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Towards maximizing biomass and lipid productivity: high-throughput screening assay for prospecting heterotrophic growth for new microalgal isolates

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Abstract

Background Microalgae have emerged as sustainable alternatives to fossil fuels and high-value petrochemicals. Despite the commercial potential of microalgae, their low biomass productivity is a significant limiting factor for large-scale production. In the photoautotrophic cultivation of microalgae, achievable cell density levels depend on the light transmittance of the production system, which can significantly decrease the photosynthetic rate and biomass production. In contrast, the mixotrophic cultivation of microalgae using heterotrophic carbon sources enables high-density cultivation, which significantly enhances biomass productivity. The identification of optimal production conditions is crucial for improving biomass productivity; however, it is typically time- and resource-consuming. To overcome this problem, high-throughput screening (HTS) system presents a practical approach to maximize biomass and lipid production and enhance the industrial applicability of microalgae.

Results In this study, we proposed a two-step HTS assay that allows effective screening of heterotrophic conditions compatible with new microalgal isolates. To confirm the effectiveness of the HTS assay, three microalgal isolates with distinctive morphological and genetic traits were selected. Suitable cultivation conditions, including various heterotrophic carbon sources, substrate concentrations, and temperatures, were investigated using a two-step HTS assay. The optimized conditions were validated at the flask scale, which confirmed a significant enhancement in the biomass and lipid productivity of each isolate. Moreover, the two-step HTS assay notably enhanced economic and temporal efficiency compared to conventional flask-based optimization.

Conclusions These results suggest that our two-step HTS assay is an efficient strategy for investigating and optimizing microalgal culture conditions to maximize biomass and lipid productivity. This approach has the potential

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to enhance the industrial applicability of microalgae and facilitate the seamless transition from laboratory to field applications.

Keywords Microalgae, High-throughput screening, Optimization, Heterotrophic carbon substrate, Lipid accumulation

Background

As primary producers, microalgae are found in most ecosystems, including polar regions [1, 2]. It is estimated that there are approximately 200,000–800,000 microalgal species, of which approximately 35,000 have been described [3]. The physiological traits and ecological functions of microalgae are known to be as diverse as their broad geographical distribution. This photosynthetic group of organisms has emerged as a sustainable feedstock for biofuel production and an effective alternative for treating wastewater [4, 5]. Microalgae are known to produce a suite of valuable metabolites, such as omega-3 fatty acids, carotenoids, and vitamins. Many of these compounds are considered promising sources of pharmaceuticals, nutraceuticals, and cosmetics [6–8]. However, the production of microalgae under typical photoautotrophic cultivation conditions is not economically feasible for a microalgae-based bioeconomy. Only extremely high-value substances, such as astaxanthin and eicosapentaenoic acid (EPA), have been identified to be economically feasible, challenging the expansion of the global market for microalgae-based products [9, 10].

Heterotrophic microalgal cultivation involves growing microalgae without light irradiation, while feeding on organic carbon sources. Mixotrophic cultivation occurs when light irradiation is provided along with an organic carbon source [11, 12]. Compared with photoautotrophic cultivation, which has a clear limit in terms of achievable biomass productivity owing to the limitation of light transmittance, mixotrophic cultivation is capable of reaching a high cell density [13]. In laboratory batch cultivation with flasks, *Chlorella sorokiniana* reaches a maximum biomass concentration of 5.08 g L^{-1} and 4.23 g L^{-1} under mixotrophic and heterotrophic cultures, respectively. These values are 7.47 and 6.22 times higher, respectively, than the 0.68 g L^{-1} obtained under photoautotrophic cultivation [14]. Compared to photoautotrophic conditions, *Scenedesmus obliquus* and *Botryococcus braunii* also produce 2 and 3.5 times more biomass, respectively, under heterotrophic cultivation, and achieve 2.09 and 4 times higher biomass production, respectively, under mixotrophic cultivation [15]. In industrial production systems, heterotrophic and mixotrophic cultivation are more favorable because they have a higher growth rate than photoautotrophic cultivation and can reduce contamination risks and operational costs [16].

However, not all microalgae perform well under conventional heterotrophic conditions, in which a limited number of organic substrates are deployed as growth substrates. Microalgal heterotrophy is highly strain dependent, and each strain is likely to exhibit a preference for different organic substrates under characteristic abiotic conditions, such as temperature and aeration [17, 18]. However, assessing strain-specific heterotrophic growth conditions using traditional methods requires considerable time and effort. Therefore, high-throughput methods are anticipated to efficiently and economically screen for optimal heterotrophic conditions [19]. To this end, ready-to-use commercial products, such as Biolog microplates, may serve as effective high-throughput screening (HTS) platforms by enabling rapid metabolic profiling of various microalgal strains [20, 21]. Moreover, further screening can be performed using PhotoBiobox, a microplate-based platform developed in a previous study, to determine the optimal temperature, substrate concentration, and light conditions [22].

This study aimed to develop a practical high-throughput method for investigating suitable mixotrophic cultivation conditions to maximize the biomass and lipid productivity of newly isolated microalgae. The validity of this method was confirmed using three isolated microalgal strains that exhibited distinct morphological characteristics: *Chlamydomonas* sp. KGG-7, *Monoraphidium* sp. KGG-9, and *Hariotina* sp. KGG-18. A total of 71 carbon substrates were tested on a microplate to identify the optimal heterotrophic organic carbon substrate. Subsequently, the optimal temperature and substrate concentration were determined using PhotoBiobox. The optimized conditions were verified at the flask scale, confirming a significant improvement in biomass and lipid productivity. Our two-step HTS assay enabled the convenient exploration of a broad range of conditions, reducing the number of final conditions that needed to be tested. Through this, it was confirmed that our approach is significantly time- and cost-efficient than the conventional flask-based optimization method. Our approach offers substantial time and cost savings, indicating its superiority as a convenient and efficient method for maximizing biomass and lipid production of microalgae. Overall, this result suggests that the two-step HTS assay serves as an efficient strategy to optimize the culture conditions of novel microalgae and promote the industrial potential of high-value-added microalgae.

Materials and methods

Isolation of three novel microalgal strains

The water sampling point for isolating microalgae was Gyeongpocheon, a stream in Gunsan, Republic of Korea (latitude 35°58'09.4"N and longitude 126°43'28.0"E). The water sample was spread onto 1.5% agar plates containing BG11 solution (Sigma-Aldrich Co., Saint Louis, MO, USA) and incubated at 25 ℃ under light condition. Colonies grown on agar plates were inoculated into liquid BG11 medium containing 500 ppm cefotaxime (Sigma-Aldrich Co., Saint Louis, MO, USA) to eliminate bacterial contamination. The inoculated cultures were grown at 25 ℃, 120 rpm, and 100 μmol photon m^{-2} s^{-1} until they exhibited a green color. Next, stepwise dilutions were spread on 1.5% agar BG11 medium to obtain single colony. Obtained single pure colonies were identified by amplifying a segment of the 18 S ribosomal RNA (*18 S rRNA*) gene using forward (5′ caagtttctgccctatcagct 3′) and reverse (5′ gctttcgcagtagttcgtctt 3′) primers according to a previously published method [23]. The *18 S rRNA* gene of *Hariotina* sp. KGG-18, which has a length of 2.8 kb was sequenced by employing the additional inner primers: inner forward, 5′ cgcctatggtgagtactgctat 3′ and inner reverse, 5′ caacttggtatcacaggctcta 3′. Subsequently, the sequences of the PCR products were analyzed using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial *18 S rRNA* sequences of the selected strains were deposited in NCBI GenBank for subsequent experiments.

