1 For Bioresource Technology as an Original article

2 **Profiling of lipid and glycogen accumulations under different growth conditions in**
3 **the sulfothermophilic red alga *Galdieria sulphuraria***

4

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12

13 **Abbreviations:** DMSO, dimethylsulfoxide; DP, degree of polymerization; TAG,

14 triacylglycerol

1 **Abstract**

2 The unicellular red alga *Galdieria sulphuraria* grows efficiently and produces a large
3 amount of biomass in acidic conditions at high temperatures. It has great potential to
4 produce biofuels and other beneficial compounds without becoming contaminated with
5 other organisms. In *G. sulphuraria*, biomass measurements and glycogen and lipid
6 analyses demonstrated that the amounts and compositions of glycogen and lipids
7 differed when cells were grown under autotrophic, mixotrophic, and heterotrophic
8 conditions. Maximum biomass production was obtained in the mixotrophic culture.
9 High amounts of glycogen were obtained in the mixotrophic cultures, while the amounts
10 of neutral lipids were similar between mixotrophic and heterotrophic cultures. The
11 amounts of neutral lipids were highest in red algae, including thermophiles. Glycogen
12 structure and fatty acids compositions largely depended on the growth conditions.

13

14 **Keywords:** biomass, *Galdieria sulphuraria*, glycogen, lipid, red algae

15

1 **Introduction**

2 Microalgae are used as commercial sources of high-value products (e.g.,
3 β -carotenoids, docosahexaenoic acid, and eicosahexaenoic acid). In the near future,
4 biofuels are expected to serve as clean and renewable forms of energy. Microalgae are
5 promising feedstocks because they produce large amounts of biomass and do not
6 compete with food production (Yoshida et al., 2012). However, microalgae often
7 become contaminated with other microorganisms in large-scale outdoor cultivations,
8 which is a major problem that inhibits algal growth and decreases the quality of bio-fuel
9 and high-value products.

10 The Cyanidiales, which are unicellular red microalgae that comprise three
11 genera, *Cyanidium*, *Cyanidioschyzon*, and *Galdieria*, usually live in acidic (pH 5) hot
12 sulfur springs at temperatures below 56°C (Seckbach, 1994). They are among the most
13 adapted eukaryotes to acidic conditions, and *Galdieria* is the predominant genus in such
14 extreme conditions. In addition to their efficient growth in extreme environments where

1 it is difficult for other organisms to grow, *Galdieria* produce a large amount of biomass
2 and many beneficial compounds (Graverholt and Eriksen, 2007). Additionally, the use
3 of *Galdieria* cells is effective for the recovery of many crucial elements, including
4 phosphate and rare earth elements, from wastewater (Selvaratnam et al., 2014; Minoda
5 et al., 2015). Furthermore, nutrient removal from urban wastewater was successfully
6 achieved using a large-scale, outdoor *Galdieria* culture (Selvaratnam et al., 2014).

7 Among the three genera in the Cyanidiales, *Galdieria* are the only algae that
8 can grow autotrophically as well as mixotrophically and heterotrophically, using over 27
9 kinds of sugars and sugar alcohols (Gross and Schnarrenberger, 1995). In this report, the
10 amounts and compositions of lipids and glycogen produced by *Galdieria sulphuraria*
11 under different growth conditions were assessed. Such knowledge is useful for
12 understanding the unique carbon metabolism of *G. sulphuraria* in extreme
13 environments and the production of beneficial compounds (Seckbach, 1994; Gross and
14 Schnarrenberger, 1995; Schönknecht et al., 2013).

2. Materials and Methods

2.1. Algal cultures

One hundred and fifty milliliters of *G. sulphuraria* 074W cells were grown in modified 2× Allen's medium (pH 2.5) in 300-ml shaking flasks at 42°C under ordinary air conditions (Minoda et al., 2015). For mixotrophic and heterotrophic cultures, cells were grown in modified 2× Allen's medium containing 25 mM glucose. The autotrophic and mixotrophic cells were incubated under continuous light (50 $\mu\text{E}/\text{m}^2\cdot\text{s}$), whereas the cells cultured under heterotrophic conditions were incubated in the dark. In Fig. 1, cells were harvested at the day indicated in the figure legend. For biomass measurements and glycogen and lipid analyses, cells were harvested in the stationary phase (optical density at 750 nm (OD_{750}) = 2). Values are averages $\pm$ SDs of three (Fig. 1, Fig. S2) or six independent experiments (Table 1, Fig. 3, Fig. S4, Table S1). Details are provided in the

1 supplementary file.

