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Full Length Research Paper

Salt-induced osmotic stress for lipid overproduction in batch culture of *Chlorella vulgaris*

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Effect of NaCl-induced osmotic stress on lipid production was investigated in batch culture of *Chlorella vulgaris*. Based on the facts that NaCl stress improved lipid production but inhibited cells growth at the same times, the novel strategies of multiple osmotic stresses with different NaCl additions (2 g/L at 80 h, 4 g/L at 100 h, and 6 g/L at 120 h) were adopted for lipid overproduction. Results show that after 180 h cultivation, lipid yield reached 3.16 g/L and intracellular lipid content was 58.6%, increased by 21.1 and 22.9%, respectively, compared to the control. Further applying the strategies to 5 L fermentor, lipid yield of 3.81 g/L was achieved at 180 h, which was 30.1% higher than the control, suggesting application of osmotic stress to lipid overproduction as being feasible.

Key words: NaCl-induced osmotic stress, heterotrophic cultivation, lipid, glucose, *Chlorella vulgaris.*

INTRODUCTION

Consumption of fossil fuel has been dramatically increased since industrial revolution. The imprudent use of fossil fuel has caused many issues in environment and economics, such as global warming and oil crisis. Due to the limitation of fossil fuel and its increased consumption rate, the need to explore alternative fuels is greatly urgent. Biodiesel, as a biodegradable and renewable fuel source, is considered as an ideal candidate (Lang et al., 2001; Antolin et al., 2002). Biodiesel is mainly made from renewable biological sources such as vegetable oils, animal fats and microalgae lipids. Compared to other two sources, microalgae biodiesel have received much attention in recent years (Xu et al., 2006; Bastianoni et al., 2008). Microalgae were considered as ideal candidates for biodiesel production because of their higher biomass and intracellular lipid content (Ginzburg, 1993; Minowa et al., 1995).

To make biodiesel production from microalgae sustainable, *Chlorella vulgaris* and *Chlorella protothecoides* were currently used on industrial scale (Chisti, 2007; Wu et al., 2008). It was demonstrated that both strains can be cultured photoautotrophically or heterotrophically (Chih and Wen, 2009). Compared to autotrophic way, heterotrophic cultivation of microalgae is considered an efficient alternative due to its higher biomass and productivity (Han et al., 2006).

More recently, many researches have been conducted on heterotrophic growth of some microalgae for efficient production of biodiesel (Wen and Chen, 2003; Miao and Wu, 2006). As an intracellular product, the ultimate goal of biodiesel production from microalgae is to obtain maximal lipid yield by increasing biomass and intracellular lipid content. Accordingly, the concerned methods or strategies have been developed (Courchesne et al., 2009; Stephenson et al., 2010). Principal researches were focused on enhancing intracellular lipid content by biochemical engineering approaches. For instance, nitrogen deprivation (Tornabene et al., 1983), silicon deficiency (Lynn et al., 2000), and iron limitation (Liu et al., 2008) were adopted.

It was reported that high salinity can stimulate microalgae to accumulate intracellular lipid (Rao et al., 2007). Although salt-induced osmotic stress can stimulate lipid accumulation, its effects on cell growth is scarcely known. Moreover, application of osmotic stress to stimulating lipid production in *C. vulgaris* and *C. protothecoides* was never reported. Accordingly, in the present study, the novel strategies of NaCl-induced osmotic stress were developed for lipid overproduction in *C. vulgaris*. To our knowledge, this is the first study on promoting lipid production by salt-induced osmotic stress.

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Initially, the effects of NaCl stress on cells growth and lipid production were studied. Then based on the results that NaCl can stimulate lipid accumulation but inhibit cells growth simultaneously, the strategies of multiple NaCl stresses were developed.