Assessment of organic substrate availability

Organic substrate utilization by the microalgal strain was assessed using a GENIII microplate (Biolog, Hayward, CA, USA). Seed cultures were prepared in a 50 mL T-flask (SPL, Pocheon, Republic of Korea) with a 15 mL working volume of 0.5 X BG11 at 25 ℃, 120 rpm, and 100 μmol photon m^{-2} s^{-1}. The seed culture broth was diluted 10-fold with 0.5 X BG11 medium in the stationary phase, and 500 ppm cefotaxime was added. Two hundred microliters of broth were transferred into each well of a GENIII microplate. The microplate was covered with a gas-permeable sealing membrane (Diversified Biotech, Dedham, MA, USA) and incubated at 25 ℃ under 100 μmol photon m^{-2} s^{-1} light intensity until the number of cells appeared sufficiently distinct. To compare cell growth, the optical density at an absorbance wavelength of 700 nm was measured using a Sunrise microplate reader (Tecan, Männedorf, Switzerland), with dilution to ensure that it did not exceed 0.8. The raw values were normalized by scaling to each negative control (without carbon substrate) using the following formula: (OD_S/OD_N), where OD_S represents the optical density of each well with different organic substrates and OD_N is the optical density without the organic substrate (negative control).

Screening of optimal mixotrophic cultivation conditions using PhotoBiobox

The optimal temperature and substrate concentration for the cultivation of microalgal strains were determined using the high-throughput bioreactor PhotoBiobox [22]. The temperature was sequentially regulated from 15.0 ℃ to 40.0 ℃ and the light intensity was set to 100 μmol photon m^{-2} s^{-1}. Seed cultures were prepared using the same method as that described for the Biolog microplate assay. Each well of the microplates for the PhotoBiobox-based screening was filled with 200 μL of 0.5 X BG11 liquid medium containing 10-fold-diluted seed culture supplemented with 500 ppm cefotaxime and the appropriate amount of organic substrates, including glucose and maltose, in the range of 0 to 30 g L^{-1}. The microplates were sealed with a gas-permeable membrane and incubated in a PhotoBiobox. After 5 days of operation, the cell concentration in each well was determined by measuring the absorbance at 700 nm using a Sunrise microplate reader.

Cell cultivation

In order to maintain microalgal strain, the strains were cultivated using a 1.5% agar plate containing BG11 medium at 25 ℃ under 100 μmol photon m^{-2} s^{-1}. To validate the screening conditions using PhotoBiobox, the cells were cultivated in a 250 mL Erlenmeyer flask with a 100 mL of working volume at 120 rpm under light intensity of 100 μmol photon m^{-2} s^{-1}. *Monoraphidium* sp. KGG-9 was incubated at 26 ℃ with glucose concentrations set at 10 g L^{-1} and 20 g L^{-1}. *Hariotina* sp. KGG-18 was subjected to incubation at 29 ℃ with maltose concentrations of 15 g L^{-1} and 30 g L^{-1}, respectively.

Growth and lipid measurements

Microalgal growth was measured by diluting until the absorbance at 700 nm was <0.8. Biomass measurements were conducted using a slight modification of a previously described method [23]. Briefly, 2 mL of culture broth was harvested by centrifugation at 3,000 rpm for 5 min and rinsed once with the same volume of phosphate-buffered saline (PBS). The cells were resuspended in 0.5 mL of PBS and filtered through pre-weighed GF/C filter paper (Whatman, Maidstone, UK) using a vacuum pump. The filter paper containing wet biomass was dried at 65 ℃ for 48 h and weighed. To ensure the accuracy, we used three filter papers filtered with equal amounts of PBS buffer as a control group, and corrected the zero value with the average value of the control group.

To measure the total lipid content of the dried biomass, the culture broth was harvested by centrifugation at 3,000 rpm for 5 min and rinsed once with the same volume of PBS. The suspension was then centrifuged at 3,000 rpm for 5 min to harvest the biomass. The

harvested cells were freeze-dried for 3 days and 10 mg of dried cells was used to extract total lipid. The quantified biomass was immersed in a 2:1 (v/v) mixture of chloroform and methanol. The mixture was tightly sealed and ultrasonicated at 65 Hz for 1 h. The solvent layer was volatilized in a pre-weighed aluminum dish and the remaining total lipids were weighed. To ensure the accuracy, three aluminum dishes volatilized with the same amount of solvent mixture were used as control group, and the zero value was corrected with the average value of the control group.

Quantitative analysis of organic substrate

The concentration of organic substrates in residual medium was analyzed using a high-performance liquid chromatography (HPLC) system (1260 Infinity; Agilent, Santa Clara, CA, USA) equipped with a refractive index detector (RID) using an Aminex ion-exclusion column HPX-87 H (300×7.8 mm, 9 µm particle size; Bio-Rad, Hercules, CA, USA). The injection volume was 10 µL and the column temperature was maintained at 65 ℃. The isocratic eluent was 5 mM sulfuric acid, which was pumped at a flow rate of 0.6 mL min^{-1} for 25 min. To prepare samples for HPLC analysis, the residual medium after cell harvest was collected at one day intervals, filtered through a 0.22 µm PTFE filter, and transferred to an HPLC vial with or without dilution. The glucose concentration in the residual medium of *Monoraphidium* sp. KGG-9 was quantified using a standard curve ranging from 0.1 to 20 ppm. The maltose concentration in the residual medium of *Hariotina* sp. KGG-18 was quantified against a standard curve ranging from 0.5 to 30 ppm.

Determination of biomass and lipid productivity

Biomass productivity was calculated as $P_{biomass}\ \left(g\ L^{-1}d^{-1}\right) = (X_b - X_a)/T$, where X_a is the biomass concentration on the final day, X_b is the biomass concentration on the initial day, and T is the number of days of cultivation.

Lipid productivity was calculated as $Q_{lipid}\left(\mathrm{mg}\ L^{-1}d^{-1}\right) = P_{biomass} \times C$, where C is the lipid content of the dried biomass harvested at the end of the cultivation.

Statistical analysis

The statistical significance of lipid productivity was determined using the Student's *t*-test. Data are expressed as the mean ± standard deviation.

Results and discussion

Isolation of three novel microalgae strains

Large-scale microalgae cultivation facilities for biodiesel production are typically exposed to the external environment to facilitate solar energy utilization, resulting in irregular cultivation conditions that are strongly influenced by abiotic factors, such as the temperature and amount of sunshine [24]. Therefore, isolating superior species with a high tolerance to various abiotic conditions is crucial for large-scale industrial applications of microalgae [25]. Previously, it has been reported that microalgae isolated from regions with environmental fluctuations have outstanding adaptability to various conditions. It is anticipated that microalgae species are well adapted to various conditions and have a robust capacity for valuable material production in environmentally dynamic region [26, 27]. Thus, we aimed to isolate a novel microalgal species with excellent adaptability and biodiesel production capacity from harsh and environmentally variable areas, such as polluted urban streams.

