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Ultrahigh-cell-density heterotrophic cultivation of the unicellular green microalga *Scenedesmus acuminatus* and application of the cells to photoautotrophic culture enhance biomass and lipid production

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Abstract

Despite production of biodiesel from microalgae is proved to be technically feasible, a commercially viable system has yet to emerge. High-cell-density fermentation of microalgae can be coupled with photoautotrophic cultivation to produce oils. In this study, through optimizing culturing conditions and employing a sophisticated substrate feed control strategy, ultra-high-cell-density of 286 and 283.5 g L^{-1} was achieved for the unicellular alga *Scenedesmus acuminatus* grown in 7.5 L bench-scale and 1,000 L pilot-scale fermenters, respectively. The outdoor scale-up experiments indicated that heterotrophically-grown *S. acuminatus* cells are more productive in terms of both biomass and lipid accumulation when they are inoculated in photobioreactors for lipid production as compared to the cells originally grown under photoautotrophic conditions. Techno-economic analysis based on the pilot-scale data indicated the cost of heterotrophic culture of

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microalgae for biomass production is comparable with that of the open pond system and much lower than that of tubular PBR, if the biomass yield was higher than 200 g L^{-1}. This study demonstrated the economic viability of heterotrophic cultivation on large-scale microalgal inocula production, but ultra-high-productivity fermentation is prerequisite. Moreover, the advantages of the combined heterotrophic and photoautotrophic cultivation of microalgae for biofuels production were also verified in pilot-scale.

Keywords high-cell-density fermentation, heterotrophy, lipid, microalgae, *Scenedesmus acuminatus*

1. **Introduction**

Microalgae is a promising biomass feedstock for renewable energy production (Hu *et al*., 2008). The biodiesel derived from the oil-enriched microalgal biomass has the potential to meet the increasing global demand for transport fuels by displacing fossil diesels (Chisti, 2007; Chisti, 2008). Despite the production of biodiesel from microalgae is proved to be technically feasible, a scalable and commercially viable system has yet to be developed (Han et al., 2012; Uduman et al., 2010).

Many microalgae can utilize organic carbon for cellular growth in dark. This feature enables growing algae in fermentor to produce biomass and bio-products. Commercial production of *Chlorella* and *Crypthecodinium* for nutritional supplements docosahexaenoic acid (DHA), has been attained in industrial-scale fermenters, respectively (Barclay et al., 2013). Recently, attempts to further extend the commercial potential of heterotrophic *Chlorella* have been focused on

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producing biodiesels (Miao and Wu, 2004; Xiong et al., 2008). Fermentation offers many advantages in terms of biomass production, including conferring higher cell growth rate, better control of culture conditions and less chance of microbial contamination as compared to photoautotrophic cultivation. Thus, a cultivation strategy combining heterotrophic and photoautotrophic culture modes, which takes advantages of both high efficiency of the former one in biomass production and low cost of the latter in oil production, has been proposed and employed for various algal species with the aim to produce biofuels (Fan et al., 2012; Han et al., 2012; Zheng et al., 2012). Although the coupled heterotrophic and photoautotrophic cultivation mode has been studied for many microalgae at different scales, its economic viability remains to be assessed, especially when utilized for large-scale biofuels production.

Fermentation, a costly and energy-intensive process, will become economically feasible only if ultra-high cell density can be achieved. To date, the reported maximum biomass concentration/productivity of most oleaginous microalgae under heterotrophic culture are still not competitive as compared to other industrial microorganisms (e.g., bacteria or yeast). Besides the inherent longer doubling time of microalgae, the lack of effective growth conditions optimization or process controlling means limited their full growth potential. On the other hand, it remains elusive regarding whether heterotrophically-grown algal cells could adapt themselves to the environmental conditions used for inducing lipid production, such as high-light and nitrogen depletion conditions after shifting from heterotrophic to photoautotrophic cultivation mode.

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A *Scenedesmus acuminatus* strain that shows potential in heterotrophic growth and lipid production was obtained in a previous study (Wang et al., 2014). Though this *S. acuminatus* strain accumulated little lipid under heterotrophic conditions, the heterotrophically-grown cells can be used as inoculum for lipid production under photoautotrophic conditions. Thus, this study aimed to improve the biomass production by optimizing heterotrophic culture conditions and process control means, and to compare their capabilities in lipid production with photoautotrophically-grown cells in pilot-scale. To evaluate the economic feasibility of heterotrophic cultivation, the techno-economic (TE) analysis for both heterotrophic and photoautotrophic processes were conducted by utilizing the pilot-scale experimental data. Based upon the results of TE analysis, this study demonstrated the economic viability of the ultra-high-cell-density fermentation in the entire chain of algal biofuels production.

2. **Materials and Methods**

2.1 Algal strain and growth conditions

The green algae *Scenedesmus acuminatus* GT-2 was isolated from South Lake of Guangzhou, China. Algal cells were maintained in a modified Endo growth medium, containing glucose 30 g L^{-1}, KNO_3 3 g L^{-1}, KH_2PO_4 1.2 g L^{-1}, $MgSO_4{\bullet}7H_2O$ 1.2 g L^{-1}, trisodium citrate 0.2 g L^{-1}, $FeSO_4{\bullet}7H_2O$: 0.016 g L^{-1}, EDTA-Na_2 2.1 mg L^{-1}, $CaCl_2{\bullet}2H_2O$ 0.03 g L^{-1}, H_3BO_3 2.86 mg L^{-1}, $ZnSO_4{\bullet}7H_2O$ 0.222 mg L^{-1}, $MnCl_2{\bullet}4H_2O$ 1.81 mg L^{-1}, $NaMoO_4$ 0.021 mg L^{-1}, $CuSO_4{\bullet}2H_2O$ 0.07 mg L^{-1}. To prepare the inoculants for fermentation, a single colony of *S. acuminatus* GT-2 was inoculated into 100 mL of modified Endo medium in a 250

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Erlenmeyer flask and grown at 30 ^{o}C for 5-6 days in a shaking incubator at the speed of 180 rpm, which was then used as inoculum for fermentation.

Bench-scale fermentation experiments were performed in a 7.5 L bioreactor (BIOFLO & CELLIGEN 310, New Brunswick, USA) with the initial working volume of 2.8 L. The pH was maintained automatically by the addition of 3 M NaOH or 1 M HCl solutions. Aeration was maintained at 1 vvm with the airflow rate of 2.8 L min^{-1}. Dissolved oxygen (DO) was controlled automatically above 40% by coupling with the stirring speed. In fermentor batch medium, KNO_3 was replaced by 0.84 g L^{-1} urea. Feeding medium used during fermentation process was the 25-fold concentrated batch medium, containing 750 g L^{-1} of glucose.

Pilot-scale fermentation was carried out in a 1,000 L stirred tank bioreactor (WKT 1000L, YANGZHONG WEIKETE BIOLOGICAL ENGINEERING EQUIPMENT CO., LTD, China) containing 300 L medium. To shorten the culture period, the 1,000-L pilot-scale fermentation was inoculated with 40 L high-cell-density culture (80 g L^{-1}) from 100-L bioreactor after four days' fed-batch cultivation, which was inoculated from 7.5 L bench-scale fermentor. The initial biomass concentration in 1000-L fermentor cultivation was approximately 10 g L^{-1}. For 1000-L pilot-scale fermentation, the aeration rate and the agitation speed were initially set at 20 m^{3} h^{-1} (1 vvm) and 80 rpm, respectively. The pressure of the inner bioreactor was kept at 0.035 Mpa.

