微細藻類バイオマス利用国際シンポジウム

International Symposium on Microalgal Biofuels and Bioproducts

中央大学駿河台記念館

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共催

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中央大学研究開発機構

International symposium on microalgal biofuels and bioproducts

21st November 2013

Surugadai Memorial Hall

Chuo University

3-11-5 Kandasurugadai, Chiyoda-ku, Tokyo 101-8324, Japan

Co-hosted by
Agriculture, Forestry and Fisheries Research Council's Secretariat;
Ministry of Agriculture, Forestry and Fisheries
&
Research and Development Initiative
Chuo University

PROGRAM

10:00	Welcome	Mr. Hirotsugu Amamiya Agriculture, Forestry and Fisheries Research Council
10:10	Towards high-efficiency microalgae biofuel systems	Professor Ben Hankamer Institute for Molecular Bioscience The University of Queensland
11:00	Conversion of CO_2 to Chemicals in Cyanobacteria.	Professor Shota Atsumi Department of Chemistry UC Davis
11:50	Lunch	
13:00	Poster session	
15:50	Molecular breeding of green algae for biofuel production.	Professor Shigeaki Harayama Department of Biological Sciences Chuo University
16:20	Cost effective outdoor cultivation of green algae for biofuel production.	Mr. Hiroaki Fukuda General Manager of Bio R&D Department Material R&D Division, DENSO CORPORATION
16:50	Food and fuel for the 21st century - Synthetic biology in micro-algae for the production of biofuels and bio-products	Professor Stephen Mayfield Director, San Diego Center for Algae Division of Biological Sciences University of California, San Diego
17:40	Closing remarks	Professor Kunio Saito Director, Research and Development Initiative Chuo University
18:30	Banquet	

Profiles of invited speakers

Ben Hankamer

Professor
The Institute for Molecular Bioscience (IMB)
The University of Queensland (UQ)

In 2002, Ben moved from Imperial College London to take up his position as a Principle Investigator at The University of Queensland's Institute for Molecular Bioscience. Ben has focused on the development of environmentally friendly high-efficiency microalgae biofuel production systems. In 2006, he established and directs the Solar Biofuels Consortium which now includes 8 international teams, ~100 researchers and ~10 industry partners.

In 2009, Ben was awarded the prestigious Eisenhower Fellowship, awarded to individuals identified as international leaders in areas of energy technology and supply. In 2013, Ben was also awarded the Discovery of Outstanding Researcher Award from the Australian Research Council.

Over the past 10 years, Ben Hankamer has focused on the development of environmentally friendly high-efficiency biofuel production systems. This area represents a rapidly expanding biotechnology. His specialisation is in the structural biology of the photosynthetic machinery, which drives the conversion of solar energy into chemical energy (fuels) and has published extensively on the water splitting Photosystem II complex, its light harvesting antenna system and V-type ATPase. Using this knowledge of the photosynthetic machinery, he embarked on the targeted engineering of the green alga *Chlamydomonas reinhardtii* for high-efficiency biofuel production. To facilitate the development of high efficiency biofuel systems, he founded the Solar Biofuels Consortium which he now directs. The consortium includes eight international teams and conducts economic analysis, bio-discovery, marine biology, structural biology, molecular biology, microbiology, genomics, metabolomics, culture optimisation and bioreactor scale up within a coordinated research program of parallel research streams. One of the biggest global challenges facing our society today is the race to discover cleaner, more affordable and sustainable energy sources. Currently, most of the world's clean energy technologies are used to produce electricity. However, 80 per cent of the global energy demand is used in the form of fuel.

Shota Atsumi

Assistant Professor
Department of Chemistry, University of California, Davis, CA, 95616

Shota Atsumi is an Assistant Professor in the Department of Chemistry at the University of California, Davis since 2009. He received his Ph.D. from Kyoto University in 2002, where he worked with Dr. Tan Inoue. He was a postdoctoral researcher with Dr. John W. Little at the University of Arizona and with Dr. James C. Liao at the University of California, Los Angeles. He was a co-recipient of the Presidential Green Chemistry Challenge Award in 2010 awarded by the US Environmental Protection Agency. In 2012, he was awarded the prestigious Hellman Fellowship, awarded to individuals identified as promising assistant professors who show capacity for great distinction in their chosen fields of endeavor.

Shota is one of the pioneers in the study of the commercial production of 1-butanol and isobutanol from *Escherichia coli*. An increased understanding of system properties underlying cellular networks enables one to construct novel systems by assembling the components and the control systems into new combinations. His group is applying this approach to the field of metabolic engineering, which strives for the optimization of desired properties and functions, such as the production of valuable biochemicals. The production of valuable chemicals from microorganisms has the potential to solve some significant challenges, such as converting renewable feedstocks into energy-rich biofuels. He and his colleagues engineered *E. coli* to produce higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-pheylethanol by taking advantage of the host's highly active amino acid biosynthetic pathway. These results have been published in *Nature*. His current research focuses on the use of synthetic biology and metabolic engineering approaches to engineer photosynthetic microorganisms to convert CO_2 to valuable chemicals using light energy. He and his colleagues engineered a model cyanobacterium, *Synechococcus elongatus* PCC 7942, to produce isobutyraldehyde and isobutanol from CO_2 published in *Nature Biotechnology* in 2009. More recently, his group developed a more efficient cyanobacterial production system published in *Proc Natl Acad Sci USA* in 2013. His group also engineered *S. elongatus* to grow without light by installing heterologous sugar transporters. The engineered strains grow continuously in light/dark conditions using saccharides such as glucose, xylose, and sucrose.

Stephen Mayfield

Director, San Diego Center for Algae Biotechnology and
John Dove Isaacs Chair of Natural Philosophy
Department of Biological Sciences
University of California, San Diego
La Jolla, CA 92037
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Stephen Mayfield is director of the San Diego Center for Algae Biotechnology, and a Co-director of the Food and Fuel for the 21st Century organized research unit at UC San Diego. He is also the John Doves Isaacs Chair of Natural Philosophy in the department of Biology. His research focuses on the molecular genetics of green algae, and on the production of high value recombinant proteins and biofuel molecules using algae as a production platform. Steve received BS degrees in Biochemistry and Plant Biology from Cal Poly State University in San Luis Obispo, and a PhD in Molecular Genetics from UC Berkeley. Following a post-doctoral fellowship at the University of Geneva Switzerland, he returned to California as an assistant professor at the Scripps Research Institute where he was the first person to achieve transformation of a green algae nuclear genome, work that allowed algae to become dominant organisms for the study of photosynthesis and gene function. Steve remained at Scripps for 22 years becoming the Associate Dean of Biology before joining UC San Diego in 2009. Over the last ten years work from the lab has identified mechanisms of chloroplast gene expression that has allowed for recombinant protein expression and metabolic engineering in algal chloroplast. Steve's lab was the first to show high levels of recombinant protein expression in algae, setting the stage for the use of algae as a platform for recombinant protein production, including the expression of human therapeutic proteins. These studies resulted in the founding of Rincon Pharmaceutical, company based on the low cost production of human therapeutics using eukaryotic algae as an expression platform. Recent studies from the lab have shown the potential of engineering algae for the production of superior biofuel molecules as a source of renewal energy, and Steve is a scientific founder of Sapphire Energy, the world's largest company developing biofuels in algae and photosynthetic bacteria. Steve's latest commercial undertaking is Trion Algae Innovations, a company developing high value recombinant proteins as animal and human nutraceuticals.

Abstracts of oral presentations

Towards High-Efficiency Microalgae Biofuel Systems

Ben Hankamer
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The development of renewable fuels is an urgent challenge facing our society, due to the importance of reducing CO_2 emissions, increase fuel security and providing a sustainable basis for economic development.

Global energy demand is ~0.5ZJ yr^{-1} (~83% used as fuels and 17% as electricity) and is rising due to the population growth and the demand for continued economic growth. Global documented 1P resources (fossil fuels with a 90% probability of recovery using current technologies and prices) and Ultimately Recoverable Resources (5% probability of recovery using current technologies and prices) of oil, gas, coal and uranium are reported to be 36.5 ZJ and 82.7 ZJ respectively (BP statistical review).

In contrast to finite fossil fuel supplies, global solar energy incident upon the Earth's surface vastly exceeds global energy demand (~3020 ZJ yr^{-1}). Of this 1300 ZJ yr^{-1} is photosynthetically active radiation that can be used for the production of biofuels. This presentation will provide an overview of the physical constraints of photo-biological biofuel production and advances made by the Solar Biofuels Consortium (www.solarbiofuels.org) in terms of developing such microalgal systems not only for fuel but also food and high value product production. Microalgae can be produced in marine systems and on non-arable land offer an opportunity to contribute to the 70% increase in food and 50% increase in fuel demand predicted by 2050.

Conversion of CO_2 to Chemicals in Cyanobacteria

Shota Atsumi

Department of Chemistry, University of California, Davis, CA, 95616

The use of photosynthetic microorganisms as a platform for biological fuel production has gained considerable popularity as an option that would avoid global energy and environmental problems. As photosynthetic microorganisms directly fix carbon dioxide as their primary carbon source, the need for a source of fermentable sugars as a carbon feedstock for biological fuel production could be eliminated. Algae and cyanobacteria have been the primary organisms of interest for this strategy of fuel production. Both can grow much faster than plants and do not need to be grown on arable land. Furthermore, such organisms are grown in water which facilitates the use of CO_2 at higher concentrations than that of ambient air and so could potentially be fed by concentrated CO_2 emissions from waste industrial sources. The great potential of the prokaryote cyanobacteria as a biofuel production platform lies in its combination of the advantages of both algae, as a photosynthetic organism, and *E. coli*, as a relatively simple naturally transformable prokaryote. Cyanobacteria have already been engineered to produce a number of different biofuel related compounds. However, synthetic pathway construction and characterization of metabolism in cyanobacteria, is still in its infancy compared with model fermentative organisms.

We systematically developed the 2,3-butanediol (23BD) biosynthetic pathway in *Synechococcus elongatus* sp. strain PCC 7942 as a model system to establish design methods for efficient exogenous chemical production in a photosynthetic host. We identified 23BD as a target chemical with low toxicity and designed an oxygen-insensitive, cofactor-matched biosynthetic pathway coupled with irreversible enzymatic steps to create a driving force toward the target, increasing titers to 2.38 g/L, which is a significant increase for chemical production from exogenous pathways in cyanobacteria. Production of 23BD appears to redirect up to 60% of biomass toward product, this leaves 40% improvement in this system. This work demonstrates that developing strong design methods can continue to increase chemical production in cyanobacteria.

All cyanobacteria are photosynthetic organisms that utilize light energy for the reduction of carbon dioxide. Many cyanobacteria, including our model system, *S. elongatus*, have been considered obligate photoautotrophs, strictly depending upon the generation of photosynthetically derived energy for biomass production. This obligate photoautotroph is incapable of product formation in the absence of light. Thus, converting an obligate photoautotroph to a heterotroph is desirable for more efficient, economical, and controllable production systems. We determined that sugar transporter systems are the necessary genetic factors to install heterotrophy in *S. elongatus* PCC 7942. After modification, continuous growth was possible under diurnal (light/dark) conditions using saccharides such as glucose, xylose, and sucrose as both energy and carbon inputs. This modified strain showed heterotrophic growth in the dark and a 2-fold growth rate increase in the presence of light. While the universal causes of obligate photoautotrophy may be diverse, installing sugar transporters provides new insight into the mode of obligate photoautotrophy for cyanobacteria. While diurnal conditions are of keen interest for cost-effective, industrial pursuits, further work with continuously dark conditions will more fully illuminate the causes of phototrophy seen in this model cyanobacterium.