To isolate novel microalgal strains, sequentially diluted environmental samples were spread onto BG11 agar plates. Separated colonies were clearly obtained in samples diluted 100- to 1,000-fold. To isolate uncontaminated strains, we selected colonies based on several criteria, including robust growth, green color, large size, and clear boundaries. The *18 S rRNA* gene was amplified and sequenced to identify each strain. The results revealed an overall predominance of the genera *Chlorella* and *Desmodesmus*. However, we focused on the other strains, because these two genera are well known to be fertile and have distinct heterotrophic properties [25, 28]. Only three strains did not belong to the genera *Chlorella* and *Desmodesmus* during the initial screening. For further experiments, we finalized three strains that have not been studied and exhibit have distinct morphological and taxonomic characteristics (Fig. 1; Table 1). KGG-7, identified as *Chlamydomonas* sp., showed morphological characteristics similar to those of the well-known laboratory strain *Chlamydomonas reinhardtii* (Fig. 1a). The KGG-9 strain showed an atypical sickle-shaped morphology and was taxonomically identified as *Monoraphidium* sp. by *18 S rRNA* sequencing (Fig. 1b). *Monoraphidium* sp. has emerged as an important strain for biodiesel production because of its high lipid content [29]. KGG-18 cells exhibited multiple unicellular characteristics, with a wrinkled cell surface and cell-to-cell clumping (Fig. 1c). It was identified as *Hariotina* sp., which has not been studied until recently [30]. It is difficult to establish initial culture conditions for newly isolated microalgae that have not been studied extensively. Thus, we considered it suitable for validation of our two-step HTS assay and utilized it in subsequent experiments.

Heterotrophic carbon source screening

To identify the optimal heterotrophic carbon source for microalgae, the preference for carbon sources must be examined for each strain. However, this process is labor-intensive and requires considerable effort. To simplify

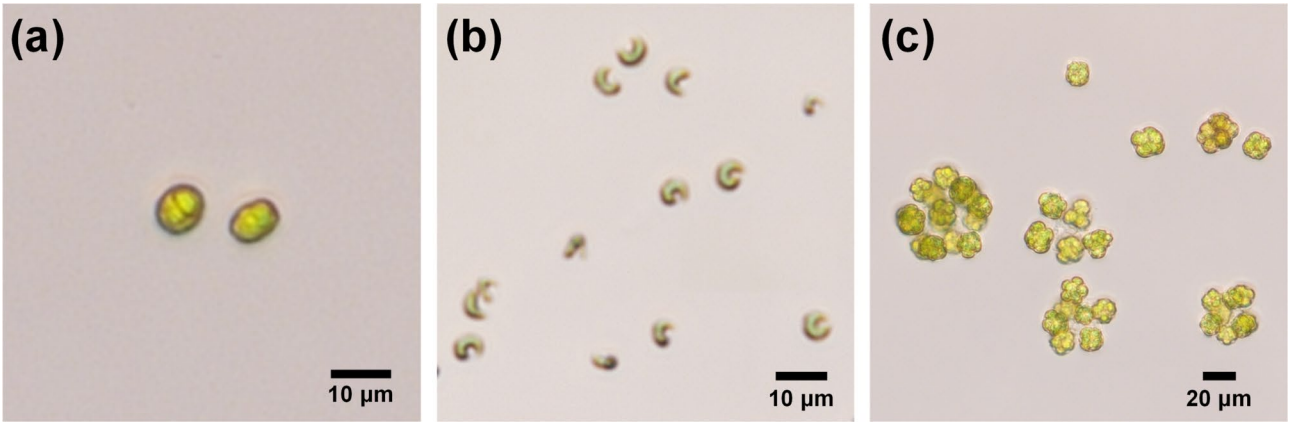

Fig. 1 Light micrograph (400X) of microalgal isolates. **(a)***Chlamydomonas* sp. KGG-7, **(b)***Monoraphidium* sp. KGG-9, and **(c)***Hariotina* sp. KGG-18

Table 1 Identification of isolated microalgal strains

Strain	Closest species (% Identity of 18 S rRNA)	Assembled 18 S rRNA length (bp)	GenBank Accession number
KGG-7	*Chlamydomonas* sp. CCAP 11/132 (99.82%)	1741	OM218994
KGG-9	*Monoraphidium convolutum* strain AS7-3 (99.94%)	1741	OM218993
KGG-18	*Hariotina* sp. QW-2010a strain FACHB-2320 (99.96%)	2792	OM218995

and perform the screening process rapidly, we used a Biolog GENIII microplate, which is a commercially developed microbial profiling system. This microplate has 71 organic substrates in each well, enabling us to analyze the availability of these substrates with a single incubation. To explore its availability, inocula of *Chlamydomonas* sp. KGG-7 and *Monoraphidium* sp. KGG-9, and *Hariotina* sp. KGG-18 were prepared in a flask culture, diluted, and dispensed into each well. After cultivation, different cell growth were observed in each well, and the cell concentrations were determined by absorbance measurements. To normalize the raw values, the absorbance values measured in the wells containing the organic carbon substrate were divided by the absorbance values measured in the wells without the organic substrate and these values are plotted in Fig. 2. The mean and standard deviation of the values are represented in a box plot. We conducted four replicate experiments and measured absorbance twice in each experiment. To identify the organic substrates favorable for heterotrophy, we focused on samples in which the final cell concentration was more than doubled. In *Chlamydomonas* sp. KGG-7, the final cell concentration increased by 2.12-fold only in the presence of α-ketoglutaric acid, whereas none of the other organic substrates caused an increase exceeding two-fold (Fig. 2a). α-Ketoglutaric acid is known as a key intermediate in the tricarboxylic acid cycle and is involved in cellular energy supply and the metabolism of carbon and nitrogen [31]. Generally, α-ketoglutaric acid has been widely studied for dietary supplementation in human health due to its clinical effects [32]. It is used as an agent that increases antioxidant activity for protection against oxidative stress in humans and animals [33]. Recently, it has also been reported that the supplementation of α-ketoglutaric acid in microbial fermentation significantly enhances the production of ε-polylysine, which is a high-value product used as a food preservative, emulsifying agent, and enhancer of anticancer agent, suggesting that α-ketoglutaric acid can be used as a supplement to promote microbial cell growth [34]. However, α-ketoglutaric acid utilization as a carbon source as well as supplements in *Chlamydomonas* has not been reported previously. Therefore, α-ketoglutaric acid was considered unsuitable as a heterotrophic organic carbon source in microalgal culture. The results also showed that similar levels of absorbance for most heterotrophic substrates, suggesting the need for further modification of the conditions for *Chlamydomonas* sp. when utilizing the Biolog GENIII microplate. To further optimize, we excluded *Chlamydomonas* sp. KGG-7 in the subsequent screening experiments.

In *Monoraphidium* sp. KGG-9, numerous organic substrates resulted in a more than two-fold increase in cell concentration. Therefore, we focused on organic substrates that increased the cell concentration by more than four times. When cellobiose, sucrose, and glucose were used, the cell concentration increased by 4.84-fold, 4.83-fold, and 4.40-fold, respectively (Fig. 2b). *Monoraphidium* sp. is considered a promising candidate for biofuel production because of its notable lipid-producing ability, indicating that the heterotrophic properties of *Monoraphidium* sp. have been relatively well studied [29, 35]. In previous studies, glucose has been frequently utilized as an organic substrate for the heterotrophic

