Acidophilic Green Alga *Pseudochlorella* sp. YKT1 Accumulates High Amount of Lipid Droplets under a Nitrogen-Depleted Condition at a Low-pH

Shunsuke Hirooka1,2*, Sumio Higuchi3, Akihiro Uzuka1,4, Hisayoshi Nozaki5, Shin-ya Miyagishima1,2,4

1 Center for Frontier Research, National Institute of Genetics, Mishima, Shizuoka, Japan, 2 Japan Science and Technology Agency, CREST, Kawaguchi, Saitama, Japan, 3 Suzaka, Nagano, Japan, 4 Department of Genetics, Graduate University for Advanced Studies (SOKENDAI), Mishima, Shizuoka, Japan, 5 Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan

Abstract

Microalgal storage lipids are considered to be a promising source for next-generation biofuel feedstock. However, microalgal biodiesel is not yet economically feasible due to the high cost of production. One of the reasons for this is that the use of a low-cost open pond system is currently limited because of the unavoidable contamination with undesirable organisms. Extremophiles have an advantage in cultivating in an open pond system because they grow in extreme environments toxic to other organisms. In this study, we isolated the acidophilic green alga *Pseudochlorella* sp. YKT1 from sulfuric acid mine drainage in Nagano Prefecture, Japan. The vegetative cells of YKT1 display the morphological characteristics of Trebouxiophyceae and molecular phylogenetic analyses indicated it to be most closely related to *Pseudochlorella pringsheimii*. The optimal pH and temperature for the growth of YKT1 are pH 3.0–5.0 and a temperature 20–25°C, respectively. Further, YKT1 is able to grow at pH 2.0 and at 32°C, which corresponds to the usual water temperature in the outdoors in summer in many countries. YKT1 accumulates a large amount of storage lipids (~30% of dry weight) under a nitrogen-depleted condition at low-pH (pH 3.0). These results show that acidophilic green algae will be useful for industrial applications by acidic open culture systems.

Introduction

Recently, microalgae have come to be seen as a promising source of next-generation biofuel feedstock that can be produced without compromising the production of food, fodder and other products derived from crops [1,2]. Microalgae have the potential to produce up to 300 times more lipids than the major oil crops, based on biofuel feedstock production per area [3], because of their rapid growth and high lipid content (20% to 50% of dry weight) [1]. Thus, microalgae have been suggested as an alternative source of green renewable energy [4]. However, industrial production of microalgal biodiesel is not yet economically feasible because of the high cost. This results mainly from the limited biological information on the oil accumulation mechanisms in microalgae [3] and problems with successful mass cultivation system at a low cost [5].

Open ponds are the simplest systems for the mass cultivation of microalgae and cost less to build and operate than enclosed, controlled photobioreactors. However, the biomass productivity of open pond systems is limited because they are easily contaminated by other, undesirable microorganisms that suppress algal growth. Sustained and reliable cultivation of a single species in open pond systems can be encouraged by cultivating extremophiles that tolerate a particular environment that is lethal for other species [3]. Among the extremophiles, *Spirulina platensis* and *Dunaliella salina* are widely cultured in open ponds and commercialized, mainly to produce nutritional supplements for humans and animal feed additives [6]. The alkaliphilic cyanobacterium *Spirulina platensis* is grown with a high concentration of bicarbonate (16 g/l) at a high pH [7]. The halophilic green alga *Dunaliella salina* is grown in high saline water (more than three times concentrated than seawater) [7]. These successful examples have led to the use of extremophiles for the mass cultivation of microalgae in open ponds.

Highly acidic environments are relatively scarce worldwide and are generally associated with volcanic activity or mining operations. In most acidic environments, acidified water facilitates metal solubility, and thus acidic environments tend to have high concentrations of heavy metals, which are toxic to organisms [8]. Despite these extreme and generally harmful environmental conditions, a number of bacteria, archaea and eukaryotes have been identified as living under them, mainly by community structure analyses [9,10]. Among the eukaryotes, microalgae are reported to exist in relative abundance in highly acidic environments, including...
the Chlorophyta, such as Chlamydomonas, Dunaliella, and Chlorella species [11]. However, the biodiversity of the validated species of acidophilic organisms is still very limited compared with that in a neutral environment [12]. Therefore, acidophilic algae also are considered to be a cultivar candidate in open pond systems since contamination by undesired organisms is limited. In industrial scale yeast fermentation, acidic conditions (pH < 5.0) have been used to reduce the risk of microbial contamination [13,14]. Thus, the isolation of fast-growing acidophilic green algae from an acidic environment is one of the key approaches to low-cost production of next-generation biofuels and supplements. In addition, as the water temperature commonly exceeds 30 °C in the outdoors in summer [15–17], a tolerance to high temperature is also an important screening index.

