

A mixotrophic preculture strategy enhances biomass and astaxanthin productivity of *Chromochloris zofingiensis*

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Abstract

Background

Chromochloris zofingiensis is a competitive potential source of natural astaxanthin, as the alternative of *Haematococcus pluvialis*. However, its intracellular astaxanthin synthesis is inhibited under heterotrophic mode, which impedes the establishment of its ultra-high-density cultivation for astaxanthin industrial production.

Results

Preculture can provide a buffer for cells to acclimate drastic condition transformations and achieve a greater physiological state. An original mixotrophic preculture (MP) strategy established in this study successfully increased the heterotrophic biomass concentration of *C. zofingiensis* up to 121.5 g L^{-1} in a 20 L fermenter, surpassing the highest high-density record. Meanwhile, the astaxanthin productivity achieved $0.111 \text{ g L}^{-1} \text{ day}^{-1}$, which is 7.3-fold higher than the best record of *C. zofingiensis*, and even exceeded that of *H. pluvialis*. The molecular mechanisms of *C. zofingiensis* under MP were further investigated using carbon partitioning model, transcriptome, and ^{13}C tracer-based metabolic flux analysis. The results revealed that MP promoted protein degradation for lipid synthesis under heterotrophic cultivation. It also enhanced the expression of genes related to central carbon metabolism, thus providing sufficient energy for biomass accumulation. The metabolic flux to lipids and carotenoids under MP was elevated, providing abundant precursors for astaxanthin biosynthesis.

Conclusions

This study firstly proposed a MP strategy to regulated the physiological state of *C. zofingiensis* cells before fermentation, thus realized its heterotrophic high-density growth and increase the astaxanthin yield on a pilot scale. This strategy exhibits great potential to be utilized in astaxanthin industrial production and more microalgal related industry.

1. Background

Astaxanthin exhibits broad market prospects in aquaculture, cosmetics, nutraceutical industries, and food supplements, with exceptional antioxidant power and pharmacological effects on diseases [1]. Among natural astaxanthin derived from various sources, microalgal astaxanthin is suitable for human absorption and utilization because its structure is consistent with that of aquatic animals [2].

Haematococcus pluvialis is the richest microalgal source and important commercial producer of natural astaxanthin, and another typical species as a potential alternative is *Chromochloris zofingiensis*, which can grow fast phototrophically, heterotrophically, and mixotrophically [3]. Its heterotrophic culture can

avoid the lack of energy caused by insufficient illumination, leading to a faster growth speed, higher cell density, and shorter fermentation period [4].

High-density growth and substance accumulation are hinged on the industrial production of astaxanthin. Astaxanthin synthesis was identified as a protective response of photo-oxidative stress stimulated by the over-reduction in the photosynthetic electron transport chain [5]. Inevitably, the heterotrophic astaxanthin content is inferior to that of the autotrophic mode, which impedes the establishment of ultra-high-density cultivation technology. Thus, current study of *C. zofingiensis* emphasizes reaching the maximal biomass concentration to achieve a more competitive yield. However, sudden changes in culture conditions may limit cell viability, and the biomass in early generations after inoculation into heterotrophic media is not satisfactory, which is known as the lag phase [6]. To eliminate this bottleneck, some researchers have chosen to change the C/N ratio of the medium or use various growth hormones [7, 8]. A two-step strategy was designed to increase heterotrophic biomass to 235 g L^{-1} ; however, it took a lengthy period [5]. Another strategy that successfully enhanced the biomass used an alternation of heterotrophic and phototrophic growth, requiring more complicated production technology and higher cost [9]. A more efficient preculture strategy is proposed to avoid defects in multistep strategies. Preculture is a measure that provides a buffer for acclimating cells to drastic transformations of nutrient sources or the environment. A preculture condition is usually a transitional state between the original and target conditions [10]. By several generations of preculture, cells can achieve a greater physiological state under target conditions, thus accelerating cell growth and shortening the lag phase in the following culture [6].

This study is the first to propose a preculture strategy to enhance the heterotrophic high-density growth of *C. zofingiensis* and utilize in astaxanthin industrial production. *C. zofingiensis* cells were precultured under autotrophic, mixotrophic, and heterotrophic modes and then transferred to heterotrophic conditions. The growth characteristics, cell composition, and astaxanthin productivity of the cells in each strategy were compared. Protein, carbohydrate, and lipid carbon fluxes in the cells were explored by establishing a carbon distribution model. The transcriptome was analyzed to investigate the effect of preculture on astaxanthin accumulation mechanism. ^{13}C tracer-based metabolic flux analysis (^{13}C -MFA) was used for exploring global relationships among astaxanthin biosynthesis, lipid biosynthesis, and central carbon metabolism. Finally, a fed-batch culture was established to amplify culture scale of *C. zofingiensis* and further improve its biomass and astaxanthin productivity.

2. Results

2.1 MP strategy increased astaxanthin productivity

In batch culture, cells were precultured and inoculated into a heterotrophic medium to investigate dynamic changes in cell growth. From the first preculture generation, MP and HP demonstrated the excellent ability to promote biomass accumulation and respectively raised final biomass concentration up to 1.95 g L^{-1} and 1.70 g L^{-1} (Fig. 1A). Figure 1B showed the growth curve of heterotrophic *C. zofingiensis* in the exponential growth phase. Different preculture strategies bred similar grow profiles,

with obvious disparities in the final biomass and growth rate. MP group maintained the highest growth rate, with final biomass almost double that of AP. The batch culture revealed that MP improved the physiological state of *C. zofingiensis* and stimulated rapid growth at laboratory level, making it an optimal strategy for biomass production.