Molecular breeding of green algae for biofuel production.

Shigeaki Harayama
Department of Biological Sciences, Chuo University

Algae biofuel is considered the third generation biofuel; however, the current costs for the production of algal biofuels are not competitive with petroleum-based fuels. It is imperative to reduce the costs of cultivation, oil extraction, and conversion oil to biofuels. We are focusing on the technology development for increased productivity and stable production of triglycerides in a green alga.

We study on the green alga, *Pseudococcomyxa ellipsoidea* that accumulates 30% (w/w) or more of triglycerides in lipid bodies inside cells upon nitrogen starvation. We mutagenized the strain by N-methyl-N'-nitro-N-nitrozoguanidine, and isolated many mutants of different phenotypes, including low-chlorophyll mutants, oil-accumulating mutants, fragile-cell-wall mutants, high-light tolerant mutants, dark-metabolism-deficient mutants, uracil-requiring mutants, nitrate-reductase deficient mutants, etc. We determined the genomic sequences of these mutants, and compared with that of the wild-type strain.

One of the low-chlorophyll mutants named strain 5P was defective both in chlorophyllide *a* monooxygenase and in one of chlorophyll a/b binding proteins. The biomass productivity of strain 5P was approximately 30% higher than that of wild-type strain when light intensity was high (300 μmol m^{-2} sec^{-1}) and the depth of the culture vessel was 10 cm or deeper. We further mutagenized strain 5P, and isolated mutants capable of accumulating triglycerides at high concentrations (>50%).

In parallel, we are developing recombinant DNA techniques to accelerate the breeding speed of this alga. We established genetic transformation methods with particle bombardment, and constructed several cloning vectors for gene expression.

Cost effective outdoor cultivation of green algae for biofuel production

Hiroaki Fukuda
General Manager of Bio R&D Department
Material R&D Division, DENSO CORPORATION

Fossil fuels are widely accepted as unsustainable energy source due to depleting oil reserves and causing global warming. Thus, interest in biofuels is increasing across the world. However, biofuels of the first generation are produced from crops, and it happened to compete with food, resulting in a rise in food prices. Currently, microalgae are recognized as a resource for the third generation biofuels. The main advantages of the microalgae are that they have a higher photon conversion efficiency (higher biomass yield) compared with terrestrial plants, and that they do not compete with food crops.

Since July 2008, DENSO has been involved in an algal research of cultivating *Pseudochoricystis* in open-type ponds. *Pseudochoricystis* is a tentative name and it may belong to the *Pseudococcomyxa* genus. We constructed pilot-scale raceway ponds in our Zenmyo factory on June 2010. As *Pseudochoricystis* can grow under acidic conditions, it was possible to cultivate this alga for several weeks in the raceway ponds without contamination of other algal species. In these cultures, flue gas from a cogeneration power system in the Zenmyo factory was used as the CO_2 source, while the effluent from an activated sludge wastewater treatment plant in the factory was used as water resource.

In the MAFF project which started on July 2010, DENSO is engaging in the development of new culture processes to lower power consumption and reduce operational cost. We are developing an automated cultivation system and water recycling technologies. In addition, we recently succeeded in developing a system to predict the triacylglycerol productivity in raceway-pond cultures.

In this presentation, I would like to introduce some of our efforts descried above.

Food and Fuel for the 21st Century - Synthetic biology in micro-algae for the production of biofuels and bio-products

Stephen P. Mayfield

Director, San Diego Center for Algae Biotechnology and Professor, Division of Biological Sciences, University of California, San Diego

Fuel, food, and all biological products are all different forms of chemical energy, and as such are closely related. All of these products are ultimately derived from photosynthesis, the process by which sunlight energy is converted to chemical energy. Over the last 100 years we have exploited cheap fossil fuels to drive unprecedented economic and agricultural growth, but in so doing we have released sequestered CO_2 into the atmosphere, which is now beginning to impact our climate. In addition, fossil fuel reserves are finite, and we are now starting to see the initial signs of depletion of these reserves, including the rising cost of fuel and food. Together these factors have provided the impetus behind the development of new renewable energy sources that can supplant fossil fuels while greatly reducing carbon emissions into the atmosphere.

Eukaryotic algae offer tremendous potential for the large scale production of biofuels and bio-products as algae require only sunlight as an energy source and sequester CO_2 during the production of biomass, and algae can be much more efficient then terrestrial plants in fixing CO_2 and producing biomass. Using “designed for purpose” photosynthetic organisms we have the opportunity to develop production platforms for fuel and food that have unmatched efficiencies and productivities.

We are developing the genetic and synthetic biology tools to enable the production of designer algae as a bio-fuels and bio-products platform. The challenges, potential, and some early successes of synthetic biology in algae for the production of high value products will be discussed.

Abstracts of poster presentations

Poster Session 1
Room 370 (3rd Floor)

P1. **Development of technologies for production of alternative fuel from microalgae: project financed by the Ministry of Agriculture, Forestry and Fisheries (2012-2015).**

P2. **Collection and screening of microalgae for lipid production – Isolation of cold-tolerant strains.**

Hideaki Miyashita[1,2], Ayako Imura[2], Yumi Tatesono[2] and Ryoma Kamikawa[1,2]

[1] Graduate School of Global Environment Studies, Kyoto University, Kyoto

[2] Graduate School of Human and Environmental Studies, Kyoto University, Kyoto

P3. **Analysis of oil-synthesizing activity in *Pseudochoricystis ellipsoidea*.**

Izumi Matsuwaki, Fumi Suzuki and **Misako Kato**

Graduate School of Humanities and Sciences, Ochanomizu University

P4. **Yieldability calculation of algae biomass.**

Hidehiko Yasui, Norihide Kurano and Hiroaki Fukuda

DENSO CORPORATION Research Laboratories

P5. **Effects of seawater addition on culture stability and oil productivity.**

Satoko Komatsu, Norihide Kurano and Hiroaki Fukuda

DENSO CORPORATION Research Laboratories

P6. **Isolation of hyper-oil-producing mutants of a unicellular green alga, *Pseudochoricystis ellipsoidea*, and identification of the hyper-oil-producing mutation by whole-genome resequencing.**

Jumpei Hayakawa, **Yoko Ide** and Shigeaki Harayama

Department of Biological Sciences, Chuo University

P7. **Isolation and characterization of mutants of *Pseudochoricystis ellipsoidea* defective in the dark metabolism of lipids.**

Jumpei Hayakawa, Eiko Sato and Shigeaki Harayama

Department of Biological Sciences, Chuo University

P8. **Isolation and characterization of a *Pseudochoricystis ellipsoidea* mutant defective in nitrate assimilation.**

Yoko Ide[1], Jumpei Hayakawa[1], Mika Sakamoto[1], Shigeaki Harayama[1], Izumi Matsuwaki[2] and Misako Kato[2]

[1]Department of Biological Sciences, Chuo University

[2]Department of Biological Sciences, Ochanomizu University

P9. **Effect of salinity on the lipid and sarch contents in a green alga *Pseudochoricystis ellipsoidea*.**

Ryushi Hasegawa, Yoko Ide and Shigeaki Harayama

Department of Biological Sciences, Chuo University

P10. **Identification of TATA-boxes in the genome of *Pseudochoricystis ellipsoidea* by chromatin immunoprecipitation sequencing.**

Yusuke Namiki, Sousuke Imamura and Shigeaki Harayama

Department of Biological Sciences, Chuo University

P11. **An instrument for the continuous monitoring of the growth of microalgae under photosynthetic and non-photosynthetic conditions.**

Shigeaki Harayama, Eiko Sato and Jumpei Hayakawa

Department of Biological Sciences, Chuo University

P12. **Solid-surface culture methods of *Pseudochoricystis*.**

Shun Nakamura, Jumpei Hayakawa and Shigeaki Harayama

Department of Biological Sciences, Chuo University

P13. **Cell harvesting with porous membrane: potential and challenges.**

Hiroshi Yamamura[1], Jumpei Hayakawa[2] and Shigeaki Harayama[2]

[1] Department of Integrated Science and Engineering, Chuo University

[2] Department of Biological Sciences, Chuo University

P14. **Influence of filamentous fungi on cultivation of alga.**

Hiroshi Yamamura[1], **Keisuke Goto**[1], Takeki Matsumoto[1], Jumpei Hayakawa[2], Shun Nakamura[2] and Shigeaki Harayama[2]

[1] Department of Civil and Environmental Engineering, Chuo University

[2] Department of Biological Sciences, Chuo University

P15. **The fundamental study of membrane filtration technology towards microalgae harvesting**.

Satoshi Ezaki and Nobukazu Suzuki
Water and Environment R&D , KUBOTA Corporation

P16. **Development of a method for extracting oil from microalgae using microwave irradiation capable of selective heating.** - Disruption effects on microalgae cells by microwave irradiation -

Keiji Kidani, Kentaro Yamamoto, Akinori Ishizuka, Yasunori Tsukahara and Iwao Yoshino
Microwave Chemical Co.,Ltd

P17. **Production of drop-in fuel from algae fxtract.**

Jun Shamoto and Tetsuya Fukunaga
Advanced Technology Research Laboratories, Idemitsu Kosan Co., Ltd.

P18. **Development of poultry feed supplied with oil-extracted algal cell residues.**

Satoshi Yamasaki, Keisuke Kogusa, Yosuke Tamaoki and Tomohiro Asano
General Research Laboratory, Research & Technical Department, Chubushiryo (Feed) Co. Ltd.

P19. **Development of aquaculture feed produced from the oil-producing microalgae, *Pseudococcomyxa* strains.**

Hiroaki Kasai
Kamaishi Research Laboratory, Kitasato University

P20. **Quantitative analysis of triacylglycerols in a unicellular red alga *Cyanidioschyzon merolae*.**

Yasuko Kawase[1], Sousuke Imamura[1,2,] Mie Shimojima[3], Hiroyuki Ohta[2,3] and Kan Tanaka[1,2]
[1]Chemical Resources Laboratory, Tokyo Institute of Technology, [2]JST CREST
[3]Center for Biological Resources and Informatics, Tokyo Institute of Technology

P21. **Development and multi-omics analysis of high-light tolerant strain of *Synechocystis* sp. PCC 6803.**

Katsunori Yoshikawa[1,2], Kenichi Ogawa[1,2] and Hiroshi Shimizu[1,2]
[1] Department of Bioinformatic Engineering, Graduate school of Information Science and Technology, Osaka University, [2] JST, CREST

P22. **Engineering marine diatom *Fistulifera* sp. JPCC DA0580 toward biodiesel fuel production.**

Masaki Muto[1], Yoshiaki Maeda[1], Masayoshi Tanaka[1], Tomoko Yoshino[1], Tsuyoshi Tanaka[1], Mitsufumi Matsumoto[2] and Akira Satoh[1]

[1]Institute of Engineering, Tokyo University of Agriculture and Technology

[2]Biotechnology Laboratory, Electric Power Development Co.