In this study, we report the identification, physiological features and lipid productivity of the acidophilic green alga Pseudochlorella sp. YKT1, which was isolated from acidic mine drainage (AMD). The optimal pH and temperature for growth of YKT1 are pH 3.0–5.0 and 20–25 °C, respectively. Further, YKT1 is able to grow at pH 2.0 and at 32 °C. In addition, we show storage lipid productivity of YKT1 under a nitrogen-depleted condition at a low-pH (pH 3.0).

**Materials and Methods**

**Isolation and maintenance of YKT1**

Fallen leaves with attached algae were collected from acid mine drainage (pH 2.13, 14.5 °C) in Nagano Prefecture, Japan; the detailed place is shown in [18]. Sampling field is located in national forest and park of Japan, and we collected samples with administrators in HOKUSHIN forest office that is responsible for management of the forest. The samples were incubated in M-Allen liquid medium [19] at 15 °C under continuous light (20 μE/m²/s). The algae were subjected to purification by serial dilution followed by plating on M-Allen medium agar. Individual colonies were isolated and inoculated into liquid M-Allen medium using the same culture conditions as described above. A pure culture of the isolated and inoculated into liquid M-Allen medium with aeration (0.25 l ambient air/min) for 24 h. To determine the optimal temperature condition, cells were cultured at 25 °C under continuous light (90 μE/m²/s) with aeration (0.25 l ambient air/min) for 24 h. For nitrogen-depleted conditions, cells were cultured until an OD750 of 0.5–1.0 in M-Allen medium at pH 3.0 and were collected by centrifugation (2,000 g, room temperature, 5 min), then gently resuspended into the M-Allen or nitrogen free medium containing the same components as M-Allen except that the 20 mM (NH4)2SO4 was substituted by 20 mM NaSO4. Cells were cultured at 25 °C under continuous light (90 μE/m²/s) with aeration (0.25 l ambient air/min) at pH 3.0 for 7 days.

**Evaluation of the growth rate**

Growth rates were determined for cell cultures at different pH levels and temperatures in order to determine their optimal values. The growth rate (µ) was calculated as the difference between the natural logarithm of OD750 or the cell number (m) at different points in time (t) during exponential growth according to the following equation:

\[
\mu = \ln \left( \frac{m_2}{m_1} \right) / t_2 - t_1
\]

Cell numbers were determined using an improved neubauer hemacytometer.

**BODIPY staining and fluorescent microscopy**

Dipyrrometheneboron difluoride (BODIPY) staining was performed as described in [26], with modifications. Briefly, 90 μl of the cell suspension was fixed by adding 5 μl of 3.5% glutaraldehyde and then stained with 5 μl of 10 μM BODIPY stock solution. Samples were observed under epifluorescence microscopy (BX51; Olympus, Tokyo, Japan) with a digital camera (DP71; Olympus, Tokyo, Japan) under green excitation (for chloroplast autofluorescence) or blue excitation (for BODIPY fluorescence). Images were processed digitally with Photoshop software (Adobe Systems, Mountain View, CA, USA).

**Quantification of storage lipids**

Quantification of storage lipids was performed as described in [27], with modifications. Briefly, the algal suspensions were treated with dimethyl sulfoxide (DMSO) at a final concentration of 10% (v/v) and ethanol at a final concentration of 10% (v/v) in a 1.5 ml tube. 10 μl of Nile Red stock solution (100 μg/ml in ethanol) were added to the tubes and then they were incubated at 37 °C for 10 min. Fluorescence intensity was quantified by a fluorescence spectrometer (F-2700; Hitachi, Tokyo, Japan) using 485 nm excitation and 570 nm emission. Glyceryl trioleate (T7140; Sigma, USA) stock solution (1 mg/ml in ethanol) was used as the lipid standard (in a range from 1 to 7.5 μg/ml) to obtain a calibration curve. For dry weight determination, cell cultures were filtered using a pre-weighed 0.45 μm HA MF-MILLIPORE MEMBRANE (Millipore Corp., Bedford, Mass.). The membrane was dried at 50 °C for 2 hour and weighed on a microbalance.