MATERIALS AND METHODS

Microorganism and culture medium

A high-lipid microalgae strain of *C. vulgaris*, which was screened and collected in our laboratory, was used in this study. Both seed and batch culture mediums were consisted of bold's basal media (BBM) supplemented with (g/L): glucose 10, yeast extract 2.0, glycine 1.0 at pH 6.0. The main components used in BBM were (g/L): $NaNO_3$ 0.25, $MgSO_4.7H_2O$ 0.075, NaCl 0.025, $K_2HPO_4.3H_2O$ 0.075, KH_2PO_4 0.175, $CaCl_2.2H_2O$ 0.025, and trace elements were (10^{-3} g/L): $ZnSO_4.7H_2O$ 8.82, $MnCl_2.4H_2O$ 1.44, MoO_3 0.71, $CuSO_4.5H_2O$ 1.57, $Co(NO_3)_2.6H_2O$ 0.49, H_3BO_3 11.42, EDTA 50.0, KOH 31.0 and $FeSO_4.7H_2O$ 4.98.

Batch cultivation in shake flask and fermentor

Flask culture experiments were performed in 250 ml flasks, each containing 50 ml medium after inoculating with 10% (v/v) of seed culture. Culture conditions of temperature, agitation rate, and growth period were fixed at 30°C, 200 rpm and 200 h, respectively. Meanwhile, batch culture with 3 L medium containing 10% (v/v) of seed culture was carried out in 5 L fermentor. The pH was controlled automatically at 6.0 by adding 3 mol/L H_2SO_4 or NaOH solutions. Agitation speed and temperature were controlled at 200 rpm and 30°C respectively.

NaCl-induced osmotic stress

The whole batch culture process for lipid production lasted 180 h. For NaCl-induced osmotic stress, the different types of NaCl additions in flask experiments were as follows: NaCl addition at the beginning of cultivation; NaCl with concentrations (2, 4, 6, 8 and 10 g/L) added to the medium at the beginning of cultivation, separated NaCl addition: NaCl with different concentrations (2, 4, 6 and 8 g/L) which were added to the medium separately at various cultivation times (40, 60, 80, 100, 120 and 140 h) and multiple NaCl addition: based on separated NaCl addition, two, three and four points additions of NaCl with three concentrations (2, 4 and 6 g/L) carried out at different times (80,100,120 and 140 h), respectively in flasks.

Analytical methods

The cell concentration was determined after drying cells at 65°C to a constant weight. Culture broths were centrifuged at 8000 rpm for 15 min and cells were washed twice with distilled water and freeze-dried. Dried cells were then pulverized into powder in a mortar and lipid was extracted by soxhlet method using n-hexane. Intracellular lipid content was obtained by the ratio of total lipid yield to cell concentration. Total nitrogen in medium was analyzed using high temperature TOC/TNb Analyzer LiquiTOC II (Elementar Analysensysteme GmBH, Hanau, Germany). Glucose residue in medium was determined according to the methods described by Miller (1959).

All experiment treatments were designed in three parallel and repeated twice and average results were used for final analysis.

RESULTS

Effects of NaCl added at initial cultivation stage on cells growth and lipid production

Although osmotic stress can result in intracellular lipid accumulation in plant, its effect on microalgae growth is scarcely known. Accordingly, to investigate the influence of salt stress on intracellular lipid accumulation and cells growth, NaCl at various levels were added at initial stage in heterotrophic cultivation of *C. vulgaris* in 5 L fermentor. Initially, the status of cells growth and lipid production without salt addition was investigated. As shown in Figure 1, according to biomass increment and lipid accumulation, the whole process was separated into two phases of cells growth and lipid synthesis. It was noticed that glucose and nitrogen concentrations decreased gradually with cultivation extending. At 120 h, nitrogen source was almost exhausted and cells stopped increasing. At this time point, maximal cell concentration of 5.78 g/L was obtained and intracellular lipid yield was 2.12 g/L.

