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Application of an in situ CO_2–bicarbonate system under nitrogen depletion to improve photosynthetic biomass and starch production and regulate amylose accumulation in a marine green microalga *Tetraselmis subcordiformis*

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Abstract

Background: Microalgal starch is regarded as a promising alternative to crop-based starch for biorefinery such as the production of biofuels and bio-based chemicals. The single or separate use of inorganic carbon source, e.g., CO_2 and $NaHCO_3$, caused aberrant pH, which restricts the biomass and starch production. The present study applied an in situ CO_2–$NaHCO_3$ system to regulate photosynthetic biomass and starch production along with starch quality in a marine green microalga *Tetraselmis subcordiformis* under nitrogen-depletion (−N) and nitrogen-limitation (±N) conditions.

Results: The CO_2 (2%)–$NaHCO_3$ (1 g L^{-1}) system stabilized the pH at 7.7 in the −N cultivation, under which the optimal biomass and starch accumulation were achieved. The biomass and starch productivity under −N were improved by 2.1-fold and 1.7-fold, respectively, with 1 g L^{-1} $NaHCO_3$ addition compared with the one without $NaHCO_3$ addition. $NaHCO_3$ addition alleviated the high-dCO_2 inhibition caused by the single CO_2 aeration, and provided sufficient effective carbon source HCO_3^- for the maintenance of adequate photosynthetic efficiency and increase in photoprotection to facilitate the biomass and starch production. The amylose content was also increased by 44% under this CO_2–bicarbonate system compared to the single use of CO_2. The highest starch productivity of 0.73 g L^{-1} day^{-1} under −N cultivation and highest starch concentration of 4.14 g L^{-1} under ±N cultivation were both achieved with the addition of 1 g L^{-1} $NaHCO_3$. These levels were comparable to or exceeded the current achievements reported in studies. The addition of 5 g L^{-1} $NaHCO_3$ under ±N cultivation led to a production of high-amylose starch (59.3% of total starch), which could be used as a source of functional food.

Conclusions: The in situ CO_2–$NaHCO_3$ system significantly improved the biomass and starch production in *T. subcordiformis*. It could also regulate the starch quality with varied relative amylose content under different cultivation modes for diverse downstream applications that could promote the economic feasibility of microalgal starch-based biofuel production. Adoption of this system in *T. subcordiformis* would facilitate the CO_2 mitigation couple with its starch-based biorefinery.

Keywords: Starch, Nitrogen depletion, pH, Bicarbonate, Amylose, *Tetraselmis subcordiformis*

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Background

Microalgae, which can photosynthetically fix CO_2 and produce a variety of compounds (carbohydrate, lipid and protein), are currently considered as sustainable feedstock for biofuel production and as high-value compounds producers due to their high photosynthetic efficiency, fast growth, robust CO_2 fixation ability, flexible and controllable cultivation modes, and no competition for arable lands [1]. Starch is the primary photosynthetic carbon sink for many microalgae, the existence of which is especially abundant in Chlorophyta [2]. Because the structure of the starch from microalgae resembles that in the higher plants, it is regarded as a promising alternative to crop-based starch for application in the fields of biofuel generation (such as bioethanol, bio-butanol, biomethane and bio-hydrogen) and bio-based chemicals production [2, 3].

Considerable microalgal starch accumulation with usually more than 50%DW stored intracellularly occurs under stressful conditions such as nutrient deprivation and high irradiance, with nitrogen depletion (−N) or nitrogen limitation (±N) being the most widely studied strategies for the improvement of starch production [2, 4–7]. In general, the −N cultivation, which in essential applies a low cell density and short cultivation time without extra or with very small amounts of nitrogen supply, can enable a relatively high light availability for an individual microalga that tends to facilitate the rapid starch accumulation with high starch productivity and content; in contrast, ±N cultivation employs a limited nitrogen supply for cell growth, which needs longer cultivation time and can get more biomass and improve starch concentration [5]. These two cultivation modes can be combined as a "two-stage" process to incorporate their respective advantages, which can maximize the starch production [8].

Another important factor affecting microalgal biomass and starch production is the carbon supply. In general, microalgae utilize CO_2 as the direct carbon source for photosynthesis. However, due to the low water solubility, gaseous CO_2 supply in air cannot meet the desired biomass productivity [9]. Moreover, although the increase in CO_2 percentage in air during aeration can improve the carbon availability in the medium, the pH will decrease, which could in turn inhibit the microalgal growth [10]. Bicarbonate is another effective carbon source that most microalgae can utilize. It can be converted to CO_2 via the action of carbonic anhydrase (CA) enzyme and then be fixed via photosynthesis [11]. $NaHCO_3$, which has high water solubility and is widely available with a low price, has been recently used to increase carbon supply and improve biomass and lipid/carbohydrate production in several microalgae such as *Tetraselmis suecica* [12], *Chlorella vulgaris* [13], *Scenedesmus* sp. [14] and *Dunaliella salina* V-101 [15]. However, the single use of $NaHCO_3$ increased the pH (usually > 10 on the final cultivation day) due to the utilization of HCO_3^- by microalgae that tended to release OH^- according to the equilibrium relationship of $HCO_3^- + H_2O \leftrightarrow H_2CO_3 + OH^-$ and $H_2CO_3 \leftrightarrow CO_2 + H_2O$, and hence, the biomass production was still limited [16, 17]. Moreover, the starch accumulation could also be influenced by the varied pH environments originated from the different carbon sources used [18, 19]. Therefore, to get an optimized biomass or starch production, suitable supply of carbon source is required to ensure a carbon-abundant environment along with a favorable pH condition.

Traditionally, pH is controlled by adding acid (including CO_2) or alkali, which usually incorporated a complex online monitoring system [20], making it difficult to be realized in large-scale cultivations especially when large open ponds are used. Recently, Zhu et al. [21] established a recycling culture in which HCO_3^- was first used for microalgal growth followed by CO_2 neutralization for medium recycle. However, this strategy required a good tolerance of microalgae to high pH (typically > 9) because in essential HCO_3^- was used solely in the cultivation stage. The combined use of CO_2 and $NaHCO_3$, which can construct a CO_2–$NaHCO_3$ buffering system and hence avoid the pH problem of the single use, had recently been demonstrated to enhance the algal growth rate and carbon utilization efficiency as well as lipid production in *Chlorella* [9, 22]. The aeration of CO_2 will in situ neutralize the OH^- derived from the uptake of HCO_3^- and regenerate HCO_3^-, and thus, stable and favorable pH can be achieved during the cultivation. However, rare attention has been paid to the effect of CO_2–$NaHCO_3$ system on the starch production under nutrient depletion or limited conditions.

Tetraselmis subcordiformis is a marine green microalga that has been demonstrated to accumulate more than 50%DW starch intracellularly under nitrogen deprivation [5, 23]. The present study aimed at further improving the biomass and starch production in this alga via the regulation of pH and effective carbon source using an appropriate in situ CO_2–$NaHCO_3$ system. The starch quality, i.e., the amylose proportion in the total accumulated starch, was also tracked to evaluate the suitability of the starch obtained under different cultivation strategies for the biofuel generation along with additional possible high-value applications that could contribute to the economic feasibility of the whole process.

Results and discussion

Biomass production and DIC under nitrogen depletion

Nitrogen depletion was an effective strategy to induce starch accumulation in *T. subcordiformis* [5]. Therefore, the impact of $NaHCO_3$ addition was investigated under nitrogen depletion. In general, microalgae can recycle the intracellular stored nitrogen (e.g., protein-derived nitrogen) to transiently support their growth when extracellular nitrogen is depleted [24]. The addition of $NaHCO_3$ in the context of 2% CO_2 aeration influenced the cell growth and biomass production under nitrogen depletion. As shown in Fig. 1a, the cell density as revealed by OD_{750} was dramatically enhanced with the addition of $NaHCO_3$, which exhibited a dose-dependent manner under the $NaHCO_3$ concentrations between 0 and 1 g L^{-1}. The final cell density in the culture with 0.2 and 1 g L^{-1} of $NaHCO_3$ addition at Day 4 was 48% and 1.1-fold higher, respectively, than that without $NaHCO_3$ addition. Similarly, the biomass accumulation was also enhanced with the addition of $NaHCO_3$. The maximum biomass production in the culture with 1 g L^{-1} $NaHCO_3$ reached 2.6 g L^{-1} at Day 3, which was 17% and 1.1-fold higher than that in the 0.2 and 0 g L^{-1} $NaHCO_3$ cultures (Fig. 1b). Noteworthily, further increasing $NaHCO_3$ concentration to 5 g L^{-1}

