Thesis for the degree of master of microbiology

Optimization of microalgae culture media for transformed *Chlorella vulgaris* PKVL7422

by

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형질전환 *Chlorella vulgaris* PKVL7422 에 대한 조류 배양 배지의 최적화

Advisor: Prof. Tae-Jin Choi

by

Ye-Lin Kim

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Abstract

As commercial applications of microalgae in many different areas such as food, feed, cosmetics, and biofuel have increased, microalgae cultivation researches have been developed all over the world. Moreover, due to limitation in expression of eukaryotic proteins in *E. coli*, microalga transformation and expression system is being used instead of bacterial system for the production of recombinant proteins. However, mass culturing techniques for transformed microalgae such as *Chlorella vulgaris* have rarely developed. Hence, in this study we aimed to increase the growth of transformed *Chlorella vulgaris* (PKVL7422) whose nitrate reductase gene was knocked out, by using NH_4 instead of NO_3 and other modifications of BG11 media. Growth of the transformed microalgae in the modified medium named as BGPK was examined by culturing in BG11 based medium containing one of the following chemicals: K_2HPO_4 (0.17mM, 1.7mM 5mM, 10mM, 15mM, 20mM, 25mM, 30mM), glucose (0%, 0.1%, 0.3%, 0.5%, 1.0%, 2.0%, 3.0%), $(NH_4)_2HPO_4$,

NH_4Cl, $(NH_4)_2SO_4$, and $CH_3CO_2NH_4$ (17mM, 5mM, 10mM, 15mM, 20mM, 25mM, 30mM). Growth of *C. vulgaris* was measured using microplate reader at OD_{680nm}. The results showed that in the presence of 20mM of $(NH_4)_2HPO_4$ and 0.5% of glucose *C. vulgaris* growth rate increased up to 49.67% compare that of in BGNK growth rate, 13.67%. Our data indicates that BGPK is a better medium for *Chlorella vulgaris* PKVL7422 transformation system than BGNK and this study can be a model for developing an efficient medium for microalga recombinant expression system.

Introduction

Microalgae value

Microalgae are photosynthetic eukaryotic cells and their simple structures and fast growth rate compare to other eukaryotic cells are one of the best properties (Ortiz Montoya et al., 2014). Because of the advantages, microalgae have been used in diverse fields, such as for food (Plaza, Herrero, Cifuentes, & Ibanez, 2009), feed (Hemaiswarya, Raja, Kumar, Ganesan, & Anbazhagan, 2011; Yaakob, Ali, Zainal, Mohamad, & Takriff, 2014), cosmetics, nutraceuticals (Borowitzka, 1999; Eom, Park, LEE, & Jin, 2005; Kathiresan & Sarada, 2009), biofuel (Varfolomeev & Wasserman, 2011), and system of recombinant protein expression system (Barrera & Mayfield, 2013; Gong, Hu, Gao, Xu, & Gao, 2011; Specht, Miyake-Stoner, & Mayfield, 2010). Especially, as the microalgae transformation system is developed, the recombinant proteins that expressed in transformed microalgae have been applicate in many areas, pharmaceutical, vaccine, and therapeutics (Rasala et al., 2010). Although many researchers have been already developed microalgae for mass culture of microalgae (Chaumont, 1993; Mandalam & Palsson, 1998), effective culture media for microalgae which express the recombinant protein is important to produce the useful recombinant proteins.

Factors that effect on microalgae growth

The main factors that affect microalgae growth are nitrogen, phosphorus, and carbon. N_2 is not common form that can be used by microalgae themselves and it can be consumed only when we are associated with other microorganisms. Usual forms of nitrogen for microalgae are mostly NO_3 and NH_4 and NO_2 can be used occasionally (Schmidt, Raven, & Paungfoo-Lonhienne, 2013; Usher, Bergman, & Raven, 2007). Phosphorus is associated with energy, signaling, and building block in the cell (Merchant & Helmann, 2012). And the major limiting nutrients in the microalga. Carbon is one of the most important factors in microalgae with nitrogen, and type and concentration of carbon are important consideration elements for microalgae growth (Daliry, Hallajisani, Mohammadi, Nouri, & Golzary, 2017). As the photosynthetic green cells, microalgae use the CO_2 for carbon source. However, most microalgae are known as mixotrophic microorganisms (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011), they can use organic carbon with CO_2 for their growth.

Problems of original transformant growth media

Transformation system that optimized from Kim (2019) target the nitrate reductase gene of *Chlorella vulgaris* PKVL7422, consequently, nitrate reductase gene is knocked out from results of transformation (Kim, 2019). As transformed microalgae cannot use the NO_3 as nitrogen source, $NaNO_3$ of BG11 is replaced with

NH_4Cl and this modified medium is named BGNK (**B**G11 containing **G**lucose, **N**H_4Cl, and **K**ClO_3).

BGNK have three kinds of problems in the culture of transformed microalgae. First, growth rate of transformed *Chlorella vulgaris* is very low. The maximum cell number is lower than wild type culture in BG11 containing glucose. Lastly, maintenance of cells in the media is not stable. In this study, we focus on the solving these problems by optimization of composition in the media for transformed *Chlorella vulgaris* PKVL7422.

Materials and methods

Microalga strain and growth condition

Microalgae strain *Chlorella vulgaris* PKVL7422 was isolated in virology laboratory, department of microbiology, Pukyong National University. Transformation system was developed from previous research (Kim, 2019) and transformed microalgae was provided form this system.

BG11 was selected for primary media of *Chlorella vulgaris* PKVL7422 wild type culture whose composition is described at Table 1. Cultivation of *Chlorella vulgaris* PKVL7422 was conducted under continuous light condition (2,800 lux), at 20 .

Table 1. Composition of BG11 and BGNK media

Compound	Concentration	
	BG11	BGNK
$Na_2MGEDTA$	0.002 mM	0.002 mM
Ferric ammonium citrate	0.02 mM	0.02 mM
Citric acid • $1H_2O$	0.03 mM	0.03 mM
$CaCl_2 • 2H_2O$	0.2 mM	0.2 mM
K_2HPO_4	0.17 mM	0.17 mM
$MgSO_4 • 7H_2O$	0.03 mM	0.03 mM
Na_2CO_3	0.18 mM	0.18 mM
Microelements[1]	1 ml/L	1 ml/L
$NaNO_3$	17 mM	-
NH_4Cl	-	17 mM
$KClO_3$	-	150 mM
Glucose	0.3% (w/v)	0.3% (w/v)

[1)] Microelements: Contain the 2.86 g/L of H_3Bo_3, 1.81 g/L of $MnCl_2 • 4H_2O$, 0.222 g/L of $ZnSO_4 • 7H_2O$, 0.079 g/L of $CuSO_4 • 5H_2O$, 0.050 g/L of $COCl_2 • 6H_2O$, and 0.391 g/L of $NaMoO_4 • 2H_2O$

Modification of NH_4 compound and concentration

Because N source in BG11 is replaced with to NH_4Cl in BGNK, we varied the N source that contain the NH_4 and used different concentration of those compound. Every composition of culture medium was same with Table 1. BGNK except N source.