Meanwhile, after nitrogen exhaustion and cell growth has stopped, the intracellular lipid increased continually from 2.12 g/L at 120 h to 2.93 g/L at 180 h, and corresponding lipid content was increased from 37 to 51%. In comparison, as indicated in Table 1, with NaCl stress, cells' growth were strongly inhibited and in turn led to a decrease in lipid yield. Moreover, the negative impacts of NaCl stress on cells growth and lipid yield were dose-dependent. For instance, as NaCl concentration was increased from 2 to 6 g/L, maximal dry cell weight (DCW) and lipid yield decreased from 5.51 and 2.87 g/L, to 5.11 and 2.77 g/L, respectively. Moreover, as salt concentration increased from 8 to 10 g/L, lipid contents decreased from 55.1 to 52.3%, indicating that the maximal addition amounts should be 8 g/L. It was evident that intracellular lipid contents in all treatments were higher than the control, indicating positive effect of NaCl stress on improving lipid synthesis. Concisely, NaCl added at the beginning of cultivation showed positive effect on intracellular lipid accumulation, but inhibited cells growth simultaneously. As a result, total lipid yields were decreased in all treatments as compared to the control.

Effects of NaCl addition times and amounts on lipid production

Based on previous results, we safely concluded that it is unsuitable to add NaCl at the initial culture time for enhancing lipid production. So it is necessary to investigate effects of addition times and amounts on lipid production. Moreover, because it is unpractical to conduct all experiments in fermentor under the same condition at one time, the following experiments were carried out in flasks.