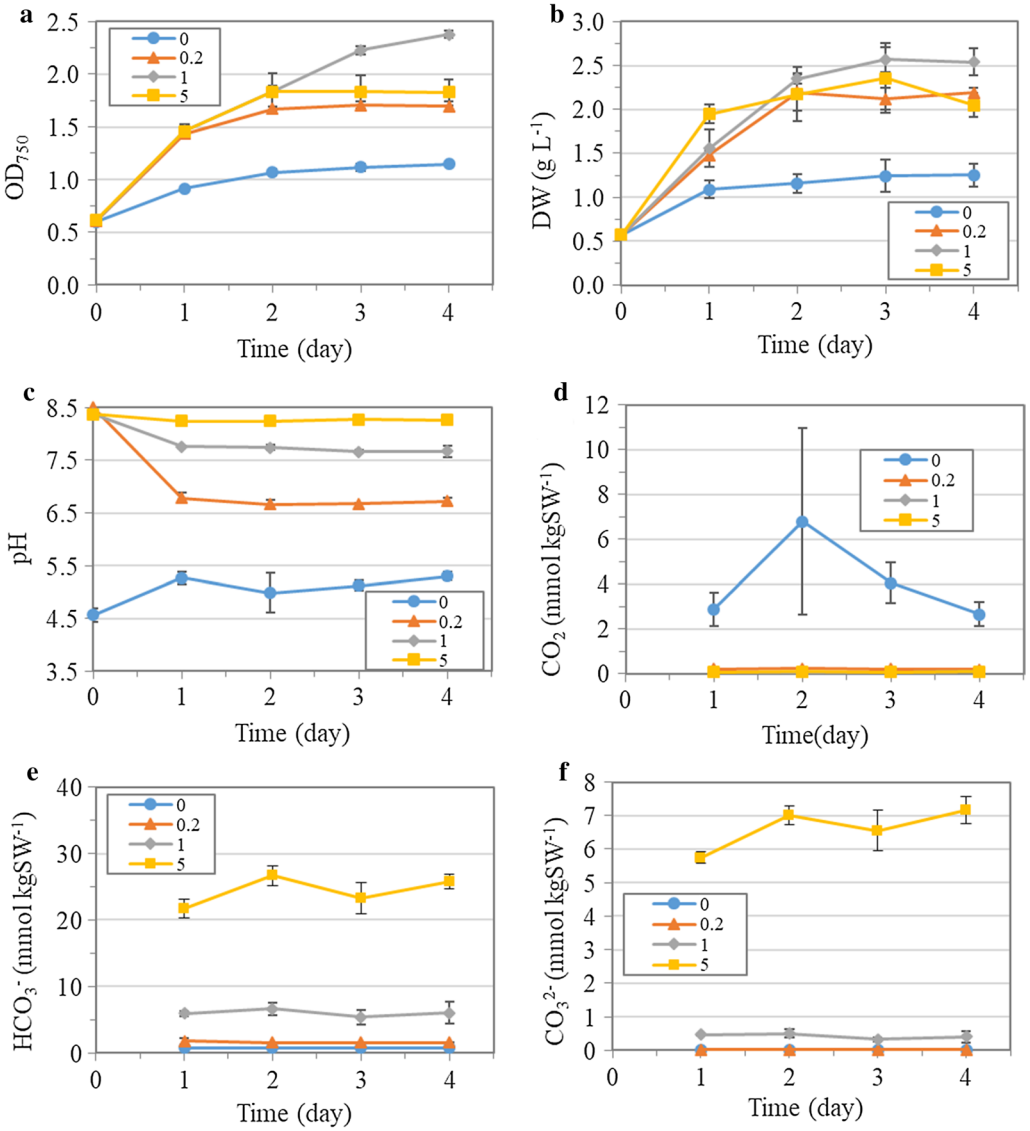

Fig. 1 Cell growth (OD_{750}, **a**), biomass production (dry weight, **b**), pH variations (**c**), dissolved CO_2 concentration (dCO_2, **d**), HCO_3^- concentration (**e**) and CO_3^{2-} concentration (**f**) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition (0, 0.2, 1 and 5 g L^{-1}) under nitrogen depletion (mean ± SD, $n = 3$)

showed negative effects in terms of both cell growth and biomass production compared with the 1 g L^{-1} $NaHCO_3$ culture, although it still improved biomass accumulation by 88% in comparison with the 0 g L^{-1} $NaHCO_3$ culture (Fig. 1b).

The addition of $NaHCO_3$ could generate a favorable pH condition for enhanced biomass accumulation in *T. subcordiformis* under nitrogen depletion. As shown in Fig. 1c, the pH of the medium generally increased evidently with the addition of $NaHCO_3$, which reached average levels of 5.2, 6.7, 7.7 and 8.3, respectively, after equilibrium for one day in the cultures with 0, 0.2, 1 and 5 g L^{-1} $NaHCO_3$ addition. The present study showed that the best biomass production was obtained with the addition of 1 g L^{-1} $NaHCO_3$ where pH was maintained at 7.7, which was in line with the optimal pH condition for biomass production in *T. suecica* [25]. The dramatic inhibition of cell growth and biomass production in the culture without $NaHCO_3$ addition could be largely ascribed to the low pH environment at around 5.2. In fact, the pH as low as 5.5 had been demonstrated to impede cell growth and reduce biomass productivity in *Tetraselmis* [25, 26]. The decrease in pH to 5–5.5 was recently shown to be the main factor contributing to the inhibition of biomass production in *Arthrospira platensis* [10], which could also apply herein. Low pH was reported to impair photosystems and inactivate some critical enzymes related to carbon assimilation (e.g., Rubisco), which caused diminished cell growth and biomass accumulation [27].

The beneficial effects of $NaHCO_3$ to biomass accumulation could also be attributed to the relieving of inhibition caused by the high dissolved CO_2 concentration (dCO_2) in the CO_2 aeration culture. It was evident from Fig. 1d that in the context of 2% CO_2 aeration, no addition of $NaHCO_3$ resulted in a dCO_2 of 2.9–4.1 mmol $kgSW^{-1}$, which was 13- to 52-fold higher than that in the cultures with 0.2 and 1 g L^{-1} $NaHCO_3$ addition. Similar dCO_2 (around 5 mmol $kgSW^{-1}$) was also found in the cultivation of *Nannochloropsis salina* with high CO_2 (20%) supply where biomass accumulation was strongly inhibited compared with the low CO_2 (0.04% and 6%) supply [17]. Li et al. [13] also found that a dCO_2 of 11.29 mM was the primary inhibitive factor for the cell growth in *C. vulgaris.* In addition, the dCO_2 accounted for averagely 79% of the total DIC in the culture without $NaHCO_3$ addition (Additional file 1: Figure S1a), further supporting the notion that the diminished biomass production could be ascribed to the inhibition caused by the high dCO_2. The addition of $NaHCO_3$ increased the total alkalinity (Additional file 1: Figure S2a) and pH (Fig. 1c) of the culture, which would convert more dissolved CO_2 into HCO_3^- according to the equilibrium relationship of $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. As a result, the dCO_2 was dramatically decreased (Fig. 1d). In fact, the predominant DIC in the cultures with the addition of $NaHCO_3$ was HCO_3^-, which accounted for approximately 88%, 92% and 78% of the total DIC in the cultures with 0.2, 1 and 5 g L^{-1} $NaHCO_3$, respectively (Additional file 1: Figure S1b–d). It indicated that HCO_3^- was the main carbon source for the growth of *T. subcordiformis* under these conditions. HCO_3^- had been shown to be an effective carbon source for *Tetraselmis* [12, 28]. Collectively, it was reasonable to conclude that cell growth and biomass production were facilitated with the addition of $NaHCO_3$ via ensuring a suitable pH, alleviating inhibition of high dCO_2 and providing sufficient effective carbon source HCO_3^-.

However, excessive addition of $NaHCO_3$ up to 5 g L^{-1} caused unfavorable effects on cell growth and biomass production relative to the 1 g L^{-1} counterpart (Fig. 1a, b). The increased pH up to 8.3 in the 5 g L^{-1} $NaHCO_3$ culture (Fig. 1c) should not be accounted for this inhibitory effect because pH ranging from 7.4 to 8.5 could not affect biomass production in *T. subcordiformis* (see the discussion in the nitrogen-limitation cultivation below). HCO_3^- was the predominant DIC in both the 1 and 5 g L^{-1} $NaHCO_3$ cultures, as discussed above, and thus, the different performance of *T. subcordiformis* could be reasonably ascribed to the difference in the HCO_3^- concentration. It should be noted that the concentration of HCO_3^- reached 26.7 mmol $kgSW^{-1}$ in the 5 g L^{-1} $NaHCO_3$ culture on Day 2 when inhibitory effects occurred, which was four times of that in the 1 g L^{-1} one (Fig. 1e). This high HCO_3^- concentration in the 5 g L^{-1} $NaHCO_3$ culture could be unfavorable for the growth of *T. subcordiformis*. The assimilation of HCO_3^- involves an active transport in microalgae which is energy consuming and therefore bio-energetically disadvantaged [11, 13]. The excessive HCO_3^- might disturb the energy supply for photosynthetic CO_2 bio-fixation and other energy-dependent metabolism for cell growth. Therefore, superfluous addition of $NaHCO_3$ caused adverse effects on biomass production.

Photosynthetic performance under nitrogen depletion

The carbon availability and pH can impact the photosynthetic performance of microalgae, leading to varied biomass production. Therefore, several chlorophyll a fluorescence kinetics parameters were tracked throughout the cultivation to check the photosynthetic efficiency.

F_v/F_m is the maximum quantum efficiency of photosystem II and represents the photosynthetic activity, the decline of which also denotes stress conditions microalgae would have been exposed to [5]. Figure 2a shows that a sharp decline of F_v/F_m (0.704 on Day 0 to 0.413 on Day 2) was present from the beginning of the cultivation in

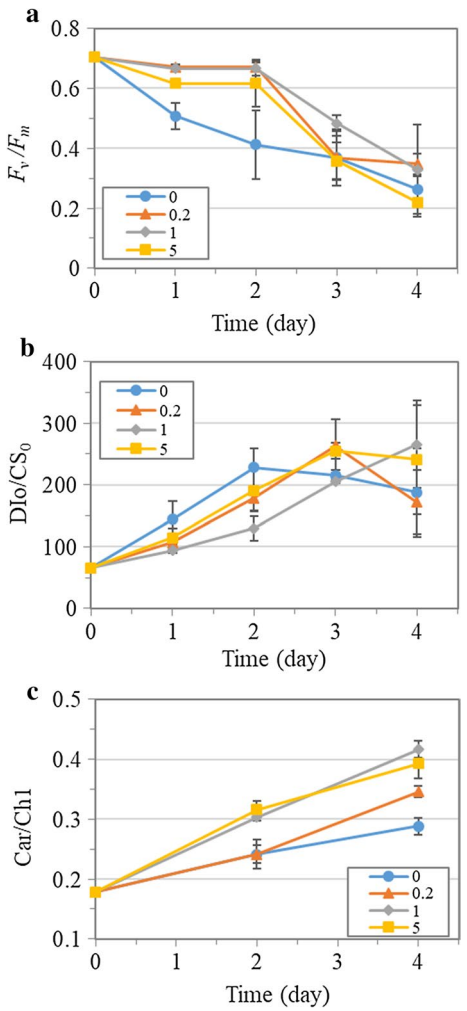

Fig. 2 Photosynthetic activity (F_v/F_m, **a**), dissipated energy flux per excited cross section (DIo/CS$_0$, **b**) and carotenoid/chlorophyll ratio (Car/Chl, **c**) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition (0, 0.2, 1 and 5 g L^{-1}) under nitrogen depletion (mean ± SD, $n = 3$)

