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# The Evaluation of UV-induced Mutation of the Microalgae, *Chlorella vulgaris* in Mass Production Systems

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The microalgae *Chlorella vulgari*s has been considered an important alternative resource for biodiesel production. However, its industrial-scale production has been constrained by the low productivity of the biomass and lipid. To overcome this problem, we isolated and characterized a potentially economical oleaginous strain of *C. vulgaris* via the random mutagenesis technique using UV irradiation. Two types of mass production systems were compared for their yield of biomass and lipid content. Among the several putatively oleaginous strains that were isolated, the particular mutant strain designated as UBM1-10 in the laboratory showed an approximately 1.5-fold higher cell yield and lipid content than those from the wild type. Based on these results, UBM1-10 was selected and cultivated under outdoor conditions using two different types of reactors, a tubular-type photobioreactor (TBPR) and an open pond-type reactor (OPR). The results indicated that the mutant strain cultivated in the TBPR showed more than 5 times higher cell concentrations (2.6 g $l^{-1}$) as compared to that from the strain cultured in the OPR (0.5 g $l^{-1}$). After the mass cultivation, the cells of UBM1-10 and the parental strain were further investigated for crude lipid content and composition. The results indicate a 3-fold higher crude lipid content from UBM1-10 (0.3%, w/w) as compared to that from the parent strain (0.1% w/w). Therefore, this study demonstrated that the economic potential of *C. vulgaris* as a biodiesel production resource can be increased with the use of a photoreactor type as well as the strategic mutant isolation technique.

**Key words** : Biodiesel resource, *Chlorella vulgaris*, mass-scale production, microalgae, mutagenesis

## Introduction

Prolonged anthropogenic activities relying on fossil fuels has created one of the most urgent global environmental issues such as climate change [2]. The search for suitable alternatives which are renewable and cleaner energy sources to mitigate carbon dioxide and greenhouse gas emissions has been one of important missions for future sustainability of our society [1]. Various types of biomass as organic matters produced by plants, microorganisms and animals have been considered as promising candidates. In particular lipid feedstock biomass derived from agricultural crops has been the main use for such an alternative [19]. However, such oil feedstocks derived from palm, corn and soybean have been created another public concerns such as ethical dilemma, competing land use issue and generating more greenhouse gas during these crops production processes [1]. Microalgae can be emphasized as one of promising alternatives since biofuels derived from the microbial biomass show relatively higher productivities based on culture area, and were also considerably less problematic to the land use issue for food and feed productions. Many researches related to the application of various microalga species to mass scale cultivation technology for biofuel production have been reported [5, 8, 12, 21, 23]. Among the microalgae, *Chlorella* sp. is considered as one of the most promising candidates due to its relatively simple and fast growth characteristics, high lipid contents, the availability of mass culture technologies, and its non-competing culturable land use for food resources [12]. However, despite of several advantages over the conventional biodiesel feedstocks, the technologies used in the microalga biomass production have been hampered by low yields of biomass and lipids. With these reasons, many research efforts have been paid to improve biomass and lipid productivities via strain developments and culturing tech-

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nology with various medium compositions, environmental conditions and photoreactors [15, 16, 18, 20]. Although hard to compare their biomass and lipid productivities from various *Chlorella* sp. so far reported due to the various important parameters considered, the highest biomass and lipid productivities ever reported were in a range of 0.17~0.23 g $l^{-1}$ $day^{-1}$ and 32~44 mg $l^{-1}$ $day^{-1}$respectively [21]. In addition, many strain developmental strategies via various random mutagenesis techniques such as UV mutagenesis or metabolic engineering have been reported to increase both productivities [3, 4, 24, 27].

Thus, in this study, we also attempted to isolate economically potential high oleaginous strain of *C. vulgaris* for the comparison of two types of mass production via the random mutagenesis technique using UV irradiation.