NaCl concentrations with 2, 4, 6, and 8 g/L were added

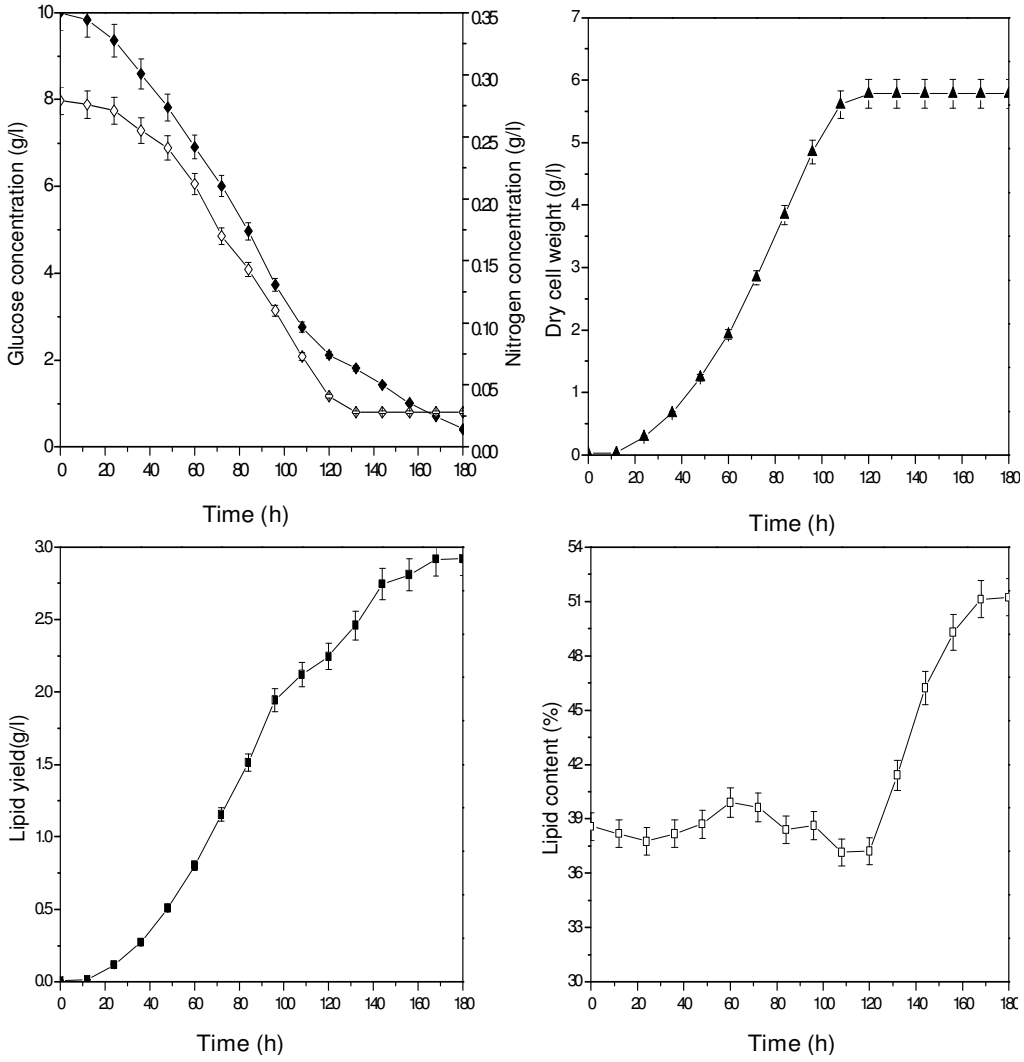

Figure 1. Effects of NaCl added at initial culture time on cells growth and lipid production. ◊, Nitrogen concentration; ◆, glucose concentration; ■, lipid yield; □, lipid content; ▲, dry cell weight.

at 40, 60, 80, 120, and 140 h, separately. As shown in Table 2, without NaCl addition, maximal DCW of 5.47 g/L was achieved at 120 h and maximal lipid yield reached 2.61 g/L at 180 h. Consequently, to better compare cells growth and lipid production in treatments with the control, cell concentration and lipid yield of all treatments were measured at 120 and 180 h, respectively, in the following works. Apparently, NaCl stress effectively induced lipid synthesis but inhibited cells growth simultaneously, especially at cell growth phase. Moreover, this inhibition was positively proportional to NaCl levels and inhibition degree accelerated with advancing of NaCl addition times. For example, with 2 g/L NaCl added at 40 and 80 h, DCW of 5.36 and 5.42 g/L were achieved at 120 h and lipid yields reached 2.66 and 2.73 g/L after 180 h cultivation. Also, 6 g/L NaCl stress addition at 120 h achieved the highest lipid yield of 2.95 g/L in all treatments.

It was fascinating that, with lower NaCl added at 40, 60, 80 and 100 h or higher added at 120 and 140 h, a comparatively higher lipid yield can be achieved. Meanwhile, the negative impact of NaCl on cells growth was insignificant with 2 g/L of NaCl added at 80 h and 4 g/L added at 100 h, indicating the inhibition degree can be minimized by adding lower NaCl at cells growth phase. Moreover, at lipid synthesis phase, lipid production was negatively proportional to NaCl addition times, which suggests that suitable times for NaCl addition perhaps were at 120 and 140 h.

Briefly, based on the phenomenon that NaCl stress can greatly promote lipid production but inhibit cell growth at the same times, we speculated that NaCl added with lower level at cells growth phase and higher one at stationary phase can maximize lipid yield while minimize cells growth inhibition. Accordingly, the types of multiple NaCl additions were further conducted.

Table 1. Effects of NaCl added at initial culture time on cells growth and lipid production.

Parameter	2 g/L NaCl	4 g/L NaCl	6 g/L NaCl	8 g/L NaCl	10 g/L NaCl
Dried cell weight (g/L)	5.51 ± 0.03	5.32 ± 0.022	5.11 ± 0.02	4.86 ± 0.01	4.61 ± 0.01
Lipid yield (g/L)	2.87 ± 0.02	2.82 ± 0.02	2.77 ± 0.01	2.68 ± 0.01	2.41 ± 0.01
Lipid content (%)	52.1 ± 0.2	53.0 ± 0.2	54.2 ± 0.3	55.1 ± 0.3	52.3 ± 0.2

Table 2. Effects of NaCl addition amounts and times on lipid production in flask.

