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The role of the storage alpha glucan in the thermophilic red microalga, *Galdieria sulphuraria*

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used anything other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Abstract

Algae are known as extremely diverse organisms and can be applied as production systems in various biotechnological applications. *Galdieria sulphuraria,* a thermo-acidophilic red microalgae, which belongs to the group of Cyanidiales differs from other algae species in the ability to grow not only auto- (perfoming photosynthesis) and mixotrophically on different carbon sources but also heterotrophically. Under certain conditions *G. sulphuraria* accumulates a storage compound called floridean starch, which shares some structural similarities with glycogen as well with amylopectin and will be degraded under stressful times, like nutrient deficiency, to supply the cell with energy.

The aim of this study was to investigate the degree of branching of floridean starch and if the survival capacity is dependent on the structure of this storage compound. Furthermore, the difference regarding the structure as well as the amount of floridean starch produced was tested by growing microalgae in medium with different types of sugars as a nutrient supply or in different kinds of nutrient deficient media. Floridean starch degradation was then analyzed following its production in *G. sulphuraria* under different growth conditions and on different substrates. Thereafter, a structural characterisation of α-glucan extracted from *G. sulphuraria* was compared to oyster glycogen, the standard used in all experiments. The average chain length was then determined with H-NMR as well as with an adapted debranching assay. Moreover, the monosaccharide composition made by acid hydrolysis as well as the chain length distribution were analyzed by HPAEC-PAC.

Results showed that *G. sulphuraria* degrades floridean starch in times of nutrient starvation. Furthermore, it was demonstrated that floridean starch is a highly branched molecule with an average chain length of around 6 glucose residues. In addition to glucose, at present unknown compounds were also identified, which might origin from impurities or some remaining di- or oligosaccharides of the debranching reaction. It was confirmed within this study that floridean starch is between 60%-80% more branched than oyster glycogen. This high degree of branching, which resembles glycogen more than to starch, could play a decisive role in the survival ability of this algae in long term periods under stressful conditions. Therefore, floridean starch is a potential candidate for an alternative, natural energy supplement for human consumption as well as for environmental friendly livestock farming.

Keywords: Rhodophyta, *G. sulphuraria*, storage polysaccharides, florideanstarch, α-glucan extraction, H-NMR, HPAEC-PAC

Kurzfassung

Algen sind bekannt als eine sehr diverse Gruppe von Organismen mit einem breitgefächerten biotechnologischen Anwendungsbereich. *Galdieria sulphuraria* SAG 108.79, der Stamm dieser Experimente, ist eine thermophile rote Mikroalge und gehört zur Gruppe der Cyanidiales. Das spezielle an *G. sulphuraria* im Vergleich zu anderen Mirkoalgenarten ist, das dieser Algenstamm sowohl auto-(Photosynthese), mixo- aber auch heterotroph auf mehr als 27 verschiedenen Kohlenstoffquellen wachsen kann. Unter heterotrophen Bedingungen bildet diese Mikroalge ein Speicherpolysaccharid, Florideenstärke, welches von der Stuktur sowohl Stärke als auch Glykogen ähnelt und auf welches in Stresssituationen, wie Nährstoffarmut, zurück gegriffen wird um das Energielevel in der Zelle, aufrecht zu halten.

Das Ziel dieser Arbeit war es die Sturktur von Florideenstärke, genauer gesagt, den Verzweigungsgrad und die Zusammensetzung dieses Moleküls zu untersuchen und herauszufinden, ob die Überlebensrate von der Struktur dieses Speicherstoff abhängig ist. Um Unterschiede in der Struktur aber auch in der Menge produzierter Florideenstärke nachzuweisen, wurde *G. sulphuraria* in Medien mit unterschiedlichen Zuckern aber auch unterschiedlichen Konzentrationen davon kultiviert, das Wachstum analysiert und Florideenstärke extrahiert. Des Weiteren wurde die Sturktur von Florideenstärke mit Austerglykogen, dem Standard aller Experiment, verglichen. Die durchschnittliche Kettenlänge wurde mittels H-NMR aber auch mittels geeigneten Debranching-Assay festgestellt. Weiters untersuchte man die Monosaccharidzusammensetzung und die Chainlength-distribution mittels HPAEC-PAC.

Die Resultate zeigten, dass Florideenstärke, unabhängig von der verwendeten Kohlenstoffquelle, bei Nährstoffarmut immer gebildet wird. Weiters zeigte sich, dass dieses Speicherpolysaccharid ein sehr verzweigtes Molekül ist, 60%-80% mehr als Austerglykogen. Die durchschnittliche Kettenlange eines Florideenstärkemoleküls lag bei 6 Glc-Einheiten, jedoch wurden neben Glucose auch andere, bis jetzt unidentifizierte Stoffe im Speicherstoff der Alge nachgewiesen. Diese Stoffe könnten Verunreinigungen aber auch verbleibende Di- oder Oligosaccharide der Entzweigungsreaktion sein. All diese Eigenschaften machen Flordieenstärke zu einem potentiellen Kanditaten als alternatives, natürliches Nahrungsergänzungsmittel sowohl für den Menschen aber auch um eine umweltfreundliche Viehzucht zu garantieren und kann entscheidend für die Überlebensdauer des Organismus sein.

Stichworte: Rhodophyta, *G. sulphuraria*, Speicherpolysaccharide, Florideenstärke, α-Glucanextraktion, H-NMR, HPAEC-PAC

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1. Introduction

Stress, excessive demand and in the worst case, burn-out are among the problems our modern society is faced with. Whether you are a business man, a sportsman or an adrenalin-junkie, they all have one goal: to keep the body`s energy level on the highest stage for as long as possible. Hence, there are several ways to supply the body with energy, like drinking juices that contain sugar or eating a protein bar.

The same effect that protein shakes have on humans, sunlight has on plants or an external carbon source for algae growing under heterotrophic conditions, which is to supply the organism with energy. Once there is an excess of energy in cells, the energy is accumulated in form of storage polysaccharides in the organisms and is released in stressful situations when the body runs out of it. The type of storage polysaccharide differs depending on the organism.

Red microalgae, for instance, produce an energy storage molecule, called floridean starch. Plants and green algae on the other hand, synthesize an α-glucan called starch and animals and bacteria, conversely, produce a storage polysaccharide called glycogen (Shimonaga et al. 2006). The only difference between all storage polysaccharides is the proportion of branching points (α-1,6 linkages). Depending on the algae species, floridean starch resembles either more to glycogen of animals and bacteria or to starch of plants and green algae.

Several studies showed, that precisely the variation in the amount of α-1,6-branches in the molecule could have an effect on the survival capacity during times of starvation, because, the more branched a storage compound is, the slower, its degradation and the longer the cells can maintain their viability (Stadnichuk et al. 2007; Wang and Wise 2011). Exactly this statement made the structure of storage polysaccharides an interesting research topic. Therefore, the goal of this experiment was to figure out what the α-glucan structure of the redmicroalga, *Galdieria sulphuraria,* looks like and if it has any impact on the survival capacity of the organism under nutrient starvation. Moreover, this experiment was carried out to check if different carbon sources (Glucose, galactose, glycerol) used as energy sources under heterotrophic growth conditions, have an effect on the growth as well as on the amount of α-glucan produced in times of energy excess.

2. Theorethical background

Depending on the organism, energy can be stored in different types of storage polysaccharides: Starch for plants and green algae, glycogen for bacteria and animals and floridean starch for red algae (Shimonaga et al. 2006).

2.1. Storage polysaccharides

2.1.1. Starch

Starch, synthesized by green algae and plants, belongs to the group of carbohydrates and is one type of energy storage compounds in cells. This type of polysaccharide consists of two components: amylose and amylopectin. Amylose consists of around 6000 glucose residues that are linear ordered and linked via α-1,4-glycosidic bonds, whereas amylopectin too consists of an α-1,4 linked glucan chain but with regular α-1,6-glycosidic branching points every 24-30 residues, with an average branching level of 6% (Cenci et al. 2014; D'Hulst and Mérida 2010; McDonald 2003; Shimonaga et al. 2006). Starch has a semicrystalline structure and is normally found in form of insoluble granules in the chloroplasts of green algae and higher plants (Viola, Nyvall, and Pedersén 2001).

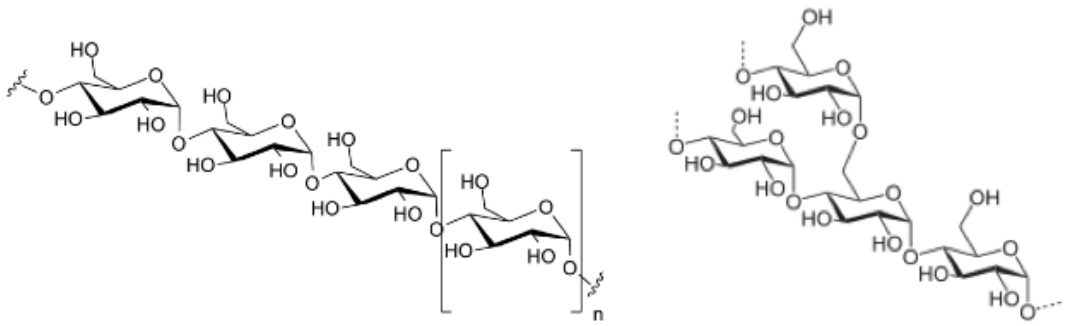

Figure 1: On the left side the structure of amylose and on the right side that of amylopectin are illustrated.[1] Amylose consists of a long glucose-chain with α-1,4 linkages, whereas amylopectin additionally contains α-1,6- branching points.

In plants, four enzymes are necessary to perform starch synthesis and consequently produce amylose and amylopectin: ADP-glucose pyrophosphorylase, starch syntase, branching enzyme and a debraning enzyme.

[1] https://www.tu-braunschweig.de/Medien-DB/agnespockelslab/download/staerke_lehrerinformation.pdf
Last access: 15.06.2015

Amylose originates from a partial degradation of amylopectin. Research shows, that the formation of amylopectin is catalyzed by a sequence of chain-elongating reactions which prior to this, were catalyzed by starch synthase and a debranching reaction carried out by a branching enzyme. These reactions lead to the arboreal structure of the molecule (BeMiller and Whistler 2009; Weiler and Nover 2008).

ADP glucose (ADPGlc) is used as a donor for the chain-elongation-reation. During this reaction glucose monomers are clinged α-1,4-glycosidic to the non-reducing ends of an α-1,4 glucanchain (BeMiller and Whistler 2009).

During the branching reaction, a monomer is released from the non-reducing end and is then linked near the nonreducing end in α-1,6-glycosidic conformation. Because of the α-1,6-glycosidic bond, a branching point is made. This branching point is extended by starch synthase until the next branching reaction takes place (BeMiller and Whistler 2009; Weiler and Nover 2008).

Amylose is made out of amylopectin. This reaction it catalyzed by isoamylase which splits the α-1,6-glycosidic linkages. Small amylose molecules are released and accumulate in the interspace between the amylopectin layers (Weiler and Nover 2008).

- ***Starch degradation***

There are two ways in which amylose and amylopectin, the two components of starch, can be degraded.

1) Phosphorolytic starch degradation via starch phosphorylase
2) Hydrolytic starch degradation via amylases

1) Phosphorolytical starch degradation:

During this type of starch degradation, an enzyme called starch phosphorylase cleaves an α-1,4-glycosdic bonds of the nonreducing ends of amylose as well as those of amylopectin under the inclusion of a phosphate molecule. The resulting products of this reaction, are a D-glucose-1-phosphat and a glucan that is one monomer shorter (BeMiller and Whistler 2009; Weiler and Nover 2008).

Glucose-1-phosphat can be directly released in the metabolic pathway, for instance via glycolysis.

Starch phosphorylase is unable to split the α-1,6-glycosidic linkages. It degrades the starch molecule until an α-1,6-branching point is reached. Exactly at this branching point a boarderdextrin remains. After an enzymatic cleavage of the α-1,6 glycosidic linkage at the periphery of a boarderdextrin, the degradation continues (BeMiller and Whistler 2009; Weiler and Nover 2008).

2) Hydrolytic degradation

This type of starch degradation forms under loss of energy disaccharides with reduced ends.

Three enzymes are responsible for the degradation of starch: α-amlyase, β-amylase and isoamylase (Weiler and Nover 2008).

- α-amylase:
  It is an endoamylase that cleaves internal α-1,4-glycosidic bonds and forms disaccharides. Amylose is cleaved by α-amylase to maltose, whereas amylopectin is broken down into maltose and isomaltose. Isomaltose, an isomere of maltose, originates from the α-1,6-glycosidic branching points, where both glucose molecules are linked via an α-1,6-glycosidic bond. Consequently, maltose as well as isomaltose hydrolyse and form two glucose molecules which can enter the metabolism without any further degradation and can be used as an energy source (Weiler and Nover 2008).
- β-amylase:
  This type of enzyme is an exoenzyme due of the fact that it separates maltose units from the non-reducing ends of amylose and amylopectin. Amylose can be degraded to glucose without any problem. Amylopectin can be degraded until a boarderdextrin at an α-1,6-branching point is reached. The α-1,6-glycosidic bond is then hydrolysed by isoamylase to form glucose (Weiler and Nover 2008).

2.1.2 Glycogen

Glycogen is a polymer and the most common storage polysaccharide for carbohydrates, which is found in the cell cytoplasm. As illustrated in figure 2, glycogen, also a type of an α-glucan, consists of a linear chain of α-1,4-linked glc residues, but with a high number of α-1,6-glycosidic branching points (Manners 1991; Preiss 1984; Viola, Nyvall, and Pedersén 2001) after every 8 to 14 residues (Voet et al. 2010,[2],McDonald 2003). Precisely this randomely branched sturcture makes this molecule a more flexible energy reserve compared to other storage compounds (Viola, Nyvall, and Pedersén 2001; Wang and Wise 2011).

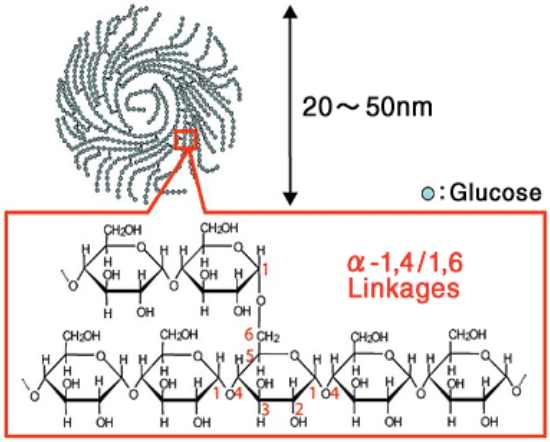

Figure 2: Structural setup of a glycogen molecule with a size between 20-50 nm. This illustration shows also the α-1,4, linked glucan chain with α-1,6 branching points (Viola, Nyvall, and Pedersén 2001).

This type of α-glucan is found in the cytoplasm of bacteria, yeast, fungi, and animals (Viola, Nyvall, and Pedersén 2001). It is an energy source that can be mobilized for ATP production in times of need.

However, Wand and Wise (2011) stated, that the rate of degradation could be influenced by a higher number of branching points. They propose that the shorter the chain length, the slower the degradation. Furthermore, they stated that the bacterial viablity is due to a slow utilization of glycogen. Therefore it can be said, that the slower the glycogen breakdown rate, the longer the survival time is in times of nutrient starvation (Wang and Wise 2011).

[2] http://www.glico.co.jp/en/material/bioglycogen/ Last access: 24.06.2015

- ***Glycogen synthesis***

Glucose is first converted into glucose-6-phosphate before it is further metabolized into glucose-1-phosphate. Glc-1-P is incorporated by glycogensynthase to the non-reducing ends of an already existing glucose-glycogen chain. This process releases energy. Consequently, a branching enzyme inserts α-1,6-glycosidic branching points in the molecule, which gives the structure of glycogen a spheric shape, the bigger the molecules become (Hallbach 2006).

- ***Glycogen degradation***

The degradation of glycogen, also called glycogenolysis, is activated by adrenalin, glucagon but also by hormones or by a change in the blood glucose level. Before the degradation of glycogen can be carried out, the enzyme responsible for this process needs to be converted into its active form. Depending on the site of action, different cofactors are needed to activate these enzymes (Jungermann and Möhler 2013).

In muscles, Ca^{2+} stimulates the phosphorylase-kinase, which then activates the phosphorylase to start the degradation. The second cofactor that can start the glycongenolysis in muscle cells is cAMP, which binds to phosphorylase kinase-kinase and as a result activates the phosphorylase kinase, that until then cannot stimulate the phosporylase to start the process (Jungermann and Möhler 2013).

In the liver only cAMP is responsible for the activation of the glycogenolysis. This can be controlled by the amount of arendalin released in times of stess, but also by glucagon levels in releaxed times (Jungermann and Möhler 2013).

In general, it can be said, that 3 enzymes are responsible for the degradation of glycogen to glucose:

- Glycogen phosporylase
- Debranching enzyme
- Phophoglucomutase (Voet et al. 2010)

In the first step, after the activation of the phosphorylase and concurrency of the deactivation of the glycogen synthase, which is responsible for the glycogen synthesis, the glycogenphoshorylase, which is linked to glycogen molecules, cleaves

α-1,4-glycosidic linkages and forms Glc-1-P. This process is repeated until the glycogenchains consist of four glc residues among two α-1,6 branching points (Hallbach 2006; Voet et al. 2010).

Thereafter, the oligo-1,4-1,4-glucantransferase transfers a trisaccharide of the α-1,6-glycosidic strain to the α-1,4-glyocosidic glycogen chain. Because of this step, the branching point is uncovered and the amylo-1,6-glucosidase splits off a glucose molecule and glc-1-P can be converted to glc-6-P by phosphoglucomutase (Hallbach 2006; Voet et al. 2010).

Afterwards phosphorlyase shortens the remaining glucose chains and a new branched glycogen molecule can be made (See 2.1.2. Glycogen; Glygogen synthesis) (Hallbach 2006; Jungermann and Möhler 2013).

Also, algae synthesize an α-glucan to store its energy. The storage polysaccharide made from red algae, called floridean starch. It is named after one group of red algae (Floridiophyceae) from which it was isolated first. Depending on the red algae species, this storage molecule resembles either more starch with amylose, amylopectin or glycogen. (Sivak and Jack 1998).

Recent research suggests that the structure of floridean starch is supposed to be more similar to glycogen than to starch produced by green algae or higher plants (Izumo et al. 2007).

2.1.3 Floridean starch

Floridean stach, which is a form of α-glucan and comprises up to 80% of the total cell volume is located in the cytosol of red microalgae. Depending whether this storage compound resembles more glycogen or starch it is stored differently in the cytosol. If it is more similar to starch, it is stored in granules called florisides (Figure 3). (Arad and Yaron 1992; Shimonaga et al. 2006; Viola, Nyvall, and Pedersén 2001).

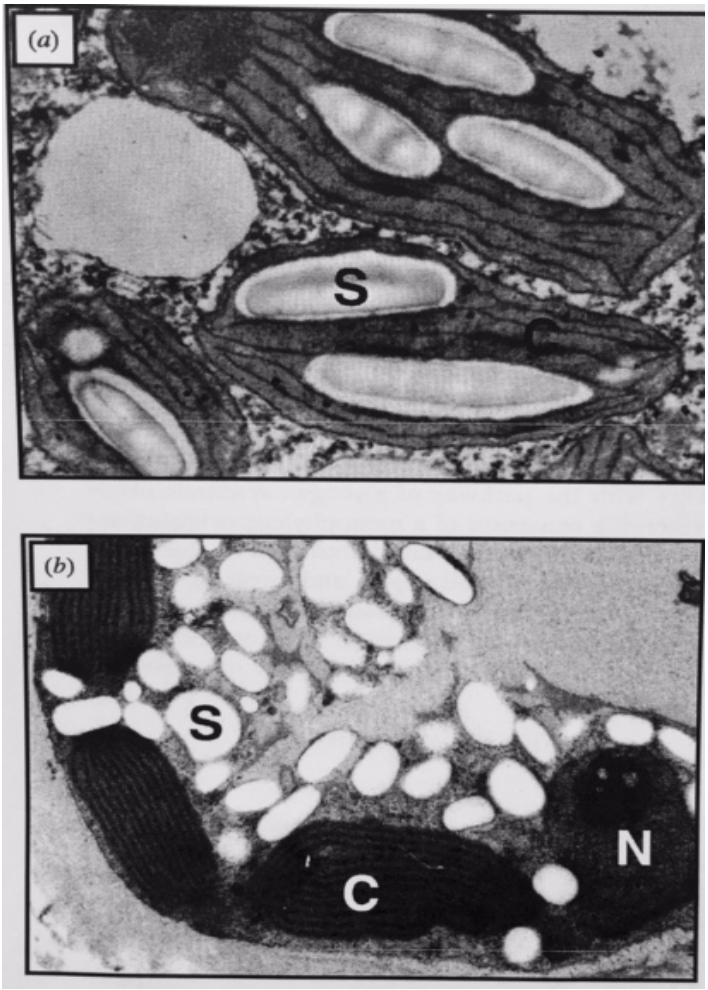

Figure 3: Picture (a) shows the occurence of starch granules in the chloroplast, typically for plants, bacteria, fungi. Picture (b) shows starch granules of redalgae which are located in the cytosol, outside of the chloroplast; S=Starch granula, C=Chloroplast, N=Nucleus (Viola, Nyvall, and Pedersén 2001).

Floridean starch consists of α-D-glucopyranose residues forming an α-1,4 linked chain with α-1,6 branching points. This type of storage compound is produced beside agar out of the fixed carbon (Garab 1998; Horton 2011). Redalgae are the main producers of this starch type. *Galdieria sulphuraria*, the microalgae I focused on during the experiments conducted for this thesis, is able to produce this starch type as well. Floridean starch uses the same precursor as glycogen does for its biosynthesis; UDPGlc. Under heterotrophic conditions *G. sulphuraria* can synthesize

large amounts of this storage poylsaccharide with a higher degree of branching in comparison to other storage polysacchrides like starch or glycogen (Horton 2011).

- ***Floridean starch synthesis:***

The synthesis of floridean starch in red algae takes place in the cytosol. As previously mentioned it differs from the synthesis of starch from plants and green algae by the precursor used in the production. Red algae use UDPglc for the synthesis, because floridean starch lacks amylose. Within the different Cyanidiales species there are some similarities as well as some differences concerning the enyzme. The comparison of *G. sulpuraria* and *C. merola,* two types of mircoalgae*,* carried out by Barbier et al. (2005) showed, that both algae species contain a putative glycogenin, which acts as a primer for the chain elongation reaction and has glycosyltransferase-actvity.

After the synthesis is initiated, chain elongation takes place catalyzed by glycogen synthase using the UDPglc precursor. For the modification of the glycan chain as well as for the implementation of α-1,6 branching points three enzymes are responsible: D-enzyme, α-branching enzyme and α-isoamylase (Barbier, Oesterhelt, and Larson 2005). As a result of the synthesis a highly branched floridean starch molecule is formed.

- ***Floridean starch degradation***

The degradation of the α-1,4 glucan chain is carried out by α-amylase as well as homologs of glycogen phoshorylase resulting in short glucan chains. *G. sulphuraria* possess an additional enzyme, called amylopullianase, which has never been reported in plants just in bacteria. This enzyme is responsible for the hydrolysis of the α-1,4 linked bonds. *G. sulphuraria* also contains a glucan-1,4-α-glucosidase which releases the β-glc units from the reducing end of a glucan chain. Thus, glucose can be used as an energy source in times of need (Barbier et al. 2005).

2.2. Algae

The name algae comes from Latin „*algae*“, which means a diverse class of seaweed and tangle. [3] The first evidence for algal life is a 1.5 billion years old fossil (Figure 4), dating back to the creation of the first eukaryotic organisms (Campbell and Reece 2006a).

Figure 4: A picture of the first algal fossil found 1.5 billion years ago in Somerset Island (Canada) (Campbell and Reece 2006a)**.**

Alge are single- or multicellular eukaryotic phototrophic organisms that can, for one, be found on traditional japanese menus and are additionally very promising organism for scientists, because they can be used in a lot of fields for instance: to kill pathogens, to produce biodiesel and as a nutrient supply. However, they can also be a plague for people with an aquarium as well as for bathers because of their colonisation of swimming pool walls.[3]

Thus, as multifaceted as its applications and developments areas are, as versatile is the size of algae. You can find all sizes within algae, from tiny algae, visible to the naked eye, to a size of several meters (Graziani et al. 2013; Milledge 2011; Spolaore et al. 2006)[3].

Until now, scientists assume that there exist more than 280 000 algae species worldwide, of which only 39 000 are know to date.[3,4] Today, 5000 of the 39 000 algae species are available in algal reference stocks. Only 20 of these species are used for industrial reasons until now.[4]

[3] http://www.planet-wissen.de/natur_technik/pflanzen/algen/ Last access: 26.6.2015
[4] http://subitec.com/de/mikroalgen-zur-kultivierung-in-photobioreaktoren Last access: 26.6.2015

Normally, algae should be green because of the chlorophyll within their cells, but this is not always true. Some algae contain additional pigments like pycocyanin (blue), phycoerythrin (red), which are dominant in the cell and overlap the chlorophyll. Exactely these addidtional pigments not only give the alga its colour, but also its name ([2,5], Arad and Yaron 1992).

Algae are divided into several classes:

- Cynaophyta-Blue green algae
- Crysophyta- Golden algae
- Englenpphyta- Photosynthetic flagelates
- Phaeophyta-Brown algae
- Chlorophyta-Green algae
- Rhodophyta- Red algae, producers of floridean starch[6]

Also the cellular structure can vary within the algae species. The ones where the plastid is surrounded by two membranes, like Rhodophyta, Glyncophyta and Chlorophyta originate from the primary endosymbiosis. During the first endosymbiosis, which occured 1 billion years ago, a cyanobacteria capable for photosynthesis was assimilated into a heterotrophic protist. This conflation resulted in an organell, called plastid, where photosynthesis takes place (Qiu, Yoon, and Bhattacharya 2013).

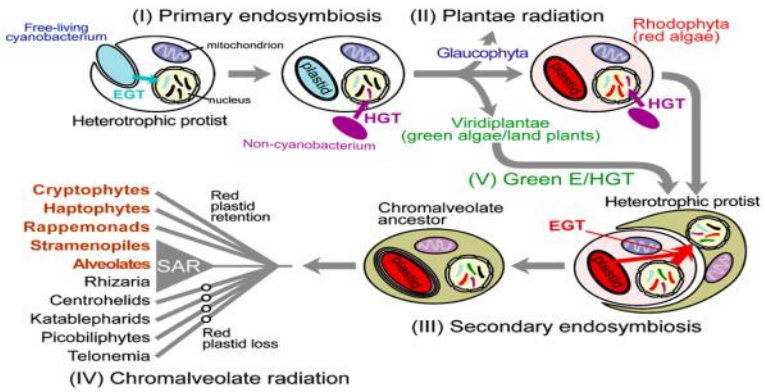

Figure 5: Schemes of the endosymbiosis. As illustrated in the picture, algae originate from the first endosymbiosis. The different species were produced from the second endosymbiosis. (Qiu, Yoon, and Bhattacharya, 2013)

[5] http://www.spektrum.de/lexikon/biologie/algen/2029 Last access: 26.6.2015
[6] http://de.slideshare.net/DithJose/phycology-lecture Last access: 26.6.2015

Algae with a plastid coated with 3 or 4 membranes, where formed during the second endosymbiosis where a plastid-containing eukaryote was gripped from another eukaryotic cell.[4]

2.2.1 Rhodophytae (Red algae)

Rhodophyta, which means red alga, are eukaryotes that are characterised by their photosynthetic pigments. This type of algae contains phycobilins and chlorophyll b as their photosynthetic pigments (Madigan and Martinko 2006a). They contain high amounts of phycoerythrin, which gives the alga its name and colour, as well as smaller amounts of phycocyanin and allophycocyanin which are arranged in phycobilisomes (J. a Raven and Giordano 2014; Seckbach and Chapman 2010). Dependent on the depth of the algae's habitat, the relation of phycoerythrin to chlorophyll can vary. Rhodophytae, living in deep waters are nearly black. This is correlated with a higher amount of phycoerythrin. Algae living at an intermediate depth of water, are of a bright red colour and the ones living in shallow waters, which have the lowest amount of phycoerythrin, are greenish-blue (Campbell and Reece 2006) (Figure 6).

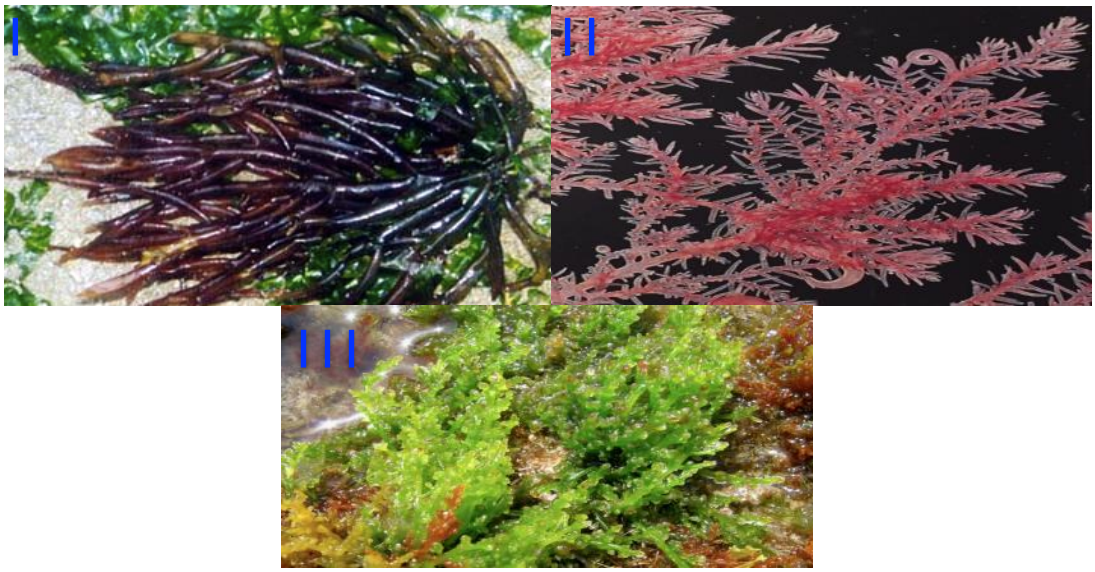

Figure 6: This picture shows 3 types of red algae, found in different water depths. Depending on the depth they live in, algal cells contain different amounts of phycoerythrin. I: *Solieria robusta* with the highest amount of phycoerythin, resulting in a brown nearly black colour[7] II: *Bonnemaisonia hamifera* found in intermediate deep water with a bright red colour.(Campbell and Reece 2006) **III:*Laurencia obtusa*, with the highest amount of chlorphyll, resulting in a dominant green colour and the lowest amount of phycoerythrin.[8]**

[7] http://palaeos.com/eukarya/plantae/plantae.html Last access: 26.6.2015
[8] http://azalas.de/blog/?page_id=264 Last access: 26.6.2015

Exactely these red pigments, which reflect red light and, as a result, blue light that drill deeper water, allow the Rhotophytae to survive in greater depths in comparison to other algae species (Owen and Daintith 2009).

2.2.2 Cyanidiales

Cyanidiales are unicellular red algae that colonize extreme environments, which where diverged about 1.3 billions years ago from the Rhotophyta. (Seckbach and Chapman 2010)

This special algae type is know since the second half of the 19th century when it was supposed to be a single species, know only under the name *Coccochloris orsiniana* (Reeb and Bhattacharya 2010).

In 1933, this algae species was renamed *Cyanidium caldarium*, which was then also officially the first alga know to be separated from Rhodophyta (Moreira et al. 1994).

In the early 1950`s, the demand for a cheap and good protein source arose due to the world`s population increase. At the same time, algae seemed to be a good option to fulfill this goal (Spolaore et al. 2006). Simultaneously, the first experiments, on algae for their use as active substances, for instance as antibiotics as well as studies where this thermophilic organism was used to exchange photosynthetic gas for space travel, were performed (Spolaore et al. 2006).

Years after, a new species, named *Cyanidioschyzon merolae*, emerged with its unique shape and size was found. Almost simultaneously, the species *Galdieria sulphuraria* was introduced. *G. sulphuraria* differs from *Cynidioschyzon merola* in its ability to grow heterotrophically (Reeb and Bhattacharya 2010).

During the energy crisis in the 1970's, algae became more and more important as a source of renewable energy (Spolaore et al. 2006).

In 1991, three new species of *Galdieria* were found in eastern Russia: *G. daedala, G. Maxima, and G. partita*, which can be distinguished by their sizes, shapes and the number of autospores produced (Ree band Bhattacharya 2010).

Finally, in 2007, a new subgroup of *Galdieria* was introduced: *G. phlegrea*. It can only be found in endolithic areas in thermal spots of Italy. Until now the Cyanidiales consists of seven species, but this number is very likely to increase in the following years (Reeb and Bhattacharya 2010).

This acidophilic way of life, which Cyanidiales have, is unique within eukaryotes. Organisms that can grow under exactely these harsh conditions, are called „acidophiles“, due to their high tolerance of acid concentration. For Cyanidiales the optimal growth conditions are found in sulphuric springs, soils as well as in endolithic environments with a pH ranging from 0.5 to 3 and temperatures from 38 to 56 °C (Barbier, Oesterhelt, and Larson 2005; Skorupa et al. 2013; Thangaraj et al. 2011), where they either dwell in hot springs or can be found on rocks, in cracks of rock walls or under rock layers (G. Pinto et al. 2003).

These harsh environmental conditions can, for instance, be found at the Yellow Stone National Park, USA.

2.2.3 Excursus

Yellow Stone National Park

The Yellow Stone National Park (YNP), which is located in the State of Wyoming, in the USA on a high volcanic plateau, astride a region of active major active crustal extension (Fournier 1989).

This unique park has a size of around 9000 km^2 and comprises around 11000 hydrothermal springs (Nordstrom et al. 2005).

These features made the Yellow Stone National Park an attraction (Fournier 1989) for many scientists, inculding Allen and Day (1935), who analysed several water samples and found that the changes in the nutrient water content and individual behavior of the springs are of common appearance in this park and have an impact on the organisms inhabiting it (Rowe, Fournier, and Morey 1973).