## Materials and Methods

### Strain and Growth medium

The microalgae *Chlorella vulgaris* (KMMCC 191) used in this study was purchased from the Korean Marine Microalgae Culture Center, Busan, Korea. The lab scale cultivation was carried out in 250 ml conical flasks with a working volume of 100 ml volume using F/2 medium [25]. The culture was maintained at 25℃ with a light intensity of 74 µmol $m^{-2}$ $s^{-1}$ in 12:12 circadian cycles.

### Growth analysis

The absorbance of *Chlorella vulgaris* was measured for cell growth at 680 nm using a UV/visible spectrophotometer (Optizen 2120UV, Mecacys Ltd, Korea). For the determination of dry cell weight (DCW), a 10 ml cell culture was filtered using a pre-weighted filter paper (Waterman No. 2), and the filtered cells were dried in a dry oven at 105℃ for 24 hr and weighed. For mass scale cultivations, the cell growth monitoring was achieved via cell number counting using Hemocytometer (Thermo Fisher Scientific, USA) under a microscope. The cell numbers was converted to dry cell weight using a conversion factor ($2.24\times10^{-11}$ g $cell^{-1}$) previously reported by Hu [14] for the comparison of biomass productivities.

### Experimental set up for Random Mutagenesis

To achieve physical mutagenesis, UV-B (312 nm) light from a UV lamp stand (15 W, 76 µM / $cm^{2}$, VILBER Lourmat, France) was used with the irradiation distance at 15 cm (Fig. 1). The ultraviolet light was irradiated to a 60-mm Petri-dish containing a 5 ml of cell culture adjusted to the initial cell absorbance ($OD_{680\ nm}$ = 0.5) with the time intervals of 1~5 min. After UV irradiation, the cells were serially diluted with F/2 medium and were spread on F/2 agar plates. The UV-irradiated plates were kept in dark for 16 hr to prevent light-induced repair. The plates were then maintained under a light intensity of 74 µmol $m^{-2}$ $s^{-1}$ for 3 weeks at 20℃ until colonies were formed. The resulting colonies were inoculated into 250 ml conical flasks containing 100 ml F/2 liquid media for further growth characteristic analysis.

### Lipid quantification analysis

The intracellular neutral lipid content of *C. vulgaris* was determined using the method previously reported by Chen *et al*. [7]. For efficient staining of nile red, the cell absorbance was adjusted to the optical density of 0.5 at 680 nm, and then the 10 µl cell culture with a mixture of 138 µl sterile

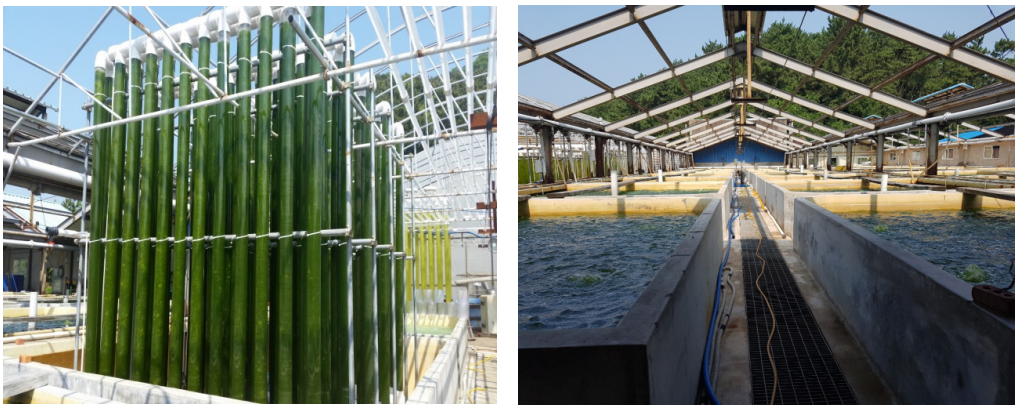

Fig 1. The outdoor microalgae cultivation systems used in this study for mass cultivation. Left, tubular-type photobioreactor (TBPR); Right, open pond type reactor (OPR)

distilled water, 2 µl nile red, and 50 µl DMSO added to a 96-well plate (Costar 3790, Corning Inc., USA). The culture was incubated at 40℃ for 10 min. After the incubation, the fluorescence intensity was measured at the wavelength of excitation at 490 nm and emission at 620 nm. The fluorescence intensity values were obtained by subtracting the auto-fluorescence values of the microalgae using a fluorescence spectrophotometer FS-2 (SCINCO, USA). The lipid content of the parental strain and the mutant strain was measured after preparing a calibration curve with Triolein (Sigma-Aldrich, USA) for lipid quantification.