the culture without $NaHCO_3$ addition, while the F_v/F_m decreased slightly to 0.67 on Day 2 in the cultures with the addition of 0.2 and 1 g L^{-1} $NaHCO_3$. This result indicated that $NaHCO_3$ addition could alleviate the stress as well as the consequent loss of photosynthetic activity caused by the combined nitrogen depletion and low pH or high dCO_2. Cell morphology analysis (Additional file 1: Figure S3a) on Day 2 also showed that the microalgal cells became abnormally round under nitrogen depletion without $NaHCO_3$ addition, whereas it remained normally elliptical in the cultures with 0.2 and 1 g L^{-1} $NaHCO_3$ addition, indicating that $NaHCO_3$ addition alleviated the stress exerted on cells, which was consistent with the F_v/F_m results. Nitrogen deprivation is considered to generate reactive oxygen species (ROS) in microalgae that will cause damage to the cellular organization and impair the photosynthesis [29]. It has been recently reported that $NaHCO_3$ addition could reduce the oxidative stress induced by nutrient (N, P or S) deficiency and consequently improve the photosynthetic activity in *D. salina* [15], which was in agreement with the present study in *T. subcordiformis*. Furthermore, the energy dissipation flux per excited cross section (DIo/CS$_0$) [30] showed an overall increase under nitrogen deprivation, with the most rapid enhancement observed in the culture without $NaHCO_3$ addition and slowest with 1 g L^{-1} $NaHCO_3$ addition (Fig. 2b). The promoted energy dissipation under nitrogen stress is a protective mechanism for microalgae coping with unfavorable conditions, which has also been observed in nitrogen-starved *Chlamydomonas reinhardtii* [31] and *Porphyridium cruentum* [32]. The lowest level of DIo/CS$_0$ in the 1 g L^{-1} $NaHCO_3$ culture during the first 2 days suggested the highest energy utilization efficiency in the microalgae and the least stress condition the microalgae were subjected to, which was in alignment with the highest photosynthetic activity (F_v/F_m, Fig. 2a). In addition, the carotenoid/chlorophyll ratio (Car/Chl) representing the status of photoprotective function against oxidative stress under nutrient-deprived conditions [29] exhibited continuous increase in all the cultures, and a more rapid increase was observed in the cultures with the addition of $NaHCO_3$ (especially with 1 and 5 g L^{-1}) relative to the non-addition one (Fig. 2c). It indicated that $NaHCO_3$ addition improved the photoprotection, which could contribute to the much better photosynthetic activity therein. Overall, owing to the best maintenance of photosynthetic efficiency in the 1 g L^{-1} $NaHCO_3$ culture on Day 2, the highest biomass productivity of 0.89 g L^{-1} day^{-1} and CO_2 fixation rate of 1.67 g L^{-1} day^{-1} were achieved therein, which were 2.1-fold higher than the 0 g L^{-1} counterpart (Table 1).

Starch production and starch quality under nitrogen depletion

Starch accumulation could be stimulated under nitrogen deprivation in *T. subcordiformis*, as had been demonstrated previously [5] and here (Fig. 3a, b). It was obvious that $NaHCO_3$ addition resulted in more pronounced starch accumulation under this stressful condition. The starch content increased rapidly from the initial level of 10.4%DW to the maximum of 60.6%DW and 56.9%DW within 3 days in the 1 and 0.2 g L^{-1} $NaHCO_3$ cultures, respectively, while it reached only 50.7%DW in the one without $NaHCO_3$ addition (Fig. 3a). As a result, the starch concentration exhibited a dose-dependent manner from the $NaHCO_3$ concentrations of 0 g L^{-1} to

Table 1 Biomass and starch productivity, CO_2 bio-fixation rate, and amylose production of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition under nitrogen-depletion (−N) and nitrogen-limitation (±N) cultivation modes (mean ± SD, *n* = 3)

$NaHCO_3$ (g L^{-1})	Cultivation mode	Biomass productivity (g L^{-1} day^{-1})	CO_2 bio-fixation rate (g L^{-1} day^{-1})	Starch productivity (g L^{-1} day^{-1})	Amylose content (% total starch)	Amylose concentration (mg L^{-1})	Amylose productivity (mg L^{-1} day^{-1})
0	−N	$0.29 \pm 0.05^{\alpha}$ (2^{a})	$0.55 \pm 0.10^{\alpha}$ (2)	$0.27 \pm 0.02^{\alpha}$ (2)	$27.8 \pm 0.3^{\alpha}$ (2)	$153 \pm 8^{\alpha}$ (2)	$54 \pm 4^{\alpha}$ (2)
	±N	0.74 ± 0.00^{A} (8)	1.39 ± 0.00^{A} (8)	0.46 ± 0.01^{A} (8)	26.7 ± 1.5^{A} (8)	984 ± 53^{A} (8)	121 ± 7^{A} (8)
0.2	−N	$0.81 \pm 0.10^{\beta}$ (2)	$1.52 \pm 0.20^{\beta}$ (2)	$0.60 \pm 0.01^{\gamma}$ (2)	$30.9 \pm 0.5^{\gamma}$ (2)	$354 \pm 2^{\gamma}$ (2)	$155 \pm 1^{\gamma}$ (2)
	±N	0.74 ± 0.01^{A} (8)	1.38 ± 0.02^{A} (8)	0.47 ± 0.03^{AB} (8)	26.7 ± 0.9^{A} (8)	1011 ± 32^{AB} (8)	125 ± 4^{AB} (8)
1	−N	$0.89 \pm 0.03^{\beta}$ (2)	$1.67 \pm 0.05^{\beta}$ (2)	$0.73 \pm 0.02^{\delta}$ (2)	$32.9 \pm 0.3^{\delta}$ (2)	$449 \pm 16^{\delta}$ (2)	$202 \pm 8^{\delta}$ (2)
	±N	0.82 ± 0.05^{B} (8)	1.55 ± 0.10^{B} (8)	0.51 ± 0.03^{B} (8)	27.7 ± 1.0^{A} (8)	1148 ± 108^{B} (8)	142 ± 14^{B} (8)
5	−N	$0.80 \pm 0.15^{\beta}$ (2)	$1.50 \pm 0.29^{\beta}$ (2)	$0.41 \pm 0.12^{\beta}$ (2)	$34.7 \pm 0.9^{\beta}$ (2)	$276 \pm 68^{\beta}$ (2)	$116 \pm 34^{\beta}$ (2)
	±N	0.69 ± 0.05 (4)	1.29 ± 0.09 (4)	0.09 ± 0.04 (4)	59.3 ± 0.5 (4)	238 ± 83 (4)	56 ± 21 (4)

The different Greek alphabets (α, β, γ and δ) represented significant difference ($p < 0.05$) between the cultures under −N cultivation mode. The different capital Latin alphabets (A and B) represented significant difference ($p < 0.05$) between the cultures under ±N cultivation mode

[a] The number in the parentheses represented the cultivation day used for calculation and comparison

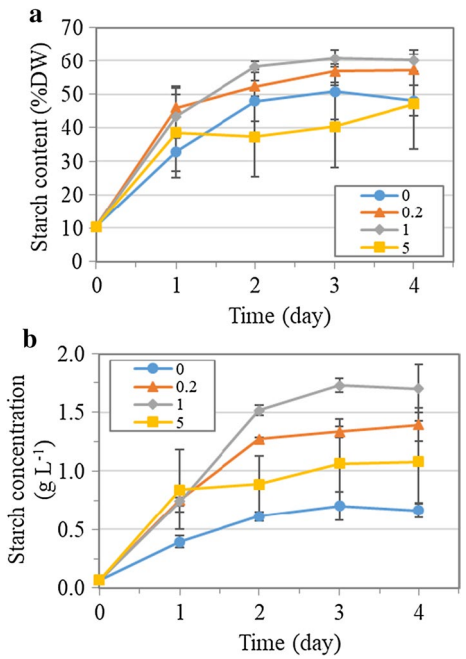

Fig. 3 Starch content (**a**) and starch concentration (**b**) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition (0, 0.2, 1 and 5 g L^{-1}) under nitrogen depletion (mean ± SD, *n* = 3)

1 g L^{-1} (Fig. 3b). The maximal starch concentration of 1.7 g L^{-1} obtained in the culture with 1 g L^{-1} $NaHCO_3$ on Day 3 was 2.5 times of that without $NaHCO_3$ addition (0.7 g L^{-1}). Similar to the case in the biomass production, addition of 5 g L^{-1} $NaHCO_3$ to the culture led to adverse effects on starch accumulation, with the lowest starch content of 40.3%DW obtained on Day 3 therein, although the starch concentration was still superior to that without $NaHCO_3$ addition due to the enhanced biomass accumulation (Fig. 3b). Starch accumulation in autotrophic microalgae relies on photosynthesis for carbon fixation and sugar-precursor (ADP-glucose) biosynthesis, both of which are energy-consuming processes [33]. Therefore, the higher photosynthetic activity under $NaHCO_3$ addition, which should generate more ATP and NADPH for these two processes, could be reasonably accounted for the enhanced starch production here in *T. subcordiformis*. Moreover, the increased Car/Chl under $NaHCO_3$ addition (Fig. 2c) suggested a more active cyclic electron flow around photosystem I, which could generate extra ATP in compensation for the loss of activity at photosystem II [34]. Consequently, the carbon fixation and starch accumulation could be facilitated. In addition, the varied pH itself could also be accounted for the difference of starch accumulation in *T. subcordiformis*. *Tetraselmis* sp. had been demonstrated to have lower starch content under alkaline medium (28%DW, pH 8) than those established under neutral pH (64%DW, pH 7) and under acidic medium (49%DW, pH 6), which coincided with the present study [35]. In *C. vulgaris*, starch content varied from 40 to 55% in the pH range of 6.5–9.0, with the maximum value obtained at pH of 7.7 [36], which was also in alignment with the present study. Collectively, the appropriate addition of $NaHCO_3$ (e.g., 1 g L^{-1}) which alleviated high dCO_2 stress along with the formation of suitable pH environment plus oxidative stress mitigation ensured adequate photosynthesis and hence supported the starch biosynthesis. Due to the maintained photosynthetic efficiency in the first 2 days, the starch productivity peaked at 0.73 g L^{-1} day^{-1} on Day 2 in the culture with 1 g L^{-1} $NaHCO_3$, which was 1.7-fold higher than the one with no $NaHCO_3$ addition (Table 1).