In order to compare the growth of transformed microalga depend on the NH_4 compounds, followed compounds were used: ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), di-ammonium phosphate ($(NH_4)_2HPO_4$), and ammonium acetate ($CH_3CO_2NH_4$) and concentration of each compound was fixed to 17Mm. Comparison of transformed microalgae growth under diverse concentration of each compound was conducted with 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM (Table 2), and 17 mM NH_4Cl as in BGNK was used as control. Measurement of algae growth is conducted by microplate reader (Varioskan LUX, Thermo scientific) on optical density(OD) at 680 nm, once every other day for 3 weeks and replicated 3 tiomes.

Table 2. Compounds and concentration of N source in modified BGNK*

	A-5	A-10	A-15	A-20	A-25	A-30
$(NH_4)_2HPO_4$	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM
	B-5	B-10	B-15	B-20	B-25	B-30
$(NH_4)_2SO_4$	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM
	C-5	C-10	C-15	C-20	C-25	C-30
NH_4Cl	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM
	D-5	D-10	D-15	D-20	D-25	D-30
$CH_3CO_2NH_4$	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM

* The modified media were named to differentiate the compound (A, B, C, D) and the concentration (-5, -10, -15, -20, -25, -30)

Variation of PO_4 concentration with fixed NH_4 concentration

One of the most important elements of microalgae growth is phosphate (Benitez-Nelson, 2000; Paytan & McLaughlin, 2007). In this part, we aim to check the effect of phosphate concentration with fixed NH_4Cl concentration of 17 mM in BGNK. We selected K_2HPO_4 as phosphate source because it is the phosphate compound in the BG11 medium. Variation of K_2HPO_4 concentration was set to 0.17 mM (control, K-0.17), 1.7 mM (10 times of control, K-1.7), 5 mM (K-5), 10 mM (K-10), 15 mM (K-15), 20 mM (K-20), 25 mM (K-25), 30 mM (K-30). Transformed microalgae growth was estimated by microplate reader (Varioskan LUX, Thermo scientific) on optical density(OD) at 680 nm, once every other day for 2 weeks with three replications.

Growth was measured at two different concentrations of $CH_3CO_2NH_4$ (5 mM (A), 10 mM (B)) with various concentration of K_2HPO_4. Control K_2HPO_4 concentration was set 0.17 mM as control (A-0.17 and B-0.17) as in BG11 and treatment concentration were set to 1.7 mM (10 times of control concentration, A-1.7 and B-1.7), 5 mM (A-5 and B-5), 10 mM (A-10 and B-10), 15 mM (A-15 and B-15), 20 mM (A-20 and B-20), 25 mM (A-25 and B-25), and 30 mM (A-30 and B-30) (Table 3). Measurement of algae growth is conducted by microplate reader (Varioskan LUX, Thermo scientific) on optical density(OD) at 680nm, once every other day for 2 weeks and with three replications.

Table 3. Abbreviation of modification BG11 medium with different concentration of K_2HPO_4 at two different concentrations of $CH_3CO_2NH_4$

K_2HPO_4 (mM)		0.17	1.7	5	10	15	20	25	30
$CH_3CO_2NH_4$	5 mM	A-0.17	A-1.7	A-5	A-10	A-15	A-20	A-25	A-30
	10 mM	B-0.17	B-1.7	B-5	B-10	B-15	B-20	B-25	B-30

Modification of NH_4 concentration with fixed PO_4 concentration

Usually, the N:P ratio of composition (Rhee, 1978) or depletion of specific component (Mandalam & Palsson, 1998) affect the microalgae growth. In this part, we aim to confirm the effect of N:P ratio on the growth of transformed chlorella. K_2HPO_4 with three different concentrations (10 mM (A), 15 mM (B), 20 mM (C)) was fixed on each condition with variation the NH_4Cl and $(NH_4)_2SO_4$ concentration (5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM) (Table 4). The growth of microalga was measured with a microplate reader (Varioskan LUX, Thermo scientific) at 680nm, once every other day for 2 weeks and with three replications.

HPO_4 and $CH_3CO_2NH_4$ are known as compound having buffering mechanism (Konermann, 2017). In order to check the buffering mechanism of HPO_4 and $CH_3CO_2NH_4$, pH was measured every day in modified media with pH meter (MP220 pH meter, METTLER TOLECO).

Table 4. Abbreviation of modified BG11 medium with different N:P ratio by varying the concentration of NH_4Cl and $(NH_4)_2SO_4$ at three different concentrations of K_2HPO_4

K_2HPO_4	Formation of NH_4 (mM)	5	10	15	20	25	30
10 mM	NH_4Cl	A-5	A-10	A-15	A-20	A-25	A-30
	$(NH_4)_2SO_4$	B-5	B-10	B-15	B-20	B-25	B-30
15 mM	NH_4Cl	C-5	C-10	C-15	C-20	C-25	C-30
	$(NH_4)_2SO_4$	D-5	D-10	D-15	D-20	D-25	D-30
20 mM	NH_4Cl	E-5	E-10	E-15	E-20	E-25	E-30
	$(NH_4)_2SO_4$	F-5	F-10	F-15	F-20	F-25	F-30

Effect of carbon source in the BG11 medium on wild type *C. vulgaris* PKVL7422

C. vulgaris PKVL7422 is newly isolated strain and the preference of carbon source has not been investigated yet. Five different type of carbon compounds, polygalacturonic acid, glucose, sucrose, glycerol, sodium acetate (Kong et al., 2011), were tested with wild type *C. vulgaris* PKVL7422. Detail condition of each compound described on Table 6.

Because the selection of transformed microalgae had been conducted in dark condition (Kim, 2019) so carbon test was accomplished without light, also. Test for carbon source was also conducted under light condition as other experiment (2,800lux) and temperature of both dark condition and light condition were 20 . BG11 without glucose was used as negative control in light condition. Measurement of microalgae growth was accomplished using the microplate reader (Varioskan LUX, Thermo scientific) at OD_{680nm}. Every condition set triple and measured every other day for 2 weeks.

Table 5. Modification of different C source in BG11

Contents	Concentration
Polygalacturonic acid(from orange)	0.3% (v/w)
Glucose	0.3% (v/w)
Sucrose	0.3% (v/w)
Glycerol	0.075% (v/w)
Sodium acetate	0.3% (v/w)

Glucose concentration optimization in modified BGNK

A culture medium called BGNK was formulated by replacing 17mM $NaNO_3$ with 17mM NH_4Cl in BG11 to culture the transformed PKVL7422 whose nitrate reductase gene was knocked-out during transformation. The BGNK was modified by replacing NH_4Cl with $(NH_4)_2HPO_4$ as N and P source. The optimum concentration of glucose in this modified media were investigate with 0% (G-0), 0.1% (G-0.1), 0.3% (G-0.3), 0.5% (G-0.5), 1% (G-1), 2% (G-2), 3% (G-3). Measurement of microalgae growth was conducted with microplate reader (Varioskan LUX, Thermo scientific) at OD680nm, every other day for 11days and cultivation condition was same with other set.

Results

Growth of transformed chlorella in BGNK containing different formation of NH_4 at 17mM.

Figure 1 shows the growth of transformed in modified BGNK containing different NH_4 compound instead of NH_4Cl. Concentration of each compounds is adjusted to 17 mM which is the same with 17 mM NH_4Cl in BGNK. The best growth of transformed microalgae was observed in the media containing $(NH_4)_2HPO_4$. That compound contains 2 times of NH_4 concentration and 100 times PO_4 concentration compare to NH_4Cl. In two media containing $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$, which contain the same concentration of NH_4 shows better growth was observed in the medium containing $(NH_4)_2HPO_4$. $CH_3CO_2NH_4$ and NH_4Cl produce the same NH_4^- ion as concentration of NH_4Cl. However, the growth of transformed chlorella was better in the modified BGNK containing $CH_3CO_2NH_4$ (line D in Fig.1) than the medium containing NH_4Cl (line C in Fig. 1).

