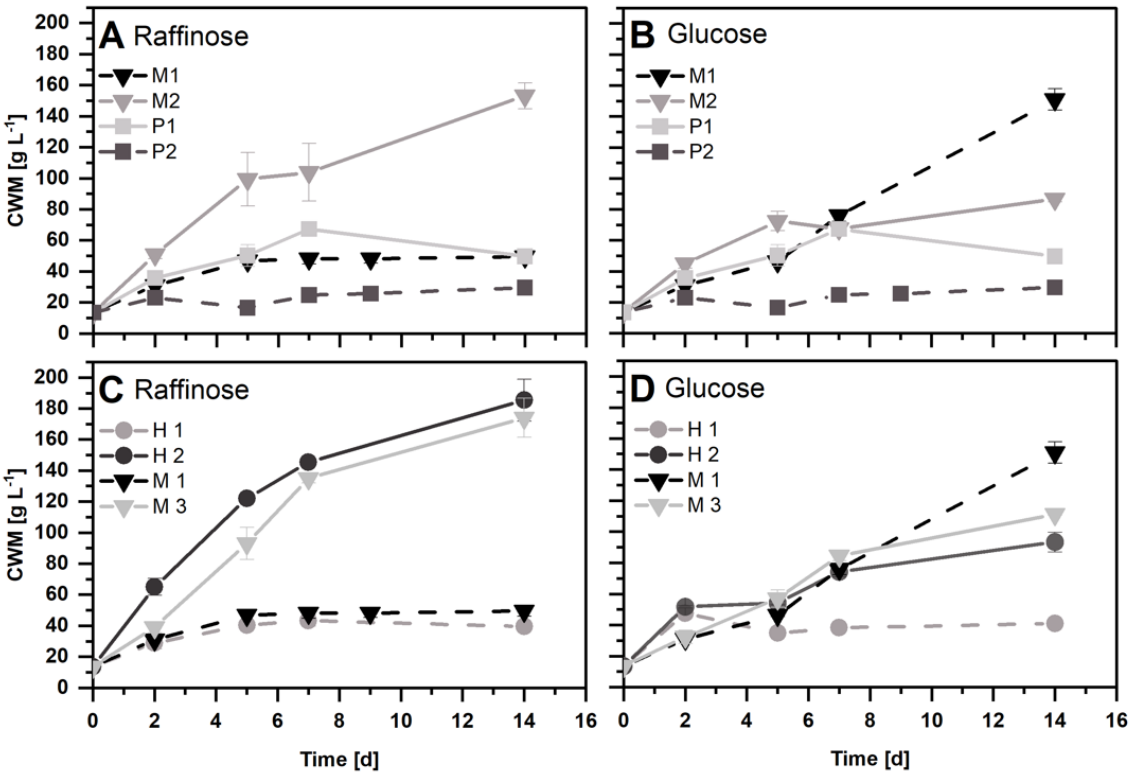

Fig. 12 Growth behaviour of *Nostoc* sp. in BG-11 medium at different light intensities and raffinose (**A**, **C**), or glucose concentrations (**B**, **D**). Initial pH 7, M1 = mixotrophic $c_{C\text{-source}}$ = 2,5 g L^{-1}, E_v = 5 $\mu mol_{photons}$ m^{-2} s^{-1}, M2 = mixotrophic $c_{C\text{-source}}$ = 2,5 g L^{-1}, E_v = 200 $\mu mol_{photons}$ m^{-2} s^{-1}, M3 = mixotrophic $c_{C\text{-source}}$ = 50 g L^{-1}, E_v = 5 $\mu mol_{photons}$ m^{-2} s^{-1}, P1 = phototrophic, E_v = 5 $\mu mol_{photons}$ m^{-2} s^{-1}, P2 = phototrophic, E_v = 200 $\mu mol_{photons}$ m^{-2} s^{-1}, H1 = heterotrophic $c_{C\text{-source}}$ = 2,5 g L^{-1}, H2 = heterotroph $c_{C\text{-source}}$ = 50 g L^{-1}. CWM = cell wet mass. Cultivation parameters: t = 14 days, T = 27 °C, n = 120 rpm, N = 3, phototrophic/mixotrophic L:D 24:0.

Not surprisingly, phototrophic growth increased along with an increase of light intensity from 5 (**Fig. 12** A/B; P1) to 200 $\mu mol_{photons}$ m^{-2} s^{-1} (P2). In case of raffinose, this effect can also

be observed at mixotrophic conditions, although the effect is much more significant (**Fig. 12** A; M1, M2). However, an interesting difference to mixotrophic cultivation with glucose can be noticed in **Fig. 12** B. Here, the increase of light intensity shows the opposite effect. While at 5 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, a cell wet mass (cwm) of about 150 g l^{-1} was reached, at 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ only about 80 g l^{-1} were obtained. In **Fig. 12** C it can be seen that *Nostoc* sp. possesses a high affinity for the metabolization of the trisaccharide raffinose. Moreover, the added concentration seems to be a crucial factor for growth. While at 2.5 g l^{-1} raffinose (**Fig. 12** C, H1) a growth rate of 0.217 d^{-1} and a maximum cwm of about 40 g l^{-1} was reached, an addition of 50 g L^{-1} (**Fig. 12** C, H2) resulted in a growth rate of 0.34 d^{-1} and a preliminary maximum cwm of > 180 g l^{-1} after 14 days of cultivation. *Nostoc* sp. showed slightly poorer growth with 5 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ light intensity and constant raffinose supply (**Fig. 12** C, M3). The opposite can be observed in **Fig. 12** D. As *Nostoc* sp. seems to have a lower affinity for the metabolization of glucose, and mixotrophic growth is thus preferred, a supply of light with 5 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and simultaneous addition of 50 g l^{-1} glucose (**Fig. 12** D, M3) results in an about 20 g L^{-1} higher cwm compared to solely heterotrophic cultivation with the same glucose concentration.

The noticed differences between cultivation of *Nostoc* sp. with raffinose or glucose could be explained by consideration of the molecular structure of the two carbohydrates. While glucose is a simple monosaccharide, raffinose constitutes a trisaccharide composed of glucose, galactose and fructose. As such, it possesses a relatively high molecular weight of 594.5 g/ml and an entrance into the cyanobacterial cell by diffusion is highly unlikely. As a consequence, it must either be extracellularly degraded, or imported by an active transport system. The first possibility includes the energy consuming synthesis and export of specialized enzymes, without a previously transmitted signal for transcription of the corresponding genes. The second option implies an active transport over the cell membrane. Although no specific raffinose transporter has been described in cyanobacteria so far, a number of ATP-binding cassette (ABC) type transporter systems have been identified. In *Anabaena* sp. ATCC 29413, the uptake of fructose is conferred by such a system (frtABC) [169] just like in *Nostoc* sp. ATCC 29133 [170]. The genome of *Synechocystis* sp. PCC 6803 contains genes coding for an ABC transporter that is responsible for the export of polysaccharides and thus for the development of exopolysaccharide layers [171]. Consequently, the import of raffinose could indeed be granted by an ATP-dependent ABC transporter in *Nostoc* sp. In phototrophically grown cells, ATP is synthesized by the electron transport chain which powers the ATP-synthase. At low light intensity, while only small amounts of ATP are produced, less of these nucleotides can be spent on the transport of raffinose. This could explain the effects seen in **Fig. 12** A, M1. By application of higher light intensities, more energy can be delivered

and raffinose gets imported and degraded in higher amounts **Fig. 12** A, M2. However, these explanations alone cannot explain the data shown in **Fig. 12** C. By supply of higher raffinose concentrations (50 g L^{-1}, M3), *Nostoc* sp. cells show a significantly better growth compared to **Fig. 12** A, M1, despite of only 5 $\mu mol_{photons}$ m^{-2} s^{-1} light intensity. This can be explained by a signal cascade, triggered by the increased carbohydrate availability in the culture medium and a consequent release of extracellularly enzymes with α-galactosidase activity. A thermostable glycosidase that also shows galactosidase activity was found in the extracellular matrix of *Nostoc commune* [172]. The resulting degradation products galactose and sucrose could enter the cell through permeases, or TonB- dependent transporters [173, 174] and promote the significantly improved growth seen in Figure 12 **C**.

In further studies regarding mixotrophic growth of cyanobacteria, cells of *Chroococcidiopsis cubana* were cultivated with addition of different carbohydrates (5 g L^{-1}, respectively; **Fig. 13**).

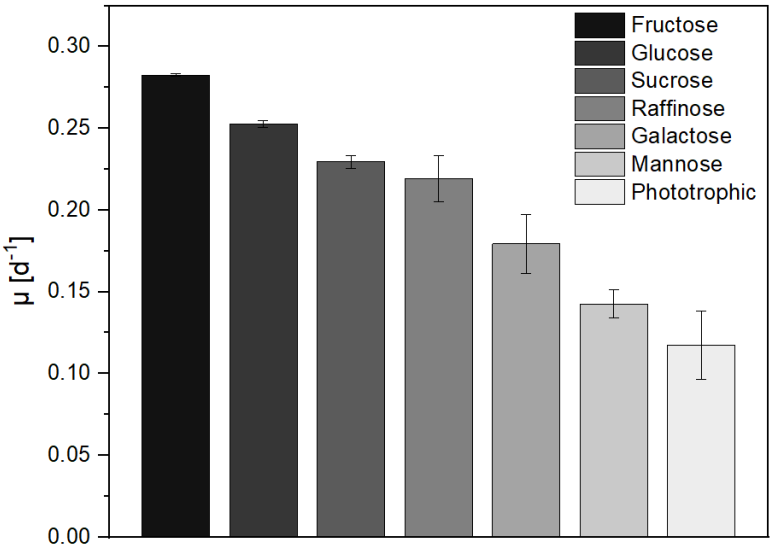

Fig. 13 Growth rates of *C. cubana* in BG-11 medium enriched with different carbon sources. Initial pH 7. E_v = 160 $\mu mol_{photons}$ m^{-2} s^{-1}, $c_{C\text{-}source}$ = 5 g L^{-1}, t = 13 days, T = 30 °C, n = 120 rpm, N = 3, phototrophic/mixotrophic L:D 24:0.

The presented results show that *C. cubana* is capable of metabolizing a wide range of carbohydrates. As seen in the growth assays with *Nostoc* sp., this terrestrial cyanobacterium also shows a significantly enhanced growth at mixotrophic conditions, compared to solely phototrophic cultivation. By supplementation of almost each organic carbon source, growth rates could be at least doubled (**Fig. 13**). As the highest amount of biomass could be gained by cultivation with fructose, further experiments for growth optimization were performed with this monosaccharide.

The reduction of light supply, or the application of light-dark-periods, can significantly diminish cultivation costs. In **Fig 12** it was shown that in some cases lower light intensities can even improve cyanobacterial cell growth when combined with mixotrophic growth. This phenomenon was also observed in cultivations with *C. cubana* (**Fig. 14**).

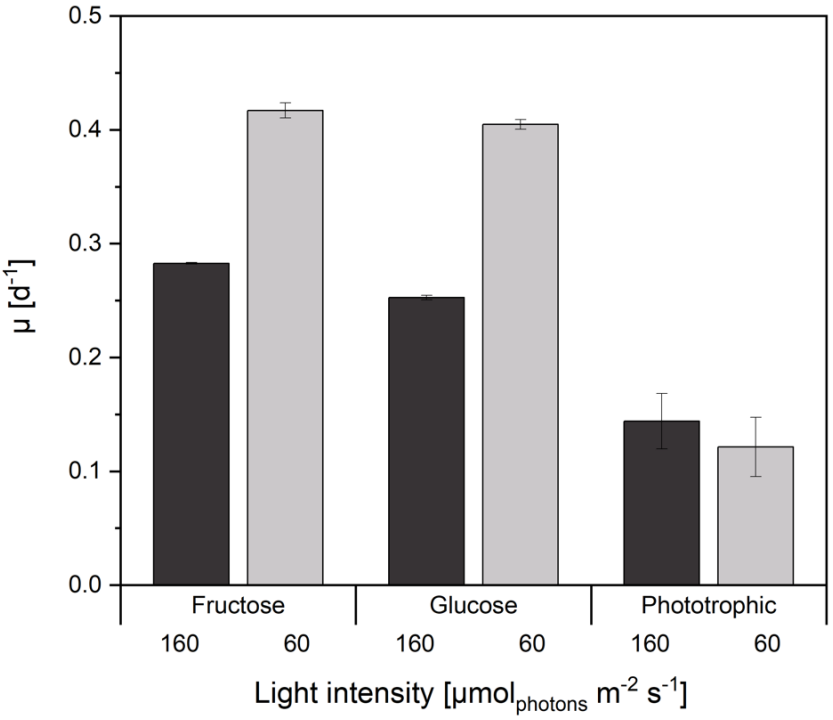

Fig. 14 Growth rates of *C. cubana* in BG-11 medium with different carbon sources and under alternating light intensities. Initial pH 7. E_v = 60/160 $\mu mol_{photons}$ m^{-2} s^{-1}, $c_{-source}$ = 5 g L^{-1}, t = 13 days, T = 30 °C, n = 120 rpm, N = 3, phototrophic/mixotrophic L:D 24:0.

While *C. cubana* only shows slight growth differences at different light intensities under solely phototrophic conditions, significant growth rate alterations were determined in mixotrophic cultivations. By decreasing the light intensity from 160 to 60 $\mu mol_{photons}$ m^{-2} s^{-1}, a growth rate increase of 15.8 % was measured for fructose; for glucose, an increase of even 25 % was observed. How can these observations be explained? Under phototrophic conditions, NADPH is generated through the photosynthetic electron transport between photosystems II and I at the thylakoid membrane. The NADPH is needed for carbon fixation in the CBB cycle. Under mixotrophic conditions, NAD(P)H can also be gained through several glycolytic pathways. The most abundantly used is the oxidative pentose phosphate pathway (OPP), which can generate 5.33 NAD(P)H per molecule of glucose [158]. This pathway can be upregulated under light limiting conditions [175]. As a result, organic carbohydrates are metabolized much more effectively, which causes an improved growth at lower light intensities. Consequently, mixotrophic cultivation of cyanobacteria does not only lead to enhanced cell growth, but also to upregulation of glycolytic pathways under certain cultivation conditions.

In recent literature, there are several promising examples of cyanobacterial bioprocesses, where a heterotrophic or mixotrophic cultivation mode greatly increased product yields (**Table 4**). Apparently, not only a general biomass productivity increase could be demonstrated for different strains, but also a significant enhancement of process productivity concerning different target products. This was, *e.g.,* found for biopolymers as poly-β-hydroxybutyrate (PHB) and the co-polymer Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], produced by *Synechocystis* sp. PCC 6803 [176] and *Nostoc muscorum* Agardh [176], respectively. With *Synechocystis*, the addition of 0.4 % acetate to a culture pre-grown in BG-11 supplemented with 0.1 % glucose, led to a 29 % higher PHB accumulation (w/w dry cell mass), compared to phototrophically grown cells. In case of *Nostoc muscorum*, a supplementation with 0.4 % (w/v) fructose, glucose or acetate led to a respective share of 19.2 %, 26 % and 28 % of total dry cell weight, compared to only 8.4 % in the phototrophic control.

Mixotrophic cultivation also showed a positive impact on the production of the phycobiliprotein phycocyanin with *Spirulina platensis* [177]. Through implementation of a fed-batch process with glucose (2 g L^{-1}), the maximum biomass concentration could be increased from 2 g L^{-1} to 10.2 g L^{-1} and the maximum phycocyanin production rose from 280 to 795 mg L^{-1}.

A more recent study dealt with the maximisation of *Leptolyngbya subtilis* JUCHE1 cell lipid concentration and lipid productivity for the production of biofuels [178]. Under photoheterotrophic (mixotrophic) conditions of 2.5 kLux (approx. 88 $\mu mol_{photons}$ m^{-2} s^{-1}) light illumination and a glycerol concentration equivalent to 5 % (v/v) CO_2, a maximum lipid productivity of 0.0702 g L^{-1} d^{-1} could be obtained – a 4.66-fold higher value than by solely phototrophic cultivation. This study could also show that not only biomass formation was enhanced by mixotrophic cultivation (1.47-fold), but also particularly the lipid productivity.

Through genetic engineering of *Synechococcus elongatus* PCC 7942, Kanno *et al*. (2016) managed to greatly improve glucose utilization under concomitant light supply [179]. The modifications in glycolytic pathways and the calvin benson cycle led to a 2,3-butanediol production rate of 1.1 g l^{-1} d^{-1}. The theoretical maximum yield, obtainable from solely glucose, was significantly exceeded by 36 %, suggesting actual mixotrophic growth with concurrent metabolization of an organic and an inorganic carbon source. Under diurnal conditions, a theoretical maximum yield increase of even 95 % was reached. These results impressively show what mixotrophic cultivation can achieve when combined with metabolic engineering strategies. Such attempts could eventually lead to an industrially relevant use of cyanobacteria in diverse biotechnological production processes.

Table 4 Examples for cyanobacterial bioprocesses enhanced by mixotrophic/heterotrophic cultivation

Strain	Product	Cultivation mode	C-source (conc.)	Max product yield [g L^{-1}]	Yield increase [%]	Literature
Arthrospira platensis	Biomass	Mixotrophic/Fed-batch	Acetate (387 mg L^{-1} d^{-1})	1.769	39	[180]
Spirulina platensis	Biomass	Mixotrophic/Batch	Molasses (0.75 g L^{-1})	2.94	n.a.	[181]
Synechocystis sp. PCC 6803	PHB	Mixotrophic/Batch	Acetate (0.4 %)	29 (% w/w)	29	[176]
Spirulina platensis	Phycocyanin	Mixotrophic/Fed-batch	Glucose (2 g L^{-1})	0.795	284	[177]
Nostoc flagelliforme	Biomass	Mixotrophic/Heterotrophic/ Batch	Glucose (2.53 g L^{-1})	1.67/0.731	499/218	[182]
Anabaena sp. PCC 7120	Biomass	Mixotrophic	Glucose (18 g L^{-1})	3.1	451	[183]
Nostoc muscorum Agardh	PHB/ P(3HB-*co*-3HV)	Mixotrophic/Batch	Acetate/Fructose/ Glucose (0.2 - 0.6 %)	0.13/0.145/0.165	283/315/359	[184]
Leptolyngbya subtilis	Lipids	Mixotrophic/Batch	Glycerol	56 (% w/w)	448	[178]
Synechococcus elongatus PCC 7942	2,3-butanediol	Mixotrophic/Batch	Glucose (15 g L^{-1})	12.6	95	[179]

The examples show that the great metabolic versatility of cyanobacteria allows a variety of possible cultivation modes. For many cyanobacterial strains, mixotrophic cultivation is described to yield the highest densities in cell mass. But solely heterotrophic processes as well show very promising results. These also have the significant advantage that cheap carbohydrates can be used in combination with regular, non-illuminated bioreactors. Cyanobacteria are potentially able to metabolize a wide range of carbohydrates. Consequently, it might be worth to test several organic carbon sources prior to coping with low cell densities in phototrophic cultivation.

7 Conclusion

Cyanobacteria offer great chances for biotechnical processes and gain more and more attention. Novel *in silico* screening possibilities and an increasing availability of sequenced genomes open new doors for genome mining attempts and lead to the detection of valuable bioactive metabolites. For a save conservation of specific strain characteristics, cryoconservation should be considered for long time storage of cyanobacteria. Contrary to common opinions, many strains are capable to survive the process, if the right cryo-protectant and -protocol is used. For a cell condition evaluation, a variety of vitality and viability tests can be chosen. As a cell viability confirmation does not necessarily result into growing cells, a vitality assay should be considered to prevent cultivation failures. These tests do not mandatorily require high-tech hardware - the determination of cell vitality by resazurin-assay, or by means of pO_2 increase, can be conducted in a quick, easy and economic way. Because of their unique chacteristics, cyanobacteria often require specialized methods and cultivation conditions. This can be challenging, but also led and leads to the development of intriguing photobioreactor systems. In this chapter, a special focus was placed on the relatively new and heterogenous group of biofilm-based cultivation systems. By an improved light supply through lower self-shading of the cells and optimal conditions for biofilm producing terrestrial cyanobacteria, the productivity could be strongly improved, and expensive cell harvest steps can be avoided. Together with the promising mixotrophic cultivation attempts, these systems could be a way to overcome the commonly low productivity rates of cyanobacteria and to prepare the ground for industrial applications.

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Chapter III

Novel method enabling a rapid vitality determination of cyanobacteria

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M. Witthohn:	Project administration, visualization, writing, investigation (all shown data).
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Practical Application

The presented method is the first approach to link the O_2 production of cyanobacteria directly to their cell vitality. By this, new conservation methods can be rapidly evaluated, without the need for long cultivation periods, expensive technical equipment or high amounts of biomass. The approach is suitable for every cyanobacterial strain and is not impaired by the commonly occurring production of viscous exopolysaccharides or the formation of cell aggregates. Further application areas are conceivable, for example the testing for antibiotic susceptibility/resistance, or the response to altered cultivation conditions.

Abstract

Cyanobacteria represent a large group of bacteria with underestimated scientific potential. Recent studies indicate them as a great reservoir of secondary metabolites with antifungal, antiviral or antibacterial activity. However, common, well established research techniques cannot be easily adapted to these organisms. Slow growth rates and irregular cell aggregates constitute challenges for researchers dealing with cyanobacteria. In this work, we present an innovative new method enabling a quick, easy and economical vitality determination of cyanobacterial strains, as *e.g.* required for the finding of optimal cryopreservation conditions. We were able to measure the vitality of previously cryopreserved and defrosted *Trichocoleus sociatus* samples within 45 minutes by means of their O_2-production. For each run, a cell wet mass of only 0.5 g was required. By application of this method, we could find DMSO (5 % v/v) and glycerin (15 % v/v) to be the most promising cryoprotectants for the conservation of *T. sociatus* cells. DMSO and glycerin guaranteed a vitality rate of 80-90 % and 60-70 % after up to four weeks of cryopreservation, compared to fresh cell material.