For photoautotrophic culture, the growth medium BG-11 was used (Rippka et al., 1979). Algal cells were grown in 750 mL BG-11 in an 800 mL column PBR (i.d. 5 cm) under continuous light (250 μmol m^{-2} s^{-1}) at 25±2 ^{o}C. Mixing and

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aeration were provided by bubbling air containing 2.0% (v/v) CO_2. The cell culture was sequentially scaled-up to a 12-L panel PBR and a 380 L tubular PBR followed by a 1,300 L tubular PBR. The inoculum size during each step was 10% (v/v) of the total volume of culture media.

2.2 Induction of lipid production

Pilot-scale lipid production experiments were conducted in a 5,300 L tubular PBR (i.d. 5 cm) outdoors from June to September in 2016 (39°97'N117°06'E, Sanhe, China). Algal cells grown in 7.5 L fermentor and 1,300 L PBR indoors was transferred to two parallel 5,300 L PBR and induced in the N-limited BG-11 medium that containing 1.1 mM nitrate for 13 days. For the outdoor experiments, CO_2 was injected into the culture during daylight hours to maintain pH in the range of 6.5-6.8. The cooling system prevented the culture temperature to exceed the value of 35 ℃. During the night, the culture temperature was allowed to equilibrate to ambient.

2.3 Analytical procedures

Cell growth was monitored by measuring the dry biomass weight according to Chini Zittelli et al (2000). Cell numbers were counted using a haemocytometer after appropriate dilution. The glucose concentration was determined with a Safe-Accu UG Blood Glucose Monitoring System (Model BGMS-1, Sinocare Inc., Changsha, China). The contents of total lipids were determined according to the method described in a previous study (Jia et al., 2015).

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2.4 Techno-economic (TE) analysis

The cost of heterotrophic cultivation for inoculum production was compared to that of conventional photoautotrophic culturing modes, including open-pond and PBR systems, both of which are widely used in algal industries and were thus used as references to evaluate the economic feasibility of heterotrophic cultivation here. The key input assumptions for TE analysis are summarized in supplemental materials tables S1 and S2. To evaluate the effect of scale on production cost, it was assumed that two different inoculum production capacities of 1,000 and 10,000 tons yr^{-1} on 300 operating days. A set of tubular photobioreactor for the seed production is 98 m^3, and the culture volume of the open pond is assumed to be 1,000 m^3. According to our pilot-scale experimental results in 1,000-L fermentor, we assumed the average harvest biomass concentration in a 120 m^3 seed production fermentor is 200 g L^{-1}, achieved within 10 days in a batch of production process. The initial biomass concentrations in open pond and tubular photobioreactor are 0.1 and 0.2 g L^{-1}, and their harvest biomass concentrations are 0.8 and 2 g L^{-1}, respectively. The collapse rates caused by biotic contamination in heterotrophic fermentation, open pond and tubular photobioreactor cultivations were assumed to be 10%, 15% and 5%, respectively. For economic assumptions, all the capital and operating costs for both heterotrophic and photoautotrophic inoculum production were estimated based on vendor quotes, previous studies, or standard engineering estimates. The biomass cost was calculated based on the model reported by Tapie and Bernard (1988). The cost structure includes two major parameters: capital investment costs and operating costs. The operating costs include fixed costs (e.g. labor, overhead, and maintenance) and variable

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costs (e.g. nutrients, power, CO_2 and water). For heterotrophic and photoautotrophic culture, overhead is 60% and 2% of the installed equipment cost for labor and maintenance, respectively. The lifetime of open pond and tubular PBR was assumed to be 10 and 15 years, respectively. For photoautotrophic culture, the CO_2 was assumed to be supplied from a nearby power plant. Water was recycled in autotrophic culture and the evaporation rate of water in open pond was assumed to be 1cm day^{-1}.

2.5 Statistical analysis

The values were expressed as mean ± standard deviation. The data were analyzed by one-way ANOVA using SPSS (version 19.0). Statistically significant difference was considered at $p < 0.05$.

3. **Results**

3.1 Optimization of heterotrophic culturing conditions for *S. acuminatus* GT-2

The effect of pH on heterotrophic growth of *S. acuminatus* GT-2 was investigated in 7.5 L fermentors, in which the culture pH was maintained at 5.0 ~ 8.0 by using pH-stat mode. As shown in Figure 1a, *S. acuminatus* GT-2 favored a weak acidic and neutral pH environment. Under the optimum pH of 6, the highest biomass concentration (205.4 g L^{-1}) was achieved at the end of fermentation (168 h). Thus, the following optimization experiments were conducted at pH 6.0.

When the culture temperature was increased from 25 to 30 ºC, *S. acuminatus* GT-2 grew more rapidly, reaching the highest cell density of 223 g L^{-1} at 30 ºC

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after 240 h (Figure 1b). Further increase in growth occurred when raising the temperature to 35 and 37 ºC during the first 168 h, after which the cellular growth leveled off and then declined. When the temperature was increased to 40 ºC, little growth was observed (Figure 1b). Although it took less time to achieve the highest biomass yield at 35 or 37 ºC as compared to at 30 ºC, longer period of time of imposing the maximum aeration rate (600 rpm) was required to maintain the dissolved oxygen level (DO) at 40% (Figure S1), which indicated more energy input. Thus, 30 ºC was chosen as the optimal culture temperature for the following experiments.

The C/N ratio of a culture medium is thought to be one of the most critical nutritional factors affecting microbial growth (Huang et al., 2010). As shown in Figure 1c, the C/N ratio of 12 sustained the highest growth rate and maximum final dry biomass concentration of 220 g L^{-1}.

3.2 A stepwise constant feeding strategy enhanced biomass production

Growth of *S. acuminatus* GT-2 cells were compared under two different glucose supply modes, i.e the traditional pulse feeding mode and a stepwise constant feeding mode proposed in this study. For the pulse feeding mode, an upper level of glucose concentration was preset (e.g. 20 g L^{-1}). When the concentration of glucose decreased from the preset level to nearly depletion, glucose was supplied into the culture according to the preset concentration within a rather short period of time (e.g., 10 min) (Figure 2a). In the proposed stepwise constant feeding mode, glucose concentrations were finely controlled at a relatively stable level (e.g. 0-5, 5-10, or 15-20 g L^{-1}, Figure 2b). Compared to the pulse feeding mode,

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more frequently sampling, measuring and adjusting feeding rate are required when the stepwise constant feeding was implemented.