### Mass scale cultivation

To evaluate UV-B induced mutant for biomass productivities and lipid contents, a tubular type photobioreactor (TBPR) with 20 ton capacity and an open pond reactor (OPR) with 30 ton capacity were used, which was previously reported [9, 17] as shown in the Fig. 1. Before the cultivation started, the culture broth with the addition of a 5 g $ton^{-1}$ of calcium hypochlorite (Nippon Soda, Japan) as a disinfectant was sterilized by aeration for 24 hr. Afterward, a 10 g $ton^{-1}$ of sodium thiosulfate (Kukdong Chemical, China) was used for neutralization. In the TBPR, an approximately 700 liter of inoculum with a cell concentration of about $3\times10^7$ cells $ml^{-1}$ was added to the neutralized seawater to 1.3 tons of seawater to achieve the initial concentration of $1\times10^7$ cells $ml^{-1}$. In the open pond experiments, a 3 liter of $100\times10^8$ cell $ml^{-1}$ inoculum was added to 30 tone media to achieve the same initial concentration of the experiments used in TBPR. The microalgae cultivation medium prepared with filtered natural seawater with supplements of 100 g of urea (Dongbu Farm Hannong, Korea) per ton of seawater and 30 g of compounds fertilizer (Dongbu Farm Hannong, Korea) per ton of seawater containing 21 g nitrogen, 5 g phosphate and 8 g potassium source. Both reactors were maintained at 20 ±2°C and pH 8. 0 under a greenhouse conditions with natural light source from April 10 to 22 for 2 weeks at Geoje Gyeonsangnam-do, South Korea (34°47′13.0"N 128°32′40.7"E). The $CO_2$ gas (Ssangyong Synthesis Gas, Korea) was supplied into both systems at 0.2 l $min^{-1}$ during cultivation using air-blowers with one horse power.

### Statistical analysis

The results of this study were expressed as mean values from 3 replicates. The statistical significance between the control and experimental groups was tested using Student's t-test at standard deviation (p-value <0.05)

## Results and Discussion

### Mutant selection

After the UV mutagenesis, each of the irradiated samples was spread onto F/2 agar medium by the plate dilution method. Single colonies were appeared after 3 weeks. The colony morphology, in particular showing large and dark green color colonies were peaked and these colonies were once again streaked on the F/2 agar plates. The isolated single colonies were transferred to 250 ml conical flasks containing 100 ml F/2 liquid media and cultured for 21 days to analyze mutagenicity characteristics. Among those mutant strains, in particular five mutant strains with excellent cell growth in the liquid medium were identified as UV-B mutants (UBM) designated as UBM 1-2, UBM1-3, UBM1-10, UBM1-15 and UBM1-18. The growth characteristics of each strain lipid contents were determined.

### Cell growth characteristics of UV-induced mutant strains

Cell growth from the 5 selected mutant strains obtained from UV mutagenesis was measured with 3-day intervals for 21 days as shown in Fig. 2. Among the mutant strains tested, the cell growth rates from the mutants UBM1-10 and UBM1-15 were higher than that of the wild-type strain. Maximum biomass concentrations from UBM1-10 (2.8±0.2 g

