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Novel photonic materials:

Isolation, purification and imaging of native chlorin based antenna from *Chloroflexus aurantiacus*

Bachelor Thesis

Institute of Chemistry

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Annotation: Pure culture of thermophilic photosynthetic green non-sulphur bacterium *Chloroflexus aurantiacus* was maintained and grown at medium scale. Chlorosome extraction and purification protocol was optimized yielding highly pure chlorosome fraction. Chlorosomes were characterized spectroscopically using UV-VIS absorption spectroscopy. Immobilized chlorosomes were imaged using advanced imaging methods in atomic force microscope and transmission electron microscope.

Declaration of Academic Honesty

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Abstract

Photonics plays a significant role in an increasing number of fields concerning human life, such as: medical applications, agriculture, optical technologies, communication networks, imaging, lighting and displays, energy conversion. The development of novel photonic materials aims to improve mankind's life quality. For this work, chlorosomes which are photosynthetic antenna complexes from the green non-sulphur bacterium *Chloroflexus* aurantiacus were studied. An important characteristic of chlorosomes is that they are composed of self-assembling pigment aggregates. This represents the main reason why chlorosomes are of great interest for constructing artificial light harvesting systems that could be consolidated into photonic materials. *Chloroflexus aurantiacus* cultures were grown under continuous light in anaerobic and aerobic conditions in order to compare the cultures grown under different conditions. The chlorosomes of these cultures were extracted, isolated, ruptured and purified by density sucrose gradients and size exclusion chromatography. Their pigments were analysed by absorption spectroscopy. BChl a was present in both anaerobic and aerobic *Chloroflexus* cultures, while the BChl c was only present in the anaerobic one, therefore the anaerobic cultures were used for further analysis. The spectroscopic analysis of the purified chlorosomes showed a peak of BChl c at 742 nm and a peak of BChl a around 796 nm indicating a successful purification process. The chlorosome structure was inspected using an imaging combination of transmission electron microscopy and atomic force microscopy which illustrated ellipsoidal chlorosome bodies different in size and shape confirming the findings of previous research.

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Abbreviations

AFM	atomic force microscopy
BChl	bacteriochlorophyll
Cfx	chloroflexus
CsmA	chlorosome protein A
DGDG	digalactosyl diacylglyceride
LED	light emitting diode
MGDG	monogalactosyl diacylglyceride
Q.dots	quantum dots
R_2A	Reasoner's 2A agar
SEC	size exclusion chromatography
TEM	transmission electron microscopy
UV/VIS	ultraviolet/visible

1. Introduction

1.1 Photonic materials

Light is an electromagnetic wave comprised of photons. In the field of physics, light usually relates to radiation in the entire electromagnetic spectrum, comprising X-rays, infrared, visible light, ultraviolet, and microwaves. The terms photonics and optics are usually used in the literature to refer to applications of light [1]. Photonics can be characterized as the science of light particles (photons) and the light-matter interaction while optics can be seen as the science that investigates the wave characteristics of light [2][3].Optical nanostructures are related to the nano-photonics technology and photonics at the nanoscale can be defined as *"the science and engineering of light-matter interactions that take place on wavelength and subwavelength scales where the physical, chemical, or structural nature of natural or artificial nanostructure matter controls the interactions*" [4][5].Optics and photonics have a substantial impact on the engineering applications, especially in the field of communications, medical imaging, solar cells, generation of modern displays. These applications are of great interest and grant the desire to investigate and extend the knowledge of the light matter interaction in the future [2][6].

1.1.1 Synthetic photonic materials

Development of synthetic photonic materials is an important study field and plays a major role in different aspects of human life. Synthetic photonic materials include for example semiconductors, dielectrics, electrodes, polymers, crystals and quantum dots. These materials require a deep understanding of their properties and structures and by combining natural sciences, material science and engineering, delivery of exceptional devices with a relevant design could be obtained. Among the major lighting discoveries in this field are the LEDs (light emitting diodes), which produce light more efficiently than the normal light bulbs. LEDs are used world-wide from television screens to automobile lights and different lighting apparatus [7].

Quantum dots (Q-dots) are especially very attractive for developing photonic materials. They are made of crystalline particles (nanometre sized) of semiconductors [8]. Q-dots exhibit high efficiency and bright fluorescence, symmetrical and sharp peak of emission, photostability and temperature insensitivity [9][10]. Because of these properties Q-dots are suitable

candidates for applications in optoelectronic devices including light-emitting diodes, photodetectors and sensors, solar concentrators and cells [8]. A major problem preventing the expansion of q-dots applications is the high cost, due to complex synthetic procedures based on high quality q-dots [11].

1.1.2 Biological photonic materials

Interaction of light with biological materials may result in its scattering, absorption and subsequent emission. The use of light for the purpose of studying biological objects (molecules, cells, tissues) in biology, agriculture, medicine, pharmaceutical science, environment science, is a combination of biology and photonics, that embraces the development of optical technologies [12].

The colour producing molecules or chromophores of plants and animals are studied in order to understand how these structures interact with light. Pigmentation is the consequence of light absorption by the chromophores, and its molecular structure characterizes the wavelength. Coloured plants and animals represent a source of interest and motivate the development of certain industrial and technological applications. A more demanding requirement is placed on understanding how to integrate these biological means of colour production into functional devices. Stretchable iridescent fibres inspired from multilayer structures found in particular pigmented tropical plant seeds have been produced utilizing advanced fabrication procedures. These structural biological colours are also very appealing for displays and sensors, lighting techniques, cosmetics and paints [13].

Some advantages regarding bio-photonics include: probing and manipulating biological objects in a minimally invasive manner, high practicality and low costs [12].

1.2 *Chloroflexus aurantiacus*

Chloroflexus (*Cfx*.) *aurantiacus* is a thermophilic green non sulphur bacterium isolated from hot springs, possessing chlorophyll-based photosynthesis, belonging to the filamentous anoxygenic phototrophs (also named green non-sulphur bacteria or green gliding bacteria). *Cfx*. *aurantiacus* shows a versatile metabolism of carbon [14][15]. It can grow chemoheterotrophically on various organic substrates in aerobic and dark conditions. *Cfx*.

aurantiacus turns to photoheterotrophic growth if provided with acetate under anaerobic and light conditions. Cells harbour unique antenna complexes, peripheral chlorosomes, which channels the captured light quanta through the base plate to a quinone type (or type-II) reaction centre, an electron transport pathway and carbon fixation pathways [16][17][18].The peripheral chlorosome complex contains characteristics of green sulphur bacteria, while the reaction centre contains characteristics of purple photosynthetic bacteria. Therefore, the photosynthesis process is a mix of the purple bacterial and green sulphur bacterial photosynthesis. It mainly consumes organic carbon sources (short-chain fatty acids, acetate, lactate) that are released by the cyanobacteria associated with it in the environment. *Cfx. aurantiacus* can however also incorporate CO_2 as a source of carbon. All these features make *Cfx.* an important system to investigate in order to understand the evolution of photosynthesis [16].

The light energy is first absorbed by the peripheral light-harvesting antenna, the chlorosome, that is a complex of self-assembled bacteriochlorophyll c (BChl c) and covered by a lipid-protein monolayer. Afterwards the energy is transmitted through the baseplate of chlorosomes to the light-harvesting core antenna complex, which is a protein-pigment complex. Lastly, the photochemical reactions occur in the reaction centre [16].

For this work the photoheterotrophically grown *Cfx. aurantiacus* has been studied. For photoheterotrophic culture of *Cfx. aurantiacus*, the growth medium requires complex nutrients such as yeast extract or casamino acids [19].

1.3 Chlorosomes of *Chloroflexus aurantiacus*

1.3.1 Composition of chlorosomes

Chlorosomes are light-harvesting complexes found in three different phyla of photosynthetic bacteria: Chlorobi, Chloroflexi and Acidobacteria. The main pigments of chlorosomes (bacteriochlorophylls c) are organized in the form of an aggregate in comparison to other photosynthetic light-harvesting complexes, which use non-covalently protein-bound pigment molecules - cyclic tetrapyrroles or covalently bound pigment molecules - open tetrapyrroles.

The chlorosome aggregates are assembled from thousands of associated BChl molecules generating the chlorosome as the largest light-harvesting complex known until now [20].

