Photo-mixotrophic Cultivation of Algae *Euglena gracilis* for Lipid Production

Tonči REZIĆ
Jelena FILIPOVIĆ
Božidar ŠANTEK (✉)

Summary

In the future, due to limited resources, a crisis of energy storing molecules (fuels), which are currently produced from crude mineral oil, is expected. One strategy to compensate a part of the oil deficiency is the production of biodiesel from microalgal lipids. As model microorganism for lipid production microalgae *Euglena gracilis* was selected and photo-mixotrophic cultivation was performed in the stirred tank photo-bioreactor. During this research, medium composition and operational conditions of photo-bioreactor were optimized in order to define adequate cultivation conditions for algae biomass and lipid production. As low-cost and available complex carbon/nitrogen source, corn steep liquor (CSL) was used to promote *E. gracilis* growth and lipid production. Due to the optimization of medium composition and cultivation conditions, lipid production was increased up to 29% of biomass dry weight in a two stage cultivation process inside one photo-bioreactor. Promising results obtained in this research encouraged us for further investigation.

Key words

photo-mixotrophic cultivation, *Euglena gracilis*, lipid production, cultivation conditions, stirred tank photo-bioreactor

University of Zagreb, Faculty of Food Technology and Biotechnology, Department of Biochemical Engineering, Pierottijeva 6/IV, HR-10000 Zagreb, Croatia
✉ e-mail: bsantek@pbf.hr

Received: December 13, 2012 | Accepted: February 14, 2013

ACKNOWLEDGEMENTS
The authors are grateful for financial support from the Ministry of Science, Education and Sport of the Republic of Croatia (Grant No. 058-0581990-1997 and 058-0581990-2004).

Introduction

A growing range of studies has been conducted to explore the techniques, procedures and processes of producing large quantities of microalgae biomass (Rodolfi et al., 2009; Spolaore et al., 2006). There are two most commonly used techniques to cultivate microalgae. These are open raceway pond systems and closed photo-bioreactor systems. The open pond system is less favorable due to limitation in controlling contaminations and cultivation parameters, while the photo-bioreactors provide an easy system of controlling growth nutrients concentration and cultivation parameters (such as temperature, dissolved O_2 and CO_2 and pH) as well as to prevent contaminations (Ugwu et al., 2008). However, photo-bioreactors have a high initial cost and their constructions are very often determined by microalgae physiological and morphological characteristics. Therefore, the facility for microalgae production is an important factor that has to be considered during scale up of these bioprocesses.

Some microalgal species contain high levels of lipids that can be extracted and converted into biofuels. The extracted lipids can further be transesterified into biodiesel (Demirbas, 2009). Furthermore, the waste biomass after lipids separation could be also used for production of other bio-fuels such as methane (Vergara-Fernandez et al., 2008), ethanol (Tsukahara and Sawayama, 2005) and hydrogen (Hankamer et al., 2007). Microalgae have also shown the potential to reduce emerging environmental problems, such as the greenhouse effect and industrial water pollution. Microalgae are able to fix carbon dioxide released from power plants by photosynthesis and to produce different nutrients at a minimal cost (Chisti, 2007). The above-mentioned scenarios show that microalgae can provide possible solutions for some of environmental problems as well as for production of valuable products.

In this investigation *E. gracilis* was cultivated in photo-mixotrophic conditions in a stirred tank photo-bioreactor. Different wavelengths and carbon/nitrogen sources were used in order to increase biomass and lipid yields. The second goal of this research was to explore the growth of *E. gracilis* in photo-autotrophic conditions with CO_2 as main carbon source.

Materials and methods

Algae strain, media and cultivation conditions

Euglena gracilis 1224-5/25 from Sammlung von Algenkulturen Göttingen was used in all experiments. Inoculums for all experiments were prepared on rotary shaker at 28°C (150 min^{-1}) in 500 mL Erlenmeyer flasks filled with 200 mL of Hutner medium (Hutner and Provasoli, 1951) for 5 days. In this research, *E. gracilis* was cultivated on modified and original Hutner media.

