# Sodium acetate promotes the growth, lipid and carbohydrate biosynthesis of Micractinium reisseri FM1 under batch and fed-batch cultivation

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# Abstract

Carbon supply and cultivation mode have important influence on the growth of microalgae and accumulation of active components in cells. The effects of exogenous sodium acetate (NaAc) and controlling the cultivation modes on the biomass production, cellular composition and lipids biosynthesis of *Micractinium reisseri* FM1 were evaluated. The algal strain showed satisfactory assimilating ability of NaAc in mixotrophic cultivation. In batch culture of the alga, at the optimal NaAc dosage of 10 g $L^{-1}$, the 8.8-fold biomass, and highest lipid content (38.85%) were attained, the maximal value of total pigments (30.83 mg $g^{-1}$) was achieved at 2 g $L^{-1}$ NaAc. Fed-batch culture mode performed the superiority in promoting the growth and total lipid accumulation by contrast with batch culture. Furthermore, carbohydrate synthesis, abundance of saturated fatty acids and polyunsaturated fatty acids were also enhanced with NaAc supplementation and transformation of cultivation strategy, while protein synthesis was restrained. These results corroborated that *M. reisseri* FM1 can realize the feasibility of high-cell density, high lipid and carbohydrate biosynthesis through acetate supplementation and manipulating the feeding mode for the production of high value-added raw materials for biorefinery.


# Introduction

Due to the shortage of resources caused by the world population growth and the greenhouse effect caused by carbon dioxide ($CO_2$) emissions from industrial production and human activities, many research teams dedicate to seeking more environmentally acceptable and sustainable bioresources. Microalgae can naturally utilize $CO_2$ and solar energy to produce lipids and other high valued biomolecules such as carbohydrates, pigments, proteins (Gee et al. 2020), which has received considerable interest in the past decades (Anonymous 2015). Several algae strains can even be cultivated using industrial flue gasses and wastewaters from industrial, agricultural and municipal sources (Hunt et al. 2010). Although algae have varieties of superiorities such as fast growth rate, short growth cycle and no competition arable land with crops, high cost of high cell density culture is still an obstacle to the commercialization of microalgae. Therefore, it is of great significance to design and develop efficient culture strategies for the low-cost production of microalgal biomass.

Compared with photoautotrophic and heterotrophic modes, mixotrophic cultivation is considered to be a preferable option for large-scale and high cell density cultivation of microalgae to synthesize lipid, polysaccharide and other bioactive substances (Lin and Wu 2015; Kong et al. 2020; Mondal et al. 2016). In mixotrophic culture, nutrient substance (carbon, nitrogen sources) (Imran et al. 2014; Rahimi and Jazini 2021), culture conditions (light, temperature and pH) (Suparmaniam et al. 2019), feeding modes (batch and fed-batch) markedly affect the growth and metabolism of microalgae, among which carbon sources and feeding modes are the main ways to improve the biomass production and lipid accumulation of microalgae.

Previous studies have studied that different carbon sources could noteworthily improve growth rate and lipid production of several microalgae species. Alam et al. (2015) found that $CaCO_3$ was more efficient in

enhancing biomass, while $NaHCO_3$ was proficient in increasing the lipid content of *Sirogonium sticticum*. Exogenous supplementation glucose led the average growth rate of *C. vulgaris* was 1.37 times higher than that of the non-glucose added control (Huang et al. 2019). As a low-cost carbon source, sodium acetate (NaAc) is highly beneficial in enhancing the biomass as well as lipid productivity of microalgae. When adding 10 g $m^{-3}$ of NaAc in cultures, 6-fold enhancement in biomass productivity and a remarkable 32-fold increment in lipid productivity were recorded in comparison to autotrophic culture (Rai et al. 2013). Furthermore, there was evidence that acetate was the limiting factor and central molecule of lipid droplet synthesis in starchless mutant of *Chlamydomonas reinhardtii* (Ramanan et al. 2013).

In addition, batch and fed-batch culture are the most common attempt to achieve high cell-density culture of microalgae (García-Cañedo et al. 2016). In batch culture, nutrients (like carbon source, nitrogen source) are directly added into medium before fermentation. However, it is easy to appear that the high initial substrate concentration inhibits the growth of microalgae cells. Fed-batch mode effectively eliminates this deficiency by intermittently adding substrates to the culture medium, which can meet the nutrient requirements of microalgae, prolong the production time of secondary metabolites and greatly reduce the pollution efficiency (Ding et al. 2013). Many accumulated evidence showed that the fed-batch culture was beneficial to the growth and the productions of secondary metabolites of many algae species, like *Crypthecodinium cohnii* (DHA) (Pei et al. 2017), *Chromochloris zofingiensis* (astaxanthin) (Sun et al. 2020), *Desmodesmus* sp. (lutein) (Xie et al. 2014), and *Galdieria sulphuraria* (phycocyanin) (Xie et al. 2015).

In the present study, the effects of different concentrations of NaAc and feeding modes on the biomass, cellular components, lipid production and fatty acids profile by *Micractinium reisseri* FM1 under mixotrophic cultivation were investigated. This study aimed were to use cheap NaAc as the main organic carbon source to cultivate the biomass of *M. reisseri* FM1 in high cell-density by regulating the culture mode, which can be used as raw materials for the production of functional oils, proteins and pigments in pharmaceutical and health food industry.

# Materials And Methods

### Microalga strain, chemicals and culture medium

The green microalga strain of *M. reisseri* FM1, isolated from pipe-type photobioreactor in a local microalgae cultivation company was used in the present study (Liu et al. 2021b). The algal strain was purified by streak plate method to satisfy the requirements of pure cultivation. The purified algal strain was cultured in liquid medium to obtain seed liquid. NaAc, $NaNO_3$, $K_2HPO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $KH_2PO_4$, NaCl, $FeCl_3 \cdot 6H_2O$, EDTA-Na, NaOH, HCl, ethanol and other reagents were all analytical reagent and purchased from domestic chemical reagent company. The modified soil extract (SE) medium referenced from Freshwater Algae Culture Collection of the Institute of Hydrobiology, China (http://algae.ihb.ac.cn/MeSearch.aspx) was adopted to cultivate the *M. reisseri* FM1, in which the soil extract (40 mL) was removed compared with normal SE. The medium consisted of (g $L^{-1}$): 0.25 g $NaNO_3$,

0.75 g $K_2HPO_4$, 0.75 g $MgSO_4•7H_2O$, 0.25 g $CaCl_2•2H_2O$, 0.175 g $KH_2PO_4$, 0.25 g NaCl, 0.05 g $FeCl_3•6H_2O$, EDTA-Fe, 1 mL of $A_5$ solution [0.22 g $ZnSO_4•7H_2O$, 1.86 g $MnCl_4•4H_2O$, 2.86 g $H_3BO_3$, 0.08 g $CuSO_4•5H_2O$, 0.05 g $Na_2Mo_7O_{24}•7H_2O$, 0.08g Co $(NO_3)_2•6H_2O$].