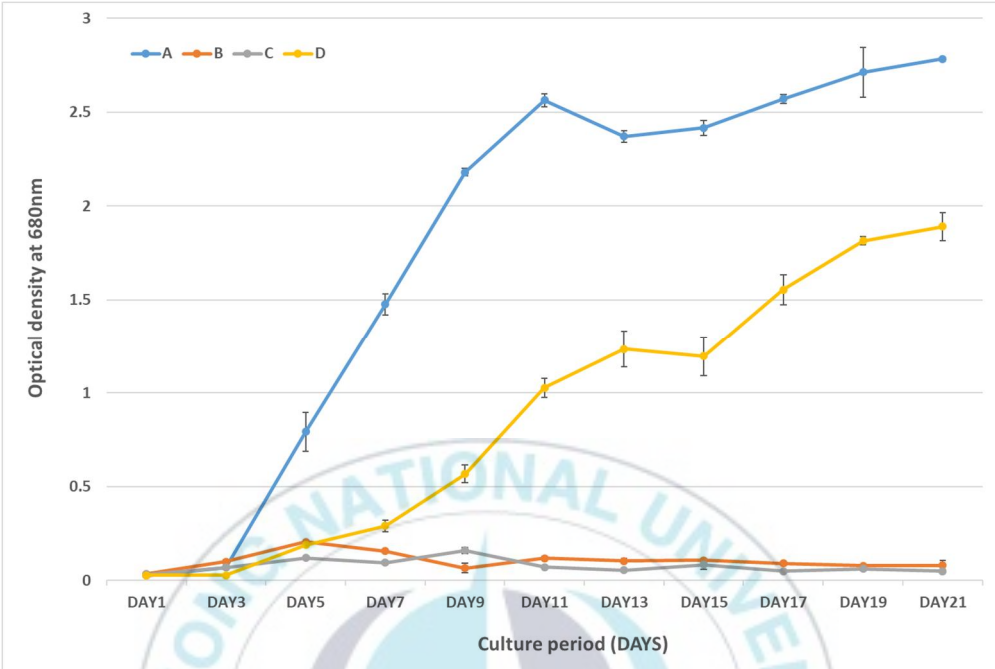

Figure 1. Growth of transformed *Chlorella vulgaris* PKVL7422 in BGNK containing modified N source.

A: BGNK containing $(NH_4)_2HPO_4$ 17 mM instead of 17 mM NH_4Cl. B: BGNK containing NH_4Cl 17 mM for N source. C: BGNK containing 17 mM $(NH_4)_2SO_4$. D: BGNK containing 17 mM $CH_3CO_2NH_4$. The results are average from three replications.

Growth of transformed chlorella in modified BGNK with different formation and concentration of NH_4

Figure 2 shows the growth of transformed microalgae in different media containing various concentration of NH_4^+ containing chemicals of different concentration. The growth of transformed chlorella increased as the concentration of $(NH_4)_2HPO_4$ increase from 5 mM to 15 mM (Fig. 2A), but there was no significant difference at 20, 25, and 30 mM.

Similar growth patterns were observed in the modified BGNK containing $CH_3CO_2NH_4$. The maximum growth was observed at the concentration of 10mM and the growth was less in higher concentration (Fig. 2B). The growth of transformed chlorella in these two modified BGNK containing $(NH_4)_2HPO_4$ or $CH_3CO_2NH_4$ continued during the culture period of 21 days.

However, the growth of transformed chlorella in the original BGNK containing NH_4Cl or $(NH_4)_2SO_4$ showed different patterns. Maximum growth was observed after 5 days at 20mM $(NH_4)_2SO_4$ or 5mM NH_4Cl, but the growth was less than the growth at day 5 in the media containing either $(NH_4)_2HPO_4$ or $CH_3CO_2NH_4$. Furthermore, the growth in the medium containing NH_4Cl or $(NH_4)_2SO_4$ sharply decreased after the maximum at day 5 regardless of the concentrations.

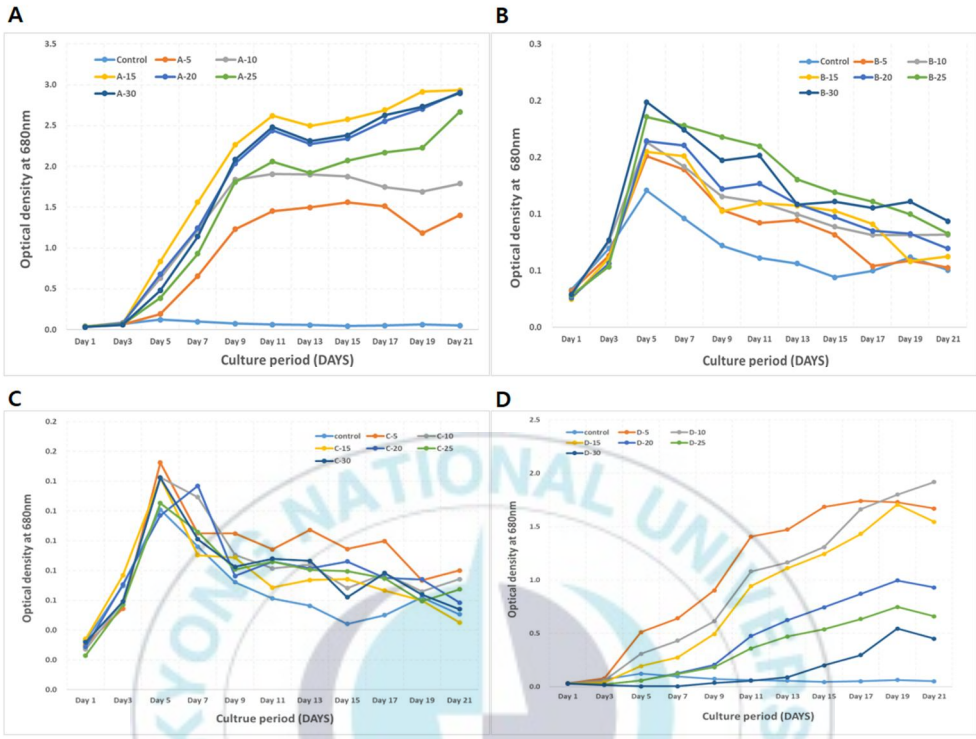

Figure 2. Growth of transformed *Chlorella vulgaris* PKVL7422 in media containing different NH_4 compound and different concentration.

A: BGNK containing $(NH_4)_2HPO_4$, B: BGNK containing $(NH_4)_2SO_4$, C: BGNK containing NH_4Cl, D: BGNK containing $CH_3CO_2NH_4$. Control is BGNK (containing NH_4Cl 17 mM). Media containing 5 mM NH_4 compound (orange line), 10 mM NH_4 compound (gray line), 15 mM NH_4 compound (yellow line), 20 mM NH_4 compound (blue line), 25 mM NH_4 compound (green line), 30 mM NH_4 compound (dark blue line). The results are average from three replications.