Chlorosomes are made of bacteriochlorophylls (BChls), proteins, lipids, carotenoids and quinones. The principal constituents of the chlorosome are BChl c, d or e that are also the major light-harvesting pigments. By dry weight, chlorosomes are composed of 50% BChl c, d or e, 30% protein and 10% lipids. The remaining weigh is composed of carotenoids, quinines, and BChl a [20].

BChl a is derived from bacteriochlorine, while BChl c, d and e are derived from chlorine, a chlorophyll skeleton that differs from bacteriochlorine by a double bond between C7 and C8 (Figure 1). BChl c, d and e posses unique structural characteristics. They self-assemble into aggregates and exist only in chlorosomes. Aggregation is mediated by the presence of a hydroxy group at the asymmetric $C3^1$ and the absence of a bulky methoxycarbonyl group at the $C13^2$ (Figure 1). The intermolecular coordination of the central Mg^{2+} ion of one BChl molecule to the hydroxy group at the $C3^1$ of a second BChl constitutes the fundamental step for aggregate formation. Furthermore, a hydrogen bond between the hydroxy group of the second BChl molecule at the $C3^1$ and the keto group at the $C13^1$ of a third BChl molecule is formed. The hydrogen bond seems to influence the exciton coupling by stabilizing the parallel orientation of the Qy transition dipole moments which is essential for strong exciton coupling. BChl a (Fig 1) which is found in a pigment-protein complex does not form aggregates and represents only 5% in Chloroflexi from the total BChl content [20].

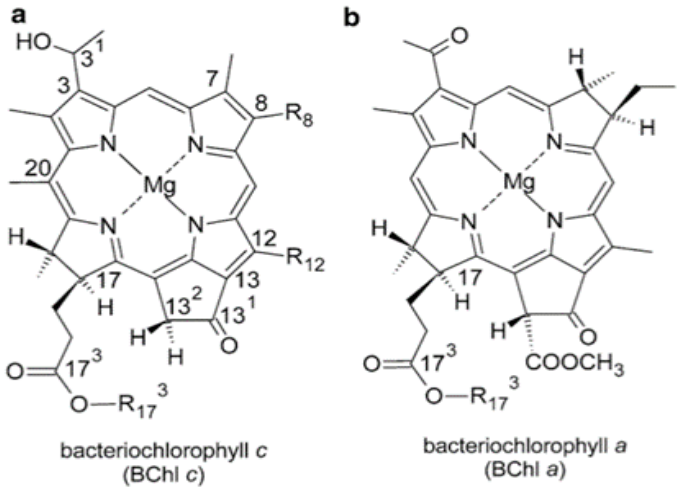

Figure 1. Structure of Bchl c and BChl a [20]

Carotenoids are found in a high amount in chlorosomes, either in the interior of the chlorosome or in the baseplate (Figure 2). They possess photoprotective, light-harvesting and structural roles. The carotenoid amount depends on several factors such as growth phase, light quality and intensity, temperature conditions and particular bacterial species. In *Cfx. aurantiacus* the carotenoid amount increases under high light conditions. Quinones, which contain a polar head group and a hydrophobic isoprenoid side chain, are also placed in chlorosomes . Their function in Chloroflexi species is still unknown [20][21].

The principal lipid found in Chloroflexi species was monogalactosyl diacylglyceride (MGDG), which is also the major lipid found in photosynthetic membranes of chloroplasts. It has been proposed that its role in chloroplasts could be to promote packing of large membrane proteins and probably could have a similar function in the protein chlorosome envelope. *Cfx. aurantiacus* also contains digalactosyl diacylglyceride (DGDG) and non-polar lipids (hydrophobic wax- esters) [20][22].

1.3.2 Structure of chlorosomes

Chlorosomes exhibit ellipsoid bodies differing in shape and dimensions among species, their structure being also influenced by the growing conditions. The chlorosomes from Chloroflexi species which consist of BChl c or d seem to have a smooth surface and an ellipsoidal shape and they are attached to the cytoplasmatic membrane via the baseplate (Fig. 2). The overall construction consists of the baseplate, envelope and interior (Figure 2). The interior comprises the pigment aggregations while the proteins are placed only in the envelope and the baseplate [20].

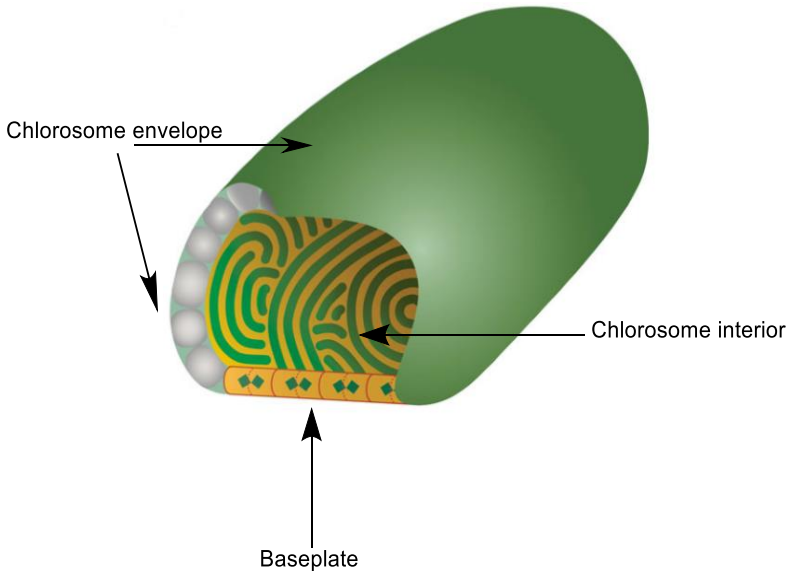

Figure 2. Basic structure of chlorosome. The green layers consist of BChl aggregates and the yellow parts are occupied by carotenoids and quinones. The baseplate contains the CsmA protein where BChl a molecule can be found (green rectangle), while the orange space is made of carotenoids and quinones. The envelope is made of proteins (the grey molecules), while lipids fill the space between them [20]

The baseplate plays a critical role in the transfer of excitonic energy from the chlorosome towards the bacterial reaction centres and is positioned on one side of the chlorosome. It is composed of multiple copies of the BChl a coordinating protein *CsmA* [23]. The envelope is made of polar lipids and represents the surface coat of the chlorosome. The polar lipids have their polar heads oriented towards the cytoplasm and it is assumed that they form a monolayer around the chlorosome covering only around 5% of the surface while more than 90% of the surface is mainly covered by proteins [24][20].

Freeze fracture micrographs showed the first structure of the chlorosome interior. It was suggested that the BChl aggregates were organized in tightly packed rods with a diameter of ~5 nm *in Cfx. aurantiacus*, but recent cryo-EM images and X-ray diffraction results showed that the pigments form a lamellar phase as shown in Figure 2. It was discovered that the packed rods structures from the freeze fracture was due to the intrinsic curvature of the lamellae [20][25].

1.3.3 Function of chlorosomes

As unique antenna complexes, chlorosomes absorb photons and transfer their energy towards photosynthetic reaction centres. The pigments of the system absorb light from higher energies and transfer the resulting excitation energy through intermediate complexes containing less pigments with decreasing their excited state energies. Excess energy and unwanted side reactions of photoprotective mechanisms also take place in the chlorosome. To understand the chlorosome energy transfer, knowledge of the spectral properties of the aggregated BChls is required [20].

BChl c has an absorption spectrum with maxima around 436 and 668 nm in CCl_4. BChls are known to have two transition bands in the visible region: Soret (B) and Q bands. The Soret band consists of two overlapping bands, B_X and B_Y. The Q band can also be decomposed into two bands, Q_X and Q_y. Due to the dense packing of the BChl c molecules in the aggregate and the alignment of their Qy dipole moments by hydrogen bonding network, strong excitonic coupling between pigments occur [20][25].

A large red-shift of BChls Qy absorption band (up to ~80 nm, ~1,600 $cm^{-1)}$ to ~715 nm (BChl e), ~730 nm (BChl d), or ~745 (BChl c) is generated by aggregation of the BChls. The Soret band dominates the blue-green region of spectra together with the carotenoids between 425 and 525 nm. The changes in the Soret band of BChl e are more emphasized and the excitonic coupling between dipole moments generates a visible splitting of the Soret and a shift of the B_y major part to ~515 nm [26]. Therefore, in the spectral region between 500 and 600 nm that corresponds to the main part of the light that gets to the habitat of this bacteria, absorption coverage is enhanced [20] [27].

