NEDO SMARTCELL PROJECT

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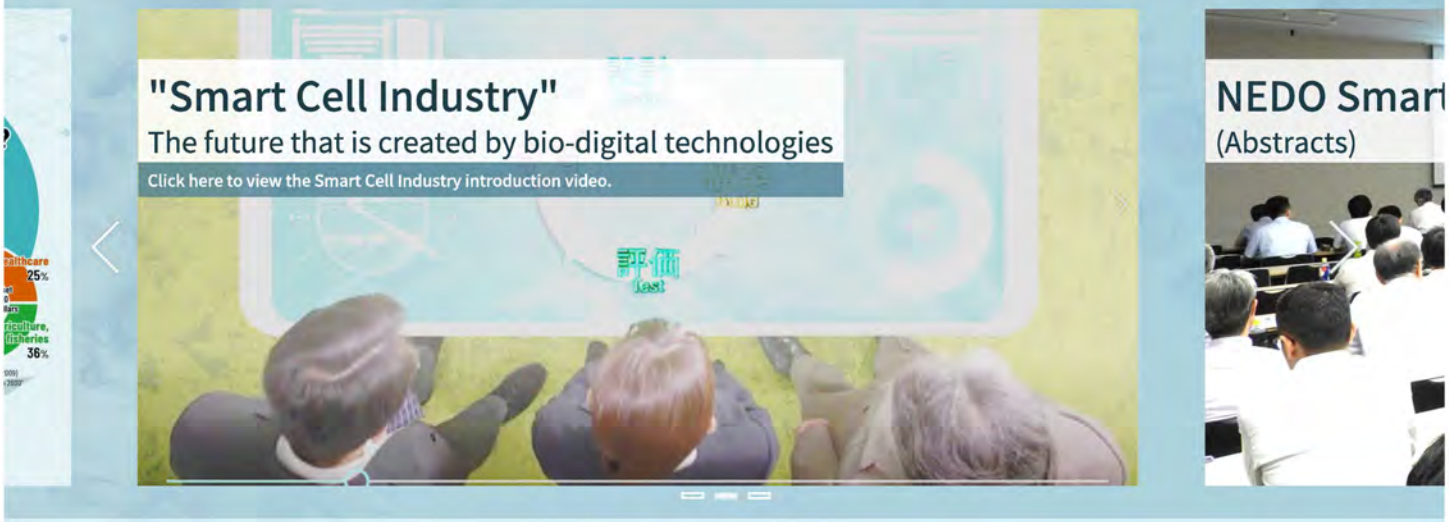

INFORMATION

October 27, 2020
The Smartcell Project Achievements Brochure was published.

October 27, 2020
Focus NEDO No. 78 was published. Feature 2 covers eco-friendly smart cell technologies.

October 01, 2020
At the NEDO booth at BioJapan 2020, we will present the latest achievements of the Smartcell Project.

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Introduction Video

The Smartcell Project Introduction Video

Japan Bioindustry Association
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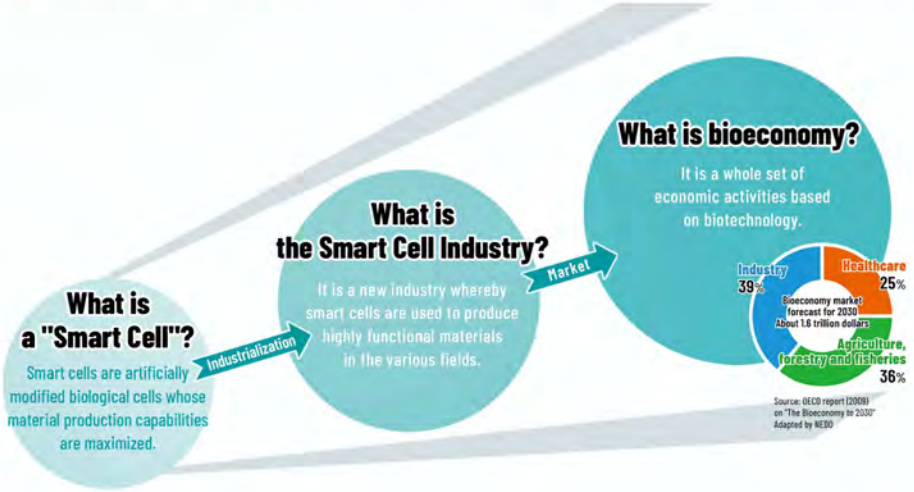
Smartcell Project

