

1 **Bioenergy Production Using *Trichormus variabilis* - A review**

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17 **Running Head:** Bioenergy from *Trichormus variabilis*

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1 **Abstract**

2 Fossil fuel processing and consumption contaminates air, soil, and water resources via
3 release of hazardous chemicals. To protect the environment, harnessing renewable
4 energy resources and development of sustainable technologies are prime targets of
5 research and increased investment. Use of bio-based feedstocks in energy production
6 inherently provides a valuable pollution-curbing pathway with sustainability credentials,
7 especially when wastewater is used to provide the nutrient requirements. The
8 filamentous cyanobacterium, *Trichormus variabilis*, has attracted substantial attention
9 from researchers due to its potential for dual industrial functions in bioenergy
10 production and bioremediation. This species can efficiently use the power of sunlight
11 energy to fix atmospheric CO₂ for generating valuable chemical compounds, such as
12 carbohydrates and fatty acids, which can be converted to biofuels. Because it grows in
13 nutrient-rich wastewater (industrial effluent), it can serve as a bio-absorbent and replace
14 costly chemical catalysts and nano-materials classically used for removal of nutrients
15 and metals. However, no recent review has presented potential for state-of-the-art *T.*
16 *variabilis*-driven phycoremediation-bioenergy production systems. Therefore, in order
17 to present possible routes from phycoremediation to energy production as a strategy for
18 developing the industrial application of *T. variabilis*, we present this review to bring
19 important research results on this species together and highlight major related
20 challenges and opportunities. The current status of applying algae in bioremediation and
21 production of liquid and gaseous fuels utilizing wildtype and mutants of *T. variabilis* is
22 explored. Finally, key points underlying potential for future research on optimization of
23 robust technologies for supplying sustainable bioenergy using this organism are
24 presented.

25
26 **Key words:** Bioenergy, Hydrogen, Bioremediation, *Trichormus variabilis*.

1 **1 Introduction**

2 Global economic growth over the last two centuries has largely been driven by
3 increased use of fossil fuels and was accompanied by significant environmental
4 pollution, particularly increased CO₂ emissions. Total world energy consumption is
5 forecast to increase by 1.4% per annum up to 2040¹, exacerbating negative
6 environmental impacts and increasing pressure on finite fossil fuel resources.
7 Additionally, both economic growth and rises in the global population are reciprocated
8 by increased demand for freshwater required for industrial processes, agriculture, and
9 domestic use. Therefore, recycling wastewater becomes an imperative for water
10 conservation. There exist a number of routes to transform the environmental challenges
11 posed by wastewater into efficient, economically viable methods for both energy
12 production and water recycling. Phycoremediation has been used since circa 1963 as a
13 cost-effective nutrient removal method using microalgae to treat municipal wastewater².
14 In addition to CO₂ capture, phycoremediation has several other benefits, including
15 removal, transformation, or degradation of nutrients, organic matter, acids, metals and
16 xenobiotic compounds, and the use of algae as an environmental monitoring system for
17 detection of toxic materials³ and production of high-value metabolites⁴. Furthermore,
18 massive biomass grown in wastewater streams can be used effectively as a promising
19 source of bio-fuels such as bioethanol, biodiesel, bio-hydrogen or biogas, which are
20 generally known as the most carbon neutral liquid and gaseous fuels, depending on their
21 generation technology.

22 Previous reviews have collated research on phycoremediation⁵, bioenergy
23 production^{6, 7}, biorefining⁸ and algal photobioreactors (PBRs)⁷. However, to our
24 knowledge, none of these reviews have been dedicated to a specific strain of algae.
25 Among many microalgae species, *Trichormus variabilis* has been studied extensively
26 owing to its dual benefits: showing high potential in industrial and municipal
27 wastewater treatment⁹⁻¹¹ and also its capability for bio-hydrogen production^{12, 13}. The
28 worldwide distribution of *T. variabilis*, high auto-flocculation capacity³, tolerance of a
29 wide temperature range, and bio-fertilizer potential¹⁴ strongly affirm that the study of this
30 species needs to be expanded in the future. Here, we present a comprehensive review,
31 focused on *T. variabilis*, and its principal enzymatic functions which make it suitable
32 for applications in biohydrogen production. Subsequently, we present the physico-
33 chemical parameters affecting the morphology, physiology and growth of *T. variabilis*
34 and evaluate how these factors might impinge on the hydrogen production process.
35 Finally challenges and opportunities in developing a route to industrial application of *T.*
36 *variabilis* as a green chemistry workhorse in effluent treatment and production of
37 sustainable bioenergy are discussed.

38 **2 Methodology**

39 Primary and secondary literature and data used in synthesis of this review were
40 obtained via searching Web of Science (<https://clarivate.com/products/web-of-science/>)
41 and PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>) databases and several sources of
42 grey literature using keywords related to *T. variabilis* in terms of various types of
43 bioenergy and nutrient removal potential. Synonyms of *T. variabilis*, such as *Anabaena*
44 *variabilis*, were also used as keywords given that authors tend to use different names for
45 this organism. Information was gathered in a two-phased process. In the first phase,
46 inclusion criteria were peer-reviewed academic research and published industrial pilot
47 studies or full-scale production systems. Because of paucity of published data specific
48

1 to *T. variabilis* no exclusion criteria were applied, except the exclusion of self-
2 published, non-peer-reviewed material. In order to capture important data from grey
3 literature, such as patents and technical reports, the second phase reversed the inclusion
4 and exclusion criteria of the first phase. The taxonomic classification of *Trichormus*
5 *variabilis* was extracted from data presented in major biological, botanical and algal
6 databanks including AlgaeBase¹⁵, World Register of Marine Species (WoRMS ID:
7 146661)¹⁶ and National Center for Biotechnology Information (NCBI taxonomy ID:
8 240292, <https://www.ncbi.nlm.nih.gov/>). Genes encoding hydrogen biosynthetic
9 enzymes (described in Section 6.3) were identified via literature and Genome Database
10 Resources of CyanoBase in Kazusa DNA Research Institute
11 (<http://genome.microbedb.jp/cyanobase/>). Vector graphics were drawn by Inkscape
12 software v0.92¹⁷. To provide images of *T. variabilis* cells, algal samples were cultivated
13 and processed for transmission electron microscopy (TEM) as described previously¹⁸.
14 All the tables in this review were constructed from the primary results reported in
15 different studies cited within each tables. Figure 1 presents a workflow chart illustrating
16 the search strategy used.

17
18 **Figure1. Flowchart of the research strategy.** To develop each section of this literature
19 review, a question was formulated and related keywords generated for interrogation of
20 the databases. The returned information hit list was divided into three broad categories
21 of algal cultivation for biomass production, algal cultivation for bioenergy production,
22 and algal cultivation for phycoremediation. Progress of innovation across these were
23 critically analysed by comparative analysis and current challenges and opportunities
24 identified. Areas of future research to ensure current investments lead to commercial
25 feasibility were finally identified and discussed.