1.3.4 Chlorosomes as components of future hybrid photonic devices

So far, a small number of photonic applications that integrate biological compounds have been announced and advertised. The number of attempts to engineer diverse biohybrid applications have however been significantly growing [28].

Organisms such as *Chloroflexus aurantiacus* or the green sulphur bacteria *Chlorobaculum tepidum* possess a particular photosynthetic-antennae, called chlorosomes that can convert solar power into chemical energy in a more efficient way than the other photosynthetic organisms or plants [29]. The photosynthetic process and the chlorosomal structure in

particular have been studied but there was no development of a proper photonic device. The coupling of the photosensitive semiconductor materials with the chlorosomes of photosynthetic bacteria was not successful up to this point. There is a need to enhance the performance of photonic devices throughout the light spectrum and to identify the anomalies in the photosensitivity of biological organisms and develop a technique to minimize or to remove these anomalies [28].

Understanding the mechanism between light and the function of the chlorosomes could be useful in delivering other systems that exhibit the same efficiency when converting the solar energy. For example, increasing the efficiency of photovoltaic solar energy conversion could be very appealing for the future applications [29]

2. Aims of the thesis

1.Maintenance and medium-scale cultivation of pure cultures of thermophilic photosynthetic green nonculture bacterium *Chloroflexus aurantiacus*

2.Optimization of cell disruption, chlorosome extraction

3.Optimization of purification of chlorosomes, spectroscopic characterization

4.Immobilization and imaging of chlorosomes using AFM

3. Materials and Methods

3.1 Overview

The *Chloroflexus aurantiacus* (Pierson and Castenholz.1974 [30]) cells were initially collected by David Kaftan in hot springs of Rupite, Bulgaria (Strunecký et al. 2019 [31])

The chlorosome isolation and purification was carried out at the Institute of Chemistry of the Faculty of Science, University of South Bohemia in České Budějovice.

Chloroflexus cells were grown under continuous light conditions in a biological incubator. The cell cultures were pelleted by centrifugation and ruptured through a pressure cell homogenizer. Sucrose gradient preparation and sample separation, absorption spectra measurements, size exclusion chromatography analysis, transmission electron and atomic force microscopy imaging were performed.

Transmission electron microscopy images were provided by Dr. Zdeno Gardian (Laboratory of EM, Biological centre, ASCR and Faculty of Science, University of South Bohemia in České Budějovice).

3.2 Growth of *Chloroflexus* cells

The *Cfx.* cells were grown in 1/2 R_2A medium supplemented by trace element solution SL-4 and vitamin solution. The 1/2 R_2A medium and the solutions were prepared according to the recipes described below:

Table I: Liquid medium (1/2 R_2A) recipe

Yeast extract	0.50 g
Casamino acids	0.50 g
Proteose Peptone	0.50 g
Glucose	0.50 g
Soluble starch	0.50 g
Na pyruvate	0.30 g
K_2HPO_4	0.30 g
$MgSO_4$ x 7 H_2O	0.05 g
Agar	15.00 g
Distilled water	2000.00 mL

Table II: SL-6 stock solution recipe

$MnCl_2$ x 4 H_2O	0.03 g
$ZnSO_4$ x 7 H_2O	0.10 g
H_3BO_3	0.30 g
$CoCl_2$ x 6 H_2O	0.20 g
$CuCl_2$ x 2 H_2O	0.01 g
$NiCl_2$ x 6 H_2O	0.02 g
Na_2MoO_4 x 2 H_2O	0.03 g
Distilled water	1000.00 mL

Table III: SL-4 stock solution recipe

EDTA	0.50 g
$FeSO_4$ x 7 H_2O	0.20 g
SL-6	100.00 mL
Distilled water	900.00 mL

Table IV: Vitamin stock solution recipe

p-Aminobenzoate	1.0 mg
Biotin	0.2 mg
Nicotinic acid	2.0 mg
Thiamine- HCl x 2 H_2O	1.0 mg
Ca-pantothenate	0.5 mg
Pyridoxamine	5.0 mg
Vitamin B_{12}	2.0 mg
Distilled water	100.0 mL

After preparation, 10 mL of the SL-4 stock solution and 2.5 mL of the vitamin stock solution were added to the 1/2 R_2A solution. The final medium solution was autoclaved and stored in a cold room for further usage. Melted 1/2 R_2A medium solidified by 1.5% agar was poured onto Petri dishes, which were used for passaging the *Cfx.* cultures.

All culture manipulation was carried out under sterile conditions of a biohazard laminal flow clean bench. Cultures were maintained in Petri dishes on 1/2 R_2A medium solidified by 1.5% agar (w:w) whereas large scale production of cells was carried out in a liquid 1/2 R_2A medium inoculated with cells from agar plates. Light was provided by custom-made LED strips light source emitting blue, red and warm white light.

The emission spectrum measured by handheld spectrometer Spectra Pen (PSI Ltd., Brno, CZ) corresponds to a light intensity of 50 µmol (photons) m^{-2} s^{-1} summed over the range from 300 to 850 nm (Figure 3).

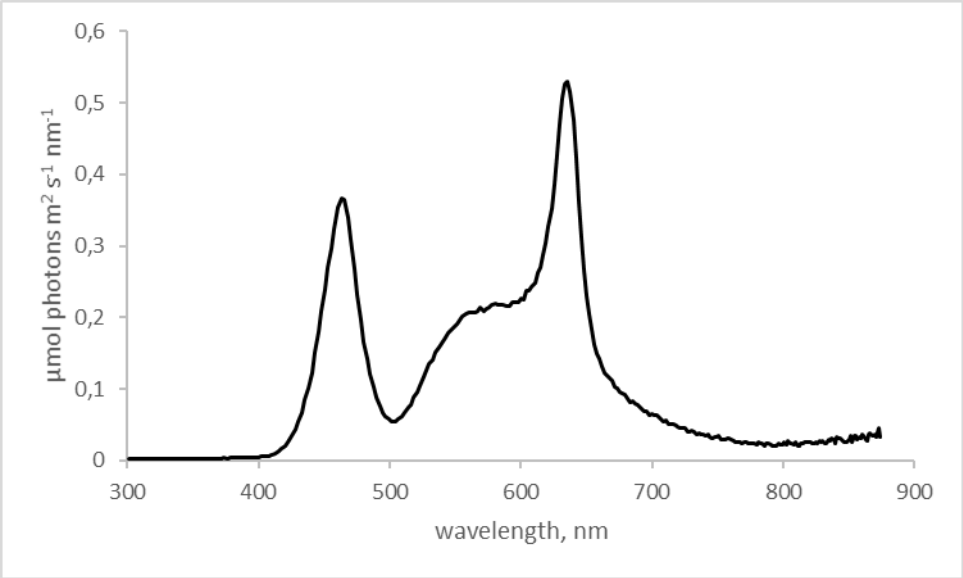

Figure 3. Emission light spectrum inside the growth chamber

New cultures of *Cfx.* cells were initiated every week by passaging the cells from the old agar plates to freshly prepared agar plates using a sterile metal inoculation loop. The new passaged agar plates were sealed with parafilm, stored in an incubator at 50°C and exposed to continuous medium light conditions of 3-50 µmol (photons) m^{-2} s^{-1}.

The *Cfx.* cells collected from agar plates using a plastic spatula were transferred into a glass flask containing the liquid 1/2 R_2A medium and a Teflon coated magnetic stirring fish. The cells in a liquid medium were grown in the incubator at 55 °C under continuous light of 100 µmol (photons) m^{-2} s^{-1}. Gas exchange inside the culture flask was provided by gentle air bubbling by air filtered through sterile filter (0.20 µm pore size) and by slow stirring (<100 rpm). Fresh liquid medium was added every week or every two weeks depending on the rate of liquid evaporation. Increased chlorosome production was induced by transferring the cell culture from aerobic condition provided by continuous aeration into anaerobic condition. Oxygen was removed from the culture by bubbling the cell culture with sterile nitrogen gas and closing the culture flask with a stopper leaving a minimal gas volume. Anaerobic conditions were initiated 1 week prior the harvest of the cells for chlorosome isolation.

3.3 Extraction of the pigments

Pigments were extracted from *Chloroflexus* cells by dilution in 50 mM Tris buffer (pH 8) containing acetone and methanol. The components of the extraction buffer were added in the following ratios(v/v/v): acetone: MeOH: Tris –> 7: 2: 1.