The contents of the significant components in *C. zofingiensis* cells were monitored during heterotrophic culture. Astaxanthin accumulation is closely related to photosynthesis, the heterotrophic cells deprived of light are destined to master a lower astaxanthin synthesis activity. The final astaxanthin content of MP and HP groups was slightly lower than that of AP (Fig. 1C), proving that their promotion effect on astaxanthin synthesis and accumulation was not as good as that of AP. However, the ultimate productivity is affected simultaneously by the intracellular astaxanthin content and biomass concentration. Due to the remarkable biomass, astaxanthin productivity under MP attained $2.03 \text{ mg L}^{-1} \text{ day}^{-1}$, reaching the highest level at laboratory scale. Once this strategy is successfully implemented at the industrial level, it holds great promise for further scaling up productivity.

Overall, protein content decreased during cultivation, whereas carbohydrate and lipid contents continued to increase over time. The final contents of the main carbon substances (Fig. 1D) showed that MP and HP both stimulated protein degradation.

2.2 MP strategy altered carbon partitioning

A kinetic model of carbon distribution was established to investigate the behavior of proteins, carbohydrates, and lipids in different preculture strategies. The model appropriately fit the experimental data ($R^2 > 0.90$), and its coefficients were listed in Table 1. The values of Y_{PX} and Y_{CX} under three strategies were maintained at a stable level, indicating that these strategies maintained similar capacity of assimilating substances for protein and carbohydrate accumulation. Under MP, D_p reached its highest value, and a_c showed remarkable improvement compared to AP, revealing the best capacity of MP to promote protein and carbohydrate degradation towards lipids or other components. μ_L was elevated to its highest level under MP, which showed a tendency to increase lipid biosynthesis. MP changed the intracellular carbon flux path, promoting rapid glucose metabolism and direct carbon flux from proteins towards lipids without experiencing carbohydrates.

Table 1
Coefficients of the model for carbon partitioning.

Coefficient	AP	MP	HP
$Y_{PX} (\text{g g}^{-1})$	0.2534	0.2469	0.2513
$\mu_P (\text{h}^{-1})$	0.0601	0.0875	0.0720
$D_P (\times 10^{-4} \text{ h}^{-1})$	1.294	2.061	1.734
$Y_{CX} (\text{g g}^{-1})$	0.3607	0.3611	0.3675
$\mu_C (\text{h}^{-1})$	0.0292	0.0330	0.0298
$\alpha_C (\text{h}^{-1})$	0.0045	0.0051	0.0076
$\mu_L (\text{h}^{-1})$	0.0155	0.0237	0.0204
$\mu (\text{h}^{-1})$	0.0165	0.0270	0.0218

The model revealed dynamic changes in intracellular carbon storage during heterotrophic batch culture, and further explained the regulatory effect of MP strategy on cell components and physiological state. MP applied to *C. zofingiensis* ensures sufficient utilization of carbon source and avoids unnecessary intermediate metabolic processes and energy loss, which endows it with the capacity to realize efficient industrial production of lipids or other valuable products, thus becoming a competitive strategy for medical care, biological health products, and biofuels.

2.3 MP strategy influence pigment and fatty acid compositions

As shown in Fig. 2A, the preculture strategies maintained a significant impact of pigment composition. While chlorophyll (Chl) a content showed a decreasing trend in AP, MP, and HP, the contents of Chl b and carotenoids increased accordingly. As the main pigment for photosynthesis, the reduced Chl a content reflects the inhibition of photosynthesis under MP. Chl a can be successively converted into Chl b and carotenoids, which can reduce reactive oxygen species (ROS) in cells when exposed to stress, and provide abundant precursors of astaxanthin [11]. However, decreased astaxanthin production under MP indicated that the transformation from carotenoids to astaxanthin was restricted due to reduced photosynthesis.

As crucial high-value components, microalgal lipids enriched with fatty acids (FAs) are important materials for various microalgae-based industries [12]. Figure 2B showed the FAs composition of heterotrophic *C. zofingiensis* cells. In all three groups, C16:0, C18:1, and C18:2 were the most common FAs; however, their specific distributions varied. FAs with the same chain length tended to have lower unsaturation under MP, which might be the consequence of increased respiration, as polyunsaturated

fatty acids (PUFAs) can act as antioxidants to effectively quench ROS formed during respiration and adjust the REDOX state of cells [13]. MP effectively promoted the oxidation of C18:0 to C18:1 (Fig. 2B). FAs, such as C16:0 and C18:1, accumulated under MP are preferred for their important biofuel properties. The saturated fatty acid content provides the biofuel a higher cetane number, and enhanced its stability. The presence of monounsaturated fatty acids (MUFAs) contribute to improving cold flow properties, while moderate PUFAs help in low-temperature properties and oxidative stability [14]. C18:2 is considered essential for human nutrition and is also an intermediate molecule of long-chain FAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The remarkable C18:2 content demonstrated the excellent potential of MP in healthcare industry. In comparison, C18:3 content of AP was prominent because its synthesis is closely related to photosynthetic carbon assimilation [15]. Analysis of fatty acids showed that *C. zofingiensis* was a potential feedstock for industrial products with strong competitiveness (biofuels, dietary, animal feeds, medication, and cosmetics).