The effect of phosphate concentration in BGNK and modified BGNK containing $CH_3CO_2NH_4$

The growth of transformed chlorella in BGNK or modified BGNK with $CH_3CO_2NH_4$ with different concentration of K_2HPO_4 was compared. As shown in Figure 3, the growth of transformed chlorella increased as the concentration of K_2HPO_4 up to 15 mM but decreased after that and no growth was observed at the concentration of 30 mM. The growth of transformed microalgae in the modified BGNK containing 5 mM $CH_3CO_2NH_4$ and 10 mM $CH_3CO_2NH_4$ is shown in Figure 4. In both concentrations of $CH_3CO_2NH_4$ the growth increased up to 15 mM K_2HPO_4 but significantly decreased in higher concentration of K_2HPO_4. Interestingly, the growth of transformed chlorella at 15 mM K_2HPO_4 showing the maximum growth did not decrease until 13 days in both concentrations of $CH_3CO_2NH_4$ (Fig 4. A, B) contract to the growth at 10 mM and 15 mM K_2HPO_4 and 17 mM NH_4Cl that reached the maximum at 11 days of culture and decreased after that shown in Figure 3.

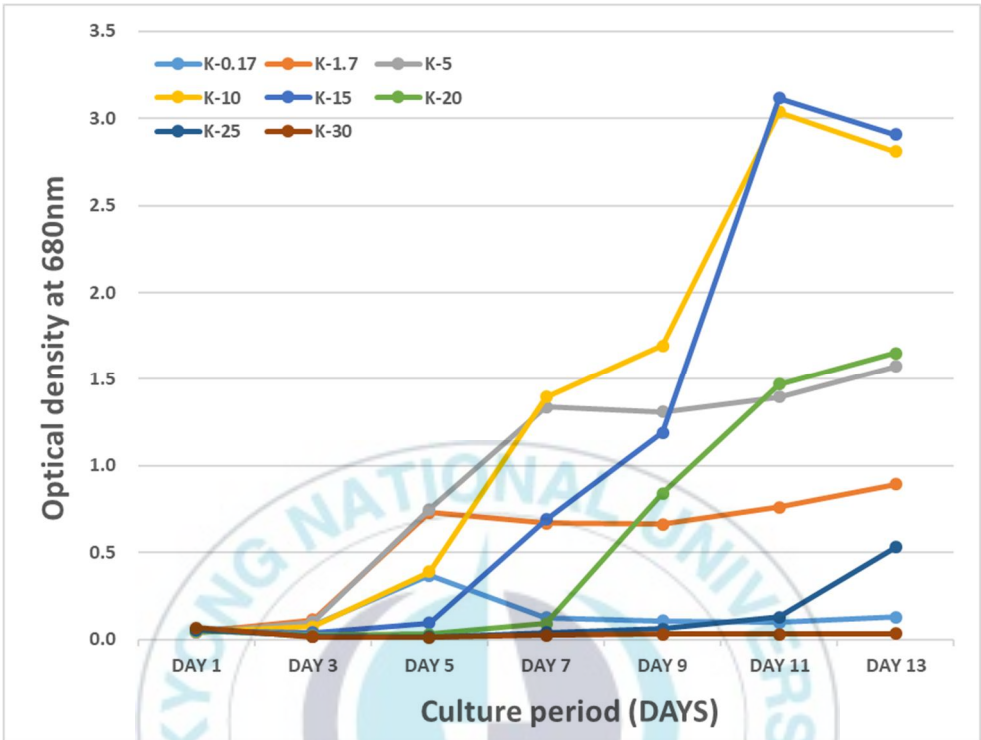

Figure 3. Growth of transformed *C. vulgaris* PKVL7422 at various concentration of K_2HPO_4 in BGNK of 17 mM NH_4Cl.

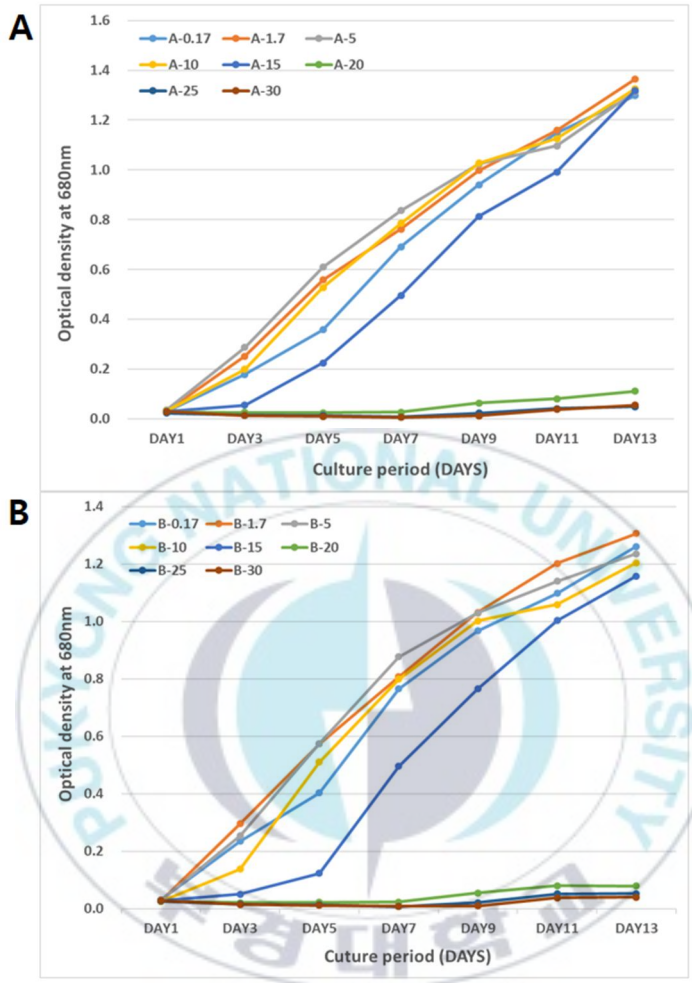

Figure 4. Growth of transformed *C. vulgaris* PKVL7422 at various concentration of K_2HPO_4 in modified BGNK that contain two different concentrations of $CH_3CO_2NH_4$ for N source.

A: Modified BGNK that contain 5mM of $CH_3CO_2NH_4$ for N source. B: Modified BGNK that contain 10mM of $CH_3CO_2NH_4$ for N source. The results are average from three replications.

Growth in modified BGNK with different concentration of K_2HPO_4, NH_4Cl, and $(NH_4)_2SO_4$.

The growth of transformed chlorella in modified media with three different concentrations of K_2HPO_4 (10, 15, 20 mM) and 7 different concentrations (1.7, 5, 10, 15, 20, 25, 30 mM) of NH_4Cl and $(NH_4)_2SO_4$ were compared.

In media containing 10 mM K_2HPO_4, the growth of chlorella in all of the different concentration of NH_4Cl (Fig.5A) or $(NH_4)_2SO_4$ (Fig. 5B) was sharply increased after 3days of culture and continued till 13 days of culture.

In both with NH_4Cl (Fig. 5C) and $(NH_4)_2SO_4$ (Fig. 5D), the growth in media containing 15 mM K_2HPO_4 showed a delay and exponential growth started at 5days of culture. However, the growth in media containing NH_4Cl reached to the stationary phase after 11days of culture (Fig. 5C) contract to continuous growth in media containing $(NH_4)_2SO_4$ (Fig. 5D). Also, the growth itself in media containing NH_4Cl was lower than the growth in media containing $(NH_4)_2SO_4$.