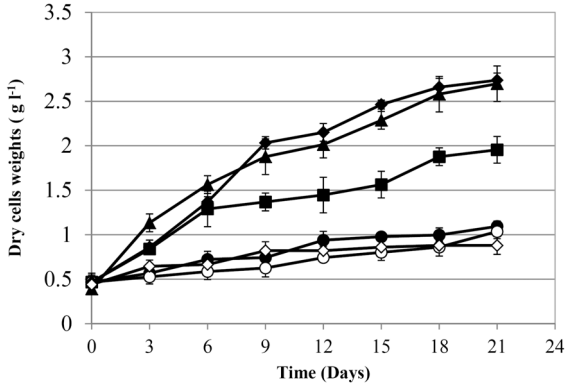


Fig. 2. Cell growth evaluation of UV-B induced mutants. The experiments were carried out at 250 ml conical flasks containing 100 ml f/2 media with a light intensity of 74 µmol $m^{-2}$ $s^{-1}$ in 12:12 circadian cycles at 25°C without air supply. Symbols: The wild type strain of *C. vulgaris* (■), the mutant strain of *C. vulgaris* UBM1-2 (●), UBM1-3 (○), UBM1-10 (◆), UBM1-15 (▲), UBM1-18 (◇). Error bars from triplicates.

$l^{-1}$) and UBM1-15, (2.8±.03 g $l^{-1}$) strains were reached at 21th days which were about 1.5 times higher than that of the control strain (1.8 g $l^{-1}$). However, the maximum biomass concentrations from other mutants such as UBM1-2, UBM1-3 and UBM1-18 were lower than that from the parental strain.

Lipid contents were measured using nile red fluorescence method mentioned in the early section on the 21st day of cultures. The results are highlighted in Table 1. Among the mutant strains, in particular the lipid content from UBM1-2 showed the highest content at 8.9%(w/w) followed by UBM1-10 (6.8%) and UBM1-18 (6.0%). The lipid contents from UBM1-2 and UBM1-10 were approximately a 2- and 1.5-fold higher than that from the wild type strain (4.5%). In particular UBM1-10 strain showed interesting characteristics. According to its growth characteristics as shown in Fig. 2, the strain showed higher biomass yield and faster growth rate as compared to those from the wild type stain whereas its lipid contents was also a 1.5 fold higher than that from the wild type strain. Rodolfi *et al*. reported [21] that the biomass and lipid contents productivities from various strains of *Chlorella sp*.. The biomass and lipid productivities from their report were in a range of 0.17~0.23 g $l^{-1}$ $day^{-1}$ and 32~44 mg $l^{-1}$ $day^{-1}$ respectively as shown in Table 1. However, the mutant strains isolated from this study were in a range of 0.04~0.13 g $l^{-1}$ $day^{-1}$ in biomass productivity and 2.6~4.2 mg $l^{-1}$ $day^{-1}$ in lipid content productivity. Such reduction in these parameters could possibly be derived from different culture conditions and strains of *Chlorella* sp. such as light intensity, $CO_2$ supply and different circadian cycles used.

Many research previously reported that microalgae can accumulate large amounts of intracellular triglycerides triggered by various environmental stresses such as nutrient starvation, UV irradiation and high concentration of salts [11, 13, 20, 22, 27]. Our research was also agreeable to the previous reports, since most of mutants isolated after UV mutagenesis showed higher lipid contents than that from the wild type strain. Zayadan *et al*. [26] reported the UV mutant isolation strategy with enhanced lipid accumulation using nitrogen source starvation. The author found the UV induced strain from *C. pyrenoidosa* C-2 showed 2-fold higher biomass yield and lipid contents as compared to those from the wild type strain. In addition, other authors [3] also reported that a UV induced mutant of *C. vulgaris* with a gene mutation involved in $CO_2$ fixation such a gene coding for acetyl CoA carboxylase showed enhanced the lipid accumulation. Although what genetic mutation caused the increased lipid content in our mutant strains remained to be further elucidated, the results indicated that the mutant strain obtained by ultraviolet irradiation can increase intracellular lipid contents under the normal cell growth conditions without nitrogen starvation, which would be economical benefits for the lipid production as a feedstocks for biodiesel production. With the economical consideration, the strain UBM1-10 showing higher cell biomass yield and lipid content was selected for mass scale production experiments.