26 27 **3 *Trichormus variabilis***

28 *Trichormus variabilis* (syn *Anabaena variabilis* ATCC 29413) is a filamentous
29 cyanobacterium from Nostocoidae subfamily^{19, 20} found in both freshwater and within
30 soil habitats. The recorded history of study of this species begins nearly two centuries
31 ago, when it was collected for the first time in 1839 from puddles and drains in
32 Hooksiel and Jadebusen, located in Wangerooge Island in Lower Saxony of Germany
33 by Kutzing²¹. Since then, its widespread distribution has been reported across almost all
34 continents, being a native species in many countries, from Asia to America, Africa and
35 Australia^{22, 23}. The taxonomy of this species has faced repeated revision owing to the
36 formation of two main cell types (akinetes and heterocysts), including differentiation
37 from the genus of *Nostoc*²⁴, *Wollea*²⁵ and *Anabaena*^{19, 20, 24, 25} on the basis of akinete
38 formation. According to the last rectified taxonomy (Fig. 2), it has been transferred
39 from *Anabaena* to the genus *Trichormus*, with the former scientific name “*Anabaena*
40 *variabilis*” proposed by Bornet and Flahault in 1886 being replaced with “*Trichormus*
41 *variabilis*” as proposed by Komarek and Anagnostidis in 1989²⁵⁻²⁷.

42 43 **Figure 2. Taxonomy of *Trichormus variabilis*.**

44 45 **3.1 Morphology and Physiology**

46 The filaments of *T. variabilis* are morphologically variable^{19, 24}. Filaments mainly
47 contain chains of barrel-shaped gram-negative vegetative cells (Fig. 3a) as a site for

1 photosynthesis with conical terminal cells (Fig. 3b) covered by a thin gelatinous
2 polysaccharide sheath (Fig. 3c). The mucilaginous sheath encapsulating the filaments
3 arises from polysaccharide secretions by the cells^{28, 29}. Vegetative cells have a primary
4 role of harnessing solar energy for fixing carbon to drive biomass production. The
5 growth in biomass can be coupled to wastewater treatment through removal of heavy
6 metal ions and nutrients. The most important cell differentiation in *T. variabilis* is the
7 formation of heterocysts, also known as heterocytes. When transferred to diazotrophic
8 conditions, some vegetative cells differentiate into heterocysts as a site for nitrogen
9 fixation²⁵. The spherical, ellipsoidal or cylindrical shape²⁴, the large size²⁵, distinctive
10 polar bodies³⁰, and hollow appearance, make heterocysts readily recognisable among
11 other cell types (Fig. 3d). Even though heterocysts represent only 5-10% of cells along
12 the filaments³¹, phylogenetic studies showed that about 45% of the genome is expressed
13 in these cells³². Cell differentiation is underpinned by changes in gene expression,
14 which trigger biochemical and structural transformations, such as formation of thick
15 multi-membranous glycolipid walls, arrest of photosynthesis and biosynthesis of the
16 nitrogenase enzyme complex³³. These modifications prepare heterocyst cells for the
17 vital role of fixing atmospheric nitrogen with hydrogen gas as a by-product of the
18 reactions. Because nitrogenase enzymes are sensitive to oxygen, heterocysts bereft of
19 oxygenic photosynthesis provide an anaerobic environment to stabilise the N-fixation
20 system in an otherwise aerobic organism. Heterocysts strongly attach to vegetative cells
21 via cyanophycin polar nodule³⁰ (Fig. 3d).

22 In response to nutrient limitation or environmental stress such as prolonged darkness,
23 dryness, freezing, and nutrient depletion^{30, 34, 35}, specialised akinete cells develop as
24 resilient archetypal spores to ensure survival of the organism in harsh environmental
25 conditions. Akinetes are oval to barrel-shaped cells with granules filled with glycogen
26 and cyanophycin³⁶, which can easily be distinguished from vegetative cells by their
27 larger size and thicker envelope (Fig. 3e). Their thick outer layer contains glycolipid
28 and polysaccharides, making them more resistant to stressful conditions in comparison
29 to vegetative cells and heterocysts²⁸. Akinetes germinate and develop into new filaments
30 when favourable growth conditions are restored. On germination, an akinete develops
31 into a short filament which is structurally distinguishable from trichorms in having 5 to
32 15 vegetative cells and lacking heterocysts (Fig. 3f). These short filaments, known as
33 hormogonia, later develop into mature trichorms possessing heterocysts. - Therefore, the
34 diverse cell functions provide an attractive system for commercial development of an
35 efficient low-cost biomass production during wastewater treatment process, which
36 could be coupled to industrial bioenergy production. The morphological appearance of
37 various cell types of *T. variabilis* is illustrated schematically in Fig. 3, with Fig. 4
38 showing actual images of the cells using transmission electron microscopy (TEM).

39
40 **Figure 3. Schematic depiction of different cell types of *T. variabilis*.** (a)
41 Vegetative cells; (b) Terminal cell; (c) polysaccharide mucilaginous sheath; (d)
42 Heterocysts, which posses: 1. Fibrous layer, 2. Laminated layers (internal layers), 3.
43 Plasmodesmata, 4. Polar nodule, and 5. Thylakoids; (e) Akinete, which has: 6. sheath, 7.
44 additional wall, 8. Cyanophycin granule, 9. Inner layer, and 10. Dense fibrillar layer; (f)
45 Hormogonium; (g) Outer membrane; (h) Polyphosphate granules.

46
47 **Figure 4. Microscopic images of *T. variabilis* cells.** (a) Low power light
48 microscope image of filaments in nitrogen-depleted growth medium showing vegetative

1 cells, heterocysts, and akinete. **(b)** Transmission electron micrograph of filaments
2 encased by a gelatinous polysaccharide sheath (PC) with terminal cells (TC) at the end
3 of the trichorms. **(c)** Transmission electron micrograph showing a detached mature
4 vegetative cell showing peptidoglycan layer (PG), carboxysome (CS), polyphosphate
5 granule(PS), cyanophycin granule (CP), thylakoid and attached phycobilisomes (TI).
6 Scale bars: **(a)** 50 μm ; **(b)** 2 μm ; **(c)** 500 nm.

