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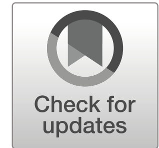

Biomass and phycocyanin content of heterotrophic *Galdieria sulphuraria* 074G under maltodextrin and granular starches–feeding conditions

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Abstract

A major disadvantage of microalgal cultivation is limited biomass yields due to the autotrophic lifestyle of most microalgal species. Heterotrophic growth on a suitable carbon source and oxygen can overcome such limitations. The red microalga *Galdieria sulphuraria* strain 074G grows heterotrophically on glucose and a number of other carbon sources while constitutively producing photopigments, including the blue-colored phycocyanin, a natural food colorant. *Galdieria sulphuraria* strain 074G grew well on maltodextrins as well as on granular starch in combination with the enzyme cocktail Stargen002. The maltodextrin cultures produced 2 mg phycocyanin per gram substrate, being slightly more than on glucose. The phycocyanin extracted from maltodextrin-grown cultures was thermostable up to 55 °C. Maltodextrins can be a cheap alternative to glucose syrups for the production of phycocyanin as natural food colorant.

Keywords *Galdieria sulphuraria* · Heterotroph · Maltodextrin · Phycocyanin · Starch

Introduction

Microalgae attract much attentions as they produce various organic compounds such as lipids, starch, and pigments that can be used as a renewable resources in the production of biodiesel, food supplements, or coloring agents (Mulders et al. 2014; Raheem et al. 2015; Rasala and Mayfield 2015; Wang et al. 2015). Large-scale cultivation of microalgae is attractive as they have a rapid life cycle, can be grown in sea, brackish water, and even waste water and in particular, do not require arable land (Chisti 2007; Li et al. 2008; Larkum et al. 2012). A major disadvantage of microalgae is that most species grow strictly autotrophic using (sun)light as energy source and carbon dioxide to form new organic matter. Autotrophic growth results in limited biomass yields as the penetration of light is inversely proportional to the cell concentration (Eriksen 2008; Liang et al. 2009; Grobbelaar 2010). Heterotrophic growth does not suffer from such disadvantages and can give substantially higher growth rates and biomass yields, especially when specific cultivation strategies like fed-batch are applied (Morales-Sanchez et al. 2013). Only a limited number of microalgae are able to grow heterotrophically depending on the strain and culture conditions (Chen and Chen 2006), examples being *Tetraselmis chuii* (Lu et al. 2017), *Chlamydomonas reindhardtii* (Zhang et al. 2019), *Nitzschia laevis* (Wen and Chen 2002), and *Neochloris oleoabundans* (Morales-Sanchez et al. 2013).

In most microalgae that grow heterotrophically, the production of photopigments is suppressed (Yamane et al. 2001; Bhatnagar et al. 2011). One of the few exceptions to this is *Galdieria sulphuraria* strain 074G, which constitutively produces photopigments when growing autotrophically in the light as well as heterotrophically in the dark (Gross and Schnarrenberger 1995). Besides chlorophyll, *G. sulphuraria* produces the blue-colored phycocyanin, a photopigment of the phycobilisomes, a light-harvesting complex found in Cyanobacteria, Cryptophyceae, and Rhodophyceae (Sekar and Chandramohan 2008). Currently the phycocyanin extracted from autotrophically grown *Spirulina platensis* is commercially available as a food colorant (Kamble et al. 2013). The

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considerably lower amount of phycocyanin per cell in heterotrophic cultures of *G. sulphuraria* 074G is compensated by much higher biomass yields (Graverholt and Eriksen 2007). An additional advantage offered by *G. sulphuraria* 074G is that it produces phycocyanin at a much higher rate than *S. platensis* (Pushparaj et al. 1997; Jiménez et al. 2003). The higher production rate and the higher yield make heterotrophic, high cell density cultivation of *G. sulphuraria* 074G attractive as an industrial-scale production system for phycocyanin.