## Culture conditions

Purified *M. reisseri* FM1 colony from the SE agar plate was transferred into sterilized SE liquid medium in 250-mL flasks to prepare the inoculum. *M. reisseri* FM1 cells of seed cultures in the exponential phase were used for subsequent experiments. To investigate the effects of NaAc on the growth, cellular components (pigments, proteins, carbohydrates), lipid content and fatty acid composition of *M. reisseri* FM1, different concentrations and feeding modes (batch and fed-batch) of NaAc were added into SE medium as carbon source. In batch culture, different concentrations (1, 2, 4, 6, 8 and 10 g $L^{-1}$) of NaAc were supplemented into 250 mL flask containing 100 mL sterile SE medium, photoautotrophic group without adding NaAc was used as the control. In the batch culture process, 4 g $L^{-1}$ and 8 g $L^{-1}$ of NaAc were directly added into SE medium. Fed-batch culture was designed that NaAc was intermittently fed three times at 1, 2 g $L^{-1}$ concentration during cultivation at 2, 4 and 6 days respectively, which ensured that the final concentration was 4 and 8 g $L^{-1}$. Initial NaAc concentrations of 4 g $L^{-1}$ and 8 g $L^{-1}$ under batch cultures were used as the control.

All the culture medium were sterilized at 121 °C and 0.1 MPa for 20 min, the purified inoculum of *M. reisseri* FM1 was inoculated with a volume of 5% (v/v). All the cultures were cultivated in a shaker incubator at 150 rpm and 25±1 ℃ under 5000 lux (around 95 μmol $m^{-2}$ $s^{-1}$), 12: 12 h light-dark period (HNY-2102C, Honour, China).

## Determination of growth curve and kinetic parameters

The growth profile of *M. reisseri* FM1 was measured at 680 nm spectrophotometrically (V-5000, Shanghai, China) after every 24 h for all test groups to plot growth curve (Li et al. 2015). The dry weight concentration of algal cells was measured by gravimetric method. The algae liquid was centrifuged at 3052 g for 10 min, and the supernatant was discarded. The algae pellet was collected after being washed twice with deionized water, then dried until constant weight. The relationship between optical density and DCW was established by linear regression. The specific growth rate ($\mu$, $day^{-1}$) and biomass productivity ($P_B$, mg $L^{-1}$ $d^{-1}$) were calculated using equation (1) and (2):

$$\mu = \ln (W_2/W_1) / (t_2-t_1) \quad (1)$$

$$P_B = (W_2-W_1) / (t_2-t_1) \quad (2)$$

where, $W_2$ and $W_1$ are the values of biomass concentration (g $L^{-1}$) on the days $t_2$ and $t_1$, respectively. Biomass productivity ($P_B$, g $L^{-1}$ $d^{-1}$) was obtained according to the following calculation (Song and Pei 2018).

## Extraction and determination of cellular components

*Pigments* Photosynthetic pigments (chlorophylls and carotenoids) were analyzed according to previous work with minor modifications (Basu et al. 2013). 5 mL of culture from each sample were collected and centrifuged at 3052 g for 10 min. The algal pellet was extracted with 95 % ethanol, mixed well and incubated at 4 °C in dark overnight. Afterwards, mixture was centrifuged at 3052 g and the supernatant was used for pigment estimation by measuring the absorbencies at 470, 649 and 665 nm, Total chlorophyll (*C*t) and carotenoid (*C*c) concentrations were calculated using Eq. (3) to (7) as follows (Kong et al. 2020):

$$C_a = 13.95 \times A_{665} - 6.88 \times A_{649} \quad (3)$$

$$C_b = 24.96 \times A_{649} - 7.32 \times A_{665} \quad (4)$$

$$C_t = C_a + C_b \quad (5)$$

$$C_c = (1000 \times A_{470} - 2.05 \times C_a - 114.8 \times C_b) / 254 \quad (6)$$

$$C\ (\text{mg g}^{-1}) = [C_t\ (\text{or } C_c) \times V_t \times D] / W \quad (7)$$

where $C$: pigment content (mg $g^{-1}$), $C_t$: total chlorophylls content (mg $mL^{-1}$), $C_c$: carotenoids content (mg $L^{-1}$), $V_t$: total volume of extract (L), $D$: dilution factor, and $W$: dry weight of algal cells (g).

*Lipids* Total lipids in the algal biomass were extracted and analyzed using the sulfo-phospho-vanillin assay described by Mishra et al (2014). Briefly, 25 mg of the lyophilized algal biomass was resuspended in 5 mL of distilled water and mixed by a vortex mixer completely. 100 µL of algal liquid, 1 mL of deionized water and 2 mL of concentrated sulfuric acid were added to fresh tube successively. The mixture was heated for 10 min in boiling water, then cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent were added into each tube and measured the optical density at 530 nm after incubation for 15 min at room temperature. Refined olive oil was used as a standard. Total lipid content was confirmed using Eq. (8) as follows:

$$\text{Total lipid content (mg mL}^{-1}) = 2.1585 \times A_{530} - 0.0159 \quad (8)$$

*Carbohydrates* The carbohydrate content was estimated by phenol-sulphuric acid method as described by Vijay et al (2021). 50 mg of disrupted and ground algal biomass was resuspended in 25 mL distilled water and kept in water bath at 80°C for 1 h for the extraction of soluble carbohydrates, and the process was repeated once again. The mixture was then centrifuged at 3052 g for 15 min, and the supernatant was collected and merged. Finally, the constant volume of the extract was set to 50 mL with distilled water. 1 mL of supernatant was mixed with 1 mL distilled water, 1 mL of 6% phenol and 98% sulphuric acid, shaken well and incubated for 20 min. The mixture was used to measure the absorbance at 490 nm.

Total carbohydrate content was quantified from the calibration curve prepared using glucose as standard and expressed as percentage of dry cell weight (%, Eq. 9).

$$\text{Carbohydrate content (\%)} = (C \times V_T \times N) * 100 / (W \times V_S \times 10^6) \quad (9)$$

where $C$: glucose content (μg), $V_T$: total volume of extraction liquid (mL), $N$: Diluted multiples, $W$: the sample quality (g), $V_S$: the volume of the sample taken for determination, and $W$: dry weight of algal biomass (g).

*Proteins* The proteins content was determined by the micro-Kjeldahl method using 100 mg of the lyophilized and ground algal powder and calculated the proteins content according to the following Eq. (10) and (11) (Aziz and Mitu 2019).

$$\text{Sample Nitrogen content (\%)} = [C \times (V_1 - V_2) \times 0.014] \times 100 / W \quad (10)$$

$$\text{Protein content (\%)} = \text{Nitrogen content (\%)} \times 6.25 \quad (11)$$

where $C$: average quantity of standard solution of hydrochloric acid when titrated sample (mol $L^{-1}$), $V_1$: average quantity of standard solution of hydrochloric acid when titrated control (mL), $V_2$: and $W$: dry weight of algal biomass (g).

### Determination of fatty acid compositions by GC-MS

Lyophilized and disrupted algal biomass was blended with chloroform/methanol (2:1, v/v). The mixture was agitated and extracted for 3 h. The chloroform phases were collected and evaporated by rotary evaporator (RE-2000E, Yarong, China) to determine the fatty acid methyl esters (FAMEs). Extracted lipids were subjected to methyl esterification reaction *via* KOH-methanol method according to *GB/T 17376-2008*. Samples were then assayed by GC-MS (Agilent, 7890B-5977A, USA). Optimized detecting conditions of GC-MS were described in our previous study (Kong et al. 2020).