It was reported by De Luca et al. (1979), that more than 30% of the hot springs are highly acidic and are all concentrated within in a geothermal area of the park. This acidic environment creates the optimal growing conditions for Cyanidiales and other acidophilic algae types. Due to the fact that hot springs of the Yellow Stone National Park are divided into two types, different algae species can grow here. The first type of hot springs have vertical wallies, which are covered by algae and sprayed with hot vapors. In these kinds of hot springs the pH ranges between 1 to 2.8 with temperatures from 25 to 40 °C. The other type of hot springs found in the YNP, are huge water pools with unicellular algae located at the bottom layer. The pH in these acidic pools is a little higher, with an accurate pH of 3 (De Luca et al. 1979).

Exactly these extreme conditions make the Yellow Stone National Park to a perfect natural habitat for Cyanidiales.

Cyanidiales are separated into 3 groups: *Cyanidium, Cyanidioschyzon* and lastly, *Galderia*, (Barbier, Oesterhelt, and Larson 2005; Seckbach and Chapman 2010; Skorupa et al. 2013), which is the focus of my experiments and which is the only species to depict the ability of growing heterotrophically (Graziani et al. 2013).

Galderia can further be subdivided into two groups (Figure 7) *Galdieria* A and *Galderia* B.

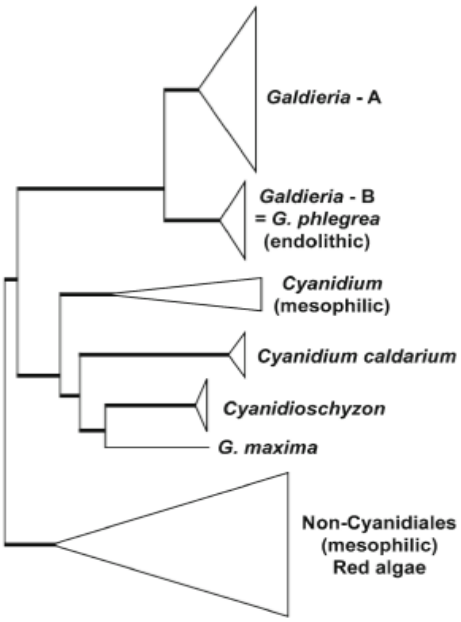

Figure 7: This phylogenetic scheme shows the three major lineages within cyanidiales. Also the seperation of Galdieria in *Galdieria A* and *Galdieria B* is shown (Reeb and Bhattacharya 2010).

The sub-group Galdieria A contains the species: *Galdieria sulphuraria, Galdieria daedala and Galdieria partita*, whereas the sub-group *Galdieria B* consists only of *Galdiera phlegrea* (Reeb and Bhattacharya 2010).

Galdieria A can be found worldwide with geographical preferences within the order of branching of group. *Galdieria B*, which can only be found in Italy, in dry and endolithic environments, where growth occurs at lower temperatures such as 25 °C (Reeb and Bhattacharya 2010).

2.2.4. Galdieria sulphuraria

Galdieria sulphuraria, first described by Merola et al. (1981), is an unicellular, acidophilic and thermophillic red microalgae (Wolfgang Gross and Schnarrenberger 1995; Selvaratnam et al. 2014; Tischendorf et al. 2007) with optimal growth conditions at a pH-level of 3 and a temperature around 40 °C, which can be found for instance in the Yellow Stone National Park (Wolfgang Gross et al. 1998).

These growing conditions make *G. sulphuraria* to a survivalist, which was described as „Alien“ by several professors of the Heine-Heine University of Düsseldorf [1] . *G. sulphuraria* is even able to grow in the form of deep blue green mats (Gross et al. 1998) in acidic waste water of mine cores, where high heavy metal- or even or high saltconcentrations are found as well as in the hot springs of YNP as mentioned above[9] (Figure 8).

Figure 8: An example of *G. sulphuraria* growing in acidophilic conditions in the YNP.[10]

To facilitate the understanding of *G. sulphuraria`s* metabolic flexability, Schönknecht et al. (2013) determinded a genome sequence of 13.7 mb.

For the morphological and structural characterisation of *G. sulphuraria,* David Moreira (1994) analyzed some *G. sulphuraria* species isolated from Rio Tinto.

All *G. sulphuraria* isoltates shared the following characteristics:

- Spherical shape with a size from 5.8 µm to 6.2 µm
- Reproduction via endospores

[9] http://derstandard.at/1363706068670/Mit-Plagiaten-zum-Erfolg-Galdieria-machts-vor
Last access: 26.6.2015
[10] http://beforeitsnews.com/strange/2013/03/creature-from-the-volcano-survives-arsenic-and-heavy-metals-2447656.html Last access:26.6.2015

- They contain several vacoules and chloroplasts
- All *G. sulphuraria* species contain photosynthetic pigments
- The number of autospores was observed to be between 8 to16
- Faculative growth: *G. sulphuraria* is able to grow heterotrophically on a medium supplied of carbohydrates, as well as autotrophically, by performing photosynthesis (Moreira et al. 1994).

2.3. Growth conditions:

A special feature about this algal type is that it is capable to grow under auto-, mixo-, or heterotrophic conditions (Wolfgang Gross and Schnarrenberger 1995; Juneja, Ceballos, and Murthy 2013; Tischendorf et al. 2007). The difference in growth also causes a distinction in the production of photosynthetic pigments. While the ones growing autotrophically have a very deep blue-green colour, *G. sulphuraria* cells of a heterotrophic culture are yellow-green, or „pale“ (Allen 1959).

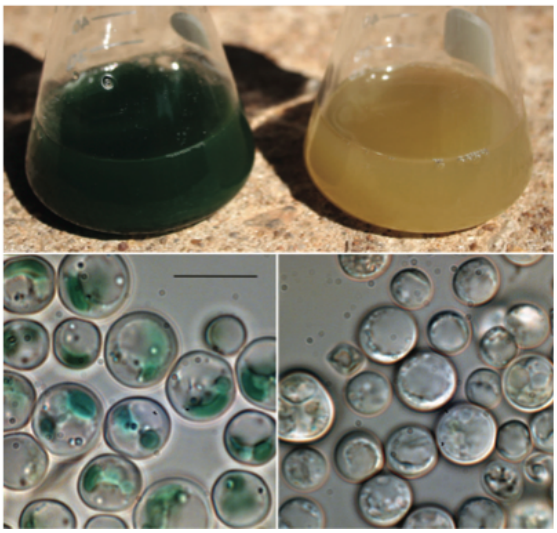

Figure 9: A comparison of *G. sulphuraria* cells growing hetero- or photoautotrophically. Cells growing heterotrophically appear pale and do not have any pigmentation nor chlorophyll production. Photoautotrophically grown cells have a nice blue-green colour, due to the higher amount of chlorophyll in the cells (Schönknecht et al. 2013)**.**

Until today, there is no proven answer as to which of these three conditions dominate in nature (Christine Oesterhelt et al. 2007).

Galdieria can grow on more than 27 different carbon sources, except myoinositol which does not support growth (Wolfgang Gross and Schnarrenberger 1995; C Oesterhelt et al. 2007). This ability was identified in the research of Allen (1952) which showed that *G. sulphuraria,* isolated from a hot sulphuric spring, is able to grow on several mono- and disaccharids.

- **Autotrophic Growth**

The word „autotrophic“ comes from the Greek language: „auto“, meaning “self” and „troph“, referring to the term “nutrition” (Campbell and Reece 2006).

Under autotrophic conditions the chlorophyll containing parts of *G. sulphuraria* use sunlight as their energy source and convert it into chemical energy through photosynthesis.

The reaction equation of the photosynthesis is:

6 CO_2 + 12 H_2O + Light (Energy) → $C_6H_{12}O_6$ + 6 O_2 + 6 H_2O (Madigan and Martinko 2006 b; Moreira et al. 1994)

Nevertheless, one disadvantage of autotrophic growth is, that the energy only comes from light, which is sometimes limited, because of the mutual shading of cells (Chen and Chen 2006).

The reaction equilibrium of the dark reaction: **$C_6H_{12}O_6$ + 6 O_2 → 6 CO_2 + 6 H_2O+ Energy**

- **Heterotrophic growth**

The name heterotrophic also has a Greek origin. „Hetero“ means “different” or “external” and „troph“, again, means nutrition (Campbell and Reece 2006a).

Heterotrophic growth is normally uncommon in algae but *Galdieria sulphuraria* uses exactely this growth conditions as its secret weapon to survive troublesome conditions (Gross et al. 1998), because whenever photosynthesis is limited and an external carbon source is available, *G. sulphuraria* can switch to heterotrophic conditions. Heterotrophy was first defined by Droop (1974) as the usage of organic compounds for growth.

Heterotrophic and autotrophic growth use the same media with one difference that the heterotrophic one contains an additionally carbon source (Perez-Garcia et al. 2011). During hetrotrophic growth we used three different carbon sources, glucose, galactose, or glycerol to replace the availability of light.

G. sulphuraria needs some essential characteristics for heterotophic growth:

- Division and metabolism carried out without light
- Usage of cheap and easily sterilizable media

- Fast adaption to a new environment
- Overlap hydrothernamic stresses (Chen and Chen 2006)

One special thing about the growth of algae under heterotrophic conditions is that some strains, including *G. sulphuraria* loose their pigmentation and become colourless. The bleaching mainly depends on the algae strain and the substrate used to support growth (Wolfgang Gross and Schnarrenberger 1995; Stadnichuk et al. 1998).

Advantages to heterothrophic growth conditions in media:

- No energy in form of light required, therefore reducing cost
- High degree of process controll
- Nearly no contamination due to the acidic media
- Defined inexpensive mineral media
- Cheaper
- Thinner cell walls and, therefore less harsh conditions can be used to break up the cells
- Easier than autotrophic cultivation to maintain on large scale
- Lower harvesting costs because of the greater amount of biomass achieved (Chen and Chen 2006; Perez-Garcia et al. 2011; Tischendorf et al. 2007)

- **Mixotrophic growth**

Mixotrophic is a version of heterotrophic cultivation, where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operate concurrently (Perez-Garcia et al. 2011).

2.4. Growth enhancers and their impacts on algae cells especially in times of starvation:

In this section, substances are described that can have a positiv or negative effect on algal growth, as well as on some metabolic pathways. Generally, it can be said, that if a nutrient limitation occurs in the cell, it is depending on the type of the limiting nutrient and to what degree the nutirent source is restricted (Juneja, Ceballos, and Murthy 2013).

- **Temperature**

For optimal algal growth, it is important to choose the optimal temperature. For *Galdieria sulphuraria* SAG 108.79, it was 40 °C.

Generally speaking, the optimal temperature is probably one of the most important factors to be considered during growth experiments. The wrong temperature can have a negative effect on the algae`s size, growth rate, and biochemical composition. Another effect of low temperature can be the decrease of the fluidity in the cell membrane, which leads to disrupted metabolic activities. Because of the fact that an increase of temperature can also inhibit protein synthesis, this can result in lower growth rates (Juneja, Ceballos, and Murthy 2013). Furthermore, it was reported by Emerson et al. (1944) that the wrong temperature leads to a degradation in the amount of floridean starch produced. This fact was further backed by the research of Nakamura and Miyachi (1983), who reported a reduced starch production of around 17% when algae were grown at 20°C instead of 38°C (Nakamura and Imamura 1983).

- **pH**

pH, similar to temperature, is a keyfactor of optimal algal growth because it is responsible for the amount of CO_2 produced and has an enormous effect on the algae's metabolism. For *G. sulphuraria*, which was used during my experiment the optimal pH was 2. Changes in pH during an experiment, can limit algal growth, due to metabolic inhibition. Either, acidic pH can reduce nutrient intake depending on the algal species used for the experiment, or lead to unimmunisation of heavy metals (Juneja et al. 2013).

- **Heavy metals**

Heavy metals, which are essential compounds of our environments, can have a negative impact on the development of algae depending on the metal and its concentration.

Low concentrations of heavy metals can occur in organic surface waters as a result of atmosperic transport and upwelling. High concentrations, on the other hand appear in coastal waters due to river runoffs (Juneja et al. 2013).

Depending on the algae species heavy metals can have different effects and are accumulated to various degrees (Jordanova et al. 1999; E. Pinto et al. 2003). The photosynthesis of *Chlamydomonas* for instance is inhibited by high metal concentrations (E.Pinto et al. 2003; Juneja et al. 2013).

In general, metals have the ability to cross membranes in two different ways. One mechanism is called molecular mimicry, during which the cell is entered by competing for binding multivalent ion carriers or by connecting to a low molecular weight thiol. In the second process cells are assimilated by endocytosis, whereby metals bind to chelating proteins (Conner and Schmid 2003; E. Pinto et al. 2003).

Considering the bigger picture, a small amount of heavy metals can be accumulated in algae, who pass them on to other organisms like fishes. High concentrations of metals, however, are a real problem for algae. They can damage the cell by an enormous increase of the ROS, which reduces the antioxitance capacity of algae cells (E. Pinto et al. 2003).

In conclusion, it can be said, that heavy metal cannot an do not necessarily have to be avoided. High levels of heavy metal concentratio, however, are very toxic.

- **Nitrogen**

Nitrogen, besides phosphor, is an essential macronutrient for algal growth and represents 7 to 20% of the algae's dry weigth. Nitrogen plays an important role in the protein and nucleic acids production.

Consequently, a nitrogen deficiency results in a reduction of the protein content, a decrease of oxygen evolution, a reduced carbon fixation, a reduced chlorophyll content due to a decrease in the chlorophyll synthesis as well as in a degradation of phycolbilisomes (Juneja, Ceballos, and Murthy 2013).

- **Phosphorus**

Phosporus, which constitues 1% of the algae`s dry weight, too is an important nutrient growth supporter. A deficiency of phosphorus can lead to a reduction of the products received from the citrat cycle, a reduction in the amount of light used for

carbon fixation, a reduction of chlorophyll a, as well a decrease of the intracellular amounts of phycobilisomes (Juneja, Ceballos, and Murthy 2013).

2.5. Carbohydrates used as growth enhancers in the experiment

During the experiment glucose, galactose, and glycerol were used as a carbon source to support the algae's growth under heterotrophic conditions. These three carbon sources were used because it was reported, in the work of Schnarrenberger et al. (1995), that they have the shortest doubling time of about 45 hours, beside mannoe and sucrose. Furthermore Schnarrenberger stated, that adonitol, l-sorbose, L-rhamnose, furctose, 2 desoxy-D-galactose , L-xylose, and meso-erythritol had the slowest growth rates.

Carbohydrates, which have to be supplied to the algal cell, are a very important growth enhancer and are used to generate other molecular compounds, which can be used for respiration, stored as an energy source, as well as act as rare material for the production of new cells.

2.5.1. Glycerol

Glycerol, a sugar alcohol, is a highly reduced carbon source which can easily absorbed into the cell by diffusion and is fragmented into its building blocks (Raghunandan et al. 2014). For a few species, including *G. sulphuraria,* it is possible to absorb glycerol from the medium, which results in an increased growth rate.

Of the intake metabolism of glycerol in algae, little is known until now and research the topic is scarce (Barbier, Oesterhelt, and Larson 2005).

2.5.2. Galactose

Galactose, as well as other hexoses, similar to glucose, are the most prefered carbon source of algae (Kwang 2007).

In comparison to higher plants, to which galactose can be toxic, *G. sulphuraria* possesses the ability to metabolize galatose. It is converted into galactose-1-phoshate and afterwards into UDP galctose via the Leloir Pathway. Thereafter, UDP galactose is epimerized to UDP glucose by UDP galactose isomerase and can be used in different metabolic pathways (Kwang 2007).

One disadvantage of using galactose for supporting heterotrophic growth is, that this monosaccharide is generally less-readily available, when compared to glucose (Perez-Garcia et al. 2011).

2.5.3. Glucose

Glucose, one of the most important carbohydrates nowadays, is a monosaccharide with a molecular formula of $C_6H_{12}O_6$ (P. H. Raven, Evert, and Eichhorn 2000). It is a polyhydroxyaldehyd and belongs to the group of hexoses (6 C Atoms) Because of the fact that the aldehydgroup of glucose can be oxidized to a acidic group, glucose is a reducing molecule. A linkages of two or more monosaccharides creates di-, or oligosaccharides, for example, galatose (Hallbach 2006).

Nowadays, glucose is the most common sugar used to support heterotrophic growth of algae. One big advantage of this carbon source is, that far higher growth and respiration rates can be achieved than with other sugars (Perez-Garcia et al. 2011). As already stated, glucose, galactose and glycerol, which were used as nutrition for *Galdieria sulphuraria* during this expiriment, can be stored in the cell in form of floridean starch until energy sources are needed. In times of lack of energy, these storage polysaccharides are degraded by endoglycosidases to monosacchrides, mostly glucose (Hallbach 2006).

It is important for the glucose concentration of our body to remain constant. This is mainly regulated by the secretion of glucagon and a hormone called insulin. It is necessary that the glucose concentration, especially in the erythrozytes and in the central nervous system (CNS) remain at a high level, because a decrease could have serious impacts on the body, from glitches of the CNS to unconciousness and even death (Hallbach 2006).

2.6. Carbohydrate metabolism

Due to the fact that *G. sulphuraria* is able to grow on more than 27 different sugars without any preference of a particular sugar, this type of microalgae represents an ideal organism to study all enzymes involved in the catabolism of sugars in plants (Wolfgang Gross and Schnarrenberger 1995).

To date, it is known, that *G. sulphuraria* needs sugar kinases and monosaccharide transporters for the intake of external substrates. Furthermore, it needs the help of enzymes to convert sugar into storage compounds and vice versa. Some enzymes that are found in *G. sulphuraira* are unique within the algae species. (Underlined in red figure 14) The produced reserves are then stored in the form of floridean starch until a lack of energy arises. Then these reserves get activated and transported to

the source of need and converted into other substances (Barbier, Oesterhelt, and Larson 2005).

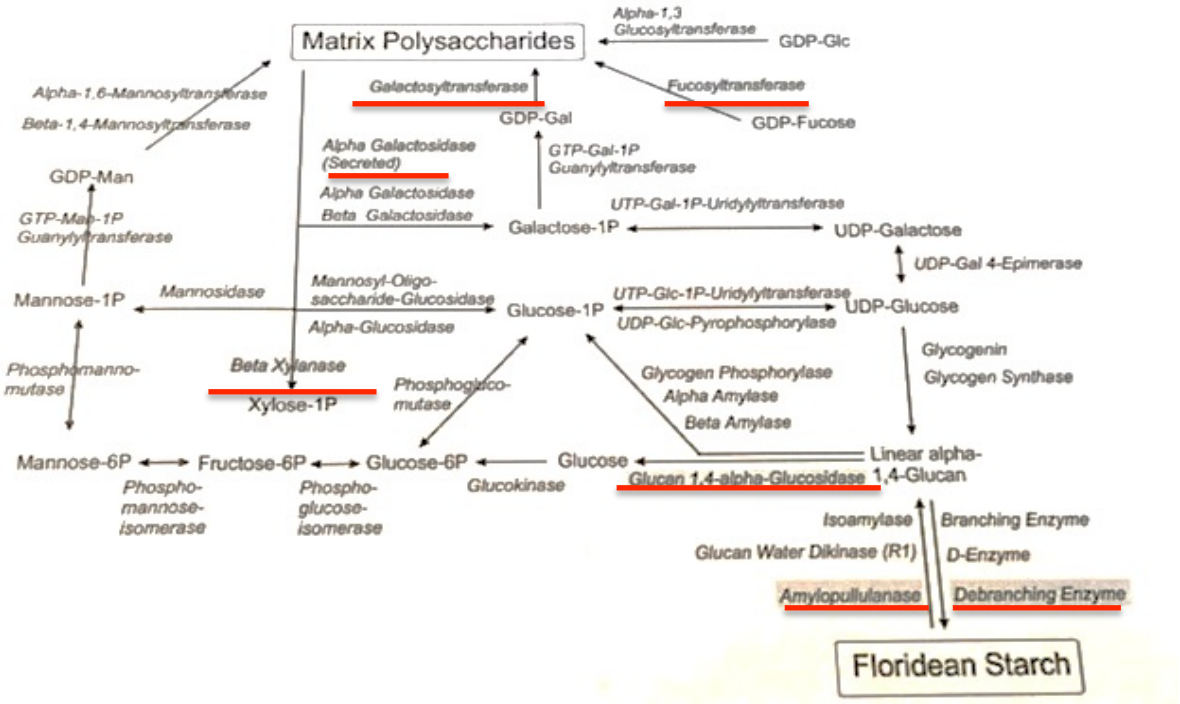

Figure 10: This illustration shows the metabolism of storage polysaccharids in *G. sulphuraria* and C. *merola.* The production as well as the degradation of this storage compound, called floridean starch, is shown with all enzymes involved. Enzymes that are underlined in red are present in *G. sulphuraria* and not in C. merolae

Now, we have come full circle, because precisely when an organism runs out of energy, the energy level needs to be kept high in order to avoid any cell damage. Algae, too, possess this kind of emergency response. In time of stress or lack of carbon sources, *G. sulphuraria* degrades its internal storage reserves, called floridean starch, to supply the cell with energy and, in turn, avoid cell damage or death.

2.7. Research questions

Under heterotrophic growth conditions *G. sulphuraria* gets its energy from additional carbon sources added to the growth medium. In times of an energy excess in cells, these carbon sources (glucose, galactose, glycerol) are converted into a storage polysaccharide named floridean starch. Harsh and stressful situations, like nutrient deficiency, lead to a degradation of these storage compounds in order to supply the cell with energy in form of sugars. As Wang and Wise (2014) and Stadnichuk (2007) stated the survival capacity of the organism is depends on the structure of the storage compound because the more branched the α-glucan is, the slower its degradation and the longer cells are able to survive in harsh conditions.

Due to this fact, the goal of this project was to identify **whether the α-glucan extracted from *G. sulphuraria* was a highly branched molecule?**

Additionally, it was tested if there is a difference in growth, as well as in the amount of α-glucan extracted, by growing *G. sulphuraria* heterotrophically on different carbon sources.

And lastly, whether or not the amount of branches of α-glucan of *G. sulphuraria* has an effect on the survival capacity of cells in times of starvation.

Three different types of experiments were performed, to research if and how different carbon sources (glucose, galactose and glycerol) affect on the growth of *Galdieria sulphuraria*, as well as if α-glucan extracted from *G. sulphuraria* was a highly branched molecule. Another part of this study consisted of testing how *G. sulphuraria* behaved in times of nutrient starvation.

3. Materials and methods

All experiments were performed at the University of Groningen in the research group of Aquatic Biotechnology and Bioproduct Engineering under the leadership of Prof. M.J.E.C. van der Maarel.

3.1. Materials

3.1.1 Strains

All experiments were performed with axenic cultures of *Galdieria sulphuraria* strain 108.79, which was obtained from the algae culture collection (SAG) of the University of Göttingen and was originally isolated from the Wyoming, a hot spring of the Yellow Stone National Park, USA.[11]

3.1.2. Chemicals

Table 1: List of chemicals used during the experiments

Chemicals	Company & headquarter
Aceton	BOOM B.V. (Meppel, Netherlands)
Anthrone	Merk Schuchardt OHG (Hohenbrunn, Germany
$CaCl_2$*H_2O	Sigma-Aldrich (St.Louis, USA)
$CuSO_4$	Sigma-Aldrich (St.Louis, USA)
Difco Agar	BOOM B.V. (Meppel, Netherlands)
DNS	Sigma-Aldrich (St.Louis, USA)
EtOH	BOOM B.V. (Meppel, Netherlands)
$FeCl_3$	Sigma-Aldrich (St. Louis, USA)
Galactose	Sigma-Aldrich (St. Louis, USA)
Glucose	Sigma-Aldrich (St. Louis, USA)
Glycerol	Sigma-Aldrich (St. Louis, USA)
Glycerol (85%)	Merck KGaA (Darmstadt, Germany)
Glycogen (Oyster)	Sigma-Aldrich (St. Louis, USA)
H_2SO_4	Sigma-Aldrich (St.Louis, USA)
H_3BO_3	Sigma-Aldrich (St.Louis, USA)
K_2HPO_4	Sigma-Aldrich (St.Louis, USA)
$KNaC_4H_4O_6$	Sigma-Aldrich (St.Louis, USA)
Mannose	Sigma-Aldrich (St.Louis, USA)
$MgCl_2$	Sigma-Aldrich (St.Louis, USA)
$MgSO_4$*$7H_2O$	Sigma-Aldrich (St.Louis, USA)
$NaCO_3$	Sigma-Aldrich (St.Louis, USA)
Na_2MoO_2*$2H_2O$	Sigma-Aldrich (St.Louis, USA)
Na_2SO_4	Sigma-Aldrich (St.Louis, USA)
$(NH_4)_2SO_4$	Sigma-Aldrich (St.Louis, USA)
NH_4VO_3	Sigma-Aldrich (St.Louis, USA)
Sodium arsenate	Sigma-Aldrich (St.Louis, USA)
$ZnSO_4$	Sigma-Aldrich (St.Louis, USA)

[11] http://www.straininfo.net/strains/821658/browser Last access: 22.2.2015

3.1.3.Water:

- MilliQ water; Purelab flex — Elga Veolia (United Kingdom)
- ddH_2O, Purelab Option S7/15 — Elga Veolia (United Kingdom)
- D_2O — Sigma Aldrich (St.Louis, USA)

3.1.4. Kits

- D-Glucose Assay (GOPOD- Kit, K-GLC 07/11) — Megazyme (Wicklow, Ireland)

3.1.5. Enzymes

- Isoamylase from *Pseudomonas sp*. (10000U/ml) — Megazyme (Wicklow, Ireland)
- Pullulanase from *B.Licheniformis* (10000U/ml) — Megazyme (Wicklow, Ireland)

3.1.6. Buffer for debraching of α-glucan

- 100 mL sodium acetate buffer 200 mM pH=5 → 70 mL 0.2M NaOAc + 30 mL 0.2 M HOAc

3.1.7. Instruments and consumables

Table 2: List of instruments used during the experiments

Instruments	Company & headquarter
Accu BlockTM Digital dry bath	Labnet International, Inc. (Edison, USA)
Algaetron AG 230	Photon Systems Instrument (Brno, Czech Republic)
Analytical balance AG204 Delta Range	Mettler Toledo (Tiel, Netherlands)
Eppifuge 5415C	Eppendorf AG (Hamburg, Germany)
Floor standing incubator	Gallenkamp (Münster, Germany)
Freeze dryer Alpha 2-4 LD plus	Martin Christ GmbH (Osterode am Harz, Germany)
Function Line Labofuge 4000	Heraeus Holding GmbH (Hanau, Germany)
Incubator	Thermo Fisher Scientific (Waltham, USA)
Labo Autoclave	Sanyo (Lier, Belgium)
Labmark T5042	Heraeus Holding GmbH (Hanau, Germany)
Mixer Mill 400	Retsch (Haan, Germany)
NMR	Brunker Biospin (Massachusetts, USA)
pH-meter	Sentron Europe BV (Lend, Netherlands)
Photometer Spectra max Plus 384	Molecular Devices (Sunnyvale, USA)
Photometer DR3900 RFID	Hach Lange GmbH (Düsseldorf, Germany)
SafeFASTlight	Faster S.r.l. (Cornaredo,Italy)

Shaker Infors HT	Labotron Heinz Sewald (Geretsried,Germany)
Sortall Legend X1R centrifuge	Thermo Scientific (Waltham, USA)
Waterbath	Memmert GmbH & Co.KG (Schwabach, Germany)

Table 3: List of consumables used during the experiments

Consumables	Company & Headquarter
Disposable plastic (1,5 mL)	Brand GmbH & Co.KG (Wertheim, Germany)
Glass beads ∅425-600 nm	Sigma-Aldrich (St.Louis, USA)
Needles; metal , ∅ 80x120 mm	B.Braun Vet Care GmbH (Tullingen, Germany)
Sterile Filter; plastic ∅ 25 mm	Carl Roth GmbH & Co.KG (Karlsruhe, Germany)
96-well plater PS-F bottom	Greiner Bio One (Alphen a/d Rijn, Netherlands)

3.1.8.Computer programs and methods

- HPAEC by DIONEX
- Mestrenova

3.1.9. Growth medium (Allen, 1959)

Table 4: Composition of Allen medium, pH=2.0

Ingredients	2L	1L	500 mL	250 mL
Allen Stock 1 $(NH_4)_2SO_4$ [50x]	40 mL	20 mL	10 mL	5 mL
Allen stock 2 $MgSO_4*7H_2O$ [100x]	20 mL	10 mL	5 mL	2,5 mL
Allen stock 3 $CaCl_2*2H_2O$ [100x]	20 mL	10 mL	5 mL	2,5 mL
K_2HPO_4 stock [1000x]	18 mL	9 mL	4,5 mL	2,25 mL
$FeCl_3$ stock [100x]	20 mL	10 mL	5 mL	2,5 mL
Trace elements stock [1000x]	2 mL	1 mL	500 µL	250 µL
H_2O	1880 mL	940 mL	470 mL	235 mL

Table 5: Trace elements stock composition

Compounds	Amount of the trace element stock ingredient [mg]
H_3BO_3	2,8
$MnCl_2$	1,8
$ZnSO_4$	218
$CuSO_4$	50
NH_4VO_3	23

$Na_2MoO4*2H_2O$	24.2
H_2O	1000 mL

After the media preparation, pH was adjusted to 2.0 by adding 4 M H_2SO_4. The culture medium was sterilized in an autoclave (Labo Autoclave, Sanyo) for 20 min at 121 °C.

3.1.9.1. Agar plates

Agar plates were prepared with Allen medium and adjusted to pH 4.0. After the pH adjustment, difco agar [20 g/L] was added and the medium was autoclaved for 20 min at 121°C. (Lab Autoclave, Sanyo)

Thereafter, the liquid agar was poured into petri dishes in the Faster "SafeFASTlight" safety cabinet (SafeFastLight, Faster S.r.l.). Plates were sealed with parafilm to avoid evaporation and contamination and stored at 10 °C upside down in the refrigerator.

3.2.Methods

3.2.1.Stock cultures

Galdieria sulphuraria cells were maintained on agar plates (Allen medium pH=4 + 1,5% (w/v) difco agar) in the incubator at 40 °C (Algaetron AG-230, Photo Systems Instruments). These stock cultures were always used to inoculate liquid pre-cultures.

3.2.2. Liquid pre-cultures

For the pre-cultures, the inoculate was prepared in 100 mL Erlenmeyer flasks containing 50 mL of Allen media with different amounts of carbon sources (glucose, galactose or glycerol) in different concentrations (0.1%, 0.5% and 1%) of the particular carbon supply, and hatched at 100 µE m^{-2} s^{-1}, 40 °C and at 150 rpm in a shaking incubator (Algaetron AG-230, Photo Systems Instruments) for three to four days, unless otherwise defined.

3.2.3. Cultures for analyzes

Cultures for analyses were grown either mixotrophically of heterotrophically.

For mixotrophic growth, the cells were grown in Allen medium with carbon source and were stored in the shaking incubator (Algaetron AG-230, Photo System Instruments) at 100 µE m^{-2} s^{-1}, 40 °C and at 150 rpm.

For heterotrophic growth, cells were grown in flasks wrapped in aluminum foil and were stored in the shaking incubator (Floorstanding incubator, Gallenkamp) 40°C, 150 rpm without light.

Cultures were made either in 250 mL flasks containing 150 mL medium or in 1 L flasks containing 500 mL medium. Cultures were adjusted to an initial OD_{800} of 0.1 by adding preculture to the medium. Flasks were stored, depending on the specific growth conditions needed, in different shaking incubators.

3.2.4. Growth curve determination

Cultures for the determination of the growth curves of *G. sulphuraria,* growing on different carbon sources (glc, gal, gly), were made in 250 mL flasks, containing 150 mL medium with a start OD_{800} of 0.1. The flasks were then stored in the shaking incubator (Algaetron AG-230, Photo System Instruments) for a week under mixotrophic conditions. At particular timepoints (Figure 11), samples were taken and then analysed. The whole experiment was made in triplicates to achieve reliable results. An average as well as the corresponding standard deviation of each point in time was calculated by using the three measured OD_{800} values.

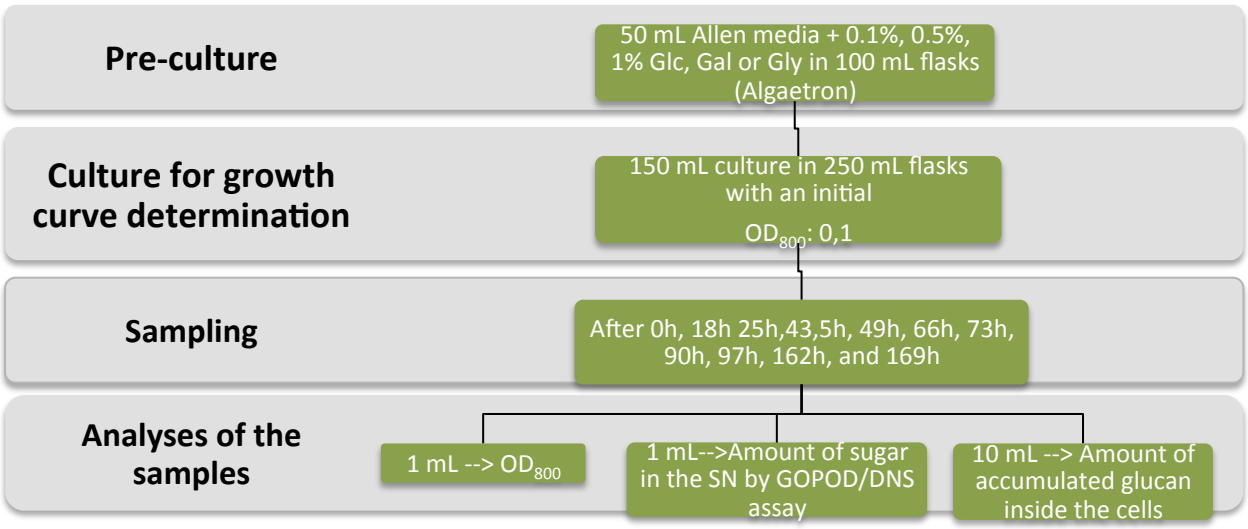

Figure 11: Scheme for the growth curve determination, starting from making a preculture until the analyses of the samples by use of different methods.