8 **4 Physico-chemical factors affect *T. variabilis* biomass production**

9 Knowing key physico-chemical factors required for promoting high rates of biomass
10 production is necessary for developing industrial-scale bioreactors for algal cultivation.
11 Zho³⁷ summarized general microalgae cultivation strategies, abiotic, biotic and
12 operational factors affecting algal growth. *T. variabilis* is capable of growing
13 phototrophically, heterotrophically/chemotrophically in the dark with exogenous sugar,
14 and also mixotrophically in light with exogenous sugar^{31, 38, 39}. This variation in growth
15 strategy makes it a potentially favourable microorganism for use in different light- and
16 dark-driven bioenergy production processes. *T. variabilis* is a mild thermophilic
17 cyanobacterium, which has photosynthetic activity in a broad temperature range (10- 35
18 °C) and light intensity (42- 562 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$)⁴⁰. It was demonstrated that the growth
19 yield declined (0.1 g dry mass.g⁻¹ CO₂) by increasing the initial CO₂ concentration from
20 4 to 18%⁴¹. Chemical nutrients, essential salts and metals directly influence different
21 metabolic modes, which consequently affects bioenergy yield and remediation potential.
22 The main growth media used for *T. variabilis* include blue-green (BG)⁴², Allen and
23 Arnon (AA)⁴³, 2-(N-Morpholino) ethane sulfonic acid-Volvox (MES)⁴⁴, Marin (MN)⁴⁵,
24 ammonium mineral salt (AMS-1)⁴⁶ and Fogg's Medium⁴⁷. Among these, BG and AA
25 are the most commonly used media, specifically when *T. variabilis* is used as a source
26 of bio-hydrogen. Higher biomass production was reported when grown in BG₁₁ enriched
27 with a nitrogen source⁴⁸. In general, BG and AA media are based on molybdenum (Mo)
28 and vanadium (V), respectively. These two metals are required for activation of two key
29 enzymes (described in section 5.3), which are responsible for hydrogen generation.
30 Investigating the effects of replacing Mo with V demonstrated that replacement of Mo
31 with V leads to decreases in the growth yield⁴³ while it improves hydrogen generation
32 yield⁴⁸ (section 5.4).

33 Experiments establishing *T. variabilis* growth profiles have classically used, as
34 proxies for biomass accumulation, parameters such as photosynthetic
35 potential/capacity⁴⁹ and cell density at specified wavelengths⁵⁰. These have been applied
36 in studying the effects of altering various physicochemical factors such as light and
37 nutrient supply, on *T. variabilis* phototrophic^{51, 52} or heterotrophic^{39, 53} growth. While a
38 large number of studies have investigated *T. variabilis* growth rate as a function of time,
39 cell density or photosynthesis components^{54, 55}, only a few have directly surveyed the
40 interrelation between multiple parameters. Such data can be used for modelling growth
41 kinetics. In prior work, a kinetic model for growth of *T. variabilis* was developed based
42 on the Monod model as a function of CO₂ and light intensity⁵⁶. Predictive models are
43 increasingly important as algal biomass generated from wastewater treatment process is
44 being used for production of biofuels when scaling-up from laboratory-scale to
45 industrial-scale cultures. Therefore, the model should capture key parameters affecting
46 growth rate so that bioreactor designs are optimised for maximal productivity.

47 Additionally, this species has variable morphology based on the type of mechanical
48 stimulation. It normally grows as filaments that attach longitudinally to neighbouring

1 filaments to form thalloid structures, but when it is grown with orbital shaking, thalli
2 formation is prevented, with a homogenous suspension of short filaments emerging⁴³.
3 High tendency of filaments for sticking to the walls blocks light penetration, which
4 causes problems when cultivating this species in photobioreactors. The membranous
5 growth form may provide an opportunity in flocculation of biomass in open raceway
6 ponds. This potential, which resolves one of the most important challenges of industrial
7 algal harvesting technologies in biofuel production, requires further investigation.

8 9 **5 Biomass production via phycoremediation**

10 Providing sufficient water and nutrients, particularly low-cost nitrogen, is a challenge
11 for large-scale microalgae cultivation for bioenergy⁷. However, nutrient-rich wastewater
12 streams can provide most of the nutrients required for algal growth. In addition to
13 utilising nitrate and phosphate, microalgae have the capacity for effective removal of
14 heavy metals. Incorporation of metals into the biomass may pose downstream risks
15 during future processing or utilisation of derivatives, depending on the uses of the
16 biomass. Owing to their rapid growth rates and potential to remove heavy metals,
17 various microorganisms have been used in phycoremediation of municipal⁵⁷, industrial⁵⁸
18 or agricultural⁵⁹ wastewaters. These microorganisms include (i) green algae;
19 *Chlamydomonas reinhardtii*⁶⁰, *Chlorella sp.*⁶¹, *Nannochloropsis oculata*⁶², *Scenedesmus*
20 *dimorphus*⁵⁹, (ii) blue-green algae; including *Synechocystis salina*⁶³, *Spirulina*
21 *platensis*⁶⁴, *Phormidium bohneri*⁶⁵, *Tolypothrix ceytonica*⁹, (iii) brown algae, including
22 *Padina sp.*⁶⁶, *Laminaria hyperborean*⁶⁷, and (iv) red algae such as *Porphyridium*
23 *cruentum*⁶⁸. Among the cyanobacteria, *T. variabilis* has received considerable attention
24 for use in bioremediation. Attraction to this species has partly been the potential for
25 coupling wastewater treatment with production of bioenergy. The growth and
26 remediation performance of *T. variabilis* in treating water contaminated with
27 ammonium⁶⁹ and biosorption of heavy metals, such as Cr, Cd, Ni and Pb⁷⁰ has been
28 demonstrated. Therefore, *T. variabilis* is already noted as an efficient microalgae for
29 actual and potential treatment of mixed industrial and municipal wastewater⁹. Table 1
30 presents a synopsis of key studies on phycoremediation using *T. variabilis* and the
31 maximum removal efficiency reported for each process.

32 A study on phenol compounds remediation illustrated notable nitrophenol compound
33 removal efficiency from an artificial supplemented wastewater using this species⁷¹.
34 However, since polyphenolic compounds produced by *Myriophyllum verticillatum* cause
35 growth inhibition of *T. variabilis* cells⁷², the feasibility and efficiency of *T. variabilis*
36 remedial action on phenolic contaminants will require further research. It was also
37 reported that nanomaterials, such as nano-titanium dioxide (nTiO₂), have negative effect
38 on the intracellular structure of *T. variabilis*⁷³⁻⁷⁵. Furthermore, *T. variabilis* growth is
39 impaired by some allelochemicals such as Harmane (1-methyl- β -carboline)⁷⁶ and
40 FischerellinB⁷⁷ causing cell lysis and photosynthesis inhibition respectively.
41 Microcystins have intense allelopathic inhibition on *T. variabilis* growth⁷⁸ and
42 differentiation of heterocysts and akinetes⁷⁹. The presence of such pollutants in
43 wastewater streams affects nutrient removal efficiency by disrupting metabolic
44 processes and consequently, disrupts bioenergy production pathways. Therefore, more
45 research on detection of growth inhibiting pollutants, their effects, and ways to deal
46 with them is required. Exposing *T. variabilis* to pollutants can gradually change gene
47 expression and acclimate the organism for growth in previously toxic compounds. For
48 example, it was demonstrated that repeated cultivation of this species in a medium with