[3]Technology Center, Yamaha Motor Co.

P23. **Tsukuba international strategic zone: Project3 - Practical use of algal biomass energy.**

Masaki Yoshida and Makoto M. Watanabe

Faculty of Life & Environmental Sciences, University of Tsukuba

P24. **NET: Next-generation Energies for Tohoku Recovery: Task2 - R&D on using algae biofuels**.

Masaki Yoshida and Makoto M. Watanabe

Faculty of Life & Environmental Sciences, University of Tsukuba

P25. **Isolation and characterization of squalaene epoxidase-like genes from *Botryococcus braunii*, Race B.**

Hidenobu Uchida, Koremitsu Sumimoto, Yusuke Fukunaga, Shigeki Matsunaga and Shigeru Okada

Graduate School of Agricultural and Life Sciences, The University of Tokyo,

CREST JST

P26. **Exploration of Cyanobacteria for biomass production and its genome analysis with next generation sequencers.**

Yuu Hirose[1], Naomi Misawa[1], Masao Nagakubo[2], Hiroaki Fukuda[2] and Masahiko Ikeuchi[3]

[1]Electronics-Inspired Interdisciplinary Research Institute, Toyohashi University of Technology

[2]DENSO CORPORATION, Research Laboratories

[3]Department of Life Sciences (Biology), The University of Tokyo

P34. **Continuous production of isoprenoids by cyanobacteria and its improvement**

Hiroshi Kiyota, Yukiko Okuda, Michiho Ito, Masami Hirai and Masahiko Ikeuchi

P35. **Microalgal Cultivation by Sewage and Evaluation as Fuel**

Kenichiro Inoue, Jun Tsumori, Yutaka Suzuki

Recycling Research Team,Incorporated Administrative Agency Public Works Research Institute

Poster Session 2
Foyer (2nd Floor)

P27. **Molecular breeding in green alga *Pseudochoricystis* sp.**
Yuya Yoshitmitsu[1], **Norihide Kurano**[1], Izumi Fukuhara[2] and Shigeaki Harayama[2]
[1] DENSO CORPORATION Research Laboratories
[2] Department of Biological Sciences, Chuo University

P28. **Structure of the *RbcS* gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxigenase and its unusual transcript in *Pseudochorysistis ellipsoidea* strain Obi.**
Yuki Kasai and Shigeaki Harayama
Department of Biological Sciences, Chuo University

P29. **Towards the construction of self-cloning system in *Pseudochoricystis ellipsoidea.***
Kohei Oshima, Yuki Kasai and Shigeaki Harayama
Department of Biological Sciences, Chuo University

P30. **Improved method for the preparation of spheroplasts from *Pseudochoricystis ellipsoidea.***
Hiroshi Tatara, Jun Abe and Shigeaki Harayama
Department of Biological Sciences, Chuo University

P31. **Exploration of regulatory factors responding to nitrogen-deficiency in *Pseudochoricystis ellipsoidea***
Masataka Seki, Jun Abe, Sousuke Imamura and Shigeaki Harayama
Department of Biological Sciences, Chuo University

P32. **Reporter genes useful in unicellular green alga *Pseudochoricystis ellipsoidea.***
Jun Abe, Satsuki Takagi, Sousuke Imamura and Shigeaki Harayama
Department of Biological Sciences, Chuo University

P33. **Transcriptome analysis of lipid metabolic genes in a unicellular green alga *Pseudochoricystis ellipsoidea***.
Saki Iwase, Yoko Ide and Shigeaki Harayama
Department of Biological Sciences, Chuo University

Development of Technologies for Production of Alternative Fuel from Microalgae

Project financed by the Ministry of Agriculture, Forestry and Fisheries (2012-2015)

This Japanese Project aims to develop innovative systems for the cultivation of microalgae, and use microalgal biomass to produce alternative fuels and other valuable materials. More specifically, this project aims to develop an integrated system of biofuel production from raw materials (i.e. algal cells) to finished products (i.e. biofuels and other valuable materials). This project consists of 6 different programs that interact with one another.

In this project, two algal stains are mainly used in our reseraches. *Pseudochoricystis ellipsoidea* (tentative scientific name) has been selected as the parental strain for biofuel development. However, since a new strain named *Pseudococcomyxa* strain KJ which is a better lipid producer than *P. ellipsoidea* was isolated in this project, we are adapting technologies developed for *P. ellipsoidea* to *Pseudococcomyxa* strain KJ. Although the generic names of these two strains are different, they belong to the Trebouxiophyceae family, and similar biochemical methods could be used for the treatment of these two strains.

Program 1: Screening and breeding of microalgal strains suitable for open pond cultivation

Program 2: Development of methods for low cost and stable cultivation

Program 3: Development of low cost harvesting technology

Program 4: Development of novel microwave-assisted oil extraction technique

Program 5: Development of esterification and hydrogenation of fatty acids for fuel production

Program 6: Development of oil-extracted cell residues as animal and fish feed

Project members (alphabetic order)

Chubushiryou Co., Ltd., Chuo Univesity, Denso Corporation, Idemitsu Kosan Co., Ltd., Kitasato University, Kubota Corporation, Kyoto University, Microwave Chemical Co., Ltd., Ochanomizu University

Collection and Screening of Microalgae for Lipid Production

– Isolation of Cold-Tolerant Strains -

Hideaki Miyashita[1,2], Ayako Imura[2], Yumi Tatesono[2], Ryoma Kamikawa[1,2]

[1] *Graduate School of Global Environment Studies, Kyoto University, Kyoto.*
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Bio-fuel production from microalgae is a sustainable green system based on the photosynthesis using solar irradiation, CO2 and water. It also has a great advantage in the lack of any serious conflictions with the supply of foods and feeds. Therefore, the algae-fuel has been actively investigated as the alternative source of energy against the oil crisis in the future, as well as for a CO2 recovering process to prevent the green house effects on the Earth. Several commercial-scale processes have been proposed and some of them are working. However, the algae-fuel is uneconomic at present, since their production costs are much higher than the current oil prices.

One choice to cut down the cost is to employ the open pond system for the production of microalgal biomass. Open pond system is more cost effective than the closed systems such as flat-panel and tubular photobioreacters. However, the biomass productivity of open pond systems is affected by the two major negative factors. One is the contamination of predators such as protozoans, rotifers and crustaceans, in addition to fungi, bacteria, virus etc. The team of DENSO found effective way. By maintaining pond at low pH (pH 3), the biomass productivity of microalgae could be maintained with reducing the growth and predation of contaminants. The other negative factor on the productivity is climate and weather. Especially, the pond temperature became up to 40-45 ˚C in the mid summer and 5-0 ˚C in the mid winter in Japan. Since most of oil-producing microalga can not grow under those temperatures, biomass productivity is drastically decrease at those seasons. Therefore, thermo-tolerant and cold-tolerant oil-producing strains are required to establish an all year production of microalgal biomass by the open pond system.

We have continued our efforts on the isolation and selection of oil-producing microalgae which tolerate low/high temperatures under low pHs. In this presentation, we would like to introduce the isolation, selection and characterization of cold-tolerant strains.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Analysis of Oil-synthesizing Activity in *Pseudochoricystis ellipsoidea*

Izumi Matsuwaki, Fumi Suzuki and Misako Kato

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Pseudochoricystis ellipsoidea is a recently isolated unicellular green alga, which is classified within the family Trebouxiophyceae. *P. ellipsoidea* accumulate large amounts of storage lipids in form of triacylglycerol (TAG) under nitrogen starvation conditions. The oil-synthesizing activity in *P. ellipsoidea* grown in nitrogen-starved medium with aeration enriched with 2% CO_2 was determined by feeding experiments using ^{14}C-compounds. $NaH^{14}CO_3$ incorporation rate into TAG increased remarkably by 13 days after inoculation. On the other hand, TAG-synthesizing activity from [2-^{14}C]acetate was highest by 7days after inoculation, subsequently, the activity decreased and remained at 30% of the maximum level. $NaH^{14}CO_3$ and [2-^{14}C]acetate incorporation rate into starch-rich fraction were highest by 4 days after inoculation and the activities drastically decreased thereafter, respectively. Hence, the allocation of carbon might be the regulatory step in TAG and starch biosynthesis during early logarithmic growth phase. TAG-synthesizing activity in *P. ellipsoidea* from [2-^{14}C]acetate in the dark was higher than that in the light (23.4μmol m^{-2} s^{-1}) and the obtained result also supported the finding from *Pseudococcomyxa* sp. KJ. TAG-synthesizing activity in the dark was cerulenin-sensitive, suggesting that this activity was derived from *de novo* fatty acid synthesis. In contrast, TAG-synthesizing activities in *Chlamydomonas reinhardtii* (Chlorophyceae) and *Dictyosphaerium pulchellum* (Trebouxiophyceae) depended on light. In parallel, we investigated the gene expression of the enzymes related to TAG biosynthesis. Transcript levels of the multiple genes homologous to the predicted diacylglycerol acyltransferase genes were high during late growth phase. Various gene expressions related to TAG biosynthesis for carbon metabolism were also discussed.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas]

Yieldability Calculation of Algae Biomass

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Prediction of lipid productivity from microalgae is very important for selection of useful mutants, biofuel cost estimation, searching adequate land for microalgae cultivation, making a technological road map of microalgae breeding for biofuel business, and so on.
We are developing a technology to produce biofuel from microalgae for the purpose of solving global warming and energy issues simultaneously. Our microalgae, "*Pseudochoricystis ellipsoidea*", can be cultured in very large scale open ponds with natural sunlight and CO_2 emissions from the industrial power plants. By the mutation breeding of *p.ellipsoidea*, we had obtained a lot of mutants. However, it takes a lot of time to select mutants with useful properties for the biofuel production process. we had to culture all of them through a trial and error evaluation by using a large scale raceway ponds.
We develop a yield simulation model of lipid productivity from microalgae with following aims.

1. Design of microalgae mutants; to clarify the guidelines for the breeding of algal strains having multiple useful properties.
2. Determination of land suitability for culturing algae; to clarify the environmental influence such as water temperature and amount of sunlight.

As a first step, we have developed a model that can simulate a yield of our microalgae in an indoor open pond.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Effects of Seawater Addition on Culture Stability and Oil Productivity

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One of the most important things to establish the algae-based oil production system is a selection of the most suitable algal strain, which has the high potential to accumulate oil inside of the cells and grows quickly. Reduction in maintenance cost of cultivation system is also significant. The open raceway ponds require low capital cost and low energy consumption compared with photobioreactors, on the other hand their open nature makes them more susceptible to contamination.