The optimization of medium composition for *E. gracilis* photo-heterotrophic cultivation

During this part of investigation, photo-heterotrophic cultivation of *E. gracilis* was also performed on the rotary shaker at 28°C (150 min^{-1}) for 5 days. In first set of experiments modified Hutner media were used for *E. gracilis* cultivation in shake flasks. These media were composed of (g/L): glucose 20, ethanol 17.6 or acetic acid 20 (as a carbon source) and CSL (corn steep liquor) 20, KH_2PO_4 0.4, $MgSO_4 \cdot 7H_2O$ 0.14, $MgCO_3$ 0.4, $CaCO_3$ 0.1, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ 0.021; and trace element solution 10 mL/L. The trace element solution contained (in g/L): $ZnSO_4 \cdot 7H_2O$ 1.1, $MgSO_4 \cdot H_2O$ 0.58, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.18, $CoSO_4 \cdot 7H_2O$ 0.024, $CuSO_4 \cdot 5H_2O$ 0.077, H_3BO_3 0.029, $NaNO_3 \cdot 4H_2O$ 0.074.

For the second set of experiments medium with glucose was selected and used for further research. In these cultivations the effect of CSL concentration was examined and it was in the range of 20 - 40 g/L. All other medium compounds had the same concentration as by the first set of experiments. In this research all media were sterilized by autoclaving at 121°C for 20 min.

Bioprocess operational conditions in a stirred tank photo-bioreactor

Photo-mixotrophic batch cultivations were performed in a 2 L bioreactor (Biostat MD, B. Braun, Germany) with a working volume of 1 L. Bioreactor was equipped with four lamps located vertically around bioreactor vessel, at the 5 cm distance from the surface of bioreactor, to provide continuous light to the system. Two different light sources were used: sun-glo lamps (Hagen-Deutschland, 15 W, lux 80, 4500 K) and aqua-glo lamps (Hagen-Deutschland, 15 W, lux 80, 12000 K). Bioreactor with 900 mL of medium was sterilized at 121°C for 20 min. After cooling, cultivation was started by the addition of the inoculum (100 mL; 10% v/v) and the cultivation temperature was maintained at 28°C. The initial *E. gracilis* cell number in the photo-bioreactor was $5 \cdot 10^5$ cell/mL determined by counting in Thoma chamber. During photo-heterotrophic cultivation of *E. gracilis* the effect of bioreactor operational conditions (stirrer speed and aeration rate) on the biomass and lipid yield was examined. The stirrer speed was altered in the range of 100 - 300 min^{-1} and aeration rate in the range of 0.2 - 0.8 v/v min, respectively. After photo-heterotrophic cultivation of *E. gracilis* (120 h) carbon sources were completely consumed and photo-autotrophic growth conditions were established. During photo-autotrophic growth of *E. gracilis* CO_2 was additionally supplied in the bioreactor air inflow. Therefore, CO_2 level in the bioreactor air inflow was increased from 0.03% (air concentration of CO_2) to 2% by mixing pure CO_2 and the air.

Analytical procedures

Cells were harvested by centrifugation at 4500 min^{-1} for 5 min at 4°C. The cell pellets were washed once with demineralized water and lyophilized. The lyophilized samples were stored at -20°C under argon gas. The lyophilized samples were extracted with chloroform - methanol - water (1:2:0.8, v/v/v) mixture with 0.5% (w/v) pyrogallol for preventing oxidation (Bligh and Dyer, 1959). The extracts were dried under vacuum at 40°C by a rotary evaporator and used for gravimetric determination of total lipid content of *E. gracilis* (Takeyama et al., 1997).

Concentrations of glucose, organic acids (acetic, glutamic, lactic and malic acid were determined separately, but they are presented as a total sum at Figures 1 and 2) and ethanol were quantified by high performance liquid chromatography (HPLC) on a Supelcogel C-610H column using a refractive index detector (RID, Schimadzu 10 A VP, Japan). Analyzed compounds were separated at a flow rate of 0.5 mL/min with 0.1% H_3PO_4 as eluent at a constant temperature (30°C). Prior to analysis, all samples were mixed with $ZnSO_4$ to a final concentration of 10% (w/w) to induce protein precipitation. Solid remains were removed by

centrifugation (4500 rpm for 20 min). Sample solutions were filtrated through a 0.20 μm filter before HPLC determination.

CO_2 concentrations were measured in the bioreactor air inflow and outflow. CO_2 concentration measurements were performed by infrared gas analyzers (Oxybaby®V range, WITT-Gastechnik, Witten, Germany) every 12 hours.

Cell number of *E. gracilis* in cultivation medium was determined by counting under microscope (400 X magnification) in Thoma chamber. Samples were continuously drawn from the stirred tank photo-bioreactor and after appropriate dilution with sterile water plated in the chamber.

