



UvA-DARE (Digital Academic Repository)

Physiological and genetic studies towards biofuel production in cyanobacteria

Schuurmans, R.M.

Publication date

2017

Document Version

Other version

License

Other

[Link to publication](#)

Citation for published version (APA):

Schuurmans, R. M. (2017). *Physiological and genetic studies towards biofuel production in cyanobacteria*. [Thesis, fully internal, Universiteit van Amsterdam].

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, P.O. Box 19185, 1000 GD Amsterdam, The Netherlands. You will be contacted as soon as possible.

Chapter 5

Physiological studies towards optimization of cellulose production in the filamentous cyanobacteria *Crinalium epipsammum*

R. Milou Schuurmans¹, Lucas J. Stal^{2,3} and Klaas J. Hellingwerf¹

¹*Molecular Microbial Physiology, Swammerdam Institute for Life Science, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands*

²*Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands*

³*Department of Marine Microbiology, Royal Netherlands Institute of Sea Research, Yerseke, The Netherlands*

Abstract

Cellulose has a great potential as a macromolecular biofuel or biofuel precursor. Consisting entirely of polymerized glucose molecules and deposited extracellularly and in a highly pure form by a wide array of bacteria, cellulose provides a non-soluble high-energy compound for a variety of biotechnological applications. One of the earliest accounts on cellulose production in a cyanobacterium describes the filamentous biofilm forming cyanobacteria *Crinalium epipsammum*, which can accumulate ~20 % of its dry weight as cellulose. In this study we aimed to improve this figure by exposing *C. epipsammum* to a variety of physiological conditions and stresses. By a combination of several conditions, which on their own showed only minor improvements, the cellulose content of *C. epipsammum* was raised to 40 % of dry weight. *C. epipsammum* was also evaluated for its potential for large-scale cultivation.

Key words:

Crinalium epipsammum, cyanobacteria, cellulose, biofuel, physiology

Introduction

Cellulose is the most abundant biopolymer on Earth and it is produced by many different organisms, ranging from α - and γ proteobacteria, cyanobacteria, firmicutes, to plants and even some animals [65]. As a macromolecular biofuel and/or a precursor of liquid biofuel, cellulose is an interesting candidate. In bacteria it is deposited just outside the cell envelope. In this way the production of cellulose does not depend on intracellular storage capacity and the cells do not have to be destroyed in order to enable harvesting. Although much is known about cellulose production in plants and heterotrophic bacteria, little research has been conducted on cellulose production in cyanobacteria. In recent years this subject has gained attention, mainly because of the increased interest in cyanobacteria for sustainability applications. Yet most of the studies on cellulose production focus on genetic modification of an organism for heterologous expression of cellulose biosynthesis genes [25,26,28]. However, one of the first detailed reports on cellulose production in cyanobacteria describes a cyanobacterial isolate, *Crinallium epipsammum* that accumulates cellulose to ~20 % of its dry weight [23]. This is still higher than currently described for transgenic organisms, with the highest cellulose content reported to be ~13 % of dry weight [28]. Therefore, in order to maximize cellulose production in *C. epipsammum*, the cellulose producing capacity was tested in this study by altering physiological conditions and the nutrient composition of the growth medium. We will also explore the potential of *C. epipsammum* for industrial-scale production.

C. epipsammum was isolated in 1990 from algal crusts that grow on the sand surface of blow-out dunes near The Hague, The Netherlands [23]. These algal crusts are characterized by harsh growth conditions, especially with respect to water availability, large temperature fluctuations, and low nutrient availability. Survival in such environments requires specific adaptations. While *C. epipsammum* cannot fix nitrogen from the air this organism has adapted to a lifestyle with low nitrogen supply - the most important nutrient after carbon. This is, for instance, reflected in the cells' low phycobiliprotein content and the absence of phycoerythrin. *C. epipsammum* also has a thick cell wall, which allows for its unusual morphology of flat rectangular cells (see further below). This cell wall presumably aids in the cells' ability to withstand desiccation [257]. The sub-cellular location of the cellulose in *C. epipsammum* is uncertain. While in other bacteria cellulose is exuded outside

the cell, this was reported not to be the case in *C. epipsammum*. It was hypothesized that the cellulose might be an integral part of the cell envelope [23]. An exopolysaccharide matrix, which may include cellulose, is important for terrestrial algae and cyanobacteria such as *C. epipsammum* for various reasons: It allows cells to adhere to a surface and it facilitates biofilm formation and increases the water-retaining capacity of the cells that helps to overcome periods of drought [40]. This also implies that cellulose production should be common in algal crusts. Therefore, we returned to the original habitat from which *C. epipsammum* has been isolated in order to attempt to re-isolate this cyanobacterium, as well as other cellulose-producing phototrophic organisms.

The cellulose biosynthesis process itself has also received a great deal of attention throughout the years. The focus in this work, however, has mostly been on the cellulose synthase protein itself, which is also the only protein known as indispensable for the production of this polymer, and not all of the supportive proteins and their function are known. Cellulose synthases and some related enzymes such as chitin synthases and class I and II hyaluronan synthases (HAS) are processive β -glycosyltransferases (PGBTs); members of the 2-glycosyltransferase family [65]. These enzymes are integral membrane proteins with multiple transmembrane domains, sharing the conserved D,D,D,QXXRW motif, with spaced aspartic acid residues, followed by the QXXRW core, within a cytosolic catalytic region [56,60]. Their catalytic domain is the only part of the protein that shows amino acid sequence conservation between related species. To allow for a quick analysis of newly identified strains it would be most convenient when they could be screened for the presence of a cellulose synthase gene. For this purpose we designed DNA primers based on the conserved regions of the cellulose synthase genes of various cyanobacteria. In 2013, *C. epipsammum* PCC 9333 was sequenced as a part of an extensive study [258]. *C. epipsammum* PCC 9333 and *C. epipsammum* SAG22.89, the latter which was used in this study, both originate from the isolate described by de Winder et al. [23]. Analysis of the PCC 9333 sequence, which was deposited at the NCBI, revealed that *C. epipsammum* possibly contains three cellulose synthase genes, one with a plant-like cellulose synthase catalytic domain plus a regulatory domain, and two with a bacterial cellulose synthase domain (see General Introduction of this thesis for details on the characteristics of these domains).

Based in part on these *Crinalium* sequences, primers were designed that should be able to identify cellulose synthase genes in other organisms.

Results

Optimization of the enzymatic assay for quantitation of the cellular amount of cellulose

In the literature several protocols have been published for cellulose detection and quantification in cyanobacteria, using an enzymatic method. We selected two different protocols and tested their suitability and efficiency. The protocol from Nobles and Brown [25] uses an aqueous solution of cellulase from *Trichoderma reesei* ATCC 26921, named Celluclast (Sigma-Aldrich) in order to degrade the extracellular cellulose of whole-cell samples overnight at 50 °C in a sodium acetate buffer at pH 5. This protocol prescribes the inclusion of a negative control without cellulose. The second, a protocol published by Kawano et al. [41] is based on partial lysis of the producing cells by lysozyme treatment and sonication, followed by overnight treatment with proteinase K. Subsequently, glycogen is degraded by glucoamylase overnight at 37 °C and finally cellulose is hydrolyzed with chromatographically purified cellulase derived from *Trichoderma reesei* for 96 hours at 37 °C in a sodium acetate buffer at pH 5.2. This latter protocol assumes that the glucose liberated after the two enzyme treatments originates from glycogen and cellulose, in that order. Both protocols use a glucose assay kit to determine the liberated glucose.

