Chapter 7

Summary &
Closing remarks
7.1 Summary

Microalgae have attracted much attention as they can be used as alternative, renewable and natural resource for the production of biofuels, pharmaceuticals, and food ingredients. Microalgae have a high productivity, grow relatively fast, and require only water, light, carbon dioxide and small amount of macro and micro-nutrients. Large-scale microalgae cultivation can even contribute to capturing carbon dioxide and reduce the green house effect. An additional advantage is that microalgae can grow in ponds on non-arable land, in brackish water, and open oceans without competing with the human food chain. Microalgae play an important role in maintaining atmospheric oxygen levels and as primary producers are the basis of our Planet’s food web.

The group of microalgae consists of more than hundred thousand species, of which many have not been characterized so far. One unique microalgae order is the Cyanidiales, or red microalgae from the phylum Rhodophyta. Cyanidiales thrive in acidic and high temperature environments. The order contains 3 genera: Cyanidium, Cyanidioschyzon, and Galdieria. The latter genus is exceptional, as it not only grows autotrophically but also heterotrophically in complete darkness using oxygen and many organic compounds as a carbon source. The Cyanidiales produce several interesting functional compounds, including photopigments, in particular phycocyanin, and the carbon/energy storage products glycogen and floridoside (Chapter 1).

This PhD thesis deals with the production of the photopigments coproporphyrinogen, an intermediate in the chlorophyll biosynthesis pathway, phycocyanin, a photopigment of the phycobilisome complex, and the functional carbohydrates glycogen and floridoside by the thermoacidophilic red microalgae Cyanidioschyzon merolae and Galdieria sulphuraria.

Phycocyanin is a light-harvesting pigment that occurs as primary phycobiliprotein in Cyanobacteria and as secondary phycobiliprotein in Rhodophyta. Phycocyanin is composed of two components: (i) an apoprotein which consists of two homologous subunits, α and β; and (ii) a chromophore or phycocyanobilin which is structurally an open chain tetapyrrole and gives the typical blue colour to phycocyanin. Commercial
phycocyanin is produced from the cyanobacterium *Spirulina platensis* that grows in open ponds under strict autotrophic conditions. The *S. platensis* phycocyanin is stable at neutral pH and moderate temperatures. Recently it was approved by the FDA and EFSA to be used in candy and ice cream as alternative to synthetic blue food colourants such as patent blue.

Heterotrophic growth offers several advantages over autotrophic growth, including higher productivity and strict control over hygiene. It has been reported that *G*. *sulphuraria* strain 074G, a mutant that still synthesizes chlorophyll under heterotrophic conditions, produces reasonable amounts of phycocyanin. **Chapter 2** reports on phycocyanin production by *G. sulphuraria* strain 074G growing heterotrophically on the potato starch maltodextrin Paselli SA2 as well as on granular starches in combination with the enzyme cocktail Stargen002.

The ability of *G. sulphuraria* 074G to grow on Paselli SA2 is explained by the production of one or more extracellular glycoside hydrolase(s) that convert Paselli SA2 into glucose, which is then taken up by cells and used as carbon and energy source. In the whole genome sequence of *G. sulphuraria* two genes encoding glucoamylases (Gasu_25520 and Gasu_25530) and one encoding a β-amylase (E.C.3.2.1.2; Gasu_04150), were identified. All three genes had a clear N terminal signal sequence, indicating that these enzymes are very likely excreted into the culture medium. The characteristics of the phycocyanin obtained from Paselli SA2 grown *G. sulphuraria* cells did not differ from the phycocyanin extracted from glucose grown cultures.