### Statistics analysis

All experiments were carried out in triplicates. The data shown in table and figure were expressed as the Mean ± SD (standard deviation). The statistical differences between experimental groups were determined by analysis of variance (ANOVA) using the SPSS Statistics (18.0, USA).

## Results

### Changes in growth characteristics of *M. reisseri* FM1

The effect of different levels and feeding modes of NaAc on the growth of *M. reisseri* FM1 for 8 days is shown in Fig. 1 and Table 1. In batch culture, NaAc promoted the growth of *M. reisseri* FM1 in a dose-dependent manner, and the growth rates of all concentrations were significantly higher than

photoautotrophic culture from the first day until the end of trial (Fig. 1a). At a transient adaptation phase of 1 day, the algae cells underwent the exponential growth for 4 days, then entered into the stationary phase on the fifth day. Obviously, 4, 6, 8 and 10 g $L^{-1}$ treatments exhibited more rapid growth, 1 g $L^{-1}$ and 2 g $L^{-1}$ showed a slight increase on the algal cell growth. Our results testified 10 g $L^{-1}$ NaAc was the most optimal concentration for mixotrophic growth of *M. reisseri* FM1 after 8 days of cultivation, while there was no significant difference from 8 g $L^{-1}$. Similarly, maximum biomass productivity of 0.56 g $L^{-1}$ $day^{-1}$ and specific growth rate ($\mu$) of 0.59 $day^{-1}$ were reached by NaAc when added 10 g $L^{-1}$ of NaAc.

Different concentrations of NaAc were added to medium three times during the period of cultivation, in order to elucidate the effects of feeding patterns on the growth characteristics of *M. reisseri* FM1. As seen in Fig. 1b, the growth of the *M. reisseri* FM1 under both cultivation conditions exhibited remarkable promotion. During the total culture stage, the treatment of fed-batch culture grew faster than batch culture. Algal growth was basically consistent with the above results in the former stage. Originally, the batch 4 g $L^{-1}$ group grew slightly faster than 8 g $L^{-1}$ and fed-batch groups, then the growth of 4 g $L^{-1}$ gradually tended to be constant and surpassed by 8 g $L^{-1}$. After fed NaAc on the second day, fed-batch groups displayed continuous rapid growth. The growth of batch 8 g $L^{-1}$ and fed-batch 2 g $L^{-1}$ showed also moderate increasing within 6-8 days, while the treatment of fed-batch 1 g $L^{-1}$ supplemented 1 g $L^{-1}$ NaAc, the stimulatory effect in growth was still evident, and received the equal growth rate with batch 8 g $L^{-1}$ until the end of test. As a result, the impact of treatment conditions on growth of algae was not statistically significant. Nevertheless, in our study, the changes of specific growth rate and biomass productivity in batch and fed-batch culture varied with the concentration of added NaAc. The specific growth rate and biomass productivity of feeding culture with low concentration of NaAc (1 g $L^{-1}$) were higher than that of batch culture (4 g $L^{-1}$), but at higher concentration, the difference was not significant.

## Biosynthesis of photosynthetic pigments

The photosynthetic pigments production of *M. reisseri* FM1 within 8 days of cultivation was depicted in Fig. 2. The utilization of NaAc led to a decrease in the carotenoid content and boosted the chlorophyll a and b production. The content of chlorophyll a and b was observed with the increasing of NaAc concentration and reached the maximum accumulation at 2 g $L^{-1}$ and 4 g $L^{-1}$, respectively, subsequently began to decline. Compared with photoautotrophic cell, the total chlorophyll content (26.46 mg $g^{-1}$) in *M. reisseri* FM1 showed a 1.23-fold increment response to NaAc-treatment. Although NaAc-treated cell mildly increased the content of carotenoid, there was a downward trend of carotenoid value compared with control in mixotrophic condition.

The effects of different cultivation strategies on the accumulation of pigments in *M. reisseri* FM1 were revealed in the current study (Fig. 2b). Pigments content in batch or fed-batch cultures was less than photoautotrophic group, except batch 4 g $L^{-1}$ contained more chlorophyll b. Batch 4 g $L^{-1}$ was the optimal dose, harvested the maximal pigments content among all assay groups. As a whole, the concentration of NaAc affected photosynthesis and pigment synthesis of the algal cells. From the pigment content per

unit mass of algae cells, the addition of NaAc can reduce the pigment content, especially at a higher concentration level. However, from the perspective of volume productivity, because acetate significantly increased the biomass of algae cells, the addition of acetate could achieve higher pigment yield of *M. reisseri* FM1. Cultivation strategies had inconspicuous impact on the different pigment fractions of *M. reisseri* FM1.

## Biosynthesis of proteins and carbohydrates

To explore the role of NaAc on the intracellular biomolecules of *M. reisseri* FM1 under diverse trophic ways, the content of protein and carbohydrate was measured after 8 days cultivation. In this study, the maximal value of proteins was achieved in photoautotrophic group and there was significantly higher than all experimental groups ($p<0.05$) (Fig. 3a). In batch culture, protein content was inclined to remarkably enhance along with the increment of NaAc concentration, till they arrived the highest content. But the growing concentration of NaAc led to a fall in protein content, indicating that protein content had a negative relation to high concentration. 1 g $L^{-1}$ NaAc achieved the highest protein content of 25.63% in all test groups, which still was an 8.31% drastic drop compared with photoautotrophic group, followed by 2 g $L^{-1}$ (22.54%). It seemed that culture strategy played a mild role in regulation of protein synthesis, Fig. 3b showed the same results that low concentration of NaAc stimulated the accumulation of protein, while high concentration had the opposite effect. The order of stimulating effect was batch 4 g $L^{-1}$>fed-batch 1 g $L^{-1}$> fed-batch 2 g $L^{-1}$> batch 8 g $L^{-1}$, batch 4 g $L^{-1}$ (20.46%) showed the unremarkable promotion impact on the protein content in contrast to fed-batch 1g $L^{-1}$ (19.40%). Interestingly, fed-batch culture remained approximative level at the further concentration of NaAc, nevertheless batch 8 g $L^{-1}$ displayed a conspicuous descent trend.

The variation trend of carbohydrate content was different from protein. It was obviously seen that the content of carbohydrate increased moderately with ascending NaAc concentration and got to the highest value of 15.86%, then it stepwise went down as the NaAc concentration continued arising (Fig. 3a). The maximal carbohydrates content was obtained at batch 4 g $L^{-1}$, followed by batch 2 g $L^{-1}$ (13.74%), which exhibited significant difference compared with photoautotrophic group. Fig. 3b showed that the regulation role of feeding modes on carbohydrate content. The consequence corroborated that low concentration was beneficial to carbohydrate synthesis under batch culture, fed-batch modes performed the better ability of maintaining the content of carbohydrate under higher concentration. Therefore, we drew a conclusion that carbohydrate content was not sensitive to variations of cultivation strategy. In a nutshell, in batch culture mode, the addition of acetate inhibits protein synthesis in general, but promotes carbohydrate synthesis to a certain extent. However, with the increase of acetate concentration, the content of protein and carbohydrate tends to decrease. The effects of feed modes on protein and carbohydrate synthesis are also related to the concentration of acetate added.