So far, *G. sulphuraria* 074G has been grown only on low molecular weight carbon sources glucose, glycerol, or sucrose (Sloth et al. 2006; Schmidt et al. 2005). Glucose syrups used for high cell density fermentations are derived from starch by cooking the starch followed by a multistep enzymatic conversion (van der Maarel et al. 2002). Starch is a mixture of the glucose polymers amylose and amylopectin (van der Maarel et al. 2002). The granular starch is first gelatinized by jet-cooking to destroy the granular structure and bring the amylose and amylopectin in solution. Subsequently, the amylose and amylopectin are degraded by heat-stable α-amylase, liquefying the suspension, and an α-amylase-glucoamylase combination, resulting in complete saccharification. Shrestha and Weber (2007) showed that *G. sulphuraria* produces an extracellular glucoamylase, a glycoside hydrolase active at pH 2 and 80 °C converting amylose and amylopectin into glucose. In this paper, the growth of *G. sulphuraria* 074G on Paselli SA2, a potato starch maltodextrin produced by liquefaction of cooked potato starch, was tested, assuming that strain 074G also produces a glucoamylase that can convert the maltodextrin completely into glucose. Strain 074G grew very well on maltodextrins and a heat-stable phycocyanin could be extracted from the heterotrophically grown cells.

Material and methods

Strain and growth media

The red microalgae *Galdieria sulphuraria* strain 074G was obtained from AlgaeBiotech (Weesp, The Netherlands). A single colony was streaked onto Allen agar plate and grown for 2 weeks. One liter Allen medium (Allen 1959) contains 1.32 g $(NH_4)_2SO_4$, 0.27 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.074 g $CaCl_2.2H_2O$, 11 mg $FeCl_3$, 2.8 mg H_3BO_3, 1.8 mg $MnCl_2$, 0.218 mg $ZnSO_4.7H_2O$, 0.05 mg $CuSO_4$, 0.023 mg NH_4VO_3, and 0.023 mg $NaMoO_4.4H_2O$. The pH was adjusted to 2.0 with 4 M H_2SO_4, and the medium was sterilized by autoclaving at 121 °C for 20 min. Stock cultures were maintained by sub-cultivation in a mineral medium without organic carbon substrates under constant light (100 μmol photons m^{-2} s^{-1}) at 150 rpm on a shaker incubator. Growth experiments were conducted in a 1 L working volume bioreactor with constant stirring at 150 rpm at 40 °C and complete darkness. Different carbon sources were used for heterotrophic growth: glucose (Sigma, USA), Paselli SA2, corn starch, sago starch, and potato starch (all from AVEBE, The Netherlands) with a final concentration of 10 g L^{-1}. The enzyme Stargen 002 (Dupont Industrial Bioscience, The Netherlands) was added (0.5%, v/v) to the cultures supplemented with corn or potato starch. Different concentrations of glucose and Paselli SA2 (10 and 50 g L^{-1}) were added for testing their effect on the biomass and pigment yield.

Paselli SA2 is a partially hydrolyzed potato starch with an average degree of polymerization of 50 (AVEBE, The Netherlands). Stargen 002 is a blend of *Aspergillus kawachi* glucoamylase and *Trichoderma reesei* α-amylase, activity minimum 570 glucoamylase unit (GAU) g^{-1}, pH 3.3–4.5, and recommended minimum temperature is 48 °C (Genencor 2008). One GAU is the amount of enzyme that will release one gram of reducing sugars per hour from soluble starch substrate under specified condition (http://www.genencor.com).

Determination of algal growth parameters

The growth rate of cultures supplemented in corn or potato starch was monitored by cell counting. The number of cells was obtained using the improved Neubauer hemocytometer counting chamber and a light microscope. Around 200 μL of the culture was diluted, and the average cell count value was recorded as cells L^{-1}. For the cultures supplemented with glucose or Paselli SA2, growth was measured by determining the optical density at 800 nm, at which pigment absorbance is negligible. The in vivo phycocyanin amount was determined by measuring the absorption at 618 nm and 652 nm. All experiments were performed in triplicate. Specific growth rates were calculated from growth curves as the slope of the linear regression of the natural log cell number versus time by Eq. 1 (Guillard and Ryther 1962):

$$\text{Growth rate} = (\ln OD_t - \ln OD_0)/(t_t - t_0) \quad (1)$$

where, OD_0 refers to the OD value of early exponential (t_0) and OD_t is the OD value of late exponential (t_t).

At the end of exponential phase, cells were harvested by centrifugation at 10,000×*g* for 5 min and subsequently dried algal biomass was obtained by freeze-drying.