There was more delay of exponential growth at 20mM K_2HPO_4 with various concentration of NH_4Cl (Fig. 5E) and $(NH_4)_2SO_4$ (Fig. 5F). Almost no growth was detected until 7 days' incubation, followed by sharp increase in growth. There was no decline of growth even after 13 days of incubation.

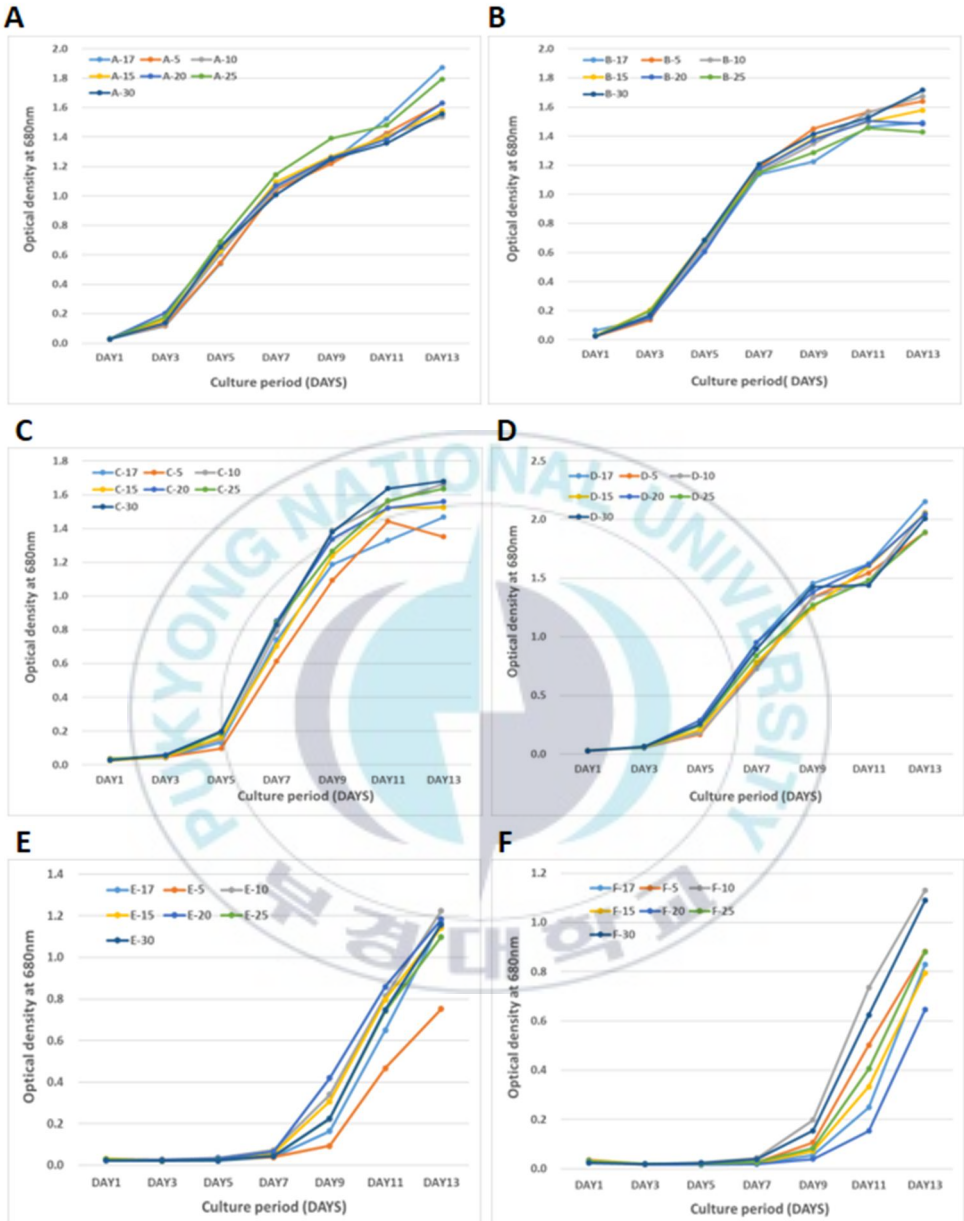

Figure 5. Growth of transformed chlorella in modified BGNK with 10, 15, 20mM K_2HPO_4 and various concentrations of NH_4Cl and $(NH_4)_2SO_4$.

A: 10mM K_2HPO_4 and various concentration of NH_4Cl, B: 10mM K_2HPO_4 and various concentration of $(NH_4)_2SO_4$, C: 15mM K_2HPO_4 and various concentration of NH_4Cl, D: 15mM K_2HPO_4 and various concentration of $(NH_4)_2SO_4$, E: 20mM K_2HPO_4 and various concentration of NH_4Cl, F: 20mM K_2HPO_4 and various concentration of $(NH_4)_2SO_4$, the results are average from three replications.

Preferred carbon source for wild type *C. vulgaris* PKVL7422.

As *C. vulgaris* PKNL7422 is known to grow heterotropically, the preferred carbon source for wild type was determined by adding different carbon source to BG11 medium at the final concentration of 0.5% in the presence of light and dark condition. As shown in Figure 6, the growth of wild type in the presence of light (A) was best in the presence of glucose followed by sodium acetate and glycerol. No growth was observed in the media containing polygalacturonic acid.

Significant growth of wild type *C. vulgaris* PKVL7422 was observed in dark condition in the present of 0.3% glucose followed by sodium acetate. Similarly, the growth of wild type *C. vulgaris* PK7422 in the presence of light was observed. There was no growth in the media containing other carbon sources in dark condition.

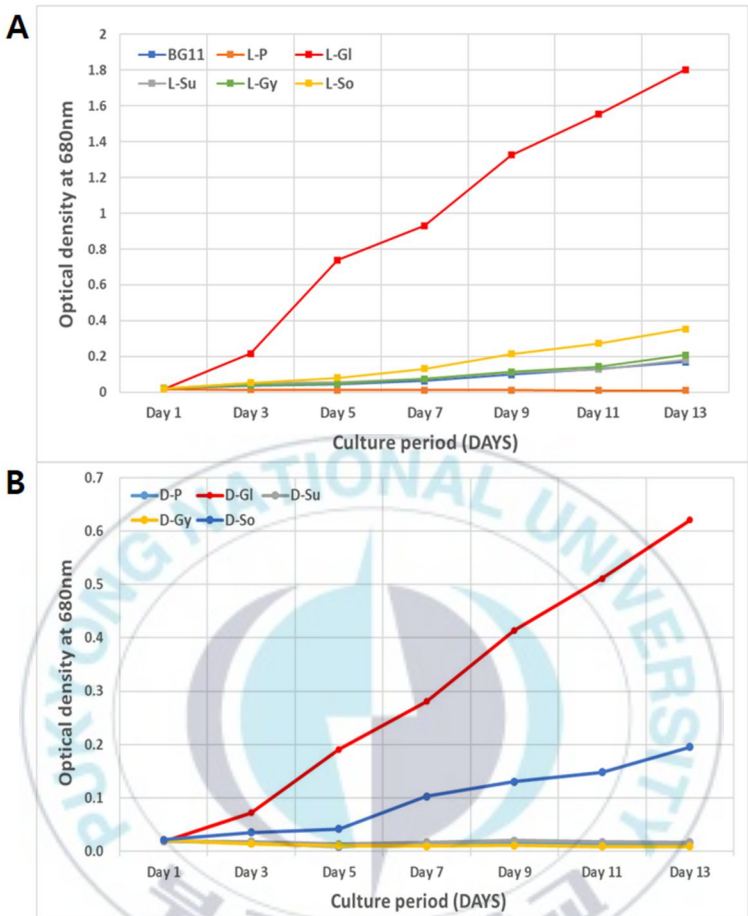

Figure 6. The growth of wild type *Chlorella vulgaris* PKVL 7422 in BG11 containing different carbon sources in light condition (A) and dark condition(B).