2

3 *2.2. Glycogen quantification*

4

5 One milliliter of cells was harvested by centrifugation ($3,500 \times g$, 10 min,
6 4°C) and suspended in 90% ethanol. Following 5 min of disruption in bead beater
7 (TOMY, Tokyo, Japan), the suspension was boiled for 5 min at 60°C and centrifuged
8 ($10,000 \times g$, 2 min, 4°C). After washing with ethanol, glycogen was extracted from the
9 pellet by boiling in water. The glycogen levels were determined using the Starch Assay
10 Kit (BioVision, Milpitas, CA, USA). Details are provided in the supplementary file.

11

12 *2.3. Glycogen preparation and capillary electrophoresis of debranched glycogen*

13

1 Glycogen was prepared using the dimethylsulfoxide (DMSO) extraction
2 method as described previously (Shimonaga et al., 2008). Aliquots (20 mg) of glycogen
3 were used for the capillary electrophoresis of debranched glycogen as described
4 previously. Details are provided in the supplementary file.

5

6 *2.4. Lipid extraction*

7

8 Total lipids were extracted from cells grown under three different culturing
9 conditions as described previously (Minoda et al., 2002). As a modification, after
10 harvesting by centrifugation, cells were dissolved in methanol and sonicated for 30 min
11 in an ice-cooled water bath with an ultrasonic cleaner (Branson Ultrasonic, Danbury, CT,
12 USA) to improve the extraction. Total lipids were separated into individual neutral lipid
13 classes by thin-layer chromatography (TLC) with a hexane/diethyl ether/acetate

1 (70:30:1, by vol.) solvent system. Each lipid class was identified by using lipid class
2 standards (Fig. S1). Details are provided in the supplementary file.

3

4 2.5. *Gas chromatography/mass spectrometry (GC/MS) analysis*

5

6 GC/MS analysis of the methyl-esterified fatty acids was conducted using an

7 Agilent 7890 Series gas chromatograph directly coupled to an Agilent 5985A mass

8 selective detector (Agilent Technologies, Santa Clara, CA, USA). Nonadecanoic acid

9 was used as an internal standard. Gas chromatographic separation was performed on

10 ULBON HR-Thermon-3000B capillary column (I.D. 0.25 mm × 25 m, Shinwa

11 Chemical Industries Ltd., Kyoto, Japan). Details are provided in the supplementary file.

12

13 2.6. *Microscopic observations*

14

1 Cells were stained by a 30-min incubation with 2 µg/ml Nile red (1:1,000
2 dilution of a 2 mg/ml Nile red stock in acetone) in water at room temperature. After
3 washing with the culture medium, stained cells were observed under a fluorescence
4 microscope (BX-FLA. Olympus Optical Co., Tokyo, Japan) using a 520–550-nm
5 excitation filter.

6

7 **3. Results and Discussion**

8

9 *3.1. Accumulations of glycogen and lipids are dependent on the growth conditions*

10 *G. sulphuraria* cells were grown under three different growth conditions
11 (autotrophic, mixotrophic, and heterotrophic). The growth factors, μ (day^{-1}), were 0.16
12 ± 0.04 , 1.1 ± 0.08 , and 1.2 ± 0.1 under autotrophic, mixotrophic, and heterotrophic
13 conditions, respectively (Fig. 1a). Although the growth factors under mixotrophic and
14 heterotrophic conditions were equal, in terms of dry weight, cell volume, and cell

number, the highest biomass production was observed under mixotrophic conditions (Table 1). There was not significant difference in cellular dry weight under all conditions (Table 1). The dry weight and cell volume were almost 3- and 1.5-fold higher than those of the autotrophic and heterotrophic conditions, respectively, and the cell number was twice those of the autotrophic and heterotrophic conditions (Table 1). The *G. sulphuraria* cell number was similar to that of another red alga, *Porphyridium cruentum*, which grows autotrophically, but the dry weight of *G. sulphuraria* was more than 100-fold larger than that of *P. cruentum* (Sheath et al., 1979; Fleck-Schneider et al. 2007). Generally, red algal biomass production is quite low compared with other algae, including green algae *Chlamydomonas* and *Chlorella*, which can grow as autotrophs, mixotrophs and heterotrophs (Sheath et al., 1979; Fleck-Schneider et al. 2007; Work et al., 2010; Liu et al., 2011; Brányiková et al., 2011; Přibyl et al., 2012; Li et al., 2013; Takeshita et al., 2014). The cell number of *G. sulphuraria* was less than one-tenth of a mixotrophic culture of green algae, while *G. sulphuraria* dry weights were almost equal

1 to or were greater than those of the latter (Work et al., 2010; Takeshita et al., 2014).