Extraction and quality test of phycocyanin

Dried biomass was resuspended in 50 mM phosphate buffer pH 7.2 and disrupted with a high pressure homogenizer (Emulsiflex-B15, Avestin) for 5 cycles at 120 psi. The cell debris was removed by centrifugation at 24,000×*g* for 90 min at 4 °C and the blue-colored supernatant was collected

in clean tubes. The phycocyanin content was measured spectrophotometrically. Phycocyanin and allophycocyanin have maximum absorption at 618 and 652, respectively. The concentration of phycocyanin in the solution was calculated using Eq. 2 (Bennet and Bogorad 1973):

$$\text{Phycocyanin}\left(\text{mg mL}^{-1}\right) = \frac{A_{618} - (0.474 \times A_{652})}{5.34} \quad (2)$$

The phycocyanin stability was evaluated as described previously with slight modification (Moon et al. 2014). 1 mL of phycocyanin solutions were incubated for 30 min at different temperatures (30, 40, 50, 60, 65, 70, and 80 °C). After incubation, the phycocyanin solutions were centrifuged to remove debris and the amount of phycocyanin in solution was measured spectrophotometrically. The remaining concentration of phycocyanin was calculated using Eq. 3 (Chaiklahan et al. 2012).

$$\text{Remaining Concentration }(C\text{R}), \% = \frac{C_1}{C_0} \times 100 \quad (3)$$

where C_0 is initial concentration of phycocyanin, and C_1 is phycocyanin concentration after treatment.

Results

Growth on maltodextrins and granular starches

As in the previous study by Schmidt et al (2005), *G. sulphuraria* was able to grow in various simple sugars as the carbon source, such as glucose, fructose, sucrose, and maltose in order to evaluate its pigmentation. In this study, effect of various complex sugar on biomass of strain 074G were investigated in a 1-L bioreactor on Allen medium under identical condition to the simple sugar substrate culture.

Growth of *G. sulphuraria* 074G on maltodextrin was compared with that with glucose at various concentrations. Figure 1 shows cell growth and substrate consumption of two comparative cultivations, using 10 g L^{-1} glucose and Paselli SA2 as growth substrate, respectively. Glucose cultures grew rapidly and within 7 days, reached their maximal cell density (OD$_{800}$ = 15.3), while cells growth at Paselli SA2 showed extension of lag phase until the fourth day. As result, Paselli SA2 cultures had a slightly longer doubling time than glucose culture (20 h versus 17 h, respectively). In the media containing 10 g L^{-1} of Paselli SA2, it was observed that the substrate was not totally consumed, in opposition to the medium with 10 g L^{-1} D-glucose, where glucose gradually decreased over time and was almost completely depleted before the end of cultivation (14 days of growth) (Fig. 1).

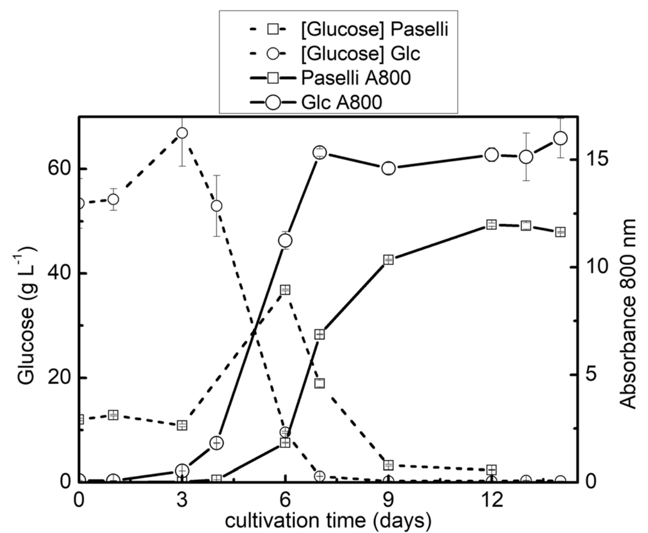

Fig. 1 Heterotrophic growth of *G. sulphuraria* 074G on 10 g L^{-1} glucose (open circle) or 10 g L^{-1} Paseli SA2 (open reverse triangle) and its consumption of C-source in 1-L bioreactor. Growth curve were determined in triplicate and symbols represent means

As shown in Table 1, when supplemented with 50 g L^{-1} glucose, the maximum biomass of 4.9 ± 0.03 g L^{-1} with the growth rate (0.49 day^{-1}) was achieved and was 1.04-fold higher than that obtained from 10 g L^{-1} glucose cultures. Interestingly, using a higher glucose concentration of 100 g L^{-1} did not provide a higher yield of biomass as the overall yield declined (data not shown). The yields (g g^{-1}) (biomass per total sugar consumed; assuming 100% conversion of Paselli to glucose) of strain 074G increased sharply when the initial Paselli SA2 concentration was increased from 10 to 50 g L^{-1}. However, no significant effect was observed in the yield of strain 074G when the glucose concentration was increased from 10 to 50 g L^{-1}. The maximum biomass concentration obtained with Paselli SA2 at 50 g L^{-1} concentration as the carbon source was 6.5 g L^{-1}.