3.2.5. Sample analyses

3.2.5.1. Optical-density

Optical density of cells was measured by taking 1 mL liquid culture under sterile conditions and putting it in an Eppendorf capped test tube. The absorption was measured by using standard cuvettes (Brand 1.5 mL) at a wavelength of 800 nm with a spectrophotometer (DR 3900 RFID, Hach Lange GmbH). A blank was always used, containing Allen medium, pH= 2.0, with the same carbon source as well as the same concentration of this carbon source as the originally culture had. If necessary, the samples were diluted with water to get an OD_{800} in a range of 0.2 to 0.9 for more precise results.[12]

3.2.5.2. Freeze drying

Before further analyses could be performed, the sample was freeze dried. After centrifugation, the SN was discarded and the sample was stored in the freezer overnight at -4 °C. The following day, the sample was dried in the freeze dryer (Alpha 2-4 LD plus, Martin Christ GmbH) at -84 °C, 1 bar overnight. Thereafter, the dry weight of the sample was determined by weighing the dried pellet (AG204 delta range, Mettler Toledo).

3.2.5.3. Determination of carbon source in culture media

Depending on the carbon source used as nutrient supply in the media, different assays had to be performed to measure the amount of sugar present in the media over time.

For the cultures growing on glucose, the amount of sugar was determined by using the Glucoseoxidase-peroxidase (GOPOD) or the Dinitrosalicylic colormetric assay (DNS), and for the ones which grew on galactose, only the DNS assay was used.

1. Glucoseoxidase-peroxidase assay in micro plate format (GOPOD assay)(Dauvillée et al. 2005)

This assay, is a colormetric method for the detection of D-Glucose in the samples. For this method the GOPOD megazyme kit (Megazyme) based on the glucose oxidase/peroxide enzymatic procedure was used. [13]

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[12] http://www.laborjournal.de/rubric/methoden/methoden/v36.lasso Last access: 19.7.2015
[13] https://secure.megazyme.com/files/Booklet/R-GLC4_1107_DATA.pdf Last access: 25.2.2015

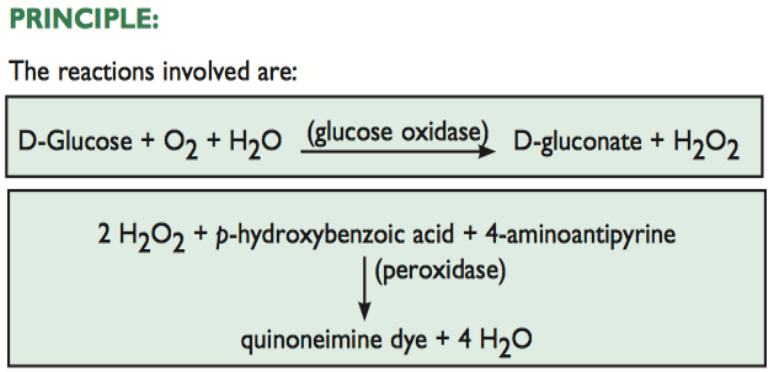

Figure 12: The reactions, which are involved in this colormetric reaction, to measure the amount of reducing sugars in a sample (Booklet for the K-GLUC 07/11 GOPOD test by Megazyme)

For this assay, 1 mL liquid culture of cells growing on glc was taken under sterile conditions. The whole assay was performed in a 96-well plate (PS-F-Bottom, Greiner Bio One) by measuring all samples in duplicates at an absorbance of 510 nm by using the platereader (Molecular Devices, Spectra max Plus). As a standard, different concentrations of glc were used [0 mg/mL, 0.02 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL]. The standards were made out of a 2 mg/mL glc stock, diluted with sterile milliQ water.

50 µL of each sample were pipetted into a well of the 96 well plate. Thereafter, 250 µL of GOPOD reagent were added to the wells containing the samples, and mixed by using an eight channel multi pipette. After that, the plate was sealed and incubated for 30 minutes at 40 °C. (Algaetron AG-230, Photo Systems Instruments) After the incubation periode, the absorbance of the samples was measured at 510 nm. (Molecular Devices, Spectra max Plus). All samples were measured in duplicates.

By absorbance exceedance of > 0.9, the samples were diluted before analysis. The right dilution for the cultures growing on different glucose concentrations are listed in table 6 bellow.

Table 6: Protocol of the right dilutions for samples taken over time, used for the GOPOD assay

Concentration of glc in the medium	Time of sampling	Dilution for GOPOD
0.1% (w/v) Glc	0 – 169.5 h	1 : 20
0.5% (w/v) Glc	0-66 h	1 : 100
	73 h-169.5 h	1 : 20
1% (w/v) Glc	0-66 h	1 : 100
	73 h-169.5 h	1 : 20

2. DNS assay (Miller 1959)

Based on the determination of free carbonyl groups, the reducing sugars[14] were analyzed by this assay. This colormetric method determines the amount of reducing sugars by using a mixture of dinitrosalicylic acid, phenol, rochelle salt, sodium hydoxide as well as sodium bisulfide (Miller 1959).

During the reaction, 3-5-dinitrosalicylicacid reacts with the reducing sugars and forms 3-amino-5-nitrosalicylic acid, which absorbs at a wavelength of 540 nm. During this reaction a colour-change from orange-brown to red can be observed. The intensity of the colour is directly proportional to the concentration of sugar in the solution.[15]

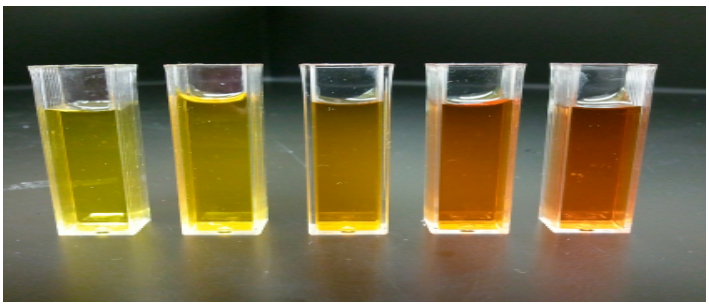

Figure 13: Glc standards in plastic cuvettes, which will be measured at an absorbance of 540 nm. (https://oxytops.wordpress.com/2012/10/23/amylase/)

First of all, a 50 µL sample was added to 50 µL of DNS reagent in a 1.5 mL capped test tube. DNS reagent was prepared by mixing 2 mL of NaOH [2M] with 0.1 g DNS. 5 mL ddH_2O were added to the reagent mixture during stirring. Also 3 g of K-Na tartarte were dissolved in 10 mL ddH_2O. Both reagent components were then mixed together and the reagent was stored wrapped in aluminum foil at -4 °C in the fridge.

Thereafter, the reaction mixture was heated to 100 °C for ten minutes to develop the above mentioned orange-brown color. After cooling the sample down to room-temperature, 900 µL of ddH_2O were added to the reaction mixture, which changed the color to a red. Then, the absorbance was recorded at 540 nm with a spectrophotometer (Photometer DR 3900 RIFD, Hach Lange GmbH). Water was used as a blank. The amount of reducing sugars was then calculated by using a galactose or glucose standard curve [0.0-0.5 mg/mL], depending on the carbon source used for the growth of *G. sulphuraria.*

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[14] http://www.eng.umd.edu/~nsw/ench485/lab4a.htm Last access: 12.2.2015
[15] https://oxytops.wordpress.com/2012/10/23/amylase/ Last access: 12.2.2015

If the OD_{540} was not in the measurable range, samples had to be diluted before performing the experiment. The right dilutions are listed in table 7.

Table 7: Right dilutions for samples taken at different timepoints for performing the DNS assay

Concentration of the sample	Time of sampling	Dilution
0.1% (w/v) Glc/Gal	0-169.5 h	Undiluted
0.5% (w/v) Glc/Gal	0h-19 h	1:10
	24h-43 h	1:5
	50h-72 h	1:2
	>72 h	Undiluted
1% (w/v) Glc/Gal	0-50 h	1:10
	50-73 h	1:5
	>73 h	Undiluted

3.2.5.3. Determination of total amount of intracellular carbohydrates by Anthrone assay (Roman dreywood, 1946)

This assay is the most commonly used and most efficient method for determining the total carbohydrate content of a sample by using sulfuric acid. The Anthrone reagent used for this colormetric method is responsible for giving the sample, containing carbohydrates, its characteristic blue-green color (Kowald and McCormack 1949; Leyva et al. 2008).

Preparation of the Anthrone reagent:

The reagent used for this assay was made of 0.2 % Anthrone diluted in sulfuric acid accoring to the protocol listed in table 8. It was important that the solution had to be prepared the same day as the assay was performed.

Table 8: Preparation protocol for making Anthrone reagent

Solution	175 mL	87.5 mL	43.75 mL	21.875 mL
A	200 mg Anthrone 100 mL H_2SO_4	100 mg Anthrone 50 mL H_2SO_4	50 mg Anthrone 25 mL H_2SO_4	25 mL Anthrone 12.5 mL H_2SO_4
B	60 mL milliQ H_2O 15 mL EtOH	30 mL milliQ H_2O 7.5 mL EtOH	15 mL milliQ H_2O 3.75 mL EtOH	7.5 mL milliQ H_2O 1.875 mL EtOH

Solution A was mixed by hand until Anthrone was completely dissolved in H_2SO_4. Thereafter, the glassbottle was placed on ice. After both solutions were prepared, solution B was carefully and very slowly added to solution A. The bottle was shaken manually, in between the gradual addition of solution B, until it was completely dissolved and had cooled down on ice. It was crucial that the bottle was wrapped in aluminum foil after the cooling step, due to the light sensitive character of the reagent. Then it was stored at room-temperature in a safety cabinet.

Before starting with the Anthrone assay, the 10 mL sample had to be purified and treated.

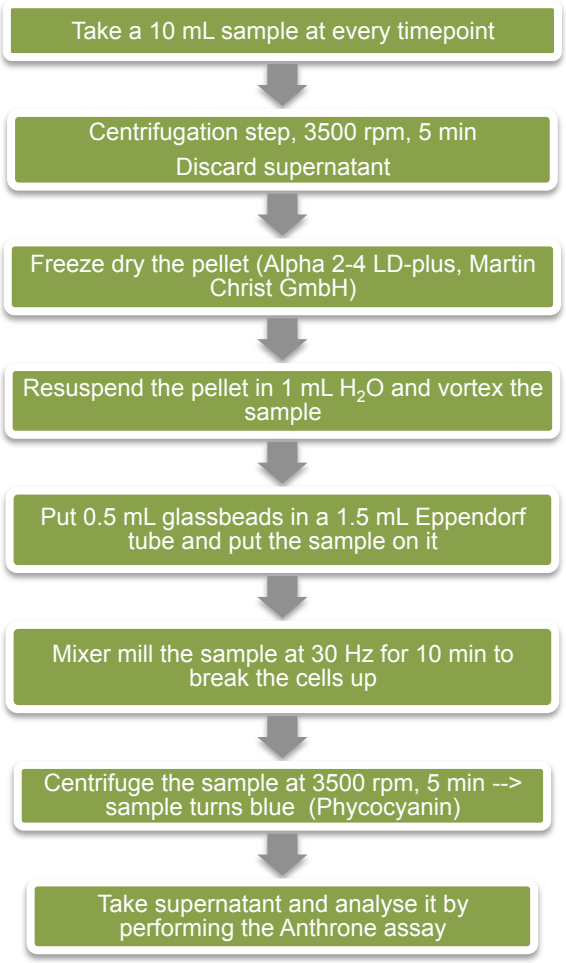


Figure 14: Purification protocol of the samples before performing the Anthrone assay

For the assay itself, 250 µL of sample were put in a glass tube. After that, 2 mL of Anthrone reagent were added to each sample before the samples were vortexed with the lid closed for 10 sec. Thereafter, the samples were incubated for 10 min at 100°C in a water bath. After the 10 minutes, the samples were cooled down to room temperature before the absorbance was measured at 620 nm (Spectrophotometer

DR 3900 RFID). MilliQ water was used as a blank for the photometric measurement. All samples were measured in triplicates.

3.2.6. Alpha glucan extraction

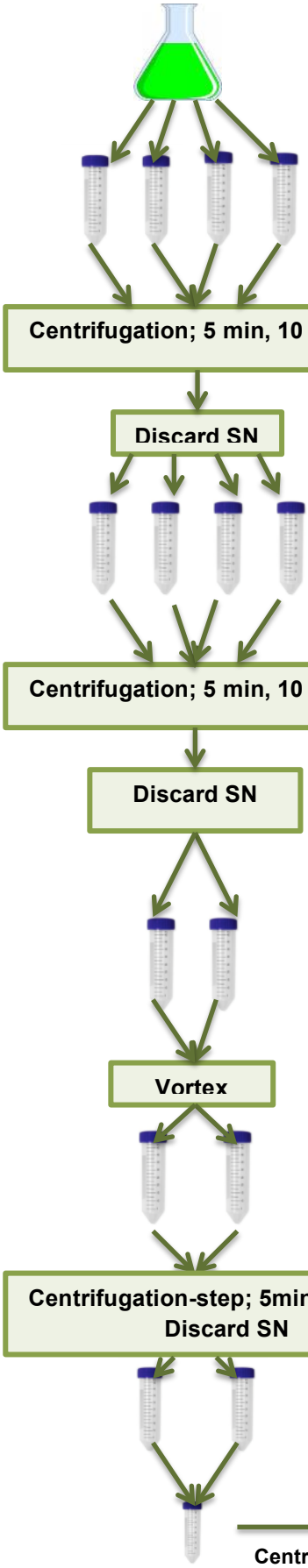
Growth determination of *G. sulphuraria* was conducted on several carbon sources (Glc, gal, gly). Consequently, α-glucan was extracted within the stationary growth phase for further analyses.

For this experiment, pre-cultures, containing 50 mL Allen medium with 1% glc, gal or gly, were made and grew for three to four days, at 40 °C, 150 rpm, heterotrophically.

For the main culture, an inoculate of 500 mL was made at an initial OD_{800}=0.1 in 1 L flasks. All cultures grew for 97 h until the stationary growth phase was reached. At this point in the stationary phase, some carbon sources were still present in the cells.

OD_{800} measurements were conducted after the desired growth was reached. Then, cells were harvested by centrifugation at 10.000 x g, 5 min (Thermo Scientific Sortall Legend X1R Centrifuge) to extract the α-glucan.

3.2.6.1. Harvesting protocol for 500ml cell suspension



Pour 50 ml cellsuspension in each of the 4 falcon tubes

Pour 50ml cellsuspension in the same falcon tube as before

Pour 50 ml cellsuspension in 2 of the 4 tubes

Put the cellsuspension with the dissolved pellet into the other 2

Add 25 mL milliQ-H_2O and vortex the samples to dissolve the pellet and put the suspensions into the final tube→ **IMPORTANT:** Weigh and label the final tube + the lid before pouring the suspension in!!!

END

After the harvesting process, the cell pellets were freeze-dried. Afterwards, the pellets were weighed and resuspended in 25 mL of milliQ H_2O before the cells were broken up in the Mixer mill (Retsch MM400). For the cell disruption, 12.5 mL glass beads (∅ 425-600 nm) and 25 mL cell suspension were combined in a 50 mL Mixer Mill jar (Retsch MM400). The sample was shaken at 30 Hz for 10 minutes. After that, the cell lysate was transferred into a new tube and centrifuged at 4000 rpm for 20 minutes at room temperature in a swinging bucket centrifuge (Function Line Labofuge 400, Heraeus Holding GmbH), to remove the unbroken cells and the cell debris. Thereafter, the supernatant was transferred into a new 50 mL falcon tube and incubated at 100 °C for ten minutes to precipitate the proteins.

To remove the precipitated proteins, samples were centrifuged for 20 min, at 10000 x g in the fixed angle rotor centrifuge (Sortall Legend X1R Centrifuge, Thermo Scientific).

If there were still some precipitated proteins floating around, the supernatants were transferred to a clean 50 mL falcon tube and the sample was then centrifuged again at 3500 rpm, 5 minutes (Function Line Labofuge 400, Heraeus Holding GmbH). After this step, the supernatant was mixed with four volumes of EtOH 100% to precipitate the glucan and incubated over night at -20 °C. The next day the precipitated glucan were recovered by centrifugation at 3500 rpm for 5 minutes (Function Line Labofuge 400, Heraeus Holding GmbH) to recover the precipitated protein. Then, the supernatant was discarded and the pellet was washed with EtOH (100%) and centrifuged again under the same conditions. Following this, the supernatant was removed and the pellet was freeze-dried. (Alpha 2-4 LD plus, Martin Christ GmbH)

The determination of the dry weight of the sample (see 3.2.5.2.freeze drying) was done after completion of the freeze-drying process by weighing the dried pellet. With these data the yield of α-glucan extracted from 500 mL *G. sulphuraria* culture could be calculated by using this formula :

$$\left(\frac{Alpha\ glucan\ [\frac{mg}{L}]}{Dryweight\ pellet\ [\frac{mg}{L}]}\right) \times 100 = Yield\ of\ alpha\ glucan\ (\%)$$

After that, the α-glucan was stored in an Eppendorf tube (1.5 mL) with the lid closed and covered with parafilm to avoid moisturization of the α-glucan until further experiments were performed.

3.2.6.2. Alpha glucan analysis

- Monosaccharide composition by acid hydrolysis and high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)

The monosaccharide composition of α-glucan of *Galderia sulphuraria* growing on different carbon sources was analysed. Oyster glycogen was used as a standard for all experiments.

For the analysis of the monosaccaride composition 20 mg α-glucan were dissolved in 2 mL of milliQ water in an Eppendorf tube (2 ml). After that, the sample was filter sterilized. 500 µL of the filter-sterilized sample were used to perform the Anthrone assay (Description 3.2.5.3.)

The Anthone assay samples were diluted to reach a final concentration of 0.2 mg/mL glucan. Furthermore, another milliliter of the filter sterilized sample was then mixed with 1 mL TFA (1M) and the whole sample was incubated at 100 °C for two hours to perform the acid hydrolysis. After two hours, the sample was cooled down and one drop of NaOH (4M) was added to make the sample slightly acid/neutral. The hydrolysed samples were then diluted 1:50 in water and analyzed by anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The samples were analysed together with glucose, galactose and mannose standards of a concentration of 1, 2.5, 5, 10, 20, 40, 80, 100 mg/mL.

- Chain length distribution by isoamylase debranching and HPAEC-PAD

For this type of experiment 60 mg of α-glucan were dissolved in 3 mL of water to achieve a final concentration of 20 mg/mL before the sample was filter-sterilized.

To measure total carbohydrate content by Anthrone assay, a 1:60 dilution with a total volume of 1 mL was made out of the filtersterilized samples.

500 µL of the 20 mg/mL glucan sample were used to perform the Nelson Somogyi assay for measuring the reducing ends of the molecule.

Another 500 µL of the filter-sterilized α-glucan sample were used for the debranching of the molecule with isoamylase.

Isoamylase reaction mix:

- 500 µL Sample [20 mg/mL]
- 1500 µL Sodium acetate buffer 200 mM pH=5 + 5 mM $CaCl_2$ (Appendix Buffer preparation)
- 1 µL Isoamylase from *Pseudomonas sp.* (1000 U/ml)
- 2 µL Pullulanase from *B. Licheniformis*

The isoamylase reaction mix was then incubated at 40 °C for 24 h.

To deactivate the enzyme after the incubation time, the samples were incubated in the heating block for 3 min at 100 °C.

A 1:50 dilution of the sample in milliQ water was made and this sample was analzyed by HPAEC-PAD (DIONEX) with the program for chain length distribution to check the length of the molecule fragments and if there were some oligosaccharide in the sample or not.

400 µL of the debranched sample were then used to make a 1:50 dilution to a final volume of 2 mL to perform the Nelson Somogyi assay.

- Nelson Somogyi assay

The Nelson Somogyi assay was performed with samples before and after the debranching of the α-glucan.

This assay is a colormetric method for the determination of reducing ends by measuring the carbohydrate activity against different polysaccharides. (Nelson 1944)

The reducing sugars were determined by measuring the absorbance at 549 nm of a colored sample containing a copper oxidized sugar and arsenomolybdate.[16] The amounts of carbohydrates in the sample were calculated by using a calibration curve with glc [0.0-0.3 mg/mL]. For the preparation of the glc standard curve samples made out of a [2mg/ml] glc stock were used.

[16] https://docs.google.com/document/d/1RW7NsSJr_2oLIJ4Rwi7OnEKutdULIT9RRrWD_j-O0DI/edit

Nelson Somogyi reagent:

- **Solution A (1 L)**

  25 g Sodium carbonate

  25 g Potassium sodium tartrate

  200 g Sodium sulphate

  All ingredients were dissolved in 1 L of milliQ H_2O

- **Solution B (200 mL)**

  19 g Copper sulphate

  The copper sulphate was dissolved in 200 mL milliQ H_2O, containing 4 drops of H_2SO_4.

- **Solution C (1 L)**

  50 g Ammonium molybdate were dissolved in 900 mL of milliQ H_2O.

  Thereafter, 42 mL of H_2SO_4 (96%) were added.

  In another beaker, 6 g of sodium arsenate heptahydrated were dissolved in 50 mL of milliQ H_2O and mixed until the sodium arsenate was completely dissolved. After that, this solution was added to the ammonium molybdate solution and filled up to 1 L with milliQ H_2O.

  The bottle was then wrapped in aluminum foil and kept at 55 °C overnight to dissolve the salts completly.

→ Solution A, B and C were stored at roomtemperature until needed.

- **Solution D**: This solution had to be prepared freshly on the day of use.

  1 part of solution B was mixed with 24 parts of solution A , 1:25 dilution.
- **Solution E**: This solution also had to be freshly prepared before performing the assay and wrapping it in aluminum foil.

  1 part of solution C was mixed with 2 parts of milliQ H_2O, 1:3 dilution.

- Usage of Nelson Somogyi assay (Gusakov, Kondratyeva, and Sinitsyn 2011)

For the Nelson Somogyi assay 500 µL of the sample was mixed with 500 µL of H_2O in a glass tube. Afterwards, 1 mL of solution D was added and the sample was vortexed subsequently. The tubes with closed lids were then put in a water bath at 100 °C for 20 min. Then the samples cooled down at roomtemerature before 3 mL of

solution E were added. The samples were vortexed again after which a color-change into blue could be observed. As a last step, the absorbance was measured at 500 nm and as a result of the measurements the amount of reducing ends could be calculated. Additionally, all samples were measured in triplicates.

With the results of the Nelson assay, performed with samples before and after debranching as well as with the samples of the Anthrone assay, the average chain length of α-glucan could be calculated by using the equation illustrated in the formula below.

Equation for calculating the ACL:

$$\frac{Anthrone\ [\frac{1mg}{ml}\ \alpha\ glucan]}{Nelson\ before\ debranching\ \left[\frac{1mg}{ml}\ \alpha\ glucan\right] - Nelson\ after\ debranching\ [\frac{1mg}{ml}\ \alpha\ glucan\ ]}$$

When the ACL is known, the degree of branching can be calculated by using the formula:

$$\frac{100}{Average\ chain\ length} = Degree\ of\ branching\ (\%)$$

- H-NMR analysis for determining the average chain length of the *G. sulphuraria*'s α-glucan

For the H-NMR analysis, 7 mg of α-glucan were dissolved in 600 µL of D_2O and afterwards freeze-dried. Thereafter, 600 µL of D_2O were, once again, added to the sample.

After that the sample was transferred into a NMR tube by using a pasteur pipette and measured by H-NMR 500 Hz (Varian) at 50 °C. Acetone (chemical shift 2.225 ppm in D_2O) was used as internal standard for peak assignment.

3.2.7. Starvation experiment

During this experiment the ability of *G. sulphuraria* growing heterotrophically in Allen media without carbon (-C) and without a carbon and nitrogen source (-C-N) was tested.

For this, a preculture (described in 3.2.2. Liquid pre-cultures) was made. Thereafter, two glucan accumulation cultures of 500 mL Allen media + 1% (w/v) glc in 1 L flasks were made. These cultures were inoculated from precultures to an initial OD_{800}= 0.1 and incubated at 40°C, 100 uE/m^2s, 150 rpm until the cells reached the late exponential/early stationary phase (≈ three days of incubation).

Afterwards, two starved cultures were made by harvesting the cells by centrifugation 3500 x g for 5 minutes. Then, the SN was discarded before the pellet was washed in 10 mL of Allen medium pH=2 without C-N source or just without C. Then, the suspension was centrifuged again and the pellet was resuspended in 500 mL of the same medium, which was used for the washing step before. The 1 L flasks were then wrapped in aluminum foil and stored in the shaking incubator at 40°C, 150 rpm (Gallenkamp). In different intervals (Figure 15), samples were taken and analyzed by the determination of their CFU, dry weigth and total carbohydrate content in the cell extract.

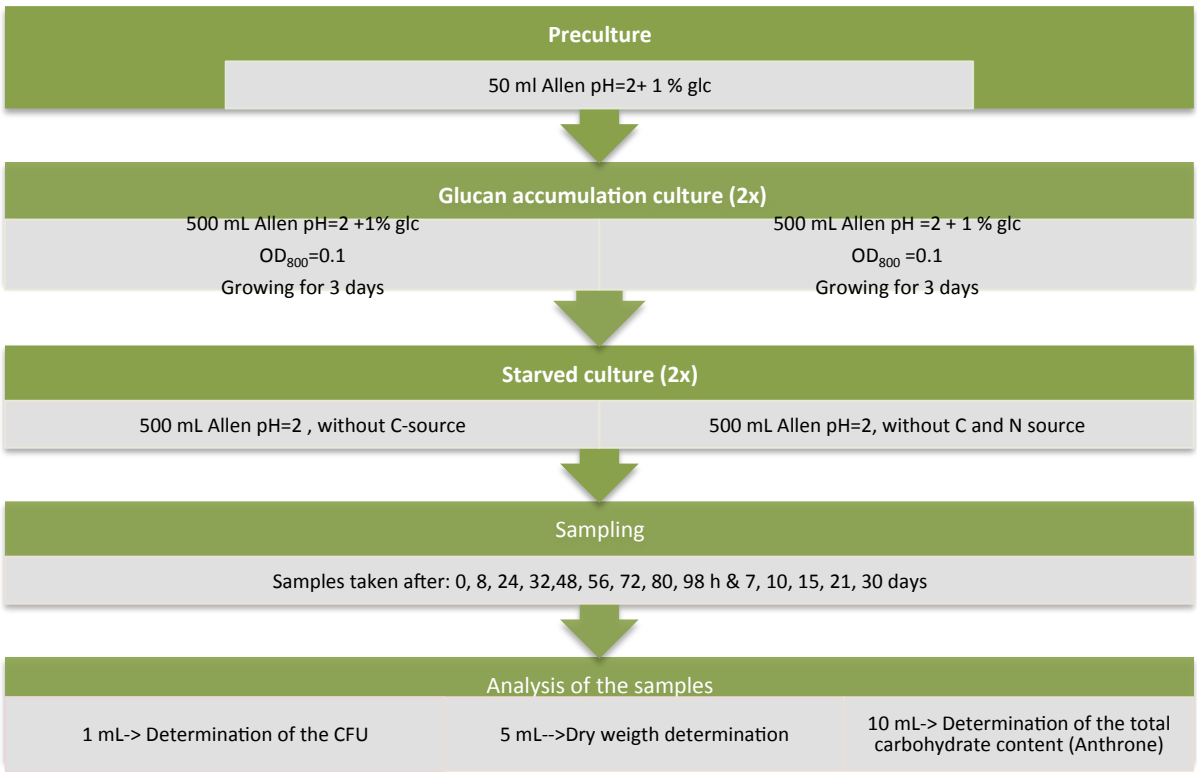


Figure 15: Starvation experiment protocol; from the precultures to the starved cultures and their analysis

3.2.7.1.Sample analysis of the starvation experiment

- Colony forming units (CFU) determination:

For the determination of the CFU, 1 mL samples were taken at defined time points. Then a 10^{-5} dilution was made out of these samples with sterile milliQ water. Later, 100 µL of this dilution was plated out on agar plates (Allen medium pH=4 + 1% glc (w/v)) and the plates were subsequently wrapped in aluminum foil and incubated at 40 °C (Thermo scientific, Heratherm) for two to three weeks.

- Dry weight determination:

For the dry weight determination 5 mL samples were taken at different times and put into the previously weighened falcon tubes. The samples were then centrifuged for 5 min at 3500 rpm. After that, the SN was discarded and the pellet was freeze-dried. After the freeze drying process was completed, the sample was weighed with the lid closed (AG 204 Delta range, Mettler Toledo).

- Anthrone assay: (See 3.2.5.3.)

All samples taken the first day (0h, 8h) were diluted 1:50 and all the others were diluted at a 1:20 ratio. All samples were measured in duplicates.

The amount of carbohydrates per dry weight was calculated for all samples.

4. Results

4.1. Chlorophyll production of *Galderia sulphuraria* over time

Regarding the color of *Galdieria sulphuraria* growing in Allen medium with nutrient supply for a defined periode, it was oberserved that cells with fewer amounts of a carbon supply turned green more quickly than the ones with either 0.5% of 1% (w/v) of sugar added. After one day of growing in the shaking incubator a light green color for cells growing in Allen media with 0.1% (w/v) glc or gal could be detected. (Figure 16) Exatly this green color appeared for cells growing in Allen medium with 0.1% (w/v) glc at an OD_{800} of 0.72 ± 0.12 and for the ones growing in medium with 0.1% (w/v) gal at an OD_{800} of 0.58 ± 0.13.

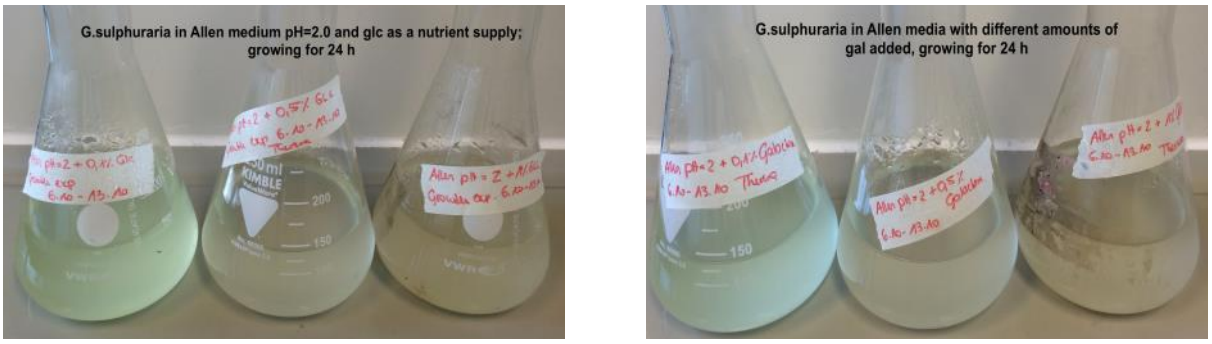


Figure 16: *G. sulphuraria* growing for 24 h in Allen media with 0.1%, 0.5 % or 1% (w/v) of glc (left side) or gal (right picture) as a nutrient supply. After 24 h of growing a slightly green color was observed in flasks where 0.1% (w/v) glc/gal was added. The other cell suspensions stayed colorless.

After two days of growth, a bright green color could be observed in *G. sulphuraria* growing in Allen medium with either 0.1% (w/v) of glc at an OD_{800} of 0.87± 0.02 or gal at an OD_{800} of 0.87 ± 0.1 added. For cells growing in media with addition of 0.1% (w/v) glc the color was more intense. Cell suspensions of *G. sulphuraria* growing in Allen media with higher sugar concentrations added as a nutrient supply remained yellowish. (Figure 17)

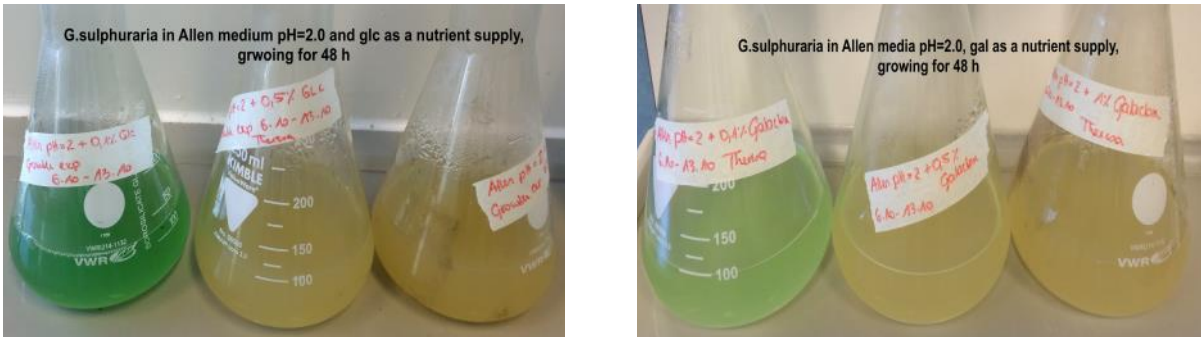


Figure 17: *G. sulphuraria* growing in Allen media with either 0.1% 0.5% or 1% (w/v) of glc/gal added as a nutrient supply. After 48 hours of incubation, cellsuspensions with the least amount of sugar were the greenest, whereas the other ones were yellowish.

After three days of growing under mixotrophic conditions, the color of all cultures turned green (Figure 18) The ones with less sugar added were still the greenest with OD_{800} values of 1.07 ± 0.19 for samples growing in Allen medium with addition of 0.1% (w/v) glc and OD_{800} values of 1.07 ± 0.06 for the ones growing in medium with 0.1% (w/v) gal as a nutrient supply. Cellsuspensions with 0.5% or 1% (w/v) of a carbon source added turned slightly green as well. *G. sulphuraria* growing in Allen medium with 0.5% (w/v) glc turned green at an OD_{800} of 4.16 ± 0.16 and with 1% (w/v) glc added at an OD_{800} of 7.44 ± 0.79. For *G. sulphuraria* growing in Allen medium with gal added as a nutrient supply, the suspension with 0.5% of this carbon source added, turned green at an OD_{800} of 4.38 ± 0.39, whereas the one with 1% (w/v) gal added, turned green after reaching a higher OD_{800} value in average of 6.39 ± 0.44.