---

**PLOS ONE** | www.plosone.org 2 September 2014 | Volume 9 | Issue 9 | e107702
Results and Discussion

Isolation and Morphology of the Acidophilic Algal Isolate

In order to isolate acidophilic algae, AMD from an abandoned sulfur mine were sampled in Nagano Prefecture, Japan on 6th September 2013. The AMD was 14.5°C and pH 2.13. The strain YKT1 was isolated by cultivation in M-Allen medium at pH 2.5 [19] from fallen leaves in the AMD. These vegetative cells are single, ellipsoidal, and measure 4.3–7.3 μm long and 2.3–5.5 μm wide (Fig. 1A). Each cell usually has a single bright green parietal bowl-shaped chloroplast, which occupies half of the cell periphery, and it does not possess a flagella (Fig. 1B). A rounded pyrenoid is present in the chloroplast (Fig. 1B). Reproduction usually occurs by producing 2, 4, or 8 autospores and hatching from the mother cell wall (Fig. 1D–G). These resemble the morphological features of Trebouxiophyceae, which contains the representative genera Chlorella and Parachlorella [28].

Molecular Phylogenetic Analyses

To specify the identity of YKT1, the nucleotide sequences of the 18S rDNA (partial, 2,555 bp) and plastid 16S rDNA (partial, 1,435 bp) genes of YKT1 were determined (GenBank accession numbers AB928019 and AB928020, respectively). The partial 18S rDNA sequence contains two introns. The obtained sequences were then compared with existing sequences in the NCBI database by the BLASTN algorithm [29]. The BLASTN search using the exon sequence of 18S rDNA was completely identical to that of Pseudochlorella pringsheimii (Chlorella ellipsoidea) SAG 211-1a, although the 18S rDNA sequence of P. pringsheimii SAG 211-1a contains only one intron. A phylogenetic analysis based on the 18S rDNA genes also indicates that YKT1 is most closely related to P. pringsheimii SAG 211-1a (Fig. 2). The BLASTN search using the plastid 16S rDNA sequence as a query showed that it is most closely related to plastid 16S rDNA of the P. pringsheimii C87 (98% identity). A phylogenetic analysis based on the plastid 16S rDNA genes further indicates that YKT1 is most closely related to P. pringsheimii C87 (Fig. 2). Cells of P. pringsheimii are ellipsoidal and measure 4–10 μm long and 3–9 μm wide, and have a parietal bowl-shaped chloroplast with a pyrenoid [30,31]. These features accord with YKT1, although size is relatively large. Based on results of morphological and molecular phylogenetic analysis, we named the strain Pseudochlorella sp. YKT1 (Trebouxiophyceae).

The chlorella-like Trebouxiophyceae species are one of the most investigated green microalgae for commercial use because of their relatively fast growth rate and value as both food supplements and potential biofuel feedstock. However, there have been only a few reports on the isolation of Trebouxiophycean algae that inhabit an acidic environment, although their existence has been confirmed by community structure analyses [10,11]. Recently, Auxenochlorella protothecoides var. acidicoa (Chlorella lineage in Figure 2) was isolated from acidic soil and drainage from abandoned copper mines, and the physiological features of A. protothecoides var. acidicoa were reported [32,33]. In contrast, there has been no report on the isolation of algae closely related to Pseudochlorella pringsheimii (the Trebouxia lineage in Figure 2) from acidic environments.

Optima and limits of pH and Temperature for the Growth of YKT1

To determine the optima and limits of pH, YKT1 that had been grown at pH 2.5 and 25°C was transferred to a fresh medium of different pH values (from pH 1.0 to 7.0) and was cultured under illumination (90 μE/m²s) with aeration (ambient air) (Figure 3A). The growth rate (change in OD 750/day and cell number/day) during a period of 1 day after the transfer was determined.
The results indicate that cells proliferated at pH 2.0 to pH 7.0 and that pH 3.0–5.0 is most suitable for both growth and cell division. Microscopic observation showed that the majority of cells cultured at pH 1.0 was bleached and did not undergo cell division (Figure 3B). Cells cultured at pH 7.0 were enlarged and aggregated, but still underwent cell division. These results indicate that the optimum is pH 3.0–5.0 and the lower limit for the growth of YKT1 is pH 2.0 (Figure 3A) in our culture conditions.