Effect of various complex sugar on biomass of strain 074G were also investigated in a 1-L bioreactor on Allen medium containing granular starches together with Stargen 002 under identical condition to the simple sugar substrate culture. The influence of various starches on cell growth is presented in Fig. 2. Following transfer into growth medium, the lag phase of *G. sulphuraria* 074G on Allen medium containing corn-Stargen 002 was significantly shorter (2 days) than that of *G. sulphuraria* 074G on potato-Stargen 002 medium (3 days). Growth on a mixture of corn and Stargen 002 produced a growth rate (μ, 0.41 day^{-1}) greater than on potato-Stargen 002 (μ, 0.38 day^{-1}), but growth rate obtained from cultures grown on corn or potato only has very low values. The total glucose consumed (1.12 g L^{-1}, conversion of starch to glucose) of corn-Stargen 002 culture was higher compared with cultures cultivated on other starches (data not shown). As a

Table 1 Heterotrophic growth and phycocyanin production of *G. sulphuraria* 074G growing on glucose, Paselli SA2, or corn/potato starch. Experiments were carried out in triplicate. Values represent mean ± standard deviation (n = 3)

Substrate	Cons. (g L^{-1})	Biomass (g L^{-1} or cells mL^{-1})	Specific growth rate,μ (day^{-1})	Yield of phycocyanin (mg L^{-1})	Yield of phycocyanin on biomass (mg g^{-1})	Efficiency of PC on substrate (mg g^{-1})
Glucose	10	4.7 ± 0.02 g L^{-1}	0.72	16 ± 9	3.4 ± 0.02	1.6
	50	4.9 ± 0.03 g L^{-1}	0.49	83 ± 5	17 ± 0.3	1.7
Paselli-SA2	10	2.3 ± 0.01 g L^{-1}	0.82	20 ± 3	8.4 ± 0.12	2.0
	50	6.5 ± 0.54 g L^{-1}	0.66	104 ± 4	16 ± 0.12	2.1
Corn starch	5	2.3 × 10^7 cells mL^{-1}	0.38	0.6 ± 0.2	nd	1.1
Corn starch + Stargen002	5	3.1 × 10^7 cells mL^{-1}	0.41	0.2 ± 0.1	nd	0.4
Potato starch	5	1.9 × 10^6 cells mL^{-1}	0.32	0.5 ± 0.1	nd	0.9
Potato starch + Stargen002	5	2.8 × 10^6 cells mL^{-1}	0.38	1.1 ± 0.2	nd	2.1

nd = not determined

result, corn-Stargen 002 showed the fastest cell growth due to fast utilization of glucose. The specific growth rate, biomass production, and PC yield on various complex sugars were compared and summarized in Table 1.

Production and quality of phycocyanin

To demonstrate further the effect of increasing glucose concentration on phycocyanin production, the amount of glucose utilized and remaining was evaluated and measured. Sloth et al. (2006) demonstrated that heterotrophic batch culture of *G. sulphuraria* accumulates phycocyanin approximately 2–4 mg g^{-1} dry weight during exponential phase and higher when grown in fed-batch system (10–30 mg g^{-1} dry weight).

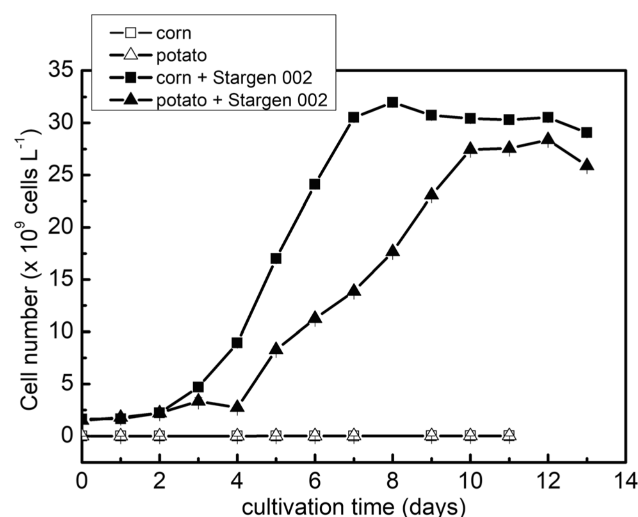

Fig. 2 Heterotrophic growth of *G. sulphuraria* 074G on 10 g L^{-1} raw starch without Stargen002 (corn, open triangle; potato, open square) and with Stargen 002 (corn, closed triangle; potato, closed square)