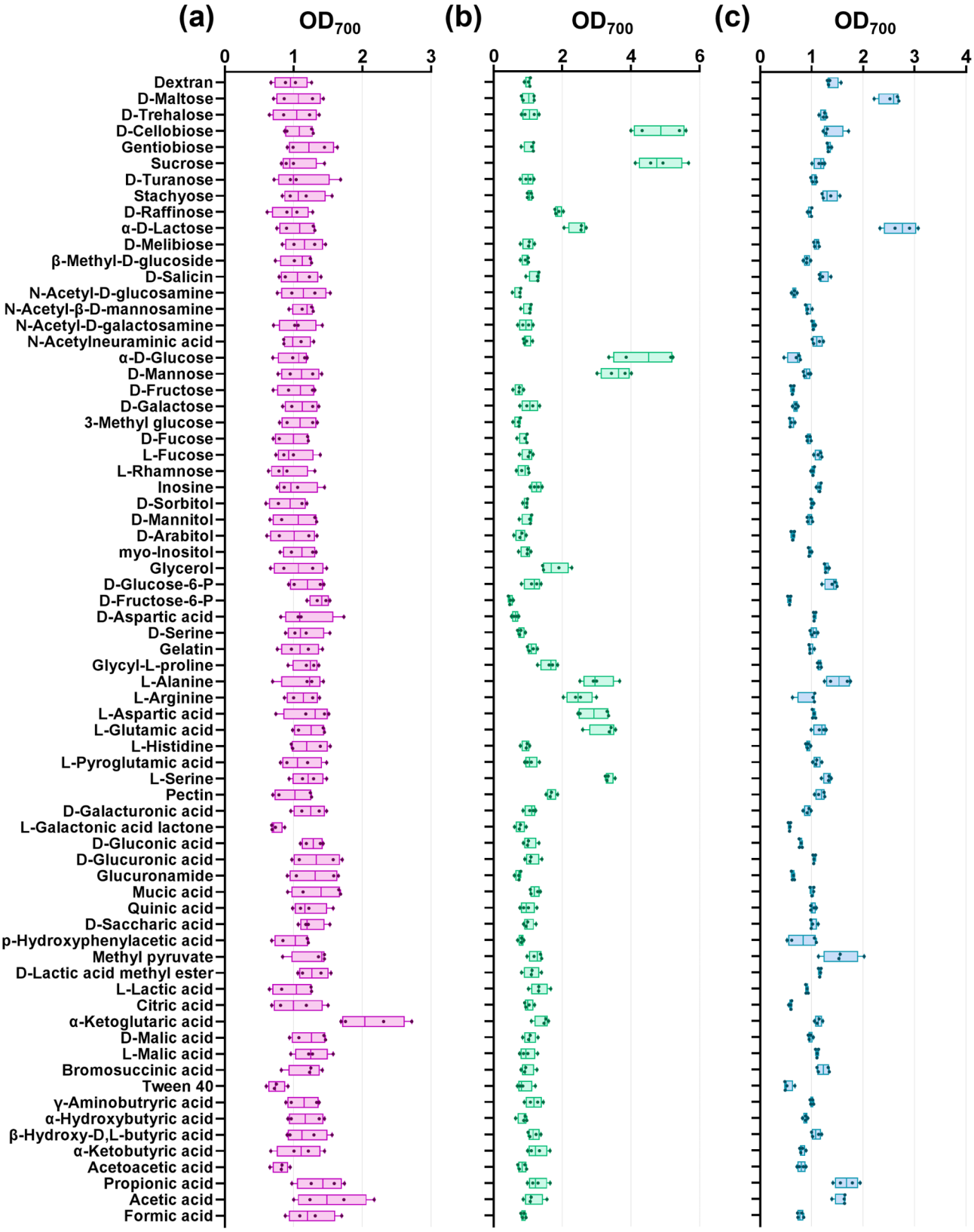

Fig. 2 Heterotrophic carbon source screening for novel microalgae strains. **(a)***Chlamydomonas* sp. KGG-7, **(b)***Monoraphidium* sp. KGG-9, and **(c)***Hariotina* sp. KGG-18. The normalization of raw values was conducted by scaling to each negative control using following formula, OD_S/OD_N. OD_S is the optical density of each well with different organic substrate, and OD_N is the optical density without the organic substrate (negative control). Experiments were conducted in four-repeated and error bars indicate standard deviation of mean

cultivation of *Monoraphidium*; however, the optimal concentration of glucose needs to be further investigated. Compared to sucrose and cellobiose, glucose has several advantages as a substrate for mixotrophic cultivation, such as broad usability, monosaccharide characteristics, and industrial applicability. Thus, we selected glucose as a screening substrate for *Monoraphidium* sp. KGG-9, and optimized its concentration in subsequent experiments.

For *Hariotina* sp. KGG-18, we filtered and selected the substrates that exhibited a double or greater increase in cell concentration. The cell concentrations showed a 2.73-fold increase in the wells supplied with lactose and a 2.53-fold increase in the wells supplied with maltose (Fig. 2c). These results indicate that lactose and maltose can be candidates for subsequent experiments. *Hariotina* was initially classified as a genus distinct from *Coelastrum* based on its morphological characteristics. However, it was later transferred to *Coelastrum* in 1899 and was reinstated as *Hariotina* in 2002 [36]. *Hariotina* sp. has not been well studied, either taxonomically or physiologically, suggesting that it needs to be examined experimentally. To accumulate an experimental dataset and precisely investigate the substrate preferences of mixotrophic cultures, we conducted additional experiments using both lactose and maltose. Overall, we successfully selected the organic substrate candidates preferred by the newly isolated microalgal strain through microplate-based screening with minimal repeated experiments, suggesting that this is a promising and time-efficient approach for the characterization of novel strains.

HTS of temperature and organic substrate concentration

Specific optimization of the cultivation conditions is necessary to maximize the productivity of microalgal biomass and valuable materials. For photosynthetic microalgal growth, abiotic parameters such as temperature, light intensity, and CO_2 supply are generally considered the most important [37]. Temperature is also an important parameter in heterotrophic and mixotrophic cultures. Determining the optimal heterotrophic carbon substrate concentration is crucial because overfeeding of organic substrates can inhibit cell growth [38]. Similar to screening for organic substrate preferences, optimizing culture conditions using a flask-by-flask approach is labor-intensive. To reduce the input labor and conduct a time- and cost-efficient optimization process, we utilized the previously developed PhotoBiobox system [22]. This system comprises a high-throughput photobioreactor that enables the precise control of temperature, light intensity, and gas supply at the microplate scale. To determine the optimal organic substrate concentration and temperature, each row of a 96-well microplate was configured with an organic substrate concentration ranging from 0 to 30 g L^{-1}, and each column of a 96-well microplate was adjusted within the range of 15 to 40 ℃ using the PhotoBiobox. After allowing sufficient time for cell growth, the absorbance of each well was measured to assess microalgal growth in response to the temperature and organic substrate concentration. When glucose was used as the carbon source by *Monoraphidium* sp. KGG-9, relatively high absorbance was observed in the temperature range of 25 to 27 °C and the glucose concentration range of 10 to 20 g L^{-1} (Fig. 3a). The maximum and minimum values over the entire plate were 1.67 and 0.47, respectively, indicating at least a three-fold increase in biomass production as a result of optimal temperature adjustment and organic substrate supplementation. These results represented that the optimal conditions for temperature and glucose concentration may be within ranges showing relatively high absorbance, suggesting that further investigations at the flask scale are necessary for a more detailed optimization.