A unicellular green alga, *Pseudococcomyxa* sp. strain KJ, which was isolated by Kyoto University and DENSO CORP. and *Pseudochoricystis ellipsoidea* (patented by DENSO CORP.) can grow under acidic condition (appropriate condition is between pH3-4). This feature is considered to have competitive merit for outdoor cultivation, because many other microalgae and protozoa cannot grow under this condition. In other words, opportunity of contamination during outdoor cultivation can be restricted under acidic conditions. We had tried to cultivate these algae in outdoor raceway ponds at Miyako island (Okinawa prefecture), where seemed to be an appropriate location for cultivating these algae. In the culture examination, unsterile natural seawater was added to some extent for further reduction in the cost of cultivation system. Although the added seawater was not sterilized, the denaturing gradient gel electrophoresis (DGGE) analysis detected no fatal contamination. In addition, the oil content of the cells was increased. Further quantitative analysis of protozoa and bacteria by Real Time PCR was also carried out. Based on these results, we will develop a low-cost and more stable mass culture system using with open raceway ponds.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Isolation of Hyper-Oil-Producing Mutants of a Unicellular Green Alga, *Pseudochoricystis ellipsoidea*, and Identification of the Hyper-Oil-Producing Mutation by Whole-genome Resequencing

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The importance of genetic and metabolic engineering of unicellular green algae for the development of a viable algal-oil industry has repeatedly been discussed in recent scientific literatures. However, the construction of breeding algae producing significantly higher yields of lipids has not yet been described. In this report, we present three mutants of *Pseudochoricystis ellipsoidea* (tentative scientific name) capable of producing lipids at a speed much higher than the wild-type strain. The whole-genome resequencing of these three mutants allowed the identification of the mutated gene responsible for the hyper-oil-productivity.

Isolation of hyper-oil-producing mutants: High-throughput cell sorting coupled with flow cytometry is a powerful tool for the enrichment of microalgae mutants exhibiting various phenotypic traits. We used fluorescence-activated cell sorting (FACS) to enrich mutants with superior productivity of lipids. First, cells of *P. ellipsoidea* were mutagenized with 1-methyl-2-nitro-1-nitrosoguanidine, and the mutagenized culture was incubated under fluorescent light for several days to allow for segregation. Cells in the culture were then stained with BODIPY 505/515, and cells with increased fluorescence were sorted and collected. The collected cells were further cultivated under fluorescent light for several days before cells were subjected to the second sorting. After consecutive three rounds of cell sorting, cells were spread on agar plates, and about 100 isolates from well separated single colonies were tested for their lipid contents. Finally, we selected three strains named JH1011, JH1012 and JH1013 for further studies.

Characterization of hyper-oil-producing mutants: The oil productivity of these strains in either small-scale flask cultivation or in indoor mesocosmic raceway-pond cultivation was determined by GC-FID and by a benchtop NMR MQC analyzer. The productivity of lipids in these mutants was much higher than that in the wild-type strain. Strain JH1013 showed the highest productivity which was 70% superior to that of the wild-type strain.

Identification of mutant gene responsible for the hyper-oil-producing phenotype: Whole-genome resequencing of these three mutants were carried out with next-generation sequencing, and the obtained sequences were aligned to the reference genome of *P. ellipsoidea*. Each of these mutants contains between 50 and 100 mutations most of them being nucleotide substitution mutations. About 80% of the mutations were located in intergenic regions, introns, or untranslated regions (5'- and 3'-UTR) of exons, while about the half of the mutations in coding sequences in exons were synonymous mutations. Mutations in the gene for a dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) were found in all the three mutants indicating that the defect in DYRK is responsible for the hyper-oil-producing phenotype. Possible roles of DYRK in the lipid biosynthesis are discussed.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas] and by a grant from NEDO (New Energy and Industrial Technology Development Organization) [Development of Biofuel Production Technology Using Microalgae].

Isolation and Characterization of Mutants of *Pseudochoricystis ellipsoidea* Defective in the Dark Metabolism of Lipids

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Introduction

Microalgae-based biofuel has been considered as an only promising resource for a sustainable energy production. However, we need to overcome major obstacles for the commercialization of biofuels. For outdoor culture with light-dark cycle, storage compounds such as starch and lipids are consumed during the night resulting in the loss of biomass; and if we can reduce night biomass loss, total biomass yield will increase. However, no study so far had been carried out to isolate microalgae mutants defective in the dark metabolism of storage compounds. In this study, we isolated mutants defective in the degradation of lipids in the dark period.

Isolation of mutants

To isolate mutants defective in the dark metabolism of lipids, cells of Pseudochoricystis ellipsoidea (tentative scientific name) were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and the mutagenized cells were segregated by cultivating them under white fluorescent light for two weeks. Two methods were subsequently used to enrich desired mutants.

Method 1: The mutagenized cells were spread onto nitrocellulose membrane filters each of which was placed on an agar plate, and grown photosynthetically. After the development of dark-green colonies of *P. ellipsoidea* onto the membrane, the membrane was transferred to a nitrogen-free agar plate, and incubated under light for 7 days. Under nitrogen-depleted conditions, the color of colonies turned light-green due to the partial breakdown of chlorophyll molecules. Subsequently, the membrane was transferred again to a normal (nitrogen-containing) plate, and incubated in dark for 2 to 4 days. Under the nitrogen repletion conditions in the dark, the biosynthesis of chlorophyll restarted by consuming storage compounds, and the colony color returned to dark-green. However, some of the colonies remained light-green suggesting that either the dark metabolism of storage compounds or the chlorophyll biosynthesis in dark is deficient in such colonies. These colonies were purified and characterized further as described below.

Method 2: Mutagenized cells were cultivated under nitrogen-depleted conditions to provoke the accumulation of lipids. Cells were next transferred to nitrogen repletion conditions in dark, and incubated for one week. Then, cells in the culture were stained with BODIPY 505/515, and strongly fluorescent cells were enriched by employing fluorescence-activated cell sorting (FACS). The collected cells were cultured and further subjected to three rounds of the FACS enrichment. Finally, cells sorted by FACS were spread onto agar plate, and about 150 colonies were isolated.

Characterizations of mutant strains

Finally, we selected four (Method 1) and eight (Method 2) mutant strains; and the lipid and starch degradation rates in dark were determined in these strains using a NMR MQC analyzer (for lipids) and by colorimetric quantification of reducing sugar (for starch). The starch consumption was rapid in both the wild-type and mutant strains; about 80-90% starch found at day 0 were consumed within one day. On the other hand, the lipid degradation was significantly suppressed in these mutants comparing to that of the wild-type strain. Several mutants isolated by “Method 2” kept more than 90% to the initial amount of lipids after the dark incubation for 4 days. Transferring these mutants under photosynthetic conditions, these mutants started to grow without significant lag phase.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Isolation and Characterization of a *Pseudochoricystis ellipsoidea* Mutant Defective in Nitrate Assimilation

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Algae biofuels are often considered to be at the forefront of biofuels. However, large-scale production of the algae biofuels raises a number of environmental concerns, one of them being the risk of the invasion of commercial algal strains into neighboring areas. Nevertheless, the assessment of the possibility of commercial strains to dominate in variable environments is not yet widely studied.

Previously, we have addressed the issue concerning the fair assessment of risks for mass cultivation of a green alga, *Pseudochoricystis ellipsoidea* (tentative scientific name), and examined the ability of this alga to survive in simulated natural environments. We found that the growth of *P. ellipsoidea* in non-eutrophic environments was observed only if nitrate was present, but its growth was not significant if nitrate was absent regardless of the presence or absence of ammonium. Thus, we concluded that nitrate is critical for *P. ellipsoidea* to survive in natural environments, and deduced that a biological containment system would be developed by isolating and using nitrate reductase-deficient mutants of *P. ellipsoidea* which would greatly diminish the risk of survival after leakage.

We have isolated *P. ellipsoidea* mutants with altered lipid accumulation patterns in cells under several different conditions. One of these strains did not grow on nitrate as the sole nitrogen source, but could grow on nitrite or ammonium. As the sequence analysis of the two nitrate reductase (NR) genes in the mutant revealed that they were intact, we performed whole-genome resequencing of this strain to identify a mutation responsible for the nitrate-negative phenotype.

A mutation was found in the gene encoding a putative molybdenum cofactor (MoCo) biosynthesis protein, CNX1G. A substitution of aspartic acid for glycine was found in the region highly conserved among various organisms. This protein is thought to catalyze the insertion of molybdenum into the pterin compound to form MoCo. As the NR is a MoCo-dependent enzyme, MoCo biosynthesis is important for nitrate assimilation pathway. Extraordinarily high concentrations (1 to 10 mM) of molybdenum supplied in medium allowed the growth of the mutant on nitrate, indicating that the nitrate-negative phenotype of this strain is caused by the mutation in CNX1G.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Effect of Salinity on the Lipid and Starch Contents in a Green Alga *Pseudochoricystis ellipsoidea*

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The single-celled green alga tentatively named *Pseudochoricystis ellipsoidea* accumulates lipids including triaclyglycerols (TAGs) in response to various environmental stresses. Especially, in nitrogen-deficiency and salinity-stressed conditions, they accumulate lipids to more than 30 % weight per cell's dry weight. However, it is not yet in situation to realize the commercialization of microalgae biofuel using *P. ellipsoidea*, and the improvement of lipid productivity in this alga is highly anticipated.

We are intended in identifying genes involved the response to salinity stress leading to the accumulation of lipids in *P. ellipsoidea*. Using two wild-type *P. ellipsoidea* strains isolated from two different regions in Japan, we examined the time course of intercellular lipids accumulation, intercellular starch accumulation, and cell growth under the salinity stress. Although the growth of these two strains was not affected by adding 200 mM NaCl, the intercellular lipid concentration increased significantly, while the intercellular starch accumulation decreased under the salinity stress.

To understand the interrelationships between the lipid and starch biosyntheses under the salinity stress, we performed transcriptome analysis (RNA-seq) using the total RNA extracted from these two *P. ellipsoidea* strains grown under normal and salt-stressed conditions. We identified genes involved in the synthesis and degradation of starch and lipids in the genome of two *P. ellipsoidea* strains referring to the isofunctional pathways in *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* and *Solanum tuberosum*. The expression of some of these genes responded to the salinity stress indicating that some of these genes are responsible to the observed changes in the lipid and starch contents in *P. ellipsoidea*.

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Identification of TATA-boxes in the Genome of *Pseudochoricystis ellipsoidea* by Chromatin Immunoprecipitation Sequencing

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Pseudochoricystis ellipsoidea (tentative scientific name) is a unicellular green alga which belongs to the *Trebouxiophyceae* family. When *P. ellipsoidea* is cultured under nitrogen starvation conditions, this alga intracellularly accumulates around 30% of lipids on a dry weight basis. However, the mechanism of the regulation of gene expression leading to the lipid accumulation in response to the nitrogen-starvation stress is largely unknown. There is a vast necessity of basic studies on aspects related to the transcriptional regulation in this organism. By this reason, we are interested in characterizing promoters and their regulation in *P. ellipsoidea*. As the first step towards the goal, we identified TATA boxes on the genome of this alga.

The gene for TATA-box Binding Protein (TBP) in the *P. ellipsoidea* genome was identified by a BLAST (tblastn) search, and the full-length TBP sequence was deduced from the corresponding cDNA sequence. An antigen peptide was synthesized chemically in accordance with the amino acid sequence of a predicted epitope, and used to immunize a Guinea pig. The high specificity of polyclonal antibodies against TBP was confirmed by western blotting.