## Accumulation of lipids and fatty acids

The lipid of *M. reisseri* FM1 on SE medium containing NaAc in mixotrophy batch culture was shown in Table 3. The supplementation of various concentrations of NaAc did significantly improve the total lipid content of *M. reisseri* FM1 compared to the control. Lipid accumulation showed a drastic rising tendency by increasing the concentration of NaAc, and the peak value of 38.85% was reached at 10 g $L^{-1}$, which was a rapid promotion of 29.92% by contrast with control. Batch 8 g $L^{-1}$ also exhibited prominent promotion on lipid contents, which was immediately followed by 10 g $L^{-1}$, and significantly higher than the photoautotrophic treatment ($p<0.05$). When the concentration of NaAc was 10 g $L^{-1}$, the maximum lipid yield and productivity achieved 0.87 g $L^{-1}$ and 109.25 g $L^{-1}$ $day^{-1}$, respectively, which was 45.3-fold and 57.5-fold higher than that of the control (0.02 g $L^{-1}$, 1.90 g $L^{-1}$ $day^{-1}$). Lipid yield and productivity abided by the similar change trend with lipid content. Fed-batch cultivation showed preferable lipid accumulation at the same concentration by contrast with batch culture. The lipid contents were enhanced by 4.5% and 3.88%, respectively, after being fed three times by NaAc at concentration of 1 and 2 g $L^{-1}$. These responses indicated that treatment conditions had a positive influence on lipid production.

When microalgal cultures were exposed to varied concentration of NaAc in batch culture, high proportion of C16-C18 fatty acid produced in *M. reisseri* FM1, including palmitic acid (C16:0), hexadecadienoic acid (C16:2), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) (Fig. 4a), which was the dominating ingredient of biodiesel. A relatively high percentage of SFAs appeared with the increasing NaAc concentration. The major SFAs of C16:0 and C18:0 increased significantly in presence of 2 g $L^{-1}$ and 6 g $L^{-1}$ NaAc compared to the control level. Overall change trend of experimental group of MUFAs (C18:1) showed a bit decline than photoautotrophic group, except 4 g $L^{-1}$ and 6 g $L^{-1}$. The fatty acid methyl ester (FAME) profile of *M. reisseri* FM1 showed a clear rise in total PUFAs, especially C18:2, 8.89% increment in 4 g $L^{-1}$ was observed. Likewise, fed-batch treatment mainly enhanced SFAs percentage, had no distinct difference in MUFAs and PUFAs (Fig. 4b). Another interesting fact that the level of PUFAs was always higher at lower concentration in both fermentation cases, for instance, batch 4 g $L^{-1}$ and fed-batch 1 g $L^{-1}$ possessed the highest value of PUFAs (34.75%, 36.87%). The abundance of SFAs and MUFAs was enhanced at high concentration by contrast with control.

Therefore, supplying of NaAc at different concentrations and feeding ways could not only significantly increase the lipid content in *M. reisseri* FM1 cells, but also regulate the proportion of major fatty acids. This means that it is a simple, effective and economical method to improve the lipid content and fatty acid composition of algae cells by controlling the content and mode of acetate.

# Discussion

### Regulation of growth and cellular components in *M. reisseri* FM1 by NaAc

**Growth and lipids** Microalgae not only could utilize the simple inorganic carbon by photosynthetic fixation, but also assimilate organic carbon sources to support growth and lipid production, such as glucose, glycerol, and NaAc (Gim et al. 2016). Liang et al. (2009) reported that glucose

and glycerol addition produced the stronger stimulating effect on cell growth, the maximum biomass density (2 g $L^{-1}$) and lipid productivity (54 mg $L^{-1}$ $day^{-1}$) were obtained with low doses of 1 % glucose when compared with autotrophic group. Supplementation NaAc in medium is an effective method to elevate the biomass and lipid accumulation. As proved in the results attained for *C. pyrenoidosa* and by Rai et al. (2013), adding different concentrations of NaAc (5, 10 g $m^{-3}$) in mixotrophic cultivation, maximum biomass production of 867 mg $m^{-3}$ was produced with 10 g $m^{-3}$, both concentrations achieved the highest lipid content of 13.5%. What's more, mixotrophic cultures possessed higher growth because they were liable to report higher chlorophyll content in comparison with photoautotrophic cultures (Mondal et al. 2016), this evidence was also supported by our results of chlorophyll contents.

Our results were in parity with above data that all tested concentration of NaAc favored the algal growth, even vastly enhanced lipid content compared to photoautotrophic algae cell. According to Spalding (2009), exogenous supplementation of NaAc could result in $CO_2$ enriched environment, which enhanced higher RuBisCO activity leading to larger growth and biomass accumulation. Interestingly, 0.5% volume fraction of glycerol produced the higher biomass by 1.26-fold and lipid content by 1.02-fold than that of 1%, which demonstrated that high carbon source concentration occurred substrate inhibition effect (Rai et al. 2013). However, in this paper, 10 g $L^{-1}$ of NaAc still showed positive effect in *M. reisseri* FM1, namely, 10 g $L^{-1}$ was not yet reached the maximum assimilation capacity of *M. reisseri* FM1. Hence, the experiment subsequent suggestively select higher NaAc concentration to get the higher growth.

Lipid content of microalga directly influences the ultimate cost of functional edible oil or biodiesel production. It is of significance to use rapid analysis assay for determination of the lipid production capacity of algae or biofuel-potential in microalgal biomass (Laurens et al. 2012). Sulfo-phospho-vanillin (SPV) is a simpler and time-intensive mean to rapidly screen the lipid production of microalgal strain. Byreddy et al. (2016) compared gravimetric quantification and SPV method, the total lipid percentages determined by two approaches showed similar quantities in all thraustochytrids. Zhang et al. (2017) verified the reliability of ensuring the total lipid content employing phosphoric acid-vanillin reaction under different cultivation conditions, the results indicated that the content of total lipid of *Scenedesmus obliquus* had a significant linear regression relationship with absorbance value of phosphoric acid - vanillin determination under different concentrations of nitrogen and phosphorus. Thus, SPV reaction can be used to determine the lipid content accurately and reliably.

Acetate was a proper carbon source to boost lipid accumulation in mixotrophic cultivation, owing to some organic macromolecular substances could be transformed into acetate and other small organic molecules easy to assimilate via anaerobic hydrolysis (Su et al. 2011). Our results corroborated that lipid production in *M. reisseri* FM1 was dose-dependent on exogenous NaAc, which the highest lipid content of 4.35-fold and the peak value of lipid productivity were obtained with 10 g $L^{-1}$ in batch culture under mixotrophic cultivation. The same effect of the acetate occurred in *C. reinhardtii*, cellular lipid content increased linearly with input acetate concentration. Moreover, acetate is the precursor of Acetyl-CoA,

which is converted into Acetyl-CoA through Acetyl-CoA synthetase (ACS), and has a crucial influence on lipid synthesis (Ramanan et al. 2013).