1 Introduction

Cyanobacteria are a group of versatile, phenologically and ecologically diverse microorganisms, which occur in most habitable areas in the world. Because of this circumstance, these organisms are of great interest for scientific research, as they are known as producers of highly valuable secondary metabolites [1-3] and can be used as phototrophic production strains of high energy compounds, used as biofuels [4]. Although cyanobacteria are not ideal production organisms, mainly due to slow growth rates, new cultivation concepts and reactor types can highly improve the productivity of promising strains [5]. Especially for terrestrial cyanobacteria, the principle of “productive biofilms” is a promising approach [6,7]. However, a reasonable exploration of these bacteria requires

stable and easy to handle strain collections. Cyanobacterial strain collections are commonly sustained as actively growing cell cultures on solid agar plates or in liquid medium [8]. This conservation mode is not only combined with much work afford, but also with high risks of contamination and genetical modifications [9]. Safe conservation methods as freeze drying, the inclusion in alginate beads [10] and, most importantly, cryopreservation [8], have already been adapted for many cyanobacterial strains. Nevertheless, it remains a challenge to define one appropriate method for all strains, due to the high phenological and ecological diversity. For the invention of possible new methods, it is necessary to compare cell vitalities after different approaches of cryoconservation. Regularly used methods are *e.g.* the determination of growth through repeated OD measurements, or the colony-forming unit assay on agar plates. There are also some promising recent approaches, as the staining of cells with a fluorescence colorant, which can only intercalate in the DNA of dead cells [5]. However, these methods are merely realizable for larger laboratories with superior equipment and resources. The classic approaches are though not easily applicable for cyanobacterial strains. Through the formation of cell aggregates or the production of exopolysaccharides, absorption measurement and colony counts often become impractical. As a possible solution on this, a new approach for the determination of cyanobacterial cell viability is presented in this work. The formation of oxygen and the consequently rising pO_2 value in *T. sociatus* samples was measured by a new method and used for vitality evaluation.

2 Materials and Methods

2.1 Strain cultivation

The cyanobacterial strain *Trichocoleus sociatus* (obtained from Prof. Dr. Burkhard Büdel, University of Kaiserslautern, Department of Plant Ecology and Systematics, Germany) was cultivated in 300 ml flasks, on a rotation shaker at 120 rpm (Multitron Pro, Infors HT, Switzerland) under phototrophic conditions (E_v = 100 µmol m^{-2} s^{-1}) at 27 °C, without addition of CO_2. The standard mineral medium BG-11 [11] was used for cultivation. The flasks were inoculated with *T. sociatus* cells sustained on solid BG-11 agar plates under phototrophic conditions (E_v = 30 µmol m^{-2} s^{-1}).

2.2 Cryopreservation

Main cultures were inoculated with about 0.25 g wet cell mass from the precultures. Cells were harvested after 4-6 weeks of growth by centrifugation at 2360 x g and 25 °C for 10 min. (Rotanta 460 R, Hettich, Germany). After cell harvest, the culture supernatant was removed, the cells were carefully mixed with a spatula and cell wet masses of 0.5 g were

respectively weighed into cryo-vials (AEH8.1, Carl Roth, Germany) with a high-resolution balance. While this, the cell mass was regularly mixed to ensure an equal composition in all tubes. Subsequently, all samples were mixed up to a concentration of 5 % (v/v) with DMSO or MeOH, or up to 15 % (v/v) with glycerin, respectively. All cryoprotectants were diluted with BG-11 medium. Each sample was done in triplicates. The cryo-vials were subsequently transferred to a precooled (8 °C) passive freezing device (Mister Frosty, Nalgene, USA) and cooled to -80 °C (New Brunswick™ U360 Innova®, Eppendorf AG, Germany) with a cooling rate of -1 °C min^{-1}. Cells were stored at -80 °C for up to four weeks. The cells were thawed quickly in a water bath at 30 °C for 10 min. Cryoprotectants were removed by three washing steps. Sedimentation was used for cell palletization instead of centrifugation because cyanobacterial cells might be sensitive towards centrifugation after cryoconservation [8]. The supernatant was removed and substituted by 1 ml of BG-11 medium. Afterwards the cryo-vials were mixed thoroughly. Cell pellets were subsequently transferred to the vitality test setup and mixed up to a volume of 35 ml with BG-11 medium.

2.3 Vitality test setup

The verification of cell vitality after cryoconservation was performed with a new measurement technique, based on the pO_2 alteration by cyanobacterial photosynthesis. The setup (Fig. 1) consists of a glass flask, wrapped with an LED strip (Article no. 9180308, Briloner, Germany) for light supply and an overlying cooling hose, connected to a cooling unit (K15, Haake, Germany).

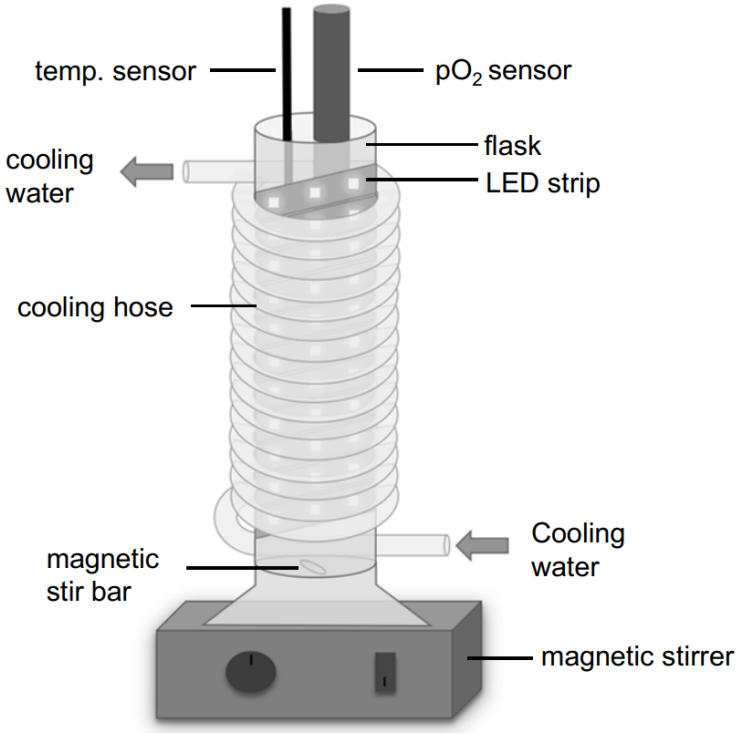

Fig 1 Vitality test setup for cyanobacteria. Cell vitality was determined after cryopreservation by means of pO_2 increase in the cultivation medium.

The setup is equipped with a temperature sensor (LT-101, TFA Dostmann, Germany) and a pO_2 sensor (OxyFerm FDA 225, Hamilton Company, USA). Data of the pO2 sensor was collected by a connected bioreactor (Minifors, Infors HT, Switzerland) and monitored with the bioprocess control software IRIS 6 (Infors HT, Switzerland). Medium circulation is assured by a magnetic stirrer (Mini MR Standard, IKA, Germany) at about 500 rpm. Measurements were done for 45 min, at a constant temperature of 27 °C. For each run, a cell wet mass of 0.5 g was used.

2.4 Determination of cell vitality

Cell vitality of *T. sociatus* was obtained by measuring the pO_2 and calculation of the highest gradient of the pO_2 curve. Values obtained from fresh cell material was determined as 100% vital and served as reference value.

2.5 Method comparison

The new pO_2 based vitality test for cyanobacteria was compared to the standard method based on cell mass increase. Therefore, samples of 0.2 g fresh *T. sociatus* biomass were cryopreserved and thawed after two weeks as specified in **2.2**. For each tested cryoprotectant, three 100 ml cultivation flasks were filled with 20 ml BG-11 medium and inoculated with the respective sample (in triplicates). The cultivation conditions are listed in **2.1**. Flasks were harvested after 3, 7 and 10 days and the cells were washed three times and centrifuged at 3000 x g (EBA 12, Hettich, Germany) with BG-11 medium. The cell dry mass was determined gravimetrically (M254Ai, BEL engineering, Italy) after drying at 60 °C for 24 h.

3 Results and Discussion

3.1 Vitality determination via pO_2 measurement

Data concerning the vitality of cyanobacterial cells was gained by measurement of increasing pO_2 values in the test setup and comparison of the gradients with data from fresh cell material (Fig. 2a). By application of the method demonstrated in this work, it was possible to determine significant differences between the three performed cryopreservation approaches, using different cryoprotectants (Fig. 2b).

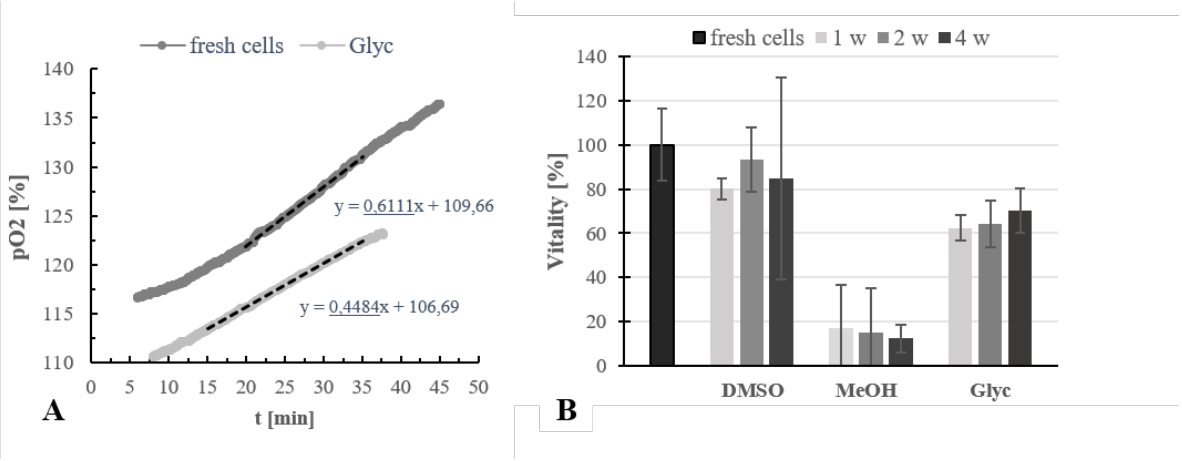

Fig. 2 A, Demonstration of data acquisition through measurement of the pO_2 increase in the setup. **B**, results of the vitality test with *T. sociatus* cells after cryopreservation with 5 % (v/v) of DMSO, 5 % (v/v) of MeOH or 15 % of (v/v) glycerin as cryoprotectant. The samples were frozen for one, two and four weeks. The pO_2 increase in the measurement setup was compared to the one of fresh cells. (Error bars = SD, n = 3)

While the samples mixed with MeOH showed a clearly diminished vitality of about 0-18 %, the ones cryopreserved with either DMSO or glycerin reached decent activity values of 80-96 % and 60-70 %. Similar results were obtained with cells of the terrestrial cyanobacterium *Nostoc muscorum*, which were equally cryopreserved and tested for vitality as *T. sociatus* (data not shown). A significant decrease in cell vitality over time was only detectable with DMSO after four weeks of cryopreservation. In case of glycerin as cryoprotectant, the freezing duration had only minimal impact on cell vitality, low standard deviations give hints on consistent amounts of intact cells.

3.2 Method validation

The developed technique was validated by comparison with the known standard method, the evaluation of growth experiments (Fig. 3).

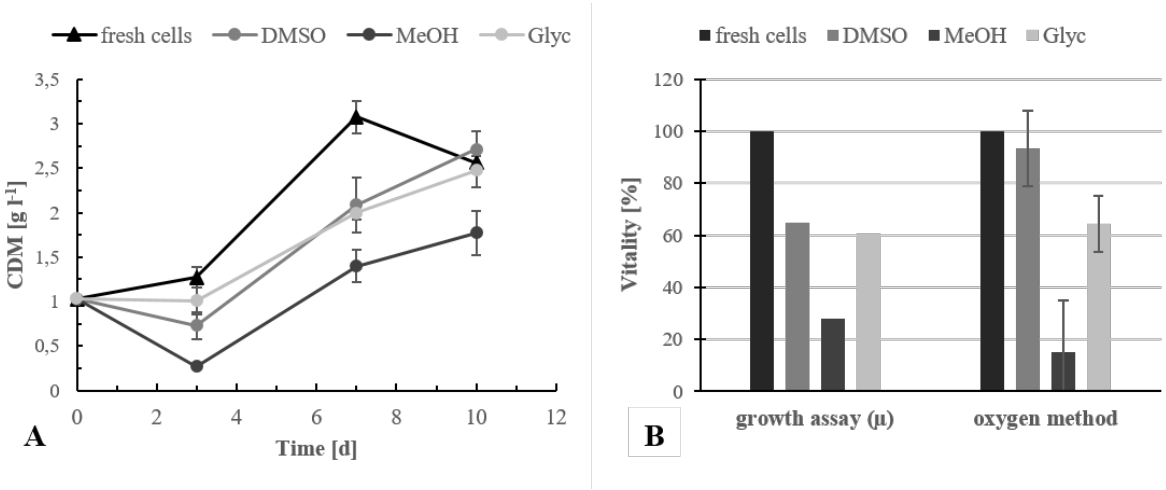

Fig. 3 A, Results of the vitality test (growth assay) with *T. sociatus* cells after two weeks of cryopreservation with 5 % (v/v) of DMSO, 5 % (v/v) of MeOH or 15 % of (v/v) glycerin as cryoprotectant. Data of fresh cells serve as reference. The increase of CDM was measured by cultivation and gravimetrical evaluation of *T. sociatus* samples after different modes of cryopreservation. The cells were harvested after 3, 7 and 10 days. For the t_0 measurement, 200 mg of fresh *T. sociatus* cells were dried at 60 °C for 24 h and the CDM was determined gravimetrically. **B**, approximate growth rates µ [h^{-1}]; CDM values of t_0 and t_7 were considered for calculation. (Error bars = SD, n = 3)

For this, the increase of *T. sociatus* cell dry weight was measured gravimetrically after different points of cultivation time (**2.5**). Prior to the vitality assay, the cells were cryopreserved under addition of DMSO, MeOH or glycerin, as done with the cells applied in the pO_2 measurements (**2.2**). This method was chosen because optical density measurements are impractical, due to inhomogeneous cell aggregates of *T. sociatus*. Cultures inoculated with 10 g l^{-1} *T. sociatus* cell wet mass (1 g l^{-1} cell dry mass, CDM) were harvested after 3, 7 and 10 days of phototrophic cultivation (**2.1**).

The results obtained from the growth assay could confirm the data from the pO_2 based vitality measurements. The obvious loss of CDM in the growth assay with cryopreserved samples indicates a diminished number of vital cells, due to the freezing process. Cells cryoconserved with MeOH showed the highest difference in CDM between t_0 and t_3. Cells frozen with glycerin seem slightly less damaged by the process, in comparison to DMSO samples. The higher CDM value after three days of cultivation can be explained by glycerin residues in the samples, which remained after the washing steps. *T. sociatus* was found to be capable of mixotrophic growth with different carbon sources, resulting in significantly higher growth rates (Schwarz et al., unpublished data). As shown in Fig. 3a, the cell vitality determined by pO_2 measurement allows a relatively precise inference to the corresponding growth rates and thus permit a quick prediction on cell growth behavior. The comparability of both data sets might be improved by more repetitions in the pO_2 measurements and more CDM values at different points of the growth curve.

4 Concluding remarks

Considering all obtained results, a simple yet effective novel method for the vitality determination of cyanobacterial cells could be established. The test can approximately predict the growth impairment caused by cryoprotective agents and can give a first hint on negative impacts, which is sufficient for a fast selection of suitable cryoprotectants. The functionality and validity of the assay was confirmed by 'classic' growth experiments after identical cell treatment. At this point it should be noted that one growth assay took ten days and 2.4 g of wet cell mass, while the vitality determination by pO_2 measurements provided results after about 30 min and only required a maximum of 1.5 g wet cell mass. The applied cell mass can also be significantly reduced when more time is invested in data acquisition. This makes the method feasible for cyanobacteria with very low growth rates, without the need for long cultivation times. Moreover, the method offers great potential for further application fields, as the testing for antibiotic susceptibilities, or reaction of cyanobacterial cells to different medium supplements.

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Chapter IV

A modified method for a fast and economic determination of nitrate concentrations in microalgal cultures

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Authorship contribution

M. Witthohn: Project administration, visualization, writing, investigation (data for fig. 1-4).

A.-K. Schmidt: Methodology, investigation (data for fig. 3).

D. Strieth: Final approval of the article, visualization.

R. Ulber: Funding acquisition, review & editing, conceptualization.

K. Muffler: Funding acquisition, supervision, conceptualization, review & editing.

Keywords: Cyanobacteria, Nitrate determination, Salicylate

Abstract

The sodium salicylate method for nitrate determination in liquids was adapted for the use in microtiter plates and the applicability of this quick, easy and economic test was examined for cyanobacterial mineral media. The assay was found to be suitable for a direct detection of nitrate ions up to a concentration of 450 mg L^{-1} within the commonly used mineral media BG-11, BBM, ASN-III and Zarrouk. The salicylate method was successfully used for the correlation of *Synechococcus elongatus* PCC 6301 cell growth and nitrate consumption over 17 cultivation days.

1 Introduction

Nitrogen (N) is essential for cyanobacterial growth, as it is a major constituent of photosynthetic pigments as chlorophyll and phycobiliproteins. It is also an important element for the production of a vast array of cyanobacteria-derived secondary metabolites. Several methods have been established for the determination of nitrate concentrations in liquids. Some, as the commonly used ion chromatography [1, 2], are based on direct detection via UV measurement at 210 [3] or 225 nm [4] and require the use of a chromatography system [5] for fractionation of complex samples, which prevents overlaying signals caused by interfering ions or larger molecules. Some require either chemical conversion/diazotisation of nitrate, mostly preceded by a reduction to nitrite or ammonia, followed by spectrometric measurement of the formed products [6–8]. There is a variety of commercial kits using a colorimetric reaction, examples are the “nitrate/nitrite Assay Kit Colorimetric” from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA), the Hach “NI-11 Nitrate Test Kit” (Hach Lange GmbH, Düsseldorf, Germany) and the “Nitrate/Nitrite Colorimetric Assay Kit” (Cayman Chemical, Michigan, USA). These tests have a price range of ~ 1,38 – 5,73 €/test (before taxes). The most common approach for on-line data recording is the use of nitrate-specific ion-selective electrodes (ISEs), based on potential difference measurements [9, 10]. All these methods have certain advantages and disadvantages, depending on the envisaged application area. In case of bacterial culture media, a nitrate detection via direct UV- or infrared spectroscopy is mostly not feasible, because of too many interfering components. ISEs are easy to use and allow continuous data acquisition, however, ionic interferences have been observed [11] and electrodes must be replaced regularly. Therefore, indirect chemical detection methods can be cheap, easy, and reliable alternatives to more time consuming, or hardware requiring approaches.

We adapted the sodium salicylate method for the use in 96-well plates and tested it for interference with the most widely used mineral media for microalgae and cyanobacteria, the

blue-green algae (BG)-11 medium [12], artificial seawater nutrients (ASN)-III medium [12], Bold's basal medium (BBM) [13] and Zarrouk's (Z) medium [14]. The salicylate method for nitrate determination [15] was chosen because of its relative insensitivity against interfering ions [16], the feasibility and the cheap and relatively low toxic ingredients. It is based on two reaction steps, first the nitration of salicylate through addition of concentrated sulphuric acid. The 5-nitro isomer is preferentially formed over the 3-nitro isomer [17]. Secondly the formation of 5-sodiumnitrosalicylate through alkalisation of the previously formed 5-nitrosalicylic acid with sodium hydroxide (**Fig. 1**).

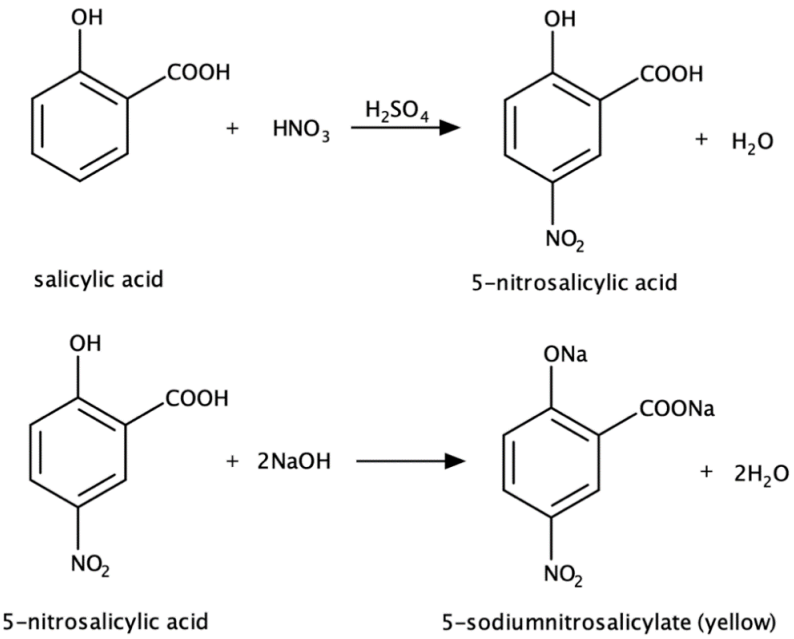

Fig. 1 Formation of 5-sodiumnitrosalicylate by nitration of salicylic acid through addition of conc. H_2SO_4 and alkalisation of the resulting 5-nitrosalicylic acid with 30 % (w/v) NaOH. For the first reaction step, water must be evaporated before the addition of H_2SO_4. The nitrate residue reacts with the H_2SO_4, resulting in the formation of nitric acid, which nitrifies the available salicylic acid. By alkalisation of the resulted compound with NaOH, the hydroxy- and carboxyl group are deprotonated. After an electrophilic addition of 2 Na^+, the yellow compound 5-sodiumnitrosalicylate is formed.

5-sodiumnitrosalicylate is a yellow compound with maximum absorbance at 412 nm (see **3.1**, **Fig. 2**). If technically necessary, a wavelength range of approx. 400 – 440 nm can as well be used for detection.

2 Materials and methods

2.1 Determination of nitrate concentration

2.1.1 Sodium salicylate assay

The adapted sodium salicylate method was performed in 96-well microtiter plates (Rotilabo® F-profile, Carl Roth GmbH, Karlsruhe, Germany). A sample volume of 25 µL was used for

each reaction. If nitrate concentrations higher than 350 mg L^{-1} were to be expected, samples were diluted 1:10 in H_2O_{demin}. Subsequently, 25 µL of 1 % (w/v) sodium salicylate solution were added to the samples. In this step, the bottom of the wells was covered evenly avoiding the formation of one single drop. The water was evaporated for 45 - 50 min at 80 °C in a drying cabinet (UT 6200, Heraeus GmbH, Hanau, Germany). The drying step is crucial, as all water must be eliminated for the formation of nitric acid from solid nitrate and sulphuric acid. The remaining salts were dissolved in 25 µL conc. H_2SO_4 by mixing on an incubation shaker at 200 rpm for 10 min at room temperature (TR-125, Infors AG, Bottmingen, Schweiz). The acidic solution was diluted with 150 µL H_2O_{demin} and then alkalised by addition of 125 µL 30 % (w/v) NaOH. After an incubation time of 5 min at room temperature, the absorption was measured at 405 nm, using a microtiter plate reader (EL808 Ultra Microplate Reader, Bio-Tek Instruments Inc., Vermont, USA). The method protocol is summarized in **Table 1**. The absorption maximum of 5-sodiumnitrosalicylate was determined by UV/VIS spectrometry (**Fig. 2**). Although 412 nm was determined as the optimal wavelength, the assay was performed at 405 nm, because of technical reasons.