Table 2 Am/Ap ratio, and Am or Ap content (%DW) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition under nitrogen-depletion (−N) cultivation modes (mean ± SD, $n = 3$)

$NaHCO_3$ ($g\ L^{-1}$)	0				0.2				1				5			
	1 day	2 days	3 days	4 days	1 day	2 days	3 days	4 days	1 day	2 days	3 days	4 days	1 day	2 days	3 days	4 days
Am/Ap	0.41 ± 0.10^{a}	0.39 ± 0.01^{a}	0.46 ± 0.06^{a}	0.48 ± 0.09^{a}	0.44 ± 0.02^{a}	0.45 ± 0.01^{b}	0.48 ± 0.02^{a}	0.49 ± 0.01^{a}	0.46 ± 0.03^{a}	0.49 ± 0.01^{c}	0.58 ± 0.01^{b}	0.62 ± 0.01^{b}	0.46 ± 0.01^{a}	0.53 ± 0.02^{d}	0.63 ± 0.06^{b}	0.60 ± 0.04^{b}
Am (%DW)	9.6 ± 3.0^{a}	13.3 ± 1.5^{a}	15.8 ± 1.7^{a}	15.5 ± 2.9^{a}	14.1 ± 2.3^{a}	16.2 ± 1.4^{ab}	18.6 ± 1.4^{ab}	18.8 ± 1.6^{ab}	13.6 ± 2.1^{a}	19.1 ± 0.6^{b}	22.2 ± 1.1^{b}	22.9 ± 1.2^{b}	12.1 ± 3.9^{a}	12.9 ± 3.8^{a}	15.7 ± 5.1^{a}	17.8 ± 5.6^{ab}
Ap (%DW)	23.4 ± 3.5^{a}	34.6 ± 4.6^{b}	35.0 ± 6.9^{b}	32.6 ± 3.0^{ab}	31.9 ± 4.2^{a}	36.2 ± 3.6^{b}	38.3 ± 2.0^{b}	38.5 ± 3.1^{b}	29.8 ± 4.5^{a}	39.0 ± 1.1^{b}	38.4 ± 1.5^{b}	37.2 ± 2.2^{ab}	26.4 ± 9.3^{a}	24.5 ± 8.2^{a}	24.6 ± 7.2^{a}	29.2 ± 7.7^{a}

The different letters (a, b, c and d) represented significant difference ($p < 0.05$) between the cultures on the same cultivation day

To further reveal the influence of nitrogen depletion and $NaHCO_3$ addition on the starch quality, the amylose (Am)/amylopectin (Ap) ratio (Am/Ap) was also measured. Generally, it appeared that as nitrogen deprivation prolonged, Am/Ap was enhanced in all the cultures (Table 2), indicating that amylose biosynthesis under nitrogen stress condition was more favored than amylopectin. This result coincided with the phenomenon found in *C. reinhardtii* 137C that 15–35% of amylose based on total starch (TS) was obtained under nitrogen starvation in against which < 5%TS of amylose under nitrogen repletion [37]. Nitrogen depletion had been found to stimulate granule-bound starch synthase (GBSS), a critical enzyme responsible for amylose biosynthesis in microalgae and plants [38, 39], which could also be applied in *T. subcordiformis*. Interestingly, the addition of $NaHCO_3$ accelerated the increase in Am/Ap under nitrogen depletion, especially in the 1 g L^{-1} and 5 g L^{-1} $NaHCO_3$ cultures ($p < 0.05$, Table 2). For example, the Am/Ap reached 0.49 (Am: 32.9%TS, Table 1) and 0.53 (Am: 34.7%TS, Table 1) in the 1 g L^{-1} and 5 g L^{-1} $NaHCO_3$ cultures, respectively, on Day 2, which was 26% and 36% higher than that in the 0 g L^{-1} one (approximately 0.39, i.e., Am: 27.8%TS, Table 1). In addition, the Am content (%DW) was significantly enhanced with the increase in $NaHCO_3$ addition in the concentration range of 0–1 g L^{-1} from Day 2 to Day 4 ($p < 0.05$), while the Ap content showed almost no significant difference ($p > 0.05$, Table 2), suggesting that the addition of $NaHCO_3$ primarily facilitated the Am accumulation. The Am content in the culture with 1 g L^{-1} $NaHCO_3$ reached 19.1%DW on Day 2, which showed 44% of improvement compared with the 0 g L^{-1} counterpart. This phenomenon, to the best of our knowledge, was rarely reported previously, the mechanism of which was poorly understood either. In fact, the enhanced Am proportion in TS was also observed in *Chlorella* under low CO_2 (air, 0.038%) conditions where CO_2-concentrating mechanisms (CCM) was induced to synthesize a pyrenoidal starch sheath [40, 41]. The addition of bicarbonate herein should also induce a CA-mediated CCM for carbon utilization [11]. Whether the improved amylose content should be ascribed to the CCM needs intensive study. Besides, the possibility of the influence of pH itself on amylose content could not be excluded, since low-CO_2 cultivation always leads to increased pH [9, 17], which mimics the effect of bicarbonate addition.

Biomass production and photosynthetic performance under nitrogen limitation

Nitrogen depletion generally led to the decline of photosynthesis and thus limited the overall biomass and starch production, although it was effective in inducing starch accumulation in microalgae. Therefore, a batch culture mode with limited nitrate supply (10 mM, nitrogen limitation) was applied in *T. subcordiformis*, in the context of which the impact of $NaHCO_3$ was further evaluated. As shown in Fig. 4a, the nitrate was almost consumed up within 2 days in all the cultures, and the overall nitrate removal rate (more than 97%) exhibited no significant difference among them. The biomass accumulation showed no significant difference between the cultures with 0, 0.2 and 1 g L^{-1} $NaHCO_3$ addition during the first 6 days, but significant improvement ($p < 0.05$) could be discerned on the 8th day in the 1 g L^{-1} $NaHCO_3$ culture where 7.1 g L^{-1} biomass was achieved, which was 10% higher than the 0 and 0.2 g L^{-1} $NaHCO_3$ cultures (Fig. 4b). The final biomass productivity and CO_2 bio-fixation rate reached 0.82 g L^{-1} day^{-1} and 1.55 g L^{-1} day^{-1}, respectively, in the 1 g L^{-1} $NaHCO_3$ culture, which exhibited 11% improvement compared with the one without $NaHCO_3$ addition (Table 1).

The photosynthetic activity (F_v/F_m) showed almost identical profile in the cultures with 0, 0.2 and 1 g L^{-1} $NaHCO_3$ addition throughout the cultivation: It increased from 0.70 to 0.74–0.75 in the first 2 days when nitrate was replete, and then gradually decreased to approximately 0.63 on Day 8 with the depletion of nitrate (Fig. 4c). In addition, DIo/CS_0 and Car/Chl showed no significant difference either in these three cultures ($p > 0.05$, Table 3). The cell morphology also suggested the little difference among the cultures with 0–1 g L^{-1} $NaHCO_3$ addition (Additional file 1: Figure S3b). Collectively, it seemed that in this nitrogen-limitation batch culture mode, the addition of $NaHCO_3$ up to 1 g L^{-1} in the context of 2% CO_2 aeration exerted little impact on the photosynthesis and biomass production under the nitrogen-repletion phase and the sequential short-term (2 to 4 days) nitrogen-depletion phase. The beneficial effects of $NaHCO_3$ addition on biomass production only occurred in the 1 g L^{-1} culture in the extended nitrogen-depletion phase (6 days). These results were quite different from those obtained under initial nitrogen-depletion cultivation in *T. subcordiformis* where suitable $NaHCO_3$ addition (e.g., 1 g L^{-1}) led to prompt and remarkable improvements of biomass production (Fig. 1b). These findings were also different from those in *Chlorella* sp. HS2 where addition of $NaHCO_3$ in the context of 1% CO_2 aeration led to a significant improvement of biomass productivity and the effect was dose dependent in the range of 0–0.75 g L^{-1} $NaHCO_3$ [9]. Notably, the addition of 5 g L^{-1} $NaHCO_3$ led to a dramatic inhibition of biomass accumulation and photosynthetic activity (Fig. 4b, c), as was also reflected by the enhanced energy dissipation (DIo/CS_0) and decreased photoprotection (Car/Chl ratio) (Table 3) as well as aberrant cell morphology (Additional file 1: Figure S3b). This inhibition was much severer