Approximately 50 mL of the *Cfx.* cells contained in the liquid medium were poured in a 50 mL plastic tube and spun down by centrifugation at 14.000 rpm for 10 min at 4°C. The supernatant was removed and the resulting pellet was transferred to 2 mL plastic tube and vortexed. Afterwards, 1 mL of the extraction buffer and 1 mL of the vortexed pellet were mixed in a 2 mL plastic tube and centrifuged at 14.000 rpm for 5 min at 4°C. The resulting pellet was mixed with approximately 100 µL of glass beads (100 µm in diameter) and 1 mL of extraction buffer, vortexed and spun down again for 5 min. Subsequently the resulted pellet was finally extracted by addition of 1 mL of extraction buffer in a new 2 mL tube. The collected supernatants were centrifuged once again (14.000 rpm for 5 min at 4°C) and finally filtered through a syringe filter (0.20 µm pore size). The filtered solution was transferred into an amber glass vials (0.5 mL per vial). The pigment solutions were gently blown by a stream of nitrogen to remove the oxygen and concentrate the solution for minimum of 5 minutes. The samples were then stored in the freezer at -20°C until further analysis.

The pigment extraction procedure for the *Chloroflexus* cells contained in the solid medium was the same with minor changes. The cells were collected with a sterile spatula from the agar plate and transferred to a 2 mL tube and mixed with 1 mL of the extraction buffer, vortexed and spun down by centrifugation. The supernatant was removed and the resulting pellet was mixed with beads and 1 mL of the buffer, vortexed and spun down. Afterwards, the pellet was diluted in 1 mL of buffer, filtered, concentrated with nitrogen and stored in the fridge.

3.4 UV/VIS absorption spectroscopy

Absorption spectroscopy technique was used in this work, in order to determine the identity and quantity of bacteriochlorophyll molecules present in *Cfx. aurantiacus* cells and in the isolated samples of chlorosomes.

UV/VIS absorption spectroscopy uses light in the ultraviolet-visible spectral region to obtain information about an analyte. Before applying light, the analyte is in its lowest energy state or ground state (HOMO). The light causes some of the analyte species to undergo transition to a

higher energy or excited state (LUMO). Every light wavelength corresponds to a certain energy. If the energy has the right amount to promote an electron from the HOMO to the LUMO this particular wavelength will be absorbed. The information about the analyte is acquired by measuring the amount of electromagnetic radiation absorbed as a result of excitation. According to Lambert-Beer's law, absorbance is directly proportional to the number of molecules that absorb radiation at a specified wavelength, the molar extinction coefficient of the analyte specific for the used solvent and a unique wavelength and to the path length of the absorbing medium [32].

Absorption spectra were measured using Lambda 35 spectrophotometer (Perkin Elmer Inc., USA). A fast scan was selected in the range of 400 – 900 nm, spectral slit width set to 1 nm. The extracted pigment samples were diluted with methanol and transferred into the cuvette. The sample volume introduced in the cuvette did not exceed more than 600 μL, usually 500 μL methanol and 100 μL extracted sample, but when the extracted pigment showed a darker color only 20 μL of the extracted sample was used.

3.5 Isolation of chlorosomes

Isolation of chlorosomes is a multi-step procedure. First step was to harvest the *Chloroflexus* cells from the liquid medium by centrifugation and washing. Second step implied homogenizing the harvested pellet using a glass homogenizer, followed by breaking the resulting cell suspension by a French press. The cell interior separated from cell walls and unbroken cells by centrifugation was loaded onto a step sucrose density gradient (first step purification). The density gradients were ultra-centrifuged and fractioned. Chlorosome containing fraction was loaded into another sucrose density gradient (second step purification) and ultra-centrifuged. The resulting fractions were separated, collected and characterized. Chlorosome containing fraction was finally purified by size exclusion chromatography and stored at 4 °C until further analysis.

3.5.1 Cell harvesting

Chloroflexus cells from the incubated liquid medium were transferred to 6 plastic Falcon tubes (50 mL each). This cell suspension was spun down at 4°C for 10 min at 4.500 g.

Afterwards the supernatant was removed from all the 6 tubes and the pellets were combined yielding 5 mL of cells. Breaking buffer (50 M Tris, pH 8, 2 M NaSCN) was used to wash the cells 2 times followed by centrifugation (4°C for 10 min at 4.500 g), supernatant was always discarded. After final wash, cells were diluted to 15 mL of breaking buffer.

3.5.2 Cell homogenization

Cell suspension was transferred into a glass homogenizer. Cells were homogenized by a teflon piston at least 5 times. The homogenized cells were then ruptured by three passages through AO412 pressure cell homogenizer (Stansted Fluid Power Products Ltd., UK) at pressures increasing from 150 to 200 MPa. The main components of the cell homogenizer consist of an air driven high-pressure liquid pump which forces the liquid media containing the cells through a back-pressure valve which regulates the controlled back pressure that controls the breakage degree in the cells. The resulted solution of ruptured cells was centrifuged at 10.000 g for 10 minutes at 4°C to remove unbroken cells and cell walls. The supernatant was stored in the dark on ice only shortly until loading onto of the sucrose gradients.

3.5.3 Density sucrose gradients

Two buffers containing 0% and 50% (w/v) sucrose were prepared and further mixed to obtain different percentages of the sucrose buffer.

Table V: Sucrose buffer formulation

	0 % Sucrose	**50% Sucrose**
50Mm Tris (pH 8)	2.8 g	1.4 g
2M NaSCN	81.7 g	40.9 g
Sucrose	0.0 g	250.0 g
Distilled water	415.5 mL	207.7 mL

The first step gradient was prepared by consecutive sub layering of 4 mL of 15% sucrose buffer (50%:0% 7:17 v:v) by 4 mL of 25% sucrose buffer (50%:0% 1:1 v:v) and by 2 mL of

35% (50%:0% 4:1 v:v) sucrose buffer respectively using a syringe with blunt tip. The step sucrose density gradients were prepared into two 14 mL open top poly-clear centrifuge tubes (Seton, 14x95 mm). Two and half of mL of ruptured cell solution (3.5.2) were added on top of each sucrose gradient. The two tubes were balanced using analytical scales to the precision better than 1 mg and ultra-centrifuged at 40.000 rpm for 16 hours at 4°C using a rotor P40ST-2113 in Himac CP90WX ultracentrifuge (Hitachi, Japan). The sample separated into bands varying in colour was fractioned using Gradient Station ip (Biocomp, Canada). The fractions collected by a fraction collector were characterized spectroscopically. The fraction with highest content of chlorosomes was immediately loaded into a second sucrose density gradient.

The second step gradient was prepared again in the 14 mL plastic tubes by sub layering 2 mL of 0% sucrose buffer by 2 mL of 15% sucrose buffer, 4 mL of 25% sucrose buffer and 4 mL of 35% sucrose buffer. The concentrated sample containing chlorosomes was diluted into 50% sucrose buffer and loaded on the bottom of the density gradient. The tubes were balanced and ultra-centrifuged at 40.000 rpm for 16 hours at 4°C. The sucrose gradients with the separated bands were fractioned and collected in separate tubes. The major bands were characterized by absorption spectroscopy and stored at 4°C in the fridge until further analysis.

3.5.4 Size exclusion chromatography

Size exclusion chromatography (SEC) separates the analysed molecules and their assemblies based on their size. Proteins, enzymes, peptides as well as molecular weight distributions of polymers can be separated by this technique. The stationary phase consists of porous particles with well-defined pore size (molecular sieve). The molecular sieves are also useful for desalting of analysed macromolecules. Molecules larger than the largest pore of the stationary phase cannot pass through the gel and therefore will pass right through the column. Small molecules that can pass through the gel particles will be eluted based on their size (in a decreasing order).

AKTA PURE M2 chromatography system (GE Healthcare, Sweden) fitted with TSKGEL G6000 PWXL 30CMX7.8MM ID (TosoHaas, Japan) size exclusion chromatographic column was used to further purify and desalt the collected bands from the second step gradient. The system was equipped with multiple angle laser light scattering detector (Wyatt Technologies Heleos II) with dynamic light scattering option (DLS) and refractive index detector (Wyatt

Technologies TRex) available in the flow path for precise characterization of mass and hydrodynamic shape of complexes ranging from 1 nm to 500 nm in size.