While investigating heterotrophic growth on maltodextrin and glucose, one of the culture media turned pink (**Chapter 3**). Closer analysis of the pink compound learned that it is coproporphyrin III (COPRO). COPRO is the oxidized form of coproporphyrinogen III (COPROGEN), one of the intermediates in chlorophyll and phycocyanobilin biosynthesis pathway. In **Chapter 3** the growth of *G. sulphuraria* 074G on glucose and the conditions at which COPRO is formed is investigated. The results show that the COPRO accumulated in the culture medium when *G. sulphuraria* 074G was growing on glucose with limited oxygen supply. A similar result was found when strain 074G was grown on galactose, sucrose, and ducitol, but only when the concentration of oxygen in the culture was limiting. In the biosynthesis of chlorophyll and phycocyanobilin, COPROGEN is converted into protoporphyrinogen IX by coproporphyrinogen III oxidase (CPO) which has two
structures: (i) the oxygen-dependent CPO (HemF) that is commonly found in eukaryotes and (ii) the oxygen-independent CPO (HemN) that is widely found in prokaryotes. In the whole genome of *G. sulphuraria* both forms of CPO are present. The accumulation of COPRO in the culture medium of *G. sulphuraria* 074G under limiting oxygen conditions is due to the HemF enzyme not functioning properly. HemF needs oxygen and a proton to convert coproporphyrinogen III into protoporphyrinogen IX.

Another alternative species for phycocyanin production is *Cyanidioschyzon merolae*. The production of the thermostable phycocyanin from *C. merolae* and its optimal cultivation conditions was described in Chapter 4. Phycocyanin can easily be extracted from *C. merolae* by an osmotic shock using ultra-pure water as this microalgae does not have a cell wall. The *C. merolae* phycocyanin has a high purity index of 9.9 without further purification. Phycocyanin with purity indexes of ≤ 1 are considered food grade while purity indexes of ≤ 4 are analytical grade. The *C. merolae* phycocyanin slightly differs from *Spirulina* phycocyanin, being stable at a pH 5 and temperatures of up to 80°C. The potential of *C. merolae* as an alternative to *Spirulina* phycocyanin lead to investigate the optimal outdoor cultivation conditions. The light intensity, the light period, and temperature are key factors that influence the growth of microalgae. The results presented in chapter 4 suggest that *C. merolae* phycocyanin production is most optimal at approximately 40°C, under low light intensity, and a constant light regime.

In Chapter 5, the effect of light, preservatives, and alcohol on the stability of *C. merolae* phycocyanin is described. Phycocyanin consists of an apoprotein and phycocyanobilin; it is sensitive to light and heat. The results show that *C. merolae* phycocyanin is very stable at normal daylight (1,000 Lux) and slightly acidic condition (pH 5). Sugars such as glucose, sucrose, fructose, and sorbitol which are added to food products as sweeteners and preservatives, have a positive effect on the stability of the *C. merolae* phycocyanin. It is not the type of sugar but the concentration that is the key factor in determining the stability of phycocyanin at higher temperatures. Sugars coat the protein part of phycocyanin and thereby maintain the protein structure. Also the effect of various ethanol concentrations on the stability of phycocyanin was investigated. Alcohol can agitate the water structure around a protein molecule, leading to instability and the subsequent protein precipitation.
The results of chapter 5 demonstrate that at alcohol concentration up to 10% the *C. merolae* phycocyanin could be an interesting alternative to synthetic colourants.

The potential application of phycocyanin and functional carbohydrates from *G. sulphuraria* and *C. merolae* persuades us to prospect local Indonesian isolates which are free of license (Chapter 6). Four Cyanidiales isolates were obtained from Rengganis crater near Bandung on West Jawa. The four isolates were characterized based on morphology and physiological characteristics of the cells. All cells have a spherical shape with multilobes plastid and 2 to 8 daughter cells. In addition, all were able to grow autotrophically as well as heterotrophically on glucose, galactose, and glycerol as a carbon source. Morphological and physiological observations showed that the four isolates had the characteristics of the genus Galdieria. All isolates produced phycocyanin when grown autotrophically, while only isolate Al009 and Al014 retained their photopigments when grown heterotrophically. The four isolates are very likely all four strains of the species *Galdieria sulphuraria*. *Galdieria* species are known to produce a highly branching glycogen and floridoside as carbon and energy store. All four isolates produced glycogen with a degree of branching of 13-15 %. They also produced floridoside when subjected to a hyperosmotic shock for 24 hours.