Maltose and lactose were selected as promising heterotrophic substrates for *Hariotina* sp. KGG-18 through GENIII-based organic substrate screening. Subsequently, we examined to simultaneously determine the optimal substrate concentration and temperature. The absorbance values were comparatively higher in the maltose concentration range of 15 to 30 g L^{-1} and the temperature range of 27 to 31 °C (Fig. 3b). The maximum and minimum absorbance values were 0.61 and 0.19, respectively, suggesting a 3.2-fold increase in biomass production due to temperature adjustment and organic substrate supplementation. These results indicated that these maltose concentrations and temperatures may be optimal for mixotrophic culture of *Hariotina* sp. KGG-18. When lactose was used as the organic carbon source, no significant differences in microalgal cell growth were observed (Additional File 1: Figure S1). This result contradicts the result shown in Fig. 2c, suggesting the potential of lactose as a carbon source. The difference between the results may be attributed to the unclear lactose concentration of the GENIII microplate and the insufficient culture conditions provided by the PhotoBiobox for lactose metabolism. For example, efficient cultivation with lactose requires adequate aeration and agitation [39], but the inner part of the PhotoBiobox is too enclosed, resulting in insufficient aeration. Due to these limitations, lactose was eliminated as a candidate and instead maltose was selected as the optimal heterotrophic substrate for mixotrophic cultivation of *Hariotina* sp. KGG-18. Despite the exceptional case, these results confirmed the optimal heterotrophic carbon source and temperature range through an HTS experiment without labor-intensive and time-consuming processes.

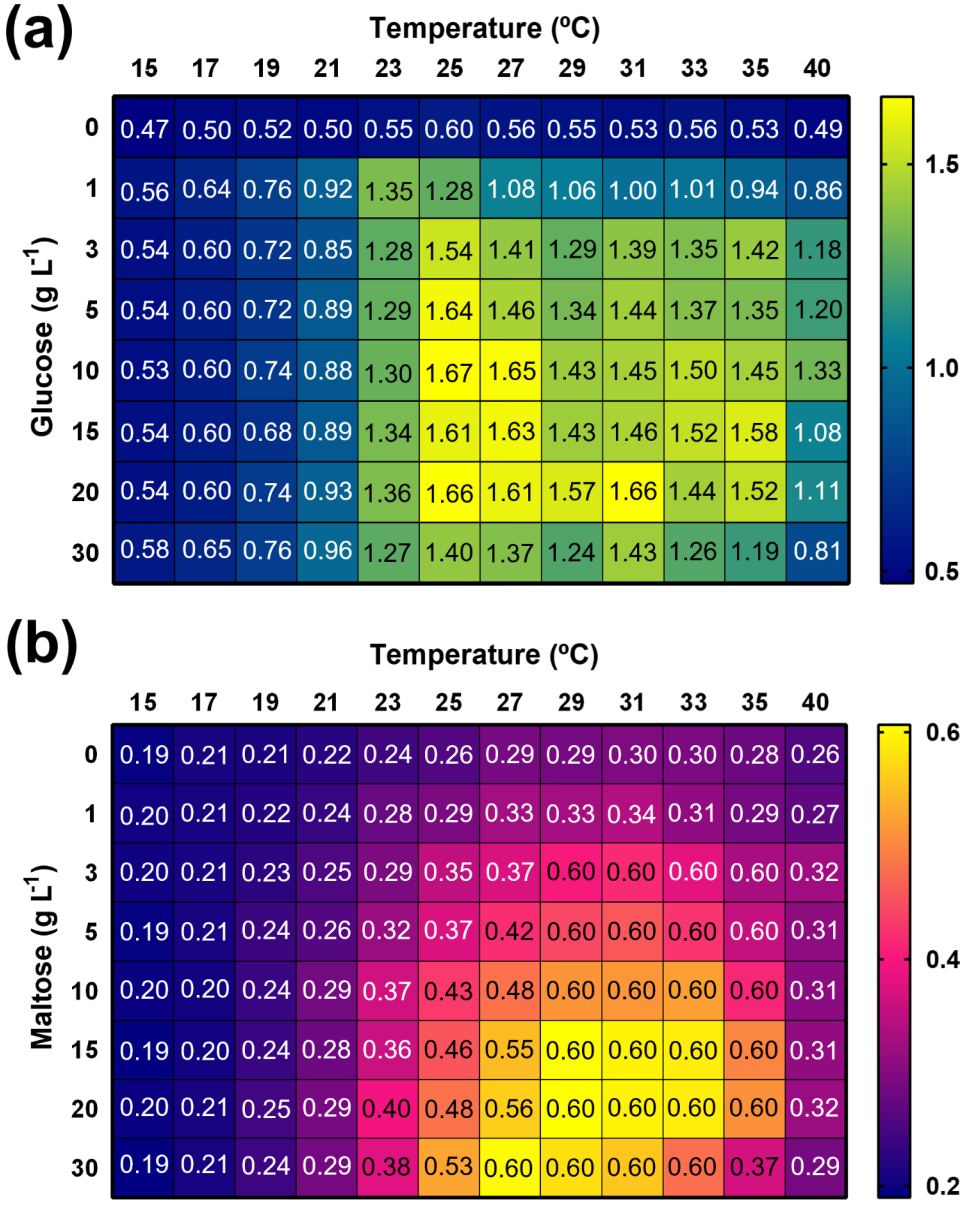

Fig. 3 High-throughput screening of optimal temperature and carbon substrate concentration using PhotoBiobox. The columns were set to range from 15.0 ℃ to 40.0 ℃ and the rows were set to range from 0 to 30 g L^{-1} of substrate concentration. The values represent optical density measured by absorbance at 700 nm. **(a)** Optimization of glucose concentration and incubation temperature for *Monoraphidium* sp. KGG-9. **(b)** Optimization of maltose concentration and incubation temperature for *Hariotina* sp. KGG-18

Validation of the two-step HTS assay

To validate the two-step HTS assay, flask cultivation was performed under the optimal conditions obtained through the two-step HTS assay. The optimal conditions were determined within a specific range; however, flask cultivations were performed at the middle point of the optimal temperature range since precise temperature control down to 1 °C is unlikely to be meaningful in typical incubators. The concentration of organic carbon substrates can be easily adjusted and is very important for cost effectiveness, so we evaluated the maximum and minimum values within the optimal range identified by the screening experiments. In addition, mixotrophic conditions were compared with photoautotrophic conditions to explore the sole effects of organic substrate addition. To investigate the optimal glucose concentration for *Monoraphidium* sp. KGG-9, glucose was supplemented into flasks at concentrations of 0, 10, or 20 g L^{-1} and the flasks were incubated at 26 °C. Microalgal cell growth in the flask culture was determined by measuring the optical density. The sample without glucose supplementation was used as a control. When glucose was provided at 10 g L^{-1} and 20 g L^{-1}, cell growth reached 4.84±0.081 and 4.7±0.177, respectively (Fig. 4a). These