2 Thus, *G. sulphuraria* is likely to use carbon sources more efficiently for cellular

3 biomass production than for cell division.

4 Total amounts of storage α -glucan and total lipids (total FAME) were determined in

5 the exponential (Day 3) and stationary (Day 13) phases (Fig. 1 b, c). *Galdieria*

6 accumulate storage α -glucan, glycogen (Stadnichuk et al., 2007; Shimonaga et al.,

7 2008), which is distinguished from the semi-amylopectin type of α -glucan, called

8 floridean starch, in red algae (Sheath et al., 1979; Nakamura et al. 2005; Shimonaga et

9 al., 2008). In the exponential phase, there was little difference in the amounts of

10 glycogen and lipids amongst the culture conditions, while their amounts did differ

11 markedly during the stationary phase. In the mixotrophic culture, the maximum

12 glycogen content per ml of culture was almost 10- and 2-fold greater than those of the

13 autotrophic and heterotrophic cultures (Fig. 1, b). The highest amounts of lipids per ml

14 of culture were observed in the heterotrophic culture, and they were almost 3- and

1 2-fold greater than those of the autotrophic and mixotrophic cultures (Fig. 1, c).

2 However, despite the large amount of dry weight under mixotrophic conditions,

3 the amounts of glycogen and lipids were significantly lower, which suggests the other

4 carbon stocks in *G. sulphuraria*. The other carbon stocks might correlate with the

5 similar cellular dry weights under all conditions (Table 1). In a previous study, as the

6 dry weight was increased by the addition of extra nitrogen and carbon sources, the

7 phycocyanin content of *G. sulphuraria* increased in a heterotrophic culture (Graverholt

8 and Eriksen, 2007). Similar to *Cyanidium*, *G. sulphuraria* also produces large amounts

9 of extracellular polysaccharides (Azúa-Bustos et al., 2009). The unique distribution of

10 carbon storage in *G. sulphuraria* under different growth conditions is an interesting

11 issue that should be examined in future studies. On the other hand, cellular glycogen

12 contents were significantly increased in mixotrophic and heterotrophic cultures of *G.*

13 *sulphuraria* by the addition of glucose (Fig. S2,), which contrasts with the fact that the

14 amount of cellular starch in *Chlamydomonas reinhardtii* was largely unaffected in

1 autotrophic, mixotrophic, and heterotrophic cultures supplied with acetate as a carbon
2 source (Ball et al., 1990). Thus, the regulation of the metabolic carbon flow into
3 glycogen and fatty acids synthesis would largely depend upon the growth conditions in
4 *G. sulphuraria*.

5

6 3.2. *The G. sulphuraria glycogen structure is dependent on the growth conditions*

7

8 Glycogen was prepared from *G. sulphuraria* cells grown under different growth
9 conditions. The chain length distribution was determined by capillary electrophoresis
10 after debranching of the glycogen by isoamylase and is represented as DP (degree of
11 polymerization of glucose) in Fig. 2. As reported previously, a highly branched structure
12 with the enrichment of short chains ($DP \leq 9$) was observed, regardless of the growth
13 conditions (Stadnichuk et al., 2007; Shimonaga et al., 2008). However, the structure
14 differed with growth conditions. Small amounts of longer chains were detected in the

1 autotrophic and mixotrophic cultures (Fig.2, a, b), while glycogen chain lengths over
2 DP15 were not detected in the heterotrophic culture (Fig. 2, c). Glycogen structure is
3 regulated by several enzymes in the biosynthesis pathways and is basically preserved at
4 the species level (Ball et al., 2015). Alterations of structure in response to environmental
5 stimuli are rarely seen. In our experimental system, the glycogen structures of the
6 autotrophic and mixotrophic culture are rather similar to that observed in an autotrophic
7 culture of *Cyanidium caldarium* than an autotrophic culture of *Galdieria sulphuraria*
8 M-8 (Shimonaga et al., 2007; 2008) (Fig. 2, a, b). In *G. sulphuraria*, the glycogen
9 structure is presumably regulated by the inner metabolic state in response to the
10 different growth conditions. Further analyses will provide new insights into the
11 regulation of the unique mechanism of α -glucan synthesis in *G. sulphuraria*.