**Pigments** Algae pigments involved in photosynthesis can protect cells from stress and oxidation, in the meantime, exhibit great commercial potential due to the beneficial health properties of antioxidants. Green algae contain abundant chlorophyll in all algae, cultivation conditions, such as nutrients and light intensity can modify the chlorophyll content (D'Alessandro and Filho 2016). The tendency to enhance the chlorophyll content of green alga *M. reisseri* FM1 when exposed to varied NaAc levels was observed in the present study. The effect of mixotrophic cultivation on pigment composition was sensitive to the species-specific and the type of carbon source. For instance, addition of diverse carbon sources (glucose, xylose, sucrose, maltose, NaAc and glycerol) hindered the photosynthesis, which resulted in the content of chlorophyll lower than photoautotrophic group in *C. vulgaris* 31 (Kong et al. 2020). *Chlorella zofingiensis* in mixotrophic cultivation treated with glucose contained less pigment than phototrophic cell (Ip et al. 2004). However, in the marine microalga *Tisochrysis lutea*, the contents of chlorophylls a, c and total carotenoids were enhanced using glycerol as the carbon source (Alkhamis and Jian 2016). Another work showed that exogenous NaAc enhanced astaxanthin accumulation and photoprotection capacity in *Haematococcus pluvialis* at the non-motile stage. Addition of acetate suppressed photosynthetic activity and facilitated respiratory activity, thus the increase of respiration promoted astaxanthin accumulation (Zhang et al. 2019). Pirastru et al. (2012) found that during astaxanthin and canthaxanthin accumulation, in the presence of NaAc and limited nitrogen supply, the photosynthetic activity of *Scenedesmus* algal cells deteriorated. A large decrease of PSII photochemical activity was evident when cultures were exposed to high concentrations of acetate. According to Fig. 2a, b, high concentration of NaAc directly inhibited photosynthesis and pigment accumulation. This change may be a consequence of higher NaAc level having an inhibitory influence in pigments synthesis, which well catered to the study of Chu et al. (1995) using glucose as carbon source.

**Proteins and carbohydrates** The contents of proteins from the algal cells changed from 6% to 70% based on dry cell weight, which are promisingly deemed as untraditional source of proteins for feed additives and food nutritional fortifiers. In addition, algae can synthesize rich carbohydrate via photosynthesis, which are mainly utilized for bioethanol production or polysaccharides for health care (Levasseur et al. 2020). Zhang et al. (2021) reported that the addition of acetate in *C. regularis* culture could not only promote cell growth and accumulation of metabolites such as lipid, carbohydrate and protein, but also improve the enzymatic degradation of amoxicillin and reduce the toxicity of antibiotic to the algal cells. While our results showed that protein biosynthesis of *M. reisseri* FM1 might be halted when submitted to NaAc by mixotrophic mode, which was in accordance with Orús et al. (1991), where the reduction of protein was observed in mixotrophic cells, and *Chlorella vulgaris* UAM 101 might be compensated by increment of the lipid under mixotrophic condition. The finding from Andersen et al. (2013) explained this behavior that the increasing of NaAc was adverse to protein production, who found that many algae generated pyrenoids due to the accumulations of the enzyme RuBisCO, the enzyme exerted a pivotal effect in photosynthesis. However, our results elucidated high concentration of NaAc presented the

inhibitory influence on photosynthetic pigment synthesis, which explained the protein content decreased with the increasing of NaAc concentration to a certain degree.

Carbohydrate content appears to be negatively correlated with lipid accumulation in our assay, this phenomenon was presumably due to microalgae appeared to deviate its original metabolic pathways to produce a higher concentration of lipid (Rosa et al. 2019). Similarly, Madani et al. (2020) reported that lipid synthesis fortified with the increasing the concentration of $GA_3$ while the carbohydrate content declined. On the other hand, excess carbon source concentration may halt carbohydrate production on account of its low pH and high dissolved $CO_2$ concentration (Ran et al. 2019; Pancha et al. 2015), which was well in close agreement with our results.

**Fatty acids profile** Microalgae mainly have two types of lipids, structural lipids and storage lipids. Structural lipids were known as an important part of a cell, like phospholipids and sterols. Lipids stored in the cell via photosynthesis, primarily triglycerides, are deemed to storage lipids. Fatty acids are a section of the storage lipids (Levasseur et al. 2020). Fatty acid composition directly affects the physical properties of biodiesel or pharmacological activity of functional edible oils. High-proportioned saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) have superior antioxygenic property, while the cold-flow property of biofuels is better in lipid abundant polyunsaturated fatty acids (PUFAs) (Liu et al. 2017). While, as a functional edible lipid raw material, consumers prefer the oil to contain more PUFAs. It exhibited that C16 and C18 fatty acids accounted for more than 97.9 % of total extracted lipids with addition of NaAc for mixotrophic culture, such fatty acids profiles were coincident with the reports of Rai et al. (2013) and Sun et al. (2016). In addition, 5 g $m^{-3}$ acetate induced the contents SFAs from 15.16% to 25.93%, a similar variation of 8.89% increase higher than photoautotrophic group presented in our study. Recently work also revealed that supplementation of iron and acetate in actual municipal wastewater could not only promote the lipid productivity of *C. pyrenoidosa*, but also improve the algal lipid quality by adjusting the composition of saturated fatty acids, thus improving its oxidation stability (Liu et al. 2021a). 4 g $L^{-1}$ NaAc triggered the maximum value of PUFAs, a possible explanation for this consequence was that PUFAs were related to chloroplasts, the primary site for photosynthesis (Yang et al. 2003), as observed in our study that 4 g $L^{-1}$ occurred the maximum chlorophyll content.

## Comparison of batch and fed-batch cultivation

Fortunately, feeding modes did observably modify the biomass and lipid synthesis of *M. reisseri* FM1 under mixotrophic cultivation. Our results elucidated that fed-batch operation was an available means to gain higher biomass on the premise of ensuring lipid production, which was in line with the previous findings (Fields et al. 2018; Nwoba et al. 2019). Gharat et al. (2018) also found that fed-batch strategy resulted in higher biomass and EPA productivity in *Nannochloropsis oculata*. Lorenz et al. (2017) established an industrially applicable process for lipid accumulation of *Rhodotorula glutinis*, which obtained higher lipid content of 63 ± 6% combined with an attractive biomass. A drastic increase from 14.5% to 38.7% of lipid content appeared in a thermotolerant microalga *Chlorella sorokiniana*, high lipid yield also was observed in fed-batch culture (Zheng et al. 2013).

However, no significant change on pigment accumulation, cellular compound and fatty acid composition was observed under both cultivation modes. As mentioned above, pigment composition showed a dependence on specific and carbon source type, in other words, supplement of NaAc through batch or fed-batch modes could not control pigment content. Fed-batch culture could maintain the higher level of protein and carbohydrate at the 2 g $L^{-1}$ by contrast with 8 g $L^{-1}$. According to Rosa et al. (2019), fed-batch cultivation facilitated an increase in the protein synthesis. This fact could be illustrated that the concentration of glycoproteins increased, which was the vital component of cell wall of green algae. Studies by Fields et al. (2018) indicated that the impact of cultivation method (batch and fed-batch cultivation) on the primary metabolite of *Chlamydomonas reinhardtii*, the carbohydrate content (galactose, glucose) of batch group was a little more than fed-batch at the same cultivation time, however with the increasing cultivation time, the value carbohydrate of batch groups appeared drop in a certain extent, fed-batch groups still kept the primary level or generated a slight drop. Finally, our results validate that it is deserved to utilize NaAc as organic carbon source to enhance the growth and lipid production under different cultivation patterns.