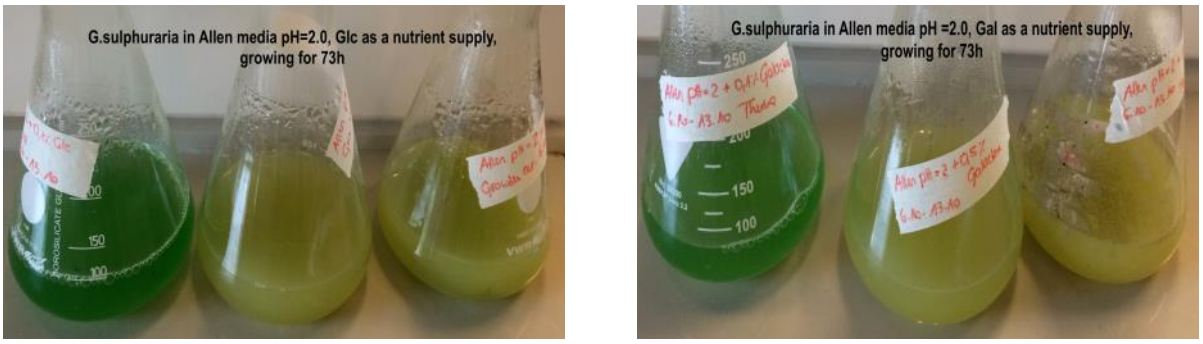


Figure 18: *G. sulphuraria* growing in Allen medium with 0.1%, 0.5% or 1% glc/gal. After three days of growing also the cultures with 0.5% or 1% (w/v) of a sugar added turned slightly green. Cellsuspensions with 0.1 % of nutirent supply had a strong green color.

After one week of incubation, a very intense colour could be observed for *G. sulphuraria* growing in Allen medium with either 0.1%, 0.5% or 1% (w/v) of a nutrient supply added for growth support (Figure 19).

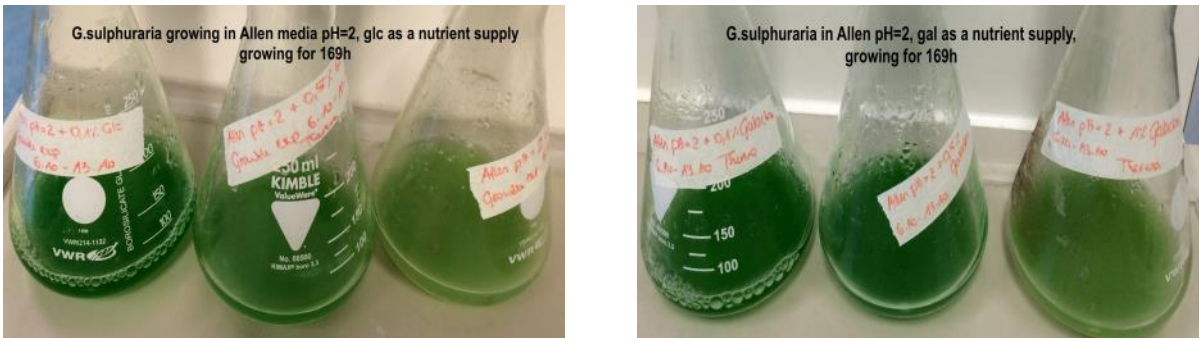


Figure 19: *G. sulphuraria* growing in Allen media with 0.1%, 0.5% or 1% (w/v) of glc/ gal. Cells were incubated for one week. All cellsuspensions had an intense green color after this incubation period.

The greenest color was achieved in *G. sulphuraria* cells growing in Allen medium with either 0.1% (w/v) gal added as a nutirent supply at an OD_{800} of 1.82 ± 0.24 or with 0.1% (w/v) glc added at an OD_{800} of 1.55 ± 0.12. Cells growing in medium with 1 % (w/v) of carbon source added, produced the lowest amount of photosynthetic pigments, which could be observed in lighter green color of the medium after one week of incubation. *G. sulphuraria* growing in Allen medium with 1% (w/v) of glc had an OD_{800} of 5.43 ± 0.35 after one week of incubation and the ones growing in medium with 1% (w/v) of gal added, measured an OD_{800} value of 5.69 ± 0.27. All other OD_{800} values measured over time are included in the appendix for further reference (Growth curve determination and measurements of the total amount of carbohydrates and sugar in cells over time).

Despite all that, it was observed that the color intensity of the cultures varied a lot depending on the amount of carbohydrates supplied to the growth medium. Furthermore, the less carbon source was added, the lower the OD_{800} values were and the faster the photosynthetic pigment production started.

4.2. Growth curve determination and analyses of the samples over time

To analyse the growth curves of *Galdieria sulphuraria*, the algae grew on Allen medium with either glc, gal or gly and different concentrations of these carbon sources over one week.

4.2.1. Glucose growth curve determination

Regarding the growth curves of *G. sulphuraria* growing on glc, it was observed, that the higher the glc concentration in the culture medium was, the longer it took to reach the stationary phase (Figure 20).

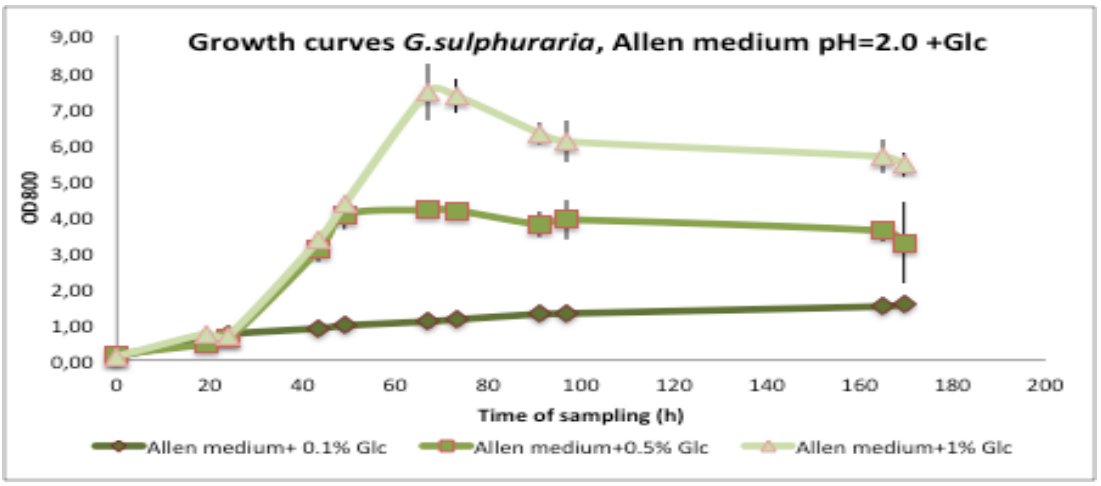


Figure 20: Growth curves of *G. sulphuraria* growing in Allen medium + either 0.1 %, 0.5% or 1 % (w/v) of glc as a nutrient supply to support the algae growth. Samples were taken over 169 h.

Cells growing in Allen medium with 0.1% (w/v) glc, reached the stationary phase after 25 h at an OD_{800} of 0.72 ± 0.12. At this point, the glc concentration measured by DNS assay had already decreased to 28.25 ± 2.73%, which is equal to 0.29 ± 0.05 mg glc/mL culture, and reached nearly zero after 49 h of growth, where it remained stable for the rest of the experiment (Figure 21). After 49 h the glc concentration was 1.44 ± 0.09%. The carbohydrate concentration was the highest at the beginning of the stationary phase with a concentration of 3.615 mg total carbohydrate/mg dry biomass and decreased rapidly afterwards to 0.166 mg total carbohydrate/mg drymass which is 22.6 ± 0.28% of the total amount of intracellular carbohydrates and stabilized at this value for the rest of the growth experiment.

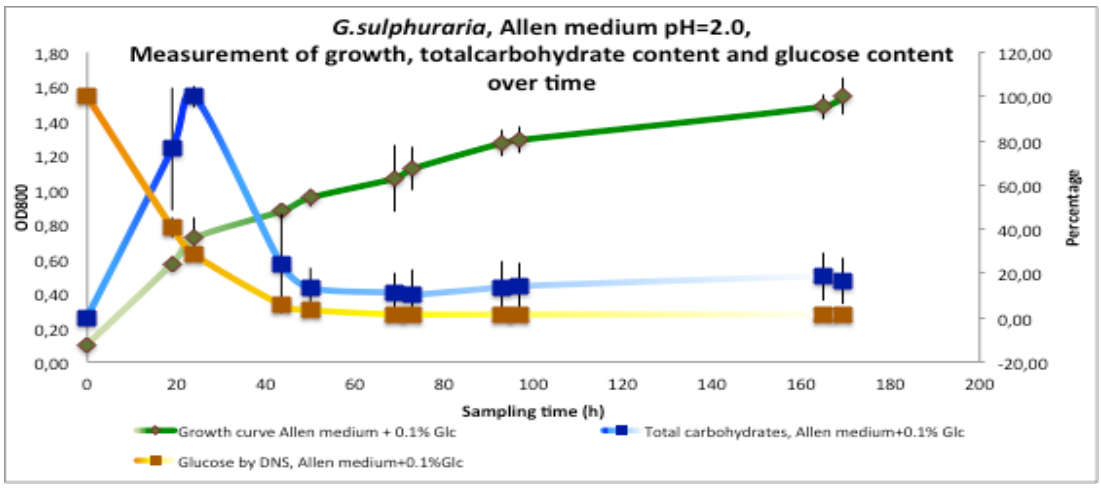


Figure 21: *G. sulphuraria* growing in Allen medium pH=2.0 with 0.1% (w/v) of glc. Comparison of growth, total carbohydrate content and sugar content over time.

Regarding the results of the GOPOD analyses (Figure 22) also a decrease of the glc concentration in cells was observed. The pink color, which is directly proportional to the glc concentration in the sample, was slightly rose after 25 h and pale after 43.5 h. After 25 h the glc concentration in the cells was 0.02 mg/mL culture which comprises to 2% of the total amount of glc in the cells. Where no change in color was observed, the complete use all the glc by the cells was evidenced.

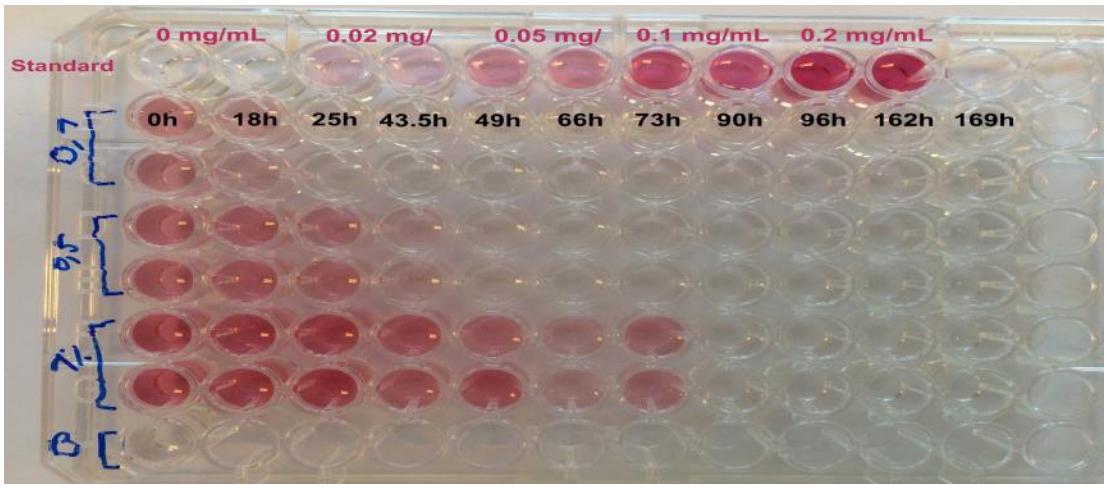


Figure 22: Results of the GOPOD assay performed in a 96-well plate; In row A the glc standard was analysed, in rows B & C. *G. sulphuraria* cells growing on Allen medium with 0.1% glc were analysed by taking samples at different points in time. Row D&E algae cells with 0.5% of nutrient supply and in rows F&G , the ones with 1% of glc supply were analysed. In the last row water was used as a blank.

For *G. sulphuraria* growing on Allen medium with 0.5% (w/v) glc similar results as the one of *G. sulphuraria* growing in medium with 0.1% glc were monitored. The stationary phase was reached a little later than for the cells growing on Allen medium with 0.1% of glc, after 49 hours at an OD_{800} of 4.01 ± 0.41. At this point in time, the

D-glucose concentration was 1.29 ± 0.24 mg glc/mL which correlates to 25.16 ± 3.6% and after 69 hours no glc was left in the sample. This effect could also be observed when regarding the plate of the GOPOD asay. After 73 hours, no color change of the sample to pink could be observed anymore (Figure 22), which means that no glucose was left in the medium anymore as a nutrient supply for the cell`s growth. At the same point, the cells reached the stationary phase, the carbohydrate content, measured by anthrone, started to decrease. After 66 hours, the amount of carbohydrates reached the lowest point with a concentration of 37.6 ± 0.02% (Figure 23).

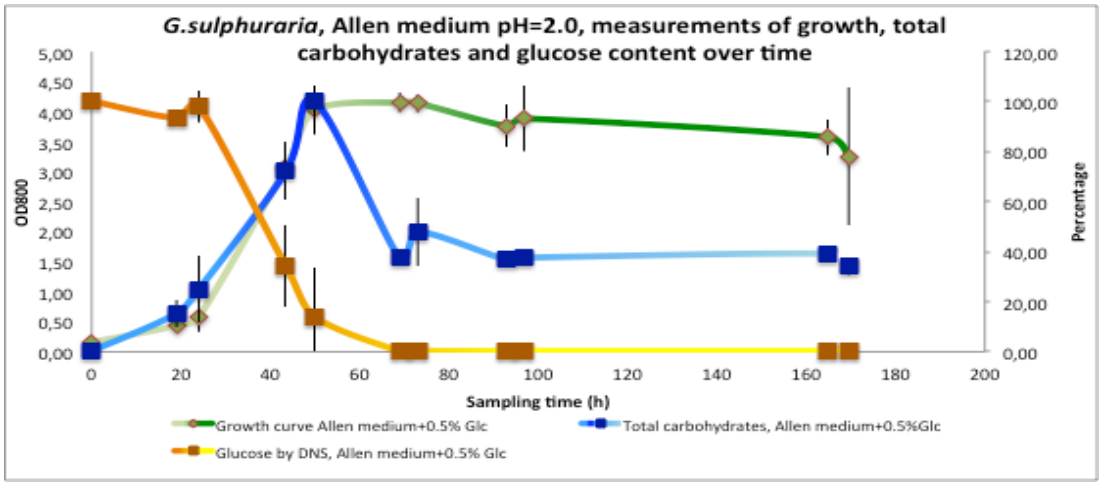


Figure 23: *G. sulphuraria* growing in Allen medium pH=2.0 with 0.5% (w/v) of glc. Comparison of growth, total carbohydrate content and sugar content over time.

For *Galderia sulphuraria* growing on 1% (w/v) glc, the stationary phase was reached after 73 hours at an OD_{800} of 7.32 ± 0.49. At this point, no glucose was left in the sample. At the same time, the amount of carbohydrates started to decrease and stayed stable at a final concentration of 23.29 ± 1,4%. This was also confirmed by the the results of the GOPOD assay. By taking a look at figure 22, it can clearly be seen, that after 90 hours, no change to pink took place anymore, which means that the glc had already been used by the cells.

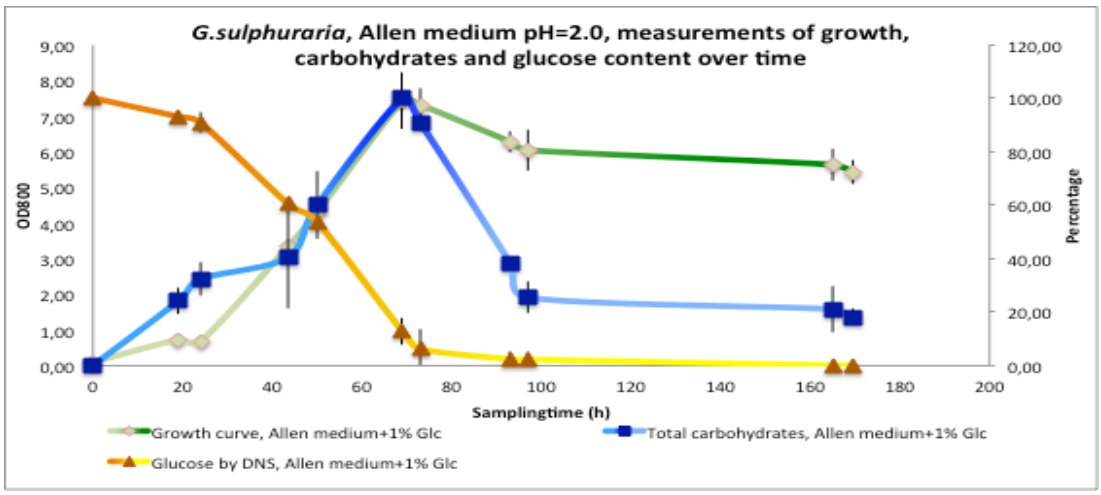


Figure 24 *G. sulphuraria* growing in Allen medium pH=2.0 with 1% (w/v) of glc. Comparison of growth, total carbohydrate content and sugar content over time.

Considering all these results, it can be said, that the more glucose the sample contained, the longer it took to use up all the nutrient supply. This was also the reason why it took longer for the algae cells to reach their stationary phase.

For the culture itself it was observed, that the less glc was present in the culture, the greener it became over time. After 24 hours the sample with 0.1% (w/v) glc had already turned green, whereas the ones with 0.5% and 1% (w/v) glc nutrient medium, were still pale.

4.2.2. Galactose growthcurve determination:

The growth curves of *G. sulphuraria* growing on different concentrated Allen media with gal followed the same scheme as the ones of *G. sulphuraria* growing on glc did.

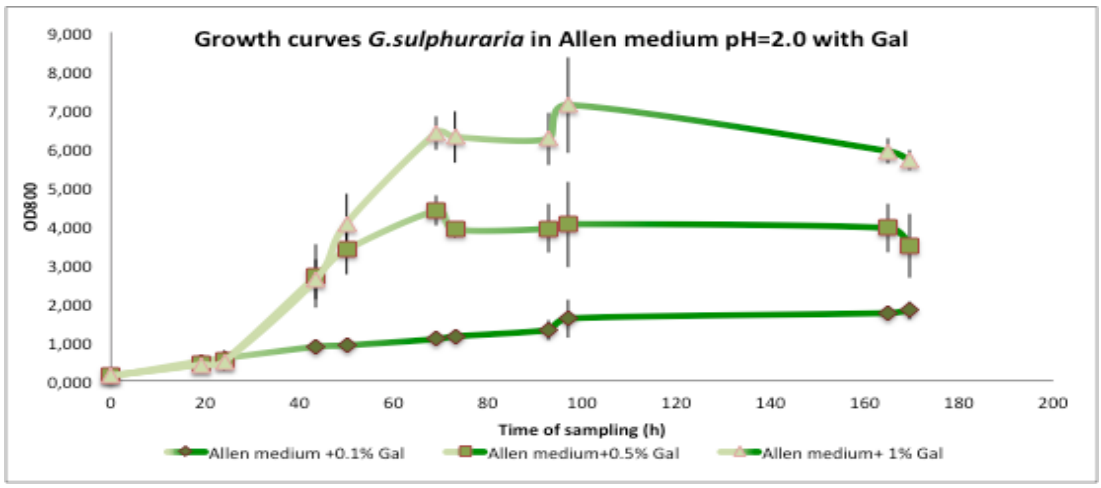


Figure 25: Growth curves of *G. sulphuraria* growing in Allen medium + either 0.1% , 0.5% or 1% (w/v) of gal as a nutrient supply to support the algae growth.

For the cells growing in culture medium with 0.1% (w/v) gal the stationary phase was reached after a day of growth, at an OD_{800} of 0.59 ± 0.11, which was lower than the OD_{800} measured for growth in medium with glc as a nutrient supply. At this point, the concentration of carbohydrates started to decrease rapidly. After an incubation time of 44 hours it more or less remained at 18.6 ± 6.79% of the total amount of carbohydrates which corresponds to 5.81 ± 2.67 mg carbohydrate / mg dry biomass. Simultaneously to the reaching of the stationary phase the galactose concentration measured by DNS, was around 31.49 ± 11.33% which corresponds to 0.3 ± 0.1 mg gal / mL medium. After 43.5 hours most of the galactose was already used up by the cells. The galactose concentration stayed stable at 2.2 ± 1.4% for the rest of the experiment (Figure 26).

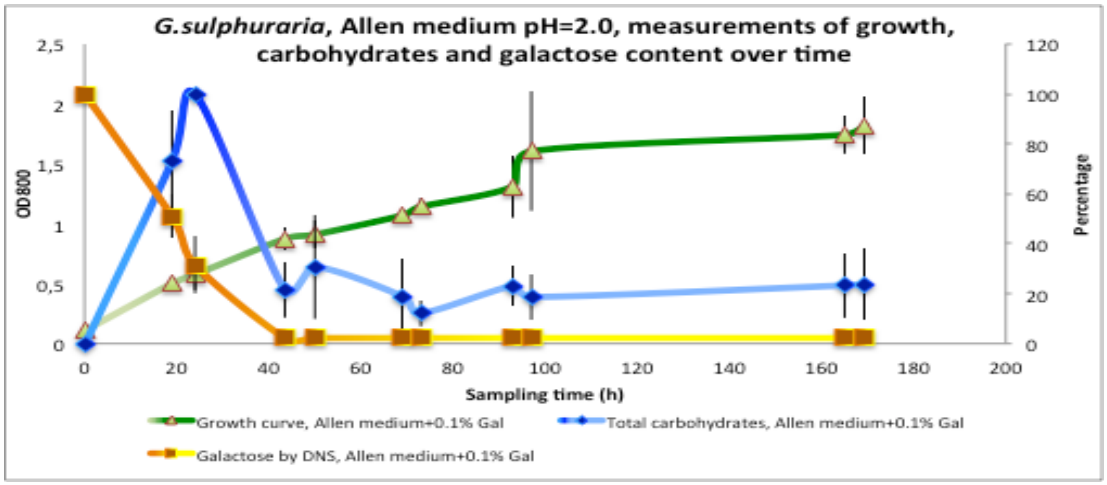


Figure 26: *G. sulphuraria* growing in Allen medium pH=2.0 with 0.1% (w/v) of gal. Comparison of growth, total carbohydrate content and sugar content over time.

For the algae growing in nutrient media with 0.5% (w/v) gal, the stationary phase was reached after 68 hours at an OD_{800} of 4.38 ± 0.39. After 68 hours the amount of carbohydrate had already decreased to 44.8 ± 16.27%. Over time this value further shrunk to 21.2 ± 7.9% of the total amount of carbohydrates in the culture which is equivalent to 0.47 ± 0.29 mg total carbohydrates / mg dry biomass. For the quantity of galactose present as a nutrient supply, it was observed that for samples growing on Allen medium with 0.5% (w/v) gal, during the time in which the cells reached their stationary phase, all gal present in the culture had already been used up.

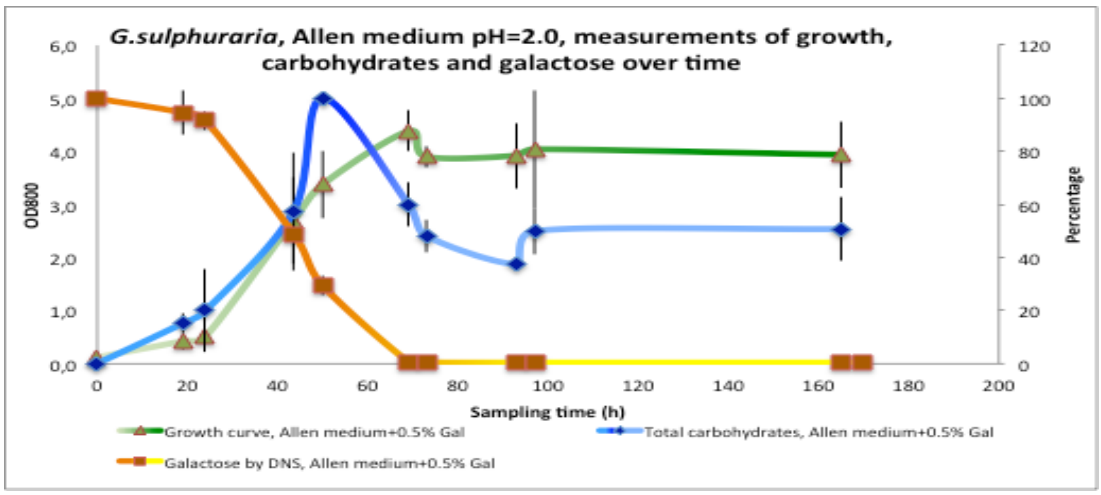


Figure 27: *G. sulphuraria* growing in Allen medium pH=2.0 with 0.5% (w/v) of gal. Comparison of growth, total carbohydrate content and sugar content over time

The highest OD_{800} of 6.53 ± 0.3 was reached for *G. sulphuraria* growing in Allen medium with 1% (w/v) gal as nutrient supply. In this case, the stationary phase was reached after around 90 hours. At that time, there was no more gal present in the sample and the amount of carbohydrates was at 20.27 ± 10.9% and stayed constant at this value for the rest of the experiment (Figure 28).

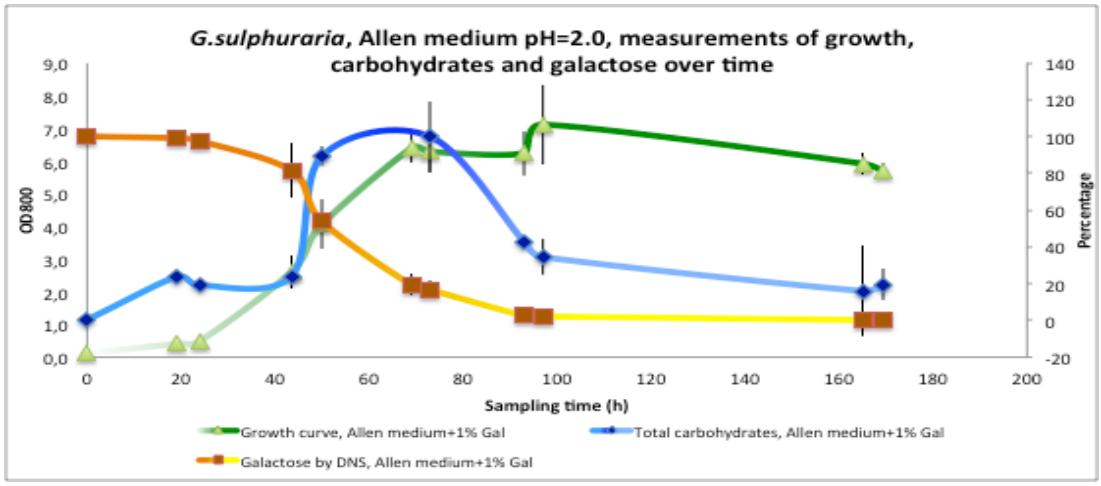


Figure 28: *G. sulphuraria* growing in Allen medium pH=2.0 with 1% (w/v) of gal. Comparison of growth, total carbohydrate content and sugar content over time.

4.2.3.Determination of glycerol growth curves

For *G. sulphuraria* growing in Allen media with gly as a nurtient supply, only 1% (w/v) of gly was used for the determination of the growth curves. This was done because after the determination of the growth curves for *G. sulphuraria* growing in nutrient medium with either glc or gal in different concentrations, it was decided, that a concentration of 1% (w/v) of a carbon source would be the best option for the α-glucan extraction.

Thus, for the growth curves of the red microalgae growing on 1% (w/v) gly it was observed, that the stationary phase was reached after 96 hours at an OD_{800} of 11.29 ± 1.56 . At this time the amount of carbohydrates had already decreased to 92.49 ± 5.73% which was equal to 0.668 ± 0.007 mg gal/ mg dry cells . After 162 hours the amount of carbohydrates present in the sample was only 17.8 ± 7.9% which is equivalent to 0.018 ± 0.005 mg gal / mg dry cells. (Figure 29).

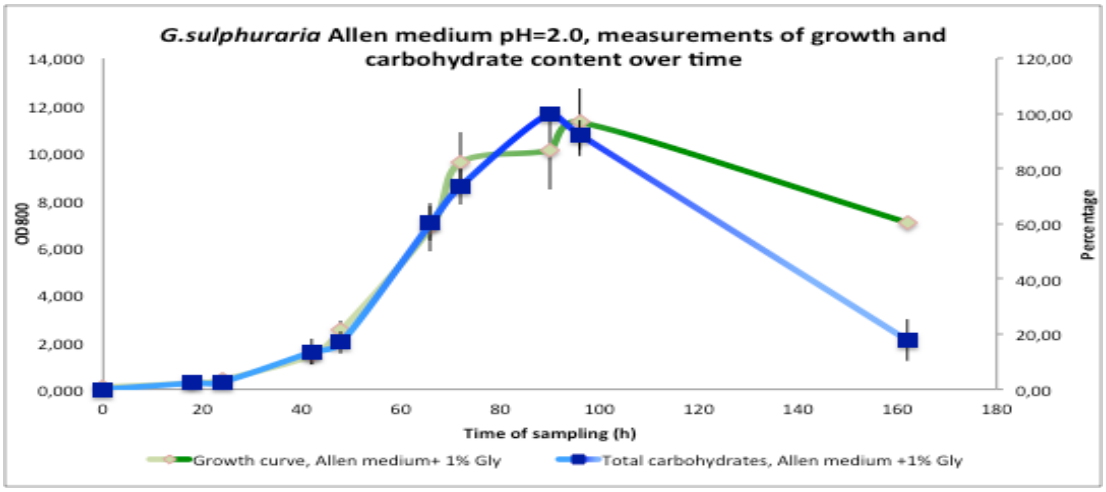


Figure 29: *G. sulphuraria* growing in Allen medium pH=2.0 with 1% (w/v) of gly. Comparison of growth, total carbohydrate content over time.

Because of the fact that at the time of performing the experiment, no efficient protocol for measuring the amount of glycerol in a samples could be found, it was assumed that, like the results of other sugar concentration measurements done before, the amount of glycerol after reaching the stationary phase was nearly 0.

4.2.4. Comparison of the three growth curves:

By comparing the three growth curves of *G. sulphuraria* growing in culture medium with either 1% glc, gal or gly it was clearly seen, that the cells growing on gly needed the longest to reach their stationary phase (Figure 30). Cells growing on gly achieved the highest optical density with a value of 11.29 ± 1.56 after 94 hours of growth. *G. sulphuraria* cells growing in Allen medium + 1% (w/v) gal reached the second highest OD_{800} with a value of 7.44 followed by algae cells growing in nutrient medium with 1% (w/v) of glc added.

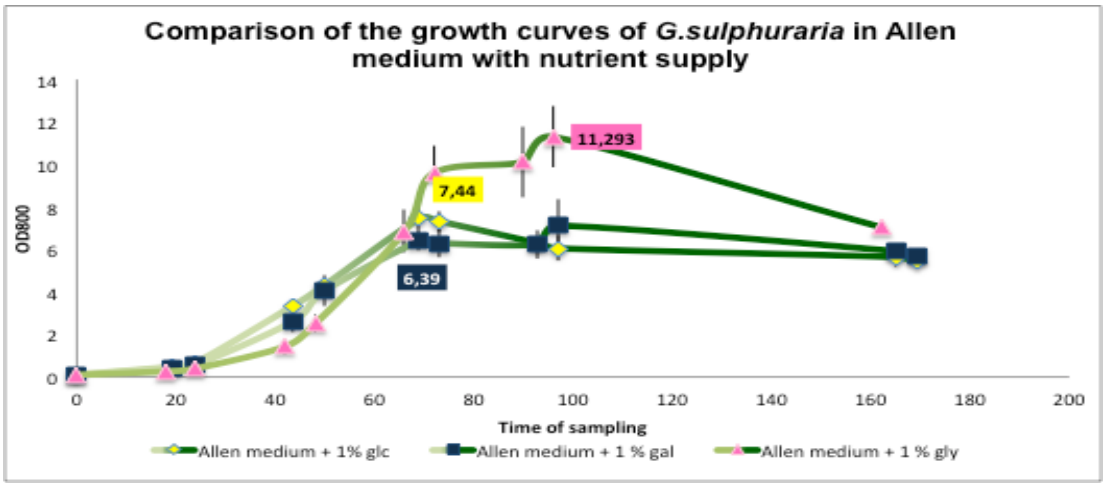


Figure 30: Comparison of the growth curbes of *G. sulphuraria* growing in Allen medium with either 1% glc, 1% gal or 1% (w/v) gly added as a growth enhancer.

Furthermore, it was also observed that synchronously to the reaching of the stationary phase, the amount of carbon source present in the samples already decreased to 50%, regardless whether the cells grew in culture medium with either 1% glc, 1% gal or 1% (w/v) gly as an additional sugar source. After one week of growing neither carbohydrates nor sugars were left in the algae samples.

By monitoring the growth of *G. sulphuraria*, it was decided to harvest the cells for the α-glucan extraction after 97 hours. That was the exact point in time, when the algae cells growing on either 1% (w/v) of glc, gal or gly had already reached the stationary phase but still contained some carbohydrates.

4.2.5. Observation during the preparation of samples for further analyses:

Before the amount of carbohydrates was determinded by Anthrone assay, the cells had to be broken up in the Mixer Mill (Mixer Mill 400, Retsch) and centrifuged (Eppifuge 5415C, Eppendorf AG). After these steps, the SN used for further analyses turned blue. The longer the algae grew, the greener the culture was at the sampling point and the more blue colour was released from the cells. After 49 hours the SN of *G. sulphuraria* growing on culture medium with 0.1% (w/v) of carbon source added, already turned light blue (Figure 31). After one week of growing the SN was intense blue. In comparison to that, the SN of cells growing on cultural medium with either 0.5 or 1% (w/v) of carbon source added, turned slightly blue just after 90 hours of growing. So the less carbon source was added as a nutrient supply, the earlier *G. sulphuraria* started to produce the blue compound, due to the fact that the carbon sources were used faster. Hence, the more chlorophyll was produced, the more bluer the SN was.