The chromatographic system and the column were equilibrated for 2 hours in a buffer solution consisting of 20 mM Tris (pH 8) and 50 mM NaCl with a flow rate of 0.1 mL min^{-1}. The buffer was sterile filtered by passing through cellulose acetate filter (0.2µm pore size) and degassed by vacuum and vigorous stirring using magnetic stirrer prior use. The equilibrated system was manually injected with 400 µL of the sample (sample loop had volume of 1 mL). The sample was separated by washing the size exclusion chromatographic column by a flow of 0.5 mL min^{-1} for 40 min. The fraction collection started 20 min after the start (0.5 mL were pooled per fraction). The collected fractions were transferred into sterile amber glass vials, gently blown with sterile nitrogen and stored in the fridge until further analysis.

3.6 Chlorosome imaging

Imaging of the chlorosomes was performed using Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) techniques. AFM imaging was conducted after the chlorosome sample was desalted and purified by SEC. TEM was used to analyse the samples from the second step gradient.

3.6.1 Atomic force microscopy

Atomic Force Microscope (AFM) allows studying surface properties of a material at an atomic scale and can generate nano-structural details and biomechanical properties of biological samples, cells or biomolecules. The AFM provides information about the samples directly in their natural environment without any sample preparation. There are no limitations for sample temperature, chemical composition or the medium type (ambient, special gas or vacuum; aqueous or non-aqueous solutions).

The instrument's principle is based on the measurements of interactions between a probing sharp tip positioned at an end of a spring-like cantilever and the material, which generates attractive or repulsive forces. The tip-sample interactions are monitored by the AFM system through measurements of the cantilever deflection. The amplitude of cantilever bending relates to the force acting on the AFM tip according to the Hook's law. The angle of cantilever

bending is amplified by an optical lever based on a laser beam reflected from a spot at the cantilever near of the AFM tip. The position of the reflected laser beam is registered by a segmented PIN photodiode. A piezoelectric XYZ scanner is used to control spatial coordinates of the AFM cantilever with the tip in respect to the sample surface. The imaging itself is performed by a raster-scanning over the surface while registering the tip-sample interactions. The AFM modes of operation include the contact mode, the non-contact mode and the tapping (intermittent contact) mode.

NanoWizard4 biological AFM system (Bruker Inc., USA) equipped with a manual sample stage and coverslip holder was used for imaging of purified chlorosomes in air and buffer solution at room temperature. Fastscan A cantilevers (Bruker Inc. USA) of nominal spring constant k = 6-6.23 N m^{-1} and resonant frequency $f_{o\ AIR}$ = 270 kHz, $f_{o\ BUFFER}$ = 120 kHz were used for quantitative imaging QI mode. The sample of chlorosomes purified by SEC (3.5.4) was diluted 1000x into a buffer containing 20 mM Tris, pH 7.8 and 0.1 mM $NiCl_2$. One hundred of µL of the diluted sample was applied onto glass cleaned by sonication (10 minutes in 1% SDS, 10 minutes in 50% acetone, 10 minutes in methanol, 10 minutes in ultrapure H_2O) and\or freshly cleaved muscovite mica. The imaging buffer contained 20 mM Tris, pH 8, 50 mM NaCl. All buffers were filtered through syringe filters (0.2 µm pore size) and sterilized by autoclaving.

3.6.2 Transmission electron microscopy

Transmission Electron Microscope (TEM) emits a beam of electrons and images the electrons passing through the thin sample allowing the projection of the sample structure with nanometre resolution. It enables a detailed micro-structural examination of crystal structures, cell sections and protein complexes. TEM makes possible the observation of the biological structures at scales that are unreachable for an optical microscope.

The working principle of TEM is based on the light microscope principle but TEM uses electrons instead of photons. Electron scattering occurs when the electron beam passes through a thin section of the material. A system of electromagnetic lenses concentrates the scattered electrons depending on the operation mode, into an image, spectrum or a diffraction pattern. The image is dark in the regions where electrons do not pass through the sample and the image is brighter where electrons are un-scattered.

The images of the chlorosomes, were obtained by Zdeno Gardian by using a TEM, JEOL 1010 (80 Kv) microscope and the negative staining method.

4. Results

4.1 *Chloroflexus aurantiacus* cultures

Cultures were maintained in Petri dishes on 1/2 R_2A medium solidified by 1.5% agar (w:w) whereas large scale production of cells was carried out in a liquid 1/2 R_2A medium inoculated with cells from agar plates. The agar plates were sealed with parafilm, stored in an incubator at 50°C and exposed to continuous medium light conditions of 3-50 µmol (photons) m^{-2} s^{-1}. The cells in a liquid medium were grown in the incubator at 55 °C under continuous light of 100 µmol (photons) m^{-2} s^{-1}. The image of a Petri dish with 1/2 R_2A agar medium covered with colonies of *Cfx. aurantiacus* can be depicted in the figure below 4(a). The second image 4(b) shows a signal of BChl fluorescence (>850 nm) excited by blue light excitation.

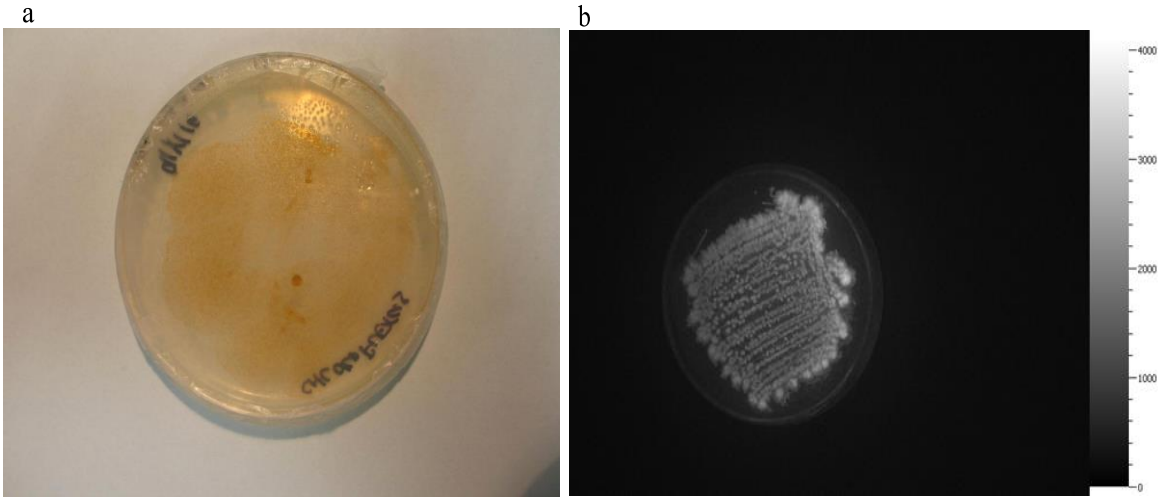

Figure 4. *Chloroflexus aurantiacus* cultures on Petri dishes. Culture under visible light (a) and culture under blue light (b)

Pigments composition of *Chloroflexus* cultures grown in aerobic conditions on the Petri dishes and *Chloroflexus* cultures grown in R_2A medium bubbled with nitrogen were characterized by absorption spectroscopy in the range of 400 – 900 nm *in vivo* in R_2A medium supplemented with 15% glycerol to minimize light scattering, spectral slit width was set to 1 nm.

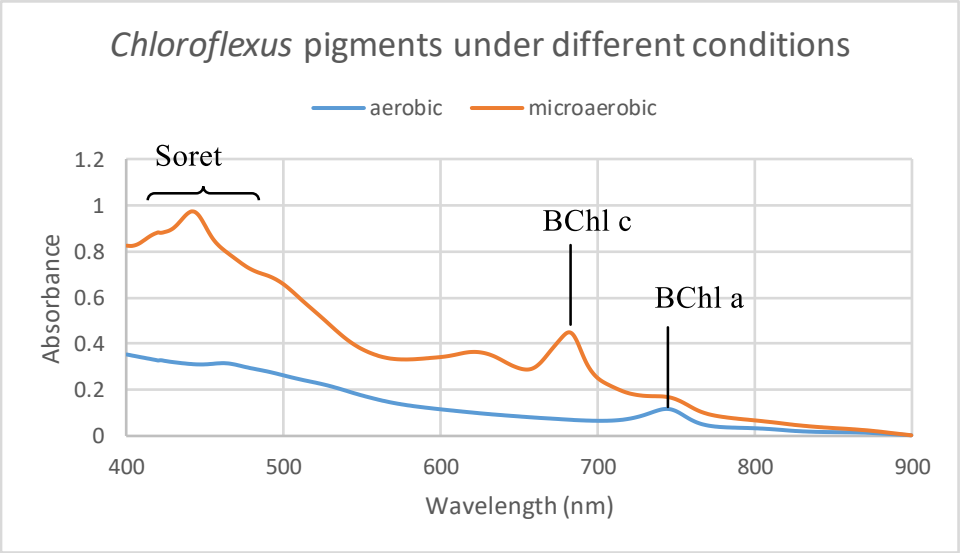

Figure 5. *In vivo* absorption spectrum of *Chloroflexus aurantiacus* cells grown aerobically (blue trace) and micro aerobically (orange trace). Markers point at the position of absorption maxima of BChl a and BChl c.