To determine the optima and limits of the temperature, YKT1 that had been grown at pH 3.0 and 25°C was transferred to different temperatures (from 10 to 35°C) and cultured for 1 day under illumination (90 μE/m2/s) with aeration (ambient air) (Figure 3C). The result showed that YKT1 is able to grow well over a wide temperature range of 20–32°C, with optimal growth at 20–25°C, although cell division at 30–32°C is delayed. Cells are capable of growing very slowly at 10°C for at least a week. By contrast, cells died on the third day at 35°C. Microscopic observation showed that cells cultured at 35°C were enlarged and dividing cells were not detected, in contrast to cells cultured at a lower temperature (Figure 3D). In conclusion, YKT1 grows well at a wide range of pH (pH 3.0–5.0) and temperature (20–32°C).

Although the pH and temperature of open pond system are not easily controlled [34], YKT1 would be suitable for culturing in open ponds.

Growth under Optimal Conditions

The microalgal maximum biomass (dry weight per culture volume) concentration is an important index in carrying out low-cost cultivation. In order to determine the maximum growth rate and maximum dry weight biomass of YKT1, cells were cultured under the optimal growth conditions (pH 3.0, 25°C) determined in this study along with M-Allen liquid medium for 7 days. The maximum dry weight biomass of YKT1 under our culture conditions is relatively lower compared with that reported in other algae (1.24–11.74 g/l) [36]. However, these other results have been obtained under different culture conditions (e.g. light intensity and media) and, in most cases, the high maximum dry weight biomass was obtained by a supply of CO2 gas [36]. Therefore, improvement of cultivation conditions such as the

Figure 2. Phylogenetic position of YKT1. Phylogenetic trees based on the 18S rDNA (A) and plastid 16S rDNA sequence (B) are shown. The trees were constructed by a maximum-likelihood method (RaxML 7.2.8) [24]. Maximum likelihood bootstrap values (ML) >50% by RaxML and bayesian posterior probabilities (BI) >0.7 by the Bayesian analysis (MrBayes 3.1.2) [25] are shown above the branches. The accession numbers of the sequences are shown along with the names of the species. The lineage designation follows [31]. The branch length reflects the evolutionary distances indicated by the scale bar.

doi:10.1371/journal.pone.0107702.g002

Figure 3A. The results indicate that cells proliferated at pH 2.0 to pH 7.0 and that pH 3.0–5.0 is most suitable for both growth and cell division. Microscopic observation showed that the majority of cells cultured at pH 1.0 was bleached and did not undergo cell division (Figure 3B). Cells cultured at pH 7.0 were enlarged and aggregated, but still underwent cell division. These results indicate that the optimum is pH 3.0–5.0 and the lower limit for the growth of YKT1 is pH 2.0 (Figure 3A) in our culture conditions.
medium, light intensity and carbon source will increase the maximum dry weight biomass of YKT1 in the future.

**Accumulation of Lipid Droplets under Nitrogen-depleted Condition**

Many microalgae accumulate storage lipids in cells as lipid droplets, mainly in the form of triacylglycerols (TAGs) in response to stress or nutrient scarcity [4] that are readily converted into biodiesel production [2]. To investigate the ability of YKT1 to accumulate lipid droplets, cells were cultured under nitrogen-replete (+N) and nitrogen-depleted (–N) conditions. The growth rate of cells cultured under the –N condition was severely decreased compared with the +N condition (Fig. 4A) and the cell culture was bleached (Figure 4B). Nitrogen-depletion induced a very fast accumulation of lipid droplets (Figure 4C) that were stained with BODIPY, a lipophilic brilliant green fluorescent dye. The storage lipid contents of cells cultured under +N and –N conditions for 7 days (Figure 4D) were determined by the Nile Red method [27] as 1.35±0.23% and 29.5±2.22% of the dry weight, respectively (Fig. 4E), which results are comparable to other neutrophilic oleaginous algae (20% to 50% of dry weight) [1].

In conclusion, *Pseudochlorella* sp. YKT1 (Trebouxiophyceae isolated from sulfuric AMD) exhibits a high tolerance to low-pH (pH 2.0) and relatively fast growth at a wide temperature range (20–32 °C) and accumulates high amount of storage lipids (~30% of dry weight). These results suggest possibility that acidophilic green algae will be useful for future industrial applications, such as a next-generation biofuel, by cultivating them in sulfuric acidic open ponds made from mine drainage, acidic hot spring or containing industrial wastes (SOx and NOx).