Table 5 Application of the sodium salicylate method for nitrate concentration determination in 96-well plates.

Step #	Ingredients	Concentration	Volume [µL]
1	Sample	100 % (v/v) or 10 % (v/v)	25
2	Sodium salicylate	1 % (w/v)	25
3	**Evaporation at 80 °C (45 - 50 min)**		
4	H_2SO_4	Conc.	25
5	**Incubation for 10 min at 200 rpm (dried residue must be fully resolved)**		
6	H_2O_{demin}		150
7	NaOH	30 % (w/v)	125
8	**Incubation for 5 min**		
9	**Absorbance measurement at 405 nm**		

2.1.2 Reference assay (Griess assay)

The Griess assay is based on a first enzymatic reduction of nitrate to nitrite, followed by diazotisation of nitrite, resulting in a deep-purple azo-compound. The concentration of the reaction product can be determined by visible spectroscopy.

The Griess assay was used for verification of the adapted sodium salicylate assay (**3.3**, **Fig. 4**). A commercially available assay kit was used for the measurements (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Michigan, USA).

2.2 Mineral media

The mineral media tested for applicability with the adapted sodium salicylate assay are listed in **Table 2**. $NaNO_3$ was omitted from the table and added to the mineral media at varying concentrations for the purpose of assay calibration (**3.2**, **Fig. 3**). For the cultivation of *Synechococcus elongatus* PCC 6301 in BG-11, the indicated $NaNO_3$ concentration at 1.5 g L^{-1} was used.

Table 6 Chemical composition of the mineral media

Ingredient [g L^{-1}]	Cyanobacteria Mineral Medium			
	BG-11	**ASN-III**	**BBM**	**Zarrouk**
NaCl		25.0	0.025	1.0
$MgCl_2 \times 6H_2O$		2.0		
KCl		0.5		
$NaNO_3$	1.5	0.75	0.25	2.5
$K_2HPO_4 \times 3H_2O$	0.04	0.02	0.098	
KH_2PO_4			0.175	0.5
K_2SO_4				1.0
$MgSO_4 \times 7H_2O$	0.075	3.5	0.075	0.2
$CaCl_2 \times 2H_2O$	0.036	0.5	0.025	0.04
Citric acid	0.006	0.003		
Ferric ammonium citrate	0.006	0.003		
EDTA-Na_2Mg	0.001	0.0005		
EDTA-Na			0.05	0.08
KOH			0.031	
$FeSO_4 \times 7H_2O$			0.00498 (+ 0,1 % H_2SO_4)	0.01
Na_2CO_3	0.02	0.02		
$NaHCO_3$				16.8
H_3BO_3	$2.86\ 10^{-3}$	$2.86\ 10^{-3}$	$11.42\ 10^{-3}$	$2.86\ 10^{-3}$
$MnCl_2 \times 4H_2O$	$1.81\ 10^{-3}$	$1.81\ 10^{-3}$	$1.44\ 10^{-3}$	$1.81\ 10^{-3}$
$ZnSO_4 \times 7H_2O$	$0.222\ 10^{-3}$	$0.222\ 10^{-3}$	$8.82\ 10^{-3}$	$0.222\ 10^{-3}$
$Na_2MoO_4 \times 2H_2O$	$0.39\ 10^{-3}$	$0.39\ 10^{-3}$		$0.39\ 10^{-3}$
MoO_3			$0.71\ 10^{-3}$	
$CuSO_4 \times 5H_2O$	$0.079\ 10^{-3}$	$0.079\ 10^{-3}$	$1.57\ 10^{-3}$	$0.079\ 10^{-3}$
$Co(NO_3)_2 \times 6H_2O$	$0.0494\ 10^{-3}$	$0.0494\ 10^{-3}$	$0.49\ 10^{-3}$	$0.0494\ 10^{-3}$
final pH	**7.4**	**7.5**	**6.6**	**8.25**

2.3 Cultivation of *Synechococcus elongatus* PCC 6301

S. elongatus PCC 6301 was cultivated at 30 °C, 150 rpm and 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (Multitron Pro, Infors AG, Bottmingen, Switzerland) in 100 mL shaking flasks containing

20 mL of BG-11 medium, respectively. Before usage, the medium was sterilized by autoclaving at 121 °C for 20 min (Systec VE-150 Systec GmbH, Linden, Germany). All flasks were inoculated to an OD_{730} of 0.1 with cells of a preculture cultivated under the same conditions. The cell dry mass (CDM) was measured by serial harvest of three flasks each 2-3 days of cultivation and subsequent desiccation and weighting of the biomass. Cell harvest was performed by centrifugation at 4600 rpm for 15 min at room temperature (Rotanta 460 R, Hettich GmbH, Tuttlingen, Germany). Desiccation of the wet biomass was carried out at 70 °C for 15 h in a drying cabinet (UT 6200, Heraeus GmbH, Hanau, Germany). The CDM was measured with a precision scale (M254Ai-M, VWR GmbH, Darmstadt, Germany).

2.4 Statistical analysis

Data of the Griess-/ sodium-salicylate assay comparison (**Fig. 4**) was analysed at a 95% confidence level using ANOVA and post-hoc test, Tukey-HSD (XLSTAT 2022.4.1).

3 Results and discussion

3.1 Spectrogram of 5-nitrosalicylic acid

To identify the possible wavelength range for the detection of 5-nitrosalicylic acid, a spectrogram of the final reaction mixture was done. The range of the yellow target product is shown in **Fig. 2**.

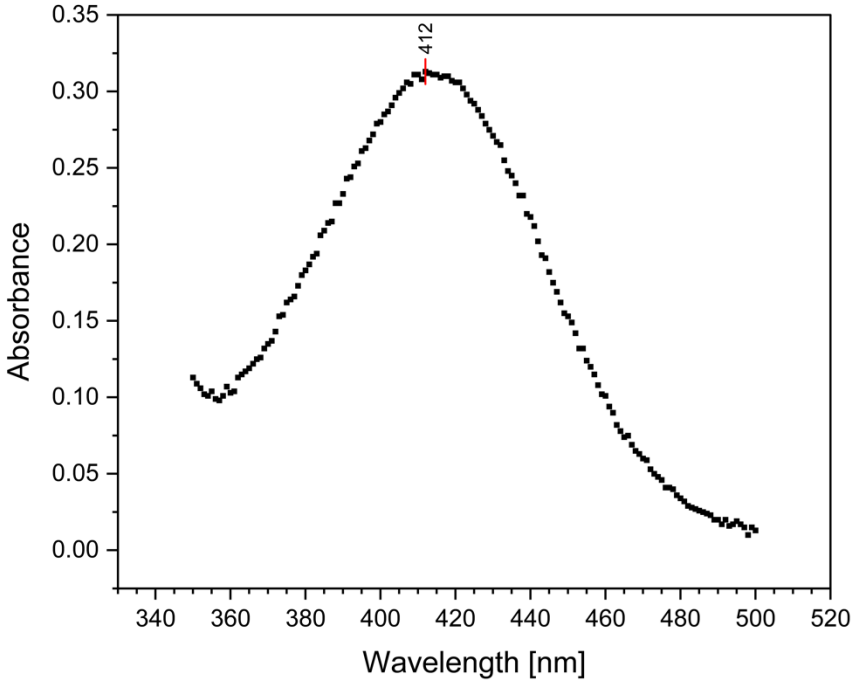

Fig. 2 Spectrogram of 5-sodiumnitrosalicylic acid, the absorption maximum is indicated at 412 nm. The absorbance scan was done using a spectrophotometer with a set scan range of 350 – 500 nm. The 5-sodiumnitrosalicylic acid was synthesized using the method shown in Table 1.

The shown UV/VIS spectrum of 5-nitrosalicylic acid shows an absorption maximum at about 410 to 420 nm, with a calculated peak at 412 nm. This is congruent with literature; a wavelength of 410 - 420 nm is mostly used for the detection of the component [16, 18–20]. The spectrogram shows that 5-nitrosalicylic acid can be detected at wavelengths between 400 and 430 nm, which expands the usability with photometers/well plate readers offering limited wavelength ranges.

3.2 Applicability for standard mineral media

The applicability of the adapted sodium salicylate method for widely used microalgae and cyanobacteria mineral media was tested with BG-11, BBM, ASN-III and Z-medium, containing nitrate concentrations between 0 and 450 µg mL^{-1} (**Fig. 3**).

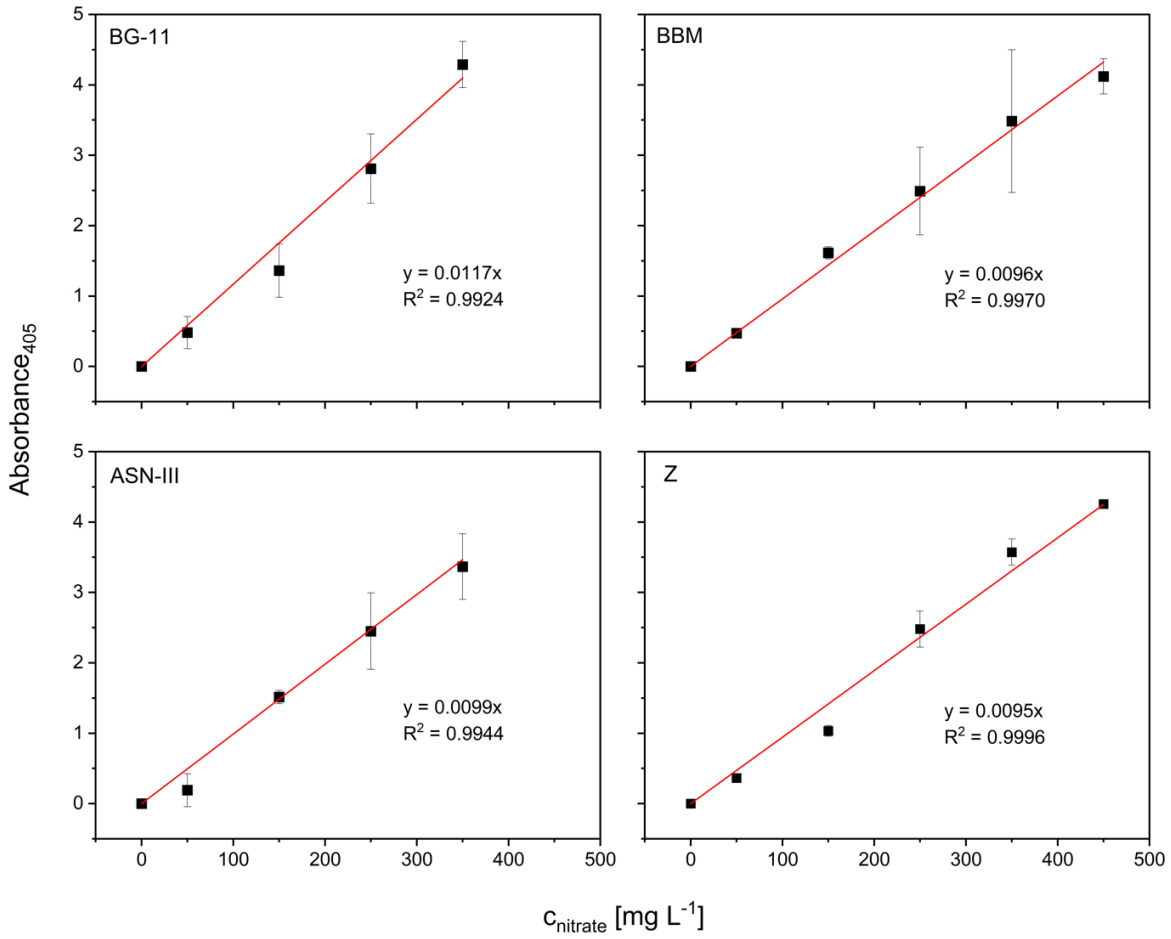

Fig. 3 Calibration curves of the sodium salicylate method adapted for 96-well plates using standard mineral media frequently used for cultivation of microalgae and cyanobacteria. $NaNO_3$ was used as NO_3 source, concentrations of 0 to 450 µg mL^{-1} were applied. The results represent the mean value (± standard deviation) of 4 replicates.

The calibration curves obtained with all four mineral media are linear until a nitrate concentration of 350 µg mL^{-1}. In case of BBM and Z-medium, a linearity until 450 µg mL^{-1} was confirmed. Concentrations exceeding the ones shown in **Fig. 3** led to non-linearity and must therefore be diluted as required. The coefficient of determination (R^2) for all calibration curves is between 0.9924 and 0.9996. Thus, the data fit the linear trend-lines with high accuracy. The slopes of all four straight lines range between 0.0095 and 0.0117. While the slopes of BBM, ASN-III and Z only differ by a magnitude of ± 0.00017, the standard deviation including the BG-11 data is ± 0.00089. Consequently, only the BG-11 slope differs more significantly from the other slopes. Nonetheless, the deviation is quite low and could be reduced by more replicates.

The data emphasize that the adapted sodium salicylate assay is suitable for the determination of nitrate concentrations in standard cyanobacteria mineral media and provides comparable data despite of diverging ingredients, salt concentrations and pH

values. It is known that high Cl^- concentrations of > 1.5 g L^{-1} and nitrite concentrations of > 10 mg L^{-1} N cause interferences in the sodium salicylate test [16, 18]. Especially the Cl^- concentration could play a role in marine microalgae cultivation. However, as only 25 µl sample is used in a total volume of 350 µl, a critical concentration is hard to reach. The application of ASN-III medium (25 g L^{-1} NaCl) had no unexpected effects on the modified assay. If in doubt, samples should be diluted accordingly, or Cl^- can be removed with silver sulphate. For the elimination of nitrite, the application of sulphamic acid was proposed [18].

3.3 Practical application of the assay

For a practical evaluation of the assay, *S. elongatus* PCC 6301 was cultivated in shaking flasks for 3-17 days and the nitrate concentration of the cell-free culture supernatants was determined. The obtained data was correlated with CDM values (**Fig. 4**).

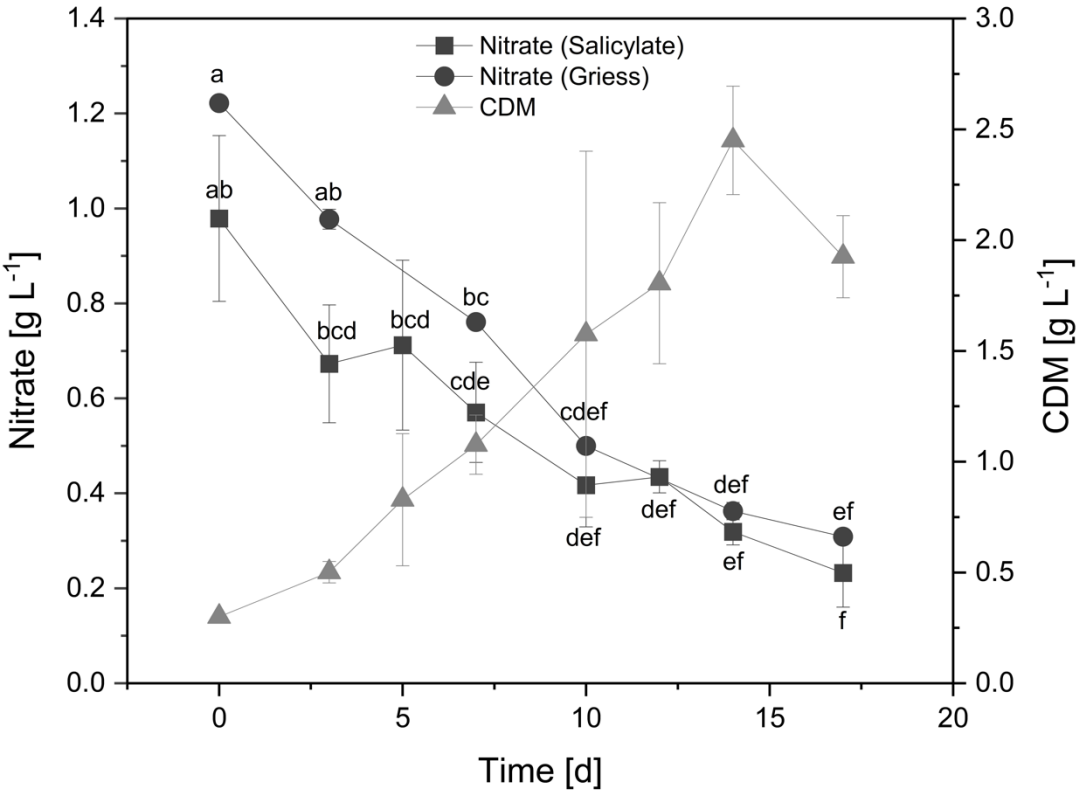

Fig. 4 Correlation of *S. elongatus* PCC 6301 CDM and nitrate concentration in the cell-free culture supernatants. Nitrate concentration was determined by the adapted sodium salicylate assay and Griess assay. Standard BG-11 with 1.5 g L^{-1} $NaNO_3$ (1.09 g L^{-1} NO_3^-) was used as cultivation medium. The results represent the mean value (± standard deviation) of 4 (nitrate) or 3 (CDM) replicates. Different letters indicate a significant difference at $p < 0.05$ (ANOVA and post-hoc with Tukey HSD).

The adapted sodium salicylate method was performed with samples of an actual *S. elongatus* cultivation, as the applicability for long-term cultivations should be shown. The obtained data was verified by Griess assay, a frequently used colorimetric low-budget test

for nitrite/nitrate analysis [7, 21, 22]. Both assays resulted into a comparable progression, the data match increases towards the end of the cultivation. The statistical analysis shows no significant differences between the corresponding values of both tests. Since standard BG-11 containing 1.5 g L^{-1} $NaNO_3$ was used, the initial nitrate concentration was 1.09 g L^{-1}. Standard deviations of the Griess assay data were considerably low, but the initially measured nitrate concentration was beyond the anticipated 1.09 g L^{-1}. Although having higher standard deviations, the adapted salicylate assay data represent the initial nitrate concentration more accurately. The nitrate depletion rate determined by means of the sodium salicylate assay is -3.0625 d^{-1}, a rate of -3.0703 d^{-1} was calculated with Griess assay data. The depletion rates show a high consistence between the two tests and are not significantly different ($p < 0.05$). It could be shown that the sodium salicylate method can be used for nitrate concentration determination in a long-term cyanobacterial culture. The assay is not interfered by any extracellular components or the typically increasing pH value. The adaption to 96-well plates offers a significantly higher sample throughput compared to the former assay, as well as to chromatographic methods. While an HPLC run takes about 30 min per sample [21], the adapted sodium salicylate assay allows the analysis of at least 100 samples in approximately 1.5 hours.

4 Conclusion

The proposed sodium salicylate method adapted for 96-well plates was shown to be fully applicable for the work with cyanobacteria and microalgae. Four different standard mineral media were tested for interfering effects, as well as samples of a long-term *S. elongatus* cultivation. Compared to the Griess assay, the initial nitrate concentration of the cultivation medium could be determined more accurately. The adapted sodium salicylate assay is quick and easy, as it does not require an additional chemical or enzymatical reduction step as the Griess method. Moreover, only minimal amounts of low-price chemicals with relatively low toxicity are needed.

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Chapter V

Continuous production of an antimicrobial metabolite with the terrestrial cyanobacterium *Chroococcidiopsis cubana*

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Keywords: Cyanobacteria, *Chroococcidiopsis cubana*, Antibiotic metabolite, Continuous production

Abstract

The terrestrial cyanobacterium *Chroococcidiopsis cubana* was identified as a producer of an antimicrobial substance inhibiting growth of the Gram-positive bacterium *Micrococcus luteus* and the yeast *Candida auris*. It was found that the production is initiated by nitrogen limitation, usually occurring during late exponential growth phase of the cyanobacterium. This effect was used for implementation of a continuous production set-up, which led to a significantly increased formation of the antimicrobial metabolite. The produced bioactive culture supernatant was purified by reverse phase chromatography; the most potent fraction caused a growth inhibition of over 90 % for both indicator organisms. The metabolite remained active until a temperature of approx. 45 °C; at increasing temperatures, the inhibiting effect decreased significantly. By identification of the trigger initiating the compound synthesis, a first scale-up could be implemented, leading to a reliable production of the antimicrobial metabolite. As *Candida auris* is an emerging pathogen causing serious infections, this work can be the first step to the development of a potent antifungal drug.

1 Introduction

Cyanobacteria are a long-known source for potent bioactive compounds [1–3]. Among these are highly toxic substances as the microcystins, which can cause severe health impacts on animals as well as on humans. But also more beneficial compounds with e.g. antibacterial [4–6], antiviral [7, 8], antifungal [4, 9, 10] and also antitumoral activity [11–13] are synthesized by cyanobacteria. However, until now, none of these substances is produced in an industrial scale or was authorised as medicinal drug, although some already passed clinical trials [14]. The reasons for this are diverse, one of them surely being the extremely tedious and costly drug approval process. Moreover, cyanobacteria are no ideal production organisms, especially for conventional bioreactor systems. As mainly phototrophic organisms, their cultivation requires specialized photobioreactors and constant light supply. In addition, the regulation and forming process of most cyanobacterial bioactive compounds is not fully understood, which results in low product yields and an unreliable process [2]. In case of fully uncovered and less complex metabolic pathways, the desired substances are commonly produced heterogeneously with standard production organisms as *Escherichia coli*, *Corynebacterium glutamicum* or *Saccharomyces cerevisiae* [15]. In case of cyanobacteria, such an approach can be challenging, as only few genomes are completely sequenced [16] and bioactive substances are mostly produced by non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS) or a combination of both [17–19]. These multi-domain enzyme complexes are encoded by large gene clusters. For example, the microcystin biosynthesis cluster from *Microcystis aeruginosa* spans 55

kilobase pairs [20]. Although it was shown that the cloning and heterologous expression of such large gene clusters is possible and can lead to functional products [21–24], such approaches are rather rare and not applied in an industrial scale. Main problems are incorrect enzyme folding, expensive precursor substrates and low product yields [23, 25]. Even though heterologous approaches could already show very promising results, it is still a long way to an economical production process.