Cells of *P. ellipsoidea* were treated with formaldehyde to reversibly crosslink chromatin-associated proteins, and the cells were disrupted by one passage through a French Press. After centrifugation of cell lysate, the cross-linked chromatin in the supernatant was sheared by sonication to obtain DNA fragments of 300 base pairs in length. The sonicated chromatin was mixed with protein A Sepharose beads and incubated for 3 hours at 4℃ to remove protein which binds to protein A nonspecifically. After the incubation, protein A Sepharose beads were removed by columns. Flow through fraction was collected and mixed with the TBP antibody to immunoprecipitate DNA fragments crosslinked to TBP. Then, (TBP-crosslinked DNA fragments) - (TBP antibody) complexes were collected by protein A magnetic beads. After overnight incubation at 65°C to reverse cross-links, DNA was purified by phenol/ chloroform/ isoamyl alcohol extraction and ethanol precipitation.

The quantity of ChIPed DNA fragments was too small to be sequenced directly. Then the ChIPed DNA fragments were amplified by Rolling Circle Amplification（RCA）with Phi29 DNA polymerase, and the amplified DNA was sequenced with Illumina Hiseq2000. The obtained sequences were mapped 109 regions onto the genome of *P. ellipsoidea*.

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An Instrument for the Continuous Monitoring of the Growth of Microalgae under Photosynthetic and Non-photosynthetic Conditions

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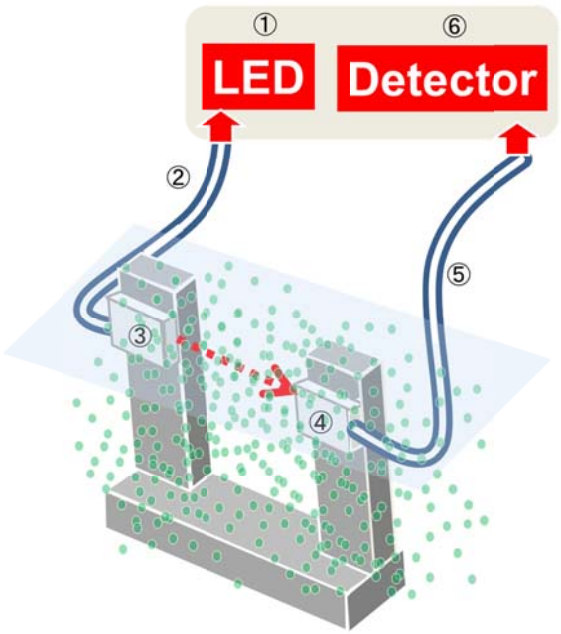
A newly designed instrument which allows the continuous monitoring of the growth of microalgae cultivated under a variety of conditions is described. This instrument consists of two modules; the first module contains a light source (①) and a light detector (⑥), while the second module is a submersible fiber optic assembly of a light transmitter (③) and a light receiver (④). The second module (③+④) is completely submerged into a liquid culture of a microalga. In this instrument, the light emitted from the light source (a light emitting diode, ①) passes through a fiber optic cable (②) to the light emitter (③) from which the light is projected toward the light receiver (④). The light from the light emitter (③) passes through the microbial culture that causes scattering and absorption of some light before reaching the light receiver (④). The distance between the light-emitter (③) and the light receiver (④) is adjustable between 0.5 to 4.5 cm. The light captured by the light receiver (④) is then transmitted, through a fiber optic cable (⑤), to the light detector (④) which converts the light energy into an electronic signal that ranges from 1 to 5 V DC.



Continuous turbidity monitoring will become increasingly popular if a non-expensive turbidity meter of this type will become commercially available because a manual sampling followed by the determination of the turbidity of the sample by a spectrophotometer is time- consuming and error-prone.

Generally, turbidity values can serve as a simple and convenient measure of the growth status of microalgae in liquid cultures. However, with the manual sampling where the sampling frequency is low, it is difficult to detect small but reproducible changes in growth behavior of microalgae in response to environmental changes. The continuous monitoring may be the only viable means to detect such microalgae responses, and thus may provide new insight into growth stage or into growth responses to environmental changes.

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Solid-surface Culture Methods of *Pseudochoricystis ellipsoidea*

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There is worldwide interest in biofuels as an alternative sustainable energy source. Among others, algae biofuels are receiving more and more attention as they will not compete with food crops for arable land use. However, algal biofuel production has many problems to be solved before its commercialization. To bring the production cost of algal biodiesel down, we are developing solid-surface culture as a new cost-effective and scalable culture method.

Solid-surface culture can be defied as a process to cultivate algal cells on the surface of solid absorbent containing nutritious water required for the growth of algal cells; and our aims are to find adsorbent materials convenient for it, and to identify the optimal conditions of solid-surface culture for the highest biomass productivity.

First, we used square agar plates to evaluate the feasibility of the solid-surface culture. Several agar plates inoculated with *Pseudochoricystis ellipsoidea* (tentative scientific name), a unicellular green alga belonging to Trebouxiophyceae, were arrayed vertically in close proximity; and light from Light Emitting Diodes（LED）(440nm/470nm/640nm/660nm/740nm) at 300 μmol/m^2/sec was illuminated parallel to the surface of the agar plates. Biomass production rate per illuminated area of *P. ellipsoidea* cultivated under the conditions was 21.2 g/m^2/day. This value was superior to that in liquid culture bubbled with air (15.5 g/m^2/day). In the solid culture, cells on the surface might assimilate CO_2 directly from the air, while in the liquid culture, CO_2 dissolved in water might only be available to cells. Thus, slower growth of cells in the air-bubbled liquid culture might be a reflection of the poor solubility of CO_2 in water.

In large-scale outdoor cultivation in sunny areas, the rate of water evaporation is high, and a way to reduce water evaporation from and/or to supply water to the solid surface should be developed. We devised a culture system consisting of a cell-supporting membrane, and a water-absorbing material attached to the membrane. We selected polytetrafluoroethylene (PTFE) membrane (pore size 0.5 μm) as the cell-supporting membrane. On the PTFE membrane attached to an agar plate (as a water-absorbing material), *P. ellipsoidea* cells grew with a biomass production rate of 13.8 g/m^2/day. We tested other water-absorbing materials including cellulose paper (Whatman 3MM), LANSEAL® (blended fabric of cellulose and polyacrylate fibers, TOYOBO) and BELL OASIS® (polyacrylate fibers, TEIJIN). With these materials, the productivity with the cellulose paper was highest being approximately 70% to that with agar plate.

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Cell Harvesting with Porous Membrane: Potential and Challenges

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Membrane filtration has now been on the rise in the field of water treatment, food processing and pharmaceutical processing because they can achieve complete separation without any phase exchange and any chemical reagents. The lower the solid content of applied solution is the better the performance of membrane filtration. So, the use of membrane is suitable for the cell harvesting in light of the value of solid contents in raceway pond cultivation (0.5 ~ 1.0mg-MLSS/L). Furthermore, the reuse of permeate culture medium for the next cultivation would be achieved through the use of membrane because it does not require any hazardous chemicals. As a whole, the membrane-based cell harvesting process has a large potential to be an essential process for establishing cost-effective and sustainable bio-fuel production.

The major drawback of membrane technology is "membrane fouling". As the fouling develops, the pure water permeability significantly decreases. Based on the research which carried out membrane filtration for drinking water production, it was demonstrated that their pure water permeability decreased to one tenth because of the fouling development. Although the membrane is periodically cleaned by air-scrubbing or backwashing to mitigate "reversible membrane fouling", some organic and inorganic substances are accumulated in and on the membrane during the long-term operation. These deposits, which can be removed only by chemical cleaning, result in the persistent loss of membrane permeability called "irreversible membrane fouling". The severe reversible fouling requires frequent physical cleaning, which leads to increase in energy consumption for air blower and poor water recovery. On the contrary, the severe irreversible fouling requires frequent chemical cleaning, which creates problems such as disposal of the used chemical cleaner and shortening of membrane lifetime. The frequency of both physical and chemical cleaning is required to be as low as possible in the actual operation. It is, therefore, of great importance to gain more knowledge on what actually causes reversible and irreversible fouling, and take measures to prevent or mitigate the membrane fouling.

In this study, the potential to develop reversible and irreversible membrane fouling was evaluated by bench-scale filtration tests. The characteristics of culture medium were also analyzed in parallel, with fluorescence spectra, infrared spectra and examined for zeta potential of micro algae. Based on the results obtained in the filtration experiments and the chemical analysis, (1) the characteristics of the organic constituents responsible for the reversible and irreversible membrane fouling and (2) potential of porous membrane for cell harvesting were discussed.

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Influence of Filamentous Fungi on Cultivation of Alga

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Membrane filtration has now been on the rise in the field of water treatment, food processing and pharmaceutical processing because they can achieve complete separation without any phase exchange and any chemical reagents. The lower the solid content of applied solution is the better the performance of membrane filtration. So, the use of membrane is suitable for the cell harvesting in light of the value of solid contents in raceway pond cultivation (0.5 ~ 1.0mg-MLSS/L). Furthermore, the reuse of permeate culture medium for the next cultivation would be achieved through the use of membrane because it does not require any hazardous chemicals. As a whole, the membrane-based cell harvesting process has a large potential to be an essential process for establishing cost-effective and sustainable bio-fuel production.

There are some researches which carry out cell harvesting with porous membranes and most of them cultivate the cells with aseptic culture. However, considering that most of practical reactors have been operated under fully opened condition (e.g., outdoor), we need to acquire the knowledge that how the contamination of the other microorganisms influence on the characteristics of culture fluid and on the performance of membrane filtration. In the present study, therefore, we cultivated a green alga, *Pseudochoricystis ellipsoidea*, in open and close systems and compared the characteristics of culture fluids in terms of molecular size, charges and hydrophobicity so as to suggest the influence of contamination on the performance of membrane filtration.

With the visual inspection, we cannot find out any contamination for the closed culture, while filamentous fungus was found in the opened culture. Based on 18S ribosomal DNA sequences, the filamentous fungus was found to be the close species of *Tricoderuma*. Comparing the characteristics of algal cells, there was no significant difference in growth rate, lipid contents and size distribution between the aseptic and contaminated cultures. Cells in both cultures accumulated the lipid around 33% of their total dry weight and had the size around 4 μm.

The characteristics of DOMs were found to be quite different between opened and closed cultures. The level of DOC was half times higher for closed culture (300 mg-C/L) than for opened culture (200 mg-C/L). Based on the LC-OCD measurement, it was implied that the filamentous fungi utilizes the extracellular polysaccharides (s-EPS) produced by algae biomass as a carbon source and yielded some hydrophobic metabolites. To confirm our assumption, we isolated the filamentous fungi from opened culture sample and carried out a batch experiment in the presence of s-EPS in closed culture sample. The experiments clearly demonstrated that the level of DOC rapidly decreased as an increase in the mass of filamentous fungi.

Based on our study, in the opened culture, we found that *P. ellipsoidea* lived in a symbiotic with the filamentous fungi and this symbiosis resulted in the elevation of organic hydrophobicity and reduction of molecular size.