Although our knowledge of the oil accumulation mechanisms of microalgae is poor at present, recent studies have yielded information on how to increase the cellular oil by genetic manipulation in tractable model algae such as *Chlamydomonas*

**Table 1. Physiological features of strain YKT1.**

| pH<sub>opt</sub> | pH<sub>limit</sub> | T<sub>opt</sub> (°C) | T<sub>limit</sub> (°C) | GR<sub>max</sub> (d<sup>−1</sup>) | Max DW Biomass (g/l) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 3.0–5.0         | 2.0             | 20–25           | 32              | 0.925           | 2.03            |

Optimal growth pH (pH<sub>opt</sub>) limit of growth pH (pH<sub>limit</sub>) optimal growth temperature (T<sub>opt</sub> in °C) limit of growth temperature (T<sub>limit</sub> in °C) maximum growth rate in exponential phase (GR<sub>max</sub> in d<sup>−1</sup>) and maximum dry weight biomass (Max DW Biomass in g/l).
reinhardtii [37,38] and the procedures for transformation in several other algal species [39] including Trebouxiophyceae [40] have been developed. These developments and a further screening of algal strains from an acidic environment will enhance oil productivity and biomass in acidophilic open ponds using AMD or other acidic soils or waste.

Acknowledgments
We thank Dr. Y. Kabeya, Ms. M. Nakamura, Dr. N. Sumiya, Dr. A. Era, and Dr. T. Fujiwara for their technical advise and Ms. A. Yamashita for technical support.

Author Contributions
Conceived and designed the experiments: SHH SYM. Performed the experiments: SHH SUH SYM. Analyzed the data: SHH SYM. Contributed reagents/materials/analysis tools: SHH SUH AU HN SYM. Contributed to the writing of the manuscript: SHH SYM.

References
1. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25: 294–306.
2. Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotech 26: 126–131.
3. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussmug JH, et al. (2008) Second generation biofuels: High-efficiency microalgae for biodiesel production. Bioenergy Res 1: 20–43.
4. Wijffels RH, Barbosa MJ (2010) An outlook on microalgal biofuels. Science 329: 796–9.
5. Brennan L, Owende P (2009) Biofuels from microalgae, a review of technologies for production, processing, and extraction of biofuels and co-products. Renewable Sustainable Energy Rev 14: 557–577.
6. Priyadarshani I, Rath B (2012) Commercial and industrial applications of microalgae—a review. J Algal Biomass Util 3: 89–100.
7. Brennemann J (2013) Microalgae for Biofuels and Animal Feeds. Energies 6: 5869–5886.
8. Pedrero F, Kelly L, Diaz M, Temporetti P, Baffico G, et al. (2001) First results on the water chemistry, algae and trophic status of an Andean acidic lake system of volcanic origin in Patagonia (Lake Caviahue). Hydrobiologia 452: 129–137.
9. López-Archilla AI, Marin I, Amils R (2001) Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. Microb Ecol 41: 20–35.
10. Amaral Zettler LA, Gómez F, Zettler E, Keenan BG, Amils R, et al. (2002) Microbiology: eukaryotic diversity in Spain’s River of Fire. Nature 417: 137.
11. Aguilera A, Zettler E, Gómez F, Amaral-Zettler L, Rodríguez N, et al. (2007) Distribution and seasonal variability in the benthic eukaryotic community of Rio Tinto (SW, Spain), an acidic, high metal extreme environment. Syst Appl Microbiol 30: 531–46.
12. Novis P, Harding JS (2007) Extreme acidophiles: freshwater algae associated with acid mine drainage, in Algae and Cyanobacteria in Extreme Environments, ed Seckbach J, editor. (Heidelberg, Germany: Springer): pp. 443–463.
11. Kádár Z, Maláha SF, Szegyelf Z, Réczey K, de Laat W (2007) Ethanol fermentation of various pretreated and hydrolyzed substrates at low initial pH. Appl Biochem Biotechnol 140: 847–58.

12. Nevogt E (2000) Progress in metabolic engineering of Saccharomyces cerevisiae. Microbiol Mol Biol Rev 72: 379–412.

13. Garcia-González M, Moreno J, Cañavate JP, Anguita V, Prieto A, et al. (2003) Conditions for open-air outdoor culture of Dunaliella salina in Southern Spain. J Appl Phycol 15: 177–184.

14. Kitto MR, Resinold M (2011) Effect of summer / winter light intensity and salt on growth kinetics and beta carotene accumulation by Dunaliella in open outdoor earthen ponds in a desert island, off UAE coast. J Algal Biomass Utd 2: 14–21.

