Functional carbohydrates from the red microalga Galdieria sulphuraria
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Chapter 6

General discussion
The primitive thermoacidophilic red microalga *Galdieria sulphuraria* displays a metabolic flexibility not matched by any other red alga species, including the other members of Cyanidiales, the order to which this microalga belongs. Not only is *G. sulphuraria* able to grow autotrophically in hot acidic environments, like those found in e.g. sulphur springs and volcanic areas, but it can also switch to a heterotrophic growth regime whenever photosynthesis is impaired, ensuring its survival in conditions where other algae would die. Horizontal gene transfer contributed to the metabolic flexibility of *G. sulphuraria* by providing it with a set of metabolite uptake facilitators (Schönknecht et al., 2013) that enable this microalga to utilize a wide range of organic compounds as carbon source (Gross & Schnarrenberger, 1995) and to colonize photosynthesis-independent areas such as internal layers of rocks (Gross et al., 1998) (Chapter 1).

The extreme conditions in which *G. sulphuraria* proliferates might have had an influence on the remarkable structure of its storage polysaccharide. While other red algae synthesize an insoluble semi-amyllopectin-like polysaccharide generally denominated floridean starch (Table 2 in Chapter 1), *G. sulphuraria* accumulates a small, highly branched glycogen with a structure that differs markedly not only from that of the storage polysaccharide of other red algae (Shimonaga et al., 2008), but also from other prokaryotic and eukaryotic glycogens (Chapter 2). The glycogen of *G. sulphuraria* is entirely composed of short chains of DP ≤ 10 and contains 18% of α-(1→6) bonds, the highest percentage of branching linkages found among all naturally occurring glycogens characterized to date (Table S1 in Chapter 2). Whether this highly branched structure is the result of the intrinsic properties of *G. sulphuraria* branching enzyme or it is a consequence of the concerted action of all enzymes involved in glycogen synthesis/degradation is still unclear. The isolation and characterization of the glycogen branching enzyme from *G. sulphuraria* would be a necessary next step to explain how this particular structure is formed.

The high branching degree of *G. sulphuraria* glycogen raises the question whether this structural trait of the storage polysaccharide gives the microalga a selective competitive advantage helping it to adapt to the extreme and variable conditions of the natural habitat.

A higher proportion of branching linkages in the glycogen molecule exposes a higher number of non-reducing ends of the side chains to the enzymes responsible for glycogen degradation, such as glycogen phosphorylase and
debranching enzyme (Wilson et al., 2010), and would thus enable *G. sulphuraria* to rapidly obtain energy from the storage polysaccharide. This energy could be used by the cells to, for example, perform the transition from autotrophy to heterotrophy as soon as photosynthesis is impaired because of light limitation. The enzymes responsible for glycogen degradation in *G. sulphuraria* must thus be adapted to such a highly branched structure. Apart from isoamylase, responsible for hydrolyzing the α-(1→6) linkages, *G. sulphuraria* also possess a 4-α-glucanotransferase–like protein (called disproportionating enzyme in plants) (Barbier et al., 2005) which could be involved in the debranching of glycogen. This enzyme catalyzes the transfer of maltooligosaccharides from one α-1,4-glucan molecule to another (Takahara et al., 1993) and could facilitate the debranching of the very short side chains in the highly branched glycogen by increasing their length to make them susceptible to hydrolysis by isoamylase. Some authors have hypothesized that a high branching degree can turn glycogen into a long-term energy storage molecule because α-(1→6) linkages are degraded by enzymes at a lower rate than α-(1→4) linkages, enabling cell survival under adverse conditions by keeping a slow, but constant supply of energy (Wang & Wise, 2011). However, this theory would imply that the enzymes of the microorganism are not adapted to the highly branched structure in order to allow its fast degradation.

The particular highly branched structure of its glycogen could also help *G. sulphuraria* in coping with changes in water activity. The high temperatures characteristic of the environments inhabited by *G. sulphuraria* can cause water evaporation from the surface or internal layers of rocks and lead to cell desiccation. Studies on the hydration and water structure of phytoglycogen particles have demonstrated that highly branched polysaccharides are able to retain large amounts of water in a more ordered structure than linear polysaccharides (Nickels et al., 2016; Grossutti & Dutcher, 2016). Thus, the high degree of branching in *G. sulphuraria* glycogen could enable the cells to retain intracellular water to cope with water stress conditions and desiccation.