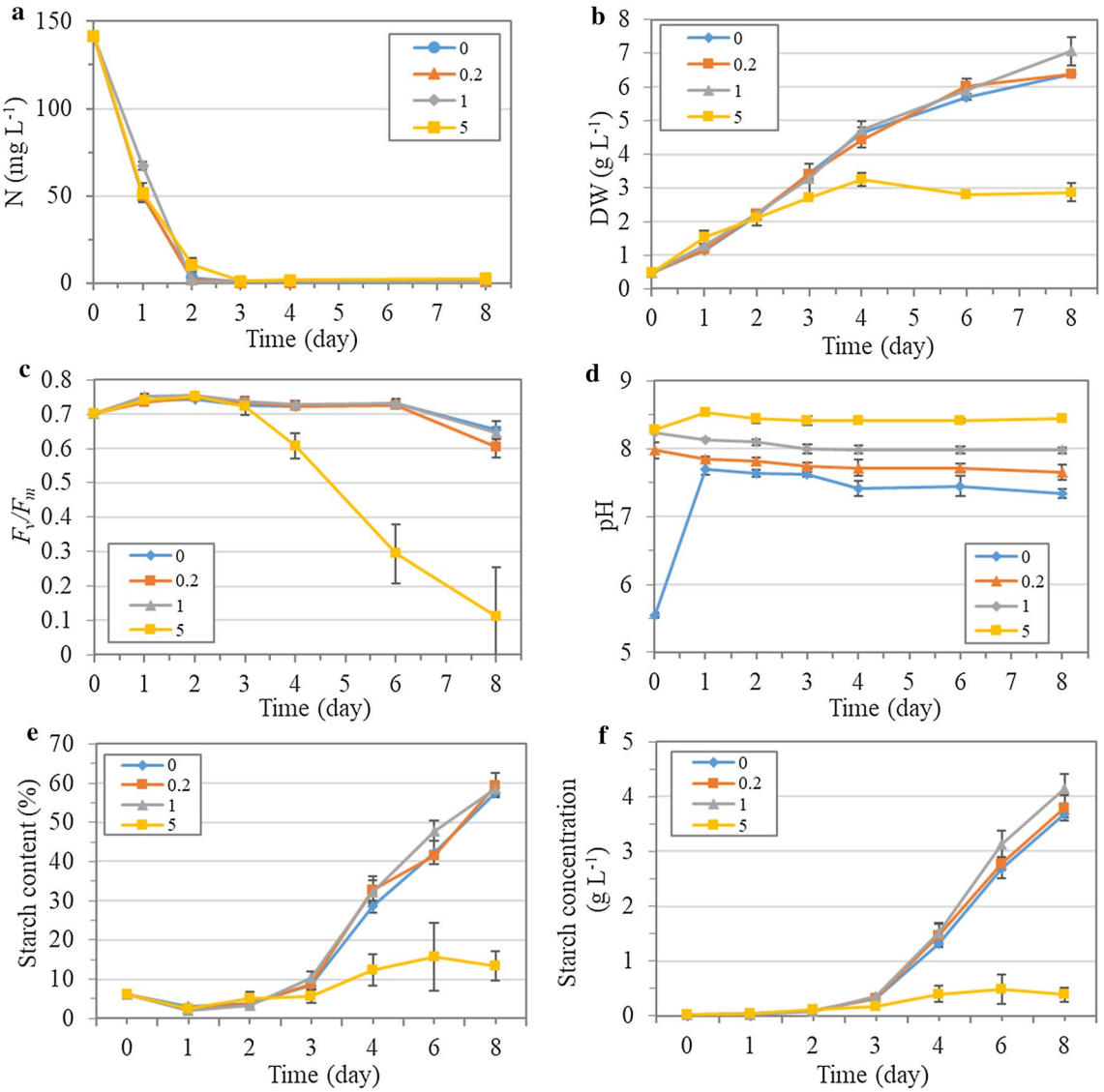

Fig. 4 Nitrate consumption (**a**), biomass production (dry weight, **b**), photosynthetic activity (F_v/F_m, **c**), pH variations (**d**), starch content (**e**) and starch concentration (**f**) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition (0, 0.2, 1 and 5 g L^{-1}) under nitrogen limitation (mean ± SD, $n = 3$)

than that under the initial nitrogen-depletion cultivation (Fig. 1b).

The pH of the medium under nitrogen limitation reached averagely 7.5, 7.7, 8.0 and 8.4 from Day 1 to Day 8 with 0, 0.2, 1 and 5 g L^{-1} $NaHCO_3$ addition, respectively (Fig. 4d), which were higher than the levels of the corresponding culture under nitrogen depletion (5.2, 6.7, 7.7 and 8.3, Fig. 1c), especially in the cultures with 0 and 0.2 g L^{-1} $NaHCO_3$ addition. The pH diversity caused by the different amounts of $NaHCO_3$ addition was also remarkably reduced (i.e., variation of 0.9 in nitrogen limitation vs. 3.1 in nitrogen depletion). Noteworthily, it was evident that the pH increased rapidly from 5.5 to 7.7 within 1 day in the culture without $NaHCO_3$ addition (0 g L^{-1}, Fig. 4d). This increase in pH could be attributed to the utilization of nitrate which led to an increase in alkalinity by releasing OH^- into the medium [17, 42, 43]. As a result, the increased alkalinity eliminated the potential acidification caused by the 2% CO_2 supply, making a favorable pH environment for biomass production. Meanwhile, the elevated pH reduced the dCO_2 and increased the HCO_3^- availability as discussed above, and thus, the high-CO_2 inhibition could be removed with simultaneously adequate effective carbon source

Table 3 Dissipated energy flux per excited cross section (DIo/CS_0), carotenoid/chlorophyll ratio (Car/Chl) and DIC (CO_2, HCO_3^- and CO_3^{2-}) concentrations in the medium of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition under nitrogen limitation (Day 8, mean ± SD, $n = 3$)

$NaHCO_3$ (g L^{-1})	DIo/CS_0	Car/Chl	CO_2 (mmol kgSW^{-1})			HCO_3^- (mmol kgSW^{-1})			CO_3^{2-} (mmol kgSW^{-1})		
			1 day	2 days	8 days	1 day	2 days	8 days	1 day	2 days	8 days
0	125 ± 22^{A}	0.301 ± 0.004^{AB}	0.03 ± 0.01	0.08 ± 0.03	0.11 ± 0.02	2.54 ± 0.07	4.84 ± 1.35	3.70 ± 0.03	0.17 ± 0.03	0.27 ± 0.04	0.11 ± 0.02
0.2	120 ± 14^{A}	0.302 ± 0.007^{AB}	0.03 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	2.90 ± 0.49	6.60 ± 0.20	4.87 ± 0.61	0.27 ± 0.05	0.57 ± 0.05	0.29 ± 0.09
1	95 ± 24^{A}	0.311 ± 0.006^{B}	0.03 ± 0.00	0.04 ± 0.02	0.07 ± 0.01	6.73 ± 0.30	8.36 ± 3.57	10.18 ± 0.73	1.24 ± 0.06	1.38 ± 0.53	1.28 ± 0.15
5	352 ± 87^{B}	0.295 ± 0.005^{A}	0.03 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	16.17 ± 2.30	24.74 ± 1.61	26.29 ± 2.86	8.36 ± 0.92	10.21 ± 1.35	10.76 ± 0.80

The different capital Latin alphabets (A and B) represented significant difference ($p < 0.05$) between the cultures

(HCO_3^-) becoming available. In fact, the HCO_3^- concentration reached 3.70 mmol $kgSW^{-1}$ during the 8-day cultivation and it accounted for 94% of the DIC in the culture with 0 g L^{-1} $NaHCO_3$ addition (Table 3, Additional file 1: Figure S1e), which could sufficiently support biomass accumulation. In a similar way, the elevated alkalization of medium in the cultures with 0.2, 1 and 5 g L^{-1} $NaHCO_3$ addition related to their nitrogen-depletion counterparts could also be attributed to the supply of nitrate. Therefore, due to the inherent alkalization nature of nitrate uptake process in microalgae, the combined supply of nitrate and CO_2 reduced the pH diversity and made the addition of $NaHCO_3$ less useful in promoting biomass production and CO_2 bio-fixation. It should be noted that the biomass production was almost identical in all the cultures before Day 2 and in the cultures with 0, 0.2 and 1 g L^{-1} $NaHCO_3$ addition before Day 6 (Fig. 4b) where the pH was at 7.4–8.5 (Fig. 4d), indicating that *T. subcordiformis* had a relatively broad suitable pH range. This result was inconsistent with other *Tetraselmis* species such as *T. suecica* [25] and *Tetraselmis* sp. [26] where biomass accumulation varied with the pH changing at 7.5–8.5. The insensitivity of *T. subcordiformis* toward pH at this range is preferable in large-scale cultivation since the biomass productivity would be less affected when exposed to pH variations, which needs less strict pH control.

Although nitrate supply minimized the pH-regulation effect of $NaHCO_3$ addition, the biomass production and CO_2 bio-fixation could still be facilitated with the addition of 1 g L^{-1} $NaHCO_3$ in the late phase (Fig. 4b). This beneficial effect might be ascribed to the relatively more abundant HCO_3^- in the medium as the effective carbon source. As shown in Table 3, the HCO_3^- concentration reached 10.18 mmol $kgSW^{-1}$ in the culture with the addition of 1 g L^{-1} $NaHCO_3$ on Day 8, which was 1.8- and 1.1-fold higher than that in the 0 and 0.2 g L^{-1} $NaHCO_3$ counterparts. The occurrence of this advantage only in the late phase of cultivation could be due to the more need of carbon source that 1 g L^{-1} $NaHCO_3$ addition was able to more easily meet when cell density reached a high level at that phase. However, excessive $NaHCO_3$ addition up to 5 g L^{-1} caused an overall inhibition on photosynthesis and algal biomass production (Fig. 4b, c), and the inhibitory effects were more remarkable compared with the nitrogen-depletion culture (Fig. 1b). The enhanced pH up to 8.5 was not the reason for this inhibition, as discussed above. It was obvious that the HCO_3^- increased up to 26.29 mmol $kgSW^{-1}$ until Day 8 (Table 3), which was comparable to that under nitrogen-depletion culture (Fig. 1e). Therefore, although high HCO_3^- concentration was unfavorable to biomass production here, it could not account for the elevated inhibitory effects in the nitrogen-limitation culture relative to the nitrogen-depletion one. It should be noted that CO_3^{2-} accounted for more than 29% of DIC in the culture with 5 g L^{-1} $NaHCO_3$ addition under nitrogen-limitation culture (Additional file 1: Figure S1h), and it reached more than 10 mmol $kgSW^{-1}$ on Day 2 (Table 3), which was 1.5 times of that under nitrogen-depletion culture in the same $NaHCO_3$ condition (Fig. 1f). Taken together, it could be speculated that high CO_3^{2-}, rather than HCO_3^- or pH, led to the severe inhibition of photosynthesis and biomass production under nitrogen-limitation culture. CO_3^{2-} is generally not a carbon source for microalgae due to the lack of membrane transportation system [13]. However, CO_3^{2-} had been found to act as a strong inhibitor to HCO_3^- assimilation in algae [44]. As a result, excessive CO_3^{2-} could give rise to carbon limitation, leading to the impaired photosynthesis and biomass production. The enhanced CO_3^{2-} could also be derived from the additional alkalization of medium as a consequence of nitrate uptake. The proportion of CO_3^{2-} in the total DIC was highly sensitive to pH variations in the range of 8–8.5 in seawater system (salinity of 36 kg m^{-3}), as demonstrated by Chen et al. [17]. The increase in pH from 8.2 in the nitrogen-depletion culture (Fig. 1c) to 8.4 in the nitrogen-limitation one (Fig. 4d) should cause a considerable enhancement of CO_3^{2-} concentration. Therefore, the nitrate supply aggravated the inhibitory effects of high $NaHCO_3$ addition.

Starch production and starch quality under nitrogen limitation

The starch accumulation occurred after the nitrate was exhausted on Day 2, and the final starch content reached approximately 58.6% on Day 8 in the cultures with 0, 0.2 and 1 g L^{-1} $NaHCO_3$ addition with no significant difference observed ($p > 0.05$), indicating that starch accumulation was unaffected with the addition of $NaHCO_3$ within this concentration range (Fig. 4e). It could be due to the relatively small variations in pH (7.5–8.0) in these cultures, as was found in *C. vulgaris* under a similar pH range [36]. As a result, the starch concentration exhibited a similar profile to biomass production where significant difference could only be discerned on the final day of cultivation. The final starch concentration and starch productivity reached 4.1 g L^{-1} and 0.51 g L^{-1} day^{-1}, respectively, in the culture with 1 g L^{-1} $NaHCO_3$ addition, which was 12% and 11% higher than the 0 g L^{-1} $NaHCO_3$ counterpart (Fig. 4f, Table 1). Like the case in the biomass production, the starch accumulation was severely inhibited in the culture with 5 g L^{-1} $NaHCO_3$ addition, with a maximum starch content of only 15.6%DW and starch concentration of 0.5 g L^{-1} obtained on Day 6 (Fig. 4e).