About the Smartcell Project

Biotechnology has remarkably evolved and developed by being fused with cutting-edge digital technologies, such as IT and artificial intelligence (AI). Along with this, material production technology using living organisms, such as plants and microorganisms, is drawing attention, and is expected to lead to a rapid expansion of related markets around the world in the future. In line with this trend, efforts are currently underway to capture the market, particularly in Europe and the United States. In order to ensure Japan's competitiveness, it is necessary to establish Japan's unique technologies that fuse rational genetic design by utilizing information technology and large-scale genetic recombination.



For 5 years from 2016 to 2020, NEDO has engaged in a project, which is called "Development of Highly Functional Materials Production Technologies Using Plants and Other Organisms." We are conducting research and development of a system for obtaining complex, large-scale bioinformation that is required for genetic design in a high-throughput manner, intracellular process design, domestic genome editing technologies, and technologies for controlling plant breeding and growing. By controlling and modifying the material producing functions of living organisms, such as microorganisms and plants, utilizing these technologies, we aim to commercialize the "Smart Cell Industry" that produces highly functional materials at low cost in an energy-efficient manner.

NEDOプロジェクトの概念図

Smartcell Project

Project Overview of the NEDO Project "Development of Highly Functional Materials Production Technologies Using Plants and Other Organisms."
https://www.nedo.go.jp/activities/ZZJP_100118.html

Focus NEDO No. 70. Smart Cell Industry Opens the Way for the Future by Bio-Digital Technologies: Challenges of Open Innovation!
https://www.nedo.go.jp/library/ZZ_focus_70_index.html

FocusNEDO 第78号が発行されました。「特集２」で環境にやさしいスマートセル技術が掲載されています。
https://www.nedo.go.jp/library/ZZ_focus_78_index.html

スマートセル・プロジェクト成果集パンフレットが発行されました。
https://www.nedo.go.jp/library/pamphlets/ZZ_pamphlets_00054.html

Nature誌（Volume 584, Issue 7819, 6 August 2020）に掲載された特集の別刷り版ができました。
Focal Point – Synthetic Biology in Japan　日本語版　(PDF 4.2MB)　／　英語版　(PDF 2.7MB)

Microorganisms

Abstracts of the NEDO Smart Cell Project Technology Seminar, "Building and Demonstration Testing of Platforms Creating Smart Cells"
https://www.jba.or.jp/activity/adv_biotech/rd_project/2220/

(一財)バイオインダストリー協会の機関誌「バイオサイエンスとインダストリー」に連載した"高生産性微生物創製技術とその応用例"を1冊にまとめました。
https://www.jba.or.jp/nedo_smartcell/outreach_bandi/HTML5/pc.html#/page/1

Genome editing

Genome Editing Industrialization Network
http://www.mls.sci.hiroshima-u.ac.jp/smg/GEIN/index.html

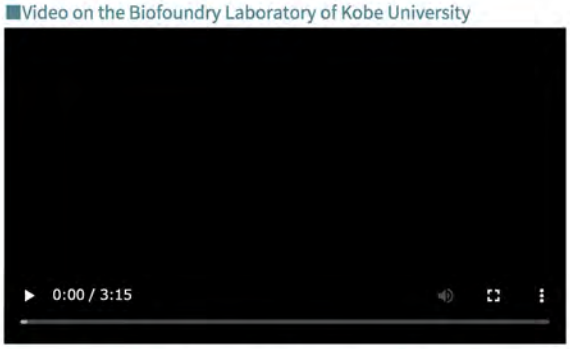
Other relevant sites

Materials Technology and Nanotechnology Department of NEDO
https://www.nedo.go.jp/activities/introduction4.html