### Outdoor experiments using TBPR and OPR

In the open pond reactors, both mutant and the wild type strains showed a similar growth rate. After 10 days of cultivation, both strains reached highest cell concentrations at 0.48 g $l^{-1}$ and 0.5 g $l^{-1}$ respectively. This indicated that both wild type and mutant strains did not show a significant difference in terms of the growth rates and biomass yields (Fig.

Table 1. The comparison of maximal biomass concentrations and lipid contents from UV induced mutants of *C. vulgaris* and other microalgae strains cultivated in 250-ml flasks under the lab conditions

| *Chlorella sp*. | Biomass (g $l^{-1}$) | Lipid content (%, w/w) | Biomass productivities (g $l^{-1}$ day $^{-1}$) | Lipid productivity ( mg $l^{-1}$ $day^{-1}$) | Ref. |
|---|---|---|---|---|---|
| *C. vulgaris* (KMMCC 191) | 1.8 | 4.5 | 0.09 | 2.1 | In this study |
| *C. vulgaris* UBM1-2 | 1.075 | 8.9 | 0.05 | 4.2 | In this study |
| UBM1-3 | 0.93 | 5.4 | 0.04 | 2.6 | In this study |
| UBM1-10 | 2.8 | 6.8 | 0.13 | 3.2 | In this study |
| UBM1-15 | 2.8 | 5.6 | 0.13 | 2.7 | In this study |
| UBM1-18 | 0.86 | 6.0 | 0.04 | 2.8 | In this study |
| *C. sorokiniana* IAM-212 | - | 19.3 | 0.23 | 44.7 | [21] |
| *Chlorella sp*. F&M-M48 | - | 18.7 | 0.23 | 42.1 | [21] |
| *C. vulgaris* F&M-M49 | - | 18.4 | 0.20 | 36.9 | [21] |
| *C. vulgaris* CCAP 211/11b | - | 19.2 | 0.17 | 32.6 | [21] |

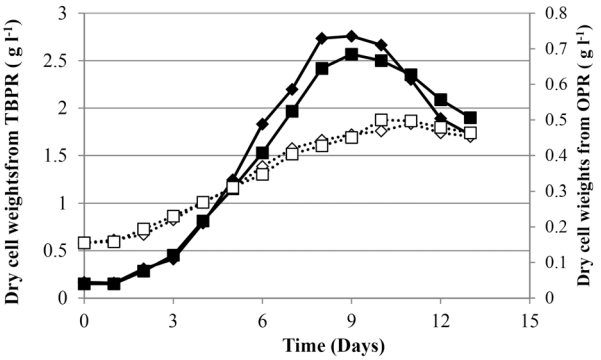


Fig. 3. Cell growth evaluation of the UV induced mutant of *C. vulgaris* UBM1-10. The experiments were carried out at either tubular type phothoreactor (TBPR) with 20 ton capacity or open pond type reactor (OPR) with 30 ton capacity under outdoor conditions. Symbols: (◆), the wild type strain of *C. vulgaris* from TBPR ; (■), the mutant stain of *C. vulgaris* UBM1-10 from TBPR; (◇), the wild type strain of *C. vulgaris* from OPR; (□), the mutant stain of *C. vulgaris* UBM1-10 from OPR.

3). However, in the TBPR system, both wild-type and the mutant strains after 9th days had reached to a highest concentration at 2.7 g $l^{-1}$ and 2.5 g $l^{-1}$ respectively, which were about 5 times higher than those from both strains as cultured in the OPR system. This result indicated that the TBPR system is much more efficient to yield higher biomass concentrations than those from using the OPR system. In particular, the results from TBPR were comparable to those results performed under small scale lab culture conditions as shown in the Table 1. This would be caused by higher light penetration rates in the TBPR than those in the OPR. The diameter of the tubular type reactor used in our system was 140mm diameter, whereas the depth of OPR was 0.8 m height, which indicates that the sunlight transmittance in the TBPR would be higher than that from OPR. Therefore, the microorganism could have more sufficiently received the sunlight required for photosynthesis as the TBPR system was used. In comparison with a simple unit productivity per area basis, the TBPR as a large-scale cultivation system has been shown to be much higher. However, industrial application for microbial biomass mass production requires an approach through various economic analyses including not only the productivity per unit area, but also other parameters such as initial facility installation and operation costs [19]. Therefore, it was difficult to conclude which method would be the best method as a suitable system for industrial application. This would require a further investigation on life cycle assessment with these economical parameters.