Table 4 Am/Ap ratio, and Am or Ap content (%DW) of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition under nitrogen-limitation (±N) cultivation modes (mean ± SD, $n = 3$)

$NaHCO_3$ ($g\ L^{-1}$)	0				0.2				1				5			
	2 days	3 days	4 days	8 days	2 days	3 days	4 days	8 days	2 days	3 days	4 days	8 days	2 days	3 days	4 days	8 days
Am/Ap	0.15 ± 0.03^{a}	0.57 ± 0.03^{a}	0.55 ± 0.16^{a}	0.36 ± 0.03^{b}	0.19 ± 0.03^{a}	0.70 ± 0.13^{a}	0.47 ± 0.04^{a}	0.36 ± 0.02^{b}	0.16 ± 0.02^{a}	0.68 ± 0.06^{a}	0.48 ± 0.05^{a}	0.38 ± 0.02^{b}	0.32 ± 0.13^{b}	0.78 ± 0.47^{a}	1.46 ± 0.03^{b}	0.23 ± 0.08^{a}
Am (%DW)	0.5 ± 0.2^{a}	3.1 ± 0.5^{ab}	10.0 ± 1.5^{ab}	15.4 ± 0.8^{b}	0.6 ± 0.0^{a}	3.6 ± 0.9^{b}	10.4 ± 0.5^{b}	15.9 ± 0.4^{b}	0.5 ± 0.2^{a}	4.1 ± 0.9^{b}	10.4 ± 0.3^{b}	16.2 ± 0.6^{b}	1.1 ± 0.2^{b}	2.2 ± 0.3^{a}	7.3 ± 2.4^{a}	2.6 ± 1.4^{a}
Ap (%DW)	3.4 ± 0.8^{a}	5.4 ± 0.6^{b}	18.6 ± 2.9^{b}	42.3 ± 1.4^{b}	3.2 ± 0.2^{a}	5.0 ± 0.3^{ab}	22.4 ± 2.8^{b}	43.6 ± 2.8^{b}	2.9 ± 0.9^{a}	6.0 ± 0.8^{b}	21.7 ± 2.9^{b}	42.3 ± 1.6^{b}	3.9 ± 1.8^{a}	3.5 ± 1.7^{a}	5.0 ± 1.7^{a}	10.7 ± 2.4^{a}

The different letters (a, b, c and d) represented significant difference ($p < 0.05$) between the cultures on the same cultivation day

Interestingly, the Am/Ap showed an increase from 0.17 on Day 2 to 0.65 on Day 3 and gradually decreased to 0.37 on Day 8 during the starch accumulation phase in the cultures with 0, 0.2 and 1 g L^{-1} $NaHCO_3$ addition without any significant difference ($p > 0.05$, Table 4), which was quite different from the case in the nitrogen-depletion cultures where Am/Ap exhibited a continuous increase and $NaHCO_3$ addition accelerated this increase (Table 2). The transient increase in Am/Ap at the start of nitrogen depletion coincided with the findings in the initial nitrogen-depletion culture (Table 2) and other microalgae such as *C. reinhardtii* 137C [37]. However, the subsequent decrease in Am/Ap was unanticipated. The most probable reason was the enhanced cell density in the nitrogen-limitation culture (3.4–7.1 g L^{-1} biomass from Day 3 to Day 8, Fig. 4b) relative to the nitrogen-depletion one (maximum of 2.6 g L^{-1} biomass on Day 3, Fig. 1b) that caused a decreased light availability because of the self-shading effects [6]. In fact, the stimulation of GBSS was shown to be light dependent [39], and low light intensity resulted in decreased GBSS activity and relative amylose content in rice [45]. Therefore, in the nitrogen-limitation culture mode, the enhanced biomass production was unfavorable for amylose production in *T. subcordiformis*. The disappeared stimulation effects of $NaHCO_3$ addition on amylose production might be due to the reduced diversity of pH and HCO_3^- concentration among the cultures with different $NaHCO_3$ addition that stemmed from the nitrate uptake (as discussed above). Unexpectedly, the Am/Ap increased dramatically from 0.32 on Day 2 to 1.46 (Am: 59.3%TS, Table 1) on Day 4 in the culture with 5 g L^{-1} $NaHCO_3$ addition (Table 4), although a weak overall starch accumulation (5.0%DW to 12.3%DW) could be observed (Fig. 4e). It was evident that the Am content increased by 5.6 times during this period, whereas the Ap content increased by only 28% (Table 4), which indicated that Ap accumulation was largely inhibited under this high-$NaHCO_3$ environment, which generated a relatively high Am/Ap.

Choice of cultivation strategy for different purposes

The present study demonstrated that the addition of 1 g L^{-1} $NaHCO_3$ in the context of 2% CO_2 aeration was preferable under both the nitrogen-depletion (−N) and nitrogen-limitation (±N) cultivation modes in terms of biomass production, CO_2 bio-fixation and overall starch production. The biomass productivity of around 0.86 g L^{-1} day^{-1} and starch content of 58.3%DW were almost the same under these two cultivation modes, and they exceeded most of the photoautotrophic microalgae under nutrient depletion (Table 5). The highest starch productivity of 0.73 g L^{-1} day^{-1} was obtained in the −N culture on Day 2, which was comparable to that in *Chlorella* sp. AE10 [8, 46], the best microalgal starch producer hitherto to the best of our knowledge, under photoautotrophic conditions in nitrogen depletion conditions, and the starch concentration of 1.52 g L^{-1} in *T. subcordiformis* was even higher, although biomass productivity and starch content were slightly lower (Table 5). These results demonstrated *T. subcordiformis* to be a good candidate for photosynthetic CO_2 bio-fixation and starch production.

Considering that the biomass production was insensitive to $NaHCO_3$ addition (Fig. 4b), the following cultivation strategy including a sequential transformation from nitrogen repletion (+N) to −N (+N → −N) can be proposed (Fig. 5a): The algae are first inoculated with low cell density (0.5 g L^{-1}) under +N (10 mM nitrate) without $NaHCO_3$ addition; after 3 days when nitrogen is completely exhausted, the algae are diluted to the initial cell density (0.5 g L^{-1}) as under +N but with nitrogen-free medium containing 1 g L^{-1} $NaHCO_3$ for starch production. The present study demonstrated that −N along with $NaHCO_3$ addition could not only improve the total starch production, but also enhance the amylose accumulation. The amylose content (19.1%DW) and amylose concentration (449 mg L^{-1}) under −N on Day 2 in the culture with the addition of 1 g L^{-1} $NaHCO_3$ (Tables 1 and 2) were 2.4- to 3.5-fold of those obtained in *Chlorella sorokiniana* [41]. The amylose content in total starch reached 33%, which was comparable to *C. reinhardtii* under nitrogen deprivation with mixotrophic cultivations, or 18% higher than *Chlorella* with photoautotrophic cultivations [37, 40]. This amylose level was even higher than most of the starch from native cereal crops where amylose accounts for about 15–32% of storage starch [47]. Amylose, which is less branched and has high gelatinization temperatures than amylopectin, has been regarded as excellent food ingredients [48]. More importantly, it has been demonstrated to be resistant to digestion and therefore is regarded as one of the contributors to resistant starch that functions for the prevention and control of colon cancer, diabetes and obesity [49]. These potential high-valued applications of amylose will contribute to the economic viability of starch-based biofuel (e.g., fermentation for liquid fuels) production. Therefore, the +N → −N cultivation strategy (two-stage mode) could be more promising from the biorefinery perspective (Fig. 5a).

For ±N cultivation, the most prominent feature was the high starch concentration (4.14 g L^{-1}). Compared with other microalgae reported, it was one of the highest levels among the cultures under ±N cultivation strategy (Table 5). Notably, the ±N cultivation led to a relatively low amylose content with finally 27.7%TS achieved (Table 1). This character, in contrast to the −N cultivation mode, should be more advantageous for starch-based

Table 5 Biomass and carbohydrate (starch, glycogen or total carbohydrate) production in microalgae under different carbon sources and nutrient stress conditions reported in literatures

Strain	Carbon source			Nutrient stress	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	Starch concentration ($g\ L^{-1}$)	Starch productivity ($g\ L^{-1}\ day^{-1}$)	Starch content (%DW)	References
	CO_2 (%)	$NaHCO_3$ ($g\ L^{-1}$)	Organic carbon						
Chlorella sorokiniana	2	0	0	−N	0.45 (2[a])	–[b]	0.17 (2)	38 (2)	[18]
Chlamydomonas reinhardtii	0.04	4.2	0	−N	–	0.79 (4)	0.18 (4)	69.3 (4)	[64]
	0.04	0	0	−N	–	0.04 (4)	–	7.3 (4)	
	5	0	0	−N	–	0.06 (4)	–	12.5 (4)	
Chlorella vulgaris CCALA 924	2	0	0	−N	0.23 (0.5)	0.10 (0.5)	0.19 (0.5)	37 (0.5)	[6]
	2	0	Urea (1.1 $g\ L^{-1}$)	−P	0.75 (0.75)	0.35 (0.75)	0.48 (0.75)	53 (0.75)	
	2	0	Urea (1.1 $g\ L^{-1}$)	−S	1.10 (0.83)	0.62 (0.83)	0.74 (0.83)	60 (0.83)	
Chlorella sp. AE10	10	0.016	0	−N	1.20 (2)	1.42 (2)	0.71 (2)	56.9 (2)	[46]
				−N (0.375 mM)	0.95 (2)	1.21 (2)	0.73 (2)	60.5 (2)	[8]
Scenedesmus obliquus CNW-N	2.5	0	0	±N (4 mM)	0.55	1.88[c] (7)	0.27[c] (7)	49.4[c] (7)	[65]
Synechococcus sp. PCC 7002	2	168	0	±N (15 mM)	1.0 (7)	3.5[d] (7)	0.50[d] (7)	49.8[d] (7)	[66]
Arthrospira platensis	0.04	16.8	0	±N (3 mM)	0.46 (3.5)	1.03[d] (3.5)	0.29[d] (3.5)	65[d] (3.5)	[67]
Tetraselmis subcordiformis	2	1	0	−N	0.89 (2)	1.52 (2)	0.73 (2)	58.1 (2)	This study
	2	1	0	±N (10 mM)	0.82 (8)	4.14 (8)	0.51 (8)	58.6 (8)	

[a] The number in the parentheses represented the cultivation day used for calculation and comparison

[b] Data unavailable

[c] Data represented the total carbohydrate

[d] Data represented the glycogen from cyanobacteria

biofuel generation because the lower amylose content in microalgal starch had been shown to have higher enzymatic hydrolysis efficiency for glucose release [41], which could improve the carbon utilization efficiency in the subsequent fermentation process. Therefore, it is more advisable to employ the ±N cultivation strategy (batch mode, Fig. 5b) where limited nitrate (10 mM) along with 1 g L^{-1} $NaHCO_3$ is supplied if fermentation efficiency was the primary target.