2.4 MP strategy promoted gene expression of lipid and carotenoid biosynthesis

Transcriptome was analyzed to illuminate the effects of preculture patterns on intracellular metabolism. Total RNA was extracted for three biological replicates from precultured cells in three groups. A total of 14,785 genes were detected. Pearson correlation coefficient and PCA analysis reflected high reproducibility among biological replicates (Fig. 3A, B). The changes in transcript abundance between groups were expressed as log2FoldChange (FC), and genes with $\log_2FC \geq 1$ or ≤ -1 , and $p_{adj} \leq 0.05$ were regarded as DEGs. 5782 and 5347 genes were identified as DEGs in AP/MP and HP/AP samples, respectively.

2.4.1 The transcriptional impact of central carbon metabolism

Genes encoding crucial enzymes of central carbon metabolism were delved. NADPH can be generated from the photosynthetic pathway or the oxidative pentose phosphate (OPP) pathway, the latter is strongly associated with FAs and astaxanthin accumulation. The expressions of 6-phosphogluconolactonase (PGLS2) and 6-phosphogluconate dehydrogenase (6PGD) in OPP pathway increased under MP (Fig. 3E). Since OPP pathway has long been recognized as the main source of NADPH and ATP under stress conditions, the up-related expression of these genes elevated the NADPH content, which led to increasing reductant pool and enhanced lipid and astaxanthin synthesis in *C. zofingiensis* [16].

Acetyl-coenzyme A (AcCoA) plays a major part in TAG accumulation. It can be synthesized in chloroplasts through pyruvate (PYR) decarboxylation with pyruvate dehydrogenase complex (PDHC) or conversed from acetate catalyzed by acetyl-CoA synthetase (ACS) [17]. Most PDHC and ACS genes were strongly up-regulated in MP (Fig. 3E), indicating them the main enzymes contributing to MP-induced TAG accumulation.

TCA cycle obtains rigidity and significance in cell metabolism, as it is the precursor source for biosynthetic pathways that provide essential substances and sufficient energy for various physiological activities [18]. The expression of related genes was generally elevated under MP, including citrate synthase (CIS2), isocitrate dehydrogenase (IDH1, IDH2), 2-oxoglutarate dehydrogenase (OGDH1, OGDH3), succinyl-CoA synthetase (SCS), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH), indicating that TCA cycle provided more energy to support the substance synthesis.

Generally, genes involved in glycolysis and gluconeogenesis got promoted expression. Enolase (ENO) catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP), and pyruvate kinase (PK) is responsible for PYR synthesis [19], their augmented expression under MP can result in higher availability of PYR, an essential precursor of carotenoid and lipid synthesis.

2.4.2 The transcriptional impact of TAG biosynthesis

The expression of the genes appertained to TAG and carotenoid synthesis were identified, and their transcriptional levels were analyzed. As substrates for TAG production, the entire de novo synthesis pathway of FAs was upregulated under MP, within which the transcription of most enzymes was stimulated, including 3-hydroxyacyl-ACP-dehydratase (HAD), enoyl-ACP reductase (ENR), and stearoyl-ACP desaturase (SAD). Thus, de novo FA synthesis is the major source of acyl substrates, and TAG is synthesized by esterification of FAs in Kennedy pathway. As two of the key enzymes limiting the rate of TAG biosynthesis, the expression level of phosphatidate phosphatase (PAP3, *Cz16g11240*) was higher under MP (Fig. 3C), and the expression of diacylglycerol acyltransferase (DGAT1A, DGTT5, DGTT6, and DGTT7) was significantly upregulated under MP among the 10 isoforms, which could further promote TAG accumulation. The differential expression of DGAT homologs is linked to functional divergence.

In contrast, glycerol-3-phosphate acyltransferase (GPAT1, *Cz11g03260*) and PAP2 (*Cz10g16040*) expressed at lower levels for MP might down-regulated the overall expression of genes involved in Kennedy pathway and inhibit the transformation of glycerol-3-phosphate towards phosphatidic acid, thus leading to the accumulation of AcCoA.

2.4.3 The transcriptional impact of astaxanthin biosynthesis

In *C. zofingiensis*, the genes of all enzymes in the 2-C-methylerythritol 4-phosphate (MEP) pathway were identified [20]. The transcription levels of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (CMS), and 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MCS) were lower under MP (Fig. 3D). Thus, the synthesis activities of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the precursors for carotenoid synthesis, were significantly suppressed. Transcription of carotenoid isomerase (CRTISO3) was elevated under MP, whereas that of geranylgeranyl diphosphate synthase (GGPPS), phytoene synthase (PSY), and phytoene desaturase (PDS) was significantly lower, indicating a decline in the synthesis of a range of pigment precursors (Fig. 3D).