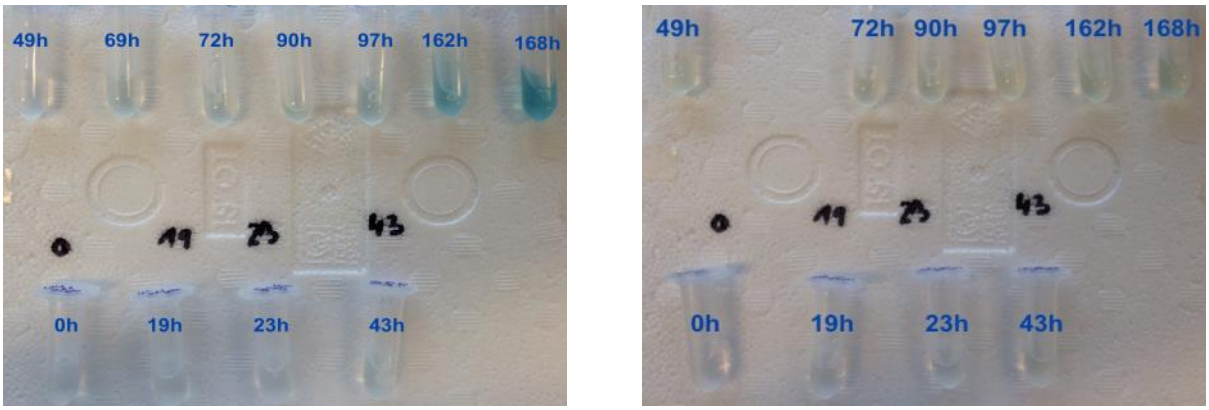


Figure 31: Supernatant of the broken down cells after centrifugation. On the left picture the SN of *G. sulphuraria* growing in Allen medium with 0.1% (w/v) of carbon source added over time is depicted. The blue colour of these samples was intenser and appeared earlier than in the samples of *G. sulphuraria* growing in nutrient medium with 1% (w/v) nutrient supply (right figure).

4.3. Alpha glucan extraction:

4.3.1. Chlorophyll production of *G. sulphuraria* growing heterotrophically

The α-glucan extracted out of 500 mL culture of *Galdieria sulphuraria*, growing heterotrophically, was harvested at the stationary phase (97 hours) and analysed afterwards.

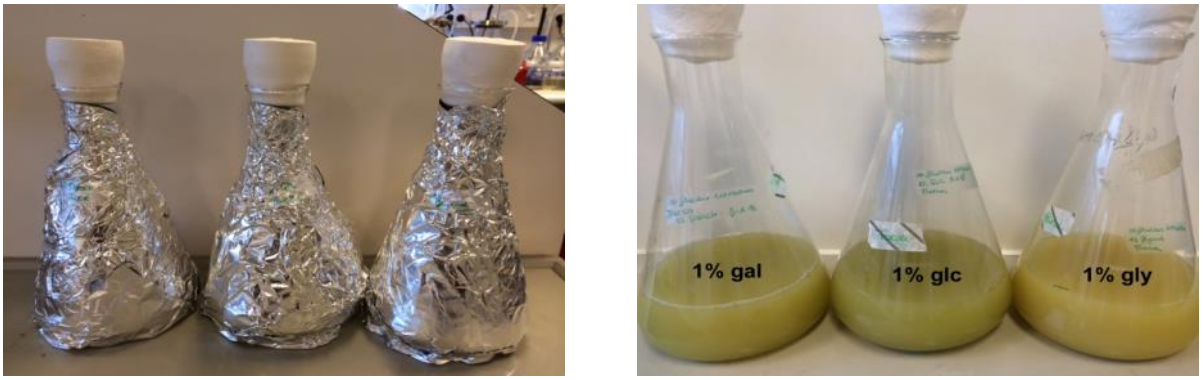


Figure 32: *G. sulphuraria* growing in 500 mL Allen medium with either 1% (w/v) glc, gal or gly heterotrophically /wrapped in aluminium foil (left figure) for 97 hours until the early stationary phase was reached. On the right picture the algae cells growing in Allen medium with 1% (w/v) glc or gal turned after 97 hours of growing slightly green, whereas the ones growing in Allen medium with 1% (w/v) gly as a carbon supply stayed pale.

The picture of the 500 mL cultures showed that the carbon source had an influence on the color of the cultures. *G. sulphuraria* cells growing on gly stayed yellowish, while the ones growing on galactose and on glucose showed a more greenish colour after 97 hours of growing. The ones growing on gal seemed to be the greenest with an average OD_{800} of 5.79 ± 1.15 after 97 hours of growth. Cells growing in nutrient medium with glc had an average of OD_{800} of 5.95 ± 1.0, a little higher than the OD_{800} of *G. sulphuraria* growing in Allen medium with galactose as an added carbon source. The lowest OD_{800} values of 5.51 ± 0.64 were achieved of *G. sulphuraria* growing in medium with gly as a nutrient supply. This effect could always be observed regardless of cells growing heterotrophically or mixotrophically (Figure 33). Of course, as seen in figure 33, the intensity of the green colour/chlorophyll produced by *G. sulphuraria* cells growing mixotrophically on different carbon sources was higher than for the ones growing heterotrophically. No matter whether the cells grew mixo- or heterotrophically, the ones growing on Allen medium + 1% (w/v) gly always remained pale.

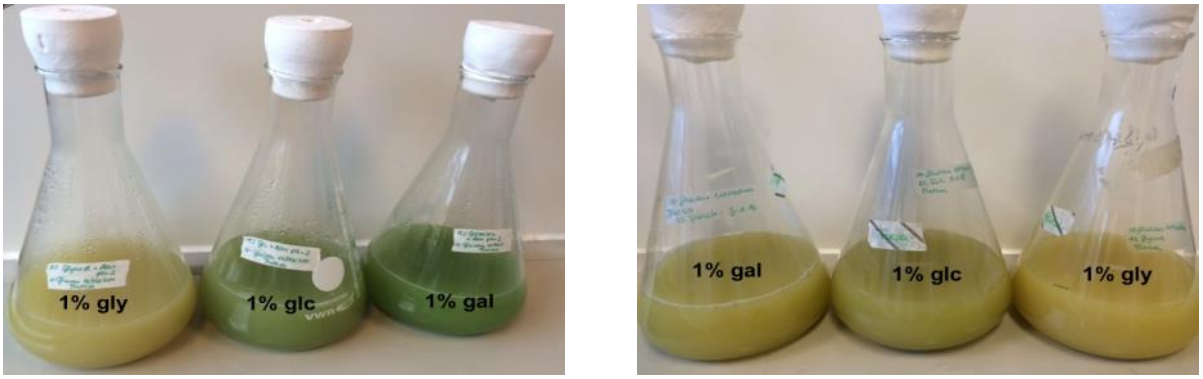


Figure 33: The left pictures shows, *G. sulphuraria* cells growing in mixotrophic conditions in Allen medium plus nutrient supply. The ones growing in nutrient medium with 1% gal were the greenest after 97 hours of incubation, whereas the ones growing with 1% (w/v) gly stayed pale. On the right picture, cultures growing under heterotrophic conditions are illustrated. The amount of chlorophyll produced under these growing conditions was less. That is the reason why the cultures stayed yellowish-green. Also here, *G. sulphuraria* growing in Allen medium + 1% (w/v) of gly stayed pale.

4.3.2. Determination of the optical density, dryweight of the pellet and of the extracted α-glucan

In table 9 the determination of the OD_{800}, the pellet`s dryweights and the weights of the extracted α-glucans is illustrated. *G. sulphuraria* cells grew under heterotrophic conditions for 97 hours before these three values were determined.

Table 9: Results of the α-glucan analysis extracted from *G. sulphuraria* growing in Allen medium with either 1% of glc, gal or gly as a nutrient supply. Determination of the OD_{800}, the pellet`s dry weight and the dry weight of the extracted α-glucan after growing *G. sulphuraria* for 97 hours.

Carbon source as a nutrient supply	OD_{800}	Dry weight pellet [mg]	Extrcted a-glucan dryweight [mg]
1% (w/v) Glc	7.15	1553.7	436.5
1% (w/v) Glc	6.37	1587.1	427.2
1% (w/v) Glc	5.88	1566	414.6
1% (w/v) Glc	4,92	1787	395.4
1% (w/v) Gal	5.99	1470.5	322.3
1% (w/v) Gal	7.33	1830.1	395.0
1% (w/v) Gal	5.15	1485.5	299.2
1% (w/v) Gal	4.72	1600.1	348.3
1% (w/v) Gly	6.27	1613.7	383.8
1% (w/v) Gly	5.26	1697.6	322.9
1% (w/v) Gly	5.02	1835.1	430.3

Regarding this table, it can be said that the biggest amount of α-glucan, 418.42 ± 17.78 mg, was extracted of *G. sulphuraria* growing on Allen medium + 1% (w/v) glc. A correlation between the OD_{800} and the dryweight of the extracted α-glucan could be

observed. The higher the OD_{800} was, the more glucan could be extracted out of 500 mL *G. sulphuraria* culture. For most of the results listed in table 9 it can be said that a correlation between the pellet`s dryweight and the amount of α-glucan extracted from *G. sulphuraria* growing on three different carbon sources could be observed.

Table 10 illustrates the yield of α-glucan extracted from 500 mL *G. sulphuraria* culture.

Table 10: Calculated yield of α-glucan extracted from *G. sulphuraria* growing until the stationary phase was reached in Allen medium with three different carbon sources (Glc, Gal, Gly) as a nutrient supply. The average of the yield is shown here.

Carbon source	Dry weight pellet [mg/mL]	α-glucan [mg/mL]	Yield (%)	Average (%)
1% (w/v) Glc	3107.4	873.0	28.09	25.9
1% (w/v) Glc	3174.2	854.4	26.92	
1% (w/v) Glc	3132	829.2	26.48	
1% (w/v) Glc	3574.2	790.8	22.13	
1% (w/v) Gal	2941	644.6	21.92	21.36
1% (w/v) Gal	3660.2	790	21.58	
1% (w/v) Gal	2971	598.4	20.14	
1% (w/v) Gal	3200	696.6	21.77	
1% (w/v) Gly	3227.4	773.6	23.97	22.15
1% (w/v) Gly	3395.2	645.8	19.01	
1% (w/v) Gly	3670.2	869.6	23.45	

The highest yield was achieved from *G. sulphuraria* growing on 1% (w/v) glc, with an average value of 25.9 ± 2.61%. The second highest yield came from the cells growing on 1% (w/v) gly with an average yield of 22.36 ± 0.82%, closely followed by the cells growing on 1% (w/v) gal with an average yield of 22.15 ± 2.73%. A yield higher than 20% was achieved for *G. sulphuraria* cells regardless of the carbon source used as a nutrient supply. Thus it seemed that nearly one fifth up to one fourth of all components in the cells were α-glucan.

4.4. Analyzes of α-glucan

4.4.1. Monosaccharide composition by acid hydrolysis and HPAEC separation

The concentration of the different monosaccharides was calculated using the linear equation of either the glc or gal standard curve (Figure 34).

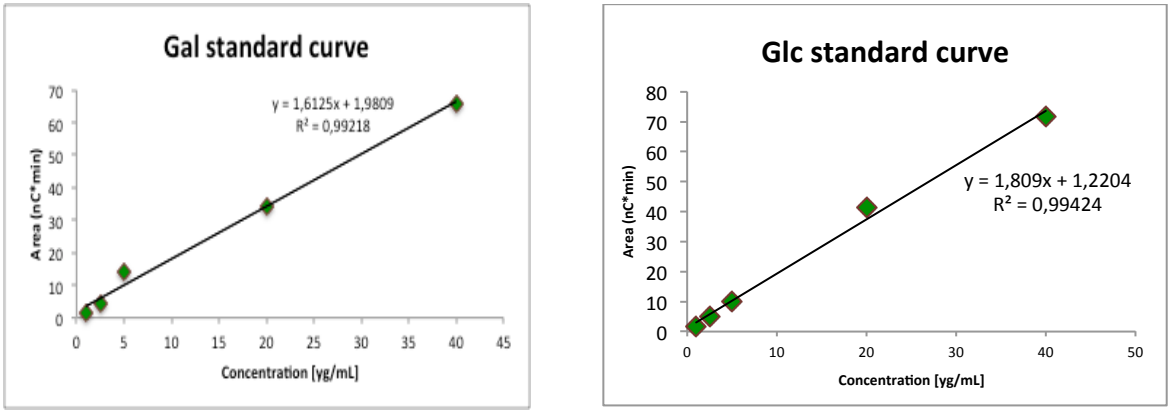


Figure 34: Left picture shows the gal standard curve for the analyses of the monosaccharide composition of α-glucan extracted from *G. sulphuraria* growing in Allen medium with three different carbon sources. In the right picture the glc standard curve is illustrated.

For the monosaccharide composition, it can be said, that not only glc was present in the samples that gave a signal. The glc peak was detected at a retention time of 15.8 minutes. As already mentioned, other substrates also gave a peak during this experiment. One signal was assumed to be galactose with a retention time of 13.9 minutes. Another signal after around 6.7 min also appeared in all analyses, except the sample of oyster glycogen. For the oyster glycogen, only the glc peak appeared. Considering the fact that the original sample should have a glc concentration of 100 µg/mL, it seemed likely, that for the glycogen extracted from oyster, as well as for all α-glucans from *G. sulphuraria*, some impurities influenced the amount of glc present in the sample. Therefore, the glc concentration of none of the measured samples was around 100 µg/mL. The highest concentration had the α-glucan of *G. sulphuraria* growing on Allen medium with 1% of glc. All analysed samples had a glc concentration between 69-76 µg/mL (Table 11).

Table 11: Results of the monosaccharides present in the extracted α-glucan of *G. sulphuraria,* as well as in glycogen from oyster, used as the standard in this experiment. The table also shows the total amount of carbohydrates before the acid hydrolysis.

Allen Medium	Monosaccaride present in the sample	Retention time (min)	Area nC*min	Concentration [µg/mL]
Oyster	Glc	15.69	128.79	72.1
1% (w/v) Glc	Glc	15.70	133.91	76.24
	Maltopentose	13.88	20.18	11.06
	Maltose	6.73	3.99	1.6
1% (w/v) Gal	Glc	15,67	125.41	68.65
	Maltopentose	13.83	40.81	24.08
	Maltose	6.73	4.671	1.67
1% (w/v) Gly	Glc	15.67	136.14	74.58
	Maltopentose	13.86	22.49	12.49
	Maltose	6.73	4.56	1.6

The “maltopentose-peak” of the samples, was assumed to be galactose by comparing its retention time with the retention time of the galactose standard. The galactose peak for *G. sulphuraria* growing on Allen medium with 1% glc or 1% (w/v) gly had a concentration of around 12 µg/mL. For the ones growing on 1% gal it was twice the amount. The signal, appeared after around 6.7 min and had a concentration of around 1.6 µg/mL in all analysed samples. So it could be identified, that the α-glucan extracted of *G. sulphuraria* did not consist only of glucose molecules. It seemed that other monosaccarides were also present and gave a signal at different times.

4.3.2. Average chain length determination by NMR and debranching assay

4.3.2.1. NMR

An example of a NMR spectrum is shown in figure 35. All other NMR spectra can be looked up in the appendix. It clearly shows that the peak of the α-1,4 linkages was bigger than the one of the α-1,6 branching points of the molecule. For α-glucan extracted from *G. sulphuraria* a small peak behind the α-1,6 braniching point peak also appeared in the spectrum, which represents impurities. So this signal is from other molecules, which are still present in the sample after the purification.

Normally the water peak, which is also measured with NMR and the peak of the α-1,6 branching points are very close together, nearly overlapping each other, which would affect the measurements. Because of the fact that this experiment was carried out at 50°C the peaks could be separated and analysed independently, without influencing each other (Figure 35) (Zang, Howseman, and Shulman 1991).

The results of the NMR (Figure 35) showed that the average chain length of the α-glucan extrated from *G. sulphuraria* growing heterotrophically in Allen medium with nutrient supply, was half of the length as compared to the ACL of oyster glycogen. To calculate the ACL, the value of both peaks was summed up.

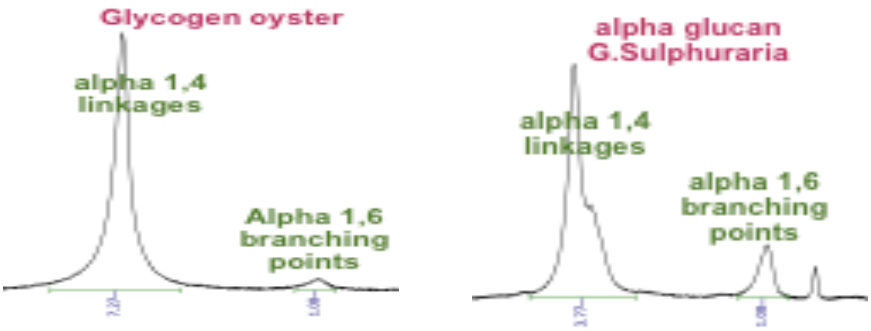


Figure 35: NMR spectra of glycogen isolated from oyster and α-glucan from *G. sulphuraria*. Two peaks are shown in each spectrum. The left one represents the α-1,4 linkages in the molecule and the right one expresses the α-1,6 branching points.

The average chain length determined by NMR, was 8.27 glc residues for oyster glycogen. In comparison to that, the average chain length of α-glucan extracted from algae had an ACL of 4.72 ± 0.07 for cells growing on Allen medium + 1% (w/v) glc, 4.97 ± 0.36 for the ones growing on Allen medium + 1% (w/v) gal and 4.49 ± 0.12 for *G. sulphuraria* cells growing on Allen medium + 1% (w/v) gly. Figure 35 also depicts that the peak of the α-1,6 branching points of oyster glycogen, which is very small, is also correlated to a longer ACL. In comparison to that, α-glucan extracted from

G. sulphuraria had bigger branching point peaks, which stated that the molecule was more branched and therefore the ACL was shorter.

After calculating the ACL of the sample, also the degree of branching was calculated by using the formula previously mentioned in 3.2.6.2. alpha glucan analysis. The standard oyster glycogen had a degree of branching of 12.09%, while α-glucan of *G. sulphuraria* growing on glc had one of 21.18% ± 0.32, for the cells growing on gal it was 20.19% ± 1.4 and the ones growing on gly had a degree of branching of 21.89% ± 0.06. The lowest standard deviation was achieved with α-glucan of *G. sulphuraria* growing on gly, which made these results more reliable than the ones of the α-glucan from algae cells with gal as a nutrient supply. Thus, also with the degree of branching measured by NMR, it was proven that α-glucan of *G. sulphuraria* was a highly branched molecule with shorter chain lengths than the oyster glycogen had.

4.3.2.2. Debranching assay

The average chain length as well as the degree of branching was also calculated through the results received from Nelson Somogyi assay, performed before and after the debranching with isoamylase, as well as of the results of the total carbohydrate determination by Anthrone. The results of Nelson Somogyi and Anthrone assay, as well as the average chain lengths and the degrees of ploymerisation of extracted α-glucan are shown in table 12.

Table 12: Results of Nelson Somogyi assay before and after the debranching with isoamlyase as well as the results of the Anthrone assay are listed in this table. With these values the average chain length and the degree of branching (of either oyster glycogen or α-glucan from *G. sulphuraria* were calculated.

Nutrient supply for Allen medium	Nelson Somogyi before DB [1mg/mL]	Nelson Somogyi after DB [1mg/mL]	Anthrone before DB [1mg/mL]	Chain length	Average chain length	Degree of branching
Oyster glycogen	0.0033	0.0839	1.03	12.81	12.72	7.8%
Oyster glycogen	0.0034	0.0813	0.983	12.62		7.9%
1% (w/v) Glc	0.0141	0.1451	0.700	5.34	6.9	18.7%
1% (w/v) Glc	0.0108	0.0993	0.700	7.91		12.6%
1% (w/v) Glc	0.0139	0.0999	0.6	6.98		14.3%
1% (w/v) Glc	0.0149	0.1278	0.833	7.38		13.5%
1% (w/v) Gal	0.0087	0.1009	1.007	10.91		9.2%
1% (w/v) Gal	0.0113	0.1009	0.94	10.49		9.5%

1% (w/v) Gal	0.0124	0.1168	0.897	8.59	10.08	11.6%
1% (w/v)Gal	0.0129	0.1060	0.963	10.34		9.7%
1% (w/v) Gly	0.0092	0.1330	0.947	7.65	9.09	13.1%
1% (w/v) Gly	0.011	0.1100	0.977	9.86		10.1%
1% (w/v) Gly	0.013	0.1189	1.033	9.76		10.2%

Regarding the results shown in table 12, it is clear that the amount of reducing ends measured with Nelson Somogyi assay increases enormously after the debranching with isoamylase. The fact that α-glucan of *G. sulphuraria* is supposed to be a highly branched molecule, was also confirmed by regarding these results, because of the fact that the extracted α-glucan had an average chain length between seven to ten molecules, depending on the sugar used for its growth. The oyster glycogen had an average chain length of around 13 molecules, which is also referenced in literature (Akai et al. 1971). According to these results it can be concluded that *G. sulphuraria* growing in Allen medium with 1% (w/v) of glc as a carbon source, led to the shortest average chain lengths with around 6.9 ± 1.1 residues. The algae cells growing in nutrient medium with 1% gal had the longest ACL within these three carbon sources used for the experiment with an ACL of 9.09 ± 1.0 residues, but were still more branched that the oyster glycogen, which was used as the standard.

Due to the fact that the α- glucan of *G. sulphuraria* is highly branched, intuitionally, its degree of branching was also a lot higher as the one from oyster glycogen.

4.3.2.3. Comparison of the results of NMR and debranching assay

- Glucan glycogen ratio

Regarding the glucan to glycogen ratio as well as the degree of branching of the samples measured by NMR and the ones calculated by using the results of the debranching assay, (Figure 36) a difference in ratios depending on the assay used, could be observed.

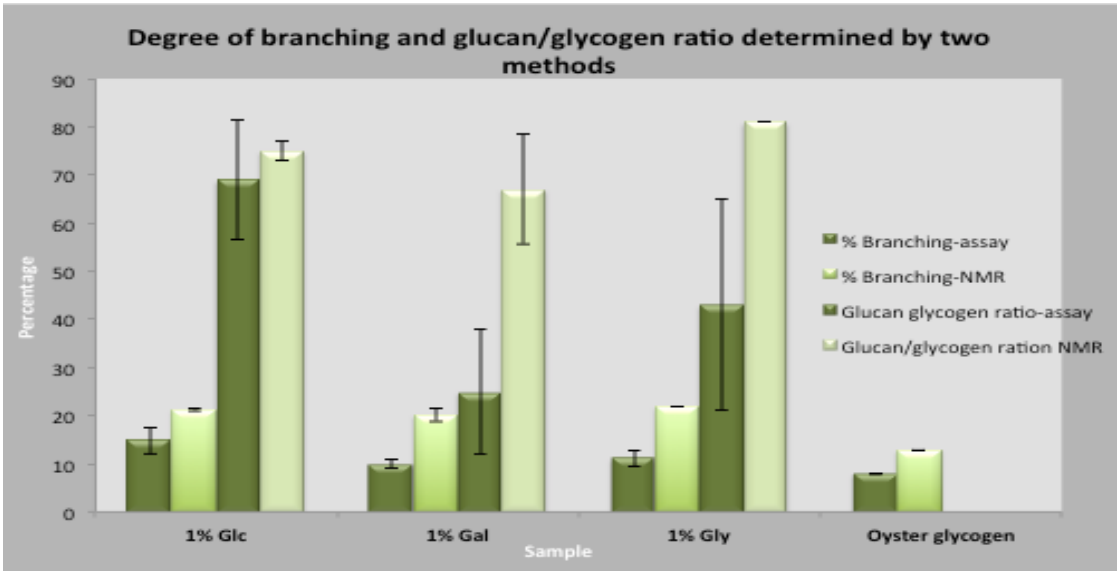


Figure 36: Comparison of the degree of branching and the glucan/glycogen ratio determined by either the NMR or the debranching assay. Dark green bars represent the results of the debranching assay and the light green ones the results of the NMR measurement. The standard variation is also shown for all samples. Values <0.5% are: Oyster glycogen (NMR & debranching assay), 1% glc (NMR), 1% gly (NMR)

For the glucan/glycogen ratio determined by NMR and calculated by using the formula it was seen, that no matter whether *G. sulphuraria* cells grew on Allen medium with either glc, gal or gly, the α-glucan extracted from these algae cells was around 70% to 80% more branched than the oyster glycogen.

The glucan to glycogen ration was calculated by use of the formula:

$$\frac{\%\ of\ branching\ (sample)}{\%\ of\ branching\ standard\ (\ Oyster\ glycogen)} = \frac{Glucan}{Glycogen}\ ratio$$

In comparison to that, the α-glucan to glycogen ration calculated from the results of the debranching assay differed quite a lot from the NMR results. Only the cells, which grew on Allen medium with 1% (w/v) glc showed nearly the same percentage of branching as the NMR did. A–glucan extracted from *G. sulphuraria* growing on Allen medium with 1% (w/v) gal or gly and further analysed by the debranching assay, had around 30% to 50% more branches in the molecule than the oyster glycogen. This ratio, confirmed without a doubt, that α-glucan extracted from *G. sulphuraria* was a

highly branched molecule. Nevertheless, the results of the glucan/glycogen ratio of the debranching assay were a lot lower and differed unexpectedly more, regarding the the different substrates used, than the ones measured by NMR.

- Degree of branching

Regarding the degree of branching of α-glucan, also a clear difference between the two methods was also detected (Figure 36, table 13). Regardless of *G. sulphuraria* growing on Allen medium with either glc, gal or gly, the α-glucan extracted from algae cells had an average degree of branching of 20% to 22%. In comparison to that, glycogen from oyster had a DB of 12%. So the algae`s α-glucan was around 10 % more branched than of the oyster glycogen.

For the degree of branching of *G. sulphuraria* determined by the branching assay, the values measured varied quite a lot depending on the nutrient supply used. Cells growing on Allen medium with 1% (w/v) glc added, had an average degree of branching of 14.7 ± 2.7%, whereas the ones growing on gal had an average degree of branching of 10 ± 1.1% and the ones growing in medium with 1% gly added had a value of around 11.1 ± 1.7%. In comparison, oyster glycogen, with an average degree of branching of 7.5% measured by Nelson Somogyi and Anthrone assay, was less branched. It was between 4% to 10% less branched than α-glucan extracted from *G. sulphuraria*.

This points to the fact that no matter which method was used for the determination of the degree of branching, both identified α-glucan extracted from *G. sulphuraria* as a highly branched molecule in comparison to glycogen from oyster. Be that as it may, the results measured by NMR were quite constant during all measurements and did not differ gravely in regards to the different carbon sources used as an additive to the growth medium. Ultimately, these results seemed to be more reliable, as the standard deviation of the samples too was a lot lower for samples measured by NMR than for the ones determined by the debranching assay.

Table 13: Illustration of the degree of branching and the calculated glucan/glycogen ratio of α-glucan extracted from *G. sulphuraria* growing on Allen medium with 1% of glc, gal or gly and from oyster glycogen. The glucan /glycogen ratio was calculated out of the degree of branching received from the NMR results as well as the results from the debranching assay.

Sample	% of branching	Glucan/gylcogen ratio	Average of the glucan/glycogen ratio
NMR-measurements			
Oyster gylcogen	12.09%	/	/
1% (w/v) Glc	20.96%	1.73	1.75
1% (w/v) Glc	21.41%	1.77	
1% (w/v) Glc	21.19%	1.75	
1% (w/v) Gal	20.79%	1.72	1.67
1% (w/v) Gal	21.19%	1.75	
1% (w/v) Gal	18.59%	1.54	
1% (w/v) Gly	21.93%	1.81	1.81
1% (w/v) Gly	21.93%	1.81	
1% (w/v) Gly	21.83%	1.81	
Debranching assay measurements			
Oyster glycogen	7.9 %	/	/
Oyster glycogen	7.8 %	/	/
1% (w/v) Glc	18.7 %	1.51	1.67
1% (w/v) Glc	12.6 %	1.67	
1% (w/v) Glc	14.3 %	1.81	
1% (w/v) Glc	13.5 %	1.71	
1% (w/v) Gal	9.2 %	1.1	1.3
1% (w/v) Gal	9.5 %	1.2	
1% (w/v) Gal	11.6 %	1.4	
1% (w/v) Gal	9.7 %	1.31	
1% (w/v) Gly	13.1%	1.68	1.47
1% (w/v) Gly	10.1 %	1.3	
1% (w/v) Gly	10.2 %	1.3	

- Average chain length

Regarding the ACL determined by these two methods, also a big difference could also be detected (Figure 37).

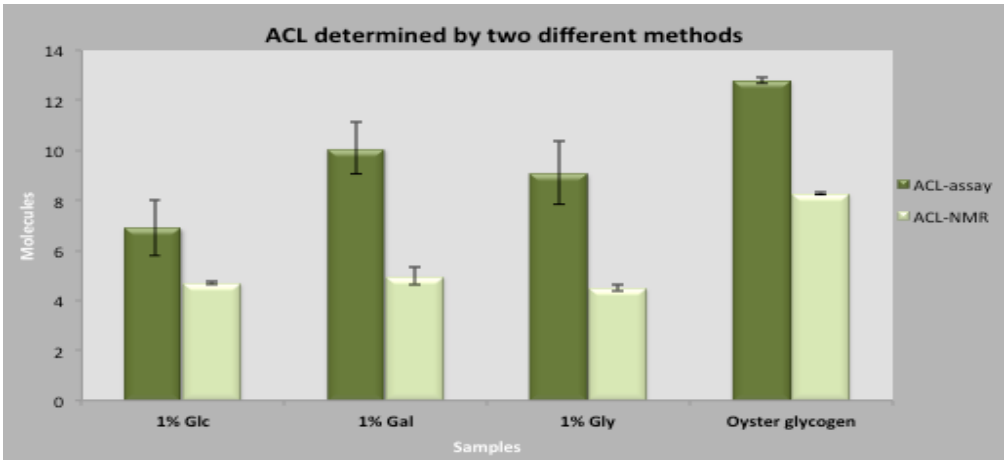


Figure 37: Comparison of the average chain length of α-glucan extracted from *G. sulphuraria* and oyster glycogen by use of two different methods. The dark green bars represent the results of the debranching assay and the light green bars the ones of the NMR measurement.

The average chain length determined by NMR was around four to five residues long, whereas the one of the oyster glycogen was around 10 residues long. Regarding these results, it could be said, that the chain lengths of α-glucan from *G. sulphuraria* was half the length of the compared oyster glycogen. It could also clearly be seen through these results, that the standard deviation of the samples measured by NMR was very small.

For the ACL measured by the debranching assay it was seen, that glycogen from oyster consisted of 13 molecules, whereas the chain length of α-glucan extracted from *G. sulphuraria* cells had an average chain length between 7 to 9 residues depending on the carbon source used as an addition to the growth medium. Considering literature, it was referenced that the average chain length of oyster glycogen lies at around 13 residues, which at least made the results of the oyster glycogen more reliable (Akai et al. 1971).

No matter the methode used, the ACL of oyster glycogen remained around five molecules longer than the one of *G. sulphuraria*, which made the α-glucan extracted from the algae cell a highly branched molecule.

4.3.2.3.Debranching with isoamylase and analyses of the chain length distribution with HPAEC by Dionex

Regarding the chain length distribution of the samples and that of the standard, it was proven, that *G. sulphuraria* had shorter chain lengths than glycogen from oyster. In figure 38 the average chain length distribution of α-glucan extracted from *G. sulphuraria* growing on three different carbon sources, as well as the gylcogen extracted from oyster is shown.

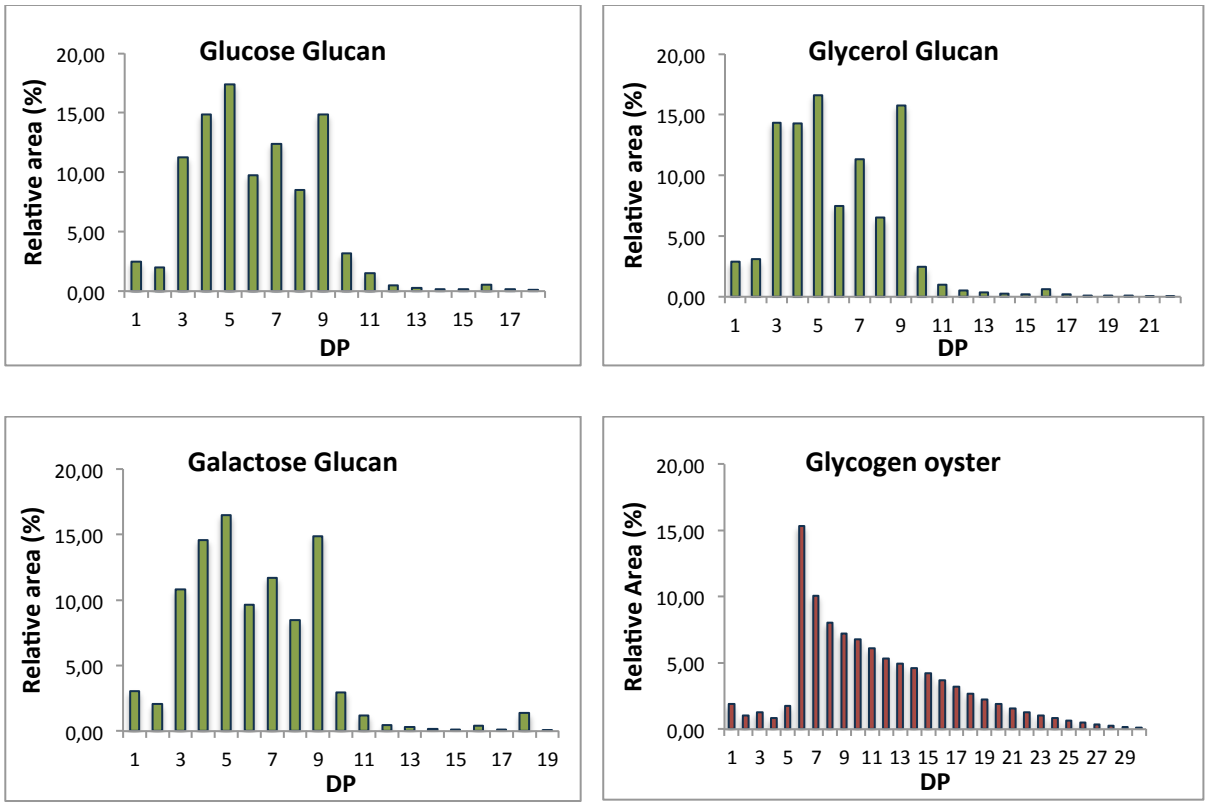


Figure 38: Comparison of the chain length distribution analysed from α-glucan extracted from *Galdieria sulphuraria* growing on Allen medium with either 1% (w/v) of glucose, galactose or glycerol as a nutrient supply or of oyster glycogen.