Moreover, the unexpected high amylose content of 59.3%TS was also achieved in the culture with 5 g L^{-1} $NaHCO_3$ addition under ±N cultivation mode (Table 1, Fig. 5c). This type of starch could be considered as high-amylose starch (more than 50% amylose), which could absolutely serve as a functional food for providing slowly digestible and resistant starch to reduce the glycaemia level in the human body [48, 50]. The present study exhibited the potential of producing high-amylose starch in microalgae simply through the manipulation of cultivation conditions, although at present it could only be achieved at the expense of the overall starch productivity (Table 1). Currently, the high-amylose starches are largely produced from the genetically modified (including transgenic or non-transgenic) cereal crops [47, 51], which may raise GMO issues for food. The production of high-amylose starch from microalga *T. subcordiformis* here had initiated a novel, simple and safe way, which needs further optimization.

Preliminary techno-economic assessment of different bicarbonate and nitrogen supply strategies

To have a clearer picture of the economic potential of these different $NaHCO_3$ and nitrogen supply strategies, the costs ($\$\ kg^{-1}$ biomass) derived from the carbon source (CO_2 and $NaHCO_3$) and nitrogen source (KNO_3) were evaluated. In addition, the biomass value ($\$\ kg^{-1}$ biomass) based on the starch quality (i.e., amylose percentage of total starch) was assessed as well. A parameter, economic index (EI) which estimated the biomass value per unit of carbon and nitrogen costs herein, was

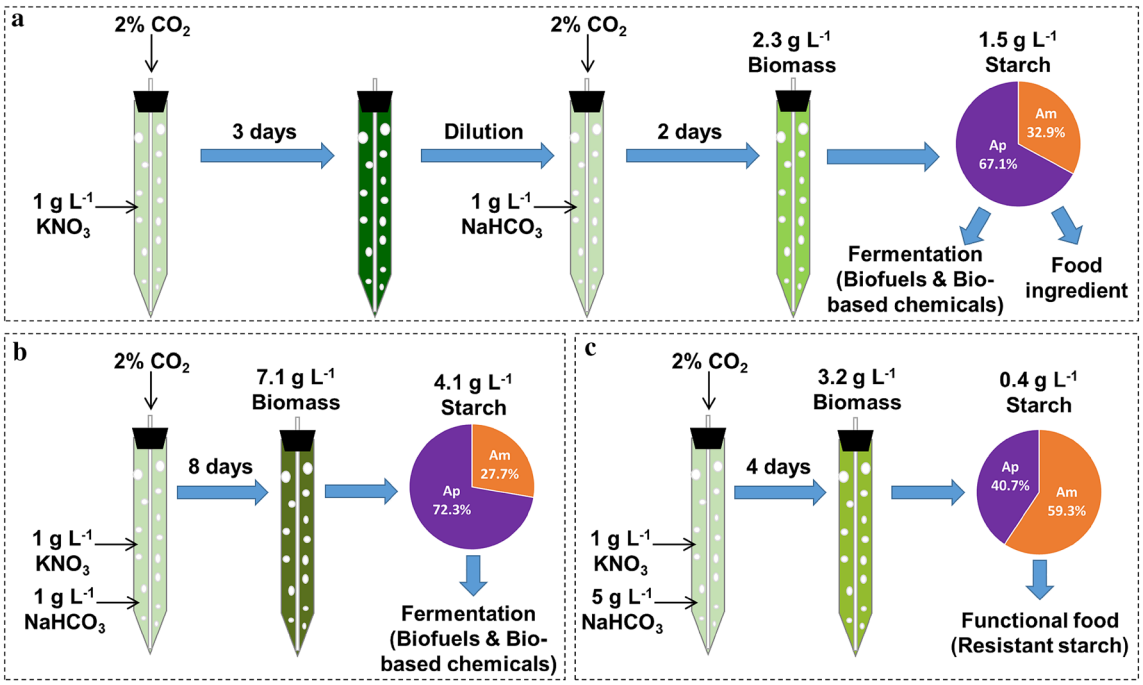

Fig. 5 Proposed cultivation strategies for starch production in *T. subcordiformis* with diverse applications. The +N → −N strategy (**a**) comprised a +N (1 g L^{-1} KNO_3) without $NaHCO_3$ addition in the first stage and a dilution for −N with 1 g L^{-1} $NaHCO_3$ addition in the second stage for short-term cultivation (2 days). The ±N strategy employed a simultaneous supply of 1 g L^{-1} KNO_3 and 1 g L^{-1} $NaHCO_3$ (**b**) or 5 g L^{-1} $NaHCO_3$ (**c**) for long- or medium-term cultivation (8 or 4 days, respectively)

introduced to partially reveal the economy. As shown in Table 6, under −N strategy, the cost of carbon and nitrogen source to produce 1 kg biomass was reduced by 62%, 57% and 5%, respectively, when supplying 0.2, 1 and 5 g L^{-1} of $NaHCO_3$ compared with the culture without $NaHCO_3$ addition, probably due to the significant improved biomass productivity. In the meantime, the biomass value in terms of starch contained increased by 1.6-, 2.8- and 2.0-fold, respectively. As a consequence, the EIs obtained by adding $NaHCO_3$ were 3.2- to 8.9-fold of that without $NaHCO_3$ addition, demonstrating the considerable improvements in economy. For ±N strategy, the cost of carbon and nitrogen source was reduced by only 4% in the culture with 1 g L^{-1} of $NaHCO_3$ addition, and it even nearly doubled with the addition of 5 g L^{-1} of $NaHCO_3$ (Table 6) due to the declined biomass productivity compared with the ±N culture without $NaHCO_3$ addition (Table 1). However, in contrast, the biomass value was enhanced by 4.7-fold in the 5 g L^{-1} $NaHCO_3$ culture because of the substantial increase in amylose percentage from 26.7%TS to 59.3%TS, which consequently promoted the EI by almost twice relative to the 0 g L^{-1} $NaHCO_3$ one (Table 6). It thus exemplified the great contribution of producing high-valued starch (high-amylose starch) to the economy of microalgal starch production. Collectively, these results further highlighted the beneficial effects of adding $NaHCO_3$ on the economic feasibility of starch production in *T. subcordiformis* under both −N and ±N cultivation modes.

Comparing the two cultivation modes, it seemed that the addition of $NaHCO_3$ under −N was generally more favorable than the ±N mode from the economic perspective. Particularly, the highest EI (1.72) was obtained under −N with the addition of 1 g L^{-1} $NaHCO_3$, which should be mainly ascribed to the relatively low nutrient (carbon and nitrogen source) cost due to the high biomass productivity (Tables 1 and 6). It is believed that improvement of photosynthetic efficiency to get enhanced productivity is the key to reduce the cost and promote economic viability of large-scale microalgal biomass production [52, 53]. In addition, the higher starch quality (32.9%TS of amylose with 1 g L^{-1} $NaHCO_3$) under −N relative to the ±N counterparts (~27%TS of amylose) further enhanced the economic viability. Moreover, the −N cultivation mode involved much less cultivation time (2 days) than the ±N one (generally 8 days), which could significantly reduce the probability of being contaminated or preyed in large-scale cultivation [54]. However, the biomass concentration in −N culture with 1 g L^{-1} $NaHCO_3$ (2.2 g L^{-1}) was 67% lower than the ±N

Table 6 The estimation of the cost required for carbon and nitrogen source used for 1 kg biomass production of *T. subcordiformis* and the potential value of biomass based on different types of starch with different amounts of $NaHCO_3$ addition under nitrogen-depletion (−N) and nitrogen-limitation (±N) cultivation modes

Cultivation mode	$NaHCO_3$ (g L^{-1})	KNO_3 (g L^{-1})	Culture time (day)	Starch (%DW)	Am (%TS)	Estimated starch price ($ kg^{-1})	Culture volume needed (L kg^{-1} biomass)	Cost of carbon and nitrogen source ($ kg^{-1} biomass)	Potential value based on starch ($ kg^{-1} biomass)	EI [biomass value/ (C+N source cost)]
−N	0	0	2	47.9	27.8	0.43	1724	1.07	0.21	0.19
	0.2	0	2	52.4	30.9	1.01	617	0.41	0.53	1.30
	1	0	2	58.1	32.9	1.37	562	0.46	0.80	1.72
	5	0	2	37.4	34.5	1.67	625	1.01	0.62	0.62
±N	0	1.01	8	57.7	26.7	0.23	169	0.58	0.13	0.23
	0.2	1.01	8	59.5	26.7	0.24	169	0.58	0.14	0.24
	1	1.01	8	58.5	27.7	0.42	152	0.55	0.24	0.44
	5	1.01	4	12.3	59.3	6.23	362	1.15	0.77	0.67

The economic index (EI) estimated from the biomass value per carbon and nitrogen cost was introduced to evaluate the relative economic viability for scale-up

counterpart (7.1 g L^{-1}) (Fig. 5), which could enhance the cost of downstream processing, especially harvesting [55]. Nevertheless, the starch-enriched *T. subcordiformis* under nitrogen depletion conditions was in fact very apt to settle (unpublished data), which should largely reduce the harvesting cost [56] and minimize the negative impact on the relative scalability under −N cultivation mode. Overall, the −N cultivation with 1 g L^{-1} $NaHCO_3$ addition could have the best scalability among all the conditions tested.

The present study demonstrated the potential of regulating amylose accumulation by acting on simple cultivation parameters such as $NaHCO_3$ addition and nitrogen supply. Of particular interest was the production of high amylose because of its high value that could contribute to the economic feasibility of the microalgae cultivation and biorefinery, as analyzed above. However, the biomass productivity and total starch content were relatively low (Table 1, Fig. 4e). The cultivation conditions should be further optimized to get as much biomass and starch as possible to improve the scalability.