In order to select the best protocol for our purpose, we tested them using a homemade solution of cellulase from *Trichoderma viride* (Sigma-Aldrich, not chromatographically purified) at 1 mg ml⁻¹ final concentration as well as the commercial Celluclast solution (Sigma-Aldrich) with a final concentration of 5 µl ml⁻¹, which corresponds to the concentrations used in the literature. We compared the yield of glucose obtained with the two different protocols on known amounts of crystalline cellulose over time and on *C. epipsammum* cell cultures, after 24 and 96 hours, respectively (Fig. 5.1A). From this comparison we concluded that the simple assay conditions from Nobles and Brown [25] gave the best results, and hence we proceeded with this assay. Next we tested the rate of cellulose degradation by the different enzymes on pure crystalline cellulose in known concentrations, the glucose background signal of the cellulase enzymes and liberation of

cellulose from *C. epipsammum* over time (Fig. 5.1B,C). We found that degradation of pure cellulose by these enzymes was always incomplete, but the amount of liberated glucose hardly increased between 48 and 96 hours with the use of Celluclast (Fig. 5.1B). Addition of more cellulase after 24 hours did not increase the amount of cellulose degradation (data not shown). Furthermore, the cellulase enzyme from *Trichoderma viride* [41] performed better on the crystalline cellulose (Fig. 5.1C), possibly because it was added in much higher concentrations (6 U/ml vs 0.005 U/ml), however, the background signal of this latter cellulase solution was also significantly higher than with the use of Celluclast, while the amount of liberated glucose from *C. epipsammum* cell cultures was similar. We decided to continue with the assay based on Nobles and Brown [25] (*i.e.* the Celluclast enzyme) at an incubation time of 48 hours.

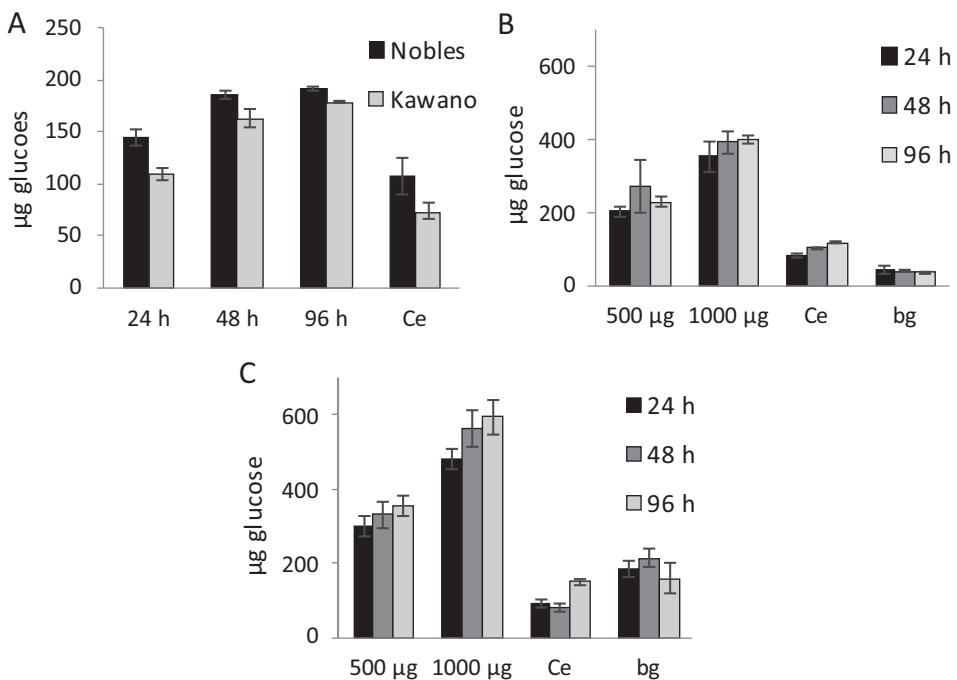


Figure 5.1. Glucose liberated from cellulose using different assays and enzymes. A, comparison of the Nobles and Brown [25] and Kawano et al. [41] protocols on 200 µg of crystalline cellulose; samples were taken 24, 48 and 96 hours after addition of cellulase. Cell pellets of *C. epipsammum* were also tested and samples were taken 24 (Nobles) or 96 (Kawano) hours after addition of cellulase, as prescribed by the respective assays. B and C, degradation (rate) and background signals of Celluclast (Nobles and Brown), and cellulase (Kawano), respectively, on 0.5 and 1 mg of crystalline cellulose and cell pellets of *C. epipsammum* after 24, 48 and 96 hours. Ce, *C. epipsammum*, data is displayed as µg

glucose per mg dry weight; bg, background. Data were derived from triplicate measurements; error bars show standard deviation.

Optimization of cellulose production in *C. epipsammum*

C. epipsammum can grow both in liquid medium and on a solid substrate. To test under which condition most cellulose is produced, the growth of *C. epipsammum* was followed in liquid culture and the cellulose content was determined in the different growth phases in cells harvested from liquid medium and after a week of incubation on a solid substrate.

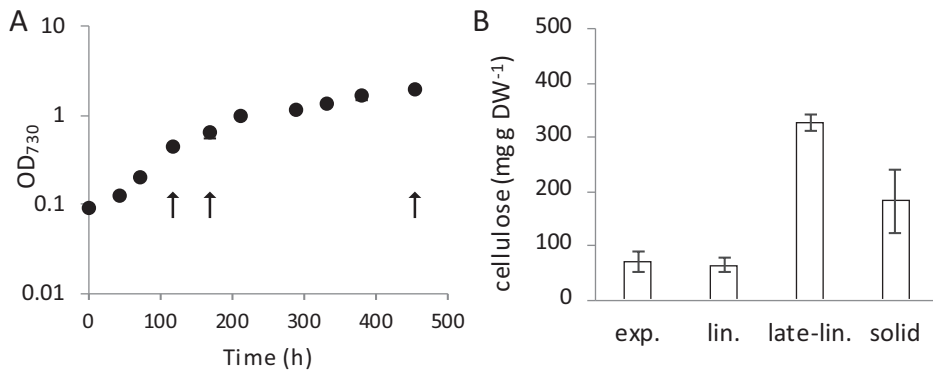


Figure 5.2. A: growth curve of *C. epipsammum* in liquid 0.5 BG-11 medium containing 2 mM sodium nitrate (see Methods section). Black arrows indicate sampling times for cellulose determination. B: Cellulose content of *C. epipsammum* during different stages of growth in liquid medium and after incubation on a solid substrate. For this latter experiment 0.5 mg DW of cells was deposited on a nylon membrane filter, which was placed on solid agar medium with the same nutrient composition as the liquid medium used in A, and was incubated for 7 days. All experiments were conducted in triplicate.