15. Heidelberg KB, Nelson WC, Holm JB, Eisenkolb N, Andrade K, et al. (2013) Characterization of eukaryotic microbial diversity in hypersaline Lake Tyrrell, Australia. Front Microbiol 4: 115.

16. Nakazawa A and Nozaki H (2004) Phylogenetic analysis of the tetrasporalean genus Asterococcus (Chlorophyceae) based on 18S ribosomal RNA gene sequences. J Plankton Res 28: 489–498.

17. Zhang J, Huss VAR, Sun X, Chang K, Pang D (2008) Morphology and phylogenetic position of a mat-forming green plant from acidic rivers in Japan. J Plant Res 116: 443–453.

18. Minoda A, Sakagami R, Yagisawa F, Kuroiwa T, Tanaka K (2004) Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, Cyanidioschyzon merolae. Plant Cell Physiol 45: 667–71.

19. Nakazawa A and Nozaki H (2004) Phylogenetic analysis of the tetrarotalcean genus Asterococcus (Chlorophyceae) based on 18S ribosomal RNA gene sequences. J Jpn Bot 79: 255–261.

20. Zhang J, Huss VAR, Sun X, Chang K, Pang D (2008) Morphology and phylogenetic position of a trebouxiophycean green alga (Chlorophyta) growing on the rubber tree, Hevea brasiliensis, with the description of a new genus and species. European Journal of Phycology 43: 185–193.

21. Larkin MA, Blackhilds G, Brown NP, Chenna R, McGtsettian PA, et al. (2007) Chlular and Chlusal X version 2.0. Bioinformatics 23: 2947–8.

22. Galier N, Goux M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12: 543–8.

23. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2681–90.

24. Roux F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–4.

25. Kuroiwa T, Ohnuma M, Imoto Y, Misumi O, Fujiwara T, et al. (2012) Lipid Droplets of Bacteria, Algae and Fungi and a Relationship between their Contents and Genome Sizes as Revealed by BODIPY and DAPI Staining. CYTOLOGIA. 77: 289–299.

26. Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods 77: 41–7.

27. Yamamoto M, Nishikawa T, Kajitani H, Kawano S (2007) Patterns of asexual reproduction in Nannochloris bacillaris and Marainuma gemmata (Chlorophyta, Trebouxiophyceae). Planta 226: 917–27.

28. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.

29. Shihira I and Krauss RW (1965) Chlorococcum: Physiology and taxonomy of forty-one isolates. Maryland: University of Maryland, College Park. pp. 1–97.

30. Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods 77: 41–7.

31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.

32. Huss VA, Caniglis C, Cenamero P, Cosulino S, Pinto G, et al. (2002) Phylogenetic relationships and taxonomic position of Chlorococcum-like isolates from low pH environments (pH<3.0). BMC Evol Biol 2: 13.

33. Nacucchio I, Barrie Johnson D (2012) Acidophilic algal isolated from mine-impacted environments and their roles in sustaining heterotrophic acidophiles. Front Microbiol 3: 325.

34. Carvalho AP, Mereles LA, Malcata FX (2006) Microalgal reactors: a review of enclosed system designs and performances. Biotechnol Prog 22: 1490–506.

35. Nielsen SL (2006) Stress-dependent growth rates in eukaryotic and prokaryotic algae exemplified by green algae and cyanobacteria: comparisons between unicells and colonial growth forms. J Plankton Res 28: 489–498.

36. Fribyl P, Cepak V, Zachleder V (2012) Production of lipids in 10 strains of Chlorococcum and Parachlorella, and enhanced lipid productivity in Chlorococcum vulgaris. Appl Microbiol Biotechnol 94: 549–61.

37. Harris EH (2009) The Chlamydomonas sourcebook: introduction to Chlamydomonas and its laboratory use, 2nd ed. Academic Press, United Kingdom.

38. Merchant SS, Kropat J, Liu B, Shaw J, Warakonjant J (2011) TAG, You’re it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation.Curr Opin Biotechnol 23: 352–63.

39. Radoszkovits R, Jinkerson RE, Darzins A, Posewitz MC (2010) Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 9: 486–501.

40. Bai LL, Yin WB, Chen YH, Niu LL, Sun YR, et al. (2013) A new strategy to produce a defensin: stable production of mutated NP-1 in nitrate reductase deficient Chlorella ellipsoidea. PLoS One 8: e54966.