1 high concentration of Cu(NO₃)₂ developed a Cu-resistant strain with broad resistance to
2 Cd, Zn and Ni⁴⁹. Thus, genomics and proteomics research could provide a clear
3 understanding of the exact metabolic changes underpinning this adaptive change.
4

5 **6 Bioenergy production using *T. variabilis***

6 Several studies focused on integration of wastewater treatment and bio-fuel
7 production using microalgal systems⁸⁰⁻⁸³. After nutrient removal, the biomass produced
8 can effectively be utilized as a resource for cost-effective bioenergy production. We
9 review the application of *T. variabilis* in bioenergy production from the perspective of
10 liquid (biodiesel, bio-ethanol) and gaseous (bio-hydrogen, bio-methane) bio-fuels.
11 Applying microalgae systems for bioenergy production has several environmental
12 advantages, such as reduction of pollutant emissions and soil erosion, and economic
13 benefits such as tax credits and grants without competing with agricultural produce for
14 freshwater and arable land^{84, 85}. In addition to these general advantages, *T. variabilis*
15 has enhanced capacity for auto-flocculation³ which is one of the most important
16 characteristics to decrease energy consumption during algae biomass harvesting. Also,
17 nutrient removal and CO₂ bio-fixation potential together with interesting cell
18 differentiations in forming heterocysts and akinetes, which provide suitable
19 opportunities for N-fixation and growth in challenging environmental conditions,
20 respectively, and availability of processed biomass as bio-fertilizer¹⁴, suggest this
21 species may present a viable solution for algae bioenergy production with valuable by-
22 products.
23

24 **6.1 Liquid bio-fuels: biodiesel and bioethanol**

25 Ethical concerns about diversion of crops from the human food chain to fuel
26 production have spurred the search for inedible crop oils and waste fats for use as a bio-
27 fuel feedstock. Inedible oilseeds, such as *Jatropha*, *Castor*, and other indigenous crops^{86,}
28 ⁸⁷ which can grow on marginal land have emerged as potential candidates. However, the
29 cost of land, labour and freshwater resources often make large-scale production
30 uneconomical when set against the yields attained. Therefore, microalgae received
31 greater attention and interest due to their capability to grow in wastewater. Since lipid
32 content is the most important key factor in utilization of oleaginous microorganisms as a
33 source of biodiesel production, considerable research focuses on increasing lipid yield.
34 Although *T. variabilis* is not considered as an oleaginous species, previous reports
35 indicate notable increases in lipid content after exposure to environmental stress or
36 nutrient limitation. For example, it was demonstrated that lipid content of this alga in N-
37 depleted Arnon medium at 30-35 °C under 12:12 h light/dark cycle was increased by ~
38 2.5-fold (yield~10.5%)⁸⁸. Furthermore, it has been reported that ultrasound at 200 W
39 after 5 minutes increased lipid content of *T. variabilis* grown in BG₁₁ medium at 25 °C
40 under continuous illumination by 1.46-fold (yield~47%)⁸⁹. Cellular stress triggered by
41 ultrasonic treatment was responsible for increased oil production while a longer
42 duration of treatment damaged the cells. Such stress or nutrient limitation leading to
43 increased lipid yield was also observed in other species, including *Nannochloropsis*
44 *sp.*⁹⁰, *Chlorella sp.*⁹¹ and *Chlamydomonas reinhardtii*⁹². These useful results can be
45 considered as a promising solution for promoting lipid accumulation in the cells and
46 consequently enhance the biodiesel production yield. However this also requires
47 accurate investigation on the profile of fatty acid methyl esters to match the biodiesel
48 quality standards.

1 From a bio-ethanol perspective, *T. variabilis* has not been widely investigated. The
2 main challenge in bio-ethanol production from this filamentous species is overcoming
3 the polysaccharide loss during processing. Research has shown that drying the biomass
4 using supercritical fluid followed by fermentation could increase the bio-ethanol yield
5 (24.1%) about 2-fold comparing with using lyophilization process followed by
6 fermentation (yield~13.6%)⁹³. A further study has confirmed that applying supercritical
7 fluid pre-treatment efficiently enhances the amount of ethanol from 1.25 g.L⁻¹ to 2.28
8 g.L⁻¹ ⁹⁴. Evidently, more research is required to fully investigate the liquid bio-fuel
9 production potential of *T. variabilis*, especially when it is grown in wastewater.

10 **6.2 Gaseous bio-fuels: bio-methane and bio-hydrogen**

11 Biogas is the oldest form of bioenergy generated by means of methanogenic bacteria
12 under anaerobic conditions containing ~50-70% bio-methane, which can be used as a
13 heat, power or transportation fuel⁹⁵. Several microalgae have been used to combine bio-
14 methane production with biorefinery approaches, for example, *Chlamydomonas*
15 *reinhardtii* and *Scenedesmus obliquus*⁹⁶. There are some reports of *T. variabilis*
16 biomass conversion into biogas under high temperature using anaerobic digesters
17 without⁹⁷ or in combination with immobilising technology to enhance gas production⁹⁸.
18 Methane production yield was recorded at 450 mL.g⁻¹ biomass using immobilised
19 methanogenic bacteria and *Rhodobacter capsulatus* on polymeric matrices in an
20 anaerobic bioreactor⁹⁸. In another research cumulative methane yield (64%) was
21 recorded at ~4 mmol.g⁻¹ biomass when anaerobic cellulolytic substrate were used
22 together with methanogenic *Archaea* from genera of *Methanoculleus* and
23 *Methanosarcina*⁹⁷. Biogas is generated via 4 successive stages: (i) hydrolysis of
24 biopolymers to monomers, (ii) fermentation (acidogenesis) of amino acids and sugars to
25 intermediary products, (iii) acetogenesis of intermediary products to acetate, CO₂ and
26 hydrogen, and (iv) methanogenesis, which transforms acetate into methane. During
27 anaerobic digestion, hydrolysis is known to be the rate limiting step which needs to be
28 optimized via efficient pre-treatment technologies. Owing to the composition and
29 structural features of microalgae resulting in changes in response to the different growth
30 conditions⁹⁹, it is assumed that biogas production yield will be affected by *T. variabilis*
31 grown in wastewaters prior to anaerobic digestion, through expression of various
32 proteins. However, no research has yet demonstrated the effect of these generated
33 macromolecules, which consequently impact bio-degradability of biomass during
34 hydrolysis. Also, more investigation is required to be undertaken on the effect of co-
35 digestion of *T. variabilis* and operational conditions such as hydraulic retention time
36 and proportion of inoculum and substrates¹⁰⁰ as the main parameters known in bio-
37 methane production. The results of previous studies indicated that variety and
38 abundance of acetate oxidizing syntrophic bacteria significantly influence conversion
39 efficiency of *T. variabilis* to bio-methane. Improving biogas conversion yield would be
40 an interesting subject particularly in industrial wastewater treatment plants for coupling
41 bioremediation with methane production.