The absorption spectra of microaerobic conditions exhibited both Bchl c and Bchl a content, while the absorption spectra from aerobic conditions showed just Bchl a. The difference regarding pigment content between the two cultures is a consequence of oxygen partial pressure, temperature and light intensity. The microaerobic cultures were grown in the incubator at 55 °C under continuous light of 100 µmol (photons) m^{-2} s^{-1}, while the aerobic cultures were stored in an incubator at 50°C and exposed to continuous medium light conditions of 3-50 µmol (photons) m^{-2} s^{-1}. Increased light intensity and lower oxygen content led to a higher pigment content in *Chloroflexus* cultures, therefore the microaerobic cultures were used for further analysis.

4.2 Absorption spectroscopy measurements of *Chloroflexus* cells

Absorption spectra of the pigments extracted from *Cfx. aurantiacus* cells grown in liquid 1/2 R_2A medium were measured in the spectrophotometer in the range of 400 – 900 nm in methanol, spectral slit width set to 1 nm. The major absorption bands of carotenoids along with the soret bands of bacteriochlorophylls were found between 400-500 nm. The Q_Y band of BChl c was prominent around 750 nm whereas the Q_Y Bchl a was found at higher wavelengths of 780-820 nm. Absorption spectra of the *Chloroflexus* cells that were broken

using a homogenizer and a French Press was also measured in the range of 400-900 nm in methanol. A difference between the unbroken cells and the broken cells (Figure 6) can be noticed in the absorbance. In the case of the broken cell the absorbance is slightly higher.

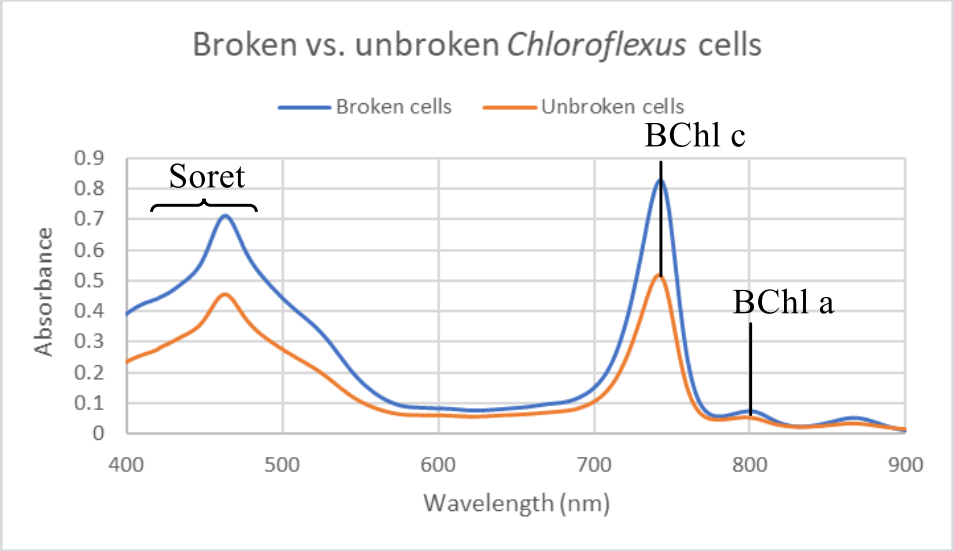

Figure 6. Absorption spectrum of broken and unbroken cells

4.3 Sucrose gradient results

Density sucrose gradients were prepared in order to further purify and separate the chlorosomes from *Cfx. aurantiacus*. The aim was now to analyse their pigment compositions and to determine their structure. The separation was done by sucrose gradient ultracentrifugation. The sample was separated into different coloured bands and further fractioned by a gradient station. The collected fractions were characterized spectroscopically and the fraction with the highest chlorosome (BChl c) composition was loaded afterwards onto a second sucrose density gradient. The 1st step sucrose gradient can be depicted in Figure 6 and it shows 4 different coloured bands. The darkest band was the richest band in chlorosomes, while the lighter ones had a higher content of carotenoids. For the 2nd step gradient, the separation of the bands was better as it can be noticed in Figure 7. The collected bands were also spectroscopically analysed. The chlorosome containing fractions were finally purified by size exclusion chromatography.

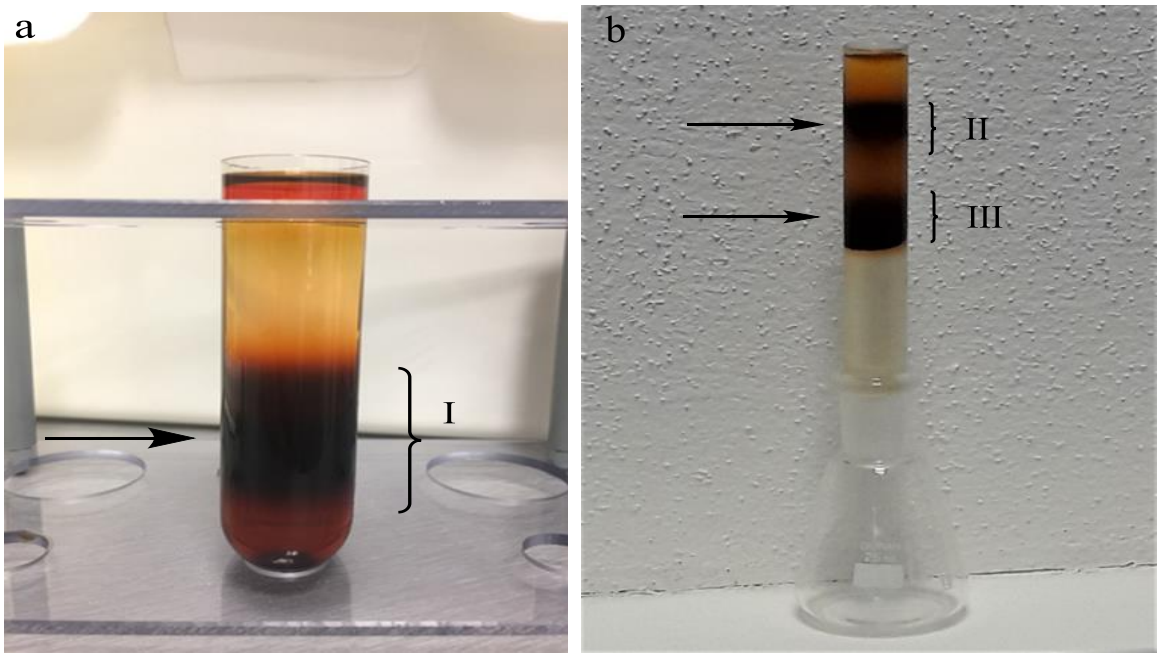

Figure 7. 1st step sucrose gradient (a) and 2nd step sucrose gradient (b). The marked bands were analysed by absorption spectroscopy

4.3.1 Absorption spectroscopy measurements of the gradient fractions

The content of the darkest band of the 1st step sucrose gradient was collected in several fractions. The absorption spectroscopy measurements of each collected fraction are summarized in the figure below:

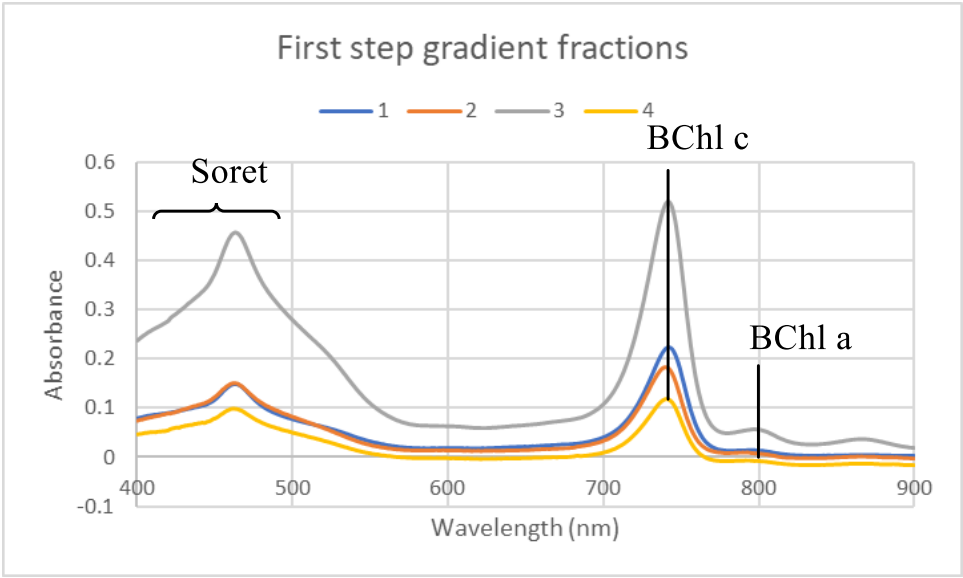

Figure 8. Spectroscopic measurements of 1st step gradient fractions

Fraction 2 and 4 were quite similar with a small difference in absorbance. However, there was a big difference between fractions 1,2,4 and fraction 3. The absorbance level of fraction 3 is higher, therefore the content of the pigments found in the chlorosomes from fraction 3 was higher.

The spectroscopic measurements of the collected fractions from the bands of the 2nd step sucrose gradient are summarized in the following figure:

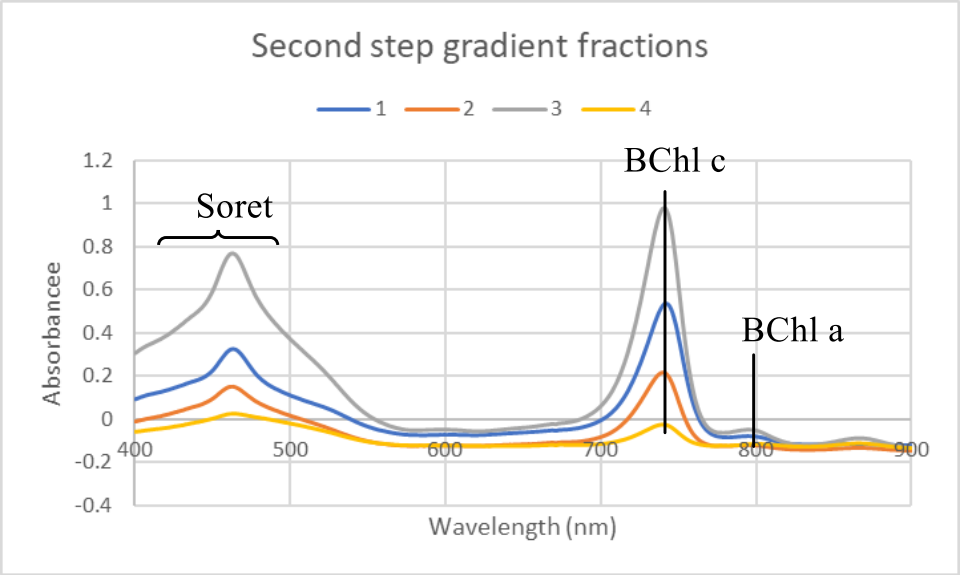

Figure 9. Spectroscopic measurements of 2nd step gradient fractions

There are differences in the absorbance among all the samples, with fraction 1 and 3 showing the highest content of pigments. Second step gradient fractions showed overall a better absorbance level than the first step gradient fractions indicating an efficient purification in the second step gradient.

4.3.2 Size exclusion chromatography analysis

Based on the data obtained from the analysis of the fractions from the 2nd step sucrose gradient, one fraction was chosen for final purification by size exclusion chromatography. The data obtained are shown in the following figure:

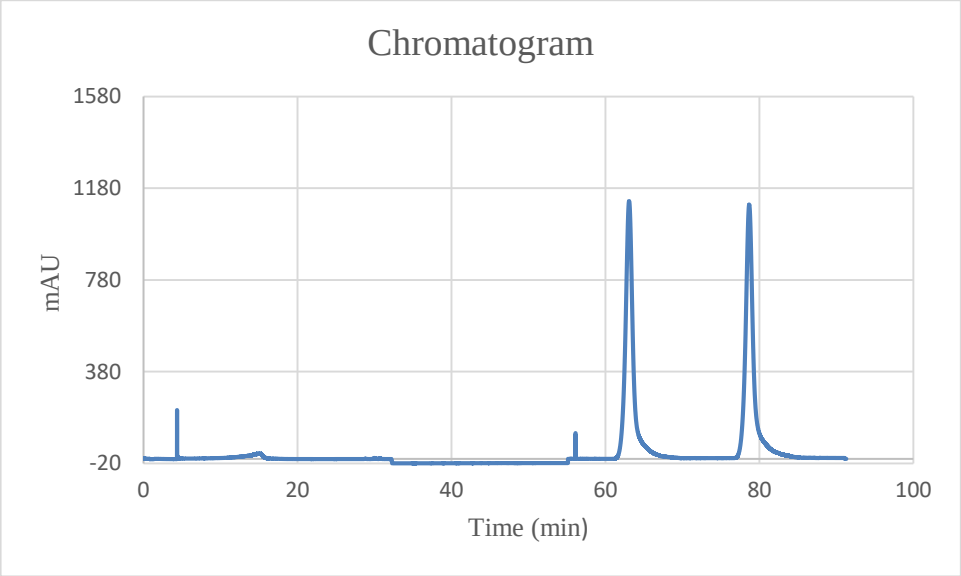

Figure 10. SEC analysis of chlorosomes at 463 nm

SEC was performed in order to collect unified fractions of chlorosomes based on their size, meaning to separate small broken chlorosomes from the complete ones. After washing the column with the running buffer for cca 55 minutes the sample was injected onto the column and eluted while the fraction collection was initiated. Once the first sample was washed out, the second one was injected onto the column and the fractions were again collected. The peak fraction was selected for imaging by AFM and TEM.

4.4 Chlorosome Imaging by TEM and AFM

Chlorosome imaging was done in order to visualize the chlorosomal ultrastructure. Chlorosomes of *Cfx. aurantiacus* imaged using TEM seemed to display elongated, ellipsoidal body shapes with a smooth surface as it can be depicted in the figure below:

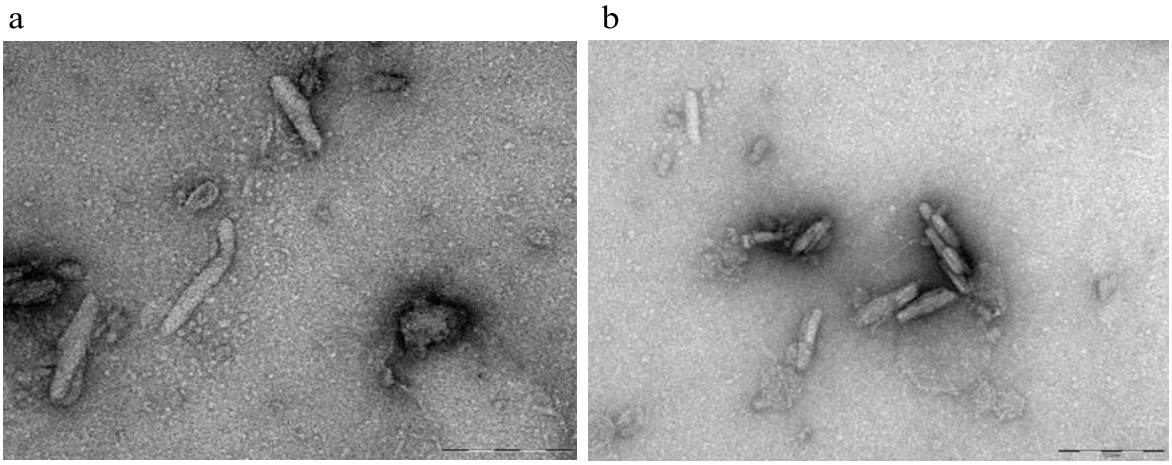

Figure 11. Chlorosome images by TEM. Closer view of the separate chlorosome bodies (a), overlapped chlorosome bodies (b)