12

13 3.3. The lipid composition of *G. sulphuraria* is dependent on the growth conditions

14

1 To analyze the accumulation of neutral lipids, cells in the exponential and
2 stationary phases were stained with Nile red (Fig. S3). Lipid droplets were detected
3 under mixotrophic and heterotrophic conditions and increased in the stationary phase,
4 whereas they were not detected under autotrophic conditions. This observation is in
5 agreement with the increase in the amount of triacylglycerol (TAG) in the mixotrophic
6 and heterotrophic cultures (Fig. S4). Additionally, diacylglycerol (DG) accounted for
7 43% of the total lipids in the heterotrophic culture (Fig. S2, Table S1). The amounts of
8 TAG were increased by the addition of glucose, and they were relatively high in
9 heterotrophic cultures ($11 \pm 6 \mu\text{g/ml}$) compared with mixotrophic cultures ($6 \pm 3 \mu\text{g/ml}$).
10 Due to the higher biomass production of *G. sulphuraria* compared with
11 *Cyanidioschyzon merolae*, the TAG amounts per ml of culture in mixotrophic and
12 heterotrophic cultures of *G. sulphuraria* were higher than that ($\sim 4 \mu\text{g/ml}$) of a
13 genetically modified strain of *C. merolae*, in which the storage lipid level was increased
14 (Sumiya et al., 2015).

1 Fatty acid compositions up to 24:1 (the number of carbon chains:the number of
2 double bonds) fatty acids were quantified by GC-MS (Fig. 3). Long-chain fatty acids
3 were not detected in *G. sulphuraria* (Lang et al., 2011) and there was little agreement on
4 the presence of long-chain fatty acids in *C. merolae* (Sato and Moriyama, 2007; Sumiya
5 et al., 2015). Under all growth conditions, 16:0, 18:1, and 18:2 fatty acids were
6 predominant, although there were increases in the levels of 18:0 fatty acids in the
7 mixotrophic culture and 18:3 fatty acids in the heterotrophic culture. Trace amounts of
8 20:0, 20:2, and 24:0 fatty acids were detected under all growth conditions, while 22:0
9 and 20:3 fatty acids were detected specifically in mixotrophic and heterotrophic
10 conditions, respectively (Fig. S5). Trace amounts of 20:1 and 22:2 fatty acids may also
11 have been present, but this needs to be analyzed further. The absence of 20:4 and 20:5
12 fatty acids, which accumulate in red algae, except for the Cyanidiales, is in agreement
13 with the lack of obvious desaturase homologs in the *G. sulphuraria* genome (Fleurence
14 et al., 1994; Schönknecht et al., 2013).

1 The percentages of saturated fatty acids were remarkably high (> 45%) in DG, free
2 fatty acids and TAG under all conditions (Table S1). In particular, they accounted for
3 more than 90% of free fatty acids and TAG in autotrophic culture and free fatty acids in
4 mixotrophic culture (Table S1). Conversely, the addition of glucose and growth in the
5 dark decreased the amounts of 16:0 and 18:0 and increased the amounts of 18:3 (Fig. 3).
6 20:0 was also increased in DG under heterotrophic conditions (Fig. 3).

7 Fatty acids are mainly synthesized *de novo* in the plastid and they become
8 unsaturated in the plastid or endoplasmic reticulum (ER) (Browse and Somerville,
9 1991). Taking into the account that the elongation of long fatty acids occurs in the ER
10 and the active synthesis of unsaturated fatty acids occurs in the ER of *C. merolae* (Sato
11 and Moriyama, 2007), in our experimental system, the fatty acid elongation and
12 unsaturation in the ER seems to have been activated under heterotrophic conditions.

13 This activation will not be ascribed to the direct influence of the absence of light but to
14 the cellular metabolic state, because 18:3 chains were also detected in autotrophic or

1 mixotrophic cultures in other *Galdieria* species (Lang et al., 2011). The regulation of
2 the metabolic flow of fatty acids is important for the production of beneficial long-chain
3 fatty acids such as docosahexanoic acids accumulated in red algae and quality control of
4 bio-fuels. However, we still do not know how it is regulated. *G. sulphuraria* is a good
5 model system to understand such regulation.