Japan Bioindustry Association
https://www.jba.or.jp/

Video on the Related Institutions

■Video on the Biofoundry Laboratory of Kobe University



微生物育種の効率化、スマートセルの高速創出に資する要素技術を集積したバイオファウンドリー研究室を、神戸大学統合拠点に整備しました。（2020年10月）

Last updated：December 25, 2023

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Project Purpose

What is the NEDO "Smartcell Project"? One answer for maintaining the global environment and a sustainable economy

1. Smart cell? What is a "smart cell"? In what way is it smart?

Microorganisms have the ability to produce various materials and products that are useful for people's lives, for example, seasonings, such as amino acids and nucleic acids, alcoholic beverages such as Japanese sake, enzymes used in food processing, and raw materials of pharmaceutical products. Over time, humans have gradually improved the capabilities of microorganisms.

Smart cells are microorganisms whose material production capabilities have been artificially enhanced. State-of-the-art technologies are enabling the modification of microorganisms to produce materials that they originally cannot produce.

For example, we ingest a substance called eicosapentaenoic acid (EPA) to improve blood circulation by smoothing blood. The EPA is a fatty acid mainly extracted from the blue-skinned fish, such as sardines, mackerel, and saurel. However, at present, there is serious concern about the depletion of resources of blue-skinned fish, which is the raw material for the production of EPA, and securing new raw materials has become an issue.

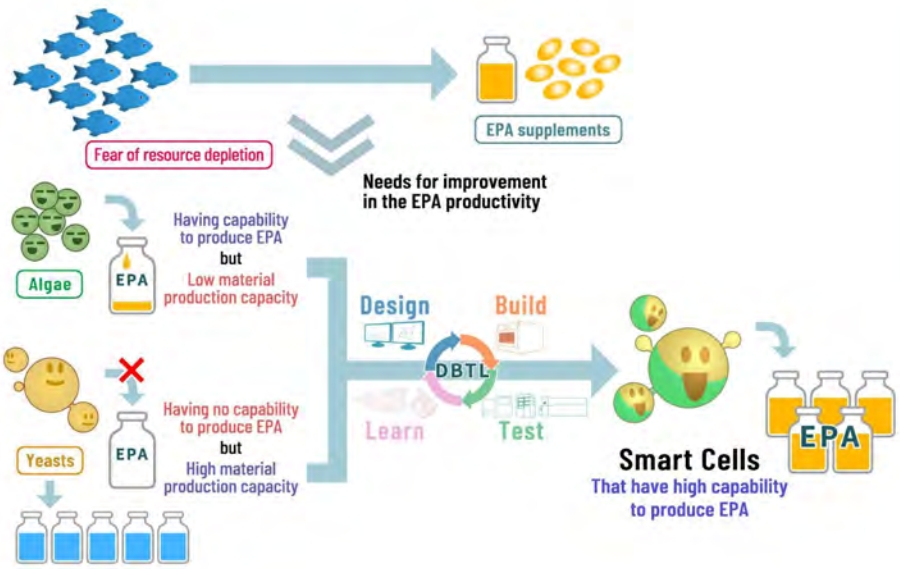
On the other hand, in addition to fish, algae also produce EPA. However, the efficiency of EPA production by algae is low. Therefore, if microorganisms such as yeasts (e.g., oleaginous yeasts) become able to mass-produce EPA utilizing the algae's function to synthesize EPA by artificial methods, this problem will be resolved. This allows us to mass-produce EPA without consuming the natural resources, such as sardines and mackerel.

In such a situation, the "smart cell" enters the scene. A "smart cell," meaning an "intelligent cell," is finely designed to induce biological functions that have not been used. For example, it refers to a cell rationally designed to enhance its function to produce a target material. Furthermore, industrialization will be realized by turning each cell into a material production factory.

Thus, "smart cells" have recently been expected worldwide as an excellent means to solve various problems facing the current industry. The expectation is about to become a reality by accumulating concrete cases as a result of the technological development by the "Smartcell Project."