This PhD thesis shows that thermoacidophilic red microalgae can be used to produce the natural blue pigment phycocyanin and functional carbohydrates. Red microalgae grow relatively easily without the risk of infections as they thrive at extremely acidic condition, at which many other microorganisms cannot survive. Strain of the species *Galdieria sulphuraria* grow in the dark on air (oxygen) on galactose and glucose, a side product from the conversion of the milk sugar lactose, and on glycerol, a side product of biodiesel production from natural oils and fats. These *Galdieria* strains can be isolated easily from acidic hot water springs found in Indonesia and other volcanic places around the world. In short, red microalgae are sustainable and simple biological production system of functional food ingredients.
7.2 Closing Remarks

The ability to grow heterotrophically is the major physiological difference between *G. sulphuraria* and *C. merolae*. Although the genome of *C. merolae* contains the genes encoding enzymes for the metabolism of carbohydrates, such as glucose, mannose, and galactose, no genes encoding known sugar transporters are present. Barbier and colleagues (2005) identified 28 different sugar transporters in *G. sulphuraria* which are not present in the *C. merolae* genome. Previous physiological studies proved the ability of *G. sulphuraria* to take up a large variety of sugars from the environment (Oesterhelt et al., 1999; Oesterhelt and Gross, 2002). The *in-vivo* study by Oesterhelt (1999) showed that the *G. sulphuraria* sugar uptake systems contain several transporters with partly overlapping substrate specificity; the transporters were grouped into hexose, polyol and pentose transporters.

The large-scale cultivation of microalgae can be costly. In the experiments presented in this thesis, the Cyanidiales were grown in artificial medium (Allen medium) containing a suitable N and P source and complex trace elements. It would economically not be feasible to use such a complex medium for industrial scale growth of Cyanidiales. Replacing the synthetic medium with environmental or wastewater-based medium could contribute in a reduction of the cultivation costs. *G. sulphuraria* strain 074G grew well on acidic hot springs water supplemented with NH$_4^+$ (Hirooka and Miyagishima, 2016). Another approach is to use waste material as growth medium. Sloth and coworkers (2017) demonstrated that *G. sulphuraria* strain 074G grows reasonably well on hydrolysed food waste from restaurants and bakeries. Extra ammonium was needed to stimulate phycocyanin synthesis. This cultivation system can be potentially used to produce high-valuable products along with reducing food waste.

To further reduce the high costs of cultivating *C. merolae*, the choice of cultivation method and location needs to be investigated. The major factor that needs to be taken into account is sufficient supply of sunlight since *C. merolae* is an obligate autotrophic organism. At laboratory scale, the optimal condition for *C. merolae* cultivation is 24 hours light supply with moderate temperatures of approximately 40°C. Tropical countries near the Equator...
such as Indonesia with constant day-night cycles and day-night temperatures could be the best choice for a large-scale production.

The result of chapter 4 showed that the thermal stability of *C. merolae* phycocyanin is higher than the commercially available phyocyanin from *Spirulina*. The difference in thermal stability between both phycocyanins might be due to the apoprotein sequence. The *C. merolae* phycocyanin protein sequence is 75% identical to the *S. platensis* (Fig. 1), 88% identical to *C. caldarium* and 83% identical to *G. sulphuraria*. The *C. merolae* phycocyanin protein sequence contains four cysteine residues, whereas the *S. platensis* has two cysteine residues (UniProtKB-P72509). These cysteine residues can form covalent disulfide bonds that contribute to a rigid structure thereby improving the thermostability of a protein (Fass, 2012).

One way to improve the stability of phycocyanin is by adding high amount of sugar (20% up to 50%) such as glucose of sucrose (chapter 5). Another method to improve the phycocyanin stability is by removing the protein part of phycocyanin as the protein part of the phycocyanin will precipitate and contribute to bleaching of the phycocyanin due to denaturation by heat. The protein part of phycocyanin can be cleaved of using methods such as acid cleavage, enzymatic treatment, or alcoholysis. Roda-Serrat et al., (2018) reported removing phycocyanobilin of the *S. platensis* phycocyanin by solvolysis in alcohol under three different conditions. The sealed vessel method gave the fastest cleavage with the same yield as the conventional reflux method, taking more time. Although this method successfully cleaved the protein part of phycocyanin, the yield of phycocyanobilin obtained was low, about 2% of phycocyanin dry weight. A suitable method to improving phycocyanin stability with high yields still needs to be developed.
Figure 1. Alignment of amino acid sequence of subunit α and β of phycocyanin from *C. merolae* and *S. platensis*. PCA: Phycocyanin subunit α; PCB: Phycocyanin subunit β.
References