An obvious alternative is the production of bioactive substances using the natural producing cyanobacteria strains. As mentioned before, the cultivation of these phototrophic bacteria requires specialized illuminated bioreactors. In some terrestrial cyanobacteria, the synthesis of pharmaceutically relevant metabolites is even desiccation induced, like the formation of the UV screening compound scytonemin [26], which requires the application of adapted reactor systems [27–29]. However, in times of increasing resource scarcity and climate change, a production process based on cheap mineral culture medium, CO_2-consumption and (sun-) light for energy supply can offer great chances over traditional bioprocesses using heterotrophic microorganisms.

For an upscaled production of valuable substances using cyanobacteria, it is crucial to have information on the regulation mechanisms that trigger the formation of the desired compounds and on how they can be exploited to maximize the product yield. Such information is generally only available to a limited degree, most research towards this subject was done for toxins from freshwater cyanobacteria, as microcystin, saxitoxin, cylindrospermopsin and anatoxin-a [30]. These molecules are all synthetised non-ribosomally by NRPS/PKS-systems or similar enzyme complexes in different cyanobacterial genera [31]. It was found that abiotic stimuli affecting the respective toxin-productivity is often strain dependent and common regulation rules are difficult to determine [31]. Very little research was done on the regulation of other, more beneficial, cyanobacterial secondary metabolites. Bloor and England examined the optimal mineral medium (BG-11) composition for the production of an antibiotic substance in *Nostoc muscorum* [32]. They discovered that a three-fold nitrate concentration of 26.4 mM, as well as a decrease in $FeSO_4 \times 6\ H_2O$ from 15 µM to 6 µM, maximized the antibiotic production. El-Sheek and colleagues (2006) also analysed the production parameters leading to an augmented antibiotic production in *N. muscorum* [6]. Their results confirm the findings of Bloor and England; a doubled nitrate concentration, while all other BG-11 components (so iron as well) are reduced by half, caused the strongest antagonistic activity in *N. muscorum* biomass extracts. Chetsumon et al. (1993) optimized the antibiotic production of immobilized *Scytonema* sp. TISTR 8208 cells. Best results were obtained by cultivation in standard mineral medium with 1.5 g L^{-1} $NaNO_3$ and an increased $FeSO_4 \times 6\ H_2O$ concentration of 0.025 g L^{-1} [33]. The presented examples show that the production

regulation of bioactive secondary metabolites in cyanobacteria depends on the specific strain and can depend on different physical and nutritional aspects. Although there is no common trigger for the formation of valuable secondary metabolites in cyanobacteria, it can still be worthwhile to test out different optimization options. Every information on regulation processes can help to establish a production process with constantly high yields of valuable bioactive substances.

The terrestrial cyanobacterium *Chroococcidiopsis cubana* attracted attention as a profound source of potentially interesting biosynthesis genes, coding for e.g. antimicrobial compounds. One of these putative bacteriocin-encoding genes was heterologously expressed in *E. coli* and showed broad growth-inhibiting activity against several Gram-positive and Gram-negative bacteria [34]. This compound was not synthesized by an NRPS or PKS and is therefore a suitable target for heterologous production. As the strain was found to also produce an antimicrobial metabolite natively, this study focused on the improvement of cultivation conditions, aiming at an increased compound production. The identification of a positive regulation trigger was intended to be used as the basis for a reliable production process, providing sufficient amounts of antimicrobial material for further analysis.

2 Material and methods

2.1 Used strains

The cyanobacterium *C. cubana* SAG 39.79 (obtained from *Culture collection of Algae* at Goettingen University) was used for the production of antimicrobial metabolites. As indicator organisms, mainly close relatives of the “ESKAPE” strains, named after *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species, were used and tested for susceptibility. These microorganisms are clinically most relevant pathogens, frequently evolving multidrug-resistances. All indicator organisms were obtained from the *German Collection of Microorganisms and Cell Cultures GmbH* (DSMZ).

- *Acinetobacter baylyi* DSM 14961
- *Bacillus subtilis* DSM 507
- *Candida auris* DSM 21092
- *Escherichia coli* DSM 498
- *Enterococcus caccae* DSM 19114
- *Micrococcus luteus* DSM 20030
- *Mycobacterium phlei* DSM 43239

- *Pseudomonas fluorescens* DSM 50090
- *Staphylococcus auricularis* DSM 20609

2.2 Cultivation and cell harvest

Cyanobacteria were cultivated in 100 mL flasks, in a photoincubator (Multitron Pro, Infors HT, Switzerland), at 120 rpm, 30 °C and a light intensity of 160 $\mu mol_{photons}$ m^{-2} s^{-1}, without addition of CO_2 or an organic carbon source. The standard mineral medium BG-11 [35] was used for cultivation. The heterotrophic organisms used in the antimicrobial assays were cultivated in 100 mL flasks at 150 rpm and 30 °C (TR-125, Infors AG, Switzerland), using Mueller-Hinton medium for all bacterial strains and Sabouraud medium for the yeast *C. auris* (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

C. cubana cultures were harvested in late exponential growth phase (approx. 14 days) by centrifugation at 4600 rpm (Rotanta 460 R, Hettich GmbH & Co. KG, Germany). Main cultures of 200 mL were inoculated with 1 g L^{-1} cell wet mass (CWM), respectively. Desiccation of the wet biomass was done at 70 °C for 15 h in a drying cabinet (UT 6200, Heraeus GmbH, Hanau, Germany). The cell dry mass (CDM) was measured with a precision scale (M254Ai-M, VWR GmbH, Darmstadt, Germany).

2.2.1 Cultivation in Bioreactors

Two identical 2.5 L bioreactors (Minifors I, Infors HT, Switzerland) were used for the comparative batch- and continuous cultivations of *C. cubana*. A respective cultivation volume of 1.7 L BG-11 medium was inoculated with 1 g L^{-1} CWM. For both processes, a constant cultivation temperature of 30 °C, an agitation speed of 150 rpm and a ventilation rate (sterile air) of 1 L min^{-1} was applied. Illumination was provided by standard (cold white) LED panels (VILTROX L116T, Shenzen Jueying Technology Co. LTD, Shenzhen, China). Light intensity was determined as 150 $\mu mol_{photons}$ m^{-2} s^{-1} (LI-250A Light Meter, LI-COR Biosciences, Germany). The cultivation processes were conducted under sterile conditions. After five days of identical batch cultivation, a dilution rate of 0.018 h^{-1} was applied to the bioreactor intended for continuous cultivation. BG-11 medium with a reduced $NaNO_3$ concentration of 0.274 g L^{-1} (≈ 0.062 g L^{-1} N) instead of 1.5 g L^{-1} was used for the continuous flow mode. By this, a condition of nitrate limitation could steadily be adjusted in the culture medium. The process was not used under chemostat conditions. Samples of both processes were taken in 2 mL triplicates each second/third day for a total cultivation time of 19 days.

2.3 Inhibition zone test

Inhibition zone tests were used for an initial, qualitatively screening of cyanobacteria for the production of antimicrobial metabolites. The applied indicator organisms (see 2.1) were

harvested in exponential growth phase, diluted to an OD_{600} of 0.1 and plated on Mueller-Hinton agar plates containing a respective volume of 25 mL. Subsequently, holes of 1 cm diameter were punched into the agar and filled with 100 µL cell-free cyanobacteria cultivation supernatant. BG-11 medium (100 µL, respectively) was taken as a negative control in order to exclude false-positive results caused by salts of the mineral medium. The plates were incubated for 24 h at 30 °C.

2.4 Checkerboard growth inhibition test

The checkerboard growth inhibition tests were performed in 96 well plates (Nunc™ MicroWell™ 96-Well Microplates, Thermo Scientific™, Waltham, USA). For each well, a volume of 50 µL *M. luteus*/*C. auris* suspension, diluted to an OD_{600} of 0.2 with the respective cultivation medium, was mixed with 50 µL of cell free *C. cubana* cultivation sample. The indicator organisms were harvested in exponential growth phase. The test plates were sealed with parafilm and incubated at 30 °C and 150 rpm for 16 h (TR-125, Infors AG, Switzerland). The OD_{595} was measured (EL 808, BioTek Instruments GmbH, Germany) and the difference of OD_{start} to OD_{end} was compared to the negative control (nc), which consisted of 50 µL of the respective indicator organism suspension mixed with 50 µL BG-11 mineral medium. The nc was cultivated exactly as the test samples. The reached OD_{595} of *M. luteus*/*C. auris* in presence of BG-11 served as 100 % growth reference. The inhibiting activity [%] was calculated as follows:

$$100 - \left(\frac{OD_{end} - OD_{start}}{OD_{nc}} \times 100\right) \qquad \text{(Eq. 1)}$$

The respective mean values of four biological replicates are shown, error bars represent standard deviation. The statistical analysis was done using a two-tailed homoscedastic Student's t-test. If no separate groups were compared (all shown data, except for Fig. 7), the inhibition data was compared to the control values using the end-OD_{595} values.

2.5 Nitrate test

The nitrate concentration in the *C. cubana* culture supernatants was measured by a modified version of the sodium salicylate method [36], using 96 well plates. All steps were exactly conducted as specified in the reference.

2.6 Purification of the antibiotic substance

The antibiotic substance was purified and concentrated by reversed phase chromatography (RPC), using an Äkta™ start FPLC system (Cytiva Europe GmbH, Freiburg, Germany) with an XK 26 column (Cytiva Europe GmbH, Freiburg, Germany), filled with Diaion HP-20 resin

(styrene-divinylbenzene, 250 – 850 µm, Mitsubishi Chemical Corp., Tokyo, Japan). The column was packed to a bed volume of 100 mL. As binding buffer, 50 mM ammoniumacetate and 2 % (v/v) acetonitrile was used, the elution buffer consisted of 20 % binding buffer and 80 % acetonitrile. For the purification and concentration of the antibiotic substance produced by *C. cubana*, 6 L of culture broth harvested in the continuous cultivation process was filtrated through 0.4 µm sterile filters (Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top Filters, Thermo Scientific™, Waltham, USA) prior to loading the column. The drained culture broth was collected over approx. 8 days; the subfractions were harvested daily, followed by filtration and storage at 4 °C until the purification step. The column was loaded with a flow rate of 5 mL min^{-1} overnight. Elution was done over 4 column volumes; the eluate was collected in 15 mL fractions. The fractions were transferred to 6-well-plates (PS, ATC0.1, Carl Roth GmbH, Karlsruhe, Germany) and evaporated to complete dryness for 24 – 48 h at 40 °C in a drying cabinet (UT 6200, Heraeus GmbH, Hanau, Germany). The drying temperature was selected on the basis of temperature stability tests (**2.7**; **Fig. 6**). The residues were resolved in 2 mL H_2O_{demin}, respectively, and subsequently used in the checkerboard growth inhibition test (**2.4**).

2.7 Temperature stability test

For the determination of the antibiotic´s temperature stability, bioactive *C. cubana* culture supernatant was heated for one hour at temperatures between 37 and 80 °C on a thermoshaker (TS-100 BIOSan, Latvia). Afterwards, the samples were tested for activity by a checkerboard growth inhibition test (2.4).

3 Results and Discussion

3.1 Screening for antibiotic substances

Several heterotrophic bacteria and one yeast were tested for susceptibility against the antimicrobial metabolite produced by *C. cubana*. As indicator organisms, primarily close relatives of the ESKAPE-strains were used in inhibition zone tests (**Table 1**).

Table 1 Indicator strains tested for susceptibility

Strain	Gram-type	Susceptibility	Max. Inhibition zone diameter [cm]
Acinetobacter baylyi DSM 14961	-	-	-
Bacillus subtilis DSM 507	+	-	-
Candida auris DSM 21092	/	+	2 (diminished cell growth)
Escherichia coli DSM 498	-	-	-
Enterococcus caccae DSM 19114	+	-	-
Micrococcus luteus DSM 20030	+	+	1.2 (clear zone)
Mycobacterium phlei DSM 43239	+	-	-
Pseudomonas fluorescens DSM 50090	-	-	-
Staphylococcus auricularis DSM 20609	+	-	-

From the tested strains, the Gram-positive bacterium *M. luteus* and the yeast *C. auris* showed growth inhibition zones on agar plates treated with *C. cubana* culture supernatant (**Fig. 1**).

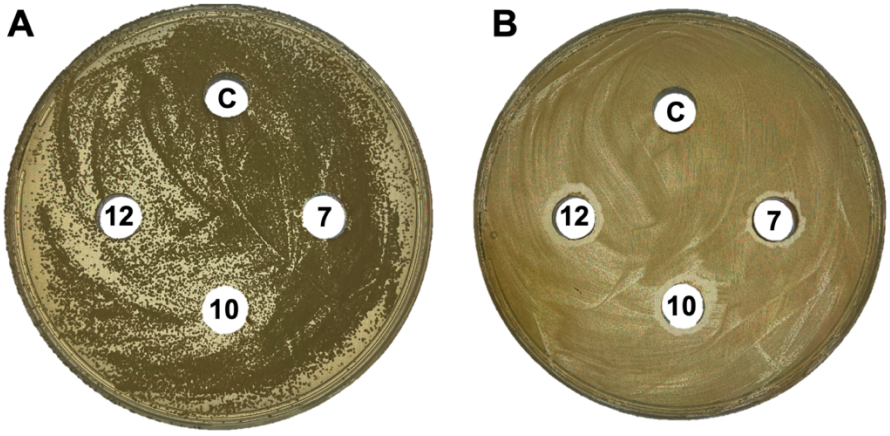

Fig. 1: Inhibition zone test with *C. cubana* culture supernatants, taken after 7, 10 and 12 days of batch cultivation using 100 mL shaking flasks, in BG-11 medium at 150 rpm and 160 µmol m^{-2} s^{-1}. *C. auris* (A) and *M. luteus* (B) served as indicator strains for verification of the antimicrobial activity. C, control. Inhibition zone diameters (including punch holes): A, day 10: 1.7 cm, day 12: 2 cm; B, day 7: 1.05 cm, day 10: 1.2 cm, day 12: 1,1 cm.

Interestingly, many antibiotic compounds produced by cyanobacteria act on both, bacterial and fungal species [37], however, not much is known about the respective mode of action [38]. Most identified antifungal metabolites from cyanobacteria are cyclic peptides and target the cell membrane, leading to lytic cell death [38]. A similar mechanism was described for an antibiotic produced by *Scytonema* sp., which showed broad spectrum activity against Gram-positive bacteria and fungal/yeast cells [39], comparable to the activity spectrum of the compound described in this work. Although the mode of action was not examined in this

study, a cell membrane affecting activity that causes changes in permeability would be thinkable.

It was observed that the inhibiting activity of the supernatant strongly depends on the cultivation time. Samples taken after 7-12 days after inoculation showed the most significant activity, while samples before and after this point showed decreased or no activity. The culture supernatant taken after twelve days had the greatest impact on *C. auris* and *M. luteus* growth (**Fig. 1**)

For further examination of the antimicrobial substance, an increased and stable production was required. Consequently, the exact conditions under which the substance was optimally produced had to be elucidated. By repeated correlation of *C. cubana* CDW increase, nitrate depletion in the cultivation medium and inhibition of *M. luteus* and *C. auris* growth, it was found that growth inhibition of the indicator organisms was always highest when nitrate levels were low, approx. beyond 0.4 g L^{-1} (**Fig. 2**).

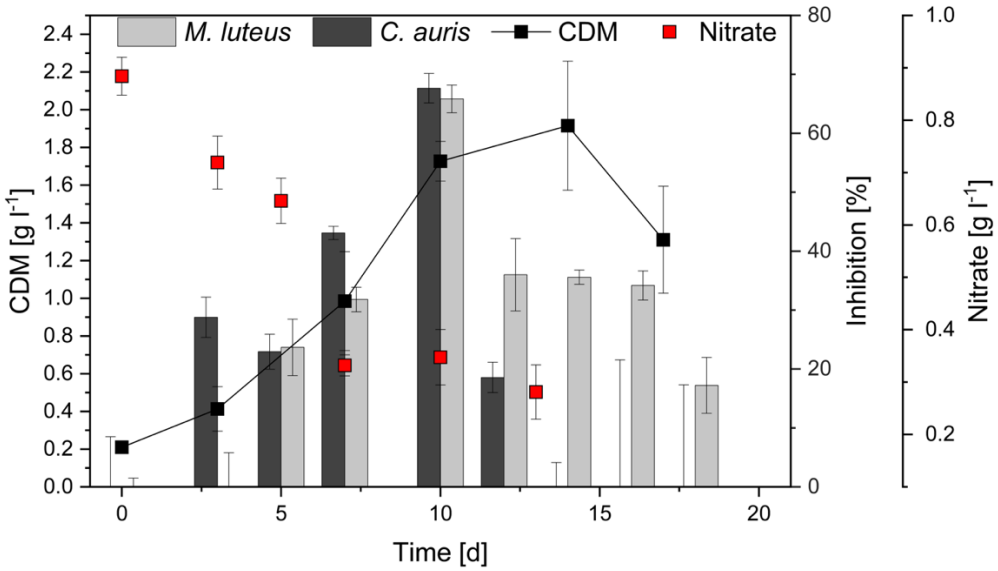

Fig. 2: Correlation of *C. cubana* cell growth under phototrophic conditions with nitrate depletion and growth inhibiting activity against *C. auris* and *M. luteus*. The cultivation was performed in 100 mL shaking flasks in BG-11 medium at 150 rpm and 160 µmol m^{-2} s^{-1}. The *C. cubana* CDM was measured by serial harvest of three shaking flasks, respectively, each 2-3 cultivation days (n = 3). The inhibiting activity was tested by growth assays in microtiter plates, using *C. cubana* cell free culture supernatant and the respective indicator organism (Start OD_{600} 0.1; mixing ratio 1:1; n = 4). The plates were incubated for 15 h at 150 rpm and 30 °C. The final OD of the indicator organism was measured at 595 nm. The nitrate concentration was measured by sodium salicylate assay, adapted for 96-well microplates (n = 6) [36]. Nitrate concentration was measured until the antimicrobial activity reached a maximum. The results represent the mean value (± standard deviation) of 3 (CDM), 4 (inhibition) or 6 (nitrate) biological replicates.

As shown in Fig. 2, the highest inhibition effects occurred with supernatant samples taken between days five and ten of *C. cubana* shaking flask cultivation. While *C. auris* growth inhibition already begins when treated with supernatant of the third *C. cubana* cultivation

day, inhibition of *M. luteus* increases more slowly. Effects were first observed after five cultivation days. In both cases, the inhibiting activity climaxes at day ten. The varying growth inhibition intensities shown by both indicator organisms after day ten are putatively caused by a diverging susceptibility against the substance produced by *C. cubana*. A hint on this can be seen in **Fig. 1**; the *C. cubana* culture supernatant causes colony-free inhibition zones with *M. luteus*, while the inhibition zones of *C. auris* show only a diminished number of colonies, but no complete cell growth inhibition.

3.2 Establishment of a continuous production setup

As the until then obtained data suggested a nitrogen limitation to be the trigger initiating the production of an antimicrobial substance by *C. cubana*, a continuous cultivation system was set up for the acquisition of more assured data (**Fig. 3**).

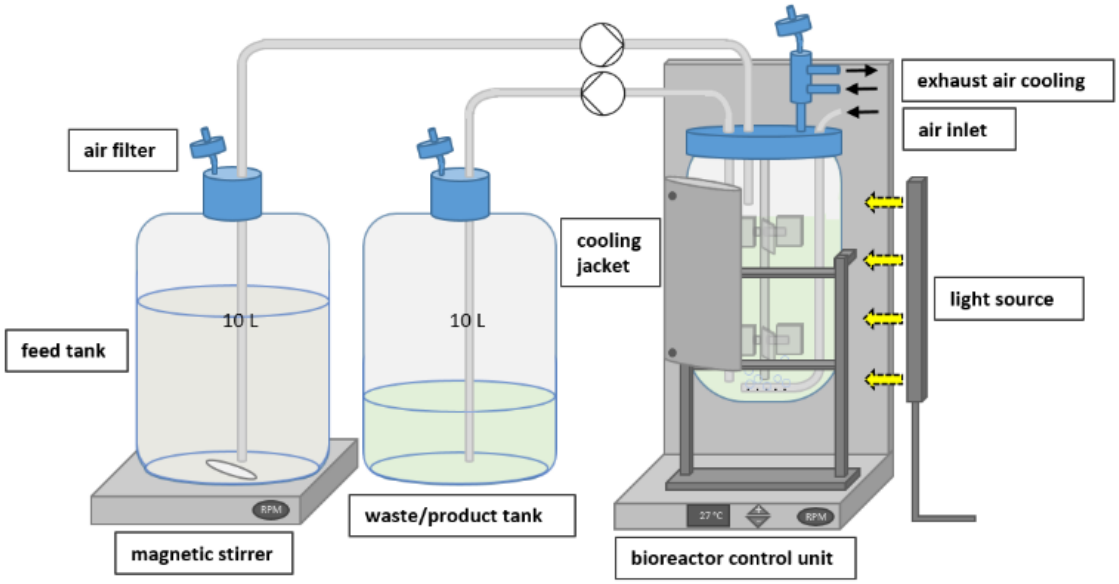

Fig. 3: Continuous cultivation of *C. cubana* in an illuminated bioreactor. (Modified) BG-11 medium with 0 – 1.5 g L^{-1} $NaNO_3$ was used as cultivation medium. Inoculum: 1 g L^{-1} CWM, stirring rate: 150 rpm, gassing: 1 L min^{-1} of sterile air, V_L = 1.7 l, T = 30 °C. Illumination (150 $\mu mol_{photons}$ m^{-2} s^{-1}) was provided by cold white LED panels (VILTROX L116T, Shenzen Jueying Technology Co. LTD, Shenzhen, China).

The continuous cultivation of *C. cubana* was used for a controlled decrease in nitrate concentration and a concomitant verification of the previously proposed N-limitation hypothesis. The cyanobacterium was first cultivated in batch mode using standard BG-11 for five days. Afterwards, the set-up was turned into continuous mode; BG-11 without a nitrogen source (BG-11_0) was used as feed medium. Over a period of ten days, samples were taken and checked for inhibiting activity against *C. auris* and *M. luteus*, as well as for remaining nitrate concentrations (**Fig. 4**).