## Conclusion

The present study demonstrated the capacity of *M. reisseri* FM1 to enhance biomass, lipid, carbohydrate and other biochemical components via assimilating NaAc efficiently in both batch culture and feed-batch culture modes under mixotrophic cultivation. Various concentration of NaAc promoted the growth, pigments and lipids in the algal cells, while protein was restrained. Fed-batch cultivation showed higher performance in regulation of growth rate and lipid accumulation compared with batch group. Besides that, carbohydrate synthesis and proportion of fatty acid were also moderately affected by acetate addition and cultivation strategy. Consequently, *M. reisseri* FM1 can realize the feasibility of high-cell density cultivation, high lipid production by feeding with acetate at appropriate concentration, which implied that NaAc can be used as a cheap and efficient regulator to promote the growth of microalgae and regulate the biosynthesis of intracellular biochemical components, especially in the biosynthesis of lipids and functional fatty acids. The *M. reisseri* FM1 biomass can be used as high quality biorefinery raw material to extract and produce functional edible oils, proteins and pigments.

## Declarations

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# Tables

**Table 1**

Effects of various concentrations of sodium acetate on the growth kinetic parameters of *M. reisseri* FM1 under batch and fed-batch culture.

| Parameters | Sodium acetate content (g $L^{-1}$) | Biomass content (g $L^{-1}$) | Specific growth rate (μ, $day^{-1}$) | Biomass productivity (g $L^{-1}$ $day^{-1}$) |
|---|---|---|---|---|
| Batch culture | 0 | 0.17 ± 0.02a | 0.32 ± 0.02a | 0.05 ± 0.01a |
| | Batch-1 | 1.27 ± 0.06b | 0.49 ± 0.03b | 0.25 ± 0.01b |
| | Batch-2 | 1.28 ± 0.08b | 0.53 ± 0.05c | 0.34 ± 0.01c |
| | Batch-4 | 1.32 ± 0.07b | 0.57 ± 0.06d | 0.45 ± 0.02d |
| | Batch-6 | 1.41 ± 0.10b | 0.57 ± 0.02de | 0.47 ± 0.01d |
| | Batch-8 | 1.68 ± 0.06c | 0.58 ± 0.06ef | 0.51 ± 0.01e |
| | Batch-10 | 2.25 ± 0.16d | 0.59 ± 0.07f | 0.57 ± 0.05f |
| Fed-batch culture | 0 | 0.17 ± 0.02a | 0.32 ± 0.02a | 0.05 ± 0.01a |
| | Fed-1 | 1.59 ± 0.06b | 0.58 ± 0.03c | 0.50 ± 0.01c |
| | Batch-4 | 1.38 ± 0.13b | 0.55 ± 0.05b | 0.45 ± 0.01b |
| | Fed-2 | 2.04 ± 0.19c | 0.58 ± 0.06c | 0.52 ± 0.02c |
| | Batch-8 | 1.98 ± 0.17c | 0.59 ± 0.04c | 0.51 ± 0.03c |

Batch-(1-10) denoted 1-10 g $L^{-1}$ of NaAc were supplemented into sterile SE medium, respectively. Fed-batch-(1, 2) denoted 1, 2 g $L^{-1}$ of NaAc were supplemented into sterile SE medium, then intermittently fed three times during cultivation at 2, 4 and 6 days respectively. Values are means ± SD ($n$=3, $P$<0.05), different minuscule denoted the significant differences between treatments.

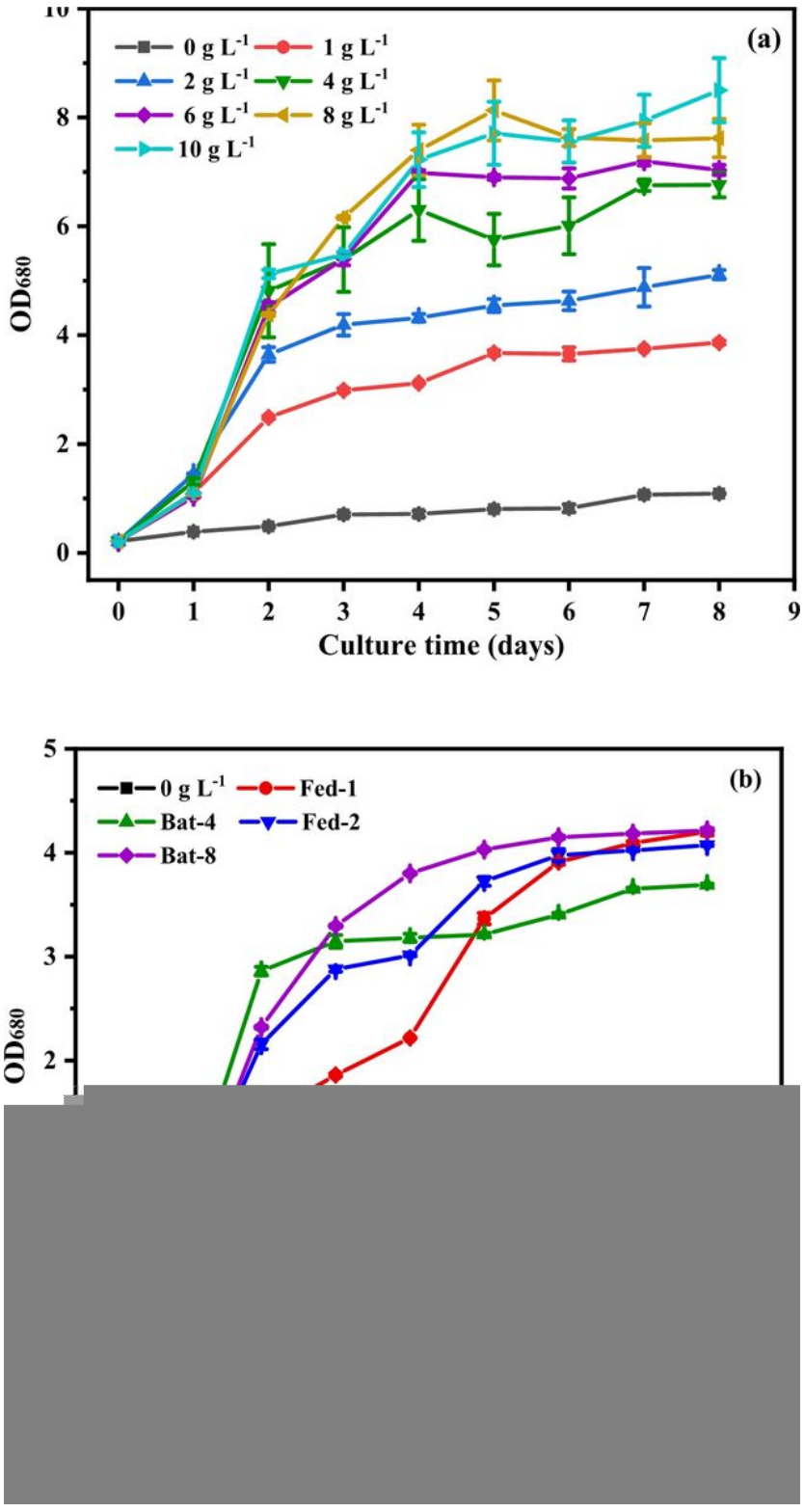
## Table 2

Effects of various concentrations of sodium acetate on the lipid production by *M. reisseri* FM1 under batch and fed-batch culture.