Astaxanthin biosynthesis requires terpenoid backbones from lycopene. Under AP, the upregulation of lycopene beta cyclase (LCYb) redirected lycopene to β -carotene, and beta-carotene hydroxylase (BKT1) reallocated zeaxanthin to astaxanthin. In comparison, the transcription levels of most identified genes in this process were decreased under MP, among which the decline of lycopene epsilon cyclase (LCYe) and cytochrome P450 beta hydroxylase (CYP) was more dramatic, which indicated the restraint of the flux of the terpenoid backbone towards astaxanthin biosynthesis. Transcript abundance of BKT1 and long-chain-alcohol O-fatty-acyltransferase (AAT) were substantially reduced under MP, which limited β -carotene finally converted into astaxanthin.

Transcriptome analysis revealed that MP strategy provided sufficient precursor materials and energy for astaxanthin and TAG synthesis; however, the synthesis activity of astaxanthin was generally lower than that of AP because of the downregulated expression of related genes, thus leading to a decline in astaxanthin content.

2.5 MP strategy altered metabolic flux of lipid and carotenoid biosynthesis

^{13}C -MFA was exploited to explore the metabolic pathway rate of *C. zofingiensis* cells under different preculture strategies. In general, the absorbed glucose is guided into the Embden–Meyerhof pathway (EMP) and pentose phosphate (PP) pathway, providing ATP and various precursors for cell growth and division. PP flux showed no significant difference under these strategies (Fig. 4). As for EMP, the conversion of 3-phospho-D-glycerate (3PG) and PEP was significantly accelerated under MP, which boosted AcCoA accumulation, potentially enhanced the carotenoid and lipid synthesis ability of *C. zofingiensis*, and channeled carbon flow rapidly into TCA cycle. The activity of each reaction within TCA cycle was obviously enhanced by MP, which indicated that *C. zofingiensis* relies on the respiration for energy production and carbon availability augmentation under the restriction of photosynthesis.

Anaplerotic reactions in cells can balance the rates of EMP, PP pathway, and TCA cycle, thus potentially promoted the accumulation of key substances. The increased fractional percentage of PYR from malate accelerated NADPH production for lipid synthesis under MP (Fig. 4). Moreover, as the key precursor of carotenoid synthesis, PYR got accelerated generation and potentially improved availability under MP. As more AcCoA accumulated, PEP can be directly converted into oxaloacetate (OAA), which then participated in TCA cycle. Since this conversion is reversible, the fractional percentage of PEP from oxaloacetate was also elevated, thus providing abundant carbon molecules for lipid biosynthesis.

Metabolic fluxes in FAs synthesis pathway varied slightly among the three groups. Compared to AP, AcCoA synthesis was more active under MP; however, the synthesis of FAs did not completely fit previous assumption (Fig. 4). The synthesis of C16 FAs was mildly inhibited by MP, whereas that of C18 did not demonstrate obvious superiority. This might be because most of the energy from TCA cycle was used to maintain cell metabolism or lost as heat dissipated by respiration, which prompted NADPH oxidase to produce ROS, and less NADPH was used for lipid synthesis.

Carotenoid synthesis activity under MP was significantly worse than that under AP (Fig. 4), which certainly limited the rapid synthesis of astaxanthin. In combination with transcriptome analysis, the lack of precursors and decreased activity of enzymes regulated by genes with lower expression levels jointly resulted in a decline of intracellular astaxanthin content under MP.

MP strengthened TCA cycle and remediation reaction performance, thus significantly increasing the synthesis rate of the precursors of lipids and astaxanthin and producing a large amount of NADPH that could be used for lipid synthesis. However, owing to the enhancement of respiration and the limitation of carbon flow direction, the synthesis rate of various FAs did not show remarkable superiority over that of AP. For astaxanthin synthesis, the MP group had limited synthesis ability caused by the dual factors of decreased gene expression and lack of precursors.

2.6 MP strategy increased biomass and astaxanthin productivity by fed-batch culture

To further increase biomass, this study designed exponential fed-batch cultures after preculture at different scales. Initial conditions were set as follows: $X_0 = 0.45 \text{ g L}^{-1}$, $V_0 = 110 \text{ mL}$ (in 250 mL Erlenmeyer flasks), $C_{FG} = 400 \text{ g L}^{-1}$, $C_{FN} = 97.6 \text{ g L}^{-1}$ and $C_{FP} = 13.1 \text{ g L}^{-1}$. As obtained in previous study, $Y_{XS} = 0.5984 \text{ g g}^{-1}$ and $\mu = 0.03 \text{ h}^{-1}$ [21]. Therefore, the Eq. (1) can be simplified as $V_{FS} = 0.006204 \exp(0.03t)$. Under these conditions, *C. zofingiensis* cells maintained the highest specific growth rate to maximize biomass production. Utilization of this model resulted in an obvious increase in biomass compared to batch culture (Fig. 5A). MP showed remarkable capability of promoting cell growth, leading to a nearly 10 times higher biomass concentration than that of AP. Laboratory-scale fermentation confirmed that MP might be the most promising preculture strategy for industrial fed-batch production of *C. zofingiensis*.

Ultimately, the fed-batch culture was developed in 20 L fermenters. As the culture volume expanded, the model converted to $V_{FS} = 0.6204 \exp(0.03t)$. At the endpoint of fermentation, MP biomass concentration successfully increased to 121.5 g L^{-1} (Fig. 5B), and the specific growth rate of exponential growth phase cells was observed (Fig. 5C). To date, the highest reported biomass has not exceeded 100 g L^{-1} , and the increased biomass obtained under MP has undoubtedly surpassed this number. MP provided sufficient material for the acquisition of astaxanthin and endowed *C. zofingiensis* with a greater value for industrialization.