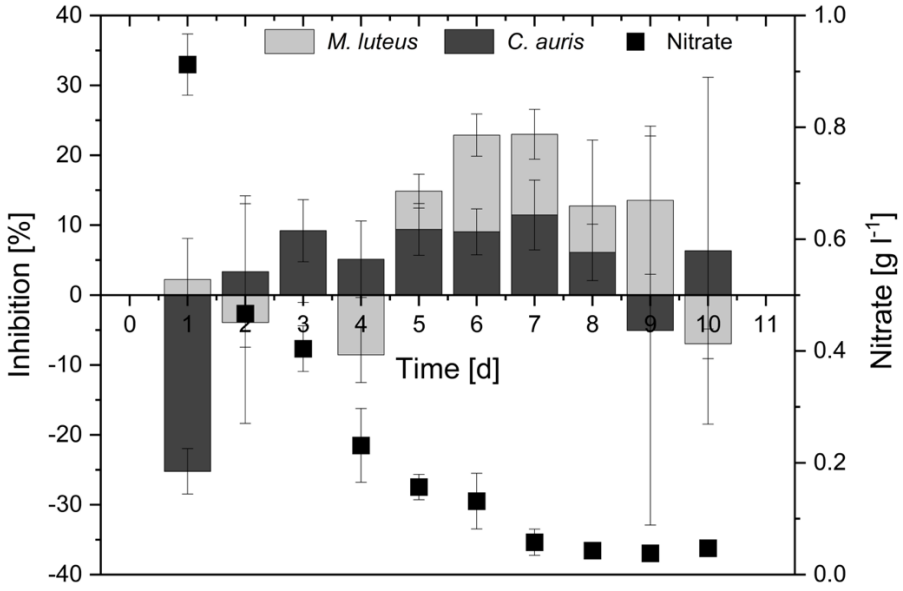

Fig. 4: Correlation of nitrate depletion in a *C. cubana* continuous cultivation (D = 0.0375 h^{-1}) and growth inhibiting activity against *C. auris* and *M. luteus*. The cultivation was performed in an illuminated 2 L stirred tank reactor at 150 rpm and 160 µmol m^{-2} s^{-1}. The inhibiting activity was tested by growth assays in microtiter plates, using *C. cubana* cell free culture supernatant and the respective indicator organism (Start OD_{600} 0.1; mixing ratio 1:1; n = 4). The plates were incubated for 15 h at 150 rpm and 30 °C. The final OD of the indicator organism was measured at 595 nm. The nitrate concentration was measured by sodium salicylate assay, adapted for 96-well microplates (n = 6) [36]. The results represent the mean value (± standard deviation) of 4 (inhibition) or 6 (nitrate) biological replicates.

The inhibiting activity of the cell-free *C. cubana* supernatants increased with decreasing nitrate concentration. Growth of the indicator organisms was most inhibited from samples taken after six and seven cultivation days, when the nitrate concentration was between 0.131 ± 0.05 and 0.059 ± 0.024 g L^{-1}. When the nitrate concentration fell below this point, the inhibiting activity of the culture supernatant decreased significantly. The observed effects were more significant with *M. luteus* than with *C. auris*, however, the general data progress remained congruent. In this experiment, a maximum growth inhibition of 22.99 ± 3.57 % for *M. luteus* and 11.46 ± 5.0 % for *C. auris* was reached with *C. cubana* culture supernatant of day seven. The significantly lower inhibition values compared to the ones obtained in the shaking flask experiment (**Fig. 3**) can be explained by the dilution rate of 0.0375 h^{-1} in the continuous run, which allowed only a short time interval with an optimal nitrate concentration for the production of the antimicrobial metabolite. The high standard deviation of some inhibition values, mostly those resulting from *C. cubana* culture supernatant samples taken towards the end of the cultivation, is probably the result of outlier replicates caused by a mix of decreasing antibiotic impact and increasingly exported extracellular polymeric substances (EPS). The latter ones mainly consists of peptides and sugars and can serve as growth enhancers for the indicator organisms.

As the optimal nitrate concentration range to produce the antibiotic substance was established, this information could be used for the setup of a continuous production system (**Fig. 5**).

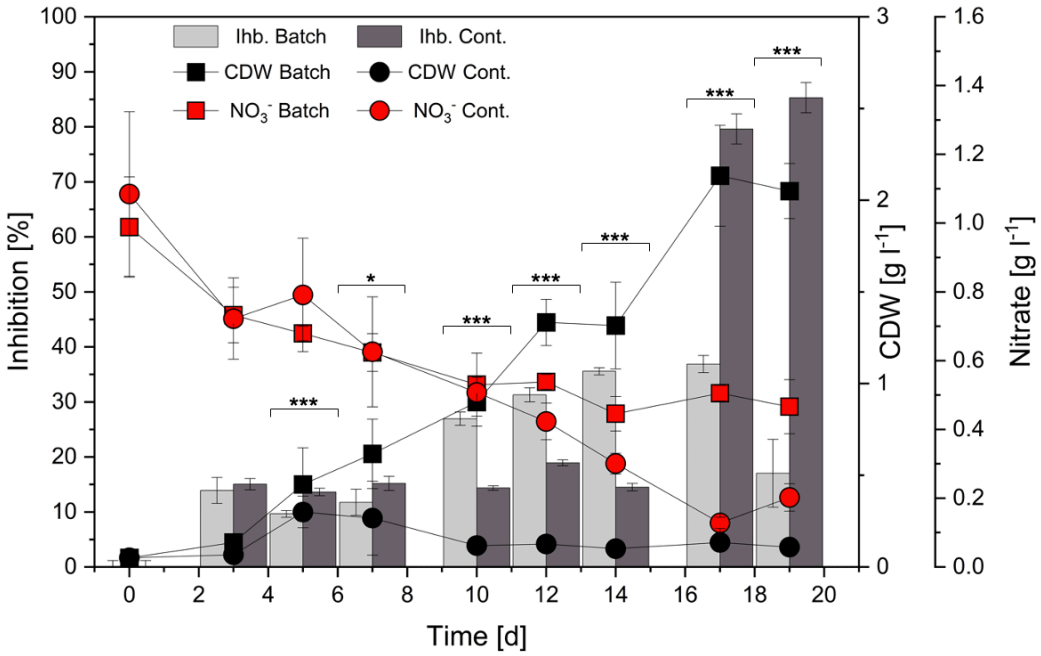

Fig. 5: Correlation of inhibiting activity, cell growth and nitrate depletion in *C. cubana* batch- and continuous cultivation (D = 0.018 h^{-1}). BG-11 with 0.3 g L^{-1} $NaNO_3$ (≙ 0.219 g L^{-1} NO_3^-) was used as cultivation medium for the continuous cultivation, standard BG-11 for batch. Preceding continuous mode, the bioreactor was run in batch mode for five days, allowing a sufficient initial cell density. Cell-free culture supernatant of *C. cubana* was tested for antimicrobial activity against *M. luteus*. The inhibiting activity was tested by growth assays in microtiter plates, using *C. cubana* cell free culture supernatant and *M. luteus* as indicator organism (Start OD_{600} 0.1; mixing ratio 1:1; n = 4). The plates were incubated for 15 h at 150 rpm and 30 °C. The final OD of the indicator organism was measured at 595 nm. The nitrate concentration was measured by sodium salicylate assay, adapted for 96-well microplates (n = 6) [36]. The CDW of *C. cubana* was measured gravimetrically after desiccation at 70 °C for 15 h (n = 3). The results represent the mean value (± standard deviation) of 3 (CDM), 4 (inhibition) or 6 (nitrate) biological replicates. Asterisks indicate significant differences in inhibition between samples from batch and continuous cultivation (*, $p \leq 0.1$; ***, $p \leq 0.001$), determined by a two-tailed homoscedastic Student's t-test.

The inhibitory activity of *C. cubana* culture supernatants from cultivation in batch- and continuous mode was compared for evaluation of the production process effectiveness. For the continuous cultivation, BG-11 with 0.3 g L^{-1} $NaNO_3$ (≙ 0.219 g L^{-1} NO_3^-) was used as feed medium, standard BG-11 for the preceding five days batch mode cultivation. Simultaneously, the *C. cubana* CDW and nitrate consumption was measured and correlated with the inhibition values. As expected from the previous experiments, the inhibitory activity of *C. cubana* batch cultivation supernatants reaches its maximum between exponential and stationary growth phase, at day 17 of cultivation, and a nitrate concentration of 0.5 g L^{-1}. By batch cultivation, a maximum *M. luteus* growth inhibition of

36.88 ± 1.55 % was reached. After that point, the inhibitory activity decreased drastically. The continuous cultivation samples from day 3 to day 14 caused a constantly low growth inhibition of 13 – 19 %. When the target nitrate concentration of 0.219 g L^{-1} was reached between cultivation days 15 and 17, the inhibiting activity significantly increased to over 80 %. A maximum of 85.29 ± 2.75 % was measured using supernatant taken after 19 days of *C. cubana* cultivation. Besides for days 3 and 7, the inhibiting activity from continuous cultivation samples was considerably higher than from batch samples, with high significance ($p \leq 0.001$). The obtained data confirm the findings of the experiment depicted in **Fig. 4** and prove that a continuous production of the antibiotic substance synthesized by *C. cubana* is possible by continuous cultivation using a feed-medium with a reduced nitrogen source. A $NaNO_3$ concentration of 0.3 g L^{-1} was shown to greatly improve the culture supernatant's inhibiting activity, which indicates a higher concentration of the antibiotic substance.

3.3 Enrichment of the antibiotic substance

Prior to purification attempts, the antibiotic substance produced by *C. cubana* was tested for temperature stability (**Fig. 6**). This test was necessary for the subsequent establishment of a purification process, as the RPC fractions had to be dried for complete evaporation of the used buffer and solvent. By this, any false-positive results could be excluded in the following inhibition assay.

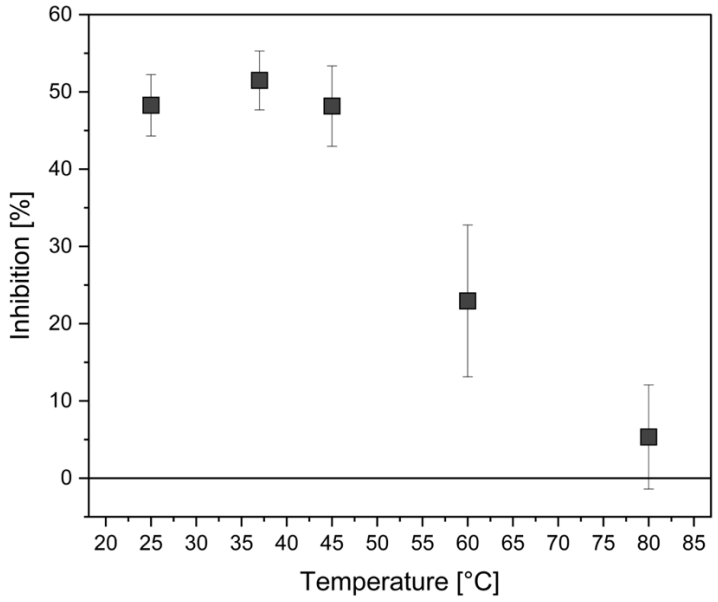

Fig. 6: Temperature stability test of the antimicrobial metabolite produced by *C. cubana*. Non-purified, cell-free culture supernatants were incubated for 1 h at the respective temperature, cooled to room temperature and tested for inhibiting activity by growth assays in microtiter plates, using *C. auris* as indicator organism (Start OD_{600} 0.1; mixing ratio 1:1; n = 4). Samples were incubated at 25 (positive control), 37, 45, 60 and 80 °C. The shown data represent relative values, referring to BG-11 as negative control. The plates were incubated for 15 h at 150 rpm and 30 °C. The final OD of

the indicator organism was measured at 595 nm. The results represent the mean value (± standard deviation) of 4 biological replicates.

The obtained data confirmed a temperature stability till 45 °C, with no or only slight decrease of inhibiting activity. An incubation of the antimicrobial compound at 60 °C led to an activity drop of about 25 %, which further decreased to 5.34 ± 6.7 % after heat treatment at 80 °C. As a result, purified fractions obtained by RPC could be evaporated safely at 40 °C, without a loss of activity.

The improved production of the antibiotic substance by the continuous cultivation of *C. cubana* at a reduced nitrate concentration enabled subsequent purification attempts. Six liters of cell free cultivation broth were purified by RPC (**2.6**). The obtained fractions were completely dried, residues were resuspended in H_2O_{demin} and used in a growth inhibition test against *M. luteus* and *C. auris* (**Fig. 7**).

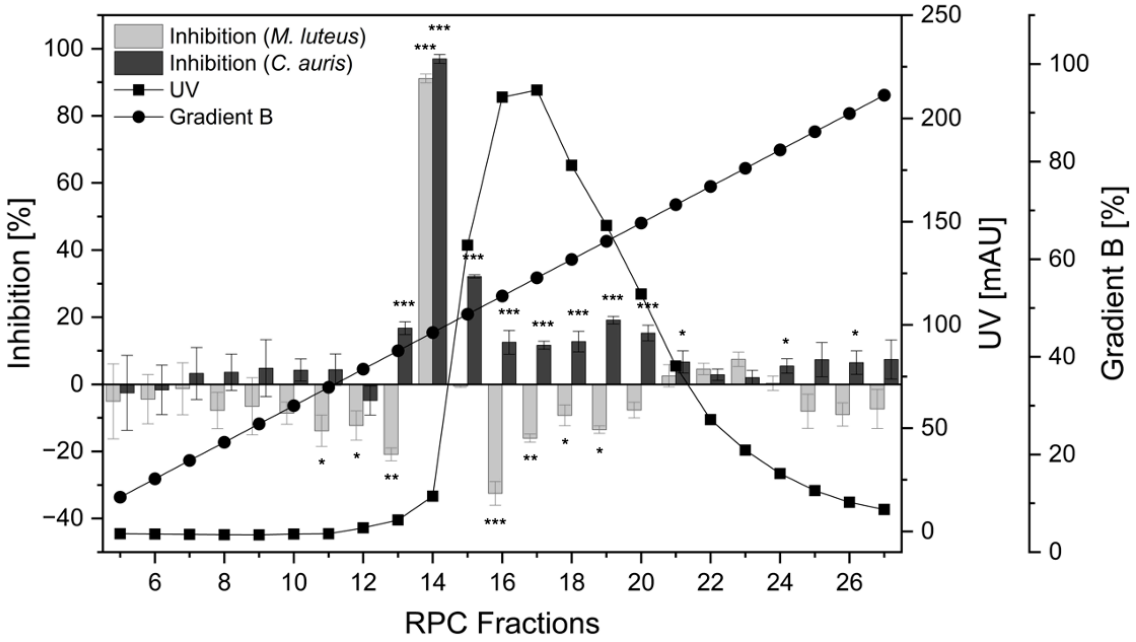

Fig. 7: Enrichment of the antimicrobial metabolite by RPC. Fractions were evaporated to complete dryness at 40 °C, resuspended in 2 mL H_2O_{demin}, respectively, and tested for inhibiting activity by growth assays in microtiter plates, using *C. auris* and *M. luteus* as indicator organisms (Start OD_{600} 0.1; mixing ratio 1:1; n = 4). The shown data represent relative values, referring to H_2O_{demin} as negative control. The plates were incubated for 15 h at 150 rpm and 30 °C. The final OD of the indicator organism was measured at 595 nm. Gradient B represents the increasing elution buffer gradient. The UV light absorbance was measured at 280 nm. The results represent the mean value (± standard deviation) of 4 biological replicates. Asterisks indicate a significant inhibition effect compared to the negative control (*, p ≤ 0.1; **, p ≤ 0.01 ***, p ≤ 0.001), determined by a two-tailed homoscedastic Student's t-test.

Fraction 14 exhibited by far the most growth inhibiting activity against both, *M. luteus* (91.15 % ± 1.32) and *C. auris* (96.95 % ± 1.32) and thus contains the antimicrobial component from *C. cubana*. A false positive effect was excluded by a negative control with

evaporated elution buffer, resolved in H_2O_{demin}. The successful enrichment of the metabolite by RPC indicates a hydrophobic nature. Purification attempts by ionic interaction chromatography did not lead to active fractions (data not shown). The antibiotic substance was eluted at an elution buffer concentration of 45 %; at this point, an UV absorbance of 17.04 mAU was measured. As the absorbance starts to rise along with the antibiotic activity, a peptidic structure could be assumed. The main peak in UV absorbance was measured after the elution of the antimicrobial compound and probably consists of protein components from EPS, produced by all terrestrial cyanobacteria [40]. The respective RPC elution fractions did not possess an inhibitory activity comparable to the one of fraction 14. As the inhibition values were calculated by means of the negative control, fractions showing negative inhibition values must contain growth-supporting elements as sugars, which are also contained in EPS [41]. These effects are probably masked by the antimicrobial compound mainly contained in fractions 14 and 15.

3.4 Putative regulation mechanism and identification of the responsible gene cluster

As nitrate starvation was clearly identified as the trigger for the production of the antibiotic metabolite, an *in silico* examination for identification of the eligible biosynthetic gene clusters was done. Several of these clusters were analysed for adjacent genes encoding transcriptional regulators and transport systems that could be involved in the regulation of expression and export of the antimicrobial metabolite. It was especially searched for already known linkages of nitrate limitation and the metabolic/transcriptomic answer it triggers in cyanobacteria.

According to the genome analysis using antiSMASH, *C. cubana* harbors five biosynthetic gene clusters, which could possibly be responsible for the synthesis of the antibiotic substance described in this work. These were chosen because of encoded transcriptional regulators in their relative proximity, which could be activated be nitrogen starvation. Also, some adjacent genes coding for transporter proteins are of interest. Among the five candidates are two NRPS clusters (ctg_56, WP_148125936.1 and ctg_5, WP_127022514.1/WP_127022517.1/RUT13761.1), two NRPS-like clusters (ctg_134, WP_241994450.1 and ctg_171, WP_106169125.1) and one lanthipeptide class v gene cluster (ctg_39, WP_106167084.1/WP_106167087.1). The product of the latter one appears to belong to the novel class v of lanthipeptides. This class was described only recently, current members are lexapeptide from *Streptomyces rochei* Sal35 [42], which shows potent activity against Gram-positive bacteria, and cacaoidin, synthesized by *Streptomyces cacaoi* [43]. Both peptides show the particularity of an N-terminal (N,N)-dimethyl phenylalanin, a C-terminal (2-aminovinyl)-3-methyl-cysteine, as well as a D-

alanine. Generally, lanthipeptides are ribosomally synthesized and post-translationally modified molecules (RiPPs), which contain thioether cross-linked (methyl-) lanthionine and often show potent antibiotic activity (lantibiotics), typically against Gram-positive bacteria. Although they are mostly produced by Gram-positive bacteria, a lantibiotic synthesized by Gram-negative *Chitinophaga pinensis* (Pinensin) was isolated, that exhibits a high inhibiting activity against filamentous fungi and yeasts [44]. Also, several class v lanthipeptide gene clusters were recently identified in cyanobacterial genomes [42]. In *C. cubana*, the corresponding gene cluster is situated next to genes encoding a sensor histidine kinase (Hik) (WP_12702384.1) and a response regulator (WP_015155273.1), respectively. Hiks are known to be responsible for the regulation of stress-induced gene expression in cyanobacteria [45, 46]. In *Synechococcus* they were shown to be involved in the response to nitrogen limitation [47]. Upstream of the NRPS-like gene cluster ctg_171, another Hik is encoded (WP_106169124.1). Solely a response regulator gene (WP_106168295.1) is situated upstream of NRPS-cluster ctg_56, without an adjacent sensor histidine kinase. Downstream of NRPS-cluster ctg_5, two AraC-family transcriptional regulators (TRs) were identified. AraC-type TRs are one of the most abundant bacterial regulators, which mostly positively regulate gene expression. AraC was first described as regulator of the L-arabinose operon in *Escherichia coli* [48]. In the cyanobacterium *Nostoc punctiforme*, an AraC-type transcription factor was shown to upregulate the expression of a PKS-cluster [49]; in *Anabaena* PCC7120 five AraC TRs were upregulated during nitrogen starvation [50]. Summarized, NRPS cluster ctg_5, together with the downstream situated *araC*, could be a suitable candidate for encoding the antibiotic metabolite produced by *C. cubana*. However, also downstream of the NRPS cluster, genes for siderophore exportation are situated. These consist of a periplasmic binding protein, a TonB-dependent siderophore receptor and a cytoplasmic membrane protein. As siderophores are often synthesized by NRPS [51], which is also described for cyanobacteria [52], it is more likely that ctg_5 encodes an NRPS responsible for the synthesis of siderophores. The expression of the respective genes is putatively regulated by the adjacently encoded AraC TR. Another interesting *C. cubana* NRPS-like gene cluster is ctg_134. In its downstream region, an EamA/DMT-family transporter was identified. Drug metabolite transporters (DMT) are often involved in amino acid metabolism [53], a member of the subfamily EamA was described as YdeD in *E. coli*, an O-acetyl-serine/cysteine exporter [54]. In *Pseudomonas aeruginosa*, an EamA-like transporter is involved in the export of antimicrobial peptides [55], the cyanobacterium *Aphanizomenon gracile* uses SxtPer, a permease of the DMT superfamily, for the export of the highly potent neurotoxin saxitoxin [56]. The transcription of ctg_134 could be regulated by a LuxR family DNA-binding response regulator, which was identified downstream of the NRPS-like cluster.

Generally, LuxR TRs are known to be involved in Quorum Sensing, the initiation of virulence factors and the synthesis of antibiotics [57]. However, to our knowledge, until now no LuxR family TR was identified in cyanobacteria that is involved in the regulation of nitrogen starvation stress.

In conclusion, several gene clusters in the genome of *C. cubana* were identified through *in silico* analysis, which could possibly be involved in the production of the detected antibiotic metabolite. For identification of the responsible genes, a transcriptomic analysis could give a more certain insight.