Concentration of NaCl (g/L)	Addition time (h)	Dry cell weight (g/L)	Lipid yield (g/L)	Lipid content (%)
Control	-	5.47 ± 0.03	2.61.3 ± 0. 2	47.71 ± 0.01
2	40	5.36 ± 0.02	2.66 ± 0.01	49.63 ± 0. 2
	60	5.38 ± 0.02	2.69 ± 0.01	50.00 ± 0. 2
	80	5.42 ± 0.03	2.73 ± 00.2	50.37 ± 0.3
	100	5.45 ± 0.03	2.76 ± 00.2	50.64 ± 0.3
	120	5.47 ± 0.03	2.78 ± 00.3	50.82 ± 0.3
	140	5.47 ± 0.04	2.65 ± 0.02	48.45 ± 0.2
4	40	5.32 ± 0.01	2.71 ± 0.02	50.94 ± 0.2
	60	5.34 ± 0.02	2.75 ± 0.03	51.15 ± 0.3
	80	5.38 ± 0.04	2.81 ± 0.02	52.23 ± 0.4
	100	5.41 ± 0.04	2.85 ± 0.03	52.68 ± 0.4
	120	5.47 ± 0.03	2.89 ± 0.03	52.83 ± 0.3
	140	5.47 ± 0.04	2.67 ± 0.01	50.64 ± 0.2
6	40	5.29 ± 0.01	2.74 ± 0.02	51.80 ± 0.1
	60	5.31 ± 0.02	2.78 ± 0.02	52.35 ± 0.2
	80	5.34 ± 0.05	2.84 ± 0.03	53.18 ± 03
	100	5.38 ± 0.05	2.88 ± 0.03	53.53 ± 0.4
	120	5.47 ± 0.04	2.95 ± 0.04	53.93 ± 0.3
	140	5.47 ± 0.04	2.79 ± 0.01	50.01 ± 0.3
8	40	5.24 ± 0.02	2.68 ± 0.03	51.15 ± 0.1
	60	5.28 ± 0.01	2.720 ± 0.02	51.52 ± 0.2
	80	5.32 ± 0.04	2.79 ± 0.02	52.44 ± 0.3
	100	5.35 ± 0.04	2.83 ± 0.03	52.90 ± 0.2
	120	5.47 ± 0.03	2.88 ± 0.03	52.65 ± 0.3
	140	5.47 ± 0.03	2.75 ± 0.02	50.27 ± 0.2

DCW and lipid yield were measured at 120 and 180 h respectively. DCW, Dry cell weight.

Multiple NaCl additions with differed times and amounts

Based on foregoing single addition results that lower NaCl levels of 2 and 4 g/L added at 80 h and at 100 h insignificantly affected cells growth while greatly promoted lipid production, and 6 g/L NaCl added at 120 h achieved the highest lipid yield, three NaCl concentrations of 2, 4 and 6 g/L were used to study impact of multiple NaCl additions on lipid production. Additional ways of two, three and four points of NaCl additions with different concentrations and times were adopted.

As indicated in Table 3, three spots NaCl stresses (2 g/L at 80 h, 4 g/L at 100 h and 6 g/L at 120) achieved a lipid yield of 3.16 g/L at 180 h and corresponding intracellular lipid content was 58.6%, which were 21.1 and 22.9% higher than the control. Meanwhile, we noticed that further frequent induction by NaCl stress at stationary phase cannot enhance lipid yield greatly. For example, four spots NaCl stresses (2 g/L at 80 h, 4 g/L at

Table 3. Effects of multiple NaCl addition on cells growth and lipid production in flasks.

No.	Addition time (h)				Experimental result		
	80	100	120	140	DCW (g/L)	Lipid yield (g/L)	Lipid content (%)
	Concentration of NaCl (g/L)						
1	2	4	0	0	5.39 ± 0.02	2.96 ± 0.02	54.9 ± 0.3
2	2	0	6	0	5.42 ± 0.01	2.99 ± 0.2	55.2 ± 0. 2
3	2	0	0	6	5.42 ± 0.01	2.92 ± 0.01	53.9 ± 0.2
4	0	4	6	0	5.41 ± 0.02	3.03 ± 0.03	56.1 ± 0.3
5	0	4	0	6	5.41 ± 0.03	2.95 ± 0.02	54.5 ± 0. 2
6	2	4	6	0	5.39 ± 0.02	3.16 ± 0.03	58.6 ± 0.3
7	2	4	0	6	5.39 ± 0.02	3.11 ± 0.03	57.7 ± 0.3
8	2	4	6	6	5.39 ± 0.02	3.18 ± 0.03	59.1 ± 0. 4

DCW and lipid yield were measured at 120 and 180 h respectively. DCW, Dry cell weight.