Although the astaxanthin content of MP cells showed the same decreasing trend as that in Fig. 1C, the final astaxanthin productivity achieved $0.111 \text{ g L}^{-1} \text{ day}^{-1}$, which was ascribed to the vast biomass accumulation (Fig. 5C). This yield was 7.3-fold higher than the best record of *C. zofingiensis* [3], and even exceeded that of *H. pluvialis* [22]. The results strongly demonstrated the superiority of the combination of MP and large-scale fed-batch culture, which has mastered the international pioneering level in industrial production of natural astaxanthin.

3. Discussion

Microalgae maintain sophisticated self-regulatory mechanisms to survive in different environments. Before heterotrophic fermentation period, the usage of MP strategy in this study facilitated physiological acclimation of *C. zofingiensis* by altering the uptake pattern of carbon source. MP improved the physiological state of *C. zofingiensis*, stimulated its rapid growth from the first preculture generation, then achieved faster growth rate and higher biomass with altered intracellular biochemical composition in heterotrophic batch culture (Fig. 1). The sharp alternation in biomass demonstrated the superiority of glucose as an organic carbon source for promoting cell growth, and MP further strengthened the absorption and metabolism of glucose (Table 1).

The dramatic change of MP-induced cellular compositions not only covered the adjustment of the ratio of proteins, lipids and carbohydrates (Fig. 1D), but also included the modification in the composition of key target products (Fig. 2). MP obtained great capacity of stimulating protein and carbohydrate degradation towards lipids and promoting the biosynthesis of affiliated high-value components. From the angle of carbon skeleton source proportion for lipid synthesis, the direct MP-induced carbon flux from proteins towards lipids partly replaced the original path flowing along proteins, carbohydrates, and lipids (Table 1). This strategy ensured sufficient utilization of carbon source, avoided unnecessary intermediate metabolic processes and energy loss, which endows it with the capacity to realize efficient industrial production of lipids or other valuable products, thus becoming a competitive strategy in commercial process.

As the culture biomass increased under MP, the Chl a content sharply decreased (Fig. 2A) and the photosynthetic system displayed damage. Meanwhile, the accumulated Chl b and carotenoids could block the oxidative damage of *C. zofingiensis* cells caused by ROS and provide abundant precursors for astaxanthin biosynthesis, but the decreased astaxanthin accumulation under MP indicated the restriction of this conversion due to reduced photosynthesis (Fig. 1C).

Under MP, the respiration was enhanced, and FAs with same chain length showed higher saturation in general, this trend presented it higher cetane number and stability when applied as materials or propellants of biofuel (Fig. 2B). When MUFAs contribute to improving cold flow properties, the moderate PUFAs help in low-temperature properties and oxidative stability [14]. Moreover, MP effectively promoted the accumulation of C18:1 and C18:2, the crucial intermediate molecules of long-chain FAs synthesis that widely used in dietary supplement and infant food. All these changes of FA composition after MP endowed *C. zofingiensis* excellent characteristics and great application value as biofuels or healthcare products, thus broadening its commercial develop domain.

Many unsaturated FAs are involved in the esterification of astaxanthin, and most of the intracellular astaxanthin is stored in lipid droplets in the form of astaxanthin ester. Therefore, the synthesis of TAG is closely related to astaxanthin synthesis and accumulation. The transcriptional levels of the genes appertained to TAG and carotenoid synthesis were analyzed, and their metabolic flux was explored by ¹³C-MFA. As shown in transcriptome results, de novo FA synthesis pathway was up-regulated under MP, thus providing abundant source of acyl substrates. In Kennedy pathway, the expression level of PAP3 and

most of DGAT enzymes were significantly upregulated, which could further promote TAG accumulation. However, the expression of GPAT1 and PAP2 were inhibited, which led to the accumulation of AcCoA. Metabolic flux results in FAs synthesis pathway did not completely fit previous assumption (Fig. 4). While AcCoA synthesis was more active under MP, the synthesis of C16 FAs was inhibited for the reason that NADPH from OPP pathway and TCA cycle was used for maintaining cell metabolism and respiration instead of lipid synthesis. Respiration outputs ROS as natural byproducts, but increasing ROS level when exposed to environmental stress, known as oxidative stress, can lead to severe damages, such as the slowdown of ATP synthesis, cell-cycle arrest, and eventually apoptosis. The synthesis pathway of FAs, especially PUFAs, serve as the electron sinks [23], and act as antioxidants to mediate oxidative stress by effectively quenching ROS and adjust the REDOX state of cells. Thus, the enhancing ROS production may be the important factor of the consumption of PUFAs under MP, which can be a reasonable explanation for the overall decrease in FAs saturation [13].