6 Our findings show that the amounts and compositions of glycogen and lipid are
7 largely regulated by growth conditions and suggest that there are large, unknown carbon
8 stores in addition to glycogen and neutral lipids in *G. sulphuraria*. Previous studies
9 showed that *G. sulphuraria* can use various carbon sources for its growth, even at high
10 concentrations that usually cause osmotic damage, and it can even grow in pure CO₂ or
11 in air containing 50 ppm of SO₂ (Sechbach and Baker, 1970; Gross and Schnarrenberger,
12 1995; Kurano et al., 1995; Graverholt and Eriksen, 2007). Understanding the
13 mechanisms underlying the results shown in this study will give us the understanding
14 the unique carbon metabolism of *G. sulphuraria* in extreme environments, which enable

1 efficient production of beneficial compounds under extreme conditions. Optimizing
2 culture conditions and establishing gene manipulation techniques will further this goal.

3

4 **4. Conclusions**

5

6 In this study, the amounts and compositions of glycogen and lipids in *G. sulphuraria*
7 were characterized under different growth conditions. The accumulation of glycogen
8 was promoted by the addition of glucose and the accumulation of neutral lipids was
9 promoted by the addition of glucose and growth in the dark. The composition of
10 glycogen and lipids were largely changed dependent on the growth condition.

11

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13

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8 **References**

- 9 1. Azúa-Bustos, A., González-Silva, C., Mancilla, R. A., Salas, L, Palma, R. E., Wynne,
10 J. J., McKay, C. P., Vicuña, 2009. Ancient photosynthetic eukaryote biofilms in an
11 Atacama desert coastal cave. *Microb. Ecol.* 58, 485-496.
- 12 2. Ball, S.G., Dirick, L., Decq, A., Martia, J.-C., Matagne, R.F., 1990. Physiology of
13 starch storage in the monocellular alga *Chlamydomonas reihardtii*. *Plant Sci.* 66,
14 1-9.

- 1 3. Ball, S., Colleoni, C., Arias M.C., 2015. The transition from glycogen to starch
2 metabolism in cyanobacteria and eukaryotes, in: Nakamura, Y. (Ed.), Starch.
3 Springer Japan, Tokyo, pp.93-158.
- 4 4. Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V.,
5 Vítová, M. 2011. Microalgae-novel highly efficient starch producers. Biotechnol.
6 Bioeng. 108, 766-776.
- 7 5. Browse, J., Someville, C. 1991. Glycerolipid synthesis: Biochemistry and regulation.
8 Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 467-506.
- 9 6. Fleck-Schneider, P., Lehr, F., Posten, C., 2007. Modelling of growth and product
10 formation of *Porphyridium purpureum*. J. Biotechnol. 132, 134-141.
- 11 7. Fleurence, J., Gutbier, G., Mabeau S., Leray, C., 1994. Fatty acids from 11 marine
12 macroalgae of the French Brittany coast. J. Appl. Phycol. 6, 527-532.
- 13 8. Graverholt, O.S., Eriksen, N.T., 2007. Heterotrophic high-cell-density fed-batch and
14 continuous-flow cultures of *Galdieria sulphuraria* and production of phycocyanin.

- 1 Appl. Microbiol. Biotechnol. 77, 69-75.
- 2 9. Gross, W., Schnarrenberger, C., 1995. Heterotrophic growth of two strains of the
- 3 acido-thermophilic red alga, *Galdieria sulphuraria*. Plant Cell Physiol. 36,
- 4 633-638.
- 5 10. Kurano, N., Ikemoto, H., Miyashita, H., Haegawa, T., Hata, H., Miyachi, S., 1995.
- 6 Fixation and utilization of carbon dioxide by microalgal photosynthesis. Energy
- 7 Convers. Mgmt. 36, 689-692.
- 8 11. Lang, I., Hodac, L., Friedl, T., Feussner I., 2011. Fatty acid profiles and their
- 9 distribution patterns in microalgae: a comprehensive analysis of more than 2000
- 10 strains from the SAG culture collection. BMC Plant Biol. 11, 124.
- 11 12. Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., Cížková, M.,
- 12 Brányiková, I., Vítová, M. 2013. The microlaga *Prachlorella kessleri*-A novel
- 13 high efficient lipid producer. Biotechnol. Bioeng. 110, 97-107.
- 14 13. Liu, J., Huang, J, Sun, Z., Zhong, Y., Jiang, Y., Chen, F., 2011. Differential lipid and