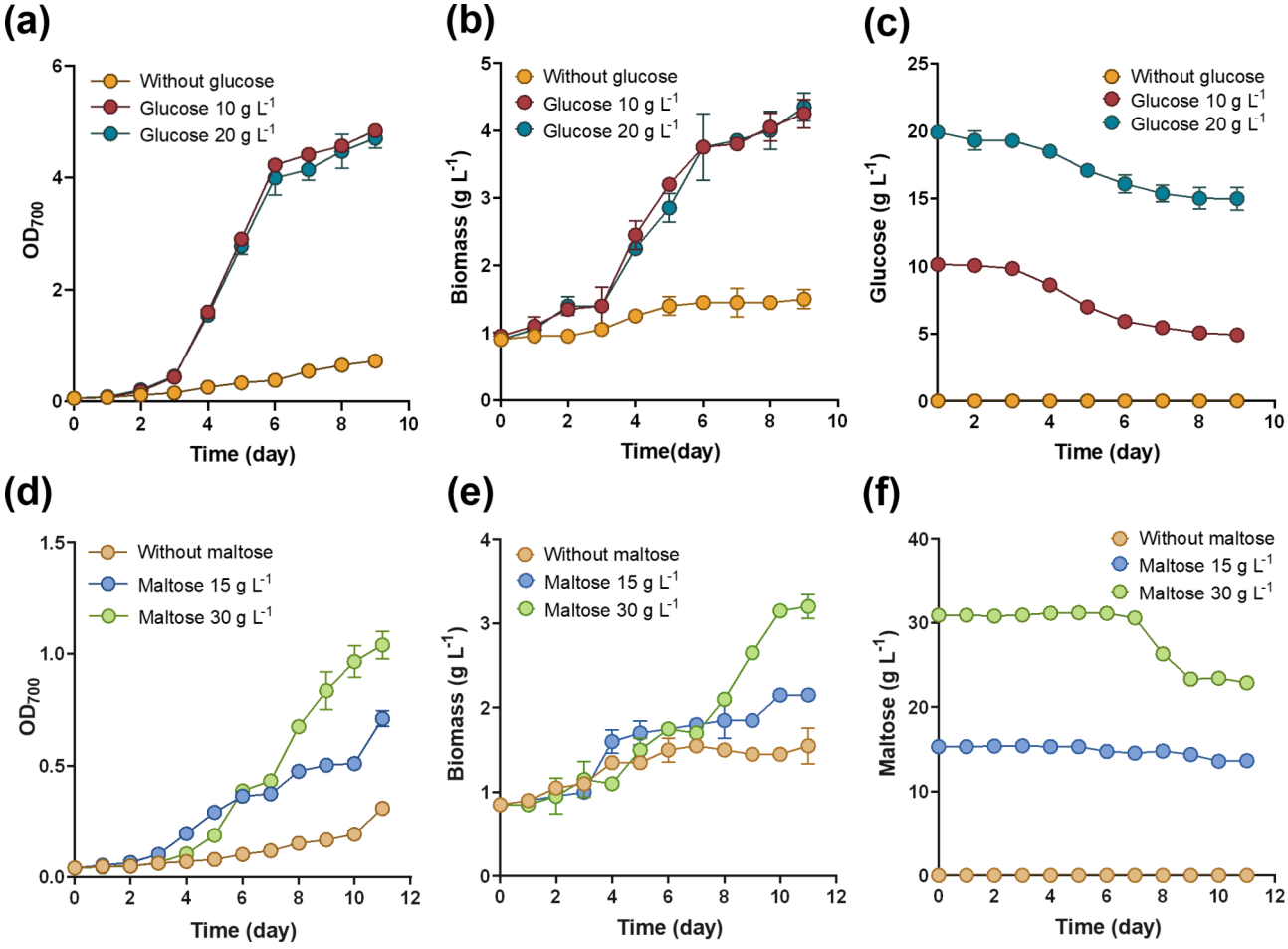

Fig. 4 Comparison of microalgal growth, biomass production, and substrate consumption on flask cultivations. **(a)** Microalgal cell growth by measuring optical density, **(b)** biomass production, and **(c)** glucose consumption in *Monoraphidium* sp. KGG-9. **(d)** Microalgal cell growth by measuring optical density, **(e)** biomass production, and **(f)** maltose consumption in *Hariotina* sp. KGG-18. Experiments were conducted in triplicate and error bars indicate standard deviation of mean

values were approximately six-fold higher than the value of 0.72±0.004 observed in the absence of glucose supplementation. The final biomass production was calculated by measuring the weight of the dried cells. When glucose was provided at 10 g L^{-1} and 20 g L^{-1}, the maximum dried cell weights reached 4.25±0.212 g L^{-1} and 4.35±0.212 g L^{-1}, respectively (Fig. 4b). These values were approximately three times higher than the concentration of 1.5±0.141 g L^{-1} measured without glucose supplementation. When glucose is added, the average time required for *Monoraphidium* sp. KGG-9 to double the biomass is 2 days. However, there was no significant difference in cell growth between the 10 g L^{-1} and glucose 20 g L^{-1} conditions. When glucose consumption was measured, the concentration of 5.09±0.03 g L^{-1} and 5.01±0.85 g L^{-1} decreased in the medium, respectively (Fig. 4c). The growth of *Monoraphidium* sp. KGG-9 dramatically increased with glucose supplementation. However, only approximately 5 g L^{-1} of glucose was consumed during cultivation, regardless of the initial glucose concentration. This result was consistent with the results shown in Fig. 3a, which showed sufficiently high growth at 25 °C and 5 g L^{-1} glucose with no significant differences when compared to glucose concentrations of 10 g L^{-1} and 20 g L^{-1}.

To optimize the organic substrate concentration for *Hariotina* sp. KGG-18, maltose was used in accordance with our preliminary results. Based on the results shown in Fig. 3b, *Hariotina* sp. KGG-18 was cultivated at 29 °C with supplementation of 0, 15, or 30 g L^{-1} of maltose. Microalgal cell growth in the flask culture was determined by measuring the optical density. The sample without maltose supplementation was used as a control. When maltose was supplied at 15 g L^{-1} and 30 g L^{-1}, the cell growth reached 0.71±0.034 and 1.04±0.062, respectively. These values were approximately 2.29-fold and 3.35-fold higher than the value of 0.31±0.012 observed in the absence of maltose supplementation (Fig. 4d). The

final biomass concentrations after supplementation with 15 g L^{-1} and 30 g L^{-1} maltose reached 2.15±0.071 g L^{-1} and 3.2±0.141 g L^{-1}, respectively. These concentrations were also 1.39-fold and 2.06-fold higher than the concentration of 1.55±0.212 g L^{-1} measured in the absence of maltose supplementation (Fig. 4e). When maltose is added at concentration of 15 g L^{-1} and 30 g L^{-1}, the time required for *Hariotina* sp. KGG-18 to double its biomass is 1.47 days and 2.23 days, respectively. When supplied with 15 g L^{-1} of maltose, only 1.65±0.25 g L^{-1} of maltose was consumed, whereas 8.01±0.86 g L^{-1} of maltose was consumed when maltose was provided at a concentration of 30 g L^{-1} (Fig. 4f). Maltose consumption was approximately five times higher when 30 g L^{-1} maltose was supplied. The performance of flask culture in the presence of 30 g L^{-1} maltose was enhanced, in contrast to the comparable growth observed for maltose concentrations of 15 g L^{-1} and 30 g L^{-1} in the PhotoBiobox screening. This was attributed to greater substrate consumption in 30 g L^{-1} maltose condition. However, the increase in biomass was relatively limited compared to the total amount of maltose provided. In this study, we focused on the optimization of organic carbon source utilization based on the general abiotic conditions only varying temperature, so it will remain as further challenges that how to improve the overall conversion yield from substrate to biomass. Several strategies, such as the alteration of microalgal physiology by modulating light, nutrients, and environmental conditions and the engineering by controlling carbon partitioning and energy route, can contributed to improve the efficient carbon conversion [40]. Overall, these findings showed a meaningful correlation between the screening results and flask validation, suggesting that our two-step HTS assay was highly effective at maximizing microalgae biomass production.