42 The most common form of bioenergy generated from *T. variabilis* is hydrogen, a
43 clean source of fuel that can be used either directly in internal combustion engines or in
44 fuel cells to generate electricity¹⁰¹. The environmental benefits offered by renewable
45 hydrogen are clear, particularly if the energy is being harnessed from microorganisms.
46 Different species of algae¹⁰² have been identified as potential sources of bio-hydrogen.
47 Basic research in this field started with investigating bacterial hydrogen production in
48

1 the 1920s and later in the 1940s, attention shifted to microalgal hydrogen production¹⁰³.
2 Development in applied research started in the early 1970s¹⁰³. Across several species
3 used as cyanobacterial sources of bio-hydrogen¹⁰⁴, *T. variabilis* has attracted much
4 attention owing to its 2-fold benefits in bio-hydrogen production and its considerable
5 potential in nutrient removal from wastewater⁹⁻¹¹. Table 2 presents hydrogen production
6 yield of *T. variabilis* under different experimental conditions. Since this species has
7 been widely investigated for hydrogen generation, here we focus on the biochemistry,
8 cultivation methods, and utilization of wildtype and mutant strains for
9 phycoremediation and/or fuel production.

10 **6.3 Biochemistry and genetics of bio-hydrogen generation using *T. variabilis***

11 Algal hydrogen production occurs via light-dependent and light-independent
12 reactions. Several articles have reviewed light- and dark-driven bio-hydrogen
13 production¹⁰⁵⁻¹⁰⁸. The former includes bio-photolysis and photo-fermentation, which
14 utilise sunlight, water, and CO₂, as energy, electron, and carbon sources, respectively.
15 Light-independent hydrogen generation includes dark fermentation, which uses
16 carbohydrate substrates, such as found in organic waste materials. Light-dependent
17 processes are mainly driven by photosynthesis¹⁰⁹. Hydrogen can be generated
18 aerobically as a product of oxygenic photosynthesis or under anaerobic conditions, as a
19 by-product of the conversion of organic substrates to acidogenic materials^{106, 110}. Here,
20 we have reviewed H₂ production methods using *T. variabilis* with specific focus on the
21 biochemistry of enzyme systems.

22 The complete genome of *T. variabilis* ATCC 29413 was sequenced by the
23 department of energy (DOE) Joint Genome Institute (JGI)³⁸ and deposited in the
24 European Molecular Biology Laboratory (EMBL)/GenBank/ DNA Data Bank of Japan
25 (DDBJ) database³⁸. The genome size is 7.1 Mbp, with a total of ~5754 predicted
26 protein-coding open-reading frames³⁸. Availability of the full genome sequence should
27 lead to a rapid increase in our knowledge of functional genes involved in growth,
28 development, and metabolic processes in this organism. Thus, key gene networks
29 supporting the different metabolic processes in various cell types are now within reach
30 for identification.

31 Two enzyme systems of hydrogenase and nitrogenase play a major role in algal
32 hydrogen production^{106, 111}. *T. variabilis* hydrogenase and nitrogenase enzymes and
33 genes encoding these proteins have been the subject of much research¹¹². Hydrogenase
34 enzymes play the main role in photolysis-dependent hydrogen production as well as
35 hydrogen utilisation. There are two kinds of hydrogenase enzymes: (i) S-Fe
36 hydrogenase (known as reversible or soluble hydrogenase), encoded by *hoxEFUYH*
37 genes¹¹³⁻¹¹⁵, function in generating molecular hydrogen to reduce NAD required for CO₂
38 absorption in the ribulose biphosphate cycle and (ii) Ni- Fe hydrogenase (known as
39 uptake or membrane hydrogenase), encoded by *hupSL* genes¹¹⁶, which catalyses
40 electron transfer from molecular hydrogen to the respiratory chain. Theoretically,
41 efficiency of H₂ production in direct photolysis is high (40.1%) but it is not practically
42 feasible due to the high inhibitory effects of O₂ on the reversible hydrogenase^{103, 106, 117}.

43 Since nitrogenase enzymes are sensitive to oxygen, this requires nitrogenase-
44 catalysed H₂-generating reactions to be separated from oxygen-containing
45 environments. Therefore, indirect photolysis evolved, in which hydrogen is generated in
46 two separate stages. In the first stage, electrons derived from water splitting are
47 consumed in CO₂ fixation to sugars. Thus, CO₂ is stored in vegetative cells as
48

1 carbohydrate and then transported to heterocysts, where the sugar is used as an electron
2 donor^{118, 119}. In the second stage, breakdown of the sugar releases electrons used to
3 produce hydrogen by nitrogenase enzymes¹⁰³. In the heterocysts of *T. variabilis*, groups
4 of *nif* and *vnf* genes are expressed to give rise to metal ion-dependent nitrogenase,
5 which is responsible for nitrogen fixing and generates hydrogen as a by-product of the
6 process. There are three main gene clusters encoding nitrogenases, depending on
7 distinct prosthetic groups: Mo-Fe (*nif1* and *nif2* genes), V-Fe (*vnf* genes) and Fe-Fe (*anf*
8 genes) nitrogenases. Mo-Fe nitrogenases are encoded by (i) groups of *nif1* genes,
9 including *nifBSUHDKENXW*^{20, 120-123} and three open reading frames expressed under
10 diazotrophic conditions, and (ii) *nif2* genes expressed under anoxic conditions. The
11 latter are essentially the same (*nifBSUHDKENXW*) genes with the exception of a few
12 differences, such as the fusion of *nifX* and *nifEN* genes into a single open reading
13 frame¹²⁴. V-Fe nitrogenase contains $\alpha\beta\delta$ -subunits and scaffold proteins encoded by
14 *vnfDKGENH*¹²⁵ which is expressed under diazotrophic conditions in the absence of Mo,
15 with or without V. The third nitrogenase known as Fe-Fe nitrogenase is encoded by
16 *anfHDGK*¹²⁶. Further reviews and reports of *T. variabilis* nitrogenase gene expression
17 and heterocyst metabolism have been published^{124, 125, 127-131}. Although indirect
18 photolysis is more feasible than direct photolysis, the H₂ production efficiency is lower
19 (16.3%) due to the multiphase process and ATP consumption required to drive the
20 nitrogenase activity¹⁰³.