3.5 Process industrialization

As already mentioned in the introduction, an industrial production of bioactive compounds from cyanobacteria is challenging, not least because of low product yields. Thus, an economic process is only feasible by high-priced products. With regard to the described growth inhibitory effect against the emerging human pathogen *C. auris*, a use as a high-value antimycotic drug would be thinkable. Of course, further studies excluding a possible toxicity towards human cells are a necessary prerequisite, especially with regard to the compound's activity against eucaryotic yeast cells, which often hinders new antimycotic therapies [58]. An advantage for a future process scale up is the elimination of cell disruption steps, because of the direct export of the compound to the surrounding culture medium. Since the cells must still be separated from the medium before the extraction process, a cultivation approach using immobilized cells would be favourable. The formerly mentioned antibiotic from *Scytonema* sp. was continuously produced using a seaweed-type bioreactor with cells attached to polyurethan foam strips. A similar approach could be feasible with *C. cubana*; a study dealing with optimization strategies for an improved biofilm production yielded a biofilm concentration of 20 g L^{-1} in 10 days, using this strain [59], which could facilitate a surface-attached growth. The application of continuous cultivation systems for pharmaceutical manufacturing offers a crucial advantage over batch or fed batch systems, as it can significantly reduce production costs and increase product yields by permanent productivity [60]. Mainly because of strict regulations and product safety aspects, the sector of continuously manufactured drugs is still slowly developing. However, between 2015 and 2017, five partly continuously produced small-molecule biosimilars were FDA approved [61], which at least constitutes a positive sign for future continuous biomanufacturing processes.

4 Conclusion

The terrestrial cyanobacterium *Chroococcidiopsis cubana* was identified as the producer of an antimicrobial metabolite with potent growth inhibitory activity against the Gram-positive bacterium *Micrococcus luteus* and the yeast *Candida auris*. The latter one is a newly emerging human pathogen, causing severe and difficultly treatable infections [62, 63]. It was found that the synthesis and export of the substance is initiated by nitrogen starvation, which sets in at about 0.2 g L^{-1} nitrate. By cultivation of *C. cubana* in a continuous cultivation system using mineral medium with a reduced nitrate concentration, a continuously high production of the target substance was achieved. The metabolite was purified and concentrated by RPC, the active fraction nearly completely inhibited growth of both indicator organisms. The metabolite is stable until 45 °C, after incubation at 60 °C, nearly half the activity was lost. By *in silico* analysis using the biosynthetic gene cluster identification/identification tool antiSMASH, several potentially involved gene clusters were identified in the genome of *C. cubana*. The most promising candidates are ctg_39, putatively encoding a class five lanthipeptide, with a sensor histidine kinase/response regulator in close proximity, and ctg_134, an NRPS-like cluster being situated adjacent to a transporter of the DMT-family encoding gene. The identification of the trigger initiating the metabolite's production and the thereby enabled set up of a continuous production system will greatly help to further elucidate the profound metabolic principles and to reveal the identity of the antibiotic substance in future studies; this next step is currently addressed by subsequent LC-MS analysis.

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Chapter VI

Heterologous production of a cyanobacterial bacteriocin with potent antibacterial activity

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Authorship contribution

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D. Strieth:	Validation, resources.
S. Eggert:	Methodology, validation, investigation (data for fig. 6), resources, review & editing, funding acquisition.
S. Kins:	Resources, review & editing, funding acquisition.
R. Ulber:	Conceptualization, resources, review & editing, funding acquisition.
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Keywords: cyanobacteria, bacteriocins, *Chroococcidiopsis cubana*

Abstract

With regard to the emerging health threat by antibiotic resistant bacteria, bacteriocins are considered as a promising way to overcome this urgent problem. In this work, a hitherto unknown bacteriocin from the terrestrial cyanobacterium *Chroococcidiopsis cubana* was heterologously expressed in *Escherichia coli*, purified to homogeneity and tested for activity against several bacteria and one yeast species. The compound showed potent bacteriolytic activity against several Gram-positive bacteria and slight activity against one Gram-negative strain. The bacteriocin had no cytotoxic impact on mouse neuroblastoma N2a cells, indicating its potential for treatment against Gram-positive bacterial pathogens in human diseases.

1 Introduction

Cyanobacteria are a rich source of different bioactive substances and valuable secondary metabolites (Carroll et al., 2019; Lakatos & Strieth, 2017; Singh et al., 2020). On the one hand there are the widely known cyanobacterial toxins as Anatoxin A from *Anabaena* (Mahmood & Carmichael, 1987) and Microcystin from *Microcystis* (Konst et al., 1965), which are very harmful for mammals and water organisms. On the other hand, cyanobacteria produce numerous valuable secondary metabolites such as pigments, long-chain fatty acids, proteins and polysaccharides, which can be, and partly already are used in food industry (Borowitzka, 1995; Burja et al., 2007). Additionally, cyanobacteria also produce a wide range of bioactive substances with potent activity against other bacteria (Ghasemi et al., 2004; Volk & Furkert, 2006), fungi (Shishido et al., 2015), viruses (Carpine & Sieber, 2021) and also tumour cells (Silva-Stenico et al., 2011). Many of these metabolites are produced by nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or PKS-NRPS hybrids, large multienzyme complexes which assemble and modify single peptides to one bioactive molecule (Jones et al., 2009; Welker et al., 2012). However, next to this mode of complex molecule synthesis, many cyanobacteria also generate ribosomal peptides with very promising biological activities – the bacteriocins. These small molecules are produced by a wide range of organisms, but especially lactobacterial bacteriocins were in focus of research for a long time (Gradisteanu Pircalabioru et al., 2021). The most prominent representative nisin, produced by *Lactococcus lactis*, is a widely applied antibiotic peptide for the prevention of bacterial growth in the food industry (Delves-Broughton et al., 1996). Most bacteriocins belong to the heterogeneous class of ribosomally-synthesized and post-translationally-modified peptides (RiPPs), with the exception of class II lactic acid bacterial bacteriocins, which are unmodified or cyclic. The superordinate term RiPPs comprises a large number of diverse subclasses which are

produced from many different prokaryotic and eukaryotic organisms and undergo various posttranslational modifications (Arnison et al., 2013). However, the mode of biosynthesis is a commonality that all RiPPs share. The ribosomally synthesized precursor peptide contains the core peptide, which is N-terminally flanked by a leader peptide and sometimes also C-terminally by a recognition sequence. The latter can be important for further processing of the core peptide. Eukaryotic precursor peptides often contain an additional signal sequence at the N-terminus of the leader peptide. During the maturation process the core peptide gets enzymatically cleaved from the leader peptide und can undergo further modifications. Whereas the leader regions of all RiPPs show significant sequence similarities, the core peptides are extremely variable (Oman & Donk, 2010).

Since the discovery of nisin in 1933 (Whitehead, 1933), Gram-positive bacteria and especially lactobacteria were in focus of bacteriocin research. However, also Gram-negative bacteria receive attention as bacteriocin producing organisms. Well-known examples are the colicins and microcins from *E. coli* and some other *Enterobacteriaceae* (Luria & Suit, 1987). More recently, the group of cyanobactins was described, which contains small cyclic peptides that possess highly diverse bioactivities and only occur in cyanobacteria (Sivonen et al., 2010; Welker & Von Döhren, 2006). A major breakthrough in the identification of novel putative bacteriocins became possible by the massive advances of the *in silico* techniques. The number of sequenced genomes is quickly growing, enabling a rapid search for conserved motifs of already known bacteriocin gene clusters. By this, a double-glycin-type motif was identified as a conserved amino acid sequence of bacteriocins in Gram-negative bacteria, among them also several cyanobacterial genera such as *Synechococcus*, *Synechocystis*, *Nostoc* and *Prochlorococcus* (Dirix et al., 2004). As subclasses of these double-glycine-type precursor peptides, the nitrile hydratase leader (NHLP) family and the N11P family, which is annotated as Nif11 nitrogen-fixing protein, were identified in cyanobacteria (Haft et al., 2010).

The cyanobacterial genus *Chroococcidiopsis* is known to include extremely desiccation and radiation tolerant strains and was therefore even treated as a possible candidate for terraforming Mars (Friedmann & Ocampo-Friedmann, 1995; Billi et al., 2011). *Chroococcidiopis* appears as single coccoid cells which duplicate through binary fission (Rippka et al. , 1979). Strain *C. cubana* SAG 39.79 was isolated from a soil sample (Pinar del Rio, Cuba) in 1966 (Komárek & Hindak, 1975). In antimicrobial screenings prior to this work, *C. cubana* showed hints for producing growth inhibiting substances effective against *Micrococcus luteus* (data not shown). Therefore, this terrestrial cyanobacterium was further investigated by bioinformatic- and molecular biological attempts.

2 Material and methods

2.1 Generation of expression strains

The DNA sequence of *b135CC* was amplified from *C. cubana* gDNA by PCR (Phusion DNA Polymerase, Thermo Scientific™, Waltham, USA), using the primers 5'-AAAAGGATCCATGAAAGCAAGTACTAACTTTAC-3' and 5'-AAAACTCGAGGCTAACTATCCCTGTCACCG-3' (restriction sites underlined). The gDNA was extracted with the NucleoSpin Tissue Mini Kit (Macherey-Nagel, Düren, Germany). The amplified DNA was purified by agarose gel electrophoresis (1 % w/v) and retrieved with the GeneJet Gel Extraction Kit (Thermo Scientific™, Waltham, USA). *b135CC* was cloned into the vector pJET1.2/blunt (CloneJET PCR Cloning Kit, Thermo Scientific™, Waltham, USA) and competent *E. coli* NEB 5-alpha cells (New England Biolabs, Ipswich, USA) were transformed with the resulting recombinant plasmid. Correctly transformed cells were identified on LB agar (1.5 % w/v) with ampicillin (100 µg ml^{-1}) and by analytical PCR. The insert was cut from pJET1.2/blunt using the restriction enzymes *Bam*H I and *Xho* I (Thermo Scientific™, Waltham, USA) and subsequently cloned into the digested expression vector pET23a (Merck, Darmstadt, Germany). The plasmid was used for transformation of competent *E. coli* LEMO21 (DE3) cells (New England Biolabs, Ipswich, USA). Correctly transformed cells were identified on LB agar (1.5 % w/v) with ampicillin (100 µg ml^{-1}) and chloramphenicol (25 µg ml^{-1}), as well as by analytical PCR. All DNA ligation steps were carried out with T4 DNA ligase (Thermo Scientific™, Waltham, USA). Isolation of plasmids was done with the NucleoSpin Plasmid Mini Kit (Macherey-Nagel, Düren, Germany). The correct orientation and sequence of the DNA insert was checked by sequencing (LGC Genomics GmbH, Berlin, Germany). All procedures were carried out according to the manufacturers' recommendations.

2.2 Protein expression

Main cultures of the expression strain were inoculated with 2 % of an overnight-grown preculture (30 °C, 200 rpm, TR-125, Infors AG, Bottmingen, Switzerland). LB supplemented with 100 µg ml^{-1} ampicillin, 25 µg ml^{-1} chloramphenicol and 1 mM L-rhamnose was used as cultivation medium. The expression was induced at OD_{600} 0.8 with 400 µM IPTG. The main culture was cultivated at 37 °C and 200 rpm for 20 h.

2.3 Cell harvest and cell disruption

Cell harvest was performed for 15 min at 2360 x g (Rotanta 460 R, Hettich Zentrifugen, Germany), followed by a wash step with 0.9 % (w/v) sterile saline. Cells were disrupted via

ultrasonic probe (Bandelin HD 70, UW 70, Bandelin, Berlin, Germany; 5 x 30 s, 100 % power; 1 min cooling on ice between each cycle).

2.4 Protein purification

The cell extract was clarified by centrifugation at 2360 x g for 100 min (Rotanta 460 R, Hettich Zentrifugen, Tuttlingen, Germany). Purification of the bacteriocin was achieved by affinity chromatography using a HisTrap™ HP 5 ml column and an Äkta™ start FPLC system (Cytiva Europe GmbH, Freiburg Germany). Binding buffer: 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 5 mM imidazole, pH 7.4; elution buffer: 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4. The bacteriocin was desalted with a HiTrap™ 5 ml desalting column (Cytiva Europe GmbH, Freiburg, Germany), using 20 mM NaH_2PO_4/Na_2HPO_4 buffer. The protein concentration was determined by Bradford assay.

2.5 Bioactivity test

The growth assays of the indicator bacteria were performed in 96 well plates (Nunc™ MicroWell™ 96-Well Microplates, Thermo Scientific™, Waltham, USA). Per well, a volume of 50 µl bacterial suspension (OD_{600} 0.2) was mixed with 50 µl prediluted bacteriocin suspension. Final B135CC concentrations of respectively 150, 100 and 50 µg ml^{-1} were used for *M. luteus*, *M. phlei* and *S. auricularis*; reduced concentrations of 25, 12.5 and 6.25 µg ml^{-1} were applied for *S. auricularis*. All used bacteria were cultivated in Müller-Hinton medium and harvested in the exponential growth phase. The bacterial suspensions were diluted with Müller-Hinton medium, the bacteriocin dilutions were done with 20 mM NaH_2PO_4/Na_2HPO_4 buffer.

2.6 Protein leakage test

Samples of *S. auricularis* (OD_{600} 1.0) were mixed with different concentrations of B135CC, followed by an incubation period of 6 h at 30 °C. Samples were taken every two hours, the protein concentrations were determined by Lowry-Assay (Pierce™ Modified Lowry Protein Assay Kit, Thermo Scientific, Massachusetts, USA). The respective share of total *S. auricularis* cell protein was determined through comparison with cells disrupted by ultrasound (Bandelin HD 70, UW 70, Bandelin, Berlin, Germany; 5 x 30 s, 100 % power; 1 min cooling on ice between each cycle).

2.7 Cytotoxicity assay

The cytotoxicity of B135CC was tested by Lactate Dehydrogenase (LDH) activity test. The assay was performed with the Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), as specified by the manufacturer. The bacteriocin was diluted with N2a medium (DMEM high glucose, 1 % penicillin/streptomycin, 1 % sodium pyruvate, 1 % L-glutamine, 1 % nonessential amino acids (all from Gibco, Thermo Fisher Scientific, Massachusetts, USA), and 10% FBS (PAN Biotech, Aidenbach, Germany) to a final volume of 100 µl and added in different concentrations to the N2a cells in a 96-well plate, followed by an overnight incubation at 37 °C and 5 % CO_2. Addition of 100 µl 10 mM HEPES buffer (vehicle of the protein) to the N2a cells (neuroblastoma mouse cells) was used as a control. Further tests for positive and negative control were carried out the following day using lysis buffer for maximum LDH release and distilled H_2O to measure spontaneous LDH activity, respectively.

3 Results and Discussion

As the genome of *C. cubana* was sequenced for phylogenetic analyses in 2019 (Will et al., 2019), an *in silico* scan for biosynthetic gene clusters could be performed using the open source online platform *antiSMASH* (Blin et al., 2019). Besides several NRPS and PKS domains, four bacteriocin precursor encoding gene clusters (locus tags ctg7_159, ctg38_34, ctg42_31/32 and ctg135_5) were identified. All four genetic sequences were amplified by PCR and used to generate *E. coli* expression strains, of which only strain *E. coli* LEMO21 pET23a::*ctg135_5* produced a significant amount of recombinant protein. The associated putative bacteriocin was designated as B135CC. The corresponding gene was identified by *antiSMASH* as an RiPP-like protein encoding sequence, which contains an NHLP family leader peptide domain (TIGR03793). Upstream of *ctg135_5* a short-chain dehydrogenase was identified as a putative additional biosynthetic gene (Fig. 1a). In a sequence alignment with four other cyanobacterial NHLP-like proteins from *Nostoc* sp. PCC 7120 (WP_010996185), *Anabaena variabilis* (WP_011317978), *Nostoc punctiforme* (WP_012409643) and *Nostoc* sp. PCC 7120 (WP_010996188), the high degree of sequence homology in the leader peptide region became evident (Fig. 1B, N-terminal sequence area up to peptidase cleavage site).

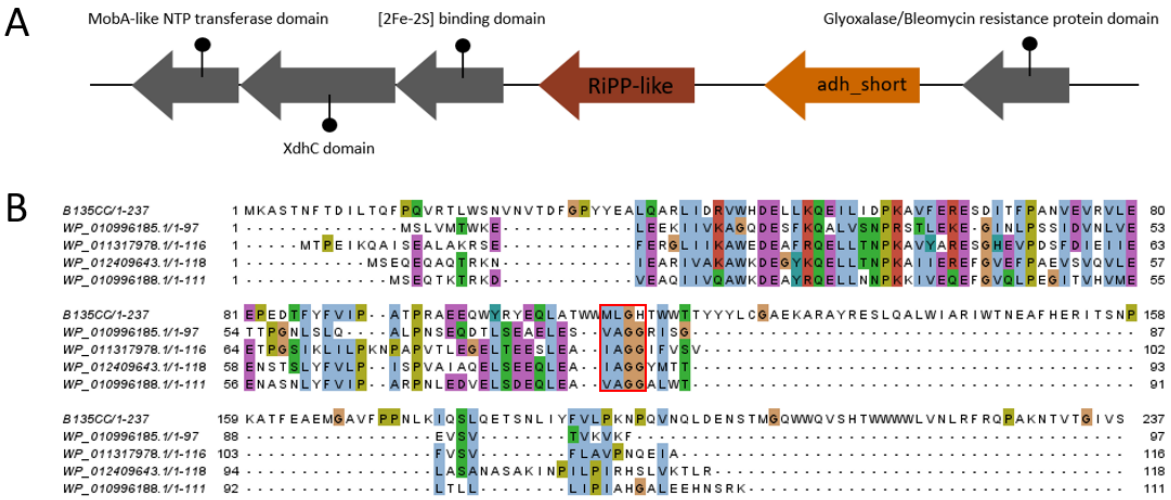

Fig. 1 **A**, Relevant genomic region of *b135CC*, putative gene products and protein domains identified by *antiSMASH*. Black pins indicate predicted protein domains. **B**, Sequence alignment of B135CC (locus tag ctg135_5) with four cyanobacterial NHLP-like proteins from *Nostoc* sp. PCC 7120 (WP_010996185), *Anabaena variabilis* (WP_011317978), *Nostoc punctiforme* (WP_012409643) and *Nostoc* sp. PCC 7120 (WP_010996188). Sequence alignment done with the online tool *MUSCLE* (Madeira et al., 2019), image created with *Jalview* (Waterhouse et al., 2009). Red box indicates the putative double glycine leader peptide cleavage site.

Interestingly, the conserved region containing the typical double glycine peptide cleavage site (Fig. 1B, red box) differs from the other sequences, as the second glycine (N to C terminus) is replaced by a histidine. However, it is known that only the first glycine is constantly conserved in this region, while the second glycine can be substituted by other amino acids (Havarstein et al., 1994). Although leader peptide sequences of double-glycine-type motif bacteriocins tend to be highly conserved, the core peptides are hypervariable (Oman & Donk, 2010). That can also be noticed in the depicted sequence alignment (Fig. 1B). However, the core peptide of B135CC is significantly longer than those of the compared precursor proteins. In case of most other bacteriocin gene clusters found in cyanobacteria, several other domains involved in bacteriocin production, maturation and transport were found in close proximity to the bacteriocin core gene(s) (Wang et al., 2011). As already elucidated in 1995, bacterial bacteriocins are transported across the cytoplasmic cell membrane by ATP-binding cassette (ABC) transporters, which contain a C39 peptidase domain concomitantly cleaving the leader peptide off the core peptide. The associated genes are mostly situated next to the precursor peptide encoding genes. NHLP like bacteriocin precursors were identified in association with thiazole/oxazole-modified microcins (TOMM) (Haft et al., 2010). These posttranslational modifications are performed by the combined action of a cyclohydratase, a dehydrogenase and a docking/scaffolding protein, which work on serine, threonine and cysteine residues in the core peptide to form the according heterocycles. The associated genes are generally located next to the

precursors. B135CC also contains an NHLP leader peptide domain and ten threonine and seven serine residues, which could be converted to oxazoles and methyloxazoles, respectively. However, there is no evidence for this assumption in case of B135CC.
The bacteriocin B135CC was heterologously produced with *E. coli* Lemo21 (New England Biolabs GmbH) and purified by affinity chromatography taking advantage of the C-terminal poly His-tag conferred by the used medium-copy expression vector pET23a (Fig. 2).

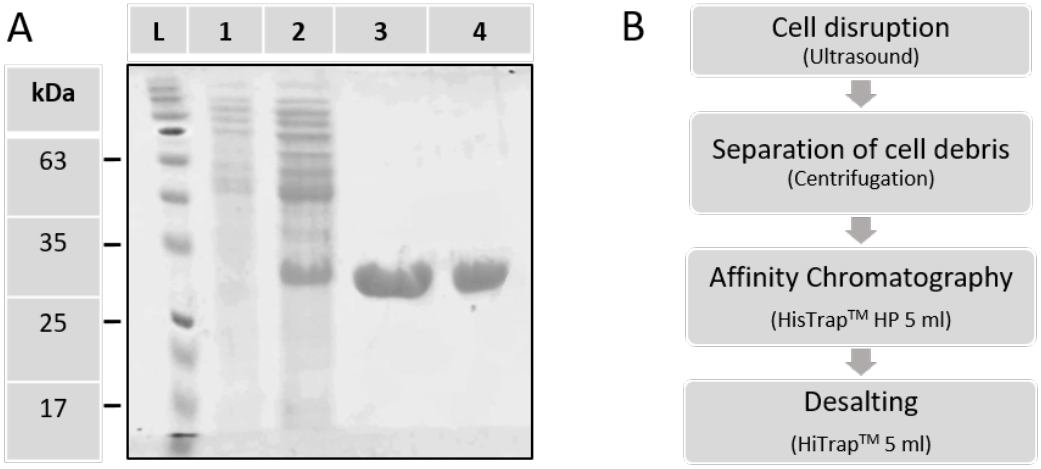

Fig. 2 A, Purification of the His-tagged bacteriocin B135CC. Protein expression was performed in *E. coli* LEMO21 (DE3) pET23a::*b135cc* (New England Biolabs GmbH). L, protein ladder (SERVA Triple Color Proteinstandard III); 1, cell-free extract, empty vector control; 2, cell-free extract; 3, HisTrap elution fraction; 4, desalted HisTrap elution fraction. Stained with Quick Coomassie Stain, Serva, Heidelberg, Germany). **B**, Purification steps.

The result of the SDS-PAGE (Coomassie staining) showed a full size protein (of approx. 29,3 kDa) that was not cleaved by an *E. coli* peptidase, although *E. coli* generally possesses several ABC transporters containing a C39 peptidase domain. The purified protein was subsequently tested for antimicrobial activity by microtiter plate growth assays against the Gram-positive bacteria *Bacillus subtilis*, *Micrococcus luteus*, *Mycobacterium phlei* and *Staphylococcus auricularis*, the Gram-negative strains *Acinetobacter baylyi*, *Pseudomonas fluorescens* and *E. coli*, as well as against the yeast *Candida auris* (Fig. 3, only results of sensitive strains shown).