The results showed that the biomass concentrations were very close among all the cell cultures with different feeding strategies before 72 h (Figure 2c). After 72 h, the biomass concentrations obtained with the glucose controlled at relatively low concentrations and within less fluctuating range (0-5 or 5-10 g L^{-1}) were obviously higher than those controlled by using the pulse feeding (0-20 g L^{-1}), or by using stepwise feeding higher glucose concentration (15-20 g L^{-1}). The highest biomass concentration reached 273.5 g L^{-1} at 192 h when the glucose concentrations were controlled at 0-5 g L^{-1}, which was 1.2-fold higher than that with the pulsed feeding strategy (226 g L^{-1}) (Figure 2c). Under such an optimal growth condition, the doubling-time and specific growth rate of *S. acuminatus* GT-2 during exponential growth phase were 16 hours and 0.043 h^{-1}, respectively.

The biomass concentration, total glucose consumption amount, biomass productivity and yield on glucose attained under different feeding strategies and glucose concentrations are listed in Table 1. It was shown that the feeding strategies and glucose concentration not only affected the growth and production performance of *S. acuminatus* cells also influenced the glucose-to-biomass conversion efficiency. Glucose-to-biomass conversion efficiency achieved 60.09% when the stepwise constant feeding strategy was employed and the glucose concentration was maintained at a steady low level of 0-5 g L^{-1}, which was significantly higher than that of the pulsed feeding mode (i.e. 55.6%, $p < 0.05$). The biomass yields obtained by using the stepwise constant feeding strategy were all higher than that of the pulsed feeding mode ($p < 0.05$). Taken together,

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the data indicated that utilization of carbon source by *S. acuminatus* cells was more efficient when glucose concentrations were controlled at a relatively steady level.

Compared to those published data, we achieved an ultrahigh cell density of 286 g L^{-1}, which was 2.4-fold higher than the highest level reported up to now (Table 2). Moreover, the maximum biomass productivity of *S. acuminatus* could reach 3.81 g L^{-1} h^{-1}, showing a great potential in commercial applications.

3.3 Heterotrophic cultivation of *S. acuminatus* GT-2 in 1,000-L fermentor

In a pilot-scale fermentation experiment carried out in a 1,000 L fermentor, the glucose concentration was maintained within the optimum range of 0-5 g L^{-1} by using the stepwise constant feeding strategy. As a result, the maximum biomass concentration reached at 283.5 g L^{-1} (Figure 3b), which was very close to the highest level (286 g L^{-1}) achieved in the 7.5 L bench-scale fermentor. Figure 3a shows the change of DO and stirring speed during the scale-up cultivation. When the biomass concentration reached a high level of about 160 g L^{-1}, the DO level cannot be maintained at a constant level and it gradually dropped to 0 at about 120 h. However, it seems that the growth of *S. acuminatus* cells were not affected by the decline of DO, and a continuous increase of biomass concentration was observed when the DO remained at 0 from 120 through 144 h (Figure 3). In addition, the increase in inoculation density shortened the lag phase and finally reduced the whole culture period. When the initial biomass concentration increased from 2 g L^{-1} in bench-scale fermentor to 10 g L^{-1} in pilot-scale fermentor, the lag phase was shortened by about 24 h, and the cell culture entered into the exponential growth stage from 24 h after inoculation (Figure 2c and 3b).

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3.4 Scale-up of lipid production in outdoor 5,300 L tubular photobioreactors

To further verify the feasibility of using heterotrophic microalgal cells for lipid production and to compare its performance with the photoautotrophic cells, pilot-scale experiments were conducted in two parallel outdoor tubular photobioreactors. One was inoculated with the algal cells from the heterotrophic late-exponential phase in 7.5 fermentors, and the other one used the algal cells from the photoautotrophic exponential growth phase in 1,300 L tubular photobioreactors. Three critical environmental parameters (temperature, light intensity and pH) were monitored during the cultivation period. The results showed these parameters were very close to each other in the two-parallel tubular photobioreactors (Figure S2).

The outdoor scale-up experiments underlined the advantage of heterotrophically-grown algal cells over the photoautotrophic counterparts in terms of lipid production, which is in accordance with the findings of many previously published work (Han et al., 2012; Zheng et al., 2012). It was observed that photoautotrophically-grown algal cells contained greater amounts of chlorophylls than the heterotrophically-grown algal cells, giving rise to a darker green color for the culture (Figure 4a). Under the N-limitation conditions, the color of the two cultures turned yellow, accompanied with accumulation of neutral lipids, as indicated by BODIPY staining and microscopic observation (Figure 4a). For the large-scale culture inoculated with the heterotrophically-grown cells, the maximum biomass concentration and lipid content were 1.38 g L^{-1} and 34.4%, respectively, which were1.3- and 1.1- fold greater than that of the culture inoculated with photoautotrophically-grown cells (Figure 4b and 4c), suggesting

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the new culture mode coupling heterotrophy and photoautotrophy was more productive than the traditional photoautotrophic mode in regard to both biomass and lipid production. With this combined culture mode, the average lipid productivity in 5,300 L pilot-scale photobioreactor reached 45.05 mg L^{-1} d^{-1}, which was the highest level in similar scale lipid production to date (Table 3).

3.5 TE analysis

To evaluate the economic feasibility of heterotrophic cultivation on large-scale for inoculum production, we conducted TE analysis based on our pilot-scale data along with several key assumptions. The cost of heterotrophic culture in fermentors was compared with that of traditional photoautotrophic culture in open ponds and tubular photobioreactors. As shown in Figure 5, to produce the same amount of biomass for lipid production, the production cost by heterotrophic culture was the lowest among the three cultivation modes. It should be pointed out that the cost of biomass production by using the industrial-scale heterotrophic cultivation was based on a high biomass concentration of 200 g L^{-1} (Table S1), which is achievable at least for *Scenedesmus acuminatus* GT-2 and *Chorella sorokinian* GT-1 (unpublished data). As an example, a much higher biomass concentration of 283.5 g L^{-1} has been obtained from the heterotrophic culture of *S. acuminatus* in a 1,000 L pilot-scale fermentor (Figure 3). With the increase of annual production capacity from 1,000 to 10,000 tons, the cost can be reduced from $1.59 to $1.07 per kilo of algal biomass (dry weight equivalent) because the increased fermentor volume (from 120 to 200 m^3) could considerably reduce both the capital and operational costs (Tables 4). Due to the high capital investment (~ 70% of the total cost), the cost of biomass production in tubular photobioreactor

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was the highest among three culture modes, about 5-fold higher compared to cultivations in fermentor and open ponds (Figure 5).

To evaluate the effect of future improvements in algal production technology and process on the overall cost, the sensitivity analysis was performed. For heterotrophic culture, the achievable maximum biomass concentration was found to have the greatest impact on the overall production cost. Moreover, the price of glucose and its conversion efficiency into algal biomass also have great influence on the cost (Figure 6a, 6b and 6c). Thus, the cost of heterotrophic culture could be reduced by improving the glucose conversion efficiency, as well as by utilizing any alternative low-cost organic carbon sources. In addition to improving biomass production, the cost for open pond cultivation could be further reduced by increasing the times of water recycling in algal cultivation. For tubular photobioreactors, the development of low-cost and highly efficient photobioreactors can simultaneously improve biomass productivity while reducing the capital cost.