Despite the augment of PYR pool with MP, the expression of most genes in MEP pathway showed no obvious promotion. IPP and DMAPP, the precursors for carotenoid synthesis, were significantly their synthesis activities were suppressed due to the lower transcription levels of DXS, CMS, and MCS (Fig. 3D). Similarly, synthesis of astaxanthin and its pigment precursors showed a decline compared to AP, which indicated the restraint of the flux of the terpenoid backbone towards astaxanthin biosynthesis. Transcript abundance of BKT1 and AAT were substantially reduced under MP, which limited β -carotene finally converted into astaxanthin. Figure 4 showed that carotenoid synthesis activity under MP was significantly declined, which certainly limited the rapid synthesis of astaxanthin. Thus, the lack of precursors and decreased activity of enzymes regulated by genes with lower expression levels jointly resulted in a decline of intracellular astaxanthin content under MP. The decrease of intracellular astaxanthin content can also be explained by ROS accumulation of enhancing respiration. The enhanced activity of TCA cycle (Fig. 4) under MP indicated that *C. zofingiensis* relies more on the respiration for energy production and carbon availability augmentation. Similar to other antioxidative carotenoids, astaxanthin has been proved to effectively protect algal cells against oxidative damage by quenching the excessive ROS and other free radicals under stress conditions. Therefore, the generation of ROS can lead to consumption of astaxanthin and its precursors. On the other hand, due to the sharp amplification of biomass in MP group, the reduction of effective light intensity caused by the shielding between cells is another reason for the block of astaxanthin accumulation. While *C. zofingiensis* can produce astaxanthin in darkness, it still shows a typical light-dependent growth and astaxanthin synthesis can still be induced by light intensity, for this process has been identified as a protective response stimulated by photo-oxidative stress [24].

Preculture strategy also plays a critical role in regulating the activity of a multitude of crucial metabolic pathways, which lays a foundation of substance and energy for the production of FA and astaxanthin. Glycolytic pathway outputs PYR and glyceraldehyde 3-phosphate, the former can realize the mutual conversion of basic ingredients central carbon metabolism. Moreover, as an irreplaceable precursor, its accessibility has a decisive influence on the production of carotenoids. AcCoA is the direct downstream of PYR, it serves as the initial block of extending acyl chain [17]. AcCoA participates in TCA circle as the

central cellular metabolite of various biochemical reactions. AcCoA is both the precursor for FA and carotenoid synthesis and a product of the degradation pathways of FA and some amino acids, its efficiency and availability are pivotal for FA and carotenoid synthesis. Overall, expression level of genes affecting glycolysis pathway and gluconeogenesis was elevated under MP (Fig. 3E), among which the augmented expression of ENO and PK is responsible for PYR synthesis and its higher availability [19]. PDHC and ACS are main enzymes contribute to AcCoA production, their significant up-regulated expression level under MP strengthened the production and availability of cellular AcCoA (Fig. 3E). Meanwhile, the down-regulated overall expression of genes related to Kennedy pathway inhibited the transformation of glycerol-3-phosphate, which further aggravated the retention of AcCoA. ^{13}C -MFA revealed the strengthened metabolic flux of EMP and anaplerotic reactions, which boosted AcCoA accumulation and NADPH production, provided abundant carbon molecules for carotenoid biosynthesis, and channeled carbon flow rapidly into TCA cycle (Fig. 4).

In addition to an abundance of substrate molecules and enzymes, astaxanthin synthesis also require NADPH and ATP to provide reducing power and energy [19]. With the photosynthesis pathway in *C. zofingiensis* was hindered, OPP pathway was likely to be the main source of NADPH under MP. OPP pathway generates 2 NADPH molecules for each molecule of glucose-6-phosphate catalyzed. The overexpression of enzymes involved in it, especially 6PGD (Fig. 3E), could catalyze NADPH-producing steps to elevate its content and consequently provide reducing power and energy for biosynthesis process, and the increased reductant pool improved anabolism.

TCA cycle is the crucial pathway that links the metabolism of three major substances and the hub of energy metabolism. As the precursor source for biosynthetic pathways that provide essential substances and sufficient energy for various physiological activities, TCA cycle produce 32 or 30 molecules of ATP in each molecule of glucose with aerobic oxidation. As carbon flow rapidly into TCA cycle and reactions within it were obviously enhanced by MP (Fig. 4), its related genes got generally elevated expression under MP (Fig. 3E), indicated that TCA cycle provided more energy to support the substance synthesis.

Modelling growth is effective for studying and adjusting the performance of microalgae growth, it helps controlling microalgae growth in artificial bioreactors [25]. Exponential fed-batch culture can maximize the growth rate of microalgae through maintaining nutrient content of the system at the most suitable level for cell growth and development. This culture pattern has been proved to be effective in promoting biomass accumulation and increasing cell density. The usage of exponential fed-batch cultures in this study provided *C. zofingiensis* the highest specific growth rate for maximized biomass production, and resulted in an obvious increase in biomass and astaxanthin productivity (Fig. 5).

As the culture volume expanded to 20 L, the biomass concentration successfully surpassed the highest reported record, MP strategy provided sufficient material for the acquisition of astaxanthin and endowed *C. zofingiensis* with a greater value for industrialization. While the astaxanthin content of MP cells showed decreasing trend, the final astaxanthin productivity achieved $0.111 \text{ g L}^{-1} \text{ day}^{-1}$, which was ascribed to the vast biomass accumulation (Fig. 5C). This yield was 7.3-fold higher than the best record

of *C. zofingiensis* [3], and even exceeded that of *H. pluvialis* [22]. The results strongly demonstrated the superiority of the combination of MP and large-scale fed-batch culture, which has mastered the international pioneering level in industrial production of natural astaxanthin as the optimal strategy for *C. zofingiensis*.