G. sulphuraria 074G grown on granular starch with or without Stargen 002 presented low growth rate compared with grown on complex sugar; therefore, phycoyanin production from these cultures was low. In cultures on granular starch, the highest phycocyanin production and efficiency of phycocyanin on substrate was potato-Stargen 002 culture, 1.1 ± 0.2 mg L^{-1} and 2.1 mg g^{-1}, respectively. While cultures on glucose and Paselli SA2, the highest efficiency of phycocyanin on substrate were figured on 50 g L^{-1} glucose and 50 g L^{-1}, 1.7 mg g^{-1} and 2.1 mg g^{-1}, respectively, and phycocyanin production were 83 ± 5 mg L^{-1} and 104 ± 4 mg L^{-1}. From these data, culture on Paselli SA2 gave highest phycocyanin production and it occurred on day 7.

Recently, phycocyanin is used as a coloring agent on food and beverages. For this application, critical temperature of phycocyanin is necessary to be determined. The effect of temperature on phycocyanin stability indicates that concentration of phycocyanin remained consistent until 50 °C, and decreased for 50% at 60 °C (Fig. 3). The decreasing of phycocyanin content in solution increased quickly after 55 °C.

Discussion

Growth on maltodextrins and granular starches

The productivity of several microalgal species when growing heterotrophically has been studied to achieve high amounts of biomass and high productivity of valuable bioproducts (Perez-Garcia et al. 2011). In this study, the phycocyanin production by *G. sulphuraria* strain 074G, a strain that maintains its photopigment production in the dark when growing on an organic carbon source, was investigated when maltodextrins or starch instead of glucose

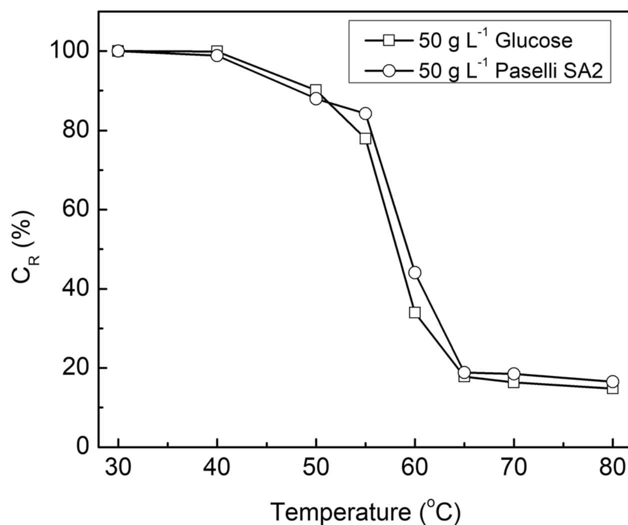

Fig. 3 Effect of different temperatures on the C_R value of phycocyanin extracted from *G. sulphuraria* 074G grown on 50 g L^{-1} glucose and 50 g L^{-1} Paselli SA2 (incubation time, 30 min)

were supplied as the substrate. On the maltodextrin Paselli SA2, a slightly longer lag phase was observed compared with glucose. In addition, the Paselli SA2 culture grew slower reaching the stationary phase after 12 days, while the glucose culture reached the stationary phase after 6 days (Fig. 1). The highest production of microalgal biomass was achieved in 50 g L^{-1} Paselli SA2, whereas 10 g L^{-1} yielded the lowest amount of biomass. The result obtained here indicates a possible adaptation of algal to Paselli SA2 and it was feasible to use Paselli SA2 as carbon source to cultivate *G. sulphuraria*. As shown in Table 1, the specific growth rates for 10 and 50 g L^{-1} of initial glucose of strain 074G cultures were 0.72 and 0.49 day^{-1}, respectively, showing that higher glucose concentrations inhibited growth. The lower specific growth was confirmed by a high amount of glucose (between 38.5 and 70.0 g L^{-1}) present in the culture supernatant at the end of cultivation (data not shown).