4. **Discussion**

This study reported ultra-high-cell-density heterotrophic culturing of the unicellular green alga *S. acuminatus* with a maximum biomass concentration of 286 and 283.5 g L^{-1} in 7.5-L bench-scale and 1,000 L pilot-scale fermenters, respectively, which is the highest level among the microbial heterotrophic cultivation up to date. It is noteworthy that when the cell density of *S. acuminatus* was 250 g L^{-1} dry weight and above, the water content of the cells was lower than 60% (Figure S3), which is dramatically different from most cells that are known to contain 80% water. Under such circumstances, the wet algal pellets occupied

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55% of the culture volume whereas the water outside the cells filled the remaining volume (ca. 45%). In addition, the viscosity of the *S. acuminatus* culture is not as high as that of many well-known industrial microorganisms. For instance, when the dry cell density of *Pichia pastoris* is above 120 g L^{-1}, the culture broth appears as slurry and hard to be stirred. On the contrast, the ultrahigh-cell-density culture (i.e. 280 g L^{-1}) of *S. acuminatus* can be mixed well, which might be attributable to little extracelluar polysacchardies secreted by this organism during cultivation.

This study demonstrated the coupled heterotrophic and photoautotrophic culturing mode is a promising technology for microalgal biodiesel production when ultra-high-cell-density heterotrophic culturing is realized through employing a sophisticated substrate feed control strategy. Such a technology was developed through two-tier optimization. Firstly, the traditional pulse addition of glucose was replaced by a stepwise constant feeding strategy, which obviously increased the biomass productivity of *S. acuminatus* in fed-batch culture. Pulse addition of carbon sources has been employed in heterotrophic cultivation for many microalgae, especially for *Chlorella* strains. However, either dramatic fluctuation or depletion of substrate could cause stress to microalgal cells. For example, it has been reported that the microalga *G. sulphuraria* metabolized intracellular components when the sugar is starved (Schmidt et al., 2005). By utilization of the stepwise constant feeding strategy, the concentration of glucose was maintained at a relatively steady level to avoid adverse effect caused by the periodic limitation and starvation of glucose.

Secondly, the highest average growth rate (1.21 g L^{-1} h^{-1}) and glucose conversion efficiency (60.09%) were achieved by maintaining the glucose at a

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relatively low constant concentration (< 5 g L^{-1}). It has been observed in a number of microalgal heterotrophic culture that keeping low concentration of glucose is essential for sustaining a higher specific growth rate. For example, a glucose concentration of 2.5 g L^{-1} is optimum for the growth of *C. saccharophila*, whereas inhibition occurred at the concentration > 25 g L^{-1} (Tan and Johns, 1991). For an eicosapntemacnioc acid-producing diatom *Nitschia laevis*, the growth rate decreased with the increase of glucose concentration from 1 to 40 g L^{-1} (Wen and Chen, 2000). In the green alga *C. kessleri*, glucose uptake is mediated by a hexose/H^+ symporter which shows saturation of behavior with a Km value of about 0.3 mM (Komor et al., 1979). This finding can explain why very low concentration of glucose is sufficient for the heterotrophic growth of many microalgae. However, understanding of the adverse effect of high concentration of glucose on microalgal growth is rather limited, although those phenomena were extensively observed in microalgal fermentation processes. There are at least three underlying mechanisms to be investigated: i) excessive glucose may interact with the symporter and inhibit uptake of glucose directly; ii) high glucose level could cause oxidative stress in microalgal cells as it does in mammalian cells; iii) inhibitory metabolic by-products (e.g., ethanol or acetate) caused by Crabtree effect under high glucose concentration may secrete into the growth medium.

This study is not only a proof of concept that combining heterotrophic and photoautotrophic cultivation for oil production at a pilot scale is economic feasible, also demonstrated that microalgal cells grown under heterotrophic conditions were conferred a superiority in terms of lipid production as compared to the photoautotrophic cells. Heterotrophically-grown cells of the green alga

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Haematococcus pluvialis were found to be susceptible to excessive light, which is attributable to the impairment of photosynthetic machinery in dark (Zhang et al., 2016). However, contradictory to the notion, *S. acuminatus* cells from the high-cell-density heterotrophic culture were acclimated to the photoautotrophic conditions while accumulating oils at a higher rate than the cells adapting photoautotrophic conditions in prior cultivation. Similar with the findings of this study, a number of previous studies have observed that the heterotrophic *Chlorella* cells showed higher growth rate and lipid productivity than the photoautotrophic cells when they are subjected to the same stress conditions (Han et al., 2012; Zheng et al., 2012).

Intriguingly, when compared to the photoautotrophic cells, enhancement in the growth rate of heterotrophic cells is coincident with the reduction in the cellular content of chlorophyll, implying that these cells could possess truncated light-harvesting antennae. It is well-known that this feature can permit a higher light penetration in high-cell-density-culture, less likelihood of photoinhibition, and reduce energy loss as heat (Melis, 2009). Our intuitive speculation, however, remains to be tested by more detailed biochemical and physiological analysis. If this is a fact, it indicates glucose could be used to tune the composition and structure of photosystems as to improve the growth capacity without changing the genetic make-up of a given algal species.

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Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

HJ, HZ participated in the design and performance of experiments, data collection and analysis, and manuscript writing. HJ, HZ, ZWZ, KPL, GLH and WHC participated in the heterotrophic cultivation. HZ, QX participated in the autotrophic cultivation. CWZ provided the experiment microalgae. GLH participated in TE analysis. DXH and QH participated in design of experiments and manuscript writing. HJ and HZ contributed equally.

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Tables

Table 1 Comparison of major fermentation performances among cultures with different feeding strategies and glucose concentration levels.

Feeding strategies	**Glucose concentration controlled (g L^{-1})**	**Maximum biomass conc. (g L^{-1}) & Time**	**Final biomass conc. (g L^{-1})**	**Total glucose consumed (g)**	**Glucose-to-biomass conversion (%)**	**Ave. productivity (g L^{-1} h^{-1})***
Stepwise constant feeding	0-5	273.5±7.4 (192 h)	262±9.2	2660	60.09±0.56	1.21±0.01
Stepwise constant feeding	5-10	258±3.5 (216 h)	258±3.5	2242	59.20±1.06	1.19±0.01
Stepwise constant feeding	15-20	244±3.9 (216 h)	244±3.9	1971	58.20±1.28	1.13±0.02
Pulsed feeding	0-20	226±3.0 (192 h)	222.5±3.5	2024	55.60±0.88	1.03±0.01

*Biomass productivity (g L^{-1} h^{-1}) = dX/dt = $(X_2-X_1)/(t_2-t_1)$, where X_1, X_2 are the biomass concentration at time t_1 and t_2 of the fermentation

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Table 2 Overview of maximum biomass concentration and productivity of microalgae under heterotrophic fed-batch cultivation.