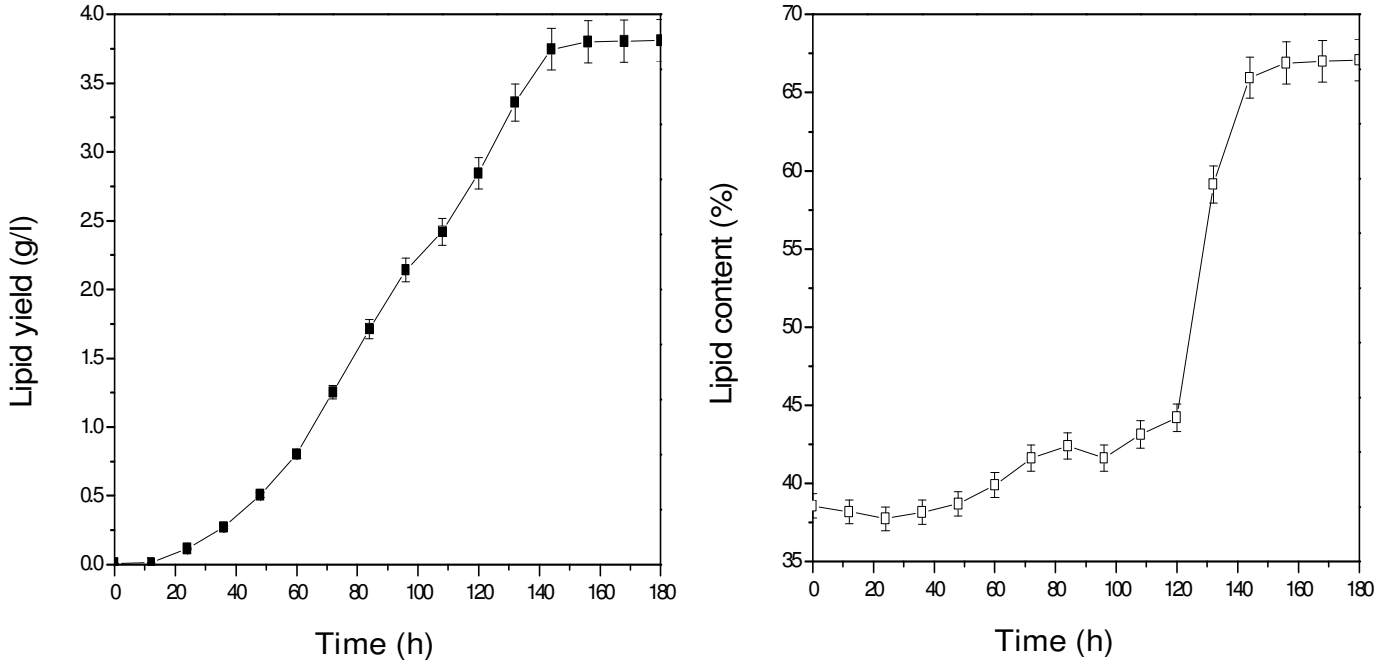

Figure 2. Enhanced lipid production by multiple NaCl addition in 5 L fermentor. ■, Lipid yield; □, lipid content.

100 h, 6 g/L at 120 and 140 h) led to a lipid yield of 3.18 g/L, which was slightly higher than three point's stresses. It was suggested that NaCl addition with three points is feasible for lipid overproduction.