Figure 5.2A shows the growth curve of *C. epipsammum* displaying a classical cyanobacterial growth curve with a lag phase, an exponential phase with a growth rate of 0.017 h^{-1} and a long light-limited phase of linear growth, which gradually transitions into the nutrient-limited stationary phase. Figure 5.2B shows that the cellulose content of the cell increases from around 10 % of dry weight in the early stages of growth to a little over 30 % of the cell dry weight in the very late linear or early stationary growth phase. This cellulose content is 1.5 times higher than previously reported [23]. Incubation of the cells on a solid substrate showed that the cells formed intermediate cellulose contents of about 20 % of dry weight. When grown in liquid culture, *C. epipsammum* tends to aggregate during the later stages of growth, causing it to stick to the side of the shake flask and form clumps (Fig.

5.3A). This may be caused by shear stress, generated by shaking, because aggregation is stronger in larger flasks in which shaking occurs with larger amplitude than in small flasks. The different aggregates were tested for their cellulose content. Samples were taken from the spent medium, of clumps that were 'fished' out and of attached cells that were scraped from the glass wall of the Erlenmeyer. Cellulose was mostly produced and/or accumulated in the free-floating aggregates (Fig. 5.3B).

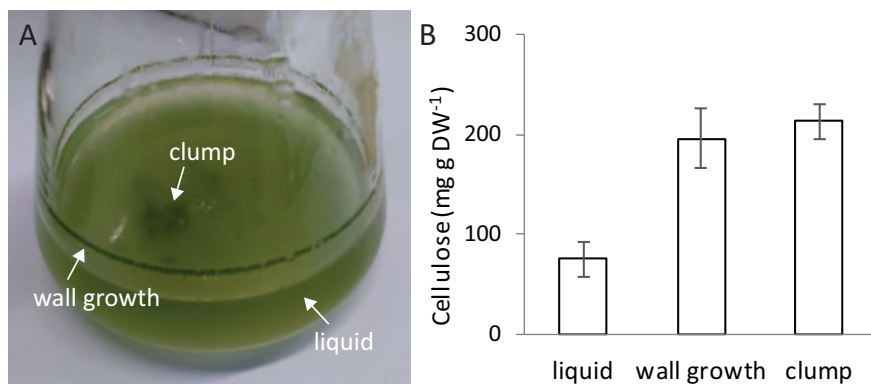


Figure 5.3: A: Liquid culture of *C. epipsammum* in stationary phase with aggregated cells. B: cellulose content of the different types of aggregate in the *C. epipsammum* culture shown in panel A.

To further increase the cellulose content of the cells and optimize growth conditions for *C. epipsammum* we tested different growth concentrations with respect to the nutrients from the BG-11 nutrient mix, the pH and inorganic carbon conditions. Also, we applied and tested several stress conditions (Table 5.1).

		end OD ₇₃₀	μ_{\max}	μ_{average}	cellulose content (mg g ⁻¹)
BG-11 (times)	0.2	1.51 ± 0.22	0.032 ± 0.006	0.018 ± 0.008	22 ± 1.0
	0.5	1.53 ± 0.37	0.029 ± 0.001	0.018 ± 0.007	18 ± 1.7
	1	1.04 ± 0.25	0.025 ± 0.002	0.015 ± 0.006	27 ± 0.6
pH	7	1.80 ± 0.18	0.047 ± 0.005	0.028 ± 0.011	16 ± 1.9
	7.5	2.42 ± 0.16	0.047 ± 0.005	0.024 ± 0.013	12 ± 0.9
	8	2.25 ± 0.24	0.043 ± 0.002	0.023 ± 0.010	13 ± 1.9
NaHCO₃ (mM)	0	1.28 ± 0.44	0.034 ± 0.002	0.023 ± 0.009	26 ± 9.3
	10	0.94 ± 0.04	0.023 ± 0.001	0.011 ± 0.007	25 ± 1.1
	20	0.40 ± 0.10	0.016 ± 0.001	0.007 ± 0.005	62 ± 21
K₂HPO₄ (μM)	0	0.20 ± 0.01	0.019 ± 0.002	0.008 ± 0.006	97 ± 11
	100	1.25 ± 0.12	0.029 ± 0.003	0.019 ± 0.005	18 ± 1.6
	200	1.32 ± 0.10	0.027 ± 0.003	0.021 ± 0.004	18 ± 2.1
NaNO₃ (mM)	0	0.16 ± 0.02	0.014 ± 0.005	0.003 ± 0.005	234 ± 9
	0.1	0.47 ± 0.05	0.022 ± 0.001	0.009 ± 0.007	41 ± 13
	1	1.31 ± 0.07	0.033 ± 0.002	0.015 ± 0.010	29 ± 1.8
	2	1.28 ± 0.11	0.025 ± 0.003	0.016 ± 0.004	18 ± 1.4
	5	1.24 ± 0.19	0.025 ± 0.001	0.015 ± 0.007	24 ± 3
NaCl (mM)	10	0.85 ± 0.08	0.032 ± 0.005	0.018 ± 0.008	31 ± 0.4
	30	0.53 ± 0.18	0.030 ± 0.002	0.018 ± 0.010	42 ± 3.3
	50	0.69 ± 0.08	0.034 ± 0.006	0.014 ± 0.008	1 ± 3
	100	0.10 ± 0.01	0.009 ± 0.001	0.001 ± 0.005	3 ± 0.32

Table 5.1. Growth and cellulose content of *C. epipsammum* under various nutrient conditions. The growth experiments ran for 7 ± 0.7 days and were performed in triplicate. By default the growth medium contained 0.5 times BG-11 with 2 mM NaNO₃, 5 mM NaHCO₃ and a starting pH of 7.5. Values in the table indicate the final concentration of the varied nutrient. Shown are final optical density, maximal growth rate, growth rate over 7 days and the cellulose content at the end of the experiment expressed in mg cellulose per gram dry weight.

Cellulose content of the cells in all these experiments with varying nutrient conditions and applied stresses were lower than the cellulose content of stationary ‘clumpy’ cultures. For logistic reasons, experiments with different nutrient conditions were conducted in 100 ml shake flasks while the other experiments were done in 300 ml shake flasks. The gentler shaking in the smaller flasks may explain the lower levels of cellulose. When looking at table 5.1 in closer detail it can be concluded that *C. epipsammum* grows best at low BG-11 concentrations, at a pH of around 7.5 and without the addition of

NaHCO₃. In fact, a bicarbonate concentration higher than 5 mM has a negative effect on growth. This negative effect on growth is not solely caused by the increased concentration of sodium ions, because addition of sodium chloride at these concentrations lowers the final density of the culture but does not affect the growth rate. When dissolved, bicarbonate equilibrates with the ambient air and the CO₂ escapes, leaving hydroxide ions behind, resulting in alkalization of the medium. Alkalization of the medium in combination with an increase in sodium ion concentration may be the cause of the impaired growth at high bicarbonate concentrations. Table 5.1 also shows that nutrient deprivation increases the cellulose content relative to nutrient replete conditions. However, it should be taken into account that nutrient deprivation prevents growth and will lead to a low cell density in the culture.