Microalgal species	Product	Maximum biomass concentration ($g L^{-1}$)	Maximum biomass productivity ($g L^{-1} h^{-1}$)	References
Aurantiochytrium sp.	DHA	31.8	0.44	(Ryu et al., 2013)
Chlorella protothecoides	Lutein	19.6	0.11	(Shi et al, 2002)
Chlorella protothecoides	Lipids	70.9	0.39	(Yan et al., 2011)
Chlorella protothecoides	Biomass	116	0.98	(Wu and Shi, 2007)
Chlorella regularis	Biomass	84	2.8	(Sansawa and Endo, 2004)
Chlorella vulgaris	Biomass	117.2	3.66	(Doucha and Livansky, 2012)
Chlorella zofingiensis	Astaxanthin	53	0.14	(Sun et al., 2008)
Chlorella sorokiniana	Lipids	103.8	0.45	(Zheng et al., 2013)
Chlorococcum sp.	Ketocarotenoid	18	-	(Zhang and Lee, 2001)
Cryptecodinium cohnii	DHA	109	0.28	(De Swaaf et al., 2003)
Euglena gracilis	α-Tocopherol	48	0.26	(Ogbonna et al., 1998)
Galdieria sulphuraria	Phycocyanin	109	0.72	(Graverholt and Eriksen, 2007)

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Galdieria sulphuraria	Phycocyanin	116	0.34	(Schmidt et al., 2005)
Haematococcus pluvialis	Astaxanthin	26	0.06	(Wan et al., 2015)
Neochloris oleoabundans	Lipids	20.9	0.08	(Morales-Sanchez et al., 2013)
Nitzschia laevis	EPA	40	0.12	(Wen and Chen, 2002)
Scenedesmus acuminatus GT-2	Lipids	286	3.81	(This study)

Table 3 Summary of lipid production performance by outdoor cultivation of microalgae under different cultural scales.

Microalgal species	**Type of cultural system**	**Seed source & culture mode**	**Culture scale (L)**	**Lipid content (%, dwt)**	**Lipid Productivity (mg L^{-1} d^{-1})**	**References**
Chlorella zofingiensi	Flat plate	P and B	60	33.80	22.30	(Feng et al., 2011)
Chlorella sp.	Bag PBR	P and B	120	23.00	13.70	(Moheimani, 2013)
Graesiella sp.	Raceway pond	P and B	40,000	31.80	14.50	(Wen et al., 2016)
Monoraphidium dybowskii	Raceway pond	P and S	40,000	30.00	27.20	(Yang et al., 2018)
Nannochloropsis sp.	Green wall	P and B	110	60.00	204.00	(Rodolfi et al., 2010)

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	panel					
Nannochloropsis gaditana	Tubular PBR	P and C	340	18.60	110.00	(San Pedro et al., 2014)
Nannochloropsis sp.	Green wall panel	P and B	590	43.00	110.00	(Biondi et al., 2013)
Nannochloropsis gaditana	Raceway pond	P and C	792	25.60	30.40	(San Pedro et al., 2015)
Nannochloropsis sp.	Raceway pond	P and B	8,000	28.00	4.69	(Zhu et al., 2014)
Scenedesmus obtusus	Tubular PBR	P and S	500	13.40	19.00	(Hulatt and Thomas, 2011)
Scenedesmus acutus	Raceway pond	P and B	2,278	21.50	9.20	(Eustance et al., 2016)
Scenedesmus acuminatus	Tubular PBR	P and B	5,300	31.04	27.45	This study
Scenedesmus acuminatus	Tubular PBR	H and B	5,300	35.68	45.05	This study

P: Seed from photoautotrophic cultivation; H: Seed from heterotrophic cultivation; B: Batch culture; C: Continuous culture; S: Semi-continuous culture; T:Two-step culture

Table 4 Comparison of annual production cost compositions and proportions with different culture modes.

No.	**Items**	**Open pond**		**Tubular photobioreactor**		**Heterotrophic culture**	
		Cost/$1	**Proporti**	**Cost/$1**	**Proporti**	**Cost/$1**	**Proporti**

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		M	on	M	on	M	on
1,000 t per year							
1	Equipment depreciation	0.5200	29.50%	4.7456	68.68%	0.2658	16.75%
2	Land use	0.0138	0.79%	0.0120	0.17%	0.0004	0.03%
3	Water	0.3024	17.15%	0.0224	0.32%	0.0070	0.44%
4	Nutrient	0.0573	3.25%	0.0503	0.73%	0.6224	39.23%
5	Power	0.6523	37.00%	1.6253	23.52%	0.5989	37.75%
6	CO_2	0.0000	0.00%	0.0000	0.00%	0.0000	0.00%
7	Maintenance	0.0000	0.00%	0.2373	3.43%	0.0053	0.34%
8	Labor	0.2170	12.31%	0.2173	3.14%	0.0868	5.47%
9	Total cost	1.7628	100.00%	6.9099	100.00%	1.5866	100.00%
10,000 t per year							
1	Equipment depreciation	5.2354	30.91%	47.6734	69.29%	0.94	8.85%
2	Land use	0.1374	0.81%	0.1196	0.17%	0.0013	0.01%
3	Water	3.0018	17.72%	0.2254	0.33%	0.0000	0.00%
4	Nutrient	0.5688	3.36%	0.5072	0.74%	6.2226	58.41%
5	Power	6.4753	38.23%	16.3760	23.80%	3.3372	31.33%
6	CO_2 cost	0.00	0.00%	0.00	0.00%	0.00	0.00%
7	Maintenance	0.00	0.00	2.3837	3.46%	0.0189	0.18%

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8	Labor	1.5191	8.97%	1.5191	2.21%	0.1302	1.22%
9	Total cost	16.9378	100.00%	68.8045	100.00%	10.6530	100.00%

Figure1. Effect of different pH (a), temperatures (b) and C/N ratios (c) on *Scenedesmus acuminatus* cellular growth under heterotrophic conditions in 7.5 L bioreactors.