As it is known, the pH value cannot be kept constant in flask experiments. As a result, relatively lower cell concentration and lipid yield were obtained as compared to that in fermentor. For example, maximal DCW and lipid yield were 5.47 and 2.61 g/L in the control of flask experiments, while the corresponding values were 5.78 and 2.93 g/L in 5 L fermentor. Accordingly, to test the effectiveness of the strategies developed in flasks, three points NaCl additions were further applied to 5 L fermentor.

Maximized lipid production by applying multiple NaCl additions to 5 L fermentor

According to foregoing results, it was known that three points NaCl additions are feasible and practical in improving lipid yield. However, since pH change was the main factor leading to a decrease in cell concentration and lipid yield in flask experiments compared to that in fermentor. Consequently, three points NaCl additions were further applied to batch culture in 5 L fermentor. As indicated in Figure 2, with three points NaCl additions (2 g/L at 80 h, 4 g/L at 100 h and 6 g/L at 120 h), maximal lipid yield and corresponding intracellular lipid content reached 3.81 g/L and 67.2% after 180 h cultivation, which

were 30.1 and 31.8% higher than the control.

Furthermore, with three points salt additions, we found that lipid yield was changed from 3.16 g/L in flask to 3.81g/L in 5 L fermentor, increased by 20.6%, which indicates that pH affects lipid synthesis as well as cells growth. In short, a significant increase was observed in lipid yield via salt stresses, suggesting the feasibility of the proposed strategies for lipid overproduction.

DISCUSSION

Microalgae were suggested as very good candidates for biodiesel production because of their rapid growth and higher lipid yield (Minowa et al., 1995). Some microalgae, such as *C. vulgaris* and *C. protothecoides*, are exceedingly rich in lipid and used for lipid production. Lipid yield can be improved by increasing cell concentration and intracellular lipid content. However, an increase in biomass can inevitably lead to lowered intracellular lipid content. As a result, some methods or strategies, such as nitrogen deprivation (Tornabene et al., 1983), were adopted to enhance lipid accumulation. Besides nutrition supply, environmental conditions such as dissolved oxygen, temperature and pH also significantly affect cells growth and products production (Elibol, 2002). Recently, much attention has been paid to the response of plants or microorganisms to osmotic stress (Ruiz and Blumwald, 2002; Liang et al., 2009). For example, investigations showed that high salinity can stimulate lipid accumulation in microalgae (Rao et al., 2007). However, up to date, effects of salinity on cells growth and lipid production in heterotrophic cultivation of microalgae were little known. As an intracellular product, lipid production was closely related to biomass and lipid content. Salt stress significantly affects cells growth and lipid formation. Accordingly, how to maximize lipid production by optimizing salt stress is the point we tried to explore.

In this study, NaCl-induced osmotic stress was adopted to stimulate lipid accumulation in *C. vulgaris*. As a result, an increase in lipid yield was observed, which can be explained by a protective mechanism that cells synthesized lipid excessively to avoid injuries caused by salt stress. However, we found that effects of single salt stress on cells growth and lipid production were time and concentration dependent. In particular, cell growth was strongly affected which in turn led to a significantly negative effect on lipid accumulation when salt was added at the beginning of cultivation. As expected, the multiple salt additions strategies with lower salt concentration added at cells growth phase and higher one at the stationary phase would maximize lipid yield, while minimizing its negative effect on cells growth. By adopting this novel strategy of multiple salt stresses (2 g/L at 80 h, 4 g/L at 100 h and 6 g/L at 120 h), a lipid yield of 3.81 g/L was achieved and the corresponding intracellular lipid content reached 67.2%.

In conclusion, a strategy based on defensive mecha-nism of *C. vulgaris* to NaCl-induced osmotic stress was adopted to enhance lipid production. To maximize intracellular lipid yield but minimize inhibition of cells growth, a multiple salt addition method was developed. It can be said that the novel strategy developed is a feasible method in lipid production. Although osmotic stress can lead to lipid accumulation in microalgae, Na^+ perhaps also play a positive role in stimulating lipid synthesis. Therefore, we will further investigate the role of Na^+ in inducing lipid accumulation in future study.

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