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The Fundamental Study of Membrane Filtration Technology towards Microalgae Harvesting

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The harvesting process, located between algae culture and oil extraction step, is absolutely necessary step for commercialization of biofuel production system. In general, sedimentation, coagulating separation and centrifugation are often used for harvesting procedure. However, these methods are not thought to be best way when we consider total cost (CAPEX, OPEX) through the system design on the basis of dispersible, small size and minimal specific density of cell. As this background, on the purpose of these business solutions, we evaluated the applicability of the membrane filtration technology by using microfiltration (MF). In particular, we selected several types (structure, material and pore size) of prospective membrane comprehending with water qualities (chemical composition and particle size distribution) of the culture solution. Furthermore, we made a bench-scale prototype machine based on performance of the each membrane, and started the fundamental study about filtration and operation method. As these results, it was revealed that the membrane filtration is not only prospective technology toward a major reduction of harvesting cost, but also confirmed that it was suitable for “efficient use of extract residue” and “water recycling of culture solution” compared with another separation methods.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Development of a Method for Extracting Oil from Microalgae using Microwave Irradiation Capable of Selective Heating -Disruption Effects on Microalgae Cells by Microwave Irradiation-

マイクロ波照射による選択的加熱を利用した藻類からの油分抽出技術の開発 -マイクロ波照射による藻体細胞に対する破砕効果-

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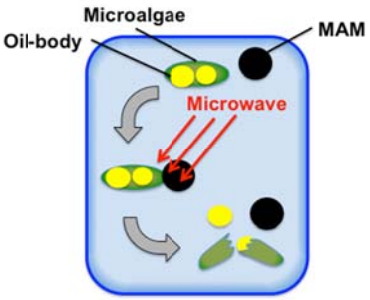
藻体保有油分の抽出・分離過程は、未だに技術的・経済的課題を解決出来る有効な方法が殆ど確立されていない。本研究課題では、 マイクロ波による藻体の破壊を促進させる電磁波吸収体を開発し、油水分離を起こした状態で油脂を抽出する技術を開発することを目的としている。

本検討系に適したマイクロ波吸収体(Microwave Absorbent Materials：MAM)を用いることにより、 マイクロ波照射下において、その近傍に電界を集中させることができる。MAM 近傍にある微細藻類は、選択的にマイクロ波と相互作用し、微小領域で局所加熱等が起こるため細胞壁が破砕され油分の抽出が可能となる。 また、 超音波などマイクロ波との組み合わせにより相乗効果の期待できる手法を組み入れることで、より効率の良い系の構築が期待できる。これまでの検討においてMAM を用いたマイクロ波照射により、120 min で藻体内に含まれる油状物質の 68 wt%を回収することを達成した。



今回の検討では、より短時間での効率的な油分抽出を達成するため、マイクロ波照射による藻体破砕効果を視覚的に確認し、定量的な評価を行った。実験は、任意の濃度に調整した供試試料に、マイクロ波を照射した後、藻体及び油分を染料により染色し、顕微鏡観察を行い、細胞を直接カウントする手法を採用した。その結果、油状物質が藻体外に放出される過程が観察され、油状物質は破砕された藻残渣に付着した状態で油滴として系内に存在している事を確認した。これら観察結果と電磁場解析ソフトによるコンピュータシミュレーションとの併用により、マイクロ波照射条件のより詳細な検討を行い、 実際の実験に反映したところ、約 30 min にて十分な藻体破砕効果が得られることが分かった。

本研究は、農林水産省委託プロジェクト研究「地域資源を活用した再生可能エネルギーの生産・利用のためのプロジェクト／微細藻類を利用した石油代替燃料等の製造技術の開発」の一環として実施された。

Production of Drop-in Fuel from Algae Extract

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Our goal is to produce drop-in biofuel, which meets existing diesel fuel quality specifications, and is ready to "drop-in" to existing engines and infrastructure. For this purpose, we have been studying and developing hydro-treating technology applicable to algae extract from *Pseudochoricystis ellipsoidea*.

Various tests revealed that critical parameters to meet the quality of drop-in fuel were catalyst type, reaction pressure and reaction temperature. An appropriate condition brought a high diesel fuel yield, over 80%. The produced hydro-treated bio diesel (HBD100), included no oxygen-containing compounds, e.g. triglycerides, free fatty-acids, etc. Very high cetane number and good distillation property as diesel oil were other features of the HBD100. We further, from the practical point of view, successfully designed a diesel oil meeting all the specifications of JIS (Japanese Industrial Standards) S No. 1, by blending the HBD100 with petroleum-based diesel oil.

The only drawback of the HBD100 was its high pour point. This can be overcome by converting the *n*-paraffin, occupying most of the hydrocarbon molecules in HBD100, to *iso*-paraffin on an isomerization catalyst. Our developed catalyst showed very high isomerization activity and selectivity in a model-reaction; showing that production of JIS No. 2 diesel (for winter season) will be realized.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Rural Biomass Research Project).

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Development of Poultry Feed Supplied with Oil-Extracted Algal Cell Residues

微細藻類油脂抽出残渣を用いた家禽用飼料の開発

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バイオ燃料の製造過程で生じる微細藻類油脂抽出残渣には、飼料原料に値する色素成分や栄養成分が残存していると考えられる。そこで、残渣の有効利用法として採卵鶏用飼料原料としての用途開発をめざす。

微細藻類（*Pseudochoricystis ellipsoidea* Obi 株及び KJ 株）には、大豆など油糧種子相当のエネルギー価値があり、粗タンパク質あたりのアミノ酸組成は大豆及び大豆加工副産物と類似し、卵黄着色効果が期待されるキサントフィル類をトウモロコシの約 90 倍含むことが栄養成分分析により明らかとなった。また、飼料安全法を含む法令等で定められた残留農薬、カビ毒、放射性物質などは検出されず安全性が確認されたことからも、本微細藻類は飼料原料として利用が可能であると思われた。

次に線形計画法（LP 計算）により微細藻類の飼料原料価値を求めたところ、キサントフィル含量の多い配合飼料においてより相対価値が高くなることから、キサントフィル給源としての利用価値が高いと思われた。また、油脂抽出残渣（模擬）を作製し、同様の手順で飼料原料価値を求めたところ、微細藻類そのものと同等の価値があるキサントフィル給源となることが期待された。

現在、油脂抽出残渣（模擬）を採卵鶏に給与し、卵質や卵黄色着色効果の詳細を調査している。

本研究は、農林水産省委託プロジェクト研究「地域資源を活用した再生可能エネルギーの生産・利用のためのプロジェクト／微細藻類を利用した石油代替燃料等の製造技術の開発」の一環として実施された。

Development of Aquaculture Feed Produced from the Oil-producing Microalgae, *Pseudococcomyxa* strains

油脂生産微細藻類 *Pseudococcomyxa* 株を利用した水産飼料の開発

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The only drawback of the HBD100 was its high pour point. This can be overcome by converting the *n*-paraffin, occupying most of the hydrocarbon molecules in HBD100, to *iso*-paraffin on an isomerization catalyst. Our developed catalyst showed very high isomerization activity and selectivity in a model-reaction; showing that production of JIS No. 2

Aquaculture continues to expand, with shift from traditional systems to more costly intensive systems, where processed feed is a major component. Almost 40% of all aquaculture production is now entirely dependent on commercial feed containing high proportions of fish meal as the primary protein source. The high costs and limited availability of fish meal has meant that plant-based protein sources have received considerable attention in recent decades, either as a partial or total substitute for fish meal in the aquaculture feed industry. Similarly, the application of microalgae as a source of biofuel has meant that numerous studies have also focused on increasing microalgal productivity, raising the possibility that microalgae could also be used to produce aquaculture feed.

However, to be used in aquaculture, a microalgal strain has to be easy to culture; non-toxic; highly nutritional, especially for protein content; have cells that are suitable in size and shape; and have a digestible cell wall so that nutrients can be made available. To evaluate the suitability of the oil-producing microalgae, *Pseudococcomyxa* strains KJ and Obi as a potential aquaculture feed, we performed feeding experiments using rainbow trout fry and juvenile sea cucumber. Feed intake and growth was comparable or higher in both species fed by *Pseudococcomyxa* biomass. The algal biomass remaining after oil extraction was also available for rainbow trout feed as a partial replacer of fish meal. The results suggested that *Pseudococcomyxa* sp. strains Obi and KJ are well suited for developing a sustainable aquaculture feed.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan [Research and development for production and utilization of renewable energy in rural areas].

Quantitative Analysis of Triacylglycerols in a Unicellular Red Alga *Cyanidioschyzon merolae*

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Microalgae are widely considered as the most promising biofuel feedstock due to their fast growth rate, high lipid yields, and ability to grow in a broad range of environments. Under stress conditions, such as nitrogen deficiency (-N), microalgae tend to form intracellular lipid droplets (LDs) composed primarily of energy-rich triacylglycerols (TAGs) that can be utilized for biodiesel production.

Cyanidioschyzon merolae is a unicellular red alga isolated from an Italian sulfur-acid hot spring (pH 1-3, 40-50 ºC), and its complete genome sequences were determined. Various analytical methods and tools, such as gene knockout and knockdown techniques, have been developed for analysis of *C. merolae*. Thus, *C. merolae* is considered to be a good model organism to understand molecular mechanisms of oil production in microalgae.

Although quantification and optimal production conditions of TAGs have been extensively investigated in many species of green algae, relevant information in red algae remains unclear. In this study, we first checked whether *C. merolae* accumulates LDs in the cells. Results indicated that accumulation of LDs, which was stained with fluorescent dipyrromethene boron difluoride (BODIPY), was observed in cytoplasmic area in response to N depletion. Quantitative analysis of TAGs after N depletion by gas chromatography revealed that TAGs began to increase at 24 hr after -N conditions, and reached a peak at 96 hr, the level being a 120-fold increase compared with 0 hr and 3.7 % of the cell dry weight. Irrespective of the nitrogen status, majority of fatty acid (FA) compositions of the TAGs were saturated FAs, 16:0 and 18:0, and unsaturated FAs, 18:1-18:3. These results indicated that *C. merolae* accumulates TAGs in response to N depeltion similarly as observed in green algae. Regulation aspects of TAGs accumulation in *C. merolae* will be discussed.

Development and Multi-omics Analysis of High-light Tolerant Strain of *Synechocystis* sp. PCC 6803

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Development of bioproduction process using cyanobacteria is important to achieve a sustainable society. Under high light condition, excess energy becomes stress for cyanobacteria and inhibits the cell growth. In this study, we developed the high-light stress tolerant strain using adaptive evolution experiment, and analyzed the high-light tolerant mechanisms of the tolerant strain using multi-omics analysis.

In this study, we used *Synechocystis* sp. PCC 6803 GT strain as the parental strain and cultivated the cells with aeration of air and continuous light illumination using point source white LED. Note that the maximum light intensity obtained by the point source LED was described in this abstract. To develop the high-light tolerant strain, serial transfer experiment was performed every one or two days under 7000 μmol/m^2/s light intensity in which the growth rate of the parental strain was decreased. Moreover, during the serial transfer experiment, the light intensity was gradually increased up to 9000 μmol/m^2/s. After 56 days cultivation, we obtained the stress tolerant strain, and the tolerant strain could grow under 9000 μmol/m^2/s in which the growth of the parental strain was severely inhibited. To understand the mechanism of the high-light tolerance of the tolerant strain, we performed transcriptome, metabolome, and whole genome sequencing analyses.