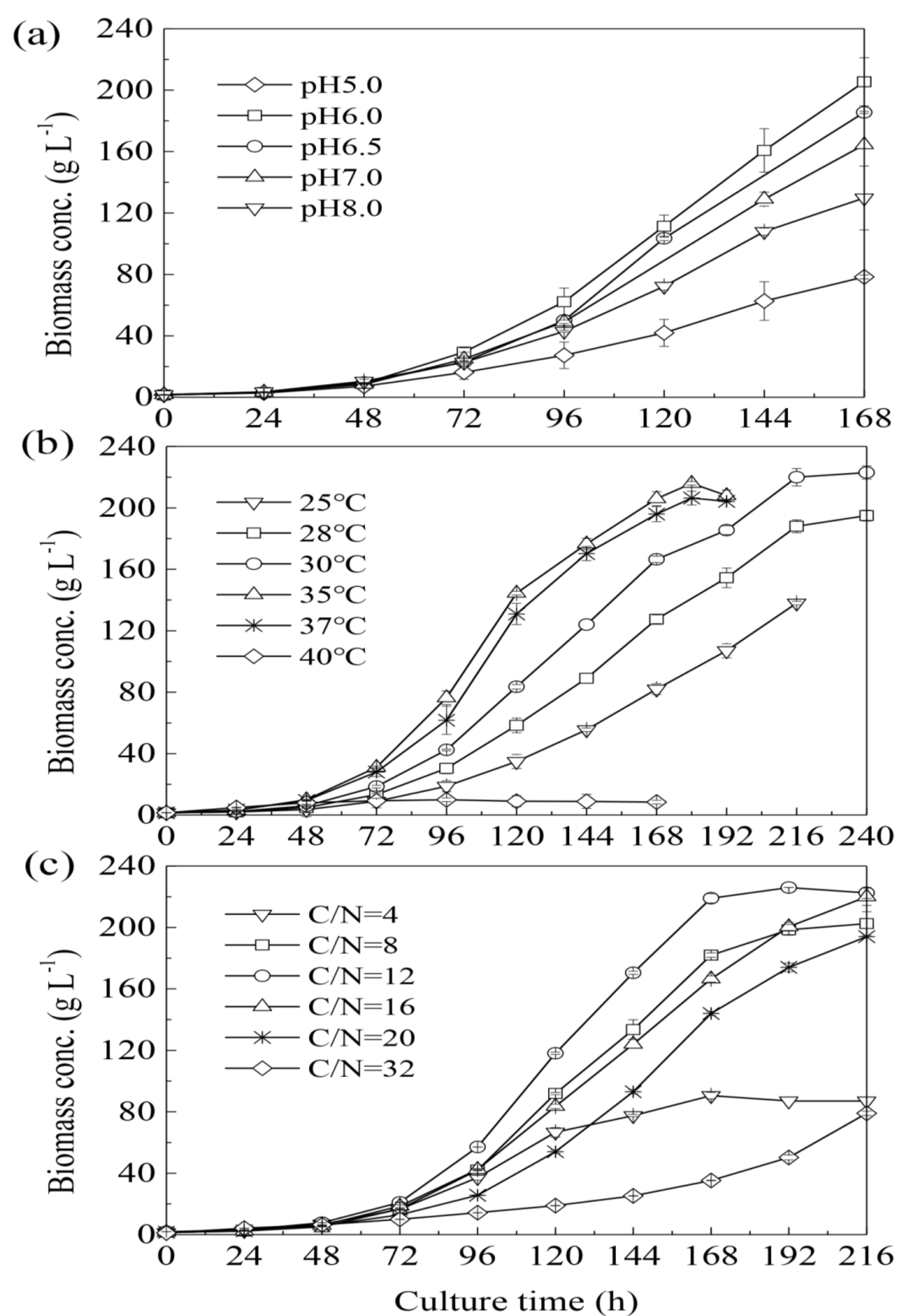

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Figure 2. Comparison of biomass production with different glucose feeding strategies. a: Pulsed feeding by controlling glucose concentration in the range of 0-20 g L-1; b: Stepwise constant feeding by controlling glucose concentration under different ranges of 0-5, 5-10 and 15-20 g L-1; c: Biomass concentration under different feeding strategies

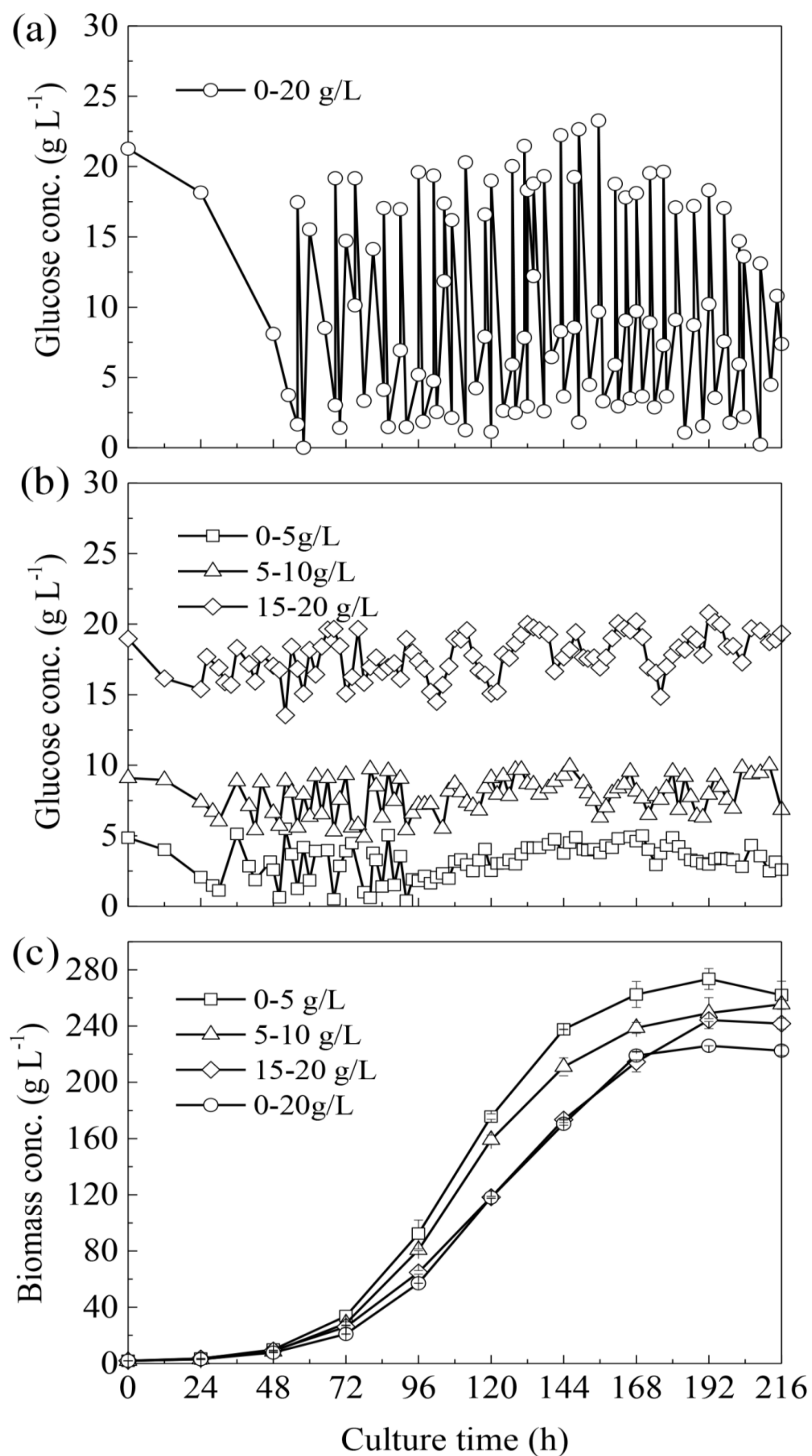

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Figure 3. Scale-up of heterotrophic culture of *S. acuminatus* in 1, 000 L fermentor with the optimal cultural conditions. a: Time courses of DO and stirring speed. b: Change of biomass and glucose concentrations with time.