However, as compared to the strain-wise, the maximal biomass concentrations and growth rates from the mutant strains cultured using either TBPR or OPR were similar to those from the wild type strain. The reason for this result is that, under the lab conditions the strain can be cultured under optimized conditions such as temperature, light intensity and medium composition. However, in the case of outdoor cultures, these cannot be controlled. In particular, under outdoor conditions, both culture systems were totally relied on the natural environments such local temperature and light source. In addition, F/2 media have been broadly used to culture marine microalgae under lab conditions [21, 25]. However this can be quite expensive due to the many trace elements such as biotin and vitamin $B_{12}$ required in the medium compositions. With the reason, many researches have been search for efficient and cheap alternatives with additional benefits for the water quality controls [10, 18]. However, in this study the plant compound fertilizer as industrial medium was applied to these purposes. Although the plant compound fertilizer as an alternative to the F/2 medium showed quite excellent industrial medium for microalgae cultivation as shown in Fig. 2 and Table 2, such an industrial medium may provide nutritional deficiency of trace elements contained in F/2 medium used for the laboratory culture experiments as mentioned by previous reports [6, 15] . This could be one of reasons leading to such poor growth of the UV-induced mutant. Therefore, it would be necessary to carry out an experiment to search for another economical culture medium that can promote cell growth in future for industrial mass culture. Many intensive review articles highlighted current status of industrial media developments of biomass feedstock production and microalgae with suitable candidates [6]. However, the information related to more than a ton unit scale was quite limited and most of results were from small lab scale productions emphasizing the importance of this study.

### Biomass composition analysis from mass culture

The mass cultivated cells of the mutant strain UBM1-10 from TBPR were harvested for the biomass composition analysis. The analysis results indicated that UBM1-10 strain showed a roughly threefold increase in crude fat content compared to that from the wild-type strain (Table 3). Interestingly, the results from the lipid analysis indicate that the concentration of docosahexaenoic acid (DHA, 0.052%) from the mutant strain cultured from TBBR showed about

Table 2. Comparison of maximal biomass yields and biomass productivities of UV induced mutant strain C. vulgaris using various types of photoreactors

| Reactor type | Stains | Maximal biomass yield ($g\ l^{-1}$) | Biomass productivities ($g\ l^{-1}\ day^{-1}$) |
|---|---|---|---|
| OPR[a] | *C. vulgaris* (KMMCC 191) | 0.48 | 0.05 |
| | *C. vulgaris* UBM1-10 | 0.50 | 0.05 |
| TBPR[a] | *C. vulgaris* (KMMCC 191) | 2.7 | 0.37 |
| | *C. vulgaris* UBM1-10 | 2.6 | 0.29 |
| 250 ml conical flask | *C. vulgaris* (KMMCC 191) | 1.8 | 0.09 |
| | *C. vulgaris* UBM1-10 | 2.8 | 0.13 |

[a]: Both strains cultured using open pond reactor (OPR) with 30 ton capacity under outdoor conditions.
[b]: Both strains cultured using tubular type reactor with 20 ton capacity under outdoor conditions.
[c]: Both strains cultured using F/2 media under lab conditons without $CO_2$ supply.