To test the effect of humidity and drought stress, cells from late linear phase liquid cultures were filtered on small (25 mm diameter) nylon filter disks, washed with nitrate-free BG-11, and placed on a 0.5 % (w/v) agarose substrate, containing a number of variations of the BG-11 medium in order to combine different stress conditions. The cells were grown for 1 week in an illuminated desiccator (30 μmol photons m⁻² s⁻¹) containing saturated aqueous solutions of CaCl₂ (to impose a humidity of 29 %) or CuSO₄ · 5 H₂O (98 % humidity) (Table 5.2). In these experiments low humidity did not affect cellulose content of the cells. The agar substrate on which the filters were placed dried out more at 29 % humidity than at 98 % humidity but did not fully desiccate. In order, however, to test the conditions of desiccation more thoroughly the filters may need to be placed on a thinner agar layer to achieve complete dehydration. In contrast, nitrogen deprivation resulted in a marked increase of cellulose content of up to 40 % of the dry weight (Table 5.2), which is higher than achieved through shear stress (Figs. 5.2 and 5.3). The cells for the humidity experiment were harvested from stationary phase cultures that were grown in large shake flasks. These cultures have been exposed to shear stress during cultivation and should already have a high cellulose content at the start of the desiccation experiment. The high cellulose content observed should therefore be interpreted as a result of combination of shear stress, nitrogen deprivation and low humidity. However, addition of extra carbon in the form of bicarbonate had a negative effect on the cellulose content. Since high bicarbonate and sodium ion concentrations have a negative effect on growth and only a

minimal effect on cellulose content (Table 5.1). The presence of high concentrations of bicarbonate may have caused damage and stress to the cells without stimulating cellulose production.

BG-11 composition	29 %	98 %
standard	145 ± 4	219 ± 62
0 mM NaNO₃	413 ± 39	392 ± 88
20 mM NaHCO₃	48 ± 13	49 ± 13
0 mM NaNO₃, 20 mM NaHCO₃	247 ± 27	232 ± 2

Table 5.2. Cellulose content (mg cellulose per gram dry weight) of *C. epipsammum* grown on solid medium at 29 and 98 % humidity under various nutrient conditions. Standard: 0.5 times BG-11 with 2 mM NaNO₃ and no NaHCO₃.

At yields of ~30 % cellulose per gram DW *C. epipsammum* would be an interesting candidate for industrial application, even though it grows slower than other cyanobacterial lab strains such as *Synechocystis*, which has a doubling time of ~8 h versus ~15 h for *C. epipsammum*. In order to test the potential of *C. epipsammum* as a commercial cellulose producing strain the culture was scaled up to 20 L with stirring and air bubbling and subsequently to 300 L in a horizontal tubular photobioreactor. In the latter case 0.5 x BG-11 medium was used with 4.25 mM NaNO₃ and 2.5 mM NaHCO₃. The 20 L culture of *C. epipsammum* grew slowly (doubling time of 45 h) to a final OD₇₃₀ of ~1. This was probably due to light limitation caused by self-shading in the large culture flask. The trichomes of *C. epipsammum* were much longer than those in the shake flasks, which was probably due to the low shear stress. The lack of shear stress was also reflected in the cellulose content (~70 mg (g DW)⁻¹) of the cells grown in the 20 L culture flask. In the tubular photobioreactor *C. epipsammum* grew well and reached a final OD₇₃₀ of ~5.5 in only 2 weeks. However, at the end of the experiment the culture became contaminated by a green alga. This contaminant increased in number and finally replaced *C. epipsammum*. The contaminant was identified as *Haematococcus*, a green alga that produces large quantities of the antioxidant astaxanthin, and is cultivated on large scale because of this property.

Identifying novel cellulose producing strains

After isolation and identification of *C. epipsammum* from the dune blowouts near The Hague, The Netherlands in 1990, the isolated strain was deposited in the Göttingen algae

collection (SAG22.89) [23]. In an attempt to re-isolate *C. epipsammum* from that environment, as well as other potentially cellulose-producing phototrophic microorganisms, we returned to the site in 2012. While we were unable to re-isolate *C. epipsammum*, 4 new strains were isolated and obtained in culture (Fig. 5.4). These strains were named dune strain (DS) 1-4. Their absorption spectra revealed that all isolated dune strains have an absorption spectrum that is similar to that of green algae, which shows peaks for chlorophyll *a* (chl *a*; 435 and 680 nm) and chlorophyll *b* (chl *b*; 450 and 660 nm) while *C. epipsammum* shows peaks for chlorophyll *a* and phycocyanin (630 nm), with a shoulder caused by the presence of carotenoids around 500 nm. The isolated strains did all produce cellulose, but not nearly as much as *C. epipsammum* (Table 5.3). The isolated strains were identified by BaseClear (Leiden, The Netherlands) based on the ribosomal ITS region. DS1 was identified as *Coccomyxa subellipsoidea*, DS3 as *Coccomyxa chodatii* and DS4 as *Pseudococcomyxa simplex*. For DS2 no ITS amplification product was formed and the strain could not be identified.