4. Conclusion

MP was an effective strategy for promoting *C. zofingiensis* cell growth and enhancing heterotrophic biomass production. It promoted protein degradation and direct lipid biosynthesis, particularly low unsaturated fatty acids, endowing higher application value in healthcare and biofuel fields. While astaxanthin synthesis in cells was restrained under MP, the productivity of the fermentation system was significantly achieved $0.111 \text{ g L}^{-1} \text{ day}^{-1}$, 7.3-fold higher than the highest record due to the prominent biomass concentration. The combination of MP and fed-batch culture maximized the efficiency of *C. zofingiensis* fermentation, thus promoting the industrialization of natural astaxanthin and other high-value products.

5. Methods

5.1 Batch culture of *C. zofingiensis*

C. zofingiensis (strain ATCC 30412, from the American Type Culture Collection) was cultured at 22°C under continuous white light in autoclaved BG-11 medium (pH 7.2, used exclusively for cyanobacteria). To motivate the cell growth, the mixotrophic preculture (MP) strategy of this study used a medium with a glucose concentration of 5 g L^{-1} , nitrate concentration of 2.02 g L^{-1} , and light intensity of $50 \mu\text{E m}^{-2} \text{ s}^{-1}$ [21]. The same nutritional condition without light was classified as the heterotrophic preculture (HP) group. The autotrophic preculture (AP) strategy maintained similar conditions to those of the MP without glucose. *C. zofingiensis* was cultivated for 5 d and then inoculated again into new corresponding media at 10% (v/v). After three generations of repeated preculture, the cells were inoculated into a heterotrophic culture at the same initial concentration for data acquisition.

5.2 Exponential fed-batch culture of *C. zofingiensis* in 20 L fermenters

In the 20 L fermenter, the pressure was set at 0.05 MPa, the rotation speed was initially set at 100 rpm and gradually increased to 600 rpm, and the dissolved oxygen was set at 40% and gradually decreased to 0. To maximize biomass concentration, the mass ratio of glucose, nitrate, and phosphate in designed medium was 23:10:1; other conditions were same as those in the heterotrophic culture [21]. The substance and energy consumption of *C. zofingiensis* for cell maintenance was assumed to be low in the exponential growth phase, and extracellular products were rare. The model was established as [26]:

$$V_{FS} = \mu X_0 \exp(\mu t) V_0 / Y_{XS} C_{FS}$$

1

where Y_{XS} represented the biomass yield on the substrate, V_{FS} was the feeding rate (mL d^{-1}), V_0 was the initial volume (mL). C_{FS} represented the nutrient concentration (g L^{-1}), for example, C_{FG} was the concentrations of glucose in the feeding medium, and C_{FN} , C_{FP} were that of nitrate and phosphate.

5.3 Model development of carbon partitioning

A carbon-partitioning model was contrived based on an existing study [27]. Nutrients in the medium are assimilated into proteins and carbohydrates after absorption. Proteins are partially converted to carbohydrates, lipids, and other functional groups. Carbohydrates were partially reallocated to lipids. Therefore, carbon flux can be streamlined into two specific fluxes, separately leading to lipid production. Based on these assumptions, the model can be established as follows:

$$dQ_P/dt = Y_{PX}\mu X_0 \exp(\mu t) - D_P Q_{P0} \exp(\mu_P t)$$

2

$$dQ_C/dt = Y_{CX}\mu X_0 \exp(\mu t) - \alpha_C Q_{C0} \exp(\mu_C t)$$

3

$$dQ_L/dt = (\mu_C/\mu_L)\alpha_C Q_{C0} \exp(\mu_C t) + (\mu_P/\mu_L)D_P Q_{P0} \exp(\mu_P t)$$

4

where Q_P , Q_C , and Q_L were the concentrations of protein, carbohydrate, and lipid (g L^{-1}), t was the cultivation time (h), μ , μ_P , μ_C , and μ_L respectively represented specific rates of cell growth, protein, carbohydrate, and lipid (h^{-1}). Y_{PX} , and Y_{CX} were yields of protein and carbohydrate on biomass concentration (g g^{-1}), X_0 was the initial biomass concentration (g L^{-1}), D_P and α_C were defined as the conversion rates of protein and carbohydrate (h^{-1}). Since specific rate was influenced by protein content, it could be described as follows:

$$\mu = \mu_{max}(1 - C_P/C_{P,max})$$

5

$$dX/dt = \mu X$$

6

where C_P was the protein concentration (g L^{-1}), and $C_{P,max}$ represented the maximum of it (g L^{-1}). μ_P , μ_C , and μ_L can also be described by Eq. (5).

5.4 Differential gene expression analysis

Total RNA was extracted using a plant RNA extraction kit (TaKaRa, Tokyo, Japan), and Agilent 2100 Bioanalyzer was used to analyze its concentration and quality. Data analysis was performed on BGISEQ-500 platform (BGI, China).

Differential gene expression was analyzed based on a previous study [18]. RSEM software was used to calculate gene expression levels and R software was used to perform statistical analyses [20]. Identified DEGs (differentially expressed genes) between samples were clustered using Deseq2 software.