2. We have built platforms for creating smart cells.

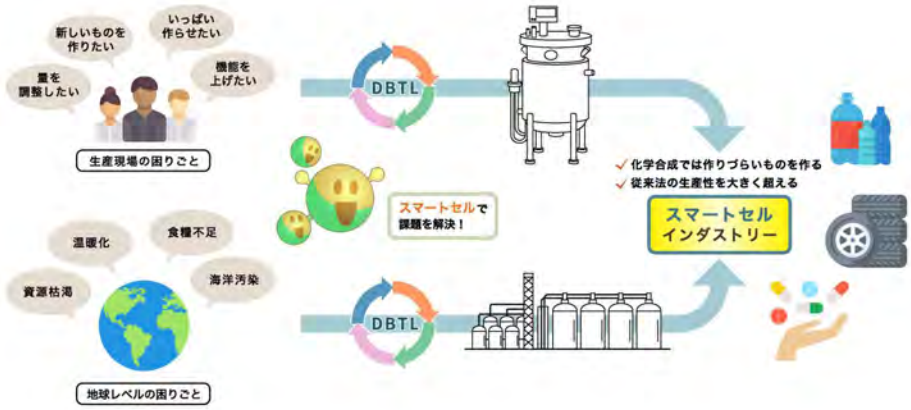
One of the achievements of the Smartcell Project is building platforms to efficiently advance a series of research and development processes to create a "smart cell."



In the smart cell project, the process of creating a smart cell is classified into 4 steps, namely, "Design" → "Build" → "Test" → "Learn." After developing technologies required for each step, we have built platforms, the "Platforms Creating Smart Cells," that integrates those technologies.

By utilizing the Platforms Creating Smart Cells, which is the achievement of our research through the project over the past 5 years, new companies and researchers who wish to enter this biological production business area will realize the commercialization in a shorter period of time than before.

3. Utilize the Platforms Creating Smart Cells.



While the modernization brought by petroleum-based industries has played a significant role, it has raised a variety of global environmental issues. The Smart Cell Industry is also expected as a next-generation industry that can solve these problems.

Even from the standpoint of the problem of plastics that pollute the world's oceans, the industrialization of biodegradable plastics that are not derived from fossil resources is an urgent challenge. Against the use of petroleum resources that accelerate global warming and environmental pollution, in exchange for industrial development, the Smart Cell Industry, which focuses on the active utilization of biological resources and functions, will support humans in the 21st century in the literal sense.

In this way, the door of the Smart Cell Industry is beginning to open. If you would like to join the members of the Smart Cell Industry, please contact the Smart Cell liaison! ⇒ Click here

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Project Summary

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The United Nations have set Sustainable Development Goals (SDGs) to realize a sustainable society. The international community has started to introduce the "bioeconomy," which aims to reconcile global-scale issues with economic development by using biological resources and biotechnology. For example, strategic efforts are being made at the national level to make effective use of renewable resources, such as biomass, and to produce functional materials and commodity chemicals that enrich people's lives through environmentally friendly bioprocesses.

In recent years, technological innovations have occurred in DNA sequencing, bioinformatics, genome synthesis, genome editing, etc., and it has become possible to extract potential biological functions that have not been available until now. Furthermore, automation and IoT by utilizing big data and robot technology are supporting the rapid development of the bioindustry.[1,2]。

In Japan, the NEDO Smartcell Project (hereinafter referred to as "this project") has been conducted since 2016, and bio-digital integration is being promoted. By defining smart cells as "biological cells whose material production capabilities are artificially maximized and optimized," basic technologies for bioinformatics, which design blueprints of smart cells, and for biotechnology, which constructs the designed cells, have been developed. For example, the developments of technologies, such as an information analysis technology for designing metabolic pathways and analyzing gene expression control networks on a computer, a long-chain DNA synthesis technology, a high-throughput material productivity evaluation technology, and a high-throughput omics analysis technology, are currently underway. These technologies have been verified to enable the creation and development of functional materials using microorganisms in a short time, at a low cost, with high performance, never before possible.