21 Other options based on fermentation of biomass have been developed. Hey *et al*¹³²
22 reviewed key factors affecting photo and dark fermentation, including substrates,
23 inoculum density, and environmental conditions. Light fermentation is similar to
24 indirect photolysis. During indirect photolysis, endogenous organic compounds are used
25 to produce hydrogen, while in photofermentation exogenous organic compounds are
26 consumed for use in generating electrons. Thus, fermentation is considered the most
27 promising method for microbial H₂ production¹⁰³. Unlike light driven processes, during
28 dark fermentation organic substrates are used both as energy and electron sources.
29 Bartacek *et al*¹³³ summarized fundamentals of fermentative H₂ generation in different
30 microorganisms. Although anaerobic bacteria are mostly used in dark fermentation, it
31 was reported that the combination of photo and dark fermentation of photosynthetic
32 bacteria and algae could significantly enhance the bio-hydrogen productivity¹³⁴. To the
33 best of our knowledge, there are no reports on application of *T. variabilis* in such a
34 hybrid system. Thus, further research on metabolic pathways of dark-driven hydrogen
35 generation using *T. variabilis* is required.

36

37 **6.4 Factors affecting H₂ generation in *T. variabilis***

38 Maximal H₂ production from algal cultures can be achieved through optimisation of
39 key physicochemical factors with a regulatory influence on the generation capacity.
40 High light intensity inhibits H₂ production rate due to up-regulation of photosynthesis,
41 which enhances oxygen accumulation and consequent inhibition of nitrogenases^{104, 135}.
42 Darkness, on the other hand, can block H₂ production via depletion of carbohydrate
43 reserves, which triggers a deficiency of energy and electrons needed for nitrogenase
44 activity^{118, 136}. The negative effects of excessive oxygen can be overcome through
45 degassing or generating a partial vacuum¹³⁷, sparging with argon gas¹³⁸ and temporal
46 separation of the photosynthetic phase from the H₂ generation stage⁴⁸. Based on the 2-
47 stage system for H₂ production, a flat panel photo-bioreactor was found to increase
48 biomass production in the first stage with N-replete medium, and the H₂ generating in

1 the second stage with N-depleted medium sparging with pure Ar⁴⁸.

2 The presence of a nitrogen source in the growth medium severely decreases H₂
3 production, particularly when the nitrogenase enzyme machinery is the dominant H₂-
4 producing complex. This is because inorganic nitrogen suppresses differentiation of
5 heterocysts, thereby removing heterocyst-dependent generation. Although H₂ could be
6 generated as a by-product of N-fixation utilizing atmospheric nitrogen as noted earlier
7 (section 5.3) and the frequency of heterocysts increases in N-free growth medium¹³⁹, the
8 innate biochemical inefficiencies of this process reduce H₂ gas production. About 75%
9 of electrons are diverted away from H₂ synthesis to ammonium reduction, and this loss
10 can be averted by the absence of atmospheric nitrogen¹¹⁸.

11 H₂ production rate diminishes with decreasing pH¹⁴⁰. A study focusing on pH effects
12 demonstrated that algal growth rate was 3 times higher in the presence of Mo- or V-
13 nitrogenase than in the presence of Fe- nitrogenase and maximum H₂ production was
14 attained in the presence of V-nitrogenase in a wide range of alkaline cultures (pH 7-
15 9)¹⁴¹. Mo and V are required as essential components in Mo-nitrogenase and V-
16 nitrogenase based H₂ production, respectively, provided in BG and AA growth media¹¹⁸,
17 ¹⁴². Although Mo significantly increases H₂ production rate, it has a specific upper limit
18 (1.6 mM) beyond which further increases do not influence productivity¹¹⁸. The
19 maximum light to hydrogen energy conversion efficiencies were achieved in Allen-
20 Arnon media, where a larger heterocyst frequency was noted⁴⁸. Temperature is another
21 parameter affecting H₂ photo-production. A short-term thermal stress (30-36 °C)
22 enhances nitrogenase activity of *T. variabilis*¹⁴³. The results of studying CO₂
23 concentration, as a carbon source, demonstrated that an optimum initial CO₂ molar
24 fraction of 0.05 is required for maximum biomass production⁵⁶. Additionally, inclusion
25 of a carbon source, such as glucose, gives rise to further hydrogen production¹¹⁸.

26 27 **6.5 Genetic approach for increasing *T. variabilis* H₂ production**

28 Manipulation of the parameters dealt with in the preceding section (e.g., pH,
29 temperature, and nutrients) can be used to increase hydrogen production by algal
30 systems. However, substantial research efforts have been directed at other key targets to
31 enhance hydrogen yield. For example, the reversible hydrogenase enzyme system,
32 which can consume as well as generate hydrogen, is an obvious target for inactivation
33 using genetic approaches. Similarly, the uptake hydrogenase, which only consumes H₂
34 can be targeted for gene deletion. Accordingly, a *T. variabilis* loss-of-function mutant
35 (AVM13) in the *hupSL* gene, which encodes the uptake hydrogenase, had at least a 3-
36 fold increase in H₂ production when compared to the wildtype¹⁴⁴. Nitroso-guanidine
37 mutagenesis was used to generate PK84 and PK17R *T. variabilis* mutants with loss-of-
38 function mutations impairing uptake hydrogenase in both mutants and an additional
39 impairment of bidirectional (reversible) hydrogenase in PK84¹⁴⁵. When compared to the
40 wildtype, H₂ production in PK17R and PK84 was increased 1.4-fold and 4.3-fold,
41 respectively¹⁴⁶. Notably, while wild-type strains produce appreciable amounts of H₂
42 only in an argon atmosphere (to preclude O₂), the mutant PK84 produced H₂ in a CO₂
43 atmosphere, which is more desirable from a process scale-up perspective¹⁴⁷. While
44 mutants are showing great promise (Table 2), environmental concerns about the release
45 of genetically modified organisms exist¹⁴⁸. Since open biomass production systems are
46 likely to be used in combined phycoremediation and bioenergy generation, use of
47 mutant algae is inevitably problematic.