Engineering Marine Diatom *Fistulifera* sp. JPCC DA0580 toward Biodiesel Fuel Production

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Over the past several decades, bioenergy production has attracted much interest due to the approaching exhaustion of fossil fuels and their negative impacts on climate. By taking advantage of higher biomass productivity and non-competition with foods, microalgae have been recognized as more promising energy producers rather than land crops. Among a number of candidate strains, we recently discovered the oleaginous diatom, *Fistulifera* sp. strain JPCC DA0580 from our marine microalgal culture collection[1]. The great features of this strain are high lipid productivity (~60% wt/wt of the dried biomass) and fast growth. Additionally, since *Fistulifera* sp. is a marine diatom, vast sea area and water is available for its cultivation. These beneficial features led us to carry out the through studies for this diatom with the aim of practical fuel production.

Establishment of the massive cultivation technique is one of the most important researches for the sustainable bioenergy production. To address this issue, we attempted bench-scale cultivation in both indoor/closed- and outdoor/open-type bioreactors. First, the theoretical maximum of the lipid productivity was evaluated using the indoor/closed photobioreactor with the optimized culture condition[2]. Then *Fistulifera* sp. was subjected to outdoor/open cultivation, by which the actual industrial production is supposed to be performed, to evaluate its energy profit ratio (EPR).

Another challenge is to understand the molecular underpinnings of such advantageous phenotypes so that we could further improve lipid productivity by means of metabolic engineering. Toward this, we conducted a whole genome analysis, revealing nuclear genome (49.7 Mbp), chloroplast genome (135 kbp)[3] and mitochondrial genome (>38.6 kbp). Based on this genetic information, the transformation technique for this strain was also established[4]. This technique was useful to identify the proteins associated with the lipid fraction[5], as well as to enhance the lipid productivity by modifying the metabolisms. We believe that these fundamental and applied studies will open doors for industrial biofuel production using this oleaginous diatom.

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[2] Satoh et al. (2013) *Bioresour Technol* 137: 132–138.
[3] Tanaka et al. (2011) *Photosynth Res* 109: 223–229.
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Tsukuba International Strategic Zone
Project3 - Practical Use of Algal Biomass Energy
つくば国際戦略総合特区
Project3 藻類バイオマスエネルギーの実用化

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藻類バイオマスの作出には潤沢な水資源とそれを利用可能な土地が必要である。日本は比較的淡水資源に恵まれ、灌漑設備の整った農地が全国に広がっている。しかし一方、耕作放棄地や東日本大震災の被災地など、農業利用が滞った土地も近年増加している。これらの土地は藻類の大量培養の候補地となり得るが、農地法の定めによりその転用は厳しく制限されている。そこでつくば市では国際戦略総合特区の制度を活用し、耕作放棄地を活用した藻類バイオマスの生産について、農地転用許可に係る特例措置を提案、平成 24-28 年度の 5 カ年にわたり実証実験を進めることとなった。

筑波大学の東に位置するつくば市栗原の耕作放棄地にボトリオコッカスのオープンポンド型培養設備を展開し、炭化水素であるボトリオコッセンを中心とした藻類オイルの生産を行う。ボトリオコッカスが生産する余剰多糖や、オイルを抽出した後の抽出残渣は、適宜処理して従属栄養性藻類であるオーランチオキトリウムの炭素源および窒素源として活用する。このオーランチオキトリウムからも炭化水素であるスクアレンが得られる。

現在は屋外の実証実験設備の建設が進められるとともに、筑波大学および連携機関において基礎研究が行われている。生産されたオイルに対する品質評価や、これを用いた自動車運用実証試験等も行われる。平成 27 年度以降はオイルの生産規模を拡大するほか、健康食品や医薬品等の高付加価値用途への展開も図り、産業としての競争力と持続可能性を高めてゆく。

http://www.tsukuba-sogotokku.jp/project3/

NET: Next-generation Energies for Tohoku Recovery
Task2 - R&D on Using Algae Biofuels
NET: 東北復興次世代エネルギー研究開発プロジェクト
課題 2 微細藻類のエネルギー利用に関する研究開発

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2011 年 3 月の東日本大震災により、東北地方沿岸部のインフラは甚大な被害を被った。仙台市宮城野区の南蒲生浄化センターも被災した施設の一つである。南蒲生浄化センターは仙台市の下水の 9 割を処理する施設であったが、津波によって水処理施設が壊滅的被害を受け、現在も復旧作業中である。処理機能は回復しきっておらず、暫定的に接触酸化法による中級処理が行われている。センターの復旧に際して、単なる浄化機能の回復に留まらず、藻類バイオマスの活用により下水処理プロセスのエネルギー効率を向上させ、エネルギーの生産すら可能にすることが、本研究開発計画の企図である。

下水処理は不溶有機物の物理的除去、溶存態有機物の生物的分解除去、窒素およびリンの回収など多段階の過程よりなる。これらの処理過程に対し、有機物を分解吸収する従属栄養性藻類のオーランチオキトリウムや、窒素・リンなど富栄養化をもたらす無機塩を吸収するボトリオコッカスなど、適切な藻類を組み込んでゆくことで、下水からのエネルギー調達を可能にする。既知のオイル産生藻類を活用する一方、新規に下水処理に適した藻類の探索および評価も進めている。また、余剰汚泥の燃焼熱や排出される CO_2 を藻類の培養に利用することで、下水処理-培養システム全体のエネルギー効率の向上を図る。

現在はセンター内に実験室を設置して、ラボスケールでの基礎研究を進めている。今後は屋外試験プラントを建設し、下水処理と藻類オイル生産との効率的な連携システムを開発する予定である。最終的には実規模プラント設計に資する基盤技術の創出を目指し、日本各地の下水処理施設への応用可能な技術を確立してゆく。

http://net-tohoku.sakura.ne.jp/wp/task2

Isolation and Characterization of Squalaene Epoxidase-like Genes from *Botryococcus braunii*, Race B.
Botryococcus braunii B 品種におけるスクアレン エポキシダーゼ様遺伝子の単離と機能解析

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多量のトリテルペン系炭化水素を合成し細胞外に蓄積する微細藻 *Botryococcus braunii* B 品種におけるスクアレンの代謝経路には、多くの生物と同様にスクアレンの 2,3-位をエポキシ化し、一次代謝産物のステロールへと変換する経路と、メチルスクアレンの 10,11-位をエポキシ化後、二次代謝産物として細胞外に分泌・蓄積する、本藻種に特異的な経路とが存在する。本研究では、本藻種のスクアレン 2,3-エポキシダーゼ様遺伝子のクローニングと機能解析を試みた。

本藻種の EST データベースからスクアレン 2,3-エポキシダーゼ様遺伝子を探索し、塩基配列のホモロジーがそれぞれ 60%以下である 3 つの contig を見出し、それらの遺伝子を *Botryococcus braunii squalene epoxidase-like genes 1,2,3* （*BbSEL1,2,3*）と名づけた。これまで、RT-PCR と 5'-,3'-RACE により、それぞれ 1.6 kb，0.4 kb，2.3 kb の cDNA 断片をクローニングしている。これらのうち、ORF 全長域を含むと考えられる *BbSEL1* の遺伝子機能を同定するため、ORF を含む断片を酵母発現ベクターpWV3 にクローニング、スクアレン 2,3-エポキシダーゼ遺伝子（*ERG1*）欠損酵母株へ導入、エルゴステロール要求性が相補されるかを解析した。ORF 領域とそれに隣接する 5'，3'非コード領域を含む断片を改変せずに導入した結果、エルゴステロール要求性の解除は見られなかった。既報（Suzuki et al. 2002）から、 2,3-エポキシダーゼの N 末端の疎水性領域を削除することにより、相補活性が上昇することが知られていたため、 BbSEL1 の N 末端疎水性領域を削除し、コドン頻度を酵母に至適化した cDNA ORF 配列を酵母細胞内で発現させたが、相補活性は検出できなかった。また、シロイヌナズナのゲノムにある 6 種類の 2,3-エポキシダーゼ様遺伝子（Rasbery et al. 2007）のアミノ酸配列の推定膜貫通領域を TMHMM Server により調べてみると 、酵母相補活性が検出される 3 種の配列には C 末端に顕著な膜貫通領域が存在し、相補活性が検出されない 3 種類にはその領域が見られなかった。さらに、BbSEL1 の C 末端には明瞭な膜貫通領域が検出されなかったため、この C 末端に異種生物 SE の C 末端疎水性領域配列を付加したキメラタンパク質と、BbSEL1 の C 末端配列を異種生物 SE の C 末端疎水性領域で置換したキメラタンパク質を酵母細胞で発現させてみた。その結果、これらの形質転換体においても、同様に相補活性が検出できなかった。そこで、現在、他のホモローグ *BbSEL3* を酵母内で発現するコンストラクトを作成している。なお、本研究は JST CREST により行われた。

Exploration of Cyanobacteria for Biomass Production and its Genome Analysis with Next Generation Sequencers

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Recently, microalgae have attracted much attention for their potential of biomass production. There are two major strategies to produce the algal biomass. One is to utilize eukaryotic algae that produce oil in nature (e. g. *Botryococcus braunii* or *Pseudococcomyxa ellipsoidea*), and the other is to make cyanobacteria (e.g. *Synechocystis* sp PCC 6803 or *Synechococcus* sp. PCC 7942) to produce biomass by genetic engineering. The former strategy has an advantage in large-scale biomass production, while the latter has an advantage in selectiveness of the products. For the latter strategy, the most important point to select cyanobacteria species that possess high photosynthetic activity, high carbon fixation activity, long lifetime in same medium, and tolerance to other contaminated bacteria. Model cyanobacteria species do not have all capacities mentioned above and therefore are hard for practical biomass production, especially in the large scale. To solve these difficulties, we are now exploring new cyanobacteria species that live various environments in Japan. We are also set up a method of DNA extraction and a pipeline of genome analysis using three next generation sequencers, illumina's MiSeq , Roche's 454 FLX+, and Life Tech's Ion proton.

Molecular Breeding in Green Alga *Pseudochoricystis* sp.

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Pseudochoricystis ellipsoidea is a unicellular green alga that accumulates a large amount of triacylglycerols within cells. This strain could be cultivated in open ponds without the interference by other algae and protozoa under acidic conditions. In addition, the total lipid content in this alga increased more than 10 times under nitrogen-starvation conditions. Thus, *P. ellipsoidea* could be a potential source of renewable diesel fuel. However, the total cost for biofuel production with this alga is still too high with current technologies; and the effort for reduction of process costs through the improvement of algal oil productivity is necessary.

To date, we isolated various mutants of *P. ellipsoidea* exhibiting beneficial phenotypes including low chlorophyll contents, increased oil productivity, and fragile cell wall. If a single strain of *P. ellipsoidea* can gain all of these useful traits, the cost of diesel fuel production would be reduced more than ever with that strain.