Results and discussion

Optimization of medium composition for *E. gracilis* photo-heterotrophic cultivation

During this investigation photo-heterotrophic cultivation of *E. gracilis* was performed in order to increase biomass and lipid yield. Therefore, different carbon sources (glucose, ethanol and acetic acid) and CSL were used for the modification of Hutner medium composition. The CSL was used to replace glutamic, malic and succinic acids, glycine, asparagine and urea as well as vitamins (B_1 and B_{12}) in order to simplify and reduce the cost of cultivation medium. In this investigation *E. gracilis* cultivation was also done on the original Hutner medium as control. Results of these photo-heterotrophic cultivations are presented in Table 1. As it can be seen in Table 1, the highest biomass yield and cell density were observed by the cultivation on the original and modified Hutner medium with glucose and CSL. This effect can be explained by the fact that glucose is more favorable carbon source compared to the ethanol and acetic acid due to enzyme induction and synthesis for glucose transport and metabolism in the algae cells (Yamane et al., 2001). However, the highest lipid yield was observed during cultivation on the modified Hutner medium with glucose and CSL what clearly shows that this medium composition is suitable for lipid accumulation in algae *E. gracilis*. On the basis of these results it is also clear that CSL is adequate complex source of carbon, nitrogen and growth factors (amino acids and vitamins) for *E. gracilis* cultivation and lipid production.

In order to examine the effect of CSL concentration on the lipid yield during photo-heterotrophic cultivation of *E. gracilis* second set of experiments was carried out on modified Hutner medium with glucose as a carbon source. In this research, CSL concentration was changed in the range of 20 - 40 g/L and therefore lipid yield was reduced as a consequence of the increase of easy metabolized nitrogen content in the medium (Table 2). It is well known that nitrogen depletion from the medium induces lipid accumulation (Evans and Ratledge, 1984; Yoon and Rhee, 1983a; Yoon and Rhee, 1983b). In the nitrogen exhausted medium, glucose conversion in lipids is occurred due to present high ATP:AMP ratio (Sheehan et al., 1998; Chen et al., 2011). In this investigation, cell number was at approximately constant level (0.22 - 0.24 · 10^8 cell/mL), but biomass and lipid yield were reduced with increase of CSL concentration in the medium. Therefore, for further study modified Hutner medium with 20 g/L of glucose and 20 g/L of CSL was selected (Table 2).

Table 1. The effect of medium composition on the *E. gracilis* growth and lipid production

Medium	Cell number (10^8 cell/mL)	Biomass yield (g/g)	Lipid yield (g/g)
Hutner medium	0.24	0.3	0.08
MHM* (glucose and CSL)	0.22	0.24	0.20
MHM* (ethanol and CSL)	0.15	0.09	0.10
MHM* (acetic acid and CSL)	0.11	0.05	0.04

*MHM - modified Hutner medium

Table 2. The effect of CSL concentration in the modified Hutner medium on the *E. gracilis* growth and lipid production

CSL (g/L)	Cell number (10^8 cell/mL)	Biomass yield (g/g)	Lipid yield (g/g)
20	0.22	0.24	0.20
30	0.23	0.15	0.15
40	0.24	0.14	0.12

Effect of bioreactor operational conditions on the photo-heterotrophic cultivation of *E. gracilis* and lipid production

At the start of research in the stirred tank photo-bioreactor the effect of bioreactor operational parameters on the algae growth and lipid production was studied. Mixing intensity is important parameter of *E. gracilis* cultivation since it affects the cells and medium compounds distribution as well as mass and heat transfer. In algae large scale production optimal degree of turbulence is required due to the circulation of cells from the dark to the light zone of the bioreactor (Mata et al., 2010). On the other hand, high liquid velocities and degrees of turbulence (due to mechanical mixing or air bubbles mixing) can damage algae cells because of high shear stress (Eriksen, 2008). The maximal level of turbulence (above which cell death occurs) is strain dependent and it has to be experimentally determined in order to avoid decline in the bioprocess productivity (Mata et al., 2010).