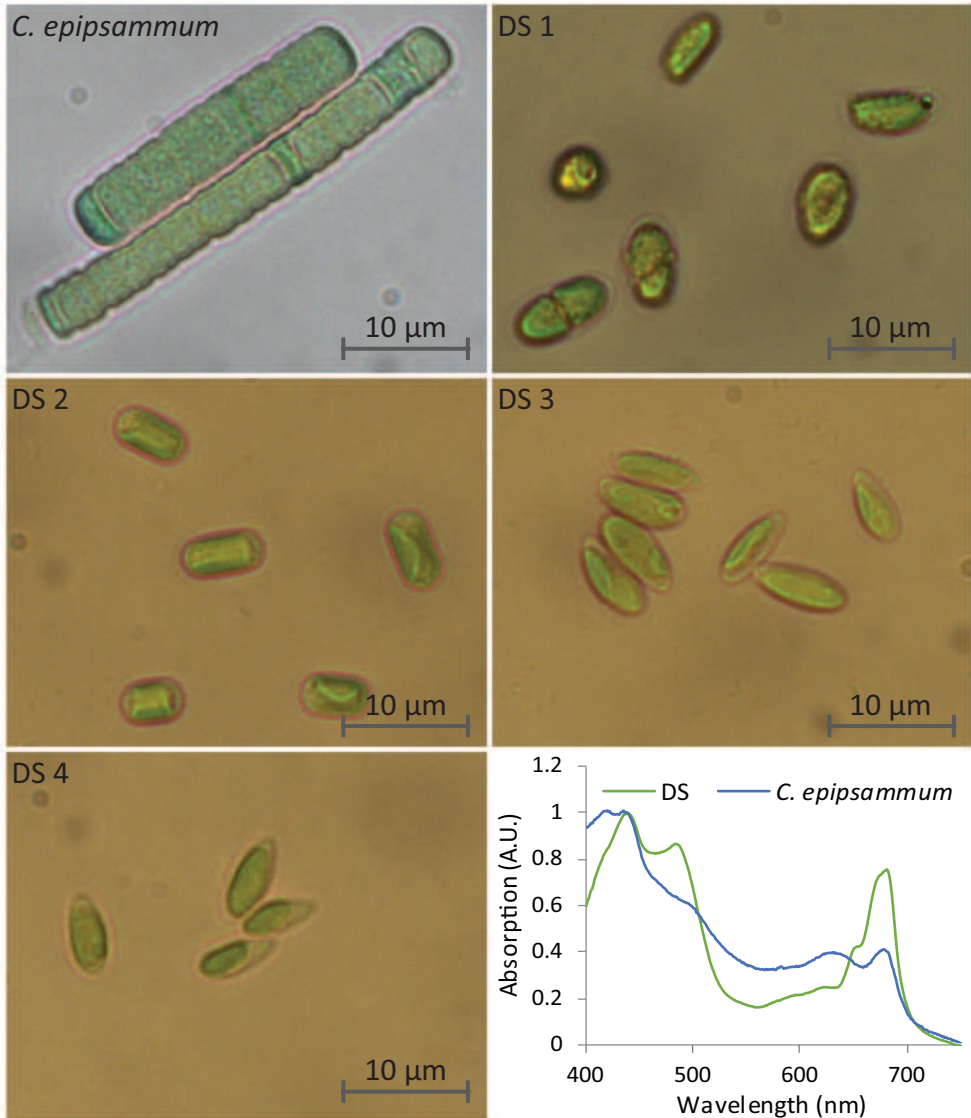


Figure 5.4. Bright field microscope images of the different strains used in this study. Magnification: 100-fold. The spectra show the room temperature absorption spectra of the dune strains and *C. epipsammum*.

	Standard		20 mM NaHCO ₃		0 mM NaNO ₃		100 mM NaCl	
	end OD ₇₃₀	cellulose content (mg g ⁻¹)	end OD ₇₃₀	cellulose content (mg g ⁻¹)	end OD ₇₃₀	cellulose content (mg g ⁻¹)	end OD ₇₃₀	cellulose content (mg g ⁻¹)
DS1	1.61 ± 0.22	46 ± 4	0.10 ± 0.03	17 ± 3	0.82 ± 0.07	112 ± 3	0.40 ± 0.04	120 ± 15
DS2	2.39 ± 0.23	67 ± 1	2.00 ± 0.24	76 ± 9	0.90 ± 0.15	132 ± 8	1.84 ± 0.22	81 ± 1
DS3	1.88 ± 0.18	38 ± 4	0.34 ± 0.03	77 ± 6	0.87 ± 0.06	140 ± 10	1.30 ± 0.08	48 ± 4
DS4	1.30 ± 0.16	44 ± 5	0.33 ± 0.08	89 ± 18	0.69 ± 0.09	127 ± 74	0.58 ± 0.17	96 ± 10

Table 5.3. Growth and cellulose content of the isolated dune strains under various nutrient conditions. The growth experiments ran for ~7 days and were performed in triplicate. The standard growth medium contained 0.5 fold BG-11 with 2 mM NaNO₃, 5 mM NaHCO₃ and a starting pH of 7.5. The final optical density and the cellulose content in mg per gram dry weight at the end of the experiment are shown.

Identifying cellulose synthase genes from cyanobacteria

As a screening method for the presence for cellulose producing strains, the presence of cellulose synthase genes was screened by PCR using DNA primers based on consensus sequences. The low overall homology and the small size of the conserved motifs make screening for cellulose synthase genes difficult in organisms that have not been sequenced and annotated. Sequence conservation of the gene coding for cellulose synthase among cyanobacteria and green alga proved to be too low to enable the design of primers. However, at the amino acid level some small conserved motifs were found (Fig. 5.5). Using amino acid sequences found through Blast searches, based on CelA4 from *Arabidopsis thaliana* and BcsA from *Gluconacetobacter xylinus*, two sets of degenerate primers were designed that correspond to consensus sequences of proteins containing a bacterial cellulose synthase domain (BcsA) and proteins that contain a plant-like cellulose synthase domain (CelA) (Table 5.4). The first generation of primers that were designed contained a considerable level of degeneracy (Table 5.4) but yielded virtually no product in any of the strains tested (data not shown). Hence, the degeneracy was minimized in the second generation of primers (Table 5.4) and PCR analysis was conducted on the isolated dune strains and *Crinalium epipsammum*. As a negative control, samples of *Synechocystis* sp. PCC

6803 were used because this organism does not contain cellulose synthase genes. However, none of the primer pairs gave an amplification product in *C. epipsammum*, even though the cellulose synthase gene of this organism was used for the primer design. Amplification occurred but the size of the amplified bands was often slightly larger than expected and a-specific amplification was frequently observed with the CelA-type primers. Further optimization of the PCR conditions failed to improve the observed results (data not shown).