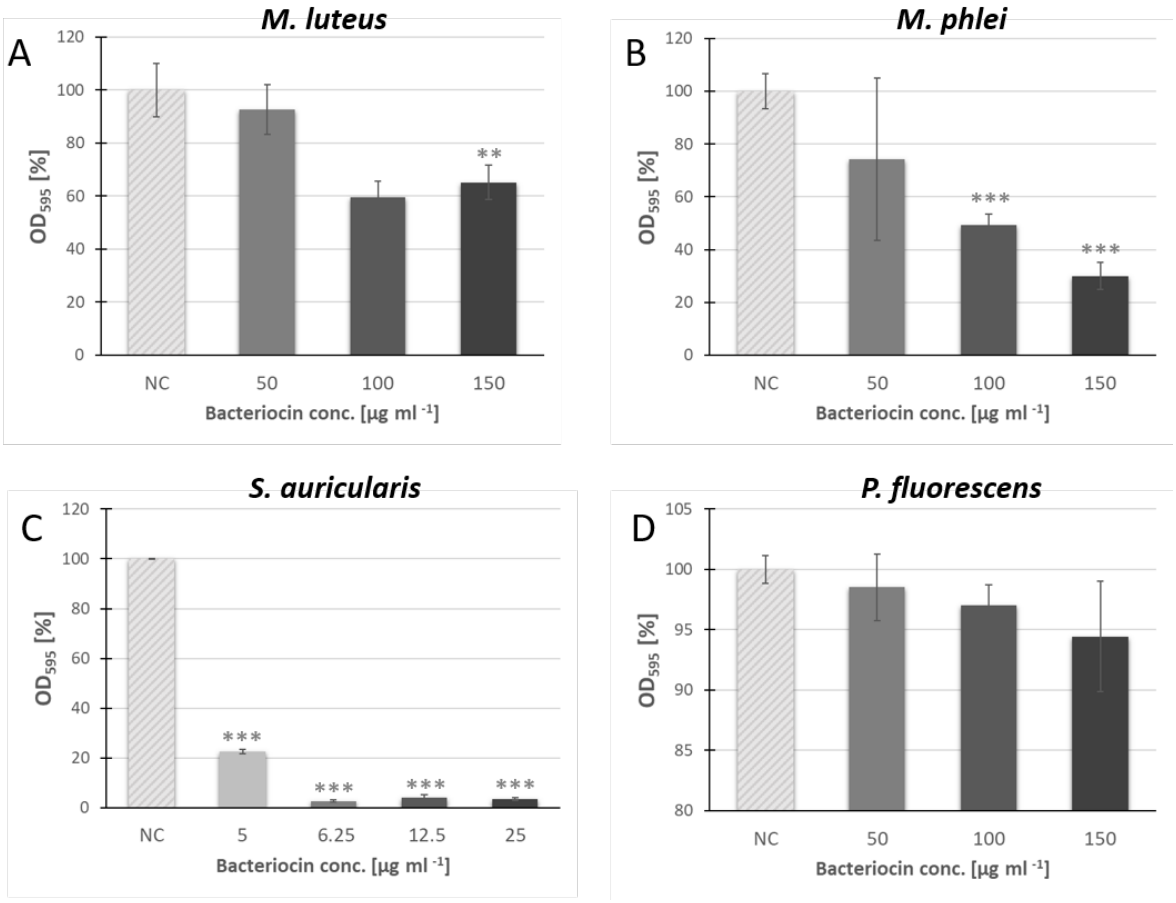

Fig. 3 Bioactivity (microtiter plate) test of B135CC against **A**, *Micrococcus luteus*; **B**, *Mycobacterium phlei*; **C**, *Staphylococcus auricularis* and **D**, *Pseudomonas fluorescens*. The bars depict the relative OD_{595} of the indicated bacterial strains after growth for 15 h at 30 °C and low agitation on a microtiter plate reader (BioTek EL808, BioTek Instruments Inc., Winooski, USA) with initial addition of different B135CC concentrations. The negative control (NC, 20 mM NaH_2PO_4/Na_2HPO_4 buffer) served as reference. Error bars indicate standard deviation, n = 3; Asterisks indicate significant differences compared to the control (**, $p \leq 0.01$; ***, $p \leq 0.001$), determined by a two-tailed homoscedastic Student's t-test.

The bacteriocin B135CC from the terrestrial cyanobacterium *C. cubana* showed a slight inhibitory impact on the growth of *P. fluorescens* and significant effects on *M. luteus* (150 µg ml^{-1}), *M. phlei* (100 and 150 µg ml^{-1}) and *S. auricularis* (5, 6.25, 12.5 and 25 µg ml^{-1}). Growth of the latter strain was strongly inhibited by B135CC, pre-tests using concentrations of 50 - 150 µg ml^{-1} completely prevented *S. auricularis* growth, therefore dilutions up to 5 µg ml^{-1} were tested for determination of the minimum inhibitory concentration (MIC). Until a bacteriocin concentration of 6.25 µg ml^{-1}, *S. auricularis* growth was completely inhibited. When the B135CC concentration was decreased to 5 µg ml^{-1}, the bacterium could grow to an OD_{595} of 20%, compared to the negative control. As a consequence, a bacteriocin concentration of 6.25 µg ml^{-1} was determined as the MIC for *S. auricularis*. The results of the bioactivity test show a strong antimicrobial activity of B135CC towards the Gram-positive strain *S. auricularis*, whereas a significantly lower

inhibition was observed against *M. luteus*. Consequently, the bacteriocin does not act as a general antibiotic compound against Gram-positive bacteria but seems to possess specificity for *S. auricularis* cells. Antibiotics like lyostaphin, a bacteriolytic enzyme produced by *Staphylococcus simulans*, specifically binds to *Staphylococcus aureus* cells, due to a cell wall targeting C-terminal sequence [267]. Lyostaphin acts as peptidoglycan hydrolase and thus damages the cell wall of *S. aureus*. Although no evidence for such a cell wall targeting sequence could be found for B135CC, a similar mechanism could explain the potent activity against *S. auricularis*.

In order to illuminate the bacteriocin's mode of action, a protein leakage test was conducted with *S. auricularis* (Fig. 4), as the release of proteins after treatment with the bacteriocin can indicate a bacteriolytic activity [268]. For this test, B135CC concentrations of 62.5 – 500 µg ml^{-1} were applied, since high concentrations lead to quick protein releases. A prolonged incubation phase could lead to falsified results because of protease activity.

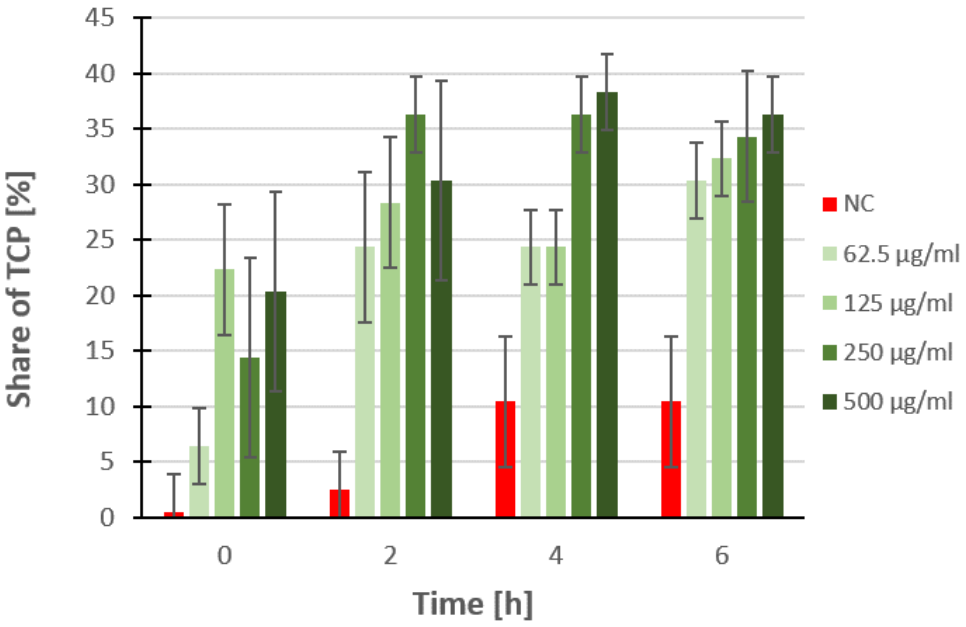

Fig. 4 Protein leakage test with *S. auricularis*. Samples of the indicator bacterium were mixed with different concentrations of B135CC and incubated at 30 °C. Samples were taken every two hours and the protein concentration was determined by Lowry-assay. NC, negative control, *S. auricularis* without addition of the bacteriocin. The share of total cell protein (TCP) was calculated on the basis of untreated *S. auricularis* cells, disrupted by ultrasound. Error bars indicate standard deviation, n = 3.

The results of the protein leakage test (Fig. 4) show a significant release of *S. auricularis* proteins, after treatment with *C. cubana* bacteriocin B135CC. After six hours of incubation, all applied concentrations (62.5 – 500 µg ml^{-1}) led to a release of about 30 – 35% of total cell protein. The negative control without B135CC caused only a minor protein release that climaxed after four hours. The obtained results emphasise that B135CC acts as a

bacteriolytic bacteriocin. For further characterisation of the bacteriocin, a temperature stability test was done with *S. auricularis* (Fig. 5).

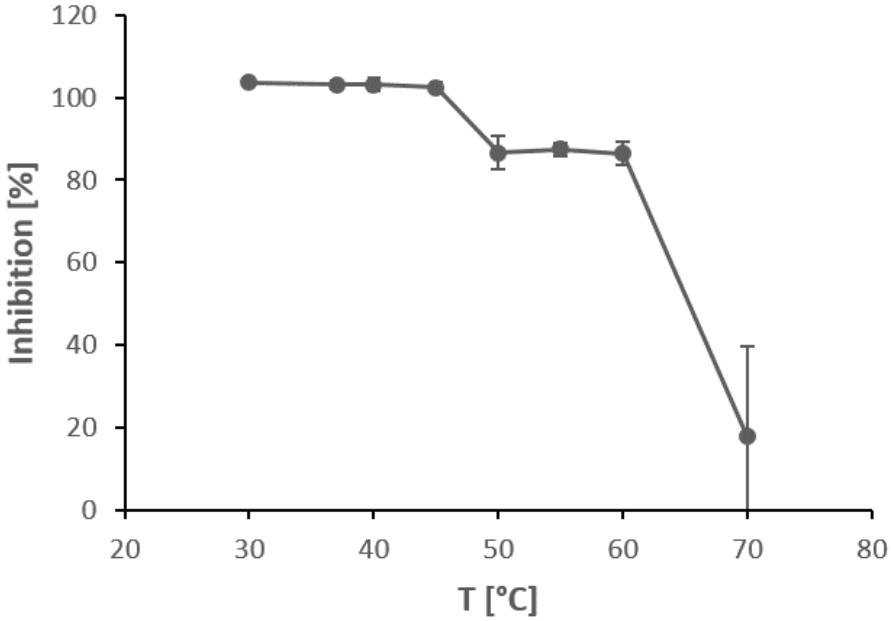

Fig. 5 Temperature stability test of B135CC. The bacteriocin was incubated at different temperatures for one hour and subsequently tested for activity. The inhibition test was done with *S. auricularis* in microtiter plates. Error bars indicate standard deviation, n = 3.

Temperature stability tests confirmed a bacteriocin activity till 60 °C. From 70 °C on, activity was significantly diminished. Together with the obtained data about the bacteriocin's activity spectrum and its size, B135CC shows similarities to class III bacteriocins as lysostaphin from *Staphylococcus simulans,* helveticin J from *Lactobacillus helveticus* or the colicin-type family from *E. coli* and some other *Enterobacteriaceae*. These bacteriocins tend to be much larger than class I or II bacteriocins, are heat labile and mainly act on Gram-positive bacteria (Bastos et al., 2010). However, all class III bacteriocins possess a common gene cluster organisation, that includes a structural bacteriocin gene, a lysis gene and an immunity gene (Luria & Suit, 1987), which is not the case for B135CC. Moreover, most known class III bacteriocins do not exhibit a double glycine leader motif, except for colicin V, a less than 10 kDa protein which does possess a conserved double glycine peptide cleavage site (Havarstein et al., 1994). For evaluation of the applicability of the bacteriocin as food preservative or as a possible treatment for human bacterial infections, B135CC was tested for toxicity against mammalian N2a-cells (Fig. 6).

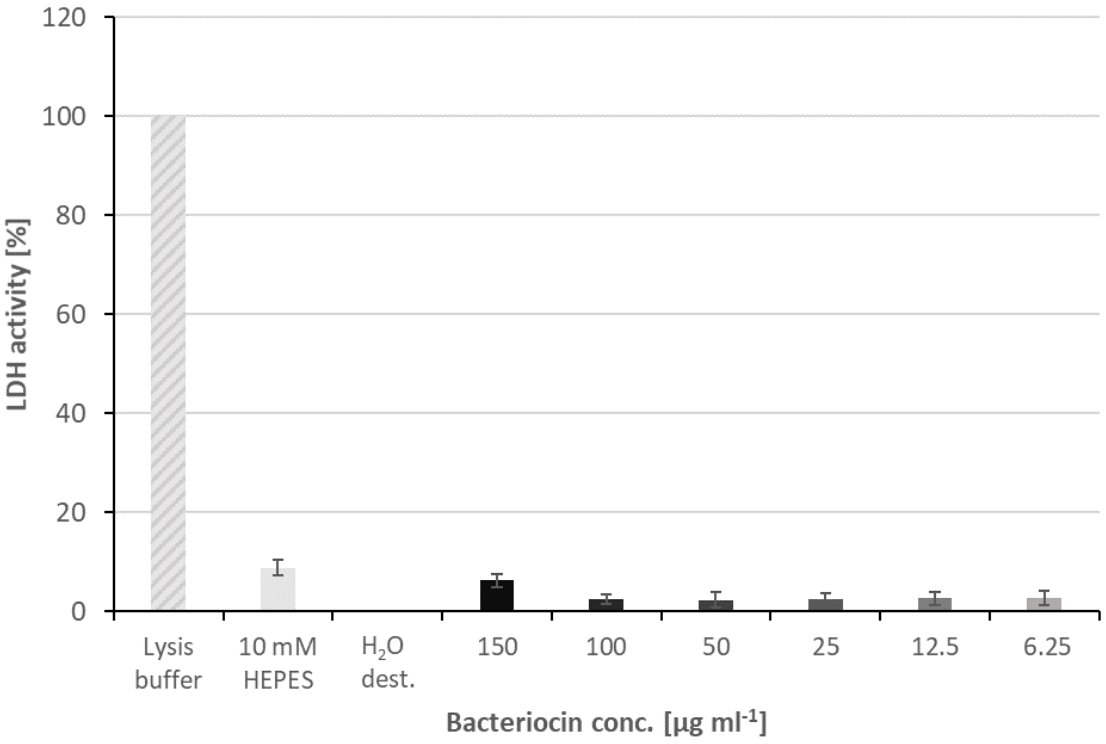

Fig. 6 LDH activity test for evaluation of B135CC-toxicity against mammalian N2a cells. For the toxicity tests, B135CC was eluted in 10 mM HEPES buffer in the desalting step after affinity chromatography. Subsequently it was diluted with N2a medium (6.25 – 150 µg ml^{-1} bacteriocin) to a final volume of 100 µl and added to the N2a cells in 96-well plates. The samples were incubated overnight at 37 °C and 5% CO_2. The negative control with only 10 mM HEPES (without N2a medium) was incubated overnight. The following day, for maximal LDH activity control, N2a cells were incubated in 10 µl lysis buffer plus 90 µl N2a medium for 45 min. The spontaneous LDH activity was tested by incubation of the cells in 10 µl distilled water plus 90 µl N2a medium for 45 min. Error bars indicate standard deviation, n = 3.

N2a cells, a mouse neuroblastoma cell line, is frequently used for the detection of neurotoxic compounds [269, 270]. As many cyanobacteria tend to produce highly (neuro-) toxic compounds, it is especially important to exclude the production of such substances. The neurotoxicity tests with B135CC did not show any negative impact on N2a cells. All applied bacteriocin concentrations (6.25 - 150 µg ml^{-1}) caused a lower LDH activity than the negative control (10 mM HEPES).

4 Conclusion

The bacteriocin B135CC from the terrestrial cyanobacterium *C. cubana* shows promising narrow range antibacterial activity mainly against some Gram-positive strains, especially against *S. auricularis*. The latter was identified as an inhabitant of the human ear (Kloos & Schleifer, 1983) and can rarely cause infections and sepsis (Hoffman et al., 2007). Susceptibility of this bacterium to B135CC can particularly be seen as a hint for further applicability on related *Staphylococcus* strains as multi-resistant *Staphylococcus aureus*, which still causes thousands of dead every year (Navidinia, 2016). While all other sensitive strains had to be treated with concentrations of at least 50 µg ml^{-1} to observe a growth inhibition, B135CC almost completely prevented growth of *S. auricularis* in concentrations as low as 6.25 µg ml^{-1}. Moreover, the bacteriocin showed no cytotoxicity against mammalian N2a cells in concentrations up to 150 µg ml^{-1}. Together, our data suggest that B135CC could be of great use for the specific treatment of different Gram-positive pathogenic bacteria with highest activity against *Staphylococci* species.

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Chapter VII

General Discussion & Outlook

1 Antimicrobial metabolites from *Chroococcidipsis cubana*

In this chapter, the main results of the thesis, regarding the identification and production of antimicrobial metabolites, are critically discussed, compared to recent findings from literature and evaluated in regard to possible proceeding research approaches.

1.1 Native production of an antimicrobial metabolite

In this chapter, the production process of the antimicrobial compound using the native producer strain, *C. cubana*, is discussed, including possible optimization strategies and approaches for the identification of responsible gene clusters and the compound identity itself.

1.1.1 Identification of the biosynthetic gene cluster

The key element for the implementation of the production process was the identification of the regulatory trigger. Generally, the regulation of cyanobacterial bioactive metabolite synthesis is not fully understood and mostly limited to cyanotoxins [1]. So, the identification of nitrogen limitation to be the necessary prerequisite for *C. cubana* to produce the substance of interest, was an interesting new finding for cyanobacterial research. This trigger was exploited for the implementation of a continuous production system, relying on a permanently low nitrate concentration in the culture medium (Chapter V). In the course of this thesis, the metabolic background of this regulation mechanism could not be revealed, but it was postulated that the synthesis of the antibiotic compound could be linked to the formation of spore-like survival cells. These cells are functionally similar to akinetes, formed by cyanobacteria strains belonging to the *Nostocales* or *Stigonematales*, and are reported to be formed by *Chroococcidiopsis* strains in response to nitrogen starvation (Chapter V). Until now, spores, akinetes, or similar cyanobacterial survival cells were not found to be linked to the formation of bioactive compounds in cyanobacteria, however, such connections were indeed described for *Streptomyces* species (Chapter V). Although the question of regulation could not be clarified, the connection of nitrogen starvation and compound production was clearly shown and could eventually lead to the identification of the responsible biosynthetic gene cluster in *C. cubana*. This, together with the identification of the responsible transcriptional factors, can then be used for an induced overexpression of the relevant genes.

For this purpose, a combined strategy of *in silico* analysis and molecular biological techniques might be used. As already reported, five biosynthetic gene clusters in *C. cubana* could likely come into question for the synthesis of the bioactive compound (Chapter V). These were identified using antiSMASH, a tool for the identification of biosynthetic gene

clusters in bacteria and fungi [2] and given a closer consideration because of proximate encoded transcriptional regulators, which might be activated by nitrogen starvation (Chapter V). As the most suitable candidates count, a lanthipeptide class V gene cluster, situated next to a histidine kinase (Hik) encoding cluster, an NRPS-like gene cluster with an adjacent *hik*, an NRPS-cluster close to an encoded response regulator gene and another NRPS-like gene cluster which is situated next to a drug metabolite transporter gene, as well as a gene coding for a LuxR family transcriptional regulator. Members of the latter one are known for the regulation of antibiotic synthesis genes [3]. By narrowing the selection to these four clusters, a subsequent reverse transcriptase (RT)-PCR can become useful [4]. For this, *C. cubana* cells should be cultivated under normal- and nitrogen starvation conditions, followed by extraction of all transcribed RNA molecules. In the RT-PCR process, these are re-transcribed to DNA, which, in turn, allows the identification and, more importantly, quantification of the formed DNA-molecules by qPCR using specific primers for the formerly chosen gene clusters. As it could be shown that the antimicrobial compound from *C. cubana* is predominantly produced at a limited nitrogen concentration, the responsible genes should be transcribed at a significantly higher rate than under normal cultivation conditions. Next to the over-expression approach, the identification of the biosynthetic gene cluster would also provide information about the nature of the produced metabolite, as NRPS-cluster consist of individual enzymatic domains, catalyzing specific reactions and modifications on the growing peptide chain. Another possibility for the cluster identification is a liquid chromatography–mass spectrometry (LC-MS)-based approach, where the respectively transcribed and translated domains are identified on the basis of their predicted molecular weight [5]. As mentioned before, the elucidation of the transcriptional regulation is an important step towards genetic manipulation of the transcription rate. The regulation of jamaicamide, a toxin produced by *Lyngbya majuscula*, was uncovered by a β-galactosidase-based reporter gene assay in *E. coli* and a subsequent protein pulldown assay [1]. For the first assay, the predicted promoter regions upstream of the gene cluster were cloned to a vector, upstream of a β-galactosidase gene, which was then used to transform *E. coli* cells. The formation of β-galactosidase was verified by chemiluminescence, taking advantage of its hydrolytic activity. The protein pulldown assay uses a target gDNA-sequence, containing the genes of interest, which is immobilized to e.g. an agarose matrix. The matrix is then incubated with the soluble crude protein fraction containing the putative transcription factors. After binding to the specific DNA target sequences, all unbound proteins can be washed out and the transcription factors are eluted for further analysis [1]. A similar approach could be used to identify the transcription factors involved in the synthesis of the bioactive metabolite from *C. cubana*.