For the construction of such a strain, the development of recombinant DNA technology applicable to *P. ellipsoidea* is prerequisite as sexual reproduction of this strain is not known. In one of our efforts, we aim at to develop a gene knockout technique. We selected the tanscription activator-like effector nuclease (TALEN) method which is capable of cleaving a specific sequence within a genome of any organisms. TALEN consists of a transcription activator-like (TAL) effector domain originally found in the plant pathogen, *Xanthomonas* spp., and the FokI nuclease. TAL effector domain recognizes and binds to a specific DNA sequence while the FokI nuclease cleaves DNA in the vicinity of the binding site.

By applying the TALEN method, we isolated uracil auxotrophs of *P. ellipsoidea* at a frequency much higher than the spontaneous mutation frequency. Next, we tried to knock out the chlorophllide *a* oxygenase (CAO) gene involved in the chlorophyll *b* biosynthesis. Based on the *CAO* gene sequence in *P. ellipsoidea*, we designed a custom-made TALENs for the inactivation of the gene. After introduction of the TALEN plasmids into *P. ellipsoidea* cells by using particle bombardment, we isolated several mutants synthesizing lower amount of chlorophyll than the wild-type strain. It is known that the defect in *CAO* causes the reduction in antenna chlorophyll content; and these low chlorophyll mutants would well be defective in the *CAO* gene. Currently, we are sequencing the *CAO* gene region in these low chlorophyll mutants.

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Structure of the *RbcS* Gene Encoding the Small Subunit of Ribulose-1,5-bisphosphate Carboxylase/Oxigenase and its Unusual Transcript in *Pseudococcomyxa ellipsoidea* Strain Obi

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Ribulose-1,5-bisphosphate carboxylase/oxigenase (RuBisCO) is an enzyme involved in carbon fixation. It is probably the most abundant protein on Earth. In *Pseudococcomyxa ellipsoidea*, oil-producing unicellular algae, RuBisCO consists of two types of protein, called the large-chain (RbcL) and the small-chain (RbcS), like higher plants and green algae. The large-chain genes are coded in the chloroplast, while the small-chain genes are coded in the nucleus. Previously, whole transcriptome shotgun sequence (RNA-seq) was applied to the *P. ellipsoidea* strain obi, and it revealed that the transcription level of rbcS gene was highest among of all. To evaluate of the transcription initiation sites, the RNA-seq data of 5' region of *rbcS* mRNA were analyzed. The result indicated that almost 50 % was transcribed from an upstream promoter. The rest had an unusual structure, their 5' region was substituted by the antisense sequence of *rbcS* mRNA. The PCR primers to amplify such chimeric regions were designed and amplified using three independent cDNA libraries as templates. The sequences of resulting PCR products were identical with those of RNA-seq data. The results showed that the 5' antisense region was not an artifact produced during the RNA-seq process, and suggested that unknown mechanisms of *rbcS* transcription will exist in *P. ellipsoidea* strain obi.

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Towards the Construction of Self-cloning System in *Pseudochoricystis ellipsoidea*

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For industrial applications of *Pseudochoricystis ellipsoidea* (tentative scientific name), it must be cultivated outdoor. Thus, the use of genetically engineered *P. ellipsoidea* strains in such cultures will be subject to governmental regulations. On the other hand, the cloning of a gene derived from the same species is called self-cloning, and this cloning procedure is subject to less stringent government restrictions in some countries including Japan, as a gene cloned into the same species is considered to be less hazardous than that cloned in another species. Therefore, we are developing a self-cloning-based positive selection system to be used for the breeding of *P. ellipsoidea* in industrial applications

First, we isolated a uracil auxotroph of *P. ellipsoide*a as the host for self-cloning by the following procedures. UV-irradiated *P. ellipsoidea* cells were spread on a medium containing uracil and 5-fluoroorotic acid (5-FOA). 5-FOA resistance generally selects for mutants deficient in uridine monophosphate synthase (UMPS). As expected, all the 5-FOA-resistant mutants were uracil auxotrophs. We also analyzed the genome of one uracil auxotroph mutant and confirmed that the gene for UMPS of the mutant contained a small deletion in it.

Next, we isolated a cDNA of UMPS from the wild-type strain of *P. ellipsoidea.* This cDNA was sandwiched between the actin promoter and tubulin terminator both derived from *P. ellipsoidea*. We introduced this plasmid to the above mutant using a particle gun, and selected for prototrophic transformants. We are still unsuccessful to isolate prototrophic transformants. Possible reasons for the failure are currently investigated.

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Improved Method for the Preparation of Spheroplasts from *Pseudochoricystis ellipsoidea*

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Pseudochoricystis ellipsoidea (tentative scientific name) is a unicellular green alga which accumulates neutral lipid under the stress of nitrogen deficiency. Currently, the research that attempts to convert this neutral lipid into biofuel is progressing. However, the commercialization of biofuels is subject to the production cost.

The molecular breeding of strains suitable for biofuel production may be effective in cost reduction; however, the efficiency of the genetic transformation of *P. ellipsoidea* was low with a DNA particle bombardment method most probably due to the existence of a rigid cell wall. In this study, we developed methods for the preparation of spheroplasts from *P. ellipsoidea* cells.

Driselase is a cell-wall-digesting enzyme. The spheroplast formation efficiency with this enzyme was about 10% at a concentration of 1 % (w/v). The efficiency was not increased even if the Driselase concentration was raised to 10% (w/v) suggesting that only 10% of cells were susceptible to the attack by Driselase.

We hypothesized that cells in a specific cell cycle phase become susceptible to Driselase. In order to test this hypothesis, we cultivated *P. ellipsoidea* under light-dark cycle (12h:12h) to synchronize cell populations. After fifth cycle of the light-dark regime, cells under light phase were sampled 0.5, 3, 6, 9 and 12 h after the onset of light, and the susceptibility of the cells to Driselase was examined. It was found that the efficiency of spheroplast formation with Driselase was higher in cells cultivated under light for 0.5 to 6 h than cells in other phases.

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Exploration of Regulatory Factors Responding to Nitrogen-deficiency in *Pseudochoricystis ellipsoidea*

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To realize commercial biodiesel production from microalgae, isolation and breeding of microalgae are required. *Pseudochoricystis ellipsoidea* (tentative scientific name) is a unicellular alga that intracellularly accumulates lipids under stressed conditions such as nitrogen deficiency. However, the transduction pathway for the nitrogen-deficiency signal leading to the lipid accumulation in this alga is not clarified yet. In this work, we aimed at to identify signaling factors for the transduction of the nitrogen-deficiency signal, since such information will provide a basis for developing strategies for genetic improvement of this alga.

As the first step towards the goal, we explored transcription factor genes in the genome of *P. ellipsoidea* by using a tblastn search with a comprehensive database of transcriptional factor motifs as a query. The expression profiles of the candidate genes for transcription factors thus detected were then examined using RNA seq data of *P. ellipsoidea* cells incubated under nitrogen-deficiency conditions.

Among 41 genes responding to nitrogen deficiency, we selected the genes for two transcription factors, NIT2-like gene which is a central regulatory gene required for nitrate signaling in many organisms, and AP2B, a subunit of a dominant negative transcription factor, for further studies as both genes were strongly induced under nitrogen deficiency conditions. In addition, during the examination of the expression profiles of many genes, we also discovered that a gene encoding a PP2C- (protein phosphatase 2C) family protein which may be involved in the signal transduction in stress responses, was also induced under nitrogen deficiency conditions.

Thus, we examined these three genes in more detail. The induction of these genes under nitrogen deficiency conditions was confirmed by RealTime PCR. Currently, we were successful to clone cDNAs of the genes between appropriate promoter and terminator sequences. We will introduce these genes in *P. ellipsoidea*, and examine the effect of the constitutive expression of these genes on the intracellular accumulation of lipids in this organism.

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Reporter Genes Useful in Unicellular Green Alga *Pseudochoricystis ellipsoidea*

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Microalgae have many advantages in promising application for sustainable biofuel production due to their high growth rate and energy productivity. A unicellular green alga, *Pseudochoricystis ellipsoidea* (tentative scientific name), is one of useful organisms for biofuel production because it can accumulate large amount of lipids in the cells under nitrogen deprivation conditions. Genetic approaches became available to this alga after the development of a DNA transformation method by particle bombardment. To develop further the genetic engineering technology applicable to this organism, we focus on the improvement of the stability of transgene expression.

As the first step towards the goal, we are developing methods to monitor the transgene expression. We constructed several plasmids in which codon-optimized green fluorescent protein gene (*PeEGFP*) is flanked by native promoters and terminators. After co-transformation with marker plasmids harboring G418-resistant gene (*nptII*), *PeEGFP*-transformed cells were screened among G418-resistant cells by PCR amplification using primers specific to the *PeEGFP* gene. Then, the expression of PeEGFP was monitored using the fluorescent activated cell sorting (FACS) system. The signals derived from PeEGFP-expressing cells were clearly separated from that of wild-type cells, indicating that transgene expression was successfully monitored in living *P. ellipsoidea* cells. Based on this technique, we are now trying to isolate strong and constitutive promoter/terminator sets.

Other approached to monitor transgene expression will also be discussed.

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Transcriptome Analysis of Lipid Metabolic Genes in a Unicellular Green Alga *Pseudochoricystis ellipsoidea*

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High lipid productivity is one of the essential characteristics of an alga for commercial production of biodiesel. It is known that algae accumulate starch and TAG (triacylglycerol) in response to environmental stresses. In fact, we have found that lipid is accumulated in cells of *Pseudochoricystis ellipsoidea* (tentative scientific name) under nitrogen deficiency or under high salinity.

We expect that clues to enhance the lipid productivity of this alga would be provided by elucidation of mechanisms underlying the regulation of lipid metabolisms. As the first step to resolve the mechanisms, we acquired amino acid sequences of lipid metabolic enzymes in the model plant *Arabidopsis thaliana* and *Chlamydomonas reinhardtii.* Using these sequences as queries, we used the Basic Local Alignment Search Tool (BLAST) to find key enzymes of lipid metabolism in the genome of *P. ellipsoidea*. Then, we examined, from RNAseq data, the expression profiles of the genes for lipid metabolic enzymes in *P. ellipsoidea*.

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Microalgal Cultivation by Sewage and Evaluation as Fuel

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Outdoor microalgal cultivation was carried out using sewage influent or effluent at an actual sewage treatment plant. The microalgae could be cultured by supplying only sewage influent or effluent without additional microalgal stock or nutrient salts. Adding air and CO_2 to the culture water promoted the consumption of nitrogen in addition to the cultivation of green algae. Increasing the hydraulic retention time from 4 days to 10 days also promoted consumption and cultivation. In particular, by adding CO_2 to the sewage influent, the cell number of specific *Scenedesmus* species useful as components for fuel, increased 59-fold. The microalgae culture advanced even in Japan autumn/winter. The higher heating values of microalgae ranged from 18 to 20 MJ kg^{-1}. There was no large difference in higher heating values between the small-sized floating microalgae and the large-sized settled microalgae. The major organic components eluted by *n*-hexane were fatty acids such as palmitic and 11-hexadecenoic acid. The ratio of unsaturated fatty acids exceeded that of saturated fatty acids, which was possibly due to the fluidity of the cell membrane. The possible application of sewage culture microalgae to biofuel was demonstrated.

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