In order to increase the biomass and lipid production, *E. gracilis* photo-heterotrophic cultivation in the stirred tank photo-bioreactor was carried out at various combinations of bioreactor stirrer speed and aeration rate and only a few characteristic combinations are presented in Table 3. The cell number and biomass yield were increased with increase of stirrer speed and aeration rate due to the fact that cell growth is affected by the substrate (carbon source and oxygen) and light distribution as well as by the shear stress in the photo-bioreactor. However, the highest lipid yield (0.25 g lipid / g cell dry weight) was observed with the stirrer speed of 200 min^{-1} and aeration rate of 0.4 v/v min. Further increase of both bioreactor operational parameters was related to the slight decrease of lipid yield as a consequence of shear stress enlargement in these conditions. However, in these conditions biomass yield was also increased what resulted in approximately similar lipid concentrations at the end of these bioprocesses (Table 3). On the basis of these results it can be concluded that for successful lipid production by *E. gracilis* aeration rate has to be in the range of 0.4 - 0.8 v/v min and stirrer speed

Table 3. The effect of bioreactor operational conditions on the *E. gracilis* growth and lipid production during photo-heterotrophic cultivation (5 days) in the stirred tank photo-bioreactor

Run	Stirrer speed (min^{-1})	Aeration rate (v/v min)	Cell number (10^8 cell/mL)	Biomass yield (g/g)	Lipid yield (g/g)	Lipid concentration (g/L)
1	100	0.2	0.19	0.16	0.23	1.71
2	200	0.4	0.22	0.23	0.25	2.25
3	300	0.8	0.25	0.29	0.22	2.47

in the range of 200 - 300 min^{-1} what is in agreement with literature data (Ogbonna et al., 1999; Šantek et al., 2009).

Photo-mixotrophic cultivation of *E. gracilis* and lipid production in the stirred tank photo-bioreactor

In this part of research in the stirred tank photo-bioreactor the effect of bioreactor operational parameters (light source type and CO_2 level in the air inflow) on the photo-autotrophic growth *E. gracilis* was studied. In first experiment with aqua-glo lamps (maximal wavelength of 160 nm) *E. gracilis* cultivation was performed on the modified Hutner medium with 20 g/L of glucose and 20 g/L of CSL. Furthermore, CO_2 level in the air inflow was increased at 2% what is considerably higher compared to the natural CO_2 level in the air (0.03%). In first 120 h of this photo-mixotrophic cultivation of *E. gracilis* glucose was used as a primary carbon source and consequently organic acids (mostly lactic acid) from CSL as a secondary carbon source. This phenomenon was indirectly confirmed by the significant pH increase (from 3.26 to 7.62) and decrease of organic acids concentration (from 5.10 to 0.05 g/L; Figure 1). Obtained results from this part of bioprocess clearly confirm diauxic growth of *E. gracilis* that is characterized by the maximal lipid concentration of 3.33 g/L (Figure 1) and lipid yield of 0.22 g/g (data not shown). After organic carbon sources were completely depleted (120 h) conditions for photo-autotrophic growth were established. In these conditions, CO_2 from the air is main carbon source and consequently its concentration was reduced in the air outflow of photo-bioreactor. During photo-autotrophic cultivation of *E. gracilis* biomass and lipid concentrations were significantly reduced (for cca. 40 - 50%) as a consequence of cultivation conditions that are characterized by considerably lower biomass growth (Mata et al., 2010; Figure 1).

In order to avoid negative effect of photo-autotrophic conditions on cell growth and lipid production, aqua-glo lamps were replaced with sun-glo lamps (maximal wavelength of 643 nm) that imitate sun light spectra (wavelength from 380 to 740 nm). Furthermore, during photo-autotrophic phase of this bioprocess CO_2 level in the air inflow was also increased at 2% in order to avoid significant reduction of biomass concentration. In this experiment, during photo-heterotrophic phase of *E. gracilis* cultivation similar bioprocess trends were observed as by the cultivation with aqua-glo lamps (Figure 2). The use of sun-glo lumps resulted in the formation of optimal conditions for the photo-autotrophic cultivation of *E. gracilis* and the promotion of light adsorption in the chlorophyll molecules. Chlorophyll reaction center with photo-system I and II is sensitive to the sun light source and therefore lipid synthesis was stimulated (Mata et al., 2010). Even so, there is still limitation by the self-shading effect, that is, light penetration decreases as the algal biomass