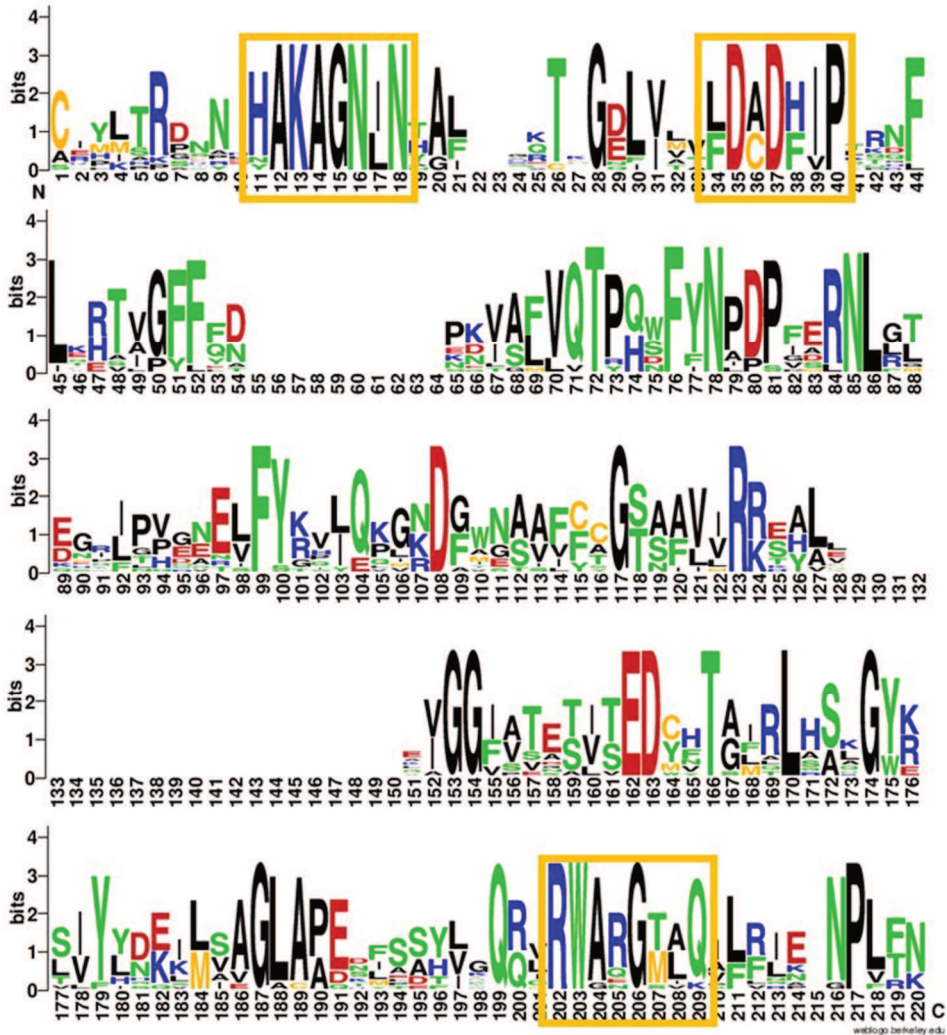


Figure 5.5. Alignment logo of part of the catalytic domain of the cellulose synthase of cyanobacteria: The larger the letter the more conserved the respective amino acid. Regions used for primer design are marked with yellow boxes. Colors: Black, aliphatic; red, acidic; blue, basic; orange, sulfur

containing; green, other. This logo is based on the catalytic domain of CelA from 14 different cyanobacteria.

name	sequence	Degen- eracy	direction	amino acid consensus
pCela1	CAYG C IAARGCIGGIAAYMTNAA Y	128	Forward	HAKAGNLN
pCela2	TTYGAYGCIGAYTTYRTICCN	128	Forward	DADFIP
pCela3	CY T GIARIG T ICCN Y TNSCCC	512	Reverse	WARGTLQ
pCela4	AATGCTAAAGCAGGCAATCTCAAT	0	Forward	HAKAGNLN
pCela5	TTTGATGCTGATTTTATTCC	0	Forward	FDADFIP
pCela6	TTTTAAAGTTCCTTCTGCC	0	Reverse	WARGTLQ
pBcsa1	GGIGGNAARKCIGNGCIYTNAAY	1024	Forward	GGKSGALN
pBcsa2	YTIGAYGCIGAYGCICARSTN	128	Forward	FDADAKV
pBcsa3	CY T GI W RIC C IC C Y T CNGCCC	64	Reverse	WAEGGLQ
pBcsa4	GGYGGTAAATCMGGSGCKCTTAAT	16	Forward	GGKSGALN
pBcsa5	TTYGATGCTGATGCTCAACT	2	Forward	FDADAKV
pBcsa6	YTGR T ATCCTCCTTCW G CCC	8	Reverse	WAEGGLQ

Table 5.4. First- (1-3) and second- (4-6) generation primers for screening of cellulose synthase encoding genes from cyanobacteria and green algae. Special nucleotides are marked in grey; N=ACTG, Y=CT, R=AG, M=AC, S=GC, K=TG, W=AT, I= inosine, which binds C A or T. Degeneracy is the number of unique oligonucleotides in the primer mixture.

Discussion

Strain selection for industrial production of algal or cyanobacterial products depends largely on the desired product, whether it is biomass, low molecular weight fuel, fatty acids or high value products, but some features are generally preferred. The ideal strain should grow fast, be resistant to shear stress and the growth of contaminating organisms, produce a valuable product, be tolerant to salt and sediment quickly when left to stand for cheap harvesting [168,259,260]. *C. epipsammum* does not meet all these criteria. It is sensitive to salt and shear stress and grows only in low salt- and low-nutrient medium, in a bubble column or airlift photobioreactor (*i.e.* not in photobioreactors that are mechanically stirred with pumps, R.M. Schuurmans, unpublished). However, *C. epipsammum* does grow reasonably fast with an average exponential growth rate of around 0.02 h⁻¹ and a maximum

of 0.047 h^{-1} (Table 5.1), corresponding to doubling times of 35 and 15 h, respectively. Also, the trichomes sink rapidly and the culture sediments within 24 h (R.M. Schuurmans, unpublished). The culture produces the high-energy extracellular product cellulose up to levels of 40 % of the cellular dry weight. A major concern in large-scale cultivation of phototrophic microorganisms is the risk of infection and subsequent consumption of the product by the invader. In nature, *C. epipsammum* grows in association with many other microorganisms yet this does not harm its growth or cellulose production. In fact, the addition of heterotrophic bacteria to axenic cultures of *Microcystis* has been reported to stimulate EPS production and reconstitute colony formation [261]. This implies that the (minor) presence of heterotrophic bacteria in *C. epipsammum* cultures may not pose a threat and an axenic culture is not a strict requirement for large scale cultivation of this organism, provided that the contamination remains minor and does not outgrow or consume *C. epipsammum*. Additionally, *C. epipsammum* favors low nutrient conditions, specifically low nitrogen conditions, which is the main constituent of the nutrients of BG-11 medium. This could lower the cost of the medium and the environmental burden for large-scale biomass production with this organism. Hence, *C. epipsammum* could be an interesting candidate for large-scale cultivation, but for which application would this organism be most suited?

The most straightforward application would be to grow *C. epipsammum* biomass to a high density in a suitable photobioreactor, followed by the optional application of stress conditions to further increase cellulose production. The biomass could then be harvested as a whole and the glucose from the cell-bound cellulose of *C. epipsammum* biomass can be released via acid hydrolysis, and after pH correction, the hydrolysate can be used as a feedstock for biofuel fermentation [27]. For biogas production via anaerobic digestion the nitrogen content of algae and cyanobacteria is often too high, leading to the production of ammonium and ammonia, which inhibit fermentation [262]. Organisms such as *C. epipsammum* that have a naturally high C:N ratio could be interesting for such feedstock applications. Manipulation of nutrient composition, and especially nutrient deprivation, can stimulate EPS production in cyanobacteria [263] and perhaps further optimize C:N ratios for fermentation [262]. In *C. epipsammum* the effect of nutrient deprivation on cellulose content was limited (Table 5.1) and the application of (moderate)

shear stress appeared to be much more effective and probably also easier to apply. However, a combination of both stresses may be even more effective.

Alternatively, *C. epipsammum* could be grown directly in a consortium with fermentative, heterotrophic bacteria. In the field of biofuel production with algae and cyanobacteria an interest in a consortia-based approach has been developing [264,265] in which an alga or cyanobacterium provide a carbon and energy source via photosynthesis to a heterotrophic organism such as *E. coli*, and the latter then produces the product of interest. Again the natural ability to exude a glucose polymer makes *C. epipsammum* an excellent candidate for the phototroph in such a consortium, provided the heterotrophic bacterium is capable of degrading cellulose, but this can be achieved – if necessary - by equipping it with an extracellular cellulase [266,267].

For the production and extraction of cellulose as a final product one will have to find a way to separate the presumed non-crystalline cellulose [23] from the *C. epipsammum* trichomes preferably without lysing the organism. No glucose was released from *C. epipsammum* supernatant samples treated with cellulase (data not shown), indicating that cellulose in *C. epipsammum* is a capsular (cell bound) polysaccharide (CPS) rather than a released polysaccharide (RPS) [268]. One could add cellulase to the cells to release the cellulose polymer, but this would result in free glucose in the medium, which is soluble and necessitates an expensive extraction procedure. The extracellular glucose will also strongly increase the risk of contamination of the culture.