Conclusions

The CO_2–bicarbonate system was crucial to ensure a suitable pH, alleviate the high-dCO_2 inhibition, and provide sufficient effective carbon source HCO_3^- for the maintenance of adequate photosynthetic efficiency and increase in photoprotection to get improved biomass and starch production as well as enhanced relative amylose content in the microalga *T. subcordiformis* under nitrogen-depletion cultivation. The biomass productivity was enhanced by 2.1-fold, and the starch productivity and concentration were both improved by more than 1.5-fold in the culture with the addition of 1 g L^{-1} $NaHCO_3$ compared with the one without $NaHCO_3$ addition in the context of 2% CO_2 aeration. The amylose content was also increased by 44% under this CO_2–bicarbonate system compared to the single use of CO_2. The +N → −N cultivation strategy (two-stage mode) could achieve high starch productivity with enhanced amylose content that was suitable for both biofuel and high-valued food production in a biorefinery scenario, whereas ±N cultivation strategy (batch mode) could get a high starch concentration and low amylose content that was promising for biofuel generation via fermentation. High-amylose starch could be produced via the addition of 5 g L^{-1} $NaHCO_3$ under ±N cultivation mode in *T. subcordiformis*, which represented a new way for the production of starch-based functional food. Considering the relatively high biomass and starch productivity as well as amylose content in *T. subcordiformis*, it could be anticipated that this excellent starch-producing microalga, as a potential substitute for agricultural crops, would play an important role in the CO_2 mitigation for the biofuel, bio-based chemicals and functional food generation in the future.

Methods

Algal strain and culture condition

Tetraselmis subcordiformis FACHB-1751 was isolated from the Huanghai Sea near Dalian, Liaoning Province, China, and maintained by the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB collection), Chinese Academy of Sciences. The microalgae were previously cultivated in artificial seawater (ASW) [5] with extra additions of 0.81 g L^{-1} Tris and 0.33 mL L^{-1} glacial acetic acid. Algal cells were harvested during the late exponential phase and washed twice with nitrogen-free artificial seawater (ASW-N) where nitrate was eliminated. For nitrogen-depletion (−N) cultivation, the washed cells were inoculated with $OD_{750}=0.6$ in ASW-N with the addition of $NaHCO_3$ to final concentrations of 0, 0.2, 1 and 5 g L^{-1}, respectively. For nitrogen-limitation (±N) cultivation, an extra of 10 mM KNO_3 was added into the medium above.

The cells were cultivated photoautotrophically in a 600-mL glass bubble column photobioreactor (50 mm diameter, 400 mm height) with a working volume of 500 mL as descried by Yao et al. [23]. An aeration of 0.4 vvm with air containing 2% CO_2 at 25 ± 2 °C was applied to the cultures. Continuous illumination from one side with cool white fluorescent lamps that provided an incident light intensity of 150 μmol m^{-1} s^{-1} was supplied. All the experiments were done in three biological replicates.

pH and growth measurement

Medium pH was measured using a standard bench top pH meter (ARK, pHS-4C$^+$, Sichuan, China). The cell growth was determined as the optical density of the culture at 750 nm on a spectrophotometer (AOE, UV/Vis A-360, Shanghai, China). The cell dry weight (DW, g L^{-1}) was determined gravimetrically according to Yao et al. [23]. Biomass productivity (P_b, g L^{-1} day^{-1}) was calculated as follows:

$$P_b = \frac{DW_t - DW_0}{t} \quad (1)$$

where DW_t and DW_0 are the cell dry weight at culture times t and 0, respectively.

Photosynthetic performance analysis

The photosynthetic performance with regard to the photosystem II (PS II) maximum photochemical efficiency (F_v/F_m) and dissipated energy flux per excited cross section (at $t=0$) (DIo/CS_0) were evaluated with chlorophyll a fluorescence determined using a chlorophyll

fluorometer Os30p$^+$ (Opti-sciences, USA). The parameters, F_v/F_m and DIo/CS$_0$, were calculated according to Strasser et al. [30] as follows:

$$F_v/F_m = (F_m - F_0)/F_m \quad (2)$$

$$\mathrm{DIo/CS}_0 = F_0(1 - F_v/F_m) \quad (3)$$

where F_v represents the variation of chlorophyll fluorescence between maximal fluorescence (F_m) induced by saturating pulse and initial fluorescence (F_0).

Estimates of dissolved inorganic carbon species and nitrate

Dissolved inorganic carbon (DIC) species [dissolved carbon dioxide (CO_2 (*aq*)), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-})] were calculated using the software CO_2calc [57]. The input data included: total alkalinity (TA), pH, temperature (T), pressure (P), and salinity (S). Total alkalinity (TA) was determined by acid–base titration in a seawater system according to Dickson et al. [58]. Salinity was estimated according to the composition of medium considering the amount of sodium bicarbonate and potassium nitrate under different systems, with the values ranging at 4.19–4.69. The CO_2 constants were taken from Millero [59] based on seawater scale. The nitrate concentration in the medium was tracked using an optical method described by Chi et al. [60].

Biochemical composition analysis

The pigments were extracted from a 1–5 mg algal pellet by ethanol and measured as described by Yao et al. [7]. The starch was extracted from alga by 30% perchloric acid and measured by sulfuric acid–anthrone method [23]. Starch productivity (P_s, g L^{-1} day^{-1}) was calculated as follows:

$$P_s = \frac{\mathrm{DW}_t C_{st} - \mathrm{DW}_0 C_{s0}}{t} \quad (4)$$

where C_{st} and C_{s0} are the starch content at culture times t and 0, respectively.

Amylose/amylopectin ratio (Am/Ap) was determined according to Hovenkamp-Hermelink et al. [61] with minor modifications. Briefly, the starch was extracted from alga by 30% perchloric acid. After staining with a diluted (1:2, v/v) Lugol's 1_2–KI solution, absorbancies at 618 and 550 nm were measured. The Am/Ap was estimated from the ratio of the absorbancies by a graph in which the specific absorptions of the two compounds were introduced. Amylose content in total starch ($C_{am/ts}$, %TS), amylose content in dry weight ($C_{am/dw}$, %DW), amylose concentration (C_{am}, mg L^{-1}) and amylose productivity (P_{am}, mg L^{-1} day^{-1}) were calculated using the following equations:

$$C_{am/ts} = \frac{1}{1 + 1/(\mathrm{Am/Ap})} \quad (5)$$

$$C_{am/dw} = C_{am/ts} \times C_s \times 100 \quad (6)$$

$$C_{am} = \mathrm{DW} \times C_{am/dw}/100 \times 1000 \quad (7)$$

$$P_{am} = \frac{C_{am(t)} - C_{am(0)}}{t} \quad (8)$$

Preliminary techno-economic assessment of different bicarbonate and nitrogen supply strategies

The cost of carbon source (CO_2 and $NaHCO_3$) and nitrogen source (KNO_3) to produce 1 kg of biomass at laboratory production scale was calculated according to Nayak [9]. Culture volume needed to produce 1 kg of biomass (V, L kg^{-1} biomass) was calculated as follows:

$$V = \frac{1000}{P_s \times t} \quad (9)$$

The amounts of CO_2 consumed for 1 L culture (Q_{CO_2}, kg L^{-1}) was calculated according to the following equation:

$$Q_{CO_2} = \frac{W_{CO_2} \times F \times \mathrm{Mr}_{CO_2} \times 60 \times 24 \times t}{24.5 \times 1000} \quad (10)$$

where W_{CO_2} (%) was the CO_2 concentration in the air (2%), F (vvm) was the aeration rate (0.4 vvm), Mr_{CO_2} (g mol^{-1}) was the relative molecular mass of CO_2 (44 g mol^{-1}) and 24.5 was the molar volume of gas (L mol^{-1}) at 25 °C (298.15 K). The cost of carbon source and nitrogen source for 1 kg of biomass (C_{C+N}, \$ kg^{-1}) was calculated according to the following equation:

$$C_{C+N} = (P_{CO_2} \times Q_{CO_2} + P_{NaHCO_3} \times Q_{NaHCO_3} + P_{KNO_3} \times Q_{KNO_3}) \times V \quad (11)$$

where P_{CO_2}, P_{NaHCO_3} and P_{KNO_3} represented the price of CO_2 (0.015 \$ kg^{-1} [9]), $NaHCO_3$ (0.2 \$ kg^{-1} [9]) and KNO_3 (0.93 \$ kg^{-1} [62]), respectively, and Q_{NaHCO_3} and Q_{KNO_3} represented the amounts of $NaHCO_3$ and KNO_3 consumed for 1 L culture (kg L^{-1}), respectively. The biomass value (V_b, \$ kg^{-1} biomass) based on starch quality (i.e., amylose percentage of total starch) was assessed as follows:

$$V_b = P_{sta} \times C_{st} \quad (12)$$

where P_{sta} was the price of starch (\$ kg^{-1} starch). Since P_{sta} is significantly affected by Am/Ap [63], it was assumed that P_{sta} was proportional to the amylose percentage of total starch ($C_{am/ts}$, %TS). The P_{sta} was then estimated by extrapolation from the price of normal starch (0.29 \$ kg^{-1} for $C_{am/ts}$ of 27%TS) and high-amylose

starch (6.35 \$ kg^{-1} for $C_{am/ts}$ of 60%TS) according to the $C_{am/ts}$ obtained under different cultivation conditions (Table 1). The economic index (EI) was defined as the biomass value per unit of carbon and nitrogen costs to partially reveal the economy:

$$EI = V_b / C_{C+N} \quad (13)$$

Statistical analysis

Results are expressed as mean ± SD from three independent experiments. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) and Fisher's LSD. Values of $p < 0.05$ were defined as statistically significant.

Additional file

Additional file 1: Figure S1. The DIC species distribution of *T. subcordiformis* cultures. **Figure S2.** The total alkalinity (TA) of *T. subcordiformis* cultures. **Figure S3.** Cell morphology of *T. subcordiformis* cultures with different amounts of $NaHCO_3$ addition under nitrogen depletion and nitrogen limitation.

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Authors' contributions

CY designed the project, analyzed the data and drafted the manuscript. MQ and BS conducted the cultivation and biochemical analysis; MQ analyzed the data and drafted the manuscript; QF and B L performed the techno-economic assessment; WR and QX carried out the cultivation. XC, YZ and XL revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file 1.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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