BG11 was modified by adding 0.3% polygalacturonic acid (from orange) (P), 0.3% glucose (Gl), 0.3% sucrose (Su), 75ul/100ml glycerol (Gy), 0.3% sodium acetate (So). The results are average from three replications.

Growth of transformed chlorella in BGNK with different concentration of glucose.

As glucose was identified as the prepared carbon source even in the presence of light, the optimum glucose concentration was determined by adding different concentrations of glucose to the modified BGNK that contains 20 mM $(NH_4)_2HPO_4$. As shown in Figure 7, the growth of transformed chlorella increased as the glucose concentration increase from 0.1 to 0.5% and decrease in the order of 1%, 2%, and 3%.

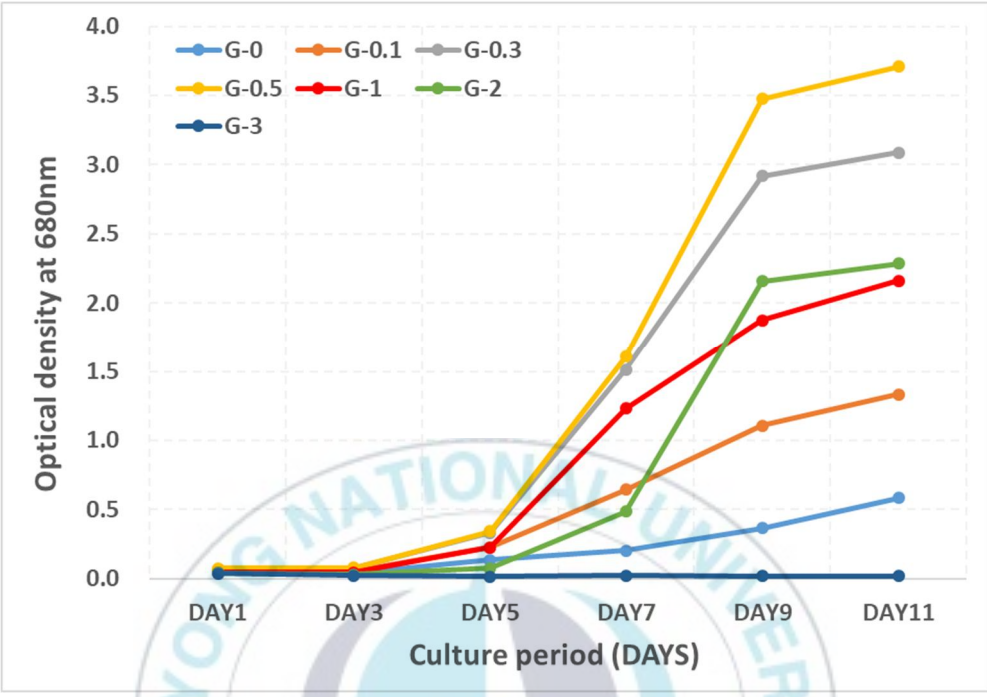

Figure 7. Growth of transformed microalgae in modified BGNK with various concentration of glucose.

The numbers after G- are percentage of glucose in media. The results are average from three replications.

pH variation according to composition of media

Figure 8 shows the pH changes during culture in different media composition. The initial pH of modified BGNK with different concentration of K_2HPO_4 is most 8.0. After 13days culture, pH in the media containing 10 mM of K_2HPO_4 and different concentrations of NH_4Cl and $(NH_4)_2SO_4$ decreased to average 5.8 (Fig. 8A and 8B). In the media containing 15 mM of K_2HPO_4 (Fig. 8C, 8D) and 20 mM of K_2HPO_4 (Fig. 8E, 8F), pH decreased from 8.0 to average of 6.3 (Fig. 8C), 6.0 (Fig. 8D), 7.0 (Fig. 8E), and 7.0 (Fig. 8F), respectively. Decrease of pH was more rapid in the media with lower concentration of K_2HPO_4.

pH changes during culture in different concentration of K_2HPO_4 with two different concentrations of $CH_3CO_2NH_4$ are shown in Fig. 9 (5 mM (A) and 10mM (B)). Initiate pH of media containing the same concentration of ammonium acetate was lower as the concentration of K_2HPO_4 is getting lower. pH decreased in all of the media containing K_2HPO_4 during the cultivation. Compare to Fig 8 results, pH variation was less and pH changes in media containing over 20 mM of K_2HPO_4 were lower than media containing K_2HPO_4 under 20 mM. In the media containing 10 mM of ammonium acetate and 0.17 mM of K_2HPO_4 (B-0.17), pH sharply decreased after day 7.

Fig. 10 shows the optical density and pH changes of BGNK and BGPK during the culture period. As the growth of the transformed microalgae increased, the pH in the media decreased.