1.1.2 Identification of the antimicrobial compound

Although the continuous production process led to a significantly higher bioactive sample supply and the RPC-based purification of these samples resulted in partially purified and concentrated fractions with clearly increased antimicrobial activity, the identity of the unknown substance could not be revealed. As the genome of *C. cubana* has already been sequenced, the afore mentioned identification of the corresponding gene cluster could help identifying the bioactive product. In case of an NRPS-cluster, the number and type of enzymatic domains allows conclusions about size, amino acid composition and possible modifications [6, 7]. Again, heavily improved *in silico* prediction tools as SeMPI 2.0 can provide precise structure predictions [8]. Next to this approach, a structure analysis of the antimicrobial compound itself is a mandatory step. For this, a functional purification strategy is needed in the first place. In this respect, in-depth information about the structure of the associated gene cluster, possibly provided by *in silico* analysis, would be useful. Information about hydrophobicity, isoelectric point or spectroscopic behavior can be a great help for planning purification approaches. However, since the purification method used in Chapter V already provided at least fractions with concentrated compound, a fine tuning of the applied RPC-protocol could eventually lead to a complete purification. In literature, mostly solvents as methanol, chloroform or ethanol are used for the direct extraction of the target substances from dried or lyophilized cells, followed by a first purification by thin layer chromatography [9–11]. But as the antimicrobial compound from *C. cubana* is directly exported to the surrounding medium, both techniques are not applicable. After a complete purification, further MS and NMR analyses can be used for complete structure elucidation.

1.1.3 Optimization of the production process

For the production of the antimicrobial compound from *C. cubana*, a continuous cultivation system, providing a specific nitrogen concentration in the cultivation medium, was used. Instead of cultivating free-floating cells, also immobilized cells would have been a thinkable option for this process. Biofilm-based photobioreactors are an interesting topic for the cultivation of cyanobacteria, since downstream processes regarding cell harvest or the isolation of exported products from the medium become a lot easier (Chapter IV). An example for a successful continuous antibiotic production using immobilized cyanobacterial cells was shown by Chetsumon *et al*. (1995). The team used a seaweed-type bioreactor with *Scytonema* sp. TISTR 8208 cells attached to polyurethan foam strips for a 20 days lasting production of a cyclic peptide antibiotic [12]. Although *C. cubana* is not an extremely biofilm forming strain, a layer of cells sticked to the cultivation vessel (glass) was always observed at cultivations. Like all terrestrial cyanobacteria, *C. cubana* produces extracellular polymeric substances (EPS), which make up the largest proportion of its biofilm [13]. The

formed polysaccharide matrix mainly consists of water, polysaccharides, proteins, lipids and nucleic acids and serves the protection against environmental impacts [14]. Importantly for a possible biofilm-based cultivation, the formation of EPS can be triggered by drought-induced stress, nitrogen starvation and changes in light- and temperature conditions [13, 15]. A study using a *C. cubana* strain even reports a media optimization strategy, yielding a biofilm concentration of 20 g L^{-1} in 10 days [16]. This was achieved by using BG-11 cultivation medium containing 13 g L^{-1} $NaNO_3$ and 6.5 g L^{-1} K_2HPO_4, which are significantly higher concentrations compared to the standard protocol [17]. Additionally to these optimization strategies, the ability of cyanobacteria to metabolize organic carbon sources alongside with photosynthesis could once again be used. For *Nostoc* sp., it was documented that a mixotrophic cultivation with 2.5 g L^{-1} potassium acetate leads to an about 3-fold increased EPS production, compared to phototrophic cultivation [18]. A comparable effect could be shown for *Nostoc flagelliforme*, where a mixture of glucose and acetate yielded the highest EPS yields [19].

To summarize, a biofilm-based cultivation approach with *C. cubana* could be well feasible. The main advantages would be the elimination of cell separation steps, as well as further possibilities for an automated production optimization. In the course of this thesis, a self-optimizing AI-controlled PBR system was created, which was used for a biomass yield maximization of the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 (**Fig.1**). The system is regulated by a continuous OD measurement and in-, or decreases medium inflow/outflow speed in response to the calculated productivity Dx (Bachelor Thesis M. Dörr). A comparable system, also using a machine learning approach, was shown to significantly improve limonene productivity of *S. elongatus* UTEX 2973 [20].

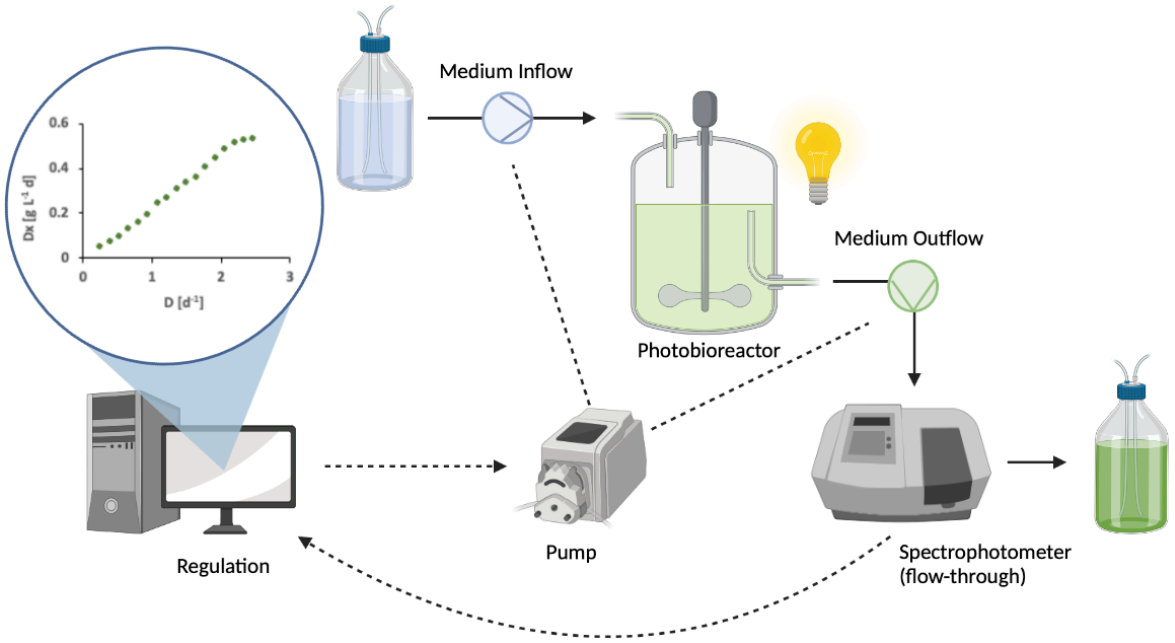

Fig. 1 AI-controlled PBR. The system works similar to a turbidostat, the cell density (*S. elongatus* UTEX 2973) is controlled by the flow rate (D), volume and composition of the culture medium remain constant. In contrast to a turbidostat, D is used to maximize the productivity (Dx), with cell mass as the product. The optical density (OD) of the bacterial culture is determined via the effluent using a flow-through spectrophotometer, after which it enters the harvest tank. The OD is transmitted to the control center, which calculates Dx and compares it to the previously measured value. A signal is then transmitted to the pump control system and the flow rate is adjusted accordingly. This continues until the maximum possible productivity has been reached.

In case of a biofilm-based cultivation with immobilized *C. cubana* cells with the aim to maximize the production of the antimicrobial metabolite, the outflow medium would not contain cells and would thus cause no interferences in the spectrometric measurements. So, if the compound would show spectrometric activity, the system could directly optimize the flow rate on the basis of antibiotic metabolite production. As the system is expendable by further regulation loops for e.g., light intensity, temperature and the addition of carbon sources, the productivity could be optimized with regard to many possibly influencing cultivation parameters.

1.2 Heterologous production of an antimicrobial metabolite

The bacteriocin B135CC from *C. cubana* was heterologously expressed in *E. coli* and showed potent activity especially against Gram-positive bacteria. From a concentration of 6.25 µg mL^{-1}, growth of *Staphylococcus auricularis* was nearly completely inhibited. As the mode of action, a bacteriolytic activity was shown. Since food spoiling bacteria as *Listeria monocytogenes*, *Clostridium botulinum*, *Enterococcus*-, *Streptococcus*-, *Staphylococcus*-, and *Bacillus* species are predominantly Gram-positive, the selectivity of the produced bacteriocin could be an advantage [21]. A possible application as a food preservant is also

indicated by the absent cytotoxicity for mammal N2a cells, and the relatively high temperature stability till 60 °C (Chapter VI). The bacteriocin nisin, produced by *Lactobacillus lactis*, is approved as a food additive since 1988. However, resistances have evolved [22], which increases the urgency for novel compounds.

For a production of B135CC on an industrial scale, the use of a heterologous host as *E. coli* is presumably inevitable, because *b135cc* seems to be a silent gene, which is the case for most cyanobacterial biosynthetic gene clusters [23]. An inhibitory activity against the bacteria susceptible for the bacteriocin was neither caused by *C. cubana* biomass nor cultivation medium. As the vector-based expression of *b135CC* led to satisfying bacteriocin concentrations and the host cells were not impaired by the formed product (shown by comparison with an empty vector control while gene expression and a subsequent growth inhibitory assay with purified bacteriocin), *E. coli* seems to be a suitable host for B135CC production. For an increase in product formation, a range of possibilities exists. Expression efficacy can vary between different expression strains and also *E. coli* strains specially adapted for the production of bacteriocins can be tried. Strain BL21 (DE3), which was used for B135CC production, is deficient in Lon and OmpT proteases and therefore frequently used for bacteriocin synthesis [24]. The plasmid copy number can as well greatly influence the expression rate. The used pET vector system is designated as a low to middle copy plasmid (about 15 – 20 copies per cell). The use of a high-copy plasmid, such as pUC vectors could be worth trying, as it leads to > 100 copies per cell. However, a high copy number can also lead to the formation of aggregation bodies or misfolding of larger peptides [25], so generally low and medium copy vectors are used for heterologous bacteriocin production [24]. Moreover, the protein expression rate can be positively influenced by codon optimization. If codons rather unusual in *E. coli* are present in the sequence of the cloned fragment, it is possible that the specific tRNA for these codons is only marginally available or completely absent, which in turn slows down the expression rate or completely prevents the translation [24]. In this case either a codon optimization can be done, or a vector as pRIL or pRARE can be used, which further expands the tRNA pool. An online tool for codon optimization attested an optimization potential of 48 % for B135CC (https://www.novoprolabs.com/tools/codon-optimization), so this approach would be worth trying.

Bacteriocins contain a double-glycine motif within the leader peptide sequence, that is recognized and cleaved by a C39 peptidase domain, which is part of an ABC transporter [26]. This transporter is also responsible for the following export of the bacteriocin to the surrounding medium. Such transporters also occur in *E. coli*, where they are e.g. responsible for the secretion of colicin-type bacteriocins. As the leader peptide of B135CC shows no clear double-glycine-motif and is significantly longer than leader peptides of other

bacteriocins, it is apparently not processed and transported by *E. coli* ABC-type transporter. However, a secretion of B135CC in *E. coli* was not tested and cannot be excluded. With regard to the high intracellular bacteriocin content after cell disruption, an export to the cultivation medium seems rather unlikely. Interestingly, it was shown for divergicin A, a bacteriocin from *Carnobacterium divergens*, that the replacement of the native leader peptide with the leader peptide of colicin V, mediated the secretion of the fusion protein in *E. coli* after heterologous expression, using the outer membrane protein TolC and an ABC-type transporter [27]. Consequently, the same principle could be tried for B135CC (**Fig. 2**).

Fig. 2 Possible generation of a fusion peptide consisting of the B135CC core peptide from *C. cubana* and the colicin V leader peptide from *E. coli*. The two fragments are fused by Gibson assembly, using overlapping homologous sequences.

With a functional secretion process in *E. coli*, the bacteriocin B135CC could be produced much more efficiently, without the need for cell separation, or disruption. As a consequence, a significantly more economic process could be implemented, which could lead to a wide field of applications for the novel bacteriocin from *C. cubana*.

2 Summary and Conclusion

The aim of this thesis was the production and identification of novel antimicrobial compounds from terrestrial cyanobacteria, associated with the establishment of a suitable purification method. In the initial screening process, the strain *Chroococcidiopsis cubana* attracted attention by causing inhibition zones at agar plates inoculated with Gram-positive *M. luteus* or the yeast *C. auris* (**Chapter V**). The following research brought light into the required conditions for the synthesis of the responsible compound. It was found that the production was initiated by nitrogen depletion, mostly in late exponential growth phase/early stationary phase when the nitrate concentration was between 0.35 ± 0.053 and 0.059 ± 0.024 g L^{-1}. Using this knowledge, a continuous production process was implemented that used a constant inflow of mineral medium with a decreased nitrate concentration of 0.219 g L^{-1}. The process provided highly active culture supernatants with a maximum of 85.29 ± 2.75 % inhibition in the peak against *M. luteus*. Consequently, a purification approach was implemented, which led to a highly active fraction nearly completely inhibiting growth of *M. luteus* (91.15 % ± 1.32) and *C. auris* (96.95 % ± 1.32). The compound was found to remain its activity until a temperature of about 45 °C.

As *C. cubana* raised our attention, its genome sequence was analyzed and searched for biosynthetic gene clusters, possibly encoding the purified antimicrobial compound. And indeed, several NRPS, PKS, and RiPP domains were identified, of which the latter ones were cloned and heterologously expressed in *E. coli* (**Chapter VI**). One of the peptides, B135CC, could be produced in sufficiently high amounts and was tested against several microorganisms. The bacteriocin showed potent antagonistic activity against the mycobacterium *M. phlei,* a bacteriocin concentration of 150 µg ml^{-1} resulted in a growth inhibition of 70 %. For Gram-positive *S. auricularis*, a minimum inhibitory concentration of 6.25 µg ml^{-1} was determined for complete growth inhibition. To clarify the mode of action, a protein leakage assay was done by incubation of *S. auricularis* with different concentrations of B135CC and a subsequent quantification of released cell proteins by Lowry-assay. The test confirmed a bacteriolytic mode of action, after six hours of incubation, all applied concentrations (62.5 – 500 µg ml^{-1}) led to a release of about 30 – 35% of total cell protein. Since the bacteriocin showed no cytotoxic activity against mammalian N2a cells and a temperature stability until 60 °C was confirmed, a future medical application could at least be imaginable.

As written in **Chapter I**, the work with cyanobacteria can be quite challenging, because standard microbiology methods often need adaptations or must be replaced. But despite, or precisely because of these difficulties, one is forced to develop alternative ways for reaching the anticipated goals. So, in the course of this work, two standard protocols,

specifically adapted for the work with cyanobacteria, were established. First, a method for a quick and easy *in vivo* vitality estimation of phototrophic cells, based on pO_2 measurements and only requiring a maximum of 1.5 g wet cyanobacterial cell mass and 30 min of reaction time per run was developed (**Chapter III**). Second, a sodium salicylate approach for a fast and economic determination of nitrate quantities in microalgal cultures was established, which was found to be suitable for a direct detection of nitrate ions up to a concentration of 450 mg L^{-1} within common microalgal mineral media (**Chapter IV**). Both methods greatly helped to move forward the whole project, the first one by enabling the identification of suitable cryopreservation protocols for individual cyanobacteria strains (outlined in **Chapter II**) and the second one by facilitating the determination of the optimal nitrate concentration for the production of the antimicrobial compound from *C. cubana*.

In the end of **Chapter I**, the ability of cyanobacteria to utilize organic carbon sources for an accelerated cell growth was linked to the (increased) production of bioactive metabolites. Although an enhanced synthesis of the antimicrobial metabolite natively produced by *C. cubana* was not noticed at mixotrophic cultivation, it could though be shown that the bacterium reaches significantly higher growth rates when cultivated with fructose or glucose. Interestingly, this effect was even further enhanced when light intensity was decreased. Under these low-light conditions, phototrophically cultivated *C. cubana* cells showed the exactly opposite reaction (**Chapter II**). This effect might be extremely useful for an economic cultivation of cyanobacteria.

In this thesis, two promising new antimicrobial compounds from the terrestrial cyanobacterium *Chroococcidiopsis cubana* were identified, using two different approaches. One was detected by “classic” antimicrobial screening, followed by targeted production and purification, and the other one was found by screening the genome of *C. cubana* for biosynthetic gene clusters, using an *in silico*-based approach, followed by a heterologous expression strategy. Both ways led to a functional molecule with strong antimicrobial activity against different microorganisms. The production strategies were shown to be suitable for providing sufficient amounts of the respective compound, in regard to further analysis. Next to structure elucidation of the natively produced compound, both antibiotics should be tested against the afore mentioned, multi-resistant ESKAPE bacteria, as well as pathogenic fungi like *Candida albicans*. Especially because the discovered compounds show antagonistic activity against the closely related strains *Staphylococcus auricularis* and *Candida auris*.

The obtained results show that especially terrestrial cyanobacteria are indeed a largely untapped source for bioactive metabolites, which is worth to be further explored.

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A Supplemental Material

A1 Betreute Arbeiten

Dann, M.: Optimization of growth and secondary metabolite production by the terrestrial cyanobacterium *Chroococcidiopsis cubana* (2020), Masterarbeit.

Gecgel, S.: Screening terrestrischer Cyanobakterien hinsichtlich der Produktion antimikrobieller Sekundärmetabolite (2020), Bachelorarbeit.

Rabbachin, L.: Analyse von Kulturüberständen des Cyanobakteriums *Chroococcidiopsis cubana* hinsichtlich ihrer antibiotischen Wirkung (2020), Projektarbeit.

Voigt, M.: Strukturaufklärung eines cyanobakteriellen Antibiotikums mittels chemischer Nachweisreaktionen und Stabilitätstests (2020), Projektarbeit.

Iseni, S.: Überprüfung cyanobakterieller Kulturüberstände auf antagonistische Wirkung gegenüber anderen Cyanobakterien-Stämmen mittels der pO_2 – Vitalitätsmessmethode (2021), Projektarbeit.

Kosanenko, A.: Kultivierung der Cyanobakterien *Chroococcidiopsis cubana* und *Chroococcidiopsis thermalis* zur Produktion antimikrobieller Substanzen (2021), Projektarbeit.

Rabbachin, L.: Gewinnung antimikrobieller Substanzen aus Überständen terrestrischer Cyanobakterien-Kulturen (2021), Praxisphase.

Rabbachin, L.: Production and Enrichment of Antimicrobial Substances from the Cyanobacteria *Synechococcus elongatus* and *Scytonema stuposum* (2021), Bachelorarbeit

Schmidt, A.-K.: Wachstumssteigerung des Cyanobakteriums *Synechococcus elongatus* UTEX 2973 (2021), Projektarbeit.

Srikanthamoorthy, G.: Mixotrophe und heterotrophe Wachstumstests mit *Synechococcus elongatus* UTEX 2973 (2021), Projektarbeit.

Stielow, C.: Expressionsoptimierung, Aufreinigung und Charakterisierung von heterolog produzierten cyanobakteriellen Bacteriocinen (2021), Bachelorarbeit.

Voigt, M.: Gewinnung antimikrobieller Substanzen aus terrestrischen Cyanobakterien (2021), Praxisphase.

Voigt, M.: Gewinnung antimikrobieller Substanzen aus dem terrestrischen Cyanobakterium *Chroococcidiopsis thermalis* (2021), Bachelorarbeit.

Börner, T.: Verbesserung der Lipidproduktion mit *Synechococcus elongatus* UTEX 2973 (2022), Projektarbeit.

Dörr, M.: Entwicklung einer digitalen Pumpensteuerung mit Turbiditäts-Rückkopplung für die Durchführung und Optimierung von kontinuierlichen Kultivierungen in Bioreaktoren (2022), Bachelorarbeit.

El Azem, S.: Analyse von Kulturüberständen des Cyanobakteriums *Chroococcidiopsis thermalis* in verschiedenen Kulturmedienzusammensetzungen hinsichtlich der Produktion antibiotischer Wirkstoffe (2022), Projektarbeit.

Gandras, L.: Optimierung der Lipidproduktion von *Synechococcus elongatus* UTEX 2973 mittels mixotropher Kultivierung (2022), Projektarbeit.

Gandras, L.: Produktion, Aufreinigung und Charakterisierung einer antimikrobiellen Substanz aus dem terrestrischen Cyanobakterium *Nostoc punctiforme* (2022), Praxisphase.

Lopes, A.: Analyse von Kulturüberständen des Cyanobakteriums hinsichtlich ihrer antimikrobiellen Wirkung (2022), Projektarbeit.

Lopes, A.: Wirkspektrum antimikrobieller Substanzen aus Überständen terrestrischer Cyanobakterien-Kulturen (2022), Praxisphase.

Ludwig, E.: Einfluss der Stickstofflimitierung bei der Produktion antimikrobieller Substanzen durch das Cyanobakterium *Scytonema stuposum* (2022), Projektarbeit.

Schmidt, A.-K.: Wirkspektrum antimikrobieller Substanzen aus Überständen terrestrischer Cyanobakterien-Kulturen (2022), Praxisphase.

Schmidt, A.-K.: Bioanalytische Untersuchung der durch *Synechococcus elongatus* produzierten antibiotischen Substanzen (2022), Bachelorarbeit.

Ternes, M.: Bioaktivitätsmonitoring einer antibiotischen Substanz aus *Calothrix desertica* (2022), Projektarbeit.

A2 Tagungsbeiträge

M. Witthohn, R. Ulber, K. Muffler: Vitalitätsbestimmung von terrestrischen Cyanobakterien über deren Sauerstoff-Produktion. 12. Bundesalgenstammtisch (2019), Kiel, Deutschland (Poster)

M. Witthohn, R. Ulber, K. Muffler: Novel pO_2-based method for a quick vitality determination of cyanobacteria. 6th Joint Conference of the DGHM & VAAM (2020), Leipzig, Deutschland (Poster)

M. Witthohn, R. Ulber, K. Muffler: Extraction and analysis of antimicrobial secondary metabolites produced by a terrestrial cyanobacterium. ProcessNet-Jahrestagung und 34. DECHEMA-Jahrestagung der Biotechnologen (2020), Aachen, Deutschland (Poster)

M. Witthohn, R. Ulber, K. Muffler: Terrestrische Cyanobakterien als Quelle neuer antimikrobieller Substanzen. DACH Algen Summit (2021), Wien, Österrreich (Vortrag)

M. Witthohn, R. Ulber, K. Muffler: Characterisation of a cyanobacterial bacteriocin heterologously produced with *E. coli*. VAAM Digitale Jahrestagung (2022), Düsseldorf, Deutschland (Poster)

A3 Lebenslauf

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation „Production, purification and analysis of novel peptide antibiotics from terrestrial cyanobacteria“ ohne unerlaubte fremde Hilfe angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Wörtlich oder sinngemäß übernommenes Gedankengut wurde als Entlehnung kenntlich gemacht.

Ort, Datum Marco Witthohn