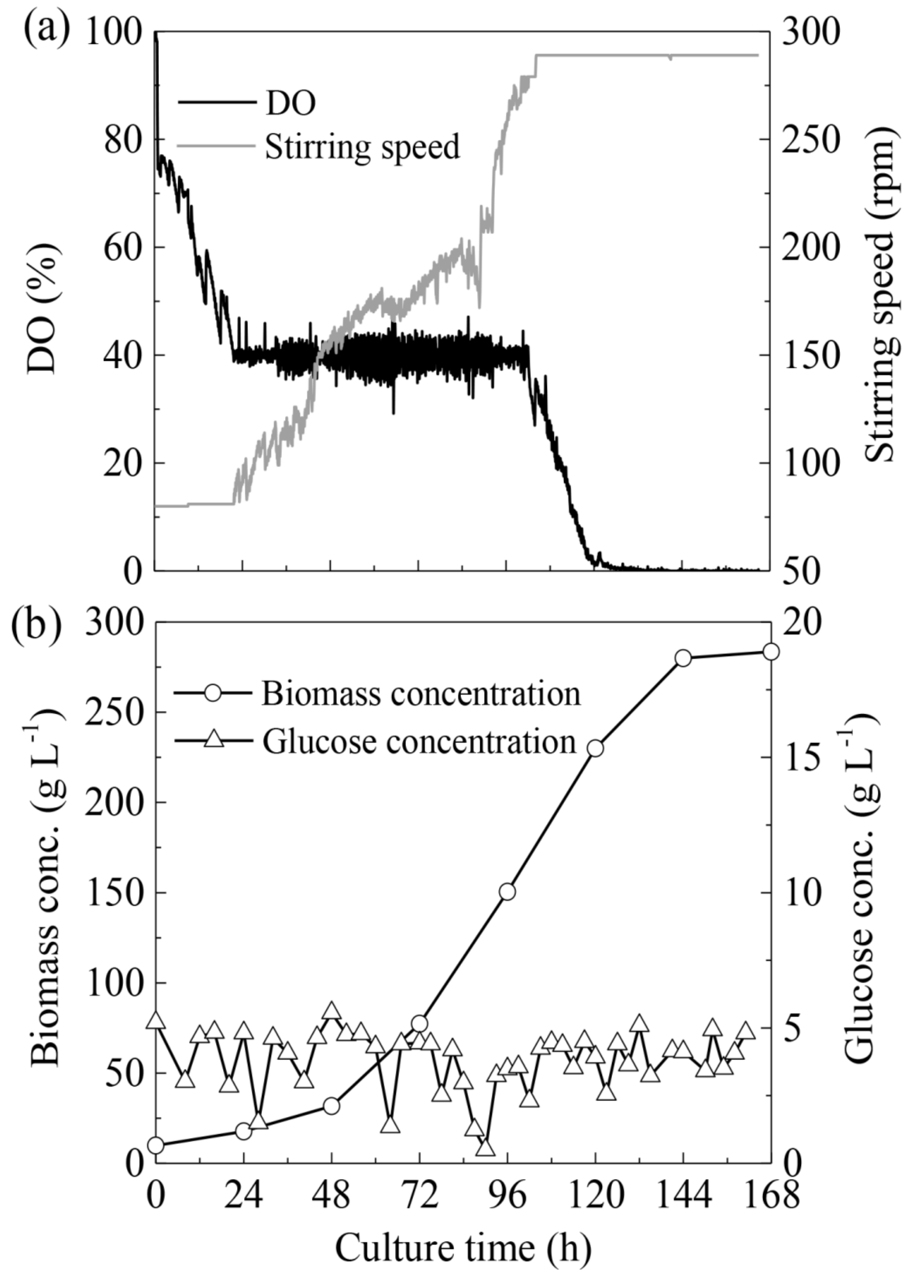

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Figure 4. Lipid production in pilot-scale tubular photobioreactors. a. Culture appearance and cell morphology; b. Cell growth; c. Lipid contents of the culture inoculated with the cells from heterotrophic (□) and photoautotrophic (○) cultures. AS: Inocula from autotrophic culture; HS: inocula from heterotrophic culture; Scale bar=10 μm.

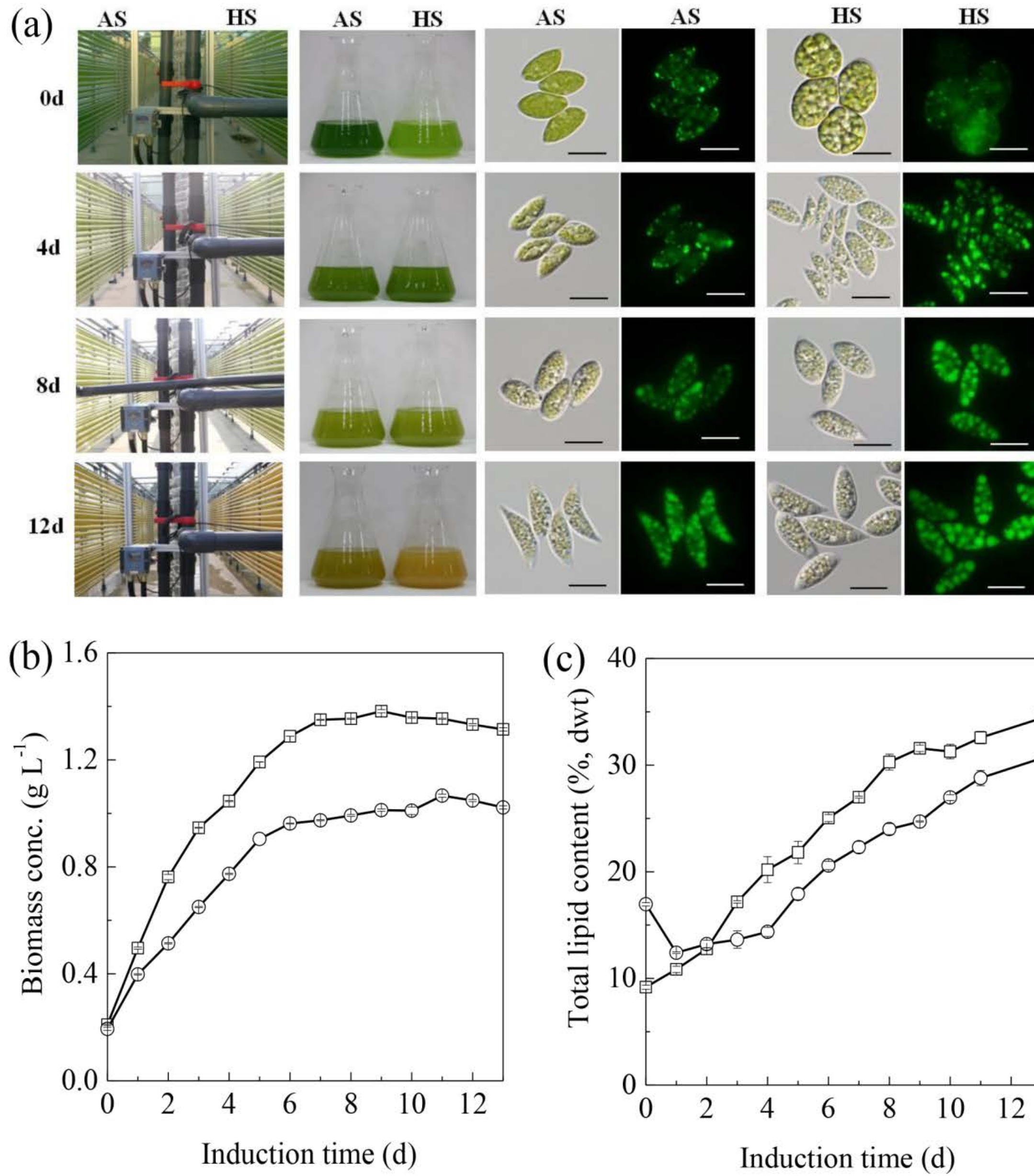

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Figure 5. Cost comparison of inocula production with different culture modes.