| Parameters | Content (g $L^{-1}$) | Lipid content (%) | Lipid yield (g $L^{-1}$) | Lipid productivity (mg $L^{-1}$ $day^{-1}$) |
|---|---|---|---|---|
| Batch culture | 0 | 8.93 ± 0.03a | 0.02 ± 0a | 1.90 ± 0.07a |
| | Batch-1 | 12.03 ± 0.01b | 0.15 ± 0.01b | 19.10 ± 1.31b |
| | Batch-2 | 13.97 ± 0.02b | 0.18 ± 0.02b | 22.35 ± 2.84b |
| | Batch-4 | 24.99 ± 0.01c | 0.33 ± 0.02c | 41.24 ± 1.72c |
| | Batch-6 | 27.09 ± 0.01c | 0.38 ± 0.02c | 47.75 ± 2.04d |
| | Batch-8 | 37.19 ± 0.02d | 0.62 ± 0.04d | 78.10 ± 4.85e |
| | Batch-10 | 38.85 ± 0.02d | 0.87 ± 0.04e | 109.25 ± 5.44f |
| Fed-batch culture | 0 | 8.93 ± 0.03a | 0.02 ± 0a | 1.90 ± 0.07a |
| | Fed-1 | 28.62 ± 0.01c | 0.46 ± 0.02c | 56.88 ± 1.99c |
| | Batch-4 | 24.12 ± 0.01b | 0.33 ± 0.01b | 41.60 ± 1.22b |
| | Fed-2 | 39.85 ± 0.02e | 0.81 ± 0.04e | 101.63 ± 5.42e |
| | Batch-8 | 35.97 ± 0.01d | 0.71 ± 0.02d | 89.02 ± 2.33d |

Values are means ± SD ($n$=3, $P$<0.05), different minuscule denoted the significant differences between treatments.

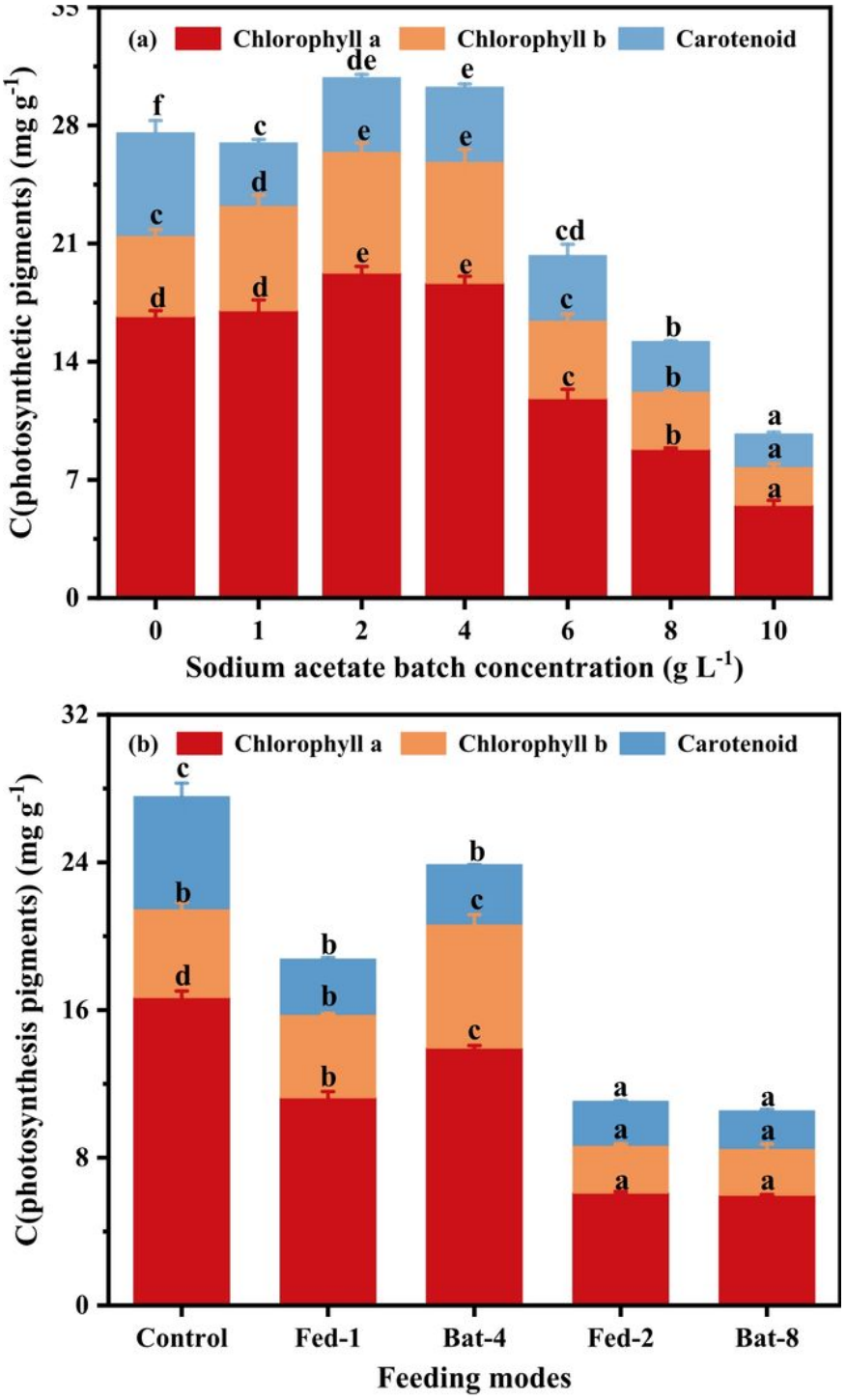
# Figures




## Figure 1

Effects of concentrations of sodium acetate and feeding modes on the growth behavior of *M. reisseri* FM1 (a: Batch-sodium acetate, b: Fed-batch).