The highly branched structure of the glycogen could also help *G. sulphuraria* in outgrowing other competitor microorganisms that might be present in the same habitats, such as heterotrophic acidophilic bacteria and fungi (Belly et al., 1973). The concentration of organic substrates in the natural habitat of *G. sulphuraria* is reported to be very low and cell survival under photosynthesis-limiting conditions is probably achieved only by means of assimilating the
compounds released by dying cells (Gross et al., 1998). Upon cell death, cellular components, including glycogen, are released in the medium and could serve as substrates to support the growth of surviving cells. The high degree of branching of \emph{G. sulphuraria} glycogen could turn it into a selective substrate that can be more rapidly degraded by this microalga species, which is expected to contain the enzymatic machinery needed to cope with such a densely branched structure, than by other microorganisms. \emph{G. sulphuraria} is reported to secrete certain catabolic enzymes such as amylases and glucoamylases (Schönknecht et al., 2013), that could be involved in the degradation and assimilation of glycogen released by dying cells as substrate.

Not only the structure of \emph{G. sulphuraria} glycogen is remarkable, but also the amounts of this glucan that can be accumulated inside the cells. In \textbf{Chapter 5} it is shown that \emph{G. sulphuraria} can accumulate 30-50\% of the cell dry weight as glycogen throughout all growth phases when growing in both low nitrogen and high nitrogen medium. This suggests that glycogen synthesis in \emph{G. sulphuraria} might be constitutively active and not triggered by growth limitation, as reported for other microorganisms (Lillie & Pringle, 1980; Aikawa et al., 2012; Möllers et al., 2014). The high amounts of glycogen accumulated by \emph{G. sulphuraria} place this microalg among other efficient carbohydrate accumulating microorganisms (Brányiková et al., 2011; Möllers et al., 2014; Song et al., 2016). The fact that glycogen is constitutively accumulated in \emph{G. sulphuraria} could indicate an essential physiological role for this polysaccharide apart from that of energy storage, such as intracellular water retention as already discussed above. However, it is important to note that the laboratory conditions used for growing \emph{G. sulphuraria} in this thesis (regarding nutrient supply) are far from similar to those present in its natural habitat, and therefore the accumulation of large amounts of glycogen in heterotrophic cells grown in the natural habitat might not be observed.

Nevertheless, since \emph{G. sulphuraria} can accumulate large amounts of the small, highly branched glycogen, this microalga could be a promising industrial source for highly branched glucose polymers. These polymers are already used for certain specific applications such as the formulation of peritoneal dialysis solutions (Deremaux et al., 2013) and sport drinks (Takii et al., 2005) and are gaining attention as a novel type of slowly digestible carbohydrate with low impact on the glycemic index (GI) that can help in the prevention of certain diseases like diabetes and obesity (Ao et al., 2007; Le et al., 2009; Lee et al.,
The production of these polymers is usually achieved by treatment of starch with enzymes such as glycogen branching enzymes, amylglucosidases, β-amylases and glucanotransferases, in order to increase the proportion of branching bonds in the structure. Because starch solubilization requires high temperatures, a considerably energy input and the use of thermostable enzymes is needed (van der Maarel et al., 2002; Kaper et al., 2004; van der Maarel & Leemhuis, 2013).

The glycogen of *G. sulphuraria* could be a promising alternative to starch as substrate for the production of highly branched glucose polymers due to its solubility in cold water, its already high proportion of branching linkages and its smaller molecular size compared to starch. As shown in Chapter 3, *G. sulphuraria* glycogen shows higher resistance to digestive enzymes and improved rheological properties compared to a highly branched polymer derived from potato starch. Its resistance to digestive enzymes, such as pancreatic α-amylase and amylloglucosidase, is conferred by its high branch density, which would limit the action of enzymes to the chains localized in the most external part of the molecule and impede the hydrolysis of the internal chain. This in agreement with a previous work that shows that the branch pattern in the internal parts of the starch molecule effectively modulates the enzymatic digestion (Lin et al., 2014). The enzymatic digestion of *G. sulphuraria* glycogen results in a population of branched limit dextrins of higher molecular weight than those obtained from the highly branched starch used in the same analysis or than the branched limit dextrins obtained by amylase hydrolysis of starches from different botanical sources (Kittisuban et al., 2014; Lee & Hamaker, 2017), and suggests that this glycogen could be used without further modification as a slowly digestible, low-glycemic carbohydrate source that is an alternative to branched glucose polymers produced from starch (Ao et al., 2007; Le et al., 2009; Lee et al, 2013). The reduced osmotic capacity and viscosity of *G. sulphuraria* glycogen could be beneficial in certain applications like e.g. the formulation of sports drinks that combine a high carbohydrate load with an optimum osmolality to achieve fast gastric emptying (Takii et al., 2005).