To assess the effects of organic substrate supplementation on lipid production, the lipid content and productivity of each microalgal strain were measured (Table 2). The sample without organic carbon substrate supplementation was used as a control. For *Monoraphidium* sp. KGG-9, the lipid content was measured at 47.49±2.11% in the absence of glucose, but 54.03±0.25% and 56.59±1.16% in the presence of 10 g L^{-1} and 20 g L^{-1} glucose, respectively. Compared to photoautotrophic cultivation, lipid content under mixotrophic cultivation slightly increased in *Monoraphidium* sp. KGG-9. In contrast, the lipid content under mixotrophic conditions was similar to or slightly lower than that under photoautotrophic conditions for *Hariotina* sp. KGG-18. When maltose was provided at 15 g L^{-1} and 30 g L^{-1}, the lipid content was measured at 35.31±0.98% and 35.86±0.96%, respectively. In the absence of maltose supplementation, the lipid content was measured at 36.09±1.13%. According to previous reports, supplementation with heterotrophic carbon sources has been shown to reduce lipid accumulation in microalgae [29, 41]. This appears to be consistent with our findings of *Hariotina* sp. KGG-18. In contrast, glucose supplementation promoted lipid accumulation and cell growth in *Monoraphidium* sp. KGG-9, resulting in a more than three-fold increase in final lipid productivity (Fig. 5a). The dramatic increase in lipid production resulting from glucose supplementation is likely due to the redistribution of photosynthetic energy and improved carbon flux. Typically, microalgae generate acetyl-CoA, ATP, and NADPH by photosynthesis in the absence of organic substrates. In contrast, when organic carbon substrates are introduced, the energy consumption responsible for photosynthesis can be redirected to lipid biosynthesis, resulting in enhanced lipid production [42]. In addition, the increased carbon flux associated with additional glucose supplementation can lead to increased levels of glycerol-3-phosphate (G3P), which further promotes lipid biosynthesis [43, 44]. Taken together, organic substrate supplementation would be either positive or negative for lipid content, but this cannot be easily determined without experimental evidence because it involves complex physiological changes in each microalgal species. Nevertheless, heterotrophic supplementation usually enhances cell growth greatly, so that the final lipid productivity would be quite improved.

Indeed, in the case of *Hariotina* sp. KGG-18, the final lipid productivity in mixotrophic cultivation using maltose was increased by up to two-fold, which was attributed to increased cell growth (Fig. 5b). Not only in the case of *Hariotina* sp. KGG-18, but also in the case of *Monoraphidium* sp. KGG-9, the lipid productivity was

Table 2 Comparison of biomass and lipid productivity in novel microalgal strains

Strain	Culture condition	Final biomass yield (g L^{-1})	Lipid content (%)	Biomass productivity (g L^{-1} d^{-1})	Lipid productivity (mg L^{-1} d^{-1})
Monoraphidium sp. KGG-9	Without glucose	1.50±0.10	47.49±2.11	0.17±0.01	78.98±3.954
	Glucose 10 g L^{-1}	4.25±0.15	54.03±0.25	0.47±0.02	255.09±11.53
	Glucose 20 g L^{-1}	4.35±0.15	56.59±1.16	0.48±0.02	273.37±7.74
Hariotina sp. KGG-18	Without maltose	1.45±0.15	36.09±1.13	0.13±0.01	47.69±8.45
	Maltose 15 g L^{-1}	2.15±0.05	35.31±0.98	0.20±0.00	69.04±4.18
	Maltose 30 g L^{-1}	3.20±0.10	35.86±0.96	0.29±0.01	104.26±1.83

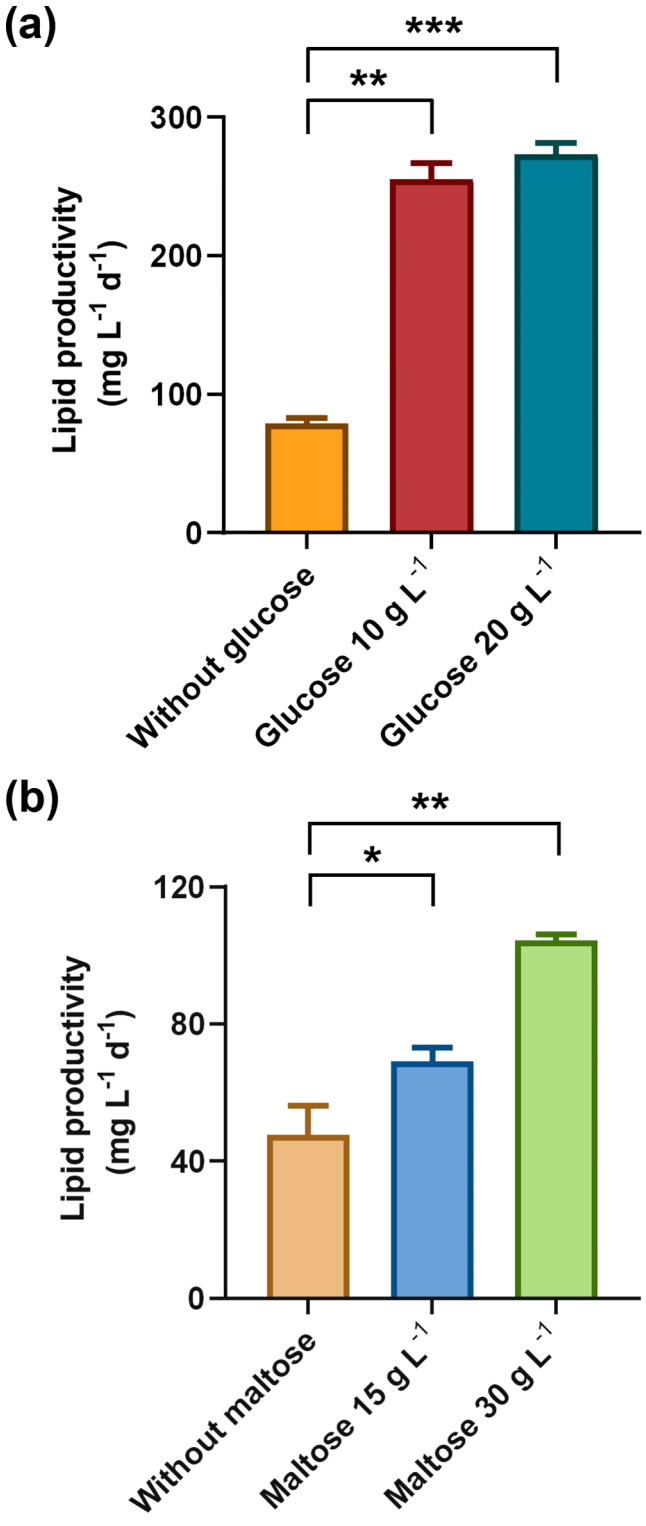

Fig. 5 Comparison of lipid productivity between photoautotrophic and mixotrophic cultivation. **(a)** Lipid productivity in *Monoraphidium* sp. KGG-9. **(b)** Lipid productivity in *Hariotina sp.* KGG-18. Experiments were conducted in triplicate and error bars indicate standard deviation of mean. Asterisks represent statistically significant difference, as determined by a Student *t*-test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