An alternative may be to grow *C. epipsammum* on a solid substrate rather than in liquid culture. This could be done in a rotating biological contactor where a contact material on which the biofilm can grow slowly rotate through a liquid medium, alternatingly exposing the material to water and to air (and light) [269,270]. This type of reactor is particularly popular in wastewater treatment applications [271,272]. This will decrease the amount of water required for growth, although it will increase the required surface area. Growth on a solid substrate may be less interesting for industrial scale production but it could be interesting for applications closer to its original biological function, such as inoculation of barren habitats [273,274] or nutrient enhancement of overused farmland [275,276] or even rooftop gardening. However, there is no information on the growth rate

of *C. epipsammum* on a solid substrate and very little information of associated cellulose production.

As the primary colonizers of dry and exposed environments, cyanobacteria often initiate biofilm formation and are the first contributors to the synthesis of EPS [263]. *C. epipsammum* is such an early colonizer, producing large amounts of EPS to bind the fine sands of its dune habitat and produces a biofilm matrix that retains moisture in the top layers of the sand, where light is available. Cellulose has a high water-retention capacity and this may very well be why *C. epipsammum* makes so much of it. The newly identified dune strains were taken from the dunes in May 2012, after the first sunny and relatively warm (≥ 15 °C) days of spring. These unicellular green algae could be secondary colonizers, taking advantage of the water-retaining matrix laid out by the cyanobacteria and producing other EPS constituents to further build the biofilm. Dune strain 3, for instance, produces visible amounts of EPS when grown on agar plates but low amounts of cellulose (3.5 % under normal growth conditions, Table 5.3).

Apart from cellulose it is not known which other sugars are present in the exopolysaccharide layer of *C. epipsammum*. Cyanobacteria produce some of the most complex and diverse mixtures of exopolysaccharides containing a higher variety of monosaccharides than most other microorganisms [268,277]. These sugars include acid sugars that are rare in other Gram-negative bacteria under nutrient replete growth conditions. Additionally, modifications such as methylation, addition of sulfate groups, acetylation and the ligation of peptide moieties further increase the EPS complexity. Nevertheless, the main constituent of most EPS layers appears to be glucose [268]. There are reports of exopolysaccharide exudations constituting of up to 60 % of the dry biomass [278,279]. Considering that up to 40 % of the biomass of *C. epipsammum* was cellulose, this polymer is most likely the main constituent of the *C. epipsammum* EPS. Although it would be interesting to further analyze the EPS layer of *C. epipsammum*, our focus was on cellulose production for industrial purposes.

In cyanobacteria two types of cellulose synthase genes are present that resemble cellulose synthase genes from either bacteria or plants. Phylogenetic analysis also shows a clear split between the bacterial cellulose synthases (e.g. BcsA) and the eukaryotic cellulose synthases (e.g. CesA; [65]). Cyanobacteria, however, contain both cellulose synthases that

group within the BcsA clade (referred to as CcsA2 for cyanobacterial cellulose synthase type 2) and cellulose synthases that form a sister clade to the CesA clade from plants (referred to as CcsA1) [65]. This supports the theory that plant cellulose synthases have a cyanobacterial origin [24]. Yet, identification of the cellulose synthase genes of the newly identified dune strains and a few cyanobacterial lab strains by PCR, using primers based on a cellulose synthase consensus sequence, was not possible. Disregarding that the isolates were green algae and that we used cyanobacteria-based primers, bands of roughly the expected size were only found in the dune strains and in *Synechocystis* sp. PCC 6803, which does not contain cellulose synthase genes. No bands were found in *C. epipsammum* or in *Synechococcus* PCC 7942 samples, even though the sequence of the genes from both strains was used in the primer design. Decreasing the annealing temperature, or using a touch-down PCR, increased the presence of a-specific bands, but did not generate positive results for *C. epipsammum* or *Synechococcus* PCC 7942. Green algae contain *cesA*-related genes [280], and although *Synechocystis* PCC 6803 does not contain cellulose synthase genes, it encodes other processive β -glycosyl transferases [65]. The obtained PCR products in the latter strain may very well be from cellulose synthase-like genes, containing the conserved β -glycosyl transferase motive. But since the technique tested here failed to amplify the cellulose synthase genes from *C. epipsammum* and *Synechococcus* it is clearly not suitable as a screening method for cellulose synthesis. Whether novel and/or selected strains of phototrophic microorganisms can produce cellulose therefore will have to be investigated using a different technique. This can, for instance, be done via the enzymatic assay described in this chapter or by staining the exopolysaccharides and extracellular cellulose with various dyes such as Congo Red and Calcofluor White, which bind to specific β -glucans like cellulose [281,282]. A more advanced technique may be tagging of the cellulose with lectins, carbohydrate-binding proteins that are a part of many biofilms and that have high specificity to different types of exopolysaccharides [283,284].

The literature describes several different methods that use enzymatic degradation followed by glucose quantitation, as the method of choice to determine the amount of cellulose produced [25,26,28,41]. These methods vary in complexity, the time required, and the necessary control experiments. The simplest of these methods proved to be best suited. Adding additional steps, especially steps that require removal of certain fractions, may lead

to the loss of product. Most importantly, it was found that addition of non-purified cellulase led to large background signals in the subsequent glucose analysis. Further inspection revealed that Celluclast contains sorbitol as a stabilizer and potassium sorbate as a preservative, while the cellulase powder from *Trichoderma viride* contains maltodextrin, sorbitol and propylene glycol as stabilizers. These additional compounds are most likely the cause of the high glucose background signal. Although chromatographic purification is an option, a simple negative control sample containing only buffer and cellulase will also suffice. However, the background signal should never be ignored, or otherwise the cellulose production of a strain may be grossly overestimated.

Materials and Methods

Strain and strain isolation

Phototrophic microorganisms were isolated from microbial crusts, collected from so-called 'blow outs' (Aeolian erosional depressions) on south-exposed slopes of sand dunes along the Dutch North Sea coast [23]. The sandy crust material was spread onto empty Petri dishes and wetted with 0.2 x BG-11 [163] and incubated at room temperature under continuous illumination with white light of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 5 days green patches were collected and spread onto agar medium containing 0.2 x BG-11 medium and 1 % (w/v) agar (SphaeroQ bacteriological agar). The different strains were isolated through several rounds of streaking until a unialgal culture was obtained. Each strain originated from a different crust. In order to minimize the growth of heterotrophic contaminants, a switch was made to plates containing 0.5 % (w/v) agarose (SphaeroQ D1 agarose) in 0.5x BG-11 medium. Strains 1, 2 and 4 were streaked until axenic, while strain 3 remained associated with some heterotrophic contaminants. Agarose plates with the strains were sent to BaseClear, Leiden, The Netherlands for identification. Based on the pigment composition (Fig. 5.4), the strains were classified as green algae and identified based on their ribosomal internal transcribed spacer (ITS) region [285]. In early stages of isolation of microorganisms crust material was examined under a microscope in an attempt to locate and isolate *C. epipsammum* through micromanipulation. However, no visible colonies formed on BG-11 plates after micromanipulation. *Crinallium epipsammum* SAG 22.89 was provided by the Culture

Collection of Algae of the Institute of Plant Physiology of the University of Göttingen, Germany.