5.5 ^{13}C tracer-based metabolic flux analysis

^{13}C -MFA was computed by designing biomass reaction with the biomass components as reference. The content of each nucleotide was quoted from published literature [20]. Samples were derivatized with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide and analyzed by GC–MS (7890B-5977B, Agilent). CO_2 statistical weight was analyzed by 100% U- ^{13}C glucose and removed to modify the labeled amino acids abundance. The metabolic flux was set with reference to a previous study [26]. Rates of reaction paths were calculated by Metran coding software [28].

5.6 Experimental methods

5.6.1 Determination of biomass

Gravimetry was used to monitored biomass growth, which was expressed as the ratio of the dry weight of cells to the volume of culture medium (g L^{-1}). The medium (1 mL) was centrifuged at 5,000 *g* and sediment pellets were filtered on dry pre-weighed filter paper at 60°C. Thereafter, the samples were cooled and weighed.

5.6.2 Determination of lipid content

Lipid content was determined by sulfo-phospho-vanillin assays. Culture fluid and concentrated sulfuric acid were heated to 100°C and shaken with vanillin phosphate at 37°C. Absorbance was measured at 530 nm and converted into total lipid content by comparison with a standard curve.

5.6.3 Determination of protein content

Cell pellets were incubated with 2 M sodium hydroxide at 80°C for hydrolysis. The mixture was centrifuged in water. Protein content was determined using a BCA kit (#5000002; Bio-Rad Laboratories Inc., Hercules, CA, USA).

5.6.4 Determination of carbohydrate content

In the phenol sulfuric acid method, cell pellets were incubated with glacial acetic acid. Then the mixture was centrifuged with acetone at 3,500 *g*. The precipitate was dissolved in 4 M trifluoroacetic acid, and the reducing sugars were released by boiling in a water bath. After centrifugation at 10,000 *g*, the supernatant was heated with phenol sulfate for 20 min. The absorbance at 490 nm was measured and converted into content by comparison with a glucose standard curve.

5.6.5 Determination of astaxanthin content

Astaxanthin content in cells was analyzed by HPLC (Waters 2695, Milford, MA, USA) equipped with a photodiode array detector, the column was Shiseido CAPCELL PAK C18 5 μm . Operational parameters were same to a pervious study [26].

5.6.6 Determination of pigment composition

Cell pellets were incubated at 45°C with 99.9% methanol in darkness for 24 h. The mixture was centrifuged, and the absorbance of the supernatant was measured at 470, 652.4, and 665.2 nm separately. The pigment concentrations were calculated based on previous study [29].

5.6.7 Determination of fatty acid composition

Biomass was extracted using chloroform and methanol. The chloroform layer was incubated with 1% sulfuric acid and converted into fatty acid methyl esters, which were analyzed using a GC-MS (Shimadzu, Kyoto, Japan) equipped with Rtx-2330 capillary column (Restek, Guangzhou, China). Operational parameters were set according to a previous study [30].

5.6.8 Determination of amino acids

Cell pellets were hydrolyzed with hydrochloric acid (6 mol L⁻¹) at 110°C, the amino acid content of hydrolysates was analyzed using an LC20AD-API TRAP 3200MD HPLC-MS/MS system (Shimadzu Corp., Kyoto, Japan).

5.7 Statistical analysis

All experiments were conducted at least three replicates. Data were statistically analyzed using SPSS software and one-way analysis of variance (ANOVA) with post hoc multiple-comparison least significant difference (LSD) tests to detect significant differences.

Declarations

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Authors' Contributions

Yuxin Wang: Conceptualization, Data curation, Validation, Writing–original draft. Han Sun: Conceptualization, Data curation, Software, Writing–original draft. Jia Wang: Data curation. Ziqiang Gu: Data curation. Feng Chen: Conceptualization, Writing – review & editing. Shufang Yang: Data curation, Software, Writing–original draft. Haijin Mou: Conceptualization, Funding acquisition, Writing – review & editing.

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Unsectioned Figure Details

A

Fig. 1

Effect of preculture strategies on biomass concentration during preculture (A) and heterotrophic batch culture (B). Effect of preculture strategies on astaxanthin (C), protein, carbohydrate, and lipid contents (D).

A

Fig. 2

Effect of preculture strategies on pigment (A) and fatty acid compositions (B).

A

Fig. 3

Pearson correlation analysis (A) and Principal component analysis (PCA) analysis (B) of all gene expression levels. Transcriptional regulation of FA and TAG biosynthesis (C), carotenoids biosynthesis (D) and central carbon metabolism (E) in response to different preculture strategies. The asterisk indicates Adjusted p value (p_{adj}) ≤ 0.05 .

A

Fig. 5

Effect of fed-batch culture on biomass concentration in Erlenmeyer flasks (A) and fermenters (B). Astaxanthin yield and specific growth rate in fermenters (C).

Tables and Figures

A

Fig. 4

Central carbon flux under different preculture strategies.

Unsectioned Paragraphs

Abbreviations

See Additional file 2.

The authors declare that they have no competing interests.

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Figure Captions

Figure 1

(A)(B)

(C)(D)

Figure 2

(A) (B)

Figure 3

(A)(B)

(C)(D)

(E)

Figure 4

Figure 5

(A)(B)

(C)

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