6.6 Feasibility of integrated biofuel production and wastewater treatment

In order to make the application of microalgae system feasible at industrial scale, it is vital to use sunlight as an energy source to significantly reduce the operation costs, relative to using artificial lighting. The mutant form of *T. variabilis* has demonstrated viable growth in ambient outdoor conditions¹⁴⁹⁻¹⁵¹. It was shown that in such conditions, this species can prolong hydrogen generation without medium refreshment for 40 days¹⁵¹. Also the feasibility of growing the wildtype of this species in wastewater had promising results⁹. Although there are some pilot studies concentrated on bioenergy production and/or wastewater treatment using *T. variabilis*, there still is lack of specific research examining performance at larger industrial scale. However, several research groups have demonstrated that under certain circumstances, large-scale H₂-photo production using microalgae is viable¹⁵². Previously, scaling up the algal systems for bioenergy production was investigated from different techno-economic and socio-environmental angles considering various parameters, such as water and energy consumption, cultivation and harvesting technologies, and associated costs and savings⁸⁴. Open raceway ponds with lower capital and operational costs, in comparison to PBRs, represent an attractive option for commercial large-scale bioenergy production¹⁵³. It was demonstrated that this technology provides a "win-win strategy"¹⁵⁴, having remarkable productivity with an average capacity of 40-70 tons biomass.ha⁻¹.year⁻¹ during bioremediation process, which can be coupled with biorefinery¹⁵³. Results of a feasibility study for integration of wastewater treatment and biogas production using microalgae systems indicated positive benefits on payback period and internal rate of return (*IRR*) for biofuel production when considering environmental revenue and cost-savings, such as bio-products income, carbon credits and wastewater treatment¹⁵⁵. The same strategy was reported as a feasible solution for using algae and bacteria cocultures to couple bioremediation and biofuel production¹⁵⁶. Economic feasibility of commercial raceway pond use in wastewater treatment and biogas production should consider the pond size, electricity requirements, and thermal capacity for bio-methane production¹⁵⁷. In such a system, the required CO₂ and thermal energy could be met by generation via combined heat and power production (305 kg CO₂.d⁻¹ and 488 kWh.d⁻¹) coupled with biogas production system¹⁵⁷. Therefore, the limited information on feasibility of bioenergy production using microalgal systems points to a beneficial outcome with significantly reduced energy consumption and costs if the system integrates with bioremediation to cover nutrient (40–100 kg of N and 3-12 kg of P) and water (11–13 ML.ha⁻¹.year⁻¹) requirements¹⁵³.

7 Challenges and opportunities

There are several studies on various aspects of *T. variabilis* biology, cultivation, and application to phycoremediation. Here, we have provided a coherent synthesis of the literature by presenting how surveying the interrelation between different studies could develop a route to industrial application of *T. variabilis* in bioremediation and production of sustainable bioenergy, discussing challenges, opportunities and research gaps as bellow:

- 1) Bioremediation performance of *T. variabilis* illustrated promising results in bioabsorption of nutrients and heavy metals such as Cu, Zn, and Ni. Remediation of different metals may influence gene expression and enzymatic function. This would be more important when the biomass harvested from remediation process

1 is used directly for bioenergy production. Thus, research into the effects of
2 remediation on metabolic pathways, which control bioenergy generation, in this
3 species needs to be undertaken.

- 4 2) There are some pilot studies showing the utility of *T. variabilis* in bioremediation
5 using artificially constituted “wastewaters”. However, actual wastewaters may
6 contain unknown hazardous compounds and allelochemicals, which can
7 negatively influence algal growth. For example, nTiO₂, Microcystins, Harmane
8 and FischerellinB restrict application of algae in wastewater streams containing
9 such pollutants. Therefore, it is necessary to examine actual biomass generation
10 in different real wastewater streams for scaling up phycoremediation at industrial
11 scale for biorefining purposes.
- 12 3) The application of *T. variabilis* for biodiesel and bio-ethanol production has not
13 been widely studied. Although *T. variabilis* is not considered an oleaginous
14 species, environmental stress can induce cells to produce significant amounts of
15 lipid. The molecular genetic basis for the enhanced lipid synthesis requires
16 further research. Also, bio-ethanol production using this organism is not viable
17 due to the challenge of polysaccharide loss together with associated costly and
18 high energy-consuming technologies required for the process. Therefore, more
19 efficient solutions are required.
- 20 4) Biogas generation using this alga is promising, particularly when coupled to
21 wastewater treatment. In order to improve bio-methane yield, first it is necessary
22 to evaluate the effect of the bioremediation process on algal cell composition,
23 protein profile and biomass bio-degradability. This would provide valuable
24 information to boost the initial hydrolysis processes via applying the most
25 appropriate pretreatment technology. Also, applying co-digestion technologies
26 can enhance anaerobic digestion kinetics and, from an economic aspect, there
27 are still several improvement methods such as optimizing the proportions of
28 microalgal cells for declining the anaerobic digestion hydraulic retention times
29 which need further investigation.
- 30 5) Bio-hydrogen production using *T. variabilis* is focused on light-driven methods
31 and the potential of using this species in dark-driven hybrid systems, particularly
32 in combination with dark fermentation, has not yet been surveyed. Although the
33 challenge of oxygen inhibitory effects on hydrogen generation can be averted by
34 degassing, partial vacuum, sparging Ar, applying two-staged H₂ generation
35 method or utilising mutants of *T. variabilis*, presented solutions are costly and
36 carry environmental risks. Therefore, still further research is necessary to
37 develop cost-effective and environmentally friendly alternatives for robust bio-
38 hydrogen production systems.

40 **8 Future prospects**

41 In this review, we have sought to collate the wide literature on *Trichormus variabilis*,
42 bringing data together from many different aspects, including morphology focusing on
43 different types of cell differentiation and physiology, together with a review of growth
44 parameters and conditions where the species may be used in remediation of wastewaters
45 coupled with production of different forms of liquid and gaseous bioenergy, such as
46 biodiesel, bio-ethanol, bio-methane and bio-hydrogen. We attempted to distil the key
47 information available on *T. variabilis* on phycoremediation and bioenergy production to
48 enable further research on optimization of robust technologies for supplying clean water

1 and sustainable energy resources. Trends in research of *T. variabilis* during the last
2 decades has focused on the main aspects of (i) biology, (ii) nutrient removal and (iii)
3 bioenergy production, and considered the effects of several stress factors on growth and
4 lipid production. There is a promising path for future application of this species even
5 though there are still major environmental and economic issues which need to be
6 resolved to optimise *T. variabilis* for industrial usage. The valuable potential of this
7 microorganism arising from the differentiation of vegetative cells into other cell types
8 for adaptive responses to different environmental conditions provides a great
9 opportunity to develop platforms for commercial exploitation of this species. It is
10 important to elucidate the mechanisms of cell differentiation and metabolic changes
11 associated with exposure to stress.

12

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16

17 **Competing interests**

18 The authors have no competing interests.

19

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Table 1. Bioremediation potential using *T. variabilis*.