As seen, the degree pf polymerization (DP) of α-glucan from *G. suphuraria* is smaller in value than the one of the oyster-glycogen. This stemmed from the fact that α-glucan had short branched chain lengths, which meant that the α-glucan of *G. sulphuraria* was composed of shorter oligosaccharides. The α-glucan extracted from *G. sulphuraria* had a DP between 1 to 9. In comparison to that, the results of the HPAEC of the glycogen from oyster showed, that it had a higher degree of polymerisation, which resulted in longer chain fragments. The chain lengths of the oyster glycogen had a DP starting from 6, which represented maltoheptaose. Fragments with a DP of 6 were the most abundant ones, which was also stated by the work of Matsui et al. (1996). For α-glucan of *G. sulphuraria,* DP of 3 to 9 were the most frequent.

As depicted in the graph in figure 39, it was observed that most of the peaks of *G. sulphuraria* appeared between 2 to 20 minutes of measuring, which represented short oligosaccharides, whereas the peaks of glycogen from oyster started to appear at after 20 minutes of measurement.

The most common oligosaccharides in α-glucan extracted from *G. sulphuraria* were maltotriose, maltotetraose, maltopentaose as well as maltononaose. So this assay was able to prove the fact that the DP of α-glucan was lower than the one of oyster glycogen. Hence, the α-glucan of *G. sulphuraria* consisted of shorter fragments than the glycogen extracted from oyster (Figure 39).

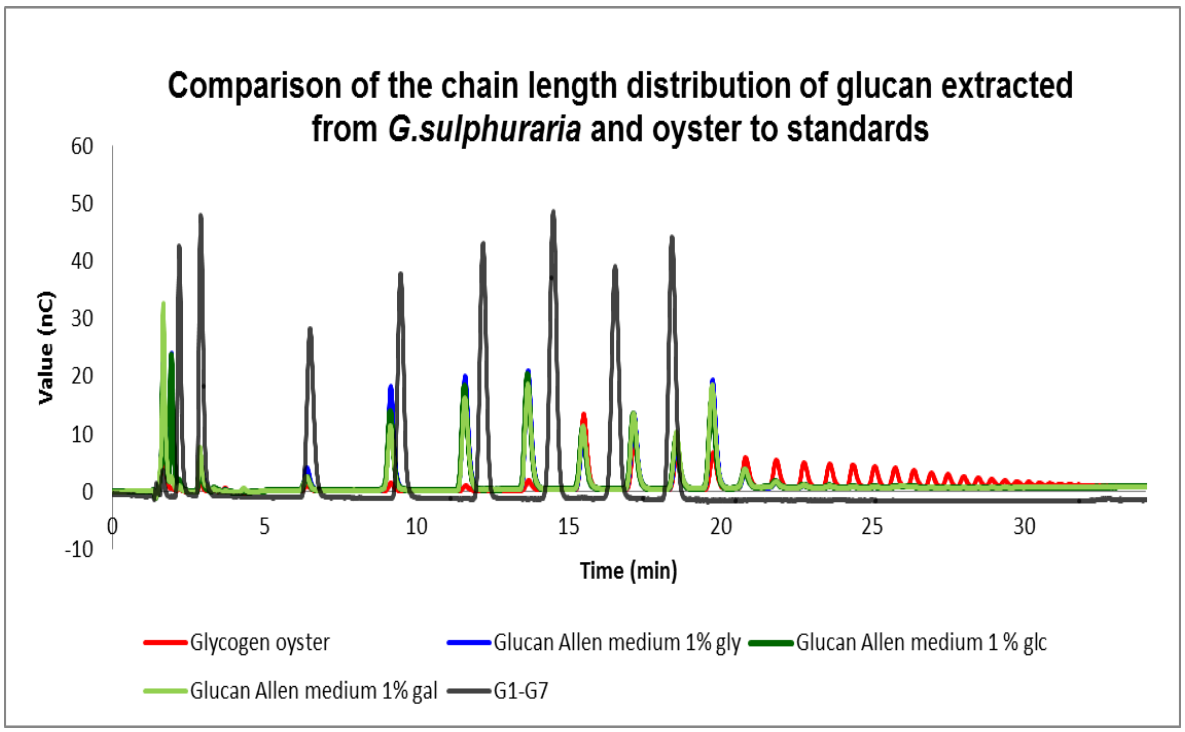


Figure 39: Comparison of the chain length distribution of *G. sulphuraria* growing on Allen medium + 1% glc, gal or gly to oyster glycogen. The grey peaks represent the standards used for this measurement.

4.4. Starvation experiment

During this project, the growth ability of *G. sulphuraria* in times of nutrient starvation was analysed. During starvation, *G. sulphuraria* can use its accumulated storage compound, to survive these harsh conditions. This was tested by growing these algae heterotrophically on Allen medium without either a nitrogen and carbon source (-N-C) or just without a carbon source (-C) over a month.

At different points in time, samples were taken and analysed with regard to their total carbohydrate content in the cells and by determination of the CFU/mL.

The total carbohydrate content of *G. sulphuraria* growing on carbohydrate deficient medium decreased a lot faster than the one of the algae growing on Allen medium without carbon and nitrogen supply. After around 24 hours the amount of carbohydrates in the sample achieved stability but it never reached zero (Figure 40). For the cells growing in Allen medium without carbon source, the amount of total carbohydrates stayed at 2.35 ± 0.38 mg carbohydrate/ml medium which is equivalent to 14.09 ± 2.94% of the total carbohydrates in cell after 24 hours of growth. For cells growing on Allen medium without nitrogen and carbon source, the total carbohydrate amount in the cells stayed higher than the one of cells growing on the Allen medium only lacking a carbon source at around 5.87 ± 1.6 mg carbohydrate / ml medium which is equal to 31.43 ± 5.58% after 32 hours of growing. So after growing *G. sulphuraria* cells incubated in cultural medium without N-C-source for a month, a lot more carbohydrates were left.

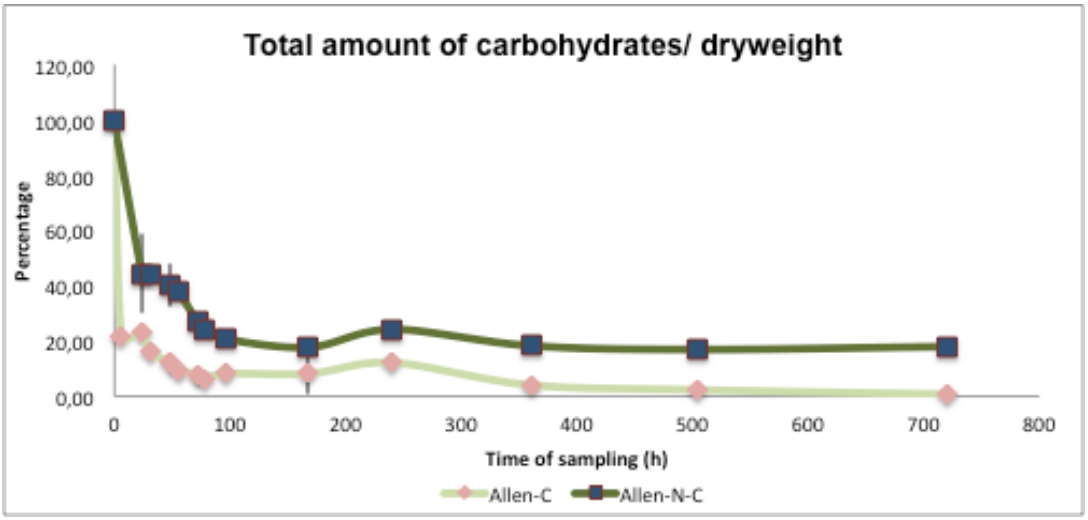


Figure 40:Total amount of carbohydrates determinded in *G. sulphuraria* growing in Allen medium either without nitrogen and carbon source or just without carbon over one month.

Concerning the CFU / mL, it was observed that the CFU was the highest with $2.18*10^8$ cells for *G. sulphuraria* growing in nitrogen and carbon deficient medium and $2.87*10^8$ for the ones growing in medium with a lack of carbon after 24 hours and afterwards decreased very fast. The CFU of *G. sulphuraria* growing on Allen medium without a nitrogen and without a carbon source was always lower than the one of *G. sulphuraria* growing on culture medium without only a carbon source. Regarding the cell number over time (Figure 41), it was seen that the CFU was never zero. For *G. sulphuraria* growing in medium without nitrogen and carbon the CFU / ml stayed stable at $37*10^6 \pm 14*10^6$ and the ones growing in medium without carbon had a CFU / ml of $32*10^6 \pm 25*10^6$. Through interpretation of the graphic of the CFU over time, it can therefore be deducted, that the cells were able to survive times of nutrient starvation.

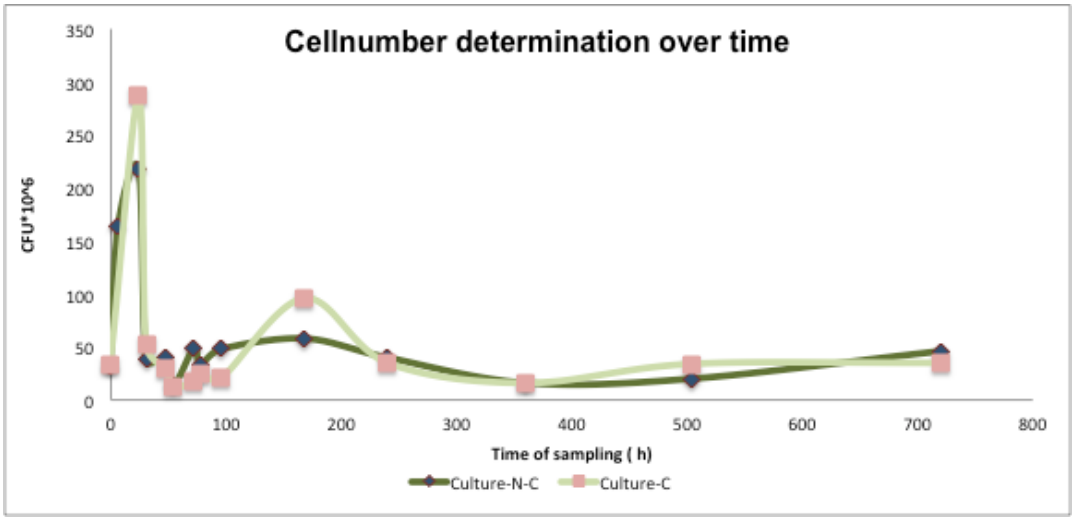


Figure 41: Comparison of the CFU/mL of *G. sulphuraria* growing in cultural medium either without nitrogen or without nitrogen and carbon source over one month.

It was proven by this experiment, that the medium composition has an effect on the survival capacity as well as on the total amount of carbohydrates in the cell.

5. Discussion:

5.1 Growth and chlorophyll production of *Galdieria sulphuraria* over time

As stated by Gross and Schnarrenberger (1995), Juneja, Ceballas and Murthy (2013) and Tischendorf et al. (2007), *G. sulphuraria* is able to grow auto-, mixo-, as well as heterotrophically on several carbon sources including glc, gal and gly - the three carbon sources used during the experiments.

G. sulphuraria was grown in medium with either 0.1%, 0.5% or 1% (w/v) of an additional carbon supply. Under these mixotrophic conditions it was observed that cells growing with the smallest amount of an additional nutrient source, turned green already after just one day of growing, whereas the ones with 1% (w/v) of carbon source turned green mere 4 days later. This can be explained by the fact that, the more additional carbon source is added, the more *G. sulphuraria* shows its metabolic flexibility and the more mixotrophic growth takes place. Under mixotrophic conditions, carbon sources as well as light can be used as energysources. This fact was also explained in the work of Oesterhelt (2007), in which glucose down regulated the number of thylakoid membranes, just as it did with the amount of photosynthetic pigments. Exactly these photosynthetic pigments are responsible for performing photosynthesis and producing photosynthetic pigments. Thus, it can be said that not only glc, but also gal regulates the production of pycobiliproteins in the same dimension (Oesterhelt et al. 2007).

This experiment showed that smaller amounts of an additional carbon source lead to a more autotrophic way of life. Because of the fact that 0.1% (w/v) of sugar is degraded very fast, photosynthesis is the main process *G. sulphuraria* gets its energy from. In comparison, *G. sulphuraria,* growing with 1% (w/v) of additional carbon source, has two possibile ways to maintain its energy over a long period of time. But before all carbon is taken up, the focus is set on the process of photosynthesis.

5.2.Growthcurve determinations and analyses of the samples over time

Due to the fact that Sloth`s experiment (2006), which identified the specific growth rates of *Galdieria* growing on either glucose or glycerol as being independent from the use of light, the results of the determination of the beginning of the stationary phase can be safely used to perform the alpha glucan extraction (Sloth, Wiebe, and Eriksen 2006).

The determination of growth curves of *Galdieria sulphuraria* growing in Allen medium with three different carbon sources showed, that the ones growing with glycerol achieved the highest optical densities with 11.29 ± 1.56 after 4 days of growing, but also took the longest time (96 h) to reach the stationary phase. This can be explained by fact that the exponential phase also starts a little later since the cells need to express the machinery for assimilation of this compound before starting to use it actively.

For glc and gal, nearly the same optical densities were achieved with only one difference. The stationary phase of cells growing with a glc supply was reached a little earlier, after only 73 hours. Generally, it can be assumed, that glc is taken in the fastest by cells, followed by gal, and last but not least gly.For glucose and galactose it can be assumed that they are more common substrates as glycerol and therefore cells can assimilate them immediately.

It could also be observed during the experiment, that at the same time as the stationary phase was reached, all of the additional carbon source added as a growth enhancer was used up. Thus, it can be concluded, that as long as cells can use both, light and external carbon source, growth increases. If there is no additional carbon source left, they start to deplete their supply of intracellularly stored carbohydrates. The results show, that at the same time when all sugar is used up and the stationary phase is reached, the degradation of intracellular carbohydrates starts to prevent cell death. This was also confirmed in the research of Gross and Schnarrenberger (1995), who explored the growth habits of *Galdieria sulphuraria*. They also observed that as long as a sugar is there as a nutrient source, cell growth will continue (Wolfgang Gross and Schnarrenberger 1995; Christine Oesterhelt, Schnarrenberger, and Gross 1999).

To sum up, the experiment was able to prove, that *G. sulphuraria* can grow in Allen medium with either an additional glc, gal or gly source. Under heterotrophic conditions cells use this sugar to synthesize storage compounds which are depleted in times of starvation. In the same moment as all sugar is used up, the degradation of carbohydrates starts. Depending on the ACL of the molecule the degradation can be either fast or slow (Wang and Wise 2011).

5.2.1. Observations during the preparation of samples for further analyses

After the cells had been broken down, a blue pigment, called pycocyanin (PC) was released. PC is a phycobilliprotein, which consists of an α and a β subunit with either one α or two β phycocyanobilin groups which are covalently bound. This blue pigment can be produced by *G. sulphuraria* heterotrophically or under mixotrophic conditions (Sloth, Wiebe, and Eriksen 2006).

As can be seen in Figure 36, the less carbon source was added to the medium and the faster the degradation was, the earlier phycocyanin was produced. For cells growing in Allen medium with 1% of carbon source, it took substantially longer until all carbon sources were degraded and the production of phycocanin commenced. This phenomen was also confirmed by Sloth (2006), who reported that for G. *sulphuraria* after all carbon was depleted, the production of pycobiliproteins started.

Under mixotrophic conditions, cells are exposed to light which can also have a negative effect on the production of phycobilliproteins. Arad and Yaron (1992) stated after studying *Pophyridium* sp., that the higher the light supplement is, the less phycobiliproteins are produced and the slower is the growth of the organism.

In summary, there are several parameters that influence the produtcion of phycocyanin but the main factor in this experiment, however, was the addition of a carbon source. Based on the literature, it was confirmed, that the more carbon sources were additionally added, the longer it took to start the production of phycobiliproteins.

At this time, it is crucial to mention that neither growth nor pigmentation experiments are reproducible (Wolfgang Gross and Schnarrenberger 1995). Their results, can slightly vary from experiment to experiment due to the metabolic flexibility of algae. It can therefore not be assumed that cells always show the same growth characteristics as in the described experiment.

5.3. Alpha glucan extraction

In reference to the culture's colour after 97 hours of growing it can be said, that cells growing on either 1% (w/v) glc or gal were slightly green, whereas the ones growing in Allen medium supplied with glycerol stayed pale. This can be explained by the fact, that *G. sulphuraria SAG* 108.79 strain being a mixed population which means it consists of green and white cells. Under heterotrophic conditions as previously stated by Allen (1959), cultures consist of both types of cells, green and white ones.

Naturally, under heterotrophic conditions, the white cells are the dominant ones. However, it has yet to be proven whether the green cells are contaminated by pale cells or if the green cells loose their pigmentation while growing heterotrophically (Gross and Schnarrenberger 1995).

As assumption that Allen medium with 1% glycerol contain less green cells, which are responsible for the production of chlorophyll, than the ones with 1% glc or gal as addition nutrition added as per the results of the experiment can be made.

A comparison of *Galdieria sulphuraria* grown in Allen medium with 1% (w/v) glc or gal showed that the ones growing on Allen medium + 1% (w/v) gal seemed a little greener than the ones growing on glc. This effect could also be observed under mixotrophic conditions, however, the results were far more intense. Cells growing in Allen medium with gal added as an additional carbon source were the greenest, followed by the ones with glc as an additional nutrient and followed by *G. sulphuraria* grown in Allen medium with 1% (w/v) gly, which stayed pale after growing for 97 hours, regardless of whether auto- or heterotrophically cultivation was used.

The same effect was also observed by Gross and Schnarrenberger (1995) who tested the chlorophyll content of *G. sulphuraria* 074 growing hetrotrophically. Also their results showed that the amount of chlorophyll was the lowest in cells growing on glycerol and the highest for the ones growing on gal.

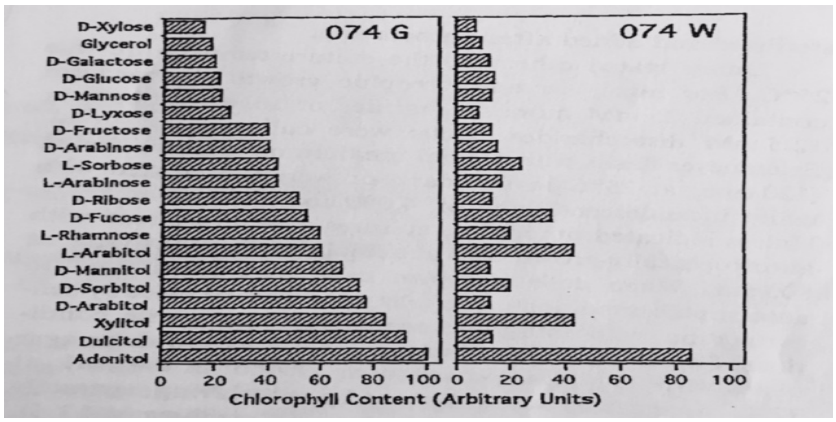


Figure 42: The amount of chlorophyll produced in the *G. sulphuraira* growing heterotrophically on different carbon sources (Gross and Schnarrenberger 1995)**.**

5.3.1. Determination of the optical density, dry weight of the pellet and of the extracted α-glucan

Regarding the ODs of *G. sulphuraria* growing heterotrophically in Allen medium with either 1% glc, gal or gly in comparison to mixotrophically growing cells, the the OD_{800} values were obviously lower after growing for 97 hours. This can be explained by a statement by Stadnichuk et al. (2007), which claims that under heterotrophic conditions, the carbon intake is faster than the increase in number of cells. This can be due to the fact that under heterotrophic conditions *G. sulphuraria* accumulates a storage compound, known as floridean starch, concurrently with cell growth (Stadnichuk et al. 2007).

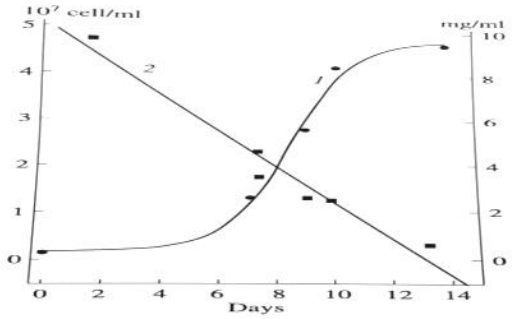


Figure 43: The correlation between carbon intake and cell growth. It is seen that the armount of carbon intake is faster than the cell number increases (Stadnichuk et al. 2007)**.**

Regarding the yield of α-glucan extraced from *Galdieria sulphuraria,* it was shown that more than 20% of the cell during the stationary phase consists of storage polysacchride. The amount of α-glucan increases with the OD_{800}. Thus, it can be concluded, the higher the OD_{800} is, the more α-glucan can be extracted.

G. sulphuraria growing on a 1% (w/v) glc containing Allen medium, formed the most α-glucan. This can be explained by the fact that *G. sulphuraria* growing on glucose reached its stationary phase as the first out of all three carbon sources used as a growth enhancer. Because of the fact that the stationary phase was already reached after 73 hours, cells can focus all energy on the formation of storage polysaccharides.

All in all, this part of the experiment was able to show that no matter if glc, gal or glycerol were added as a carbon source for heterotrophic growth, floridean starch was always produced which engage up to a quarter of the cell mass.

5.4. Analyses of alpha glucan

5.4.1. Monosaccharide composition by acid hydrolysis and HPAEC separation

A-glucan consists of glucose monomeres. With this experiment it was tested if α-glucan extrated form *G. sulphuraria* contains just glucose molecules or if there are still some other molecules still present after the filtration. Furthermore, a goal of this experiment was to see if *G. sulphuraria* really accumulates storage polysaccharides.

For this experiment a glc concentration of 100 µg/ml was used, but the measured glc concentration of the sample was at around 80%, for oyster, as well as *G. sulphuraria* samples. This can be explained by the fact that not all polysaccharides precipitated at 80% EtOH. Therefore it could be that the sample still contains some oligosaccharides, which of course decrease the pureness of the sample.

Regarding the results of the acidic hydrolysis and the DIONEX measurements for the monoaccharide composition of the samples, it was also seen that not only glc, which is the building block of an α-glucan chain, was detected. Beside the glc signal, two other signals were detected. These peaks appeared in all samples except oyster glycogen.

One of this peak is supposed to be galactose because of the fact that it gave a signal after 13.9 minutes, which was the retention time of the gal standard. This gal peak can be derived from the accumulation of gal from the biofilm algae growing on or as part of a heteropolysaccharide, which was divided into glc and gal. To check if this gal is intra- or extracellularly stored, it would be possible to introduce an additional centrifugation step to the protocol. If the peak also appears after centrifugation, it is proven that it is an intracellular compound. Another idea to check if this signal really belongs to gal, is to perform a GOPOD assay to measure gal. If it is really supposed to be galactose maybe a more purified enzyme can be used to convert galactose to glucose or to improve the extraction protocol.

The second peak could be a signal of a RNA or DNA, which is present in the cells, because the program used for the analyses can detect C_5-sugars as well as C_6 sugars. It could also be that this peak belongs to isomaltose, which α-1,6-linkages bound to a maltose molecule. In this case, maybe the hydrolysation was not completely done.

To really check if the signals are different monomers or if it is just the signal of a glc molecule which is differently branched, it would also be possible to perform a gelelectrophorses to check their molecular weights. It is also possible to perfom a HPLC for monosaccharide analysis.

The study of Stadnichuk et al. (2007) showed the same results. During his experiment he hydrolized *G. maxima* cells. The hydrolysates also contained three types of sugars; glc, gal and mannose, whereas glc was the dominant sugar. He found out that under heterotrophic conditions in comparison to autotrophic ones, glucose concentration increased by a factor of four and was at around 80 %, the same, as we measured, which indicates the storage of a α-glucan.

In summation, it can be said, that this experiment showed, that *G. sulphuraria* produced a storage polysaccharide under heterotrophic conditions. Regarding the monosaccharide composition of the storage compound it was seen that it mainly consists of glc, up to 80%, but also some other compounds. One compound is supposed to be gal, the other one is not clear yet but to date, it is known, that several possibilities exist on how to identify which compound these peaks belong to if it is not just a glucose molecule with different conformation.

5.4.2. Average chain length determination by NMR and debranching assay

This experiment was done to determine the average chain length of α-glucan extracted from *G. sulphuraria* cells in comparison to the glycogen isolated from oyster. Furthermore, we wanted to see if α-glucan extracted from algae was a highly branched molecule and if it can increase the survival ability of cells. The determination of the average chain length was done by two different methods: NMR and debranching assay using the results of Anthrone and Nelson Somogyi measuremets for the calculation of the ACL.

For NMR the average chain length for oyster was at around 8 glc residues and the ones of *G. sulphuraria* growing in Allen medium with an addidional carbon source, either glc, gal or gly was at around 4. The NMR results showed that α-glucan extracted from glycogen is 70% more branched as the glycogen from oyster.

Compared to that, the debranching assay gave us an ACL of 13 glc residues for oyster glycogen, which is similar to the ACL referenced in literature (Akai et al. 1971)

and an ACL for α-glucan of *G. sulphuraria* between 6 to 10, depending on the carbon source used.

It can be seen that the results vary substantially. It seems that the values of the NMR exhibited an ACL only half of the length of those measured by the debranching assay. This could due to the fact that not all of the chains had been debranched. On the other hand it could also be explained by the fact by a possibles approach problem in the NMR computer program. The latter assumption derives from the fact that, former analysis performed of the research group of the Aquatic Biotechnology Institute showed an ACL for oyster glycogen of 12. Nonetheless, both experiments were able to show that the glucan extrated from *G. sulphuraria* is a highly branched molecule.

As can be seen in the results of the ACL measured by the debranching assay as well as the glucan glycogen ratio of *G. sulphuraria* grown on three different carbon sources, these values too depict grave variation. Normally, regardless if it is growing on glc, gal or glycerol, the same average chain lengths should be detected. The variation within the results of the debranching assay can be explained by neither the Anthrone nor the Nelson Somogyo assay being as precise as the NMR. Negative effects of an older Anthone or Nelson reagent, as well longer or shorter incubation periods can influence the results. Bubbles in the cuvetts or samples that have not been completely cooled down prior to measurement, can also influence the results. Furthermore it could be that the debranching of the glucan chains did not work for all samples at the same level.

Regarding the glucan to glycogen ratio, especially for glc, a 70% increase in branching was observed. Also for *G. sulphuraria* growing on glc, gal and on gly it was proven, that α-glucan is a highly branched molecule, regardless of the method used for the determination.

Due to the fact that α-glucan extracted from *G. sulphuraria* has a smaller ACL and is therefore a highly branched molecule with a lot of α-1,6-linkages makes the storage compound into a more stable molecule in comparison to the less branched oyster glycogen and consequently more durable when undergoing glucan degradation (Wang and Wise 2011). Also the relation of the ACL to the survival ability was also tested by Wang and Wise (2011), who found that the longer the chain length of a molecule was, the shorter the survival time. For instance, as seen in Table 14, the

ACL of *Anthrobacte*r is 7 with a survival time of 80 days, whereas *E. coli* with an ACL of 12 has a survival time of 36 hours, a mere half of that of *Anthrobacter* (Wang and Wise 2011).

Table 14: A comparison of the ACLs of different organisms and their survival time. It can be clearly seen that the longer the ACL is, the shorter the survival time is (Wang and Wise 2011).

Bacterial	ACL	50% Survival time
***Anthrobacter* spp.**	7-9	80 d
E.coli	12	36 h
Mycobacterium tuberculosis	7-9	52 d
Thermococcus	7	24,5 d

In conclusion, it can be said, that both experiments confirmed that α-glucan extracted from *G. sulphuraria* is a highly branched molecule which increases the survival capacity of algae in times of starvation. Generally, the results of the NMR are more precise, because the standard deviation is a lot lower. The only problem that was observed during the experiment was that the values received from the NMR were half the size of the ones of the debranching assay, which could be due to a misadjustment in the NMR program. Looking at the gulcan to glycogen ratios, both type of experiments showed that α-glucan of *G. sulphuraria* was around 70% more branched than oyster glycogen. Hence the problem of the differences in the ACL of the molecule could be neglected.

5.4.3. Debranching with isoamlyase and analyses of the chain length distribution with HPAEC by Dionex

As literature stated a common enzyme for the debranching reaction of algae`s glucan is isomaylase of *Pseudomonas* (Shimonaga et al. 2006).

To performing the experiment oyster glycogen was used as a standard with an average DP of 15. The same results were achieved by Akai et al (1972) during his experiment.

The results showed that oyster glycogen has a broad distribution of chain lengths, ranging from a length of 3 up to 30 glc residues. The major peak was at a DP of 6, which was also stated by Matsui et al (1996). In comparison to that, the chain lengths of *G. sulphuraria* consists of 1-11 C-sugars. There was no difference

detectable by observing the chain length distribution of growth on different carbon sources. This indicates that the storage polysaccharide accumulated by *G. sulphuraria* consists of short chain lengths, which makes it a highly branched molecule.

Comparing the results of the chain length distribution of Shimonaga et al. (2006) to results received from the experiments conducted for this thesis, same values were achieved. The chain length distribution of *G. sulphuraria* showed similarities to the glycogen profile of cyanobacteria or *C. Caldarium* (Shimonaga et al. 2006), which hinted that floridean starch is more a glycogen-like than a amylopectin-like structure. Exactly these results showed that the DP of *G. sulphuraria* was lower than the probe of oyster glycogen or other Rhodophyta, which were tested in the experiments of Shimonaga et al. (2006). Figure 44 shows the relation of long and short glucose chains. Depending on the degree of branching of the tested organisms, the storage polysaccharide type, to which they belong, could be characterized.

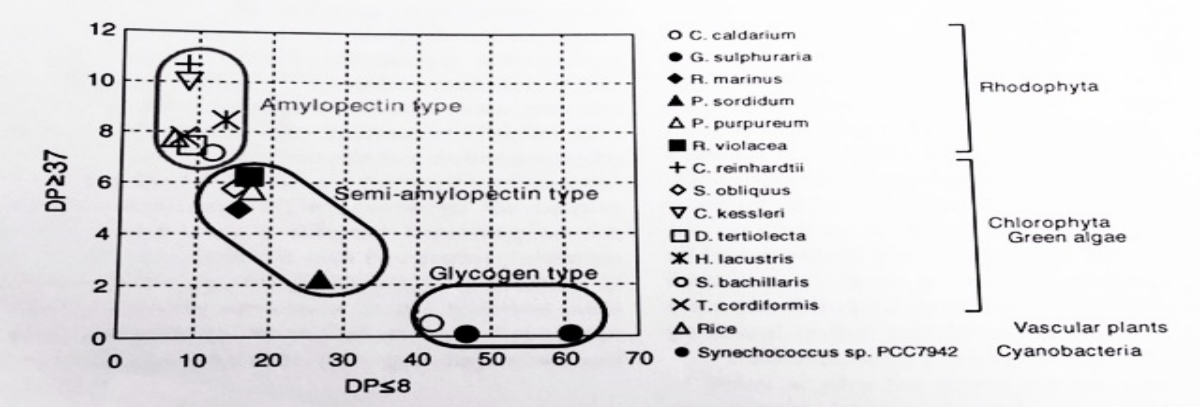


Figure 44: Correlation between the DP and the type of storage polysaccharide they belong to. The lowest DP have organisms with a glycogen type of storage compound. The highest DP is found in organisms with amylopectin as their storage molecule (Shimonaga et al. 2006).

Furthermore, figure 44 shows that the α-glucan of *Galdieria sulphuraria* consists of lot of many short α-1,4 chains which makes it to a highly branched molecule.

To sum up, it can be said that the studies of Shimonaga et al. (2006) in compared to the results received from these experiments, showed the same results. *G. sulphuraria* is a highly branched molecule with short average chain lengths in comparison to oyster glycogen, but also to other Rhodophyta. It was also recorded that the storage compound of this algae type is more similar to glycogen than to amylose.

5.5. Starvation experiment

Considering the results of the starvation experiment, both the nitrogen deficient, as well as the nitrogen and carbon deficient growth-medium had a significant influence on *G. sulphuraria*. Hence, it can be concluded that nutrient limitation has a enormous effect on the biochemical composition and growth habits of algae, depending on the limiting nutrient and to what degree this nutrient is restrictive (Ankita Jujena et al. 2013, Kilham et al. 1997).

Generally, the algae`s growth rate is commensurate to the uptake rate of the most limited nutrient under optimal conditions. For *G. sulphuraria:* pH = 2 and T = 40 °C and normally follows the Michaelis Menten equation (Titman and Kilham 1976).

As previously mentioned in the introduction, nitrogen as well as carbon or phosphor can influence the algaes metabolism.

Nitrogen, which is an essential marconutrient to support the algae's growth (Juneja, Ceballos, and Murthy 2013) is also imperative for nucleic acids and protein synthesis. Moreover, nitrogen is one of the biggest growth limiting factors for eukaryotic organisms and is additionally one of the first nutrients to be used up during the cultivation (El-Kassas 2013). Hence, when nitrogen is limited, the amount of nitrogen storage materials produced, is limited as well (Hu et al. 2008).