AFM images of chlorosomes imaged on freshly peeled mica in buffer 20 mM Tris pH 8, 50 mM NaCl using a cantilever fast scan-A having spring constant of k = 6.23 N/m and a resonant frequency of fo = 120.7 kHz are shown in the figure below:

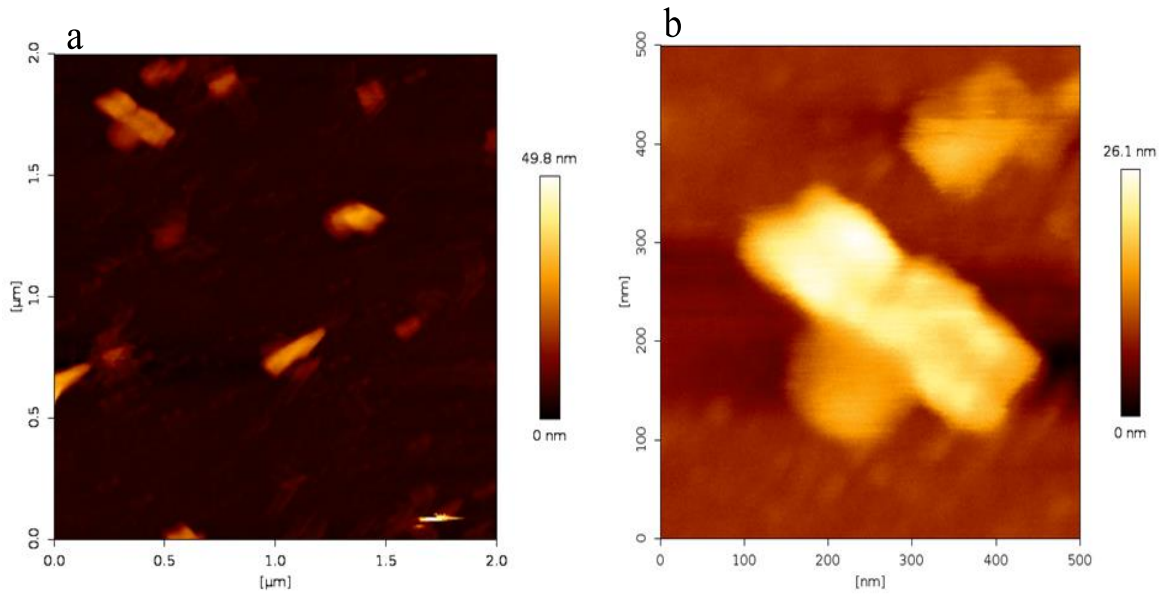

Figure 12. Chlorosome imaging by AFM. Multiple chlorosome bodies (a), individual chlorosome body (b)

AFM images showed that the chlorosomes have an oblong body form of variable size, therefore each chlorosome has an individual size or shape. Overall the obtained chlorosome dimensions were estimated around 150-180 nm long, 25-35 nm wide, 15-25 nm high.

5. Discussion

Compared to other photosynthetic light harvesting complexes, chlorosomes represent unique antenna complexes and their main pigment (bacteriochlorophylls) are built in aggregate forms making the chlorosome the largest light harvesting complex known to date. Chlorosomes structures of *Chloroflexus aurantiacus* and their pigments were studied in this work.

In the initial part of the work *Cfx.aurantiacus* cultures were grown on Petri dishes in 1/2 R_2A medium solidified by 1.5% agar and then new cultures were inoculated with cells from agar plates in a liquid 1/2 R_2A medium which were further grown under aerobic and anaerobic conditions. Pigment analysis of cells grown in anaerobic conditions, showed both BChl a and BChl c content in their absorption spectra. The cells grown under aerobic conditions showed BChl a content, contrary to the previous research stating that the *Chloroflexus* cells lack BChl a, BChl c, and chlorosomes under aerobic conditions and start to synthesize BChl a and BChl c just when shifted to semi aerobic conditions [33][34]. However, it is not clear what generated the presence of BChl a, therefore more analysis are required to further explain this matter.

The pigments extracted from broken cells of *Chloroflexus* exhibited a higher absorption level than the unbroken ones. This is due to a higher yield of pigment extraction in the broken cell sample, due to rupturing of rigid and thick cell walls making the interior of the cells more accessible for the extraction solvents and making the extraction process more efficient.

The purified chlorosome fractions of *Chloroflexus* cells from the 1^{st} and 2^{nd} step gradient showed similar absorption spectra. Some differences were noticed in the absorbance that was influenced by the pigment intensity present in each fraction. Overall, the peak of BChl c was obtained around 742 nm, and the BChl a peak at 796 nm. According to the previous literature [33], dialysis was performed on the chlorosomes obtained from the 1^{st} step sucrose gradient against 0.01 M Tris buffer (pH 8.0), and afterwards loaded on the 2^{nd} step sucrose gradient. For this work dialysis was not performed and the chlorosomes from the 1^{st} step sucrose gradient were directly loaded on the 2^{nd} step sucrose gradient. In spite of the difference between the used purification methods, the values obtained in this work support the ones obtained in the literature for the purified chlorosome fractions: BChl c peak at 740 nm and BChl a peak at 790 nm [33].

The structure of chlorosomes was first analysed by TEM of negatively stained samples. They appeared as smooth oblong structures of similar dimensions. Previous research of P.G Adams

et al. 2013 [35] compared chlorosomes of different species using the negative stained method. All chlorosomes seemed to have an oblong structure, nearly homogeneous without other unpredictable structure. Nonetheless, the chlorosomes of *Ca.C termophilium* resembled an undulating surface. The chlorosomes of *Cfx. aurantiacus* appeared to be relatively smooth and of similar size [35]. According to the data obtained by other study, it was suggested that the chlorosomes can form different structures in different organism due to the arrangement of bacteriochlorophyll molecules. However, the understanding and the functional differences between the distinct arrangements of the BChls are still unexplained but the structural differences are likely to be associated with differences in the absorption and fluorescence features of chlorosomes from different organisms [36].

The three-dimensional images provided by AFM showed that the chlorosomes of *Cfx. aurantiacus* are ellipsoid bodies that vary in size and shape. Thus, the structure of each chlorosome is unique. The surface of the imaged chlorosomes was not smooth but found to be deformed in certain areas of the chlorosme. Zhu et al. 1995 [37] claimed that the chlorosomes of *Cfx. aurantiacus* were illustrated as smooth bodies by AFM imaging. Similar results were reported by Martinez-Planells et al. 2002 [38] for the chlorosomes isolated from *Cfx. aurantiacus*. However, the chlorosomes from other species like *Chl. phaeobacteroides* and *Chl. vibrioforme* showed a rough surface with protrusions on the entire surface. The two different types of chlorosome structures between the species could be caused either by artefacts produced during isolation of chlorosome or generated by intrinsic discrepancies in the lipid or protein composition of the chlorosome [38]. Another research [39] suggested that AFM could cause deformation of the soft biological samples but Martinez-Planells et al. 2002 [38] reported that these deformations are negligible and that also different arrangements of BChls molecules inside the chlorosomes could lead to development of various chlorosome structures. The obtained values regarding the length of the chlorosomes were quite different when compared to the ones obtained by P.G Adams et al. 2013 [35] (120 nm long). However, the width and the height values yielded a closer match (44 nm wide, 30 nm high).

6. Conclusion

Cultures of *Cfx. aurantiacus* grown under anaerobic conditions contained the higher amount of pigments: BChl a and BChl c, while the ones grown under aerobic conditions showed just BChl a. For this reason, cultures grown under anaerobic conditions were further investigated. Pigments extracted from ruptured cells by the French press exhibited a higher absorbance level when compared to pigments extracted from the unbroken cells, therefore a higher pigment extraction efficiency was achieved after cell wall disruption. Isolation and purification of chlorosomes by a 1^{st} and 2^{nd} step sucrose gradient and by size exclusion chromatography that yielded purified chlorosomes fractions according to the spectroscopic measurements. Chlorosome structure was imaged by TEM and AFM. Both imaging techniques showed ellipsoid structures of chlorosomes varying in shape and size, which were similar to previous research.

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