Table 3. The biomass composition anaylsis of *C. vulgaris* UBM1-10 and its parental strains drived from mass cultures using the TBPR system

| Contents | Chlorellar vulgaris | |
|---|---|---|
| | UBM1-10 (% w/w) | Parental strain (%) |
| Crude lipid | 0.3 | 0.1 |
| Crude protein | 0.7 | 0.7 |
| Ash | 3.2 | 2.1 |
| Docosahexaenoic acid | 0.052 | 0.018 |
| Eicosapentaedecanoic acid | ND | 0.026 |

a 3-fold higher concentration than that from the wild type (0.018%), although its concentration was relatively small. In addition, the mutant strain did not produce eicosapentaedecanoic acid (EPA) whereas the wild produced such an unsaturated fatty acid at 0.021%. This result indicated that the UV induced mutagenesis could have affected the metabolic pathway of lipid biosynthesis. However, what genetic mutation in such metabolic pathway could led to such results remained to be answered. In addition, such low productivity from the mutant cultured in the TBPR system indicates current stage of mass culture production system used in this study. Many review articles highlight various reactor types can be used for the production of biofuel feedstocks and their possible limitation parameters to improve biomass and lipid content productivities have been emphasized [15]. The limitation from our results was also agreeable to those previous reports.

In this study, the mutant strains of *Chlorella vulgaris* induced by UV irradiation were isolated with the industrial application purpose. Among the mutants isolated, in particular, the mutant strain UBM1-10 isolated showed excellent growth characteristics under the lab conditions with an increase in biomass yield (35% increase) and lipid contents (34% increase) as compared to those from the wild type strain. In addition, as this strain was applied to two types of photoreactors for the production of biodiesel feedstock, our study clearly demonstrated that the biomass productivities under outdoor conditions can be significantly increased with the choice of type of cultivation technologies. In particular, the biomass composition analysis indicated that the UV-induced *C. vulgaris* mutant UBM1-10 as an excellent mutant showing about 3 times increase in the lipid contents as compared that from the wild type strain. Although their biomass and lipid productivities were quite low as compared to others that previously reported. This indicated that further researches are required to improve such important parameters for biofuel feedstock production such as industrial medium development and more sophisticated mass-scale production systems. In this respect, this study will possibly contribute to useful data for the industrial production of biofuel feedstock using microalgae with a consideration for mass culturing technologies.

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## 초록 : 자외선에 의해 유도된 *Chlorella vulgaris* 돌연변이 균주의 대량 생산 시스템에서의 평가

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미세조류 *Chlorella vulgaris*는 바이오 디젤 생산을 위한 중요한 대체원료 중 하나로 간주되어 왔으나, 이러한 미생물의 산업적 적용은 낮은 바이오 매스와 지질 생산성에 의해 제약을 받아왔다. 따라서 이러한 문제를 극복하기 위해 본 연구는 자외선을 이용한 무작위 돌연변이 유발 기술을 통해 높은 지질 및 바이오 매스 생산성을 가지는 *C. vulgaris* 균주를 분리하고 그 특성을 규명하였으며, 두 가지 유형의 대량 생산 시스템을 이용하여 바이오 매스 및 지질 함량의 산출량을 비교하였다. 분리된 돌연변이 균주 중, 특히 실험실 조건에서 UBM1-10으로 명명된 돌연변이 균주는 야생형 균주에 비해 약 1.5배 높은 세포 수율 및 지질 함량을 보였다. 이러한 결과를 바탕으로 UBM1-10을 선택하여 TBPR (tubular photobioreactor)과 OPR (open pond type reactor)의 두 가지 유형의 반응기를 사용하여 실외 배양 조건에서 배양하였다. 그 결과 TBPR에서 재배된 돌연변이 균주의 세포 수율은(2.6 g $l^{-1}$) OPR에서 배양된 균주의 세포 수율(0.5 g $l^{-1}$)과 비교하였을 때 약 5배 이상의 높은 세포 수율을 나타내었으며, 대량 배양 후, UBM1-10 및 모 균주의 조 지방 함량 및 조성 등에 대해 추가로 조사를 실시하였다. 그 결과 *C. vulagris* UBM1-10균주의 지질함량(0.3% w/w)이 모 균주의 지질함량(0.1%)에 비해 약 3배 이상의 조 지방 함량을 보유함을 확인하였다. 따라서 이 연구는 바이오 디젤 생산 자원으로서 *C. vulgaris*의 경제적 잠재성이 photoreactor type의 선택 및 전략적 돌연변이 분리 기술을 통해 증가 될 수 있음을 보여 주었다.