Carbon, which has to be supplied to algae cells to support it`s growth has an enormous influence on growth and reproduction. The carbon absorbed by algae can be used for three different metabolic functions:

1. Respiration
2. Energy source
3. Raw material for formation of additional cells

Thus, when carbon is limited, it results in a reduction of the algae's growth because of the lack of this energy source (Juneja, Ceballos, and Murthy 2013)

Looking at the results received from the starvation experiment, it can be derived that nitrogen deficient, just as nitrogen and carbon deficient mediums, influence *G. sulphuraria*.

By taking a look at the total intracellular carbohydrate content of *G. sulphuraria*, a clear difference between cells growing in nitrogen deficient or nitrogen and carbon

deficient medium could be observed. The same phenomena was reported by Zevenhuizen`s (1966) experiment using *Arthrobacter* strains growing under the same conditions. The carbohydrate content of cells growing in a medium lacking a nitrogen was very high (Figure 45), which was also true for *Arthrobacter*. (Zevenhuizen 1966)

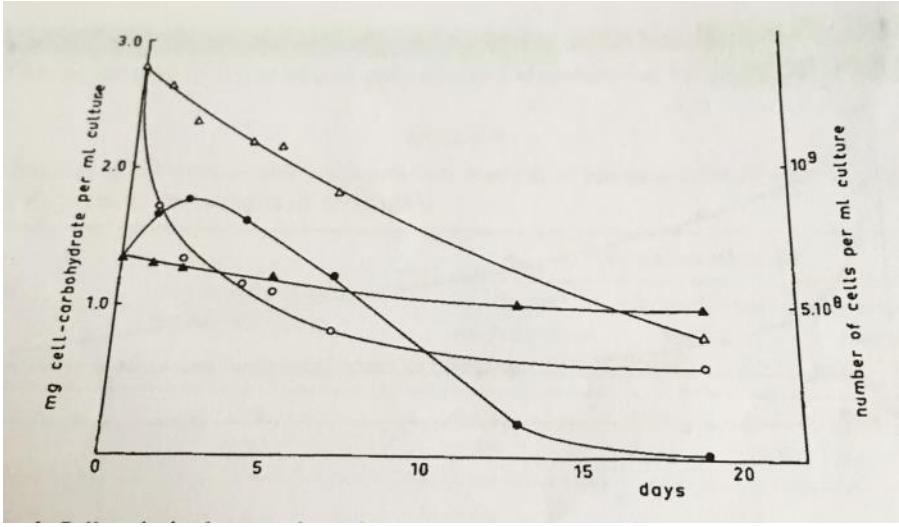


Figure 45: Zeverhuizen's results of the cell carbohydrate and cell number over time of Arthrobacter aerated in the presents or in the absence of nitrogen; o = carbohydrate content of cells in carbon deficient medium, △ = Carbohydrate content of cells in nitrogen and carbon deficient medium; ▲= Cell number in –N-C medium,• = Cellnumber in –C medium (Zevenhuizen 1966)

Cells growing in carbon deficient medium had a higher intracellular carbohydrate content over time than the ones growing in nitrogen and carbon deficient medium. This phenomen can be explained by the fact that in Allen medium lacking of carbon, a marconutrient was still available and growth was not yet limited. As mentioned above, *G. sulphuraria* uses nitrogen together with intracellular carbon to synthesize proteins. Due to the fact that all the energy goes into the synthetization process, the degradation of intracellular cabohydrates was faster than for cells growing in Allen medium without a carbon and nitrogen source.

- **Cellnumber**

The number of cells growing in either nitrogen or nitrogen and carbon deficient medium resulting from the starvation experiment, was the same as in Zeverhuizen`s experiment. (Figure 45) The cellnumber was influenced by the source and the amount of the limiting nutrient. The CFU of *G. sulphuraria* growing in Allen medium either without N or without C and N, increased during the first two days of cultivation but declined rapidly afterwards. Also, Zeverhuizen (1966) stated that the cell number of Arthrobacter growing in a nitrogen deficient medium, decreased faster than the ones growing in a carbon and nitrogen deficient medium.This phenomenon could

also be observed for *G. sulphuraria* cells, which can be explained by the fact, that all the energy had already been used to synthesize proteins (Zeverhuizen 1966).

It was also reported that after two days of growing the CFU of cells growing in carbon deficient Allen medium was higher because of the fact that cells still used nitrogen as an energy source for splitting and synthesizing proteins.

Moreover, it has to be mentioned that the cell number received from counting the cells had to be regarded with care, because as it is known, *G. sulphuraria* is metabolically flexible. Thus, it could be that the cells were in the division state and still appear as one colony on the plate. As can be seen in figure 41, neither the cell number in the starvation experiment, nor in the one reported by Zeverhuisen (1966) reached zero. The reason for this phenomenon could be that if all the carbon / nitrogen were used, cells were supplied with carbon released from dead cells and so some cells can survive these nutrient limiting conditions. This was also reported by the work of W. Gross and Oesterhelt (1999) by finding out that *G. sulphuraria* grows crytoendolithically and forms cell mats under a layer of silicous sinter. During winter time, light can not come through this layer and limit the photosynthesis process. This condition leads to the death of *G. sulphuraria* cells, which is then partly hydrolysed under the acidic conditions present in the favorable environment of this type of algae. Consequently, neigboring cells are able to take in these hydrolysed compounds. Provided with these carbon sources, *G. sulphuraria* can survive periods of starvation.

Thus, it can be concluded that *Arthrobacter* spp*,.* as well as *G. sulphuraria* are able to survive longer periods of nutrient limitation due to the degradation of the stored energy reserve, floridean starch. This phenomen can be explained by the fact that in the absence of exogenous nutrient supply, the degradation of the stored polysaccharide takes place. Endogenous respiration supplies the cells with energy to survive these harsh and stressing conditions. Due to the fact that floridean starch is a highly branched molecule, cells can survive longer times of nutrient starvation in comparison to other storage compounds.

6. Conclusion:

The thermophilic red micoalga *G. sulphuraria* is able to store a polysaccharide, called floridean starch, under heterotrophic conditions. Under these conditions most of the pigment production is suppressed and cells stay pale during the whole growth experiment (Allen 1959).

The fact that *G. sulphuraria* can accumulate high amounts of α-glucan under heterotrophic conditions was tested by taking a look at the structural composition and by performing an acid hydolysis and taking a look at the monosacchride composition of the samples. Under heterotrophic conditons, 80% of the α-glucan is built up of glc residues and the other 20%, can be some impurities or some still branched sugars. Because 80% of the α-glucan consists of glc, and also the results of the studies from Stadnichuk et al. (1998) were able to scientifically prove this. *G. sulphuraria* is able to produce this storage compound. Furthermore it was proven that this α-glucan extracted from *G. sulphuraria* is a highy branched molecule in comparison to the standard oyster glycogen. With an average chain length of 4 to 6 glucose residues, it is a lot more branched than oyster glycogen with an ACL of 13, which makes it an important energy source for algae and an interesting molecule for further research. Because of this highly branched character, floridean starch from *G. sulphuraria* resembles glycogen more than starch, and therefore the name floridean starch is not an accurate term for this storage compound isolated from *G. sulphuraria*.

As known to date, the more branched a storage compound is, the longer it can be used as an energy source and the longer the survival ability of cells. This is due the longer degradation periods of the polysaccharide. Exactly this fact makes floridean starch such an interesting candidate for the production of this natural energy reserve.

7. Future prospects

The main goal for scientists and a desire for most people is the quest for a naturally based economy. This, and their enormous field of application is why microalgae are becoming more and more the focus of research studies. From the production of natural dies received through the extraction of the pycobiliproteins, to biodiesel production to nutrition, the applications of algae are wide-ranging.

The results of the experiment have shown that floridean starch is a highly branched molecule which makes it an interesting subject for researchers. Regarding the fact that glycogen extracted from the human liver has an ACL of 10 (Manners 1991), which is around 5 glc residues longer than the ACL of α-glucan extracted from *G. sulphuraria*, makes floridean granulas into an useful storage compound with great future potential, once, it is possible to extract floridean starch in huge amounts and commercialize it. This natural substance could then be used as an alternative to protein shakes, unhealthy sugar drinks or proteinbars to refuel the body's energy level.

Exactly due to the highly branched character of α-glucan from *G. sulphuraria,* the energy level can be sustained for a longer period of time on the highest level possible. Because the molecule is so highly branched, it is more slowly degraded and can act as an energy reserve for much longer periods.

In conclusion, algae are an essential part of our ecosystem and have gained great attraction among research studies, which cannot be overlooked anymore. In the near future, more algae-based substances definitely will be commercially available due to its enormous field of applications. Who knows, perhaps soon we will forgo protein bars and, instead fill up our energy reserves with the new highly branched "wondermolecule", floridean starch?!

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List of abbreviations

– ACL	Average chain length
– ADPGlc	Adenosindiphosphate- glucose
– ATP	Adenosintriphosphate
– $CaCl_2*H_2O$	Calcium chloride dihydrate
– cAMP	Cyclic adenosine monophosphate
– CFU	Colony forming units
– CL	Chain length
– $CUSO_4$	Copper sulphate
– ddH_2O	Double distilled water
– D_2O	Deuterium oxide
– DNS	3,5 Dinitrosalicylic acid
– DP	Degree of polymerization
– $FeCl_3$	Iron(III) chloride
– Gal	Galactose
– Glc	Glucose
– Glc-1-P	Glucsose-1-phosphate
– Gly	Glycerol
– h	Hours
– H_2SO_4	Sulphuric acid
– H_3BO_4	Boric acid
– HPAEC	High performance anion exchance chromatography
– $KNaC_4H_4O_6$	Potassium sodium tartrate
– K_2HPO_4	Potassium hydrogen phosphate
– $MgCl_2$	magnesiumchloride
– $MgSO_4*7H_2O$	Magnesium sulphate heptahydrate
– Min	Minutes
– mM	Millimolar
– $Na_2MoO_2*2H_2O$	Sodium molybdate dihydrate
– NADH	Nicotinamide adenine dinucleotide
– NADPH	Nicotinamideadenine dinucleotide phosphate
– $(NH_4)_2SO_4$	Ammonium suphate
– NH_4VO_3	Ammonium metavanadate
– NMR	Nuclear magnetic resonance
– OD	Optical density

- PAC — Pulsed Amperometric Detection
- PC — Phycocyanin
- PPP — Pentose-phosphate-pathway
- PS I — Photosystem I
- PS II — Photosystem II
- ROS — Reactive oxygen species
- SN — Supernatant
- (w/v) — Weight per volume
- YNP — Yellow Stone National Park
- $ZnSO_4$ — Zinc sulphate

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Appendix

- **Allen medium**

Table 15: This table shows a preparation protocol of the components used to make Allen medium.

Macroelements				Trace elements			
$(NH_4)_2SO_4$	10 mM	$(NH_4)SO_4$	1.32 g/L	H_3BO_3	46 µM	H_3BO_3	2.8mg/L
	2 mM	KH_2PO_4	272 mg/L		9.1 µM	$MnCl_2*4H_2O$	1.8 mg/L
$MgSO_4$	1 mM	$MgSO_4*7H_2O$	246.5 mg/L	$ZnSO_4$	760 nM	$ZnSO_4*7H_2O$	0.218 mg/L
	0.5 mM	$CaCl_2*2H_2O$	73.5 mg/L		310 nM	$CuSO_4$	0.05 mg/L
$FeCl_3$	71 µM	$FeCl_2$	11mg/L	NH_4VO_3	200 nM	NH_4VO_3	0.023 mg/L
					100 nM	$Na_2MoO_4*2H_2O$	0.0242 mg/L

- **Preparation of the media weighting the ingredients**

Table 16: A description of the right amounts of components used for the preparation of 1 L, 500 mL or 200 mL of Allen medium.

Ingredients	1L	500 mL	200 mL
$(NH_4)_2SO_4$	1.32 g	660 mg	264 mg
KH_2PO_4	272 mg	136 mg	54.4 mg
$MgSO_4$	246.5 mg	123.25 mg	49.3 mg
$CaCl_2*2H_2O$	73,5 mg	36.75 mg	14.7 mg
$FeCl_3$	84 µl stock[12x] $FeCl_3$	42 µl stock [12x] $FeCl_3$	21 µl stock [12x] $FeCl_3$
Trace elements	500 µl stock [2000x]	250 µl stock [2000x]	100 µl stock [2000x]

- **Trace elements stock solution [2000x]:**

Table 17: Protocol for the preparation of the trace element stock solution used to make Allen medium

Ingredients	1L	500 ml	100 ml	50 ml
H_3BO_3	5.6g	2.8g	560 mg	280 mg
$MnCl_2*4H_2O$	3.6g	1.8g	360 mg	180 mg
$ZnSO_4*7H_2O$	436 mg	500 µl stock [1000x] $ZnSO_4*7H_2O$ (218 mg)	100 µl stock [1000x] $ZnSO_4*7H_2O$	50 µl stock [100x] $ZnSO_4*7H_2O$
$CuSO_4$	100 mg	500 µl stock [1000x] $CuSO_4$ (50 mg)	100 µl stock [1000x] $CuSO_4$	50 µl stock [1000x] CuSO4
NH_4VO_3	46 mg	500 µl stock [1000x] NH_4VO_3 (23 mg)	100 µl stock [1000x] NH_4VO_3	50 µl stock [1000x] NH_4VO_3
$Na_2MoO_4*4H_2O$	55.6 mg	500 µl stock [1000x] $Na_2MoO_4*4H_2O$ (27,8 mg)	100 µl stock [1000x] $Na_2MoO_4*4H_2O$	50 µl stock [1000x] $Na_2MoO_4*4H_2O$
$Na_2MoO_4*2H_2O$	48.4 mg	500 µl stock [1000x] $Na_2MoO_4*2H_2O$ (24,2 mg)	100 µl stock [1000x] $Na_2MoO_4*2H_2O$	50 µl stock [1000x] $Na_2MoO_4*2H_2O$

Prestock solutions of trace elements:

- Stock [1000x] $ZnSO_4*7H_2O$ → 6.540 g $ZnSO_4$ + 15 ml H_2O
- Stock [1000x] $CuSO_4$ → 500 mg CuSO + 5 ml H_2O
- Stock [1000x] NH_4VO_3 → 46 mg NH_4VO_3 + 100 ml H_2O
- Stock [1000x] $NaMoO_4*2H_2O$ → 242 mg $NaMoO_4$ + 5 ml H_2O

Growth curve determination and measurements of total amount of carbohydrates and sugar in cells over time

- *G. sulphuraria* growing in Allen medium + glc

Table 18: Results of the OD_{800} measurements, the determination of the amount of intracellular carbohdyrates by Anthrone assay as well as the amount of sugars in *G. sulphuraria* growing in Allen medium with either 0.1 %, 0.5 % or 1 % of glucose as a nutient , determinded by GOPOD assay. Samples were taken and measured at different timepoints.

Glucose 0,1% (08.09.2045)				Glucose 0,1% (22.09.2015)				Glucose 0,1% (06.10.2015)			
Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)
0	0,101	0	100,000	0	0,099	0	100,000	0	0,1	0	100
18	0,619	45,4	90,317	19	0,538	100	59,591	19	0,557	100	59,93
25	0,851	100	18,358	23	0,692	63,1	42,883	22	0,617	91,5	49,07
43,5	0,902	49,5	17,232	43,5	0,868	20,6	16,118	43,5	0,858	6,2	16,94
49	1	19,9	17,429	49	0,915	/	15,668	49	0,935	7	17,19
66,5	1,29	21,5	21,560	67	0,95	7,7	15,650	69	0,96	6	17,41
73	1,21	23,1	17,409	73	0,975	5,9	15,668	73	1,185	3,1	16,74
90	1,23	22,3	17,409	90	1,36	/	15,848	92	1,225	5,2	16,87
96,5	1,315	25,6	17,666	97	1,35	14,4	15,650	96,5	1,205	5,3	17,41
162	1,48	29,4	17,824	165	1,56	23,2	15,848	165	1,412	7,4	17,61
169,5	1,5	22,8	17,963	168	1,665	26,7	15,848	166,5	1,464	4,6	17,35

Glucose 0,5% (08.09.2014)				Glucose 0,5% (22.09.2014)				Glucose 0,5% (06.10.2014)			
Time (h)	OD800	% intracellular carbo (mg carbo./mg dry	% glc in the media (mg glc /ml)	Time (h)	OD800	% intracellular carbo (mg carbo./mg dry	% glc in the media (mg glc /ml)	Time (h)	OD800	% intracellular carbo (mg carbo./mg dry	% glc in the media (mg glc /ml)
0	0,1	0,0	100,00	0	0,114	0,0	100	0	0,1	0,0	100
18	0,456	11,5	92,65	19	0,525	19,2	95,87	19	0,328	82,7	110,34
25	0,737	14,0	70,91	23	0,724	21,5	89,09	22	0,292	39,7	103,24
43,5	3,136	83,6	35,52	43,5	3,396	32,7	37,92	43,5	2,695	100,0	98,69
49	4,145	100,0	17,91	49	4,365	100,0	15,47	49	3,575	88,9	66,74
66,5	4,34	37,6	12,82	67	4,1	/	14,68	69	4,03	/	26,33
73	4,13	32,5	2,67	73	4,3	53,4	2,80	73	4	58,3	5,23
90	3,89	37,9	2,70	90	4,03	38,3	2,78	92	3,36	34,0	5,05
96,5	3,5	41,2	2,70	97	4,51	35,1	2,79	96,5	3,65	36,7	4,83
162	3,255	37,5	2,71	165	3,63	40,4	2,78	165	3,85	39,7	4,97
169,5	3,3	36,7	2,72	168	2,08	31,6	2,76	166,5	4,38	/	4,98

Glucose 1% (08.09.2014)				Glucose 1% (22.09.2014)				Glucose 1%			
Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)
0	0,1	0,0	100,0	0	0,108	0,0	100,0	0	0,1	0,0	100,00
18	0,511	11,1	96,7	19	0,512	4,2	94,2	19	0,427	57,3	96,15
25	0,826	20,7	100,2	23	0,693	11,7	45,2	22	0,531	64,9	99,17
43,5	3,268	33,7	68,8	43,5	3,38	18,0	70,4	43,5	3,37	70,0	70,40
49	4,51	51,1	69,8	49	4,135	/	53,9	49	4,31	68,9	71,38
66,5	8,34	100,0	30,7	67	7,11	66,4	19,4	69	6,86	100,0	27,39
73	7,74	84,9	9,9	73	7,44	100,0	4,9	73	6,78	88,2	1,47
90	6,2	41,9	1,8	90	6,63	31,4	1,5	92	6,03	42,2	1,37
96,5	5,89	32,9	1,8	97	6,69	6,5	1,4	96,5	5,58	37,1	1,42
162	5,11	35,1	1,8	165	5,87	9,6	1,4	165	5,93	18,8	1,37
169,5	5,14	24,2	1,8	168	5,81	8,1	1,5	166,5	5,33	22,9	1,40

- *G. sulphuraria* growing in Allen medium + gal

Table 19: Results of the OD_{800} measurements, the determination of the amount of intracellular carbohdyrates by Anthrone assay as well as the amount of sugars in *G. sulphuraria* growing in Allen medium with either 0.1 %, 0.5 % or 1 % of glucose as a nutient, determinded by GOPOD assay. Samples were taken and measured at different timepoints.

Galactose 0.1% (22.09.2014)				Galactose 0.1% (06.10.2014)				Galactose 0.5% (22.09.2014)				Galactose 0.5% (06.10.2014)				Galactose 1%				Galactose 1% (06.10.2015)				Galactose 0.1% (13.10.2015)				Galactose 0.5% (13.10.2015)				Galactose 1% (13.10.2015)			
Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg carbo./mg dry	% glc in the media (mg glc./ml)	Time (h)	OD800	% intracellular carbo (mg carbo./mg dry biomass)	% glc in the media (mg glc./ml)	Time (h)	OD	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD800	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)	Time (h)	OD	% intracellular carbo (mg carbo./mg dry biomass)	% glc in the media (mg glc./ml)	Time (h)	OD	% intracellular carbo (mg glc equiv/ml)	% glc in the media (mg glc equiv/ml)
0	0,121	0,0	100,0	0	0,1	0,0	100,0	0	0,163	0,0	100,0	0	0,1	0,0	100,0	0	0,136	0,0	100,0	0	0,1	0,0	100,0	0	0,099	0	100,0	0	0,102	0,0	100,0	0	0,1	0,0	100,0
19	0,534	86,5	44,2	19	0,424	82,8	69,8	19	0,607	19,3	102,7	19	0,311	15,2	95,2	19	0,544	8,5	98,4	19	0,276	3,1	99,8	21	0,545	/	47,6	21	0,363	/	85,9	21	0,41	/	99,1
24	0,705	100,0	18,5	21	0,439	100,0	39,5	24	0,809	37,7	91,2	21	0,345	7,8	95,4	24	0,714	11,7	93,6	21	0,321	9,2	99,9	24	0,584	100	36,5	24	0,391	11,9	88,3	24	0,451	/	99,0
43,5	0,826	11,3	3,8	43,5	0,811	20,0	1,5	43,5	3,632	33,6	19,8	43,5	2,23	100,0	65,9	43,5	3,192	20,3	70,0	43,5	2,205	8,7	98,0	45	0,985	24,7	1,4	45	2,225	/	61,3	45	2,445	9,1	76,4
50	0,87	/	3,8	49	0,815	16,0	1,5	50	3,94	100,0	1,7	49	2,68	91,7	46,1	50	4,075	/	54,3	49	3,305	100,0	49,6	50	1,045	20,9	1,4	50	3,51	100,0	40,7	50	4,815	100,0	57,6
67	1,005	6,1	3,8	68	1,115	15,1	1,5	67	4,3	/	1,7	68	4,8	27,0	0,3	67	6,71	64,0	22,5	68	6,58	69,1	12,4	70	1,09	28,6	1,4	70	4,04	23,9	0,3	70	5,89	75,5	22,7
73	1,165	6,1	3,8	73	1,06	15,5	1,5	73	3,91	53,5	0,9	73	4,1	15,4	0,3	73	6,24	100,0	13,2	73	6,97	58,4	14,5	73	1,2	3,7	1,4	73	3,69	22,5	0,3	73	5,65	75,3	22,2
93	1,44	/	3,8	93	1,01	17,3	1,5	93	3,76	37,7	0,8	93	3,39	19,1	0,3	93	7	15,1	0,8	93	6,09	31,9	0,8	94	1,485	23,4	1,4	94	4,61	18,3	0,3	94	5,65	13,8	4,9
97,5	1,49	11,6	3,8	96,5	1,175	15,2	1,5	97,5	4,51	35,1	0,8	96,5	2,77	19,0	0,3	96,5	7,18	10,0	0,6	97,5	8,32	6,3	1,0	97,5	2,15	17	1,4	97,5	4,84	17,8	0,3	97,5	5,85	19,5	4,8
163	1,844	19,8	3,8	163	1,56	12,4	1,5	163	4,16	42,1	0,8	163	3,232	15,8	0,3	163	5,74	6,1	0,1	163	5,72	10,1	0,4	165	1,825	21,9	1,4	165	4,42	/	0,3	165	6,31	6,1	0,1
167	2,005	22,8	3,8	165	1,55	10,1	1,5	167	2,865	30,4	0,8	165	3,14	20,2	0,3	167	5,38	8,1	0,8	165	5,81		0,1	168	1,91	21,2	1,4	168	4,41	17,6	0,3	168	5,87	/	0,1

- *G. sulphuraria* growing in Allen medium + 1 % glycerol

Table 20: Results of the OD_{800} measurements, the determination of the amount of intracellular carbohdyrates by Anthrone assay as well as the amount of sugars in *G. sulphuraria* growing in Allen medium with either 0.1 %, 0.5 % or 1 % of glucose as a nutient, determinded by GOPOD assay. Samples were taken and measured at different timepoints.

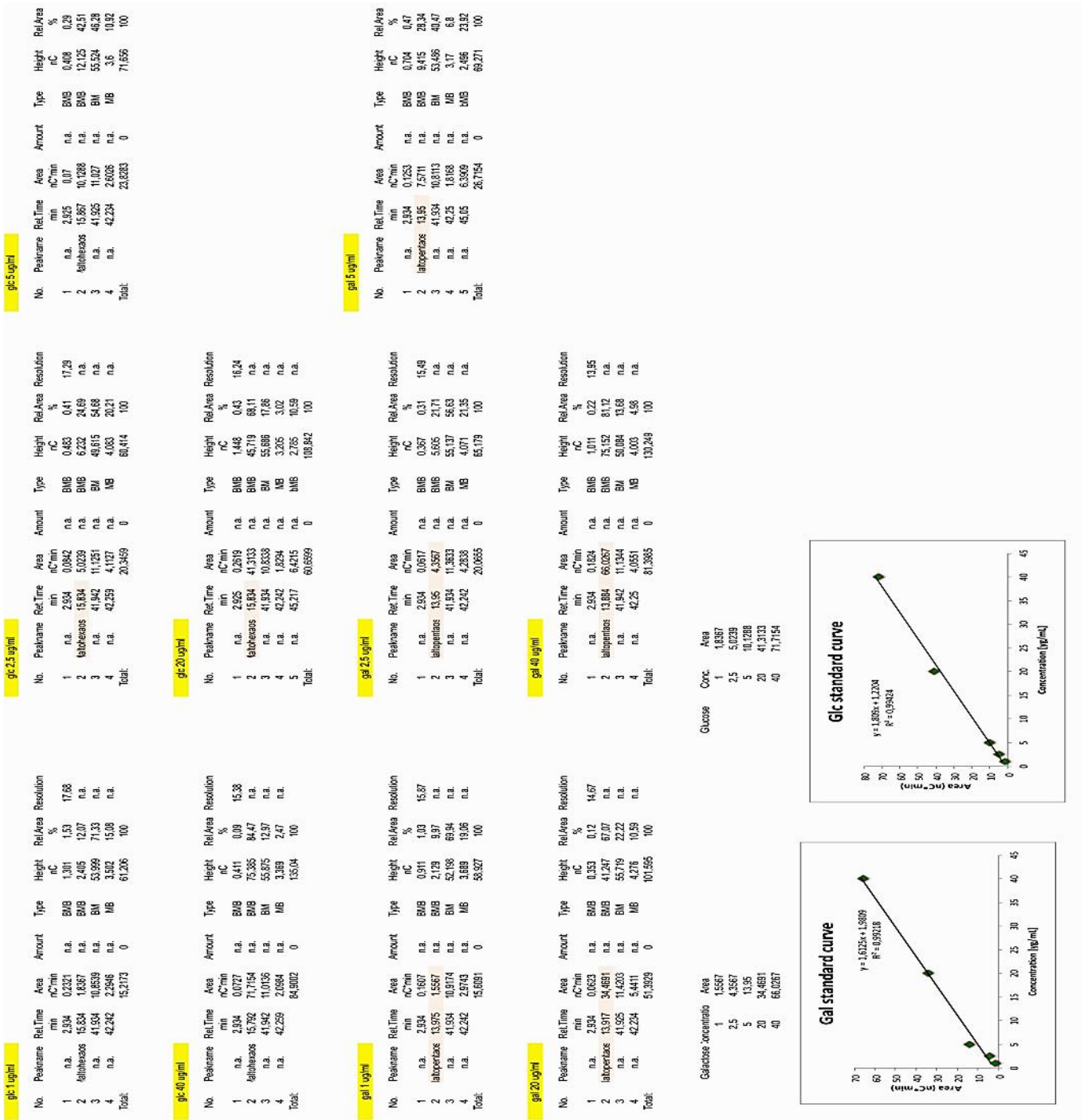
Time	OD800					mg glc equiv in cell extract/mg dry cells				
	061014-A	061014-B	131014-3	Avergae	std ddev	061014-A	061014-B	131014-3	Avergae	std dev
0	0,094	0,092	0,094	0,093	0,001	0,020	0,008	0,092	0,040	0,037
18	0,282	0,28	0,227	0,263	0,025	0,038	0,024	0,066	0,043	0,017
24	0,421	0,424	0,365	0,403	0,027	0,030	0,029	0,069	0,043	0,018
42	1,444	1,438	1,428	1,437	0,007	0,043	0,043	0,088	0,058	0,021
48	2,524	2,376	2,588	2,496	0,089	0,040	0,039	0,067	0,048	0,013
66	7,16	7,7	5,7	6,853	0,845	0,064	0,055	0,067	0,062	0,005
72	10,22	9,26	9,3	9,593	0,443	0,064	0,056	0,065	0,062	0,004
90	10,56	10,84	8,98	10,127	0,819	0,062	0,074	0,083	0,073	0,009
96	13,06	11,54	9,28	11,293	1,553	0,066	0,062	0,077	0,068	0,007
162	6,86	7,24		7,050	0,190	0,024	0,013		0,018	0,005

Alpha glucan extraction

- ***Monosaccharide composition***

Standards

Table 21: Results of the DIONEX analysis of the standards used for the determination of the monosaccharides present in the *α*-glucan extracted from *G. sulphuraria*.



Samples

Table 22: Results of the DIONEX analysis for the determination of the monosaccharides of the alpha glucan extracted from *G. sulphuraria* growing in Allen medium with either 1 % glc, gal or gly as well as the monosaccharde compositioon of oyster glycogen, the standard used for the experiment.