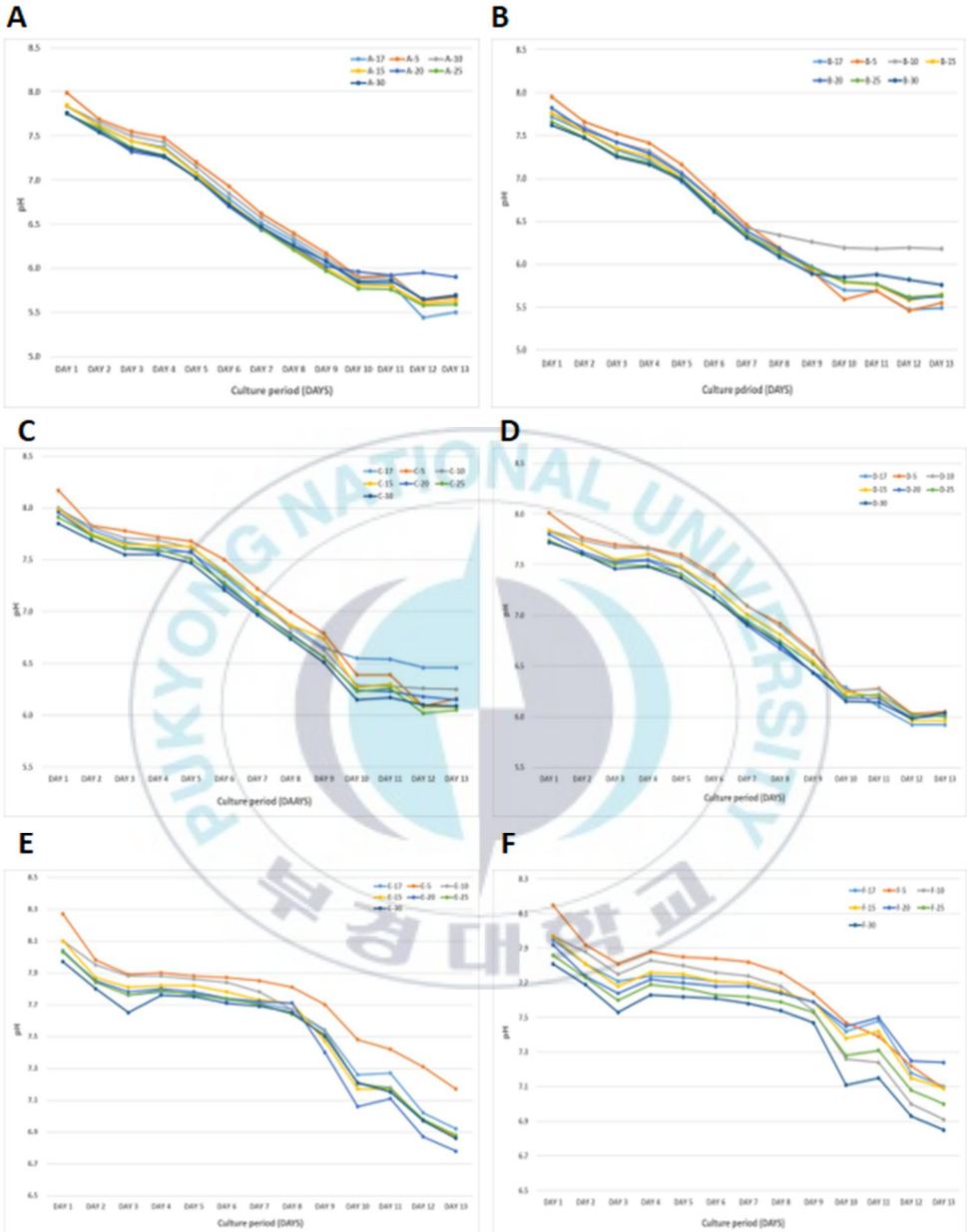

Figure 8. Variation of pH in modified BGNK containing K_2HPO_4 10mM (A, B), 15mM (C, D), and 20mM (E, F) during the transformed microalgae cultivation.

pH changes during the culture of transformed chlorella in modified BGNK with 10mM K_2HPO_4 and various concentrations of NH_4Cl (A, C, E) and $(NH_4)_2SO_4$ (B, D, F).

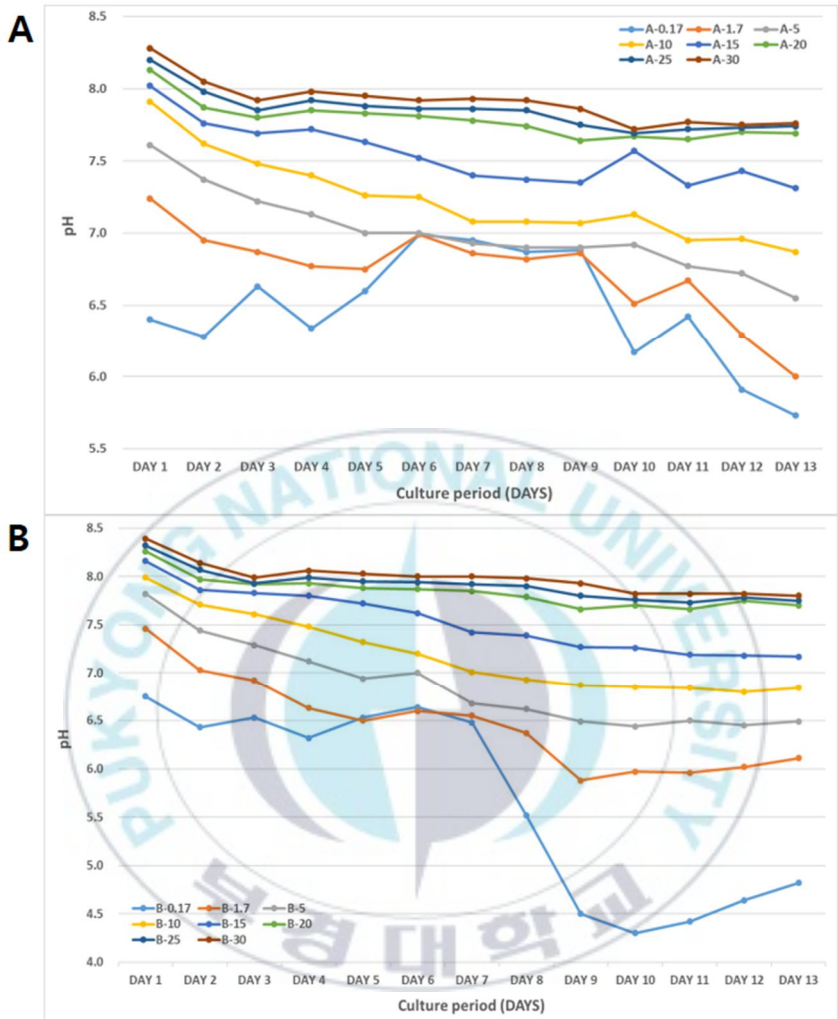

Figure 9. Variation of pH in modified BGNK with various concentration of K_2HPO_4 and contain two different concentrations of $CH_3CO_2NH_4$.

A: Modified BGNK that contain 5 mM of $CH_3CO_2NH_4$ for N source. B: Modified BGNK that contain 10 mM of $CH_3CO_2NH_4$ for N source.

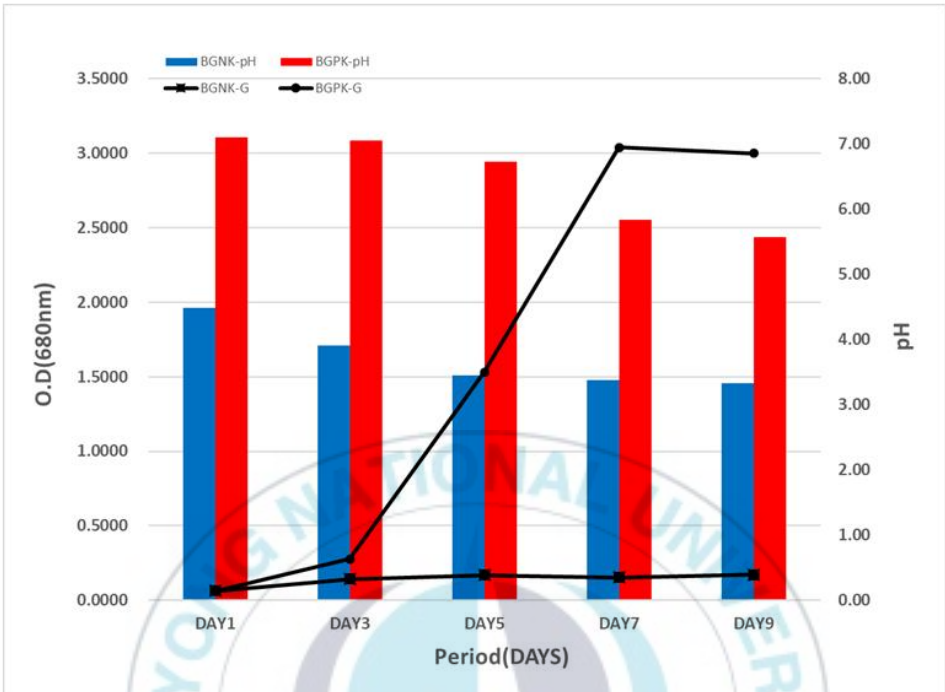

Figure 10. pH variation and growth of transformed microalgae in BGNK and modified BGNK containing 20 mM of $(NH_4)_2HPO_4$.