- 1 fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*:
2 Assessment of algal oils for biodiesel production. Bioresource Technol. 102,
3 106-110.
- 4 14. Minoda, A., Sato, N., Nozaki, H., Okada, K., Takahashi, H., Sonoike, K., Tsuzuki,
5 M., 2002. Role of sulfoquinovosyl diacylglycerol for the maintenance of
6 photosystem II in *Chlamydomonas reinhardtii*. Eur. J. Biochem. 269, 2353-2358.
- 7 15. Minoda, A., Sawada, H., Suzuki, S., Miyashita, S., Inagaki, K., Yamamoto, T.,
8 Tsuzuki, M., 2015. Recovery of rare earth elements from the sulfo-thermophilic
9 red alga *Galdieria sulphuraria* using aqueous acid. Appl. Microbiol. Biotechnol.
10 99, 1513-1519.
- 11 16. Nakamura, Y., Takahashi, J., Sakurai, A., Inaba, Y., Suzuki, E., Nihei, S., Fujiwara, S.,
12 Tsuzuki, M., Miyashita, H., Ikemoto, H., Kawachi, M., Sekiguchi, H., Kurano, M.,
13 2005. Some cyanobacteria synthesize semi-amylopectin type -polyglucans
14 instead of glycogen. Plant Cell Physiol. 46, 539-545.

- 1 17. Přibyl, P., Cepák, V., Zachleder, V. 2012. Production of lipids in 10 strains of
2 *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella*
3 *vulgaris*. *App. Microbiol. Biotechnol.* 94, 549-561.
- 4 18. Sato, N., Moriyama, T., 2007. Genomic and biochemical analysis of lipid
5 biosynthesis in the unicellular rhodophyte *Cyanidioschyzon merolae*: Lack of a
6 plastidic desaturation pathway results in the coupled pathway of galactolipid
7 synthesis. *Eukaryot. Cell* 6, 1006-1017.
- 8 19. Schönknecht, G., Schönknecht, G., Chen, W.H., Ternes, C.M., Barbier, G.G.,
9 Shrestha, R.P., Stanke, M., Bräutigam, A., Baker, B.J., Banfield, J.F., Garavito,
10 R.M., Carr, K., Wilkerson, C., Rensing, S.A., Gagneul, D., Dickenson, N.E.,
11 Oesterhelt, C., Lercher, M.J., Weber, A.P., 2013. Gene transfer from bacteria and
12 archaea facilitated evolution of an extremophilic eukaryote. *Science* 339,
13 1207-1210.
- 14 20. Seckbach J., 1994. Evolutionary Pathways and Enigmatic Algae: *Cyanidium*

- 1 *caldarium* (Rhodophyta) and Related Cells. Kluwer Academic Publishers,
2 Dordrecht.
- 3 21. Seckbach, J., Baker, F.A, 1970. Algae thrive under pure CO₂. Nature 227, 744-745.
- 4 22. Selvaratnam, T., Pegallapati, A.K., Montelya, F., Rodriguez, G., Nirmalakhandan, N.,
5 Voorhies, W.V., Lammers, P.J., 2014. Evaluation of a thermo-tolerant acidophilic
6 alga, *Galdieria sulphuraria*, for nutrient removal from urban wastewaters.
7 Bioresor. Technol. 156, 395-399.
- 8 23. Sheath, R.G., Hellebust, J.A., Sawa, T., 1979. Floridean starch mechanism of
9 *Porphyridium purpureum* (Rhodophyta) I. Changes during ageing of batch
10 culture. Phycologia, 18, 149-163.
- 11 24. Shimonaga, T., Fujiwara, S., Kaneko, M., Izumo, A., Nihei, S., Francisco Jr., P. B.,
12 Satoh, A., Fujita, N., Y., Nakamura, Y. Tsuzuki, M. 2007. Variation in storage
13 -polyglucans of red algae: amylose and semi-amylopectin types in *Porphyridium*
14 and glycogen type in *Cyanidium*. Marine Biotechnol. 9, 192-202.