Experimental conditions	Removed compounds	Efficiency (%)	Ref
Preliminary growth: MBL medium, 25–35 °C, day light for 14 days. Treatment process: Mixed domestic-industrial wastewater, 25–35 °C, daylight.	BOD	89.3	9
	COD	52.6	
	TSS	44.6	
	TDS	38.8	
	Fats, Oil and Grease	68.2	
	Zn	81.2	
	Cu	91	
Preliminary growth: BG ₁₁ medium, 30 °C for 15 days. Treatment process: ~16% of BG ₁₁ medium mixed with textile industry effluent wastewater for 25 days, 30 °C, aerobic condition.	BOD	83	10
	COD	75	
	SO ₄	55.4	
	Ni	63	
	Zn	67	
	Ca	17.5	
	Mg	28	
Preliminary growth: MDM medium, 27.5 °C, ~ 65 μmol.m ⁻² .s ⁻¹ , aerated containing 1% CO ₂ for 7 days. Treatment process: using supplemented medium, phenols concentration 40 μM, 25 °C for 120 h.	<i>o</i> -Nitrophenol	100	71
	<i>m</i> -Nitrophenol	100	
	<i>p</i> -Nitrophenol	4	
	2,4-Dinitrophenol	95	
	2,4,6-Trinitrophenol	51	
	Bisphenol A	23	
Preliminary growth: Fogg medium, 24 ± 1 °C, ~ 55 μmol.m ⁻² .s ⁻¹ , 16 h light / 8 h dark cycle for 22 days. Treatment process: supplemented medium, Standard stock solution of Zn ²⁺ ions (1000 ± 2 mg.L ⁻¹)	Zn	85.1	158

<p>Preliminary growth: calcium alginate immobilized cell Treatment process: supplemented medium, 11-10 ppm concentration of lead</p>	Pb	96	159
<p>Preliminary growth: Chu-10 medium, 28 ± 2 °C, daylight $\sim 45 \mu\text{mol m}^{-2} \text{s}^{-1}$ Treatment process: supplemented medium, Stock solutions of chromium concentrations of (10 - 100 mM) prepared by dissolving $\text{K}_2\text{Cr}_2\text{O}_7$.</p>	Cr	54	160
<p>Preliminary growth: Allen and Arnon medium, 25 °C, $15 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ Treatment process: Ammonium ions were added in concentration of 0.5mg.L^{-1}, immobilized cells in the photobioreactor for 25 days.</p>	Ammonium	90	13

Table 2. Hydrogen production yield using *T. variabilis* under different experimental conditions.

Strain (wild/mutant)	Experimental conditions	Medium	Hydrogen production rate	Ref
ATCC 29413	Growth: 30 °C, 60 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ H ₂ : 5% CO ₂ , Ar (25 ml min ⁻¹), 35 °C, 70 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, (a) 1.6 mM Mo, (b) 10 mM glucose	BG ₀	(a) 44 $\mu\text{mol.mg chla}^{-1}.\text{h}^{-1}$ (b) 49 $\mu\text{mol.mg chla}^{-1}.\text{h}^{-1}$	118
	Growth: 95% air + 5% CO ₂ , 30 °C, 65 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ H ₂ : Ar (45 ml min ⁻¹), 30 °C, 150 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$	BG ₀	0.9 mL.g dry cell ⁻¹ .h ⁻¹	48
	Growth: 30 °C, 35-161 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 190 rpm H ₂ : 100% Ar	BG ₁₁ AA BG ₁₁	1 mL.g dry cell ⁻¹ .h ⁻¹ 5.6 mL.g dry cell ⁻¹ .h ⁻¹ ~6 mL.g dry cell ⁻¹ .h ⁻¹	41
	Growth: Ambient air, 25 °C, 5 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ (bottom), 15 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ (surface) H ₂ : 100% Ar, Hydrophilic cuprammonium rayon hollow fibers PBR, medium was heated at 50°C prior injection	AA	17- 20 mg.g dry cell ⁻¹ .h ⁻¹	13
	Growth: ~ 67.5 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ H ₂ : Ar + 5% CO ₂ , 77 mM Tween 85	AA	0.44 ± 0.03 mL.mg dry cell ⁻¹ .h ⁻¹	161
CCAP 403/4B	Growth: 1.7% CO ₂ , 28 °C, continues 45-55 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 140 rpm H ₂ : Vacuum degassed (270-300 torr), 170-180 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ for 5 hr	AA ₀	12 .5 mL.g dry cell ⁻¹ .h ⁻¹	140
	Growth: Air without CO ₂ , 28 °C, 15 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 140 rpm H ₂ : No gas phase, cells immobilized on hollow fibers; 25 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ (surface), 13 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ (bottom), medium was heated at 50 °C prior injection	AA ₀	20 mL.g dry cell ⁻¹ .h ⁻¹	137

PK84	Growth: Air + 2% CO ₂ (500 mL.min ⁻¹), 12 h light (36 °C)/ 12 h dark (14- 30 °C) H ₂ : (a) air, (b) air+ 2% CO ₂ , (c) 80% Ar + 20% O ₂ , (d) 100% Ar, 332 μmol.m ⁻² .s ⁻¹	AA ₀	(a) 106.0 ± 7.0 μmol.mg chl a ⁻¹ .h ⁻¹ (b) 81.0 ± 10.0 μmol.mg chl a ⁻¹ .h ⁻¹ (c) 190.0 ± 8.0 μmol.mg chl a ⁻¹ .h ⁻¹ (d) 191.0 ± 11.0 μmol.mg chl a ⁻¹ .h ⁻¹	149
	Growth: Air+ 2% CO ₂ (500 mL.min ⁻¹) H ₂ : Outdoor PBR, ~ 1.84 mol.m ⁻² .s ⁻¹	AA ₀	80 mL.h ⁻¹ .PBR v ⁻¹	150
	Growth: air + 2% CO ₂ (500 mL.min ⁻¹), 30 °C H ₂ : Outdoor PBR, sun light	AA ₀	60-140 mL.h ⁻¹ .PBR v ⁻¹	151
(a) ATCC29413 (b) PK84	Growth: air + 2% CO ₂ (0.5 L.min ⁻¹), 30 °C, 113 μmol.m ⁻² .s ⁻¹ H ₂ : 100% Ar, 30 °C, 200 μmol.m ⁻² .s ⁻¹	AA	(a) 39.4 nmol.μg chl a ⁻¹ .h ⁻¹ (b) 32.3 nmol.μg chl a ⁻¹ .h ⁻¹	135
(a) ATCC29413 (b) PK84 (c) PK17R	Growth: 25% N ₂ , 2% CO ₂ , 73% Ar (250 mL.min ⁻¹), 90 μmol.m ⁻² .s ⁻¹ H ₂ : Ar, 30 °C, 140 μmol.m ⁻² .s ⁻¹ , N/C starvation	AA ₀	(a) 1.62- 3.07 nmol.μg prot ⁻¹ .h ⁻¹ (b) 6.91-12.6 nmol.μg prot ⁻¹ .h ⁻¹ (c) 2.24-4.10 nmol.μg prot ⁻¹ .h ⁻¹	146
AVM13	Growth: Air, 1% CO ₂ , 30 °C, 100 μmol.m ⁻² .s ⁻¹ H ₂ : Washed with N-free medium	BG ₀	68 nmol.μg ⁻¹ .chl a ⁻¹ .h ⁻¹	144