Blue bar means pH variation of BGNK and red bar means pH variation of modified BGNK containing 20 mM $(NH_4)_2HPO_4$ and 0.5% glucose (BGPK) during the cultivation of transformed microalgae. Line with quadrilateral sign shows the growth of transformed microalgae in BGNK and line with circle sigh shows the growth of transformed microalgae in modified BGNK containing 20 mM $(NH_4)_2HPO_4$ and 0.5% glucose (BGPK). The results are average from three replications.

Discussion

As application of microalgae is getting more attention, mass culture techniques for useful microalgae are asked in many microalgae industrial area. because of Living Modified Organism (LMO) nature of transformed microalgae, open pond culture method cannot be used for the culture of transformed microalgae (Sudhakar, Premalatha, & Rajesh, 2014). Biomass production using closed photobioreactors have already been developed (Lee & Palsson, 1994), and many reports have suggested the optimization of microalgae culture system. However, optimization of media composition is different microalgae strains. There has been no report on the optimization of culture media for the mass culture of transformed *C. vulgaris* PKVL7422 and we have studied the optimum composition of culture media for this specific strain.

In the analysis for the effect of NH_4 compound and concentration, the growth of transformed *C. vulgaris* PKVL7422 was better in media containing $(NH_4)_2HPO_4$ and $CH_3CO_2NH_4$ than BGNK. Media contain $(NH_4)_2HPO_4$ or $CH_3CO_2NH_4$ have the same concentration of NH_4^+. As media containing $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$. This results suggest phosphate has more effect on the growth of transformed microalgae than ammonium. In fact, phosphorus is known as the limiting nutrient for microalgae growth (Merchant & Helmann, 2012). Also, increase of phosphorus concentration improved the growth of transformed microalgae as shown in Fig. 3 and Fig. 4. However, media containing more than

specific concentration of PO_4, no difference of microalgae growth was observed.

Growth of transformed *C. vulgaris* in modified BGNK containing different concentrations of $(NH_4)_2SO_4$ (Fig 2B) and NH_4Cl (Fig 2C) show unexpected growth patterns with rapid after 5 days. This change might be due to the depletion of some nutrients or significant change of the media. When phosphate was added to these modified BGNK containing $(NH_4)_2SO_4$ or NH_4Cl, the growth of transformed chlorella continued (Fig. 5), which suggested that phosphorous can be the limiting nutrient. However, wild type *C. vulgaris* showed normal growth pattern in BG11 with the same concentration of phosphorus as the modified BGNK described above (Fig. 6L-Gl).

One factor that can affect the growth of microalgae is the N:P ratio. Fig. 3 show the N:P ratio can affect the growth of transformed microalgae growth. When the phosphate concentration was fixed and varied the concentration of nitrogen, there was no difference in the growth transformed microalgae as shown in Fig. 4 and Fig. 5. This suggest that at the given concentration of phosphate of 10, 15, 20 mM, the N:P ratio doesn't affect the growth C. vulgaris PKVL7422. The optimal N:P ratio suggested by Redfield (1963) was 16:1 but, transformed microalgae showed normal growth at the N:P ratio range of 5:3 to 1:1 (Fig. 3).

The other fact that was considered was pH of the culture media. The result shown in Fig. 10 suggested that rapid changes of pH may inhibit the transformed

microalgae growth after 5 days. As shown in Fig. 4 and Fig. 9, the growth of transformed *C. vulgaris* PKVL7422 in medium containing more than 20 mM of K_2HPO_4 decreased or stopped and similar growth was observed in media containing less than 20 mM of K_2HPO_4. The pH of media containing more than 20mM of K_2HPO_4 was higher than pH 7.5 (Fig. 9). This results suggest pH higher than 7.5 inhibit the growth of *C. vulgaris* PKVL7422, which is different from the results by Rachlin and Grosso (1991) which showed the optimal growth pH of *C. vulgaris* is pH 7.5 - 8.0. In our study, it was found that the optimal pH range of *C. vulgaris* PKVL7422 is pH 6.5 - 7.5.

Usually carbon dioxide produced by the respiration of microalgae increase the pH of environment in the presence of NO_3^- (Gatamaneni, Orsat, & Lefsrud, 2018). However, the results in Fig. 5 and Fig. 8 showed that the pH of the culture media decreased as the microalgae grow. von Wirén, Gojon, Chaillou, and Raper (2001) and Ninnemann, Jauniaux, and Frommer (1994) showed that NH_4 transporter release H^+ to out of cell when it takes NH_4^+ from outside. In this case, released H^+ can decrease pH of the media and cell growth may be inhibited by low pH. BGNK contains NH_4^+ as the nitrogen source instead of NO_3^- in BG11.

Ammonium acetate and HPO_4 have buffering mechanism (Konermann, 2017), and the results shown in this study suggest that low growth of transformed microalgae in BGNK was not due to the depletion of nutrients in the media but by the pH change during the growth.

The growth of transformed microalgae in the new medium was more than 30 times than in BGNK and transformed cell could have maintained more than three weeks in the new medium.

국문 초록

다양한 분야에서 미세조류의 상업적 사용이 늘어남에 따라 미세조류의 생장을 최적화시키기 위한 선행 연구들이 많이 수행되고 있다. 또한, 대장균을 이용한 외래 단백질 발현과 현재 개발된 진핵 세포 단백질 발현 시스템이 가지는 한계와 비교하여 미세조류 형질전환 시스템은 많은 이점이 있어 미세조류를 이용한 외래 단백질 발현에 대한 개발이 점차 확대되고 있다. 선행 연구원들에 의해 개발된 *Chlorella vulgaris* PKVL7422의 경우 대량 배양을 위한 기술이 아직 개발된 바 없으므로 본 연구를 통해 형질전환 *Chlorella vulgaris* PKVL7422의 생장을 최적화시키고자 하였다. 이전 연구를 통해 형질 전환된 미세조류의 경우 질산염 환원 효소를 생산하는 유전자를 넉아웃시키므로 BG11의 NO_3를 NH_4로 대체하였다. 형질전환 미세조류의 생장 최적화를 위해 본 연구에서는 다음의 화합물에 대한 최적 농도를 조사하였다. K_2HPO_4 (0.17mM, 1.7mM 5mM, 10mM, 15mM, 20mM, 25mM, 30mM), glucose (0%, 0.1%, 0.3%, 0.5%, 1.0%, 2.0%, 3.0%), $(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$, and $CH_3CO_2NH_4$ (17mM, 5mM, 10mM, 15mM, 20mM, 25mM, 30mM). 형질전환된 *C. vulgaris*의 생장은 마이크로 플레이트 리더를 이용하였으며 680nm 파장대의 흡광도 측정을 통해 조사하였다. 결과적으로 $(NH_4)_2HPO_4$ 20mM, 0.5%의 glucose가 포함된 수정된 BGNK 배지에서 *C. vulgaris* 의 생장이 최대로

증가하였으며 기존의 BGNK와 비교하여 생장률이 13.67%에서 49.67%로 증가되었다. 최종 배지 조성을 $(NH_4)_2HPO_4$ 20mM, glucose 0.5%, $KClO_3$ 150mM로 확립하였으며 이 배지를 BGPK로 명명하였다. 형질전환 미세조류의 생장 증가를 통해 최적화된 배지 조성을 확립하였으며 이는 재조합 단백질 생산을 위한 형질전환 미세조류 생장 최적화의 모델 시스템이 될 수 있을 것으로 보인다.

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