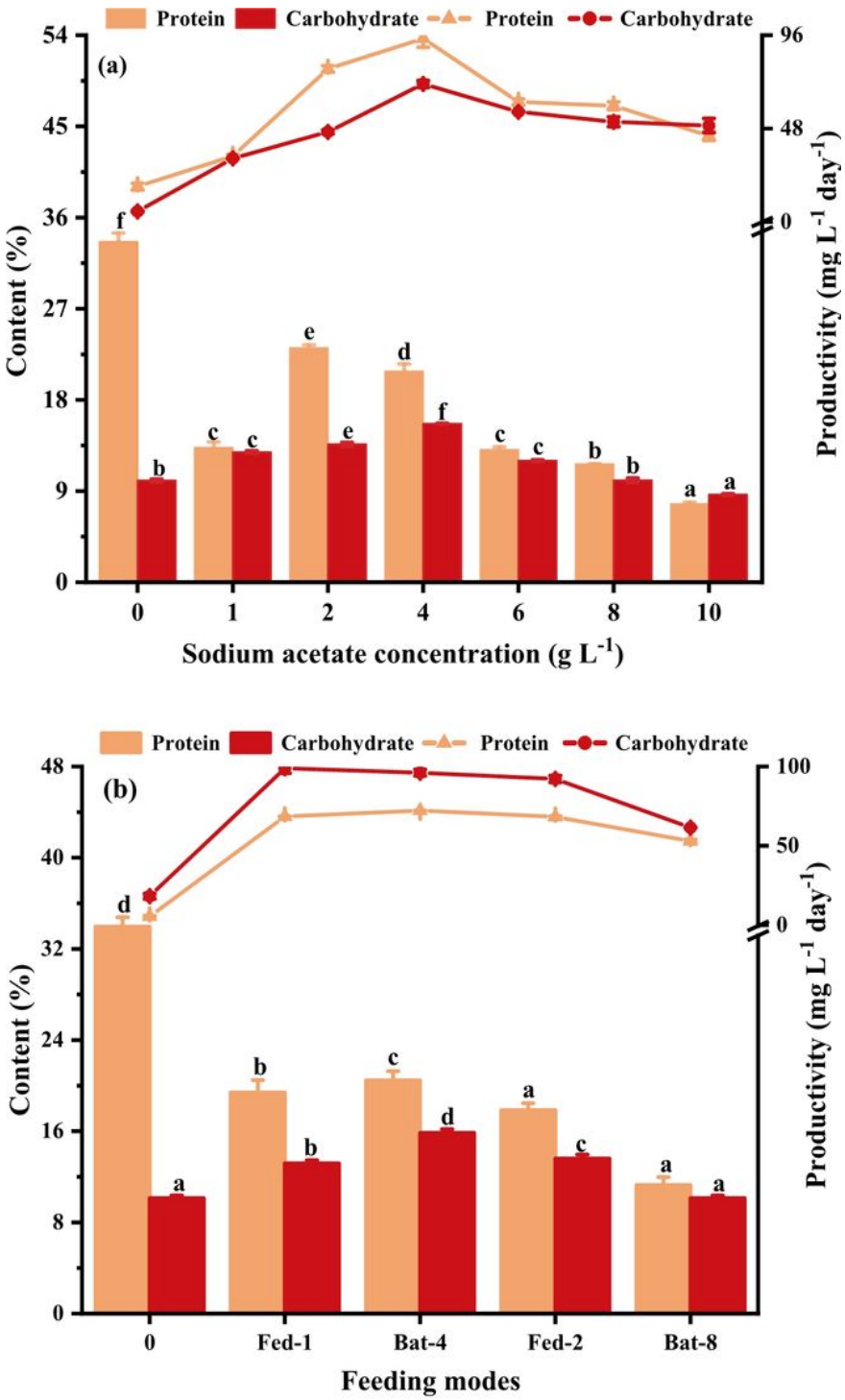
Values are means ± SD ($n$=3, $P$<0.05)




## Figure 2

Effects of concentrations of sodium acetate and feeding modes on the Photosynthesis pigments of *M. reisseri* FM1 (a: Batch-sodium acetate, b: Fed-batch).

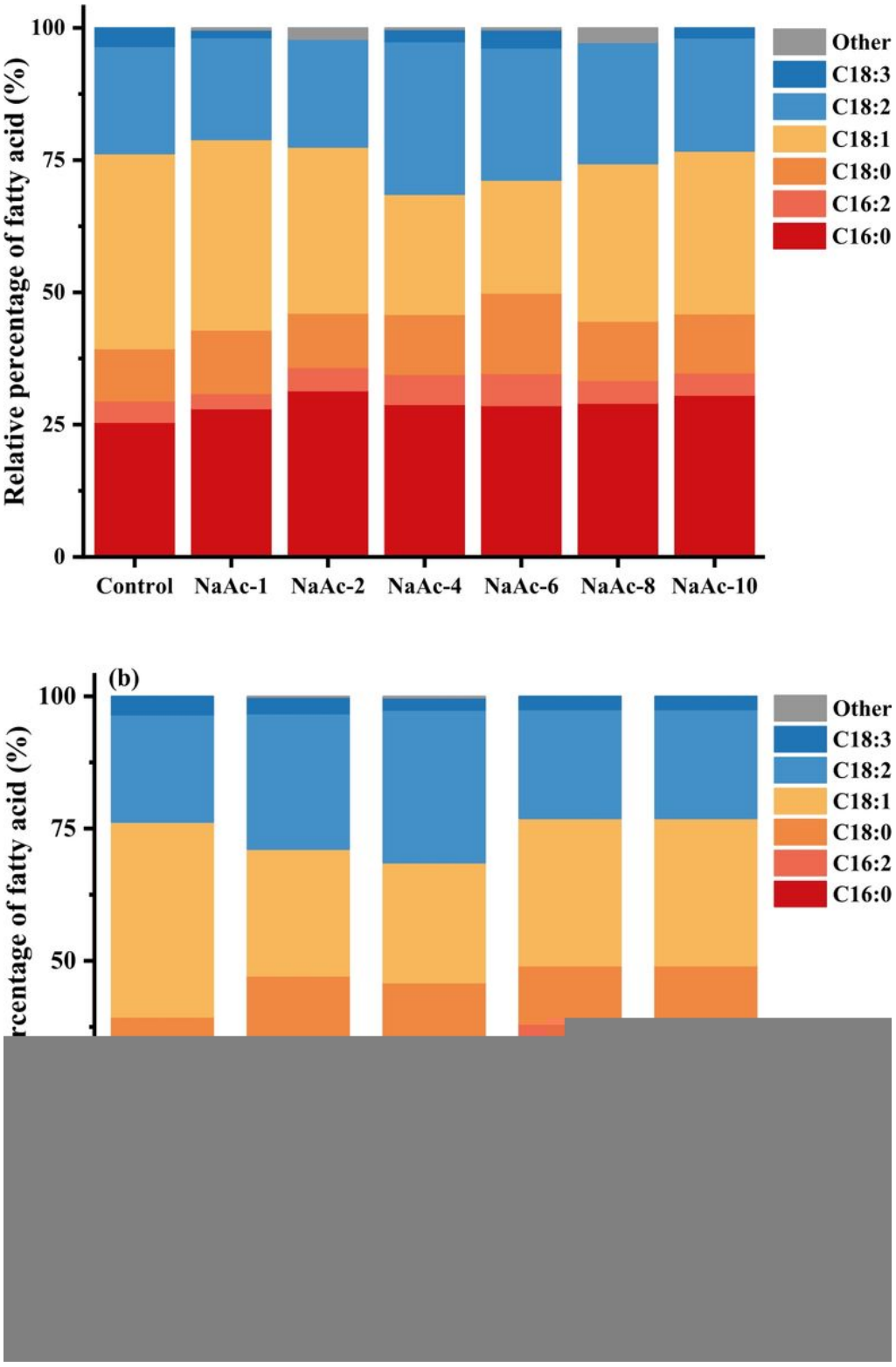
Values are means ± SD ($n$=3, $P$<0.05), different minuscule denoted the significant differences between treatments.




## Figure 3

Effects of concentrations of sodium acetate and feeding modes on the contents of carbohydrate and protein of *M. reisseri* FM1.

The bar chart shows the content and the line chart shows the productivity. Values are means ± SD (*n*=3, *P*<0.05), different minuscule denoted the significant differences between treatments.




## Figure 4

Effects of various concentrations of sodium acetate on the fatty acid profile of *M. reisseri* FM1 under batch and fed-batch culture.