Conventionally, materials produced by microorganisms, such as amino acids, nucleic acids, organic acids, alcohols, gases, lipids, vitamins, and antibiotics have been used in a wide range of industries, such as foods, pharmaceuticals, enzymes, chemicals, and energy.

In this project, we aim to accelerate the solving of conventional issues, such as the production of new compounds that could not be produced using conventional microorganisms and the enhancement of microbial productivity, by building the "Platforms Creating Smart Cells" that create cells with highly enhanced material productivity (smart cells), using an approach combining information science and synthetic biology.

The Design-Build-Test-Learn (DBTL) cycle shown in Fig. 1 is adopted as the basic concept of the platforms. In the "Design" domain, a smart cell design system is being developed that incorporates an information analysis system for the purpose of metabolic pathway design, enzyme selection and modification, and gene expression control. In the "Build" domain, techniques are being developed, such as long-chain DNA synthesis and high-throughput construction of DNA modified microorganisms, in order to realize the production of designed smart cells. In the "Test" domain, the constructed microorganisms are subject to a productivity analysis and various omics analyses. The obtained data are used for feature value extraction, which is a technology used in the "Learn" domain, and the information is used again in the "Design." By running this DBTL cycle, the microbial breeding process is streamlined and smart cells are being created.

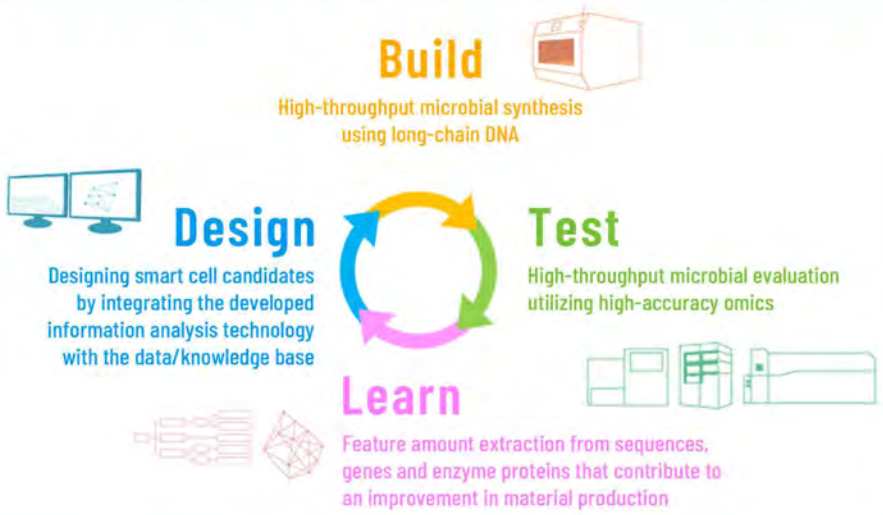

Figure 1. Overview of the DBTL cycle

The platforms are expanding the scope of its application not only to conventional host microorganisms, such as yeasts and Escherichia coli, but also to other industrial microorganisms. While each of these basic technologies is individually developed, verification tasks that are actually applied to specific materials targeted by companies or other parties are executed, in order to build the Platforms Creating Smart Cells incorporating a consistent DBTL cycle. By feeding back the verification results and various data, we are contributing to the sophistication of the platforms, and also developing a practical technology while demonstrating its effectiveness.

The next-generation industry that produces highly functional bioproducts using smart cells is called the "Smart Cell Industry." It is expected to expand into various fields, such as industrial, agricultural, and medical/healthcare fields, in the future.

⇒Click here for DL domain.

⇒Click here for BT domain.

Framework of the Project

This project consists of the following research and development theme.

(1) Development of high-throughput synthesis, analysis, and evaluation methods

By upgrading the speed of long-chain DNA synthesis, which is the unique technology of Japan, to ultra-high-speed, this theme aims to develop a technology for building microorganisms with high diversity in a short time, and an analysis/evaluation technology that allows productivity data and omics data necessary for information analysis to be obtained in a highly accurate and high-throughput