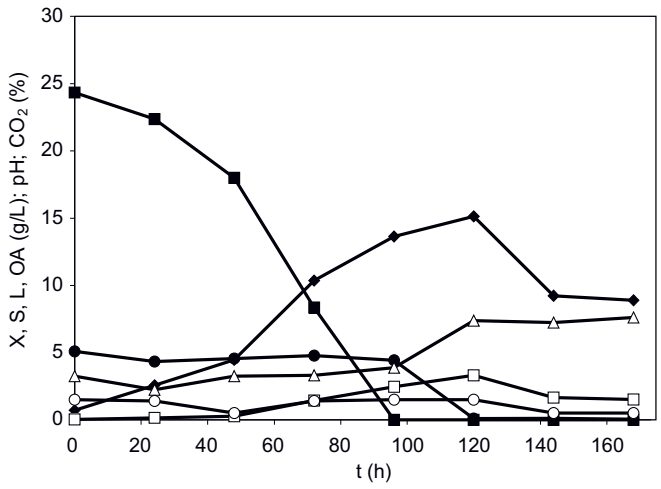

Figure 1. Changes of biomass (X; ♦), glucose (S; ▪), organic acids (OA; ●) and lipid concentration (L; □), pH (Δ) and CO2 level in the bioreactor air outflow (CO2; ○) during photo-mixotrophic cultivation of *E. gracilis* in the stirred tank photo-bioreactor with aqua-glow lamps

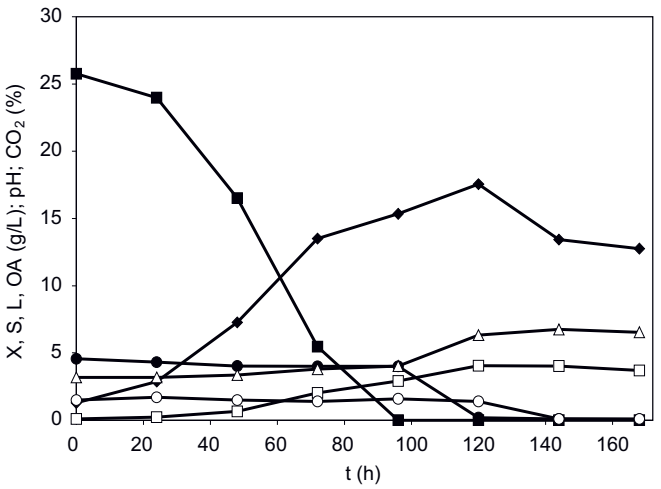

Figure 2. Changes of biomass (X; ♦), glucose (S; ▪), organic acids (OA; ●) and lipid concentration (L; □), pH (Δ) and CO2 level in the bioreactor air outflow (CO2; ○) during photo-mixotrophic cultivation of *E. gracilis* in the stirred tank photo-bioreactor with sun-glow lamps

Table 4. The effect of light source type on the *E. gracilis* growth and lipid production during photo-mixotrophic cultivation (8 days) in the stirred tank photo-bioreactor

Run	Type of light source	Cell number (10^8 cell/mL)	Biomass yield (g/g)	Lipid yield (g/g)
1	aqua-glow lamps	0.28	0.25	0.17
2	sun-glo lamps	0.38	0.36	0.29

concentration increases in the photo-bioreactor. Therefore, during photo-autotrophic phase biomass concentration was reduced for 23.5%, but lipid concentration remains at approximately constant level (3.70 - 4.03 g/L). At the same time, CO_2 in the air outflow of photo-bioreactor was almost completely consumed, what clearly pointed out that it is used for biomass growth and lipid production (Figure 2.).

Comparison between these two photo-mixotrophic cultivations on the basis of bioprocess efficiency parameters clearly shows that considerably higher biomass and lipid yields as well as cell number were observed during *E. gracilis* cultivation with sun-glo lumps (Table 4). This effect can be explained by the fact that during cultivation with sun-glo lumps CO_2 is more efficiently used for biomass growth and lipid production (CO_2 concentration in the air outflow was only 0.1%). Therefore, it is clear that for photo-autotrophic cultivation of algae *E. gracilis* light source has to reproduce sun light in order to avoid significant reduction of biomass and lipid yields. Results obtained in this research clearly indicate *E. gracilis* potential for photo-mixotrophic cultivation and lipid production. However, reduction of biomass yield during photo-autotrophic cultivation gives additional challenge for the further research of *E. gracilis* growth in these conditions.