The ability to grow on Paselli SA2 confirms the assumption that strain 074G produces one or more extracellular glycoside hydrolase(s) that converts the Paselli SA2 maltodextrin into glucose, which is then taken up by the cells and converted into energy and organic matter. Shrestha and Weber (2007) showed by proteome analysis that 11 extracellular proteins are present in the culture medium of *G. sulphuraria* including a putative glucoamylase (E.C. 3.2.1.3; 1,4-α-D-glucan glucohydrolase). This glucoamylase is active at pH 2–2.5 and 80 °C and showed activity towards starch and maltodextrin. In the whole genome sequence of *G. sulphuraria*, a range of genes with high similarity to various glycoside hydrolases are present, including two glucoamylases (Gasu_25520 and Gasu_25530) and one β-amylase (E.C. 3.2.1.2; Gasu_04150), all three with a clear signal sequence, indicating that these enzymes are excreted.

Paselli SA2 is produced by treating cooked potato starch for a few minutes with a thermostable α-amylase. The advantage such maltodextrin offers is that cooked starch only has to be treated very briefly during passage from the jet-cooker to the fermentation tank containing *G. sulphuraria* instead of degrading the starch all the way to glucose. This way, less equipment is needed, thereby saving costs. The process would even be more straightforward if *G. sulphuraria* could be grown on uncooked, granular starch which is fed directly into the fermentation tank. *Galdieria sulphuraria* 074G did not grow on granular corn or potato starch (Fig. 2). However, when the raw starch degrading enzyme cocktail Stargen 002 was added together with the granular starch, growth was clearly observed (Fig. 2), with the growth rates only slightly lower than those on glucose or maltodextrins (Table 1).

Production and quality of phycocyanin

Glucose has been used as a substrate to grow *G. sulphuraria* 074G and produce phycocyanin (Sloth et al. 2006; Graverholt and Eriksen 2007; Sørensen et al. 2013). Sloth et al. (2006) demonstrated that heterotrophic *G. sulphuraria* 074G grown in batch accumulates approx. 2–4 mg phycocyanin g^{-1} dry weight during the exponential growth phase; much higher yields of phycocyanin were found when a fed-batch system with glucose as feed was used (10–30 mg g^{-1} dry weight). As was shown in this research, *G. sulphuraria* 074G is capable of growing on the maltodextrin Paselli SA2 and even granular potato or corn starch when the enzyme cocktail Stargen 002 was added (Table 1). On Paselli SA2, *G. sulphuraria* 074G produced equal amounts of phycocyanin (16 mg g^{-1} dry weight) as on glucose, being in the range of what Sloth et al. (2006) reported (Table 1). The volumetric productivity on Paselli SA2 (104 mg L^{-1}) was slightly higher than that on glucose (83 mg L^{-1}) (Table 1).

The phycocyanin productivity on granular starch is much lower than on maltodextrin or glucose (Table 1). However, the efficiency defined as the amount of phycocyanin per gram of substrate added on granular potato starch with Stargen 002 is similar to that of maltodextrin or glucose (Table 1). Although the number of cells on granular corn starch with Stargen 002 is comparable to potato starch with Stargen 002, much less phycocyanin could be extracted from the corn starch culture. The phycocyanin extracted from maltodextrin-grown cells did not differ from that of glucose-grown cells; the overall absorption spectrum from 300 to 800 nm showed no differences and a clear absorption maximum was found at 618 nm. These absorption spectra are very similar to those found for phycocyanin

extracted from *G. sulphuraria* (autotrophic; Moon et al. 2014), *S. platensis* (Patel et al. 2005), *Calothrix* sp (Santiago-Santos Ma et al. 2004), and *Anabaena* sp. (Ramos et al. 2009).

As *G. sulphuraria* grows in acidic hot springs up to 56 °C (Toplin et al. 2008), it is likely that the phycocyanin is stable at higher temperatures. The phycocyanin extracted from cultures grown on glucose and on Paselli SA2 was exposed for 30 min at temperatures varying from 30 to 80 °C (Fig. 3). Up to 55 °C, both phycocyanin solutions remained clearly blue, with 90% remaining in soluble. At 60 °C, most of the phycocyanin precipitated and the solution turned almost colorless. This finding is consistent with Moon et al. (2014), since phycocyanin is a protein conjugate pigment, the heat-induced irreversible process of phycocyanin denaturation.

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