Culturing conditions

C. epipsammum and the newly isolated strains were grown in liquid 0.5 x BG-11 (or 0.2 x BG-11) medium (i.e. BG-11 diluted 1:1 and 1:4 (v/v) with distilled water, respectively) with 2 mM NaNO₃ and 5 mM NaHCO₃, unless specified otherwise, at room temperature under continuous illumination at 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Cultures were also grown and streaked on 0.5 % (w/v) agarose plates containing 0.5 x BG-11 medium at room temperature under ambient light conditions.

Humidity stress experiments

Desiccators containing over-saturated solutions of CuSO₄·5H₂O (Sigma Aldrich, 98 % humidity) or CaCl₂ (Merck, 29 % humidity) [286] were prepared. Cell suspensions were placed on Nylaflo filter disks (PALL life sciences, 0.45 μm pore size) via filtration to a final concentration of 0.5 mg DW. The filters were washed with 5 ml of NaNO₃-free 0.5x BG-11 medium and placed on 3 mm thick 0.5 % agarose plates containing BG-11 medium with varying compositions (see Table 5.2). The agarose plates were placed without a lid in the desiccators and the cells were grown under these conditions for 7 days at room temperature under continuous illumination at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Dry weight determination

50 ml cultures at 6 different optical densities (OD₇₃₀ = 0.25, 0.5, 0.75, 1, 2 and 4) were harvested by centrifugation (10 min, 3,500 g). Pellets were dried overnight at 90 °C and the weight difference between empty and full tubes was determined on the same precision scale (Mettler Toledo AB204). The measurements were performed in triplicate and a calibration curve based on the optical density at 730 nm was constructed. This calibration curve was then used to determine the dry weight of cell samples in different experiments, based on optical density measurements. Calibration curves were constructed for *C. epipsammum* and the newly isolated strains. At an OD₇₃₀ of 1 all organisms contain approximately 120 μg of dry weight per ml.

Bright field microscopy

A thin layer of 1 % agar containing BG-11 medium was placed on a glass slide and 5 μ l of cell suspension was spotted onto the agar layer. The samples were sealed with a coverslip and the coverslip was secured with nail polish. The cell samples were examined under a Zeiss Axiovert 40 CFL microscope equipped with an AxioCam Mrc (Zeiss) photo camera. Pictures were taken and size bars were added using Axiovision 4 software.

Enzymatic assay for cellulose quantitation

A: Based on Nobles and Brown [25]: 5 ml of culture was harvested by centrifugation (10 min, 3,500 g). To the pellet 1 ml of sodium acetate buffer (20 mM, pH 5.0) was added and the cell suspension was transferred to a 2 ml Eppendorf tube. As a control samples containing only sodium acetate buffer (20 mM, pH 5.0) were also prepared. 5 μ l Celluclast suspension (from *Trichoderma reesei*, Sigma) was added to the samples and controls. All tubes were incubated for 24 h on a rotating platform at 50 °C. All tubes were centrifuged (5 min, 6,000 g) and the glucose concentration in the supernatant was determined using an enzymatic glucose assay kit (Megazyme).

B: Based on Kawano et al. [41]: 5 ml of culture was harvested by centrifugation (10 min, 3,500 g) in 15 ml tubes. To the pellet 5 ml of TE buffer (10 mM Tris-HCl at pH 8.2, 1mM EDTA) and 1 mg of lysozyme (Sigma Aldrich) was added. Samples were incubated at 37 °C for 30 min with shaking (200 rpm). SDS was added to a final concentration of 1% (w/v) and cells were disrupted by sonication for 1 min. Insoluble material was collected by centrifugation (10 min, 3,350), resuspended in 1 ml of 0.5% (w/v) SDS in TE buffer and transferred to Eppendorf tubes. Proteinase K (Agilent) was added to a final concentration of 0.5 mg ml⁻¹ and samples were digested overnight at 55 °C with shaking (100 rpm). Insoluble material was collected by centrifugation (10 min, 19,500 g), washed in 0.5 ml of sodium acetate buffer (20 mM, pH 5.2) and resuspended in 1 ml of the same buffer. Negative controls containing only sodium acetate buffer were added at this point. Amylo-glucosidase (Boehringer Mannheim) was added to a final concentration of 1 mg ml⁻¹ and samples were incubated overnight at 55 °C with shaking (100 rpm). Insoluble material was collected by centrifugation (10 min, 19,500 g) and the supernatant was stored at -20 °C until analysis, marked as glycogen sample. The pellet was washed in 0.5 ml of sodium acetate buffer (20 mM, pH 5.0) and resuspended in 1 ml of the same buffer. Cellulase from *Trichoderma viride*

(Sigma Aldrich) was added to a final concentration of 1 mg ml^{-1} and samples were incubated at 37°C on a rotating platform for 96 h. Samples were spun down (10 min, 19,500 g) and the glucose concentration of both the glycogen and cellulose samples was determined using an enzymatic glucose assay kit (Megazyme).

Design of cellulose synthase screening primers

Protein sequences of cyanobacterial cellulose synthases and potential cellulose synthases were obtained through blast searches in the NCBI database using the BcsA protein from *Gluconacetobacter xylinus* and the CelA4 protein from *Arabidopsis thaliana* as query. 99 unique hits were selected and complemented with the three potential cellulose synthase enzymes from *C. epipsammum*. All sequences were aligned and the three regions with the highest level of conservation were selected (see Fig. 5.5). Comparison of the amino acid sequences in the three regions resulted in the formation of two groups with different consensus sequences. Analysis of the protein sequences using the Conserved Domain Database (CDD) tool on the NCBI website revealed that the grouping corresponded to proteins that contained a bacterial cellulose synthase catalytic domain (short name; COG1215, BcsA) and proteins that contained a plant-like cellulose synthase domain (short name; TIGR03030, CelA), respectively, while proteins that contained only a glycosyltransferase domain (short name; pfam1364) were found in both groups. Based on the consensus sequences of the different groups DNA primers with high and low degeneracy were designed (Table 5.3).

PCR for cellulose synthase screening

Screening PCRs were performed using 2x Taq polymerase mix (My Taq Mix, Bioline). The reactions contained 10 μl Taq mix, 2 μl of each primer, 6 μl water and one cell colony taken from plate. PCR protocols ran for 35 reaction cycles and started with 5 minutes at 94°C to break the cells, denaturation, annealing and elongation times were selected in accordance with the instructions for the Taq polymerase. The annealing temperature was set at 43°C for the CelA primers and 47°C for the BcsA primers for 35 reaction cycles. Alternatively, 15 cycles at 43°C or 47°C followed by 20 cycles at 51°C was used. PCR reactions were analyzed on 1 % agar gels.