The ability of *G. sulphuraria* to grow heterotrophically to high cell densities - reaching more than 100 g dry biomass/L in fed-batch cultivation (Graveholt & Erikssen, 2007) or around 20 g dry biomass/L in batch cultivation (with potential for improvement) (Chapter 5) - and to cope with different stresses
(e.g. high osmotic pressure), could be exploited for the production of not only glycogen, but also floridoside (Chapter 4). This glycoside is accumulated in G. sulphuraria and other red algae cells as response to osmotic pressure (Pade et al., 2015) and has raised interest for its potential antifouling (Hellio et al., 2004) and therapeutic properties (Courtois et al., 2008; Li et al., 2009; Kim et al., 2013; Ryu et al., 2015), but more analyses are needed for further properties characterization and possible improvement by the development of structural analogues. Because a production process yielding high amounts of floridoside was not developed yet, the conditions to optimize floridoside accumulation by G. sulphuraria were analyzed in Chapter 4.

This chapter reveals that optimum floridoside accumulation by G. sulphuraria is achieved by using glycerol as carbon source for cell growth and by subjecting late exponential phase cells to an hyperosmotic shock for only 24 h, which results in higher floridoside yields than sustained growth in the presence of the osmotic stress-causing agent. This strategy is more advantageous since it allows to cultivate G. sulphuraria to high cell densities without delaying growth and compromising cell physiology before inducing floridoside accumulation, and has been used in a previous work for the production of the compatible solute mannosylglycerate (Ergorova et al., 2007).

The method chosen for extraction of floridoside from G. sulphuraria cells can also have an effect on the yield of this glycoside, as shown in Chapter 5. Milking of the cells, a method successfully used in the production of other valuable, low molecular weight compounds from other microorganisms (Sauer & Galinski, 1998; Hejazi & Wijffels, 2004; Ergorova et al., 2007), did not produce satisfactory results when applied to G. sulphuraria, although the attempt performed in this thesis was very preliminary and certainly needs further investigation. Ethanolic extraction and cell disruption by bead-beating yielded similar amounts of floridoside and choosing for one or the other would depend on different factors that should be assessed, such as process costs or the possibility to integrate floridoside extraction as a part of a process producing other valuable compounds from G. sulphuraria, like the highly branched glycogen (Chapter 2, Chapter 3) or the blue pigment phycocyanin (Graveholt & Erikssen, 2007). Although still not very high, floridoside yields from G. sulphuraria reported in Chapter 4 are higher than those obtained from other red alga species evaluated as possible industrial source for this glycoside (Kerjean et al., 2007) and could be potentially increased by further
improvement of the extraction method. The ability of \textit{G. sulphuraria} to grow using a by-product such as crude glycerol from biodiesel production as carbon source (\textbf{Chapter 5}) can add extra value to the production of compounds with potential industrial prospects from this microalga.

Altogether, the results presented in this thesis show the potential of the red microalga \textit{G. sulphuraria} as a source of valuable compounds other than the already well-studied pigment phycocyanin (Graveholt & Erikssen, 2007), such as a highly branched glycogen and floridoside. Its ability to grow heterotrophically in acidic conditions places \textit{G. sulphuraria} in the selective group of microalgae that can be cultivated to high cell densities (reviewed in Bumbak et al., 2011) with a minimal risk of contamination and can be exploited for obtaining carbohydrate-enriched biomass. \textit{G. sulphuraria} cells accumulating large amounts of carbohydrates could be used not only for the extraction of highly branched glycogen and floridoside, but could also be used as potential feedstock alternative to plant-based material for the production of bioethanol (Aikawa et al., 2014; Möllers et al., 2014) or biogas (Mussgnug et al., 2010).