greatly enhanced by the synergistic effect of increased lipid content and cell growth resulting from optimized organic substrate supplementation. In previous studies, it was reported that lipid production was regulated through the combination of temperature and light intensity in *Monoraphidium dybowskii* Y2, achieving a biomass yield of 1.79 g L^{-1} and a lipid productivity of 66.17 mg L^{-1} d^{-1} under autotrophic conditions [45]. *Monoraphidium* sp. FXY-10, which was newly isolated in Lake Fuxian, also exhibited biomass yield at 3.96 g L^{-1} and lipid productivity at 148.74 mg L^{-1} d^{-1} in heterotrophic culture conditions using glucose [29]. Compared to *Chlorella* strains, which are well-known for biofuel production strains, *Monoraphidium* sp. KGG-9 show the higher biomass yield and lipid productivity. *Chlorella zofingiensis* was cultivated in in 60 L flat panel photobioreactors and exhibited a biomass yield of 1.587±0.016 g L^{-1} and lipid productivity of 22.30±0.90 mg L^{-1} [46]. *Chlorella protothecoides* showed a lipid productivity of 177.3 mg L^{-1} through two-stage fed-batch culture using optimized major nutrient conditions involving carbon, nitrogen, and phosphorus sources [47]. In this study, we achieved the biomass yield of 4.35 g L^{-1} and the lipid productivity of 273.37 mg L^{-1} d^{-1} in *Monoraphidium* sp. KGG-9 through the comprehensive optimization of organic carbon substrate supplementation, considering the type and concentration of substrate and temperature at the same time. In addition, fortunately, the novel strain KGG-9 also seems to have its own inherently superior performance, which allows achieving the highest lipid product record in microalgal species ever reported. These results indicate that our two-step HTS assay is practical for investigation of optimal culture conditions which can maximize the productivity of biomass and target products. *Hariotina* sp. has not been extensively studied so far; however, our results provide valuable experimental data for further research. This suggests that our two-step HTS assay can be utilized to rapidly investigate the cultivation characteristics of unexplored microalgae. In summary, the two-step HTS assay was effective at enhancing lipid productivity by optimizing mixotrophic culture conditions, suggesting the enhancement of the industrial applicability of novel microalgae.

Table 3 Comparative analysis of two-step HTS assay and flask-based conventional method

Parameters	Two-step HTS	Conventional*
Number of materials for 71 different substrate types screening (in triplicate)	3	213
Number of materials for substrate concentration screening (in triplicate)	3	24
Number of incubators for 12 different temperature screening	1	12
Volume of media required for overall process (mL)	28.8	8350
Estimated time of total screening for overall process (day)	8	150
Estimated cost of consumables for overall process [$] (USD)	21.30	599.60
Estimated cost of workforce for overall process[#] (USD)	464	8700

[*] Calculated based on a volume of 50 mL in a 125 mL flask, which could accommodate 25 flasks in one incubator, JSSI-200CL (JSR, Gongju, South Korea)

[$] For consumables for the overall process, BG-11 medium (Sigma-Aldrich Co., Saint Louis, MO, USA), a 125 mL Erlenmeyer flask with a vent cap (Corning, Corning, NY, USA), a GENIII plate (Biolog, Hayward, CA, USA), and 96 well plate (Thermo Fisher Scientific, Waltham, MA, USA) were used

[#] Calculated based on US federal minimum hourly wage (US$ 7.25)

Typically, the capacity of microalgae to utilize heterotrophic carbon sources is highly dependent on the microalgal strain [48]. In industrial-scale production, light independence through the supplementation of organic sources can considerably reduce production costs and space requirements [49]. This suggests that the novel microalgal strains isolated in this study have the potential to be utilized as industrial microalgal strains due to they showed excellent growth and lipid production using heterotrophic sources. To further minimize the costs of industrial bioprocesses using microalgae, it is essential to substitute the current heterotrophic carbon substrate with low-cost carbon sources, such as waste feedstocks. Food wastes, including wastewater from food processing, anaerobic digestion wastewater, and food residues, are considered sustainable growth media for microalgal biorefinery production [50]. *Monoraphidium littorale* exhibited significantly higher biomass production and lipid content when cultured with vegetable waste, such as digested rotten potato supernatant, than with the control medium [51]. The newly isolated *Monoraphidium* sp. SVMIICT6 has also been used to efficiently treat dairy wastewater, resulting in substantial biomass accumulation and elevated proportions of lipids and carbohydrates [52]. The nutrient-rich microalgae biomass produced through wastewater treatment can be utilized in various applications, such as biofertilizers and value-added products. Based on these findings and our study, *Monoraphidium* sp. KGG-9 is a suitable novel microalgal strain due to its high biomass and lipid productivity, as well as its potential for utilizing low-cost and sustainable substrates. It is also expected that further research utilizing sustainable low-cost substrates will additionally enhance the industrial applicability of newly microalgae strains.

To confirm the economic and temporal efficiencies of the HTS assay, we compared the two-step HTS assay with the conventional flask-based optimization method in terms of consumable materials and costs (Table 3). In conventional methods, a substantial number of materials, including experimental equipment, media, and incubators, are required to optimize the different carbon substrate types, concentrations, and temperatures. However,

the two-step HTS assay can handle diverse conditions simultaneously, thereby significantly reducing the amount of material and equipment required. The total duration of the optimization process using the two-step HTS assay was 8 days, whereas the flask-based method took 150 days. The estimated consumable cost for the entire optimization of the HTS assay was USD 21.30, whereas the flask-based strategy incurred a cost of USD 599.60. Additionally, the workforce cost was calculated based on time considerations for the overall optimization process. The two-step HTS required USD 464, indicating a significant reduction compared to the workforce cost of USD 8,700 for flask-based optimization. The HTS assay can effectively address several issues associated with conventional flask-based methods, including the management of a large number of flasks, demands for labor and resources, limited cultivation space, and the risk of contamination due to prolonged cultivation periods [53, 54]. It also reduces the time required for sample preparation and enables the rapid detection of cell growth. Furthermore, these advantages also suggest that HTS assays are highly beneficial for strain selection. The HTS method allows for the rapid identification of optimal nutrient conditions across a large number of strains, reducing the number of experiments by more than 2,000 times [19]. Through this, the method allows for rapid and comprehensive exploration of different experimental conditions, which can minimize the overall experimental time and lead to significant cost savings [55]. Several challenges, such as variations between 96-well conditions and difference arising from scale-up processes, remain to extend the HTS assays and optimized conditions to industrial applications. To address these, integration of HTS assay and continuous automated experimental operations can be minimize error and increase consistency between experiment [56]. Machine learning-integrated hybrid optimization method can also be used to compensate for differences between experimental conditions and perform more fine-grained condition exploration [57]. Based on these results, the two-step HTS assay has spatial, temporal, and cost advantages over the conventional method, indicating that it is expected to be effectively utilized in the pre-optimization of industrial processes. Furthermore, this practical strategy can be applied to other industrial microorganisms and microalgae, indicating the potential to expand its usage across various industrial sectors.

Conclusions

The two-step HTS assay described herein was shown to be an effective strategy for optimizing microalgal mixotrophic conditions to achieve optimal production of biomass and lipids. Using this assay, we optimized the mixotrophic culture conditions for novel microalgal strains, confirming a significant enhancement in biomass and lipid productivity. While this approach is partly dependent on the microalgal species, the variety of organic substrate, and the instruments employed, it is undoubtedly a time- and cost-effective strategy compared to conventional optimization methods. Furthermore, it can simplify the transition from laboratory-scale experiments to field applications, suggesting its potential to contribute significantly to the industrial applicability of microalgae.

Supplementary Information

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Supplementary Material 1

Author contributions

Y.J.L. and H.-S. K. jointly supervised the project and reviewed the manuscript. S.-B.P., Y.R.L., J.-H.Y., and H.I.C. contributed equally to this work by designing and performing experiments and writing the manuscript. E.J.S. performed flask cultivation and statistical analyses. D.-H.C. and D.-Y.C. performed material preparation, data curation, and visualization. All authors provided remarks on the work and reviewed and proofread the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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