glc glucan 271114

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,1234	n.a.	BMB	1,112	0,06	2,07
2	n.a.	2,925	0,7656	n.a.	BM	3,631	0,4	3,54
3	n.a.	3,659	0,0463	n.a.	MB	0,291	0,03	3,39
4	n.a.	4,825	0,0269	n.a.	Ru	0,203	0,02	n.a.
5	n.a.	5,034	0,7307	n.a.	BM	1,969	0,38	3,47
6	n.a.	5,9	0,334	n.a.	Ru	1,091	0,18	n.a.
7	Maltose	6,734	3,1032	n.a.	MB	8,015	1,63	8,42
8	Maltopentaose	13,884	16,574	n.a.	Ru	24,699	8,72	n.a.
9	Maltohexaose	15,7	140,8	n.a.	BMB	125,07	74,05	29,29
10	n.a.	41,525	15,268	n.a.	BM	85,67	8,03	3,6
11	n.a.	41,934	7,8232	n.a.	M	160,38	4,11	n.a.
12	n.a.	42,217	4,3788	n.a.	MB	6,166	2,3	n.a.
13	n.a.	42,575	0,0743	n.a.	Rd	0,667	0,04	n.a.
14	n.a.	42,867	0,014	n.a.	Rd	0,123	0,01	n.a.
15	n.a.	44,425	0,0683	n.a.	bMB	0,209	0,04	n.a.
Total:			190,13	0		419,31	100	

glc glucan 051214

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,1094	n.a.	BMB	1,01	0,05	2,09
2	n.a.	2,925	0,8156	n.a.	BM	3,855	0,38	3,61
3	Fructose	3,159	0,0242	n.a.	Rd	0,165	0,01	n.a.
4	n.a.	3,859	0,0475	n.a.	MB	0,291	0,02	n.a.
5	n.a.	4,817	0,2998	n.a.	BM	1,519	0,14	n.a.
6	n.a.	5,025	0,6677	n.a.	M	2,267	0,31	n.a.
7	n.a.	5,667	0,5334	n.a.	Ru	1,712	0,25	n.a.
8	Maltose	6,734	4,8927	n.a.	MB	12,464	2,28	8,1
9	Maltopentaose	13,867	35,711	n.a.	BM	45,399	16,66	1,29
10	Maltohexaose	15,684	135,91	n.a.	M	127,77	63,4	n.a.
11	Maltoheptaose	17,617	7,1116	n.a.	MB	6,83	3,32	n.a.
12	n.a.	41,525	16,073	n.a.	BM	93,547	7,5	3,58
13	n.a.	41,934	8,5697	n.a.	M	172,56	4	n.a.
14	n.a.	42,209	3,4799	n.a.	MB	6,373	1,62	n.a.
15	n.a.	42,575	0,1128	n.a.	Rd	0,99	0,05	n.a.
16	n.a.	42,859	0,0061	n.a.	Rd	0,074	0	n.a.
Total:			214,36	0		476,83	100	

gal glucan 211114

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,475	0,1268	n.a.	BMB	1,142	0,06	2,08
2	n.a.	2,934	0,814	n.a.	BM	3,717	0,39	3,58
3	Fructose	3,175	0,0458	n.a.	Rd	0,377	0,02	n.a.
4	n.a.	3,859	0,0438	n.a.	bMB	0,285	0,02	n.a.
5	n.a.	4,817	0,4432	n.a.	BM	2,169	0,21	n.a.
6	n.a.	5,025	0,6791	n.a.	M	2,362	0,32	n.a.
7	n.a.	5,892	0,4763	n.a.	Ru	1,537	0,23	n.a.
8	Maltose	6,734	5,4878	n.a.	MB	14,099	2,61	7,88
9	Maltopentaose	13,825	49,915	n.a.	BM	61,344	23,75	1,33
10	Maltohexaose	15,692	115,04	n.a.	M	112,06	54,75	n.a.
11	Maltoheptaose	17,684	9,2373	n.a.	MB	7,519	4,4	n.a.
12	n.a.	41,525	14,336	n.a.	BM	77,225	6,82	n.a.
13	n.a.	41,934	8,2258	n.a.	M	154,74	3,91	n.a.
14	n.a.	42,217	5,0642	n.a.	MB	6,555	2,41	n.a.
15	n.a.	42,575	0,1404	n.a.	Rd	1,205	0,07	n.a.
16	n.a.	42,875	0,0092	n.a.	Rd	0,106	0	n.a.
17	n.a.	44,634	0,0481	n.a.	bMB	0,148	0,02	n.a.
Total:			210,13	0		446,59	100	

gal glucan 241114

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,0632	n.a.	BMB	0,593	0,03	2,13
2	n.a.	2,934	0,6927	n.a.	BM	3,35	0,37	3,56
3	n.a.	3,859	0,0363	n.a.	MB	0,235	0,02	3,65
4	n.a.	5,025	0,5326	n.a.	BM	1,669	0,28	3,64
5	n.a.	5,884	0,3912	n.a.	Ru	1,195	0,21	n.a.
6	Maltose	6,725	3,0803	n.a.	MB	7,917	1,64	8,33
7	Maltopentaose	13,85	14,813	n.a.	Ru	22,138	7,9	n.a.
8	Maltohexaose	15,659	139,65	n.a.	BMB	124,49	74,63	29,16
9	n.a.	41,525	15,66	n.a.	BM	91,699	8,36	3,64
10	n.a.	41,934	7,5399	n.a.	M	160,46	4,24	n.a.
11	n.a.	42,209	4,2482	n.a.	MB	6,411	2,27	n.a.
12	n.a.	42,575	0,0877	n.a.	Rd	0,747	0,05	n.a.
Total:			187,39	0		420,9	100	

gal glucan 271114

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,475	0,3007	n.a.	BMB	1,799	0,14	1,94
2	n.a.	2,934	0,8291	n.a.	BMB	3,961	0,39	3,56
3	Fructose	3,167	0,0221	n.a.	Rd	0,175	0,01	n.a.
4	n.a.	3,859	0,0613	n.a.	BMB	0,371	0,03	n.a.
5	n.a.	4,817	0,3178	n.a.	BM	1,576	0,15	n.a.
6	n.a.	5,025	0,7125	n.a.	M	2,41	0,33	n.a.
7	n.a.	5,884	0,4957	n.a.	Ru	1,576	0,23	n.a.
8	Maltose	6,734	5,0921	n.a.	MB	12,805	2,38	8,06
9	Maltopentaose	13,85	36,164	n.a.	BM	45,776	16,9	1,29
10	Maltohexaose	15,675	134,31	n.a.	M	126,47	62,76	n.a.
11	Maltoheptaose	17,617	7,5661	n.a.	MB	6,87	3,54	n.a.
12	n.a.	41,534	15,45	n.a.	BM	88,069	7,22	n.a.
13	n.a.	41,934	8,1449	n.a.	M	155,2	3,81	n.a.
14	n.a.	42,217	4,4378	n.a.	MB	6,815	2,07	n.a.
15	n.a.	42,575	0,1094	n.a.	Rd	0,968	0,05	n.a.
16	n.a.	42,859	0,0039	n.a.	Rd	0,058	0	n.a.
Total:			214,02	0		454,9	100	

gal glucan 051214

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,2432	n.a.	BMB	1,459	0,11	1,94
2	n.a.	2,925	0,7704	n.a.	BMB	3,669	0,36	3,56
3	Fructose	3,167	0,0246	n.a.	Rd	0,207	0,01	n.a.
4	n.a.	3,859	0,0577	n.a.	BMB	0,346	0,03	n.a.
5	n.a.	4,809	0,3432	n.a.	BM	1,986	0,16	n.a.
6	n.a.	5,017	0,6843	n.a.	M	2,356	0,32	n.a.
7	n.a.	5,875	0,5105	n.a.	Ru	1,591	0,24	n.a.
8	Maltose	6,725	4,1766	n.a.	MB	10,426	1,93	7,96
9	Maltopentaose	13,834	42,611	n.a.	BM	53,158	19,73	1,29
10	Maltohexaose	15,667	130,33	n.a.	M	123,45	60,34	n.a.
11	Maltoheptaose	17,617	8,1996	n.a.	MB	7,187	3,8	n.a.
12	n.a.	41,517	15,114	n.a.	BM	86,072	7	3,67
13	n.a.	41,934	8,2397	n.a.	M	156,39	3,81	n.a.
14	n.a.	42,209	4,5336	n.a.	MB	7,073	2,1	n.a.
15	n.a.	42,567	0,1344	n.a.	Rd	1,21	0,06	n.a.
16	n.a.	42,842	0,0128	n.a.	Rd	0,126	0,01	n.a.
Total:			215,99	0		456,42	100	

gly glucan 271114

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,0825	n.a.	BMB	0,761	0,04	2,09
2	n.a.	2,925	0,8523	n.a.	BM	3,993	0,42	3,52
3	n.a.	3,85	0,0455	n.a.	MB	0,265	0,02	3,13
4	n.a.	4,817	0,0361	n.a.	Ru	0,255	0,02	n.a.
5	n.a.	5,025	0,7522	n.a.	BM	2,028	0,37	3,25
6	n.a.	5,875	0,4663	n.a.	Ru	1,536	0,24	n.a.
7	Maltose	6,725	5,8013	n.a.	MB	14,637	2,87	8,13
8	Maltopentaose	13,85	29,772	n.a.	BM	37,987	14,74	1,31
9	Maltohexaose	15,684	128,89	n.a.	M	122,49	63,81	n.a.
10	Maltoheptaose	17,584	7,4707	n.a.	MB	6,652	3,7	n.a.
11	n.a.	41,525	15,088	n.a.	BM	84,367	7,47	n.a.
12	n.a.	41,934	8,0655	n.a.	M	150,87	3,99	n.a.
13	n.a.	42,217	4,5161	n.a.	MB	6,762	2,24	n.a.
14	n.a.	42,575	0,1151	n.a.	Rd	0,958	0,06	n.a.
15	n.a.	44,625	0,0289	n.a.	BMB	0,122	0,01	n.a.
Total:			202	0		433,7	100	

gly glucan 051214

No.	Peakname	Ret.Time min	Area nC*min	Amount	Type	Height nC	Rel.Area %	Resolution
1	n.a.	2,467	0,1172	n.a.	BMB	1,053	0,06	2,08
2	n.a.	2,925	0,8315	n.a.	BM	4,093	0,42	3,6
3	n.a.	3,859	0,0498	n.a.	MB	0,302	0,03	7,09
4	n.a.	5,025	0,6149	n.a.	Ru	1,837	0,31	n.a.
5	n.a.	5,85	0,4425	n.a.	Ru	1,366	0,22	n.a.
6	Maltose	6,734	4,7838	n.a.	BMB	11,903	2,43	8,21
7	Maltopentaose	13,867	22,895	n.a.	BM	29,659	11,61	1,3
8	Maltohexaose	15,675	139,68	n.a.	MB	125,92	70,83	28,92
9	n.a.	41,525	16,218	n.a.	BM	90,946	8,22	3,6
10	n.a.	41,934	7,6219	n.a.	M	157,96	3,86	n.a.
11	n.a.	42,217	3,8437	n.a.	MB	6,123	1,95	n.a.
12	n.a.	42,575	0,0929	n.a.	Rd	0,795	0,05	n.a.
13	n.a.	44,6	0,0209	n.a.	BMB	0,111	0,01	n.a.
Total:			197,22	0		432,07	100	

Debranching assay→ Results of the Nelson Somogyi and Anthrone assay before and after debranching of the alpha glucan

Table 23: In this table the results of the Nelson Somogyi assay before and after the debranching with isoamylase as well as of the Anthrone assay of *α*-glucan extracted of *G. sulphuraria* growing in Allen medium with either 1 % glc, gal or gly as a nutrient supply, are shown. With the results of these three measurements the ACL of the *α*-glucan extracted from *G. sulphuraria* as well as of the glycogen from oyster could be calculated and are as well showen in this table.

Degree of branching

	Nelson before (10mg/ml)	Nelson before (1mg/ml)	Nelson after (1mg/ml)	Anthrone before (1mg/ml)	Chain lenght assay	Average chain length	Stand variation cL
oyster	0,04547	0,00455	0,0790	0,767	10,30		
Oyster	0,03258	0,00326	0,0839	1,03	12,81	11,55	1,78
Glc 73h	0,13415	0,01342	0,1297	0,887	7,63	7,63	1,11
Glc 97h (1)	0,1411	0,01411	0,1451	0,700	5,34	6,90	
Glc 97 (2)	0,108350046	0,01084	0,0993	0,700	7,91		
Glc 27.11 97h	0,13937	0,01394	0,0999	0,6	6,98		
Glc 5.12 97h	0,14924	0,01492	0,1278	0,833	7,38		
Galactose 73h	0,1194	0,01194	0,1244	0,833	7,41	7,41	1,03
Gal 21.11 97h	0,086657824	0,00867	0,1009	1,007	10,91	10,08	
Gal 24.11. 97h	0,112505527	0,0113	0,1009	0,94	10,49		
Gal 27.11 97h	0,123626841	0,0124	0,1168	0,897	8,59		
Gal 5.12 97h	0,128830391	0,0129	0,1060	0,963	10,34		
Glycerol 73h	0,1092	0,01092	0,1349	0,800	7,00	7,00	1,25
Gly 24.11 97h	0,091759344	0,0092	0,1330	0,947	7,65	9,09	
Gly 27.11 97h	0,110226848	0,011	0,1100	0,977	9,86		
Gly 5.12 97h	0,129952726	0,013	0,1189	1,033	9,76		

H-NMR profiles of oyster glycogen, *G. sulphuraria* growing on Allen medium with 1% of either glc, gal or gly as a nutrient supply

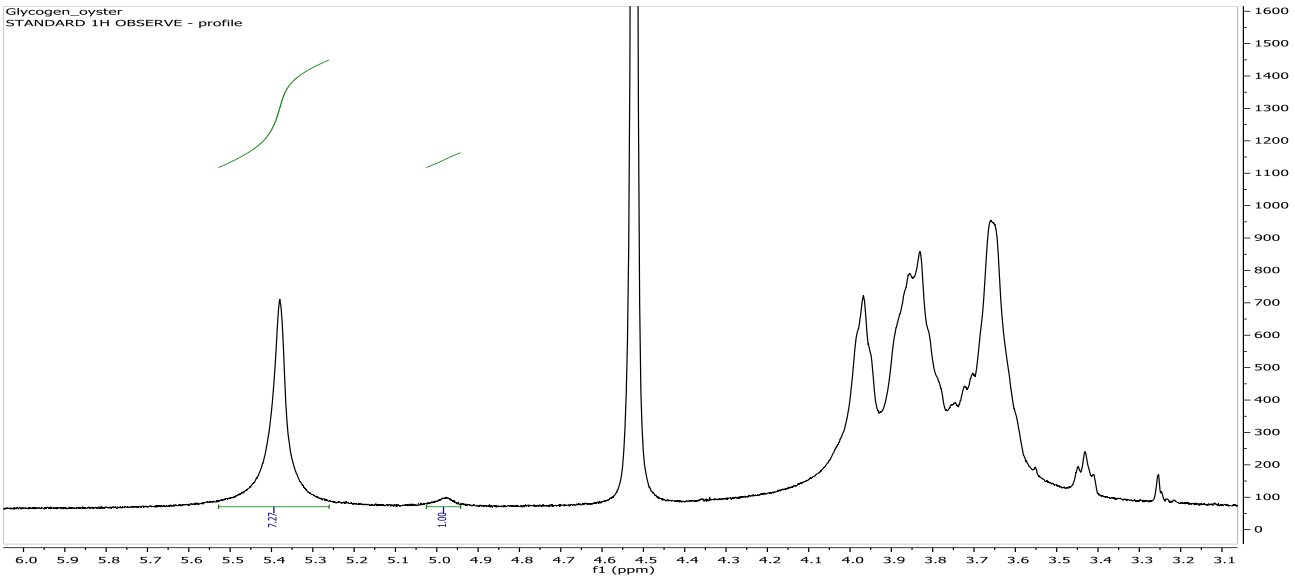


Figure 46: H-NMR spectrum of oyster glycogen shows an average chain length of 8.27 glc residues. The peak of the *α*-1,6 branching points has always a size of one to put the *α*-1,4 linkages in relation to the *α*-1,6 branching points. The peak size of the *α*-1,4 likages is 7.27.

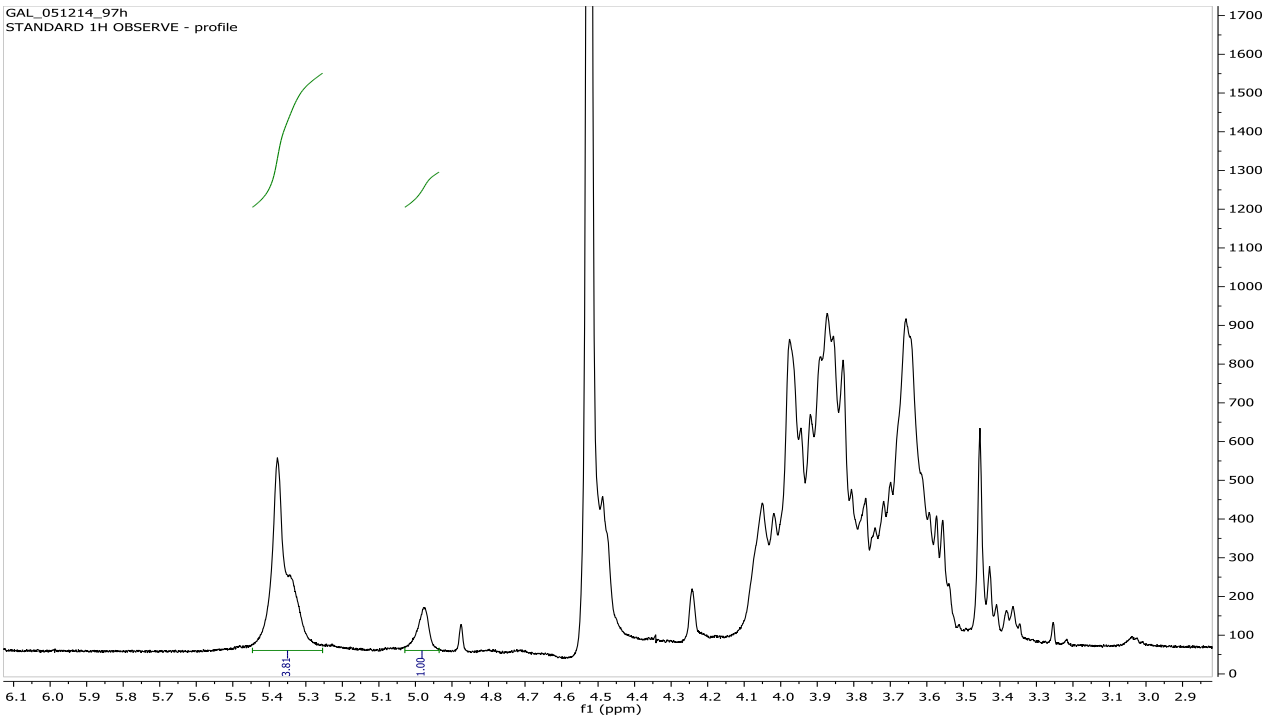


Figure 47: The H-NMR spectum of alpha glucan extraced from *G. sulphuraria* growing in Allen medium + 1 % gal as a nurierent supply is hown in this figure. The peak of the *α*-1,4 linkages has a size of 3.81 which results in an average chain length of 4.81 glucose residues for the *α*-glucan extracted from this microalga.

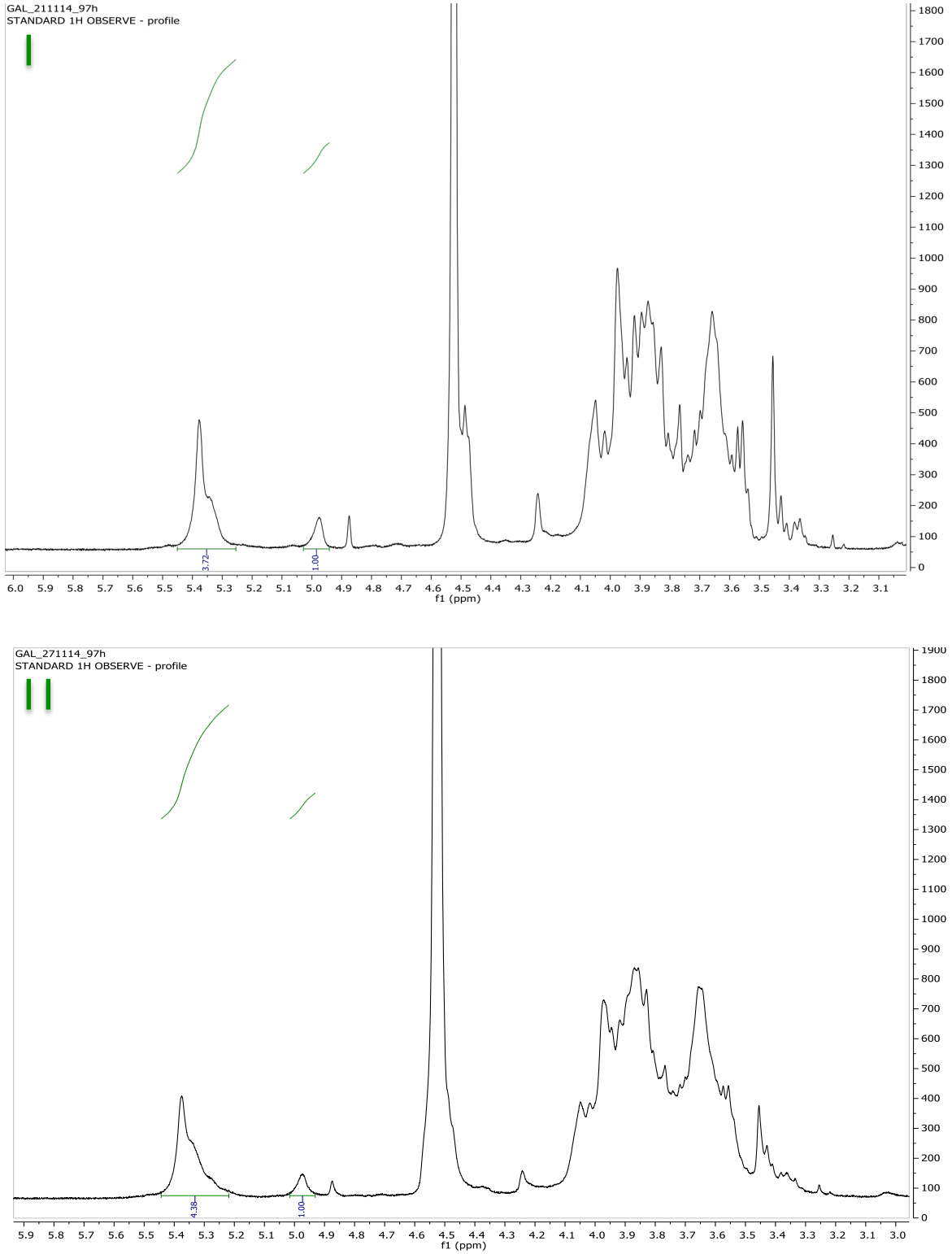


Figure 48: These two pictures show the H-NMR spectra of *α*-glucan extracted from *G. sulphuraria* growing in Allen medium 1% of galactose. In picture I the average chain length is 4.72 glucose residues long and the ACL of G. sulphuraria in spectrum II is 5.38 glc residues.

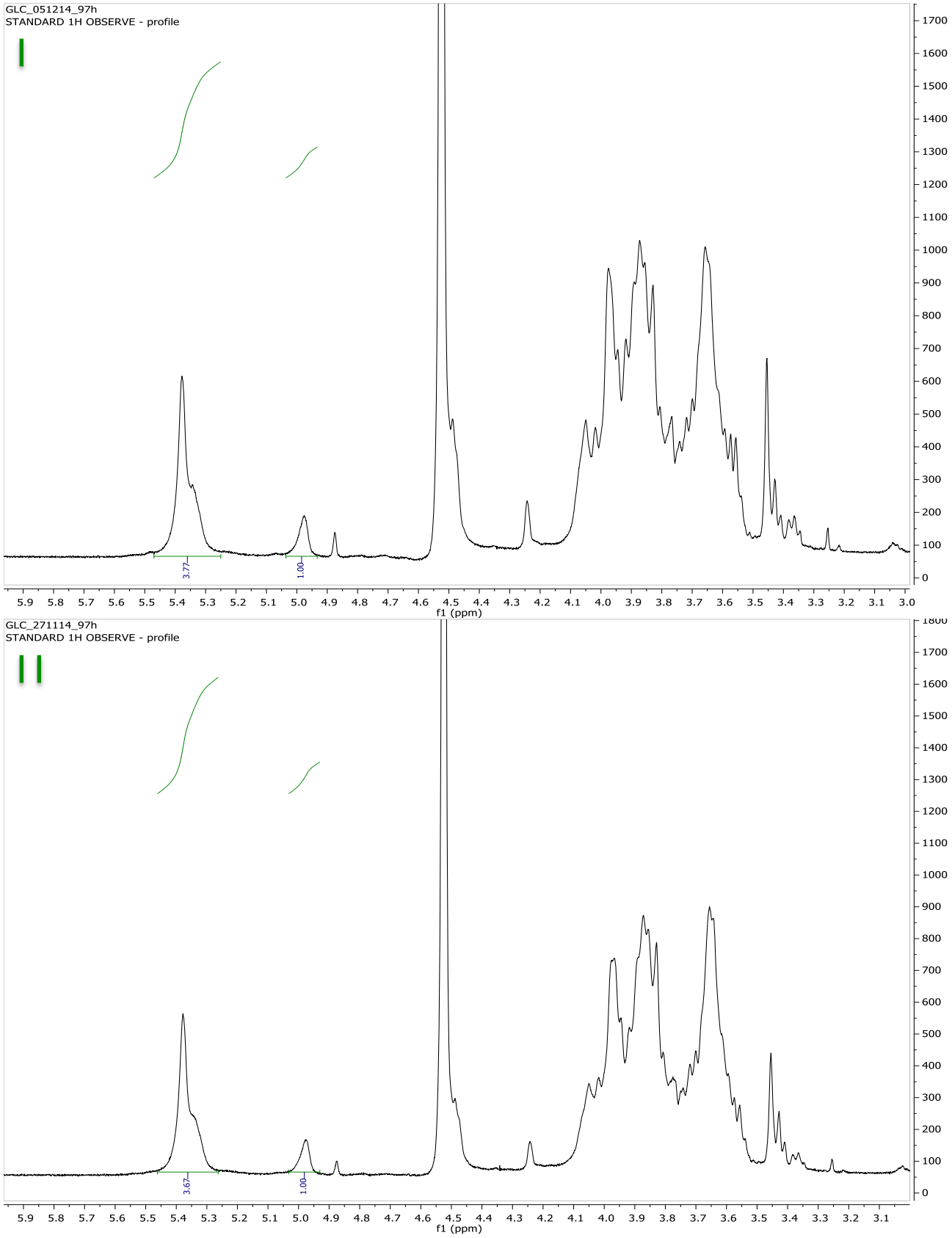


Figure 48: The H-NMR spectra of the extracted *α* -glucan of *G. sulphuraria* growin in Allen medium with 1 % glc as a nutrient supply for 97 h are shown. In picture I the ACL of *α*-glucan was 4.77 glc residues, the one in picture B 4.67.

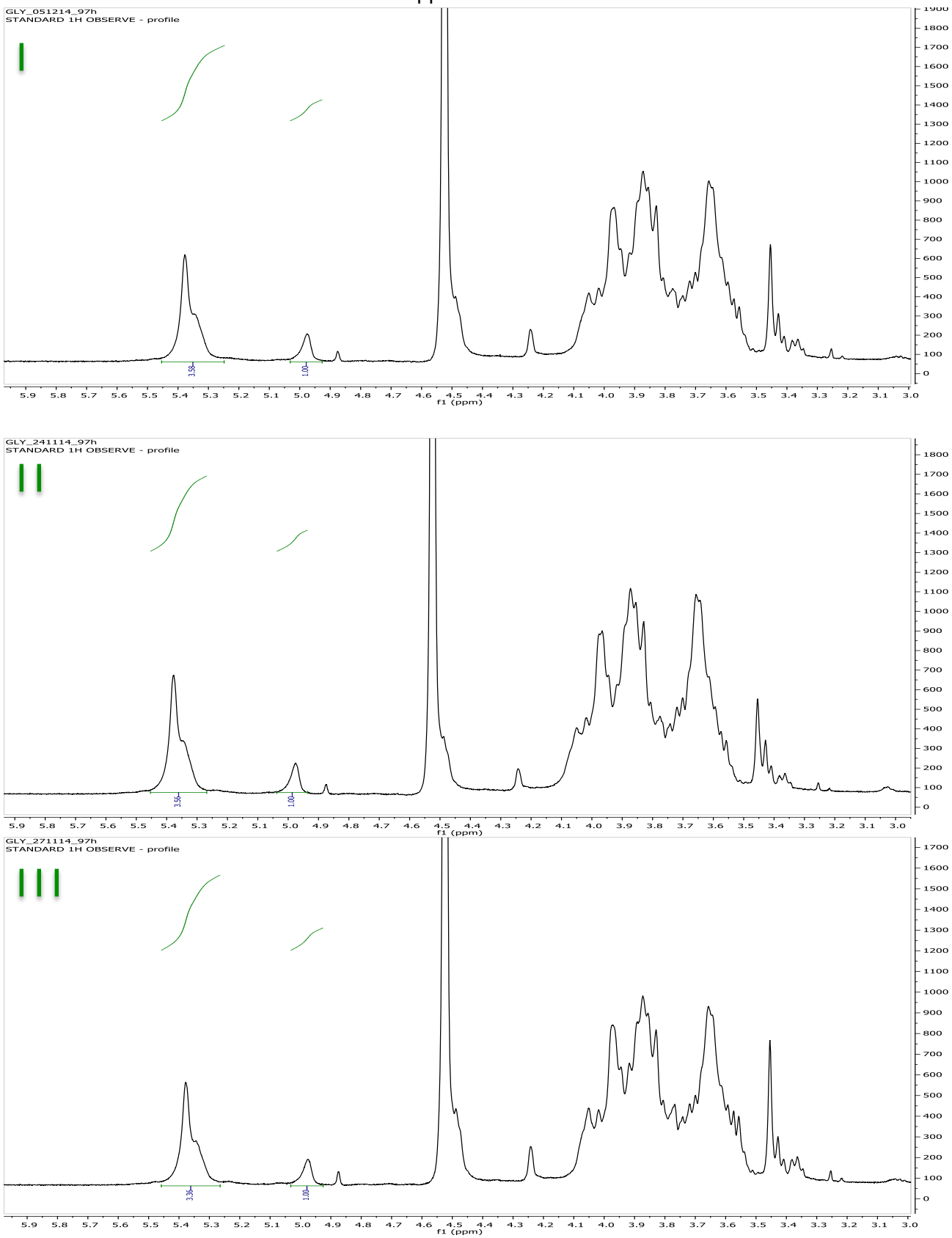


Figure 49: The H-NMR spectra of the extracted *α* -glucan from *G. sulphuraria* growing in Allen medium with 1 % gly as a nutrient supply for 97 h are shown. In picture I the ACL of *α*-glucan was 4.58 glc residues, the one in picture II 4.56 and the ACL of *α* glucan in spectra III was 4.36.

Chain length distribution results of the measurements over time

Table 24: The results of the chain length distribution measured by HPAEC by DIONEX are shown in this table. The chain length distribution was analysed of *α*-glucan extracted from *G. sulphuraria* growing in Allen medium with either 1 % of glc, gal or gly as a nutrient supply as well as of oyster glycogen.

Chainlength distribution profiles of the standard (oyster glycogen) and of *G. sulphuraria* growing in Allen medium with either glc, gal or glycerol as a nutrient supply

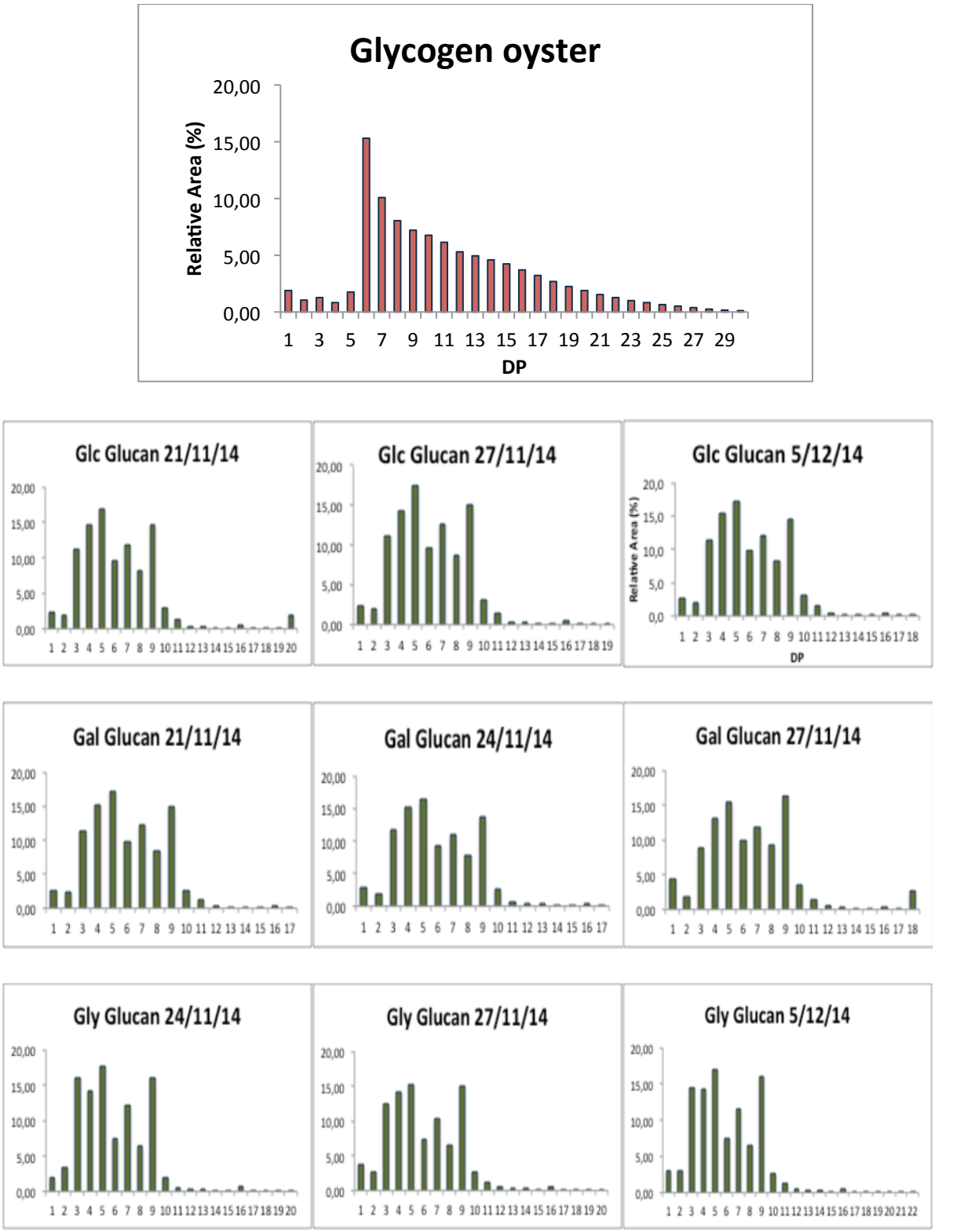


Figure 50: This figure shows the chain length distribution of oysterglycogen as well as of *α*-glucan exctracted from *G. sulphuraria* growing in Allen medium with either 1% glc, gal or gly as a nutrient supply. It is seen that the *α*-glucan extracted from microalgae consists of shorter oligosaccharides with a DP between 1 to 9 whereas the glycogen from oyster has a DP ranging from 9 to 20.

Starvationexperiment → Results of the total carbohydrate content over time

Table 25: The results of the determination of the amount of intracellular carbohydrates by Anthrone assay of *G. sulpuruaria* growing in either carbon deficient or carbon and nitrogen deficient Allen medium over time, are shown.

Samples concentrated 10X fro breaking the cells up

Cell extracts diluted 1:50 (the OD sample) or 1:20 (the rest of the samples) for measuring Anthrone

Allen Media-C

Hours		Abs	x dilution	mg/ml	Average	% respect initial
0	A	0,454	22,70	15,22	15,46	100,0
	B	0,468	23,40	15,69		
9	A	0,28	14,00	9,38	9,58	62,0
	B	0,292	14,60	9,78		
24	A	0,202	4,04	2,69	2,82	18,3
	B	0,222	4,44	2,96		
32	A	0,199	3,98	2,65	2,64	17,1
	B	0,198	3,96	2,63		
48	A	0,223	4,46	2,97	2,68	14,4
	B	0,18	3,60	2,39		
56	A	0,182	3,64	2,42	2,41	12,9
	B	0,181	3,62	2,41		
72	A	0,169	3,38	2,24	2,22	14,4
	B	0,166	3,32	2,20		
80	A	0,161	3,22	2,14	2,11	13,7
	B	0,157	3,14	2,08		
98	A	0,158	3,16	2,10	2,16	14,0
	B	0,167	3,34	2,22		
168	A	0,151	3,02	2,00	1,97	12,7
	B	0,146	2,92	1,94		
240	A	0,165	3,30	2,19	3,00	19,4
	B	0,286	5,72	3,82		
360	A	0,132	2,64	1,75	1,75	9,36
	B	0,132	2,64	1,75		
504	A	0,183	3,66	2,43	2,16	11,59
	B	0,143	2,86	1,90		
720	A	0,173	3,46	2,30	2,10	11,23
	B	0,143	2,86	1,90		

Allen Media -N-C

Hours		Abs	x dilution	mg/ml	Average	% respect initial
0	A	0,544	27,20	18,24	18,68	100,0
	B	0,57	28,50	19,12		
9	A	0,541	27,05	18,14	16,26	87,1
	B	0,429	21,45	14,38		
24	A	0,738	14,76	9,89	10,28	55,1
	B	0,797	15,94	10,68		
32	A	0,677	13,54	9,07	8,03	43,0
	B	0,522	10,44	6,99		
48	A	0,557	11,14	7,46	7,58	40,6
	B	0,576	11,52	7,71		
56	A	0,613	12,26	8,21	8,40	44,9
	B	0,641	12,82	8,58		
72	A	0,464	9,28	6,21	6,20	33,2
	B	0,463	9,26	6,19		
80	A	0,437	8,74	5,84	6,01	32,2
	B	0,461	9,22	6,17		
98	A	0,435	8,70	5,82	5,56	29,8
	B	0,397	7,94	5,31		
168	A	0,367	7,34	4,90	4,89	26,2
	B	0,365	7,30	4,88		
240	A	0,444	8,88	5,94	5,89	31,5
	B	0,437	8,74	5,84		
360	A	0,29	5,80	3,87	4,01	21,47
	B	0,311	6,22	4,15		
504	A	0,277	5,54	3,70	3,68	19,68
	B	0,274	5,48	3,66		
720	A	0,331	6,62	4,42	4,34	23,23
	B	0,319	6,38	4,26		