Conclusions

Results of this research clearly show that the photo-mixotrophic cultivation of *E. gracilis* for lipid production can be successfully carried out during two stage cultivation inside one stirred tank photo-bioreactor. Modification of Hutner medium was successfully done by the replacement of organic acids, amino-acids and vitamins (B_1 and B_{12}) with corn steep liquor (CSL) as complex source of these compounds. Modified Hutner medium with 20 g/L of glucose and 20 g/L of CSL provided the highest lipid content in *E. gracilis* cells and confirmed potential of CSL as alternative and low-cost medium compound for the *E. gracilis* cultivation. The lipid production of *E. gracilis* depended highly on the type of light source and CO_2 level in the bioreactor air inflow. During photo-mixotrophic cultivation of *E. gracilis* with sun-glo lamps and 2% CO_2 in the air inflow the lipid yield reached the highest level (0.29 g/g). However, in this photo-mixotrophic cultivation the reduction of biomass concentration during photo-autotrophic cultivation was also observed what requires further research in order to define optimal conditions for *E. gracilis* cultivation in these conditions.

References

Bligh E. G., Dyer W. J. (1959). A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917

Chen M., Tang H., Ma H., Holland T. C., Ng K. Y. S. Salley S. O. (2011). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour Technol 102: 1649-1655

Chisti Y. (2007). Biodiesel from microalgae. Biotechnol Adv 25: 294-306

Demirbas A. (2009). Production of biodiesel from algae oils. Energ Sources Part A 31: 163-168

Eriksen N. T. (2008). The technology of microalgal culturing. Biotechnol Lett 30: 1525- 1536

Evans C. T., Ratledge C. (1984). Effect of Nitrogen Source on Lipid Accumulation in Oleaginous Yeasts. J Gen Microbiol 130: 1693-1705

Hankamer B., Lehr F., Rupprecht J., Mussgnug J. H., Posten C., Kruse O. (2007). Photosynthetic biomass and H_2 production by green algae: From bioengineering to bioreactor scale-up. Physiol Plantarum 131: 10-21

Hutner S.H., Provasoli L. (1951). The phytoflagellates. In: Biochemistry and Physiology of Protozoa, Vol. 1 (A Lwoff eds.), Academic Press, New York, 27-35

Mata T. M., Martins A. A., Caetano N. S. (2010). Microalgae for biodiesel production and other applications: A review. Renew Sust Energ Rev 14: 217-232

Ogbonna J. C. (1999). Tomiyama S., Tanaka H., Production of alpha-tocopherol by sequential heterotrophic-photoautotrophic cultivation of *Euglena gracilis*. J. Biotechnol 70: 213-221

Rodolfi L., Chini-Zittelli G., Bassi N. Padovani G., Biondi N., Bonini G., Tredici M. R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 102: 100-112

Šantek B., Felski M., Friehs K., Lotz M., Flaschel E. (2009). Productin of paramylon, a beta-1,3-glucan, by heterotrophic cultivation of *Euglena gracilis* on a synthetic medium. Eng Life Sci 9: 23-28

Sheehan J., Dunahay T., Benemann J., Roessler P. (1998). A look back at the U.S. Department of Energy's Aquatic Species Program-biodiesel from alge http://www1.eere.energy.gov/biomass/pdfs/biodiesel_from_algae.pdf

Spolaore P., Joannis-Cassan C., Duran E., Isambert A. (2006). Commercial applications of microalgae. J Biosci Bioeng 101: 87-96

Takeyama H., Kanamaru A., Yoshino Y., Kakuta H., Kawamura Y., Matsunaga T. (1997). Production of Antioxidant Vitamins, β-Carotene, Vitamin C and Vitamin E, by the Two-Step Cultured *Euglena gracilis Z*. Biotechnol Bioeng 53: 185-190

Tsukahara K., Sawayama S. (2005). Liquid fuel production using microalgae. J Jpn Petrol Inst 48: 251-259

Ugwu C. U., Aoyagi H., Uchiyama H. (2008). Photobioreactors for mass cultivation of algae. Bioresour Technol 99: 4021-4028

Vergara-Fernandez A., Vargas G., Alarcon N., Velasco A. (2008). Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass Bioenerg 32: 338-344

Yamane Y., Utsunomiya T., Watanabe M., Sasaki K. (2001). Biomass production in mixotrophic culture of *Euglena gracilis* under acidic condition and its growth energetics. Biotechnol Lett 23: 1223-1228

Yoon S. H., Rhee J. A. (1983a). Quantitative physiology of *Rhodotorula glutinis* for microbial lipid production. Process Biochem. 18: 2-4

Yoon S. H., Rhee J. S. (1983b). Lipid from yeast fermentation: effects of cultural conditions on lipid production and its characteristics of *Rhodotorula glutinis*. J. Am. Oil Chem Soc 60: 1281-1286

acs78_09