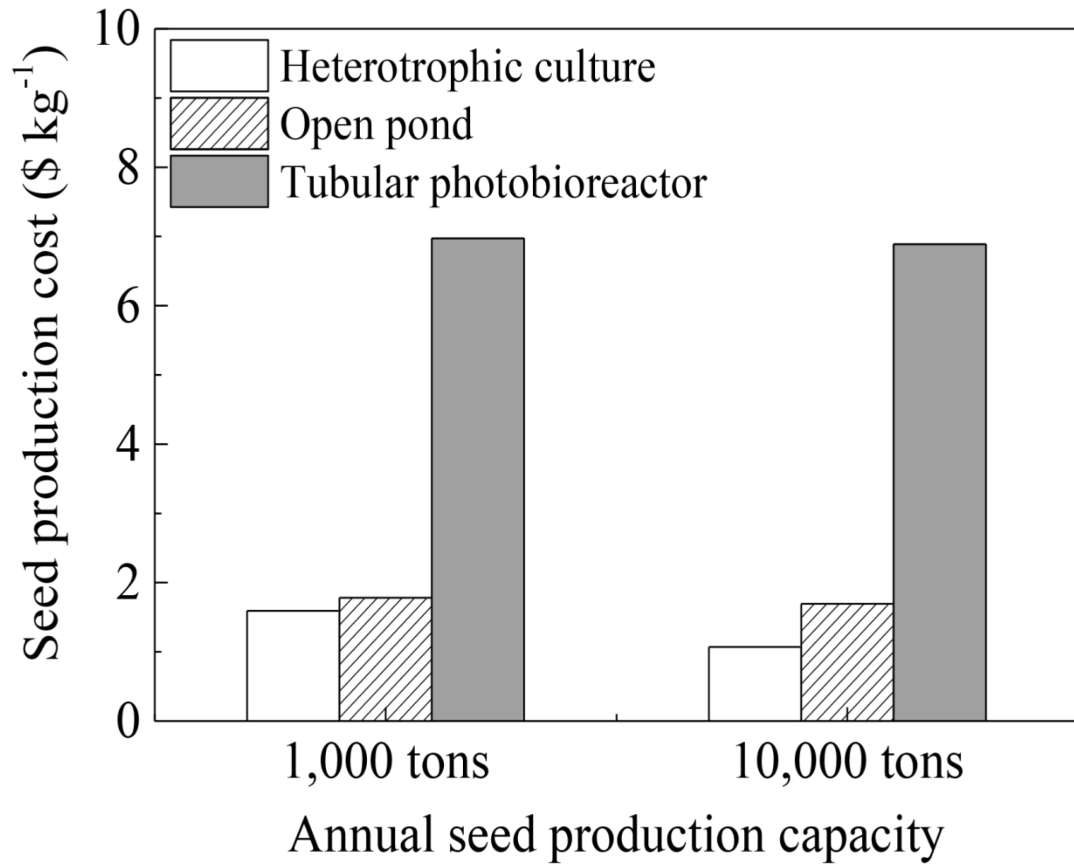

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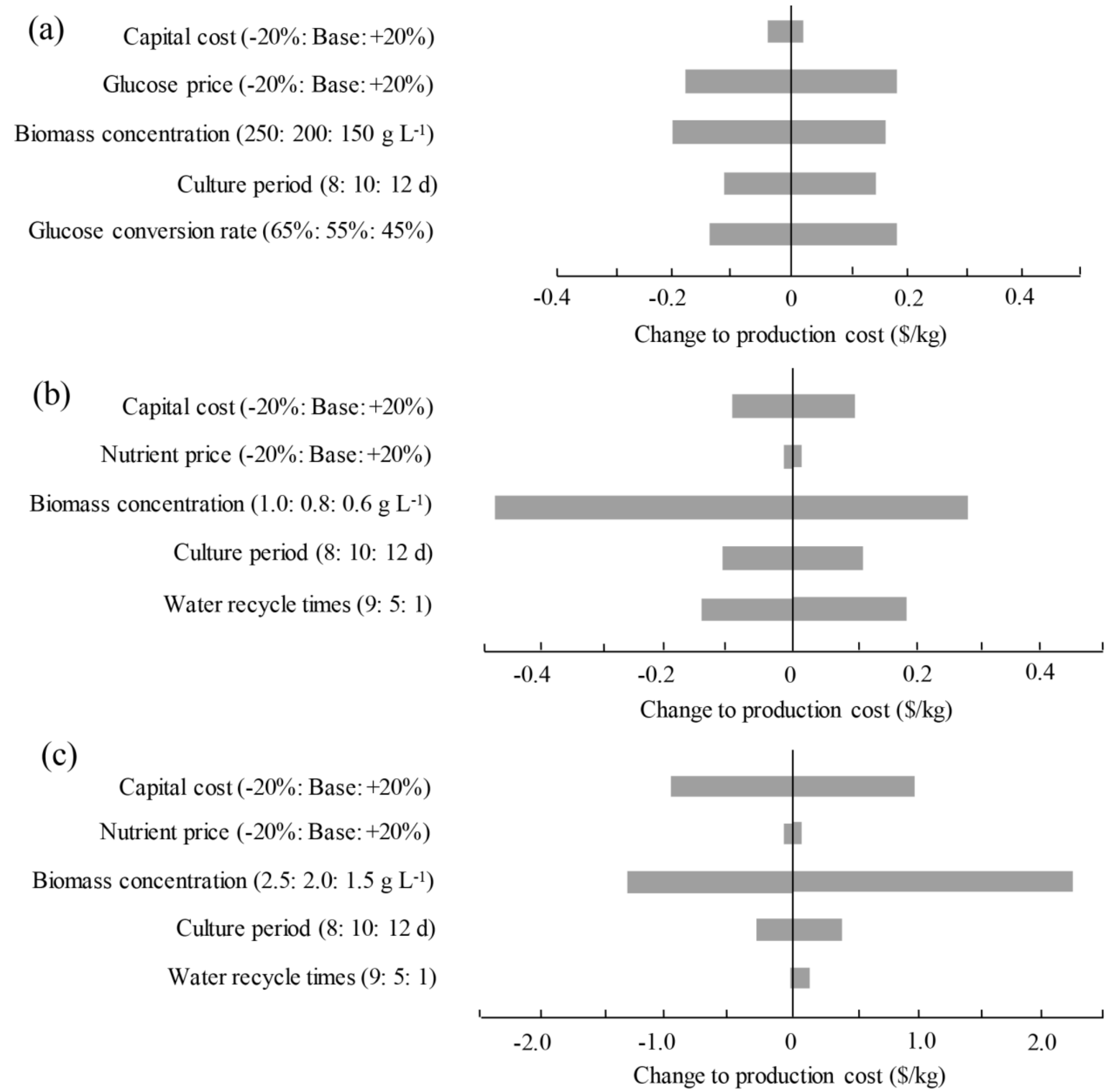
Figure 6. Sensitivity analysis of heterotrophic (a), open pond (b) and tubular photobioreactor (c) cultivation system.



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