- 1 25. Shimonaga, T., Konishi, M., Oyama, Y., Fujiwara, S., Satoh, A., Fujita, N., Colleoni,
2 C., Buléon, A., Putaux, J.-L., Ball, S.G., Yokoyama, A., Hara, Y., Nakamura, Y.,
3 Tsuzuki, M., 2008. Variation in Storage -glucans of the Porphyridiales
4 (Rhodophyta). *Plant Cell Physiol.* 49, 103-116.
- 5 26. Stadnichuk, I.N., Semenova, L.R., Smirnova, G.P., Usov, A.I., 2007. A highly
6 branched storage polyglucan in the thermoacidphilic red microalga *Galdieria*
7 *maxima* cells. *Appl. Biochem. Microbiol.* 43, 78-83.
- 8 27. Sumiya, N., Kawase, Y., Hayakawa, J., Matsuda M., Nakamura M., Era, A., Tanaka,
9 K., Kondo, A., Hasunuma, T., Imamura, S., Miyagishima, Sy., 2015. Expression
10 of cyanobacterial acyl-ACP reductase elevates the triacylglycerol level in the red
11 alga, *Cyanidioschyzon merolae*. *Plant Cell Physiol.* doi:10.1093/pcp/pcv120.
- 12 28. Takeshita, T., Ota, S., Yamazaki, T. Hirata, A., Zachleder, V., Kawano, S., 2014.
13 Starch and lipid accumulation in eight strains of six *Chlorella* species under
14 comparatively high light intensity and aeration culture condition. *Bioresource*

- 1 Technol. 158, 127-134.
- 2 29. Work, V.H., Radakovits, R., Jinkerson, R.E., Meuser, J.E., Elliott, L.G., Vinyard,
3 D.J., Laurens, L.M., Dismukes, G.C., Posewitz, M.C., 2010. Increased lipid
4 accumulation in the *Chlamydomonas reinhardtii* sta7-10 starch isoamylase mutant
5 and increased carbohydrate synthesis in complemented strains. Eukaryot. Cell 9,
6 1251-1261.
- 7 30. Yoshida, M., Tanabe, Y., Yonezawa, N., Watanabe, M.M., 2012. Energy innovation
8 potential of oleaginous microalgae. Biofuels. 3, 761-781.

9 **Figure Captions**

10 Fig. 1. Growth curve and accumulations of glycogen and lipids in *G. sulphuraria* under
11 different growth conditions. Cells were grown under autotrophic, mixotrophic, and
12 heterotrophic conditions (a) and harvested in the exponential (Day 3) and stationary
13 (Day 13) phases. Glycogen (b) and lipids (c) were quantified per ml of culture. Values
14 are averages $\pm$ SDs of three independent experiments.

1

2 Fig. 2. Chain length distributions of glycogen in *G. sulphuraria* under different growth
3 conditions. Each glycogen sample obtained from autotrophic (a), mixotrophic (b), and
4 heterotrophic (c) conditions was debranched with *P. amyloclavus* ISA. The
5 distributions of reduced debranched glycogen with different chain lengths were
6 analyzed as described in the Materials and Methods.

7 Fig. 3. Fatty acid composition (percentage of total fatty acids) of total lipids and lipid
8 classes of *G. sulphuraria* under different growth conditions. Total lipids were extracted
9 from cells grown under autotrophic (a), mixotrophic (b), and heterotrophic (c)
10 conditions. Individual lipid classes were separated using TLC and quantified by GC/MS
11 after methyl-esterification, as described in the Materials and Methods. The fatty acid
12 composition is shown on the side legend as the number of carbon atoms:the number of
13 double bonds. The percentages were calculated from six independent experiments. Total,
14 total lipids; DG, diacylglycerol; FA, free fatty acids; TAG, triacylglycerol

Fig. 1.

Fig. 2.

Fig. 3.

Table 1

Table 1 Biomass production of *G. sulphuraria* under different growth conditions

	Autotroph			Mixotroph			Heterotroph		
dry weight (mg/ml)	1.1	±	0.1	3.8	±	0.2	2.4	±	0.2
cell volume (µl pcv*/ml)	4.9	±	1.5	11.1	±	0.6	7.8	±	0.2
cell number (10 ⁶ cells/ml)	2.9	±	1.5	5.1	±	0.3	3.2	±	0.1
cellular dry weight (ng/cell)	0.7	±	0.2	1.0	±	0.2	0.8	±	0.1

***pcv, packed cell volume**

***Values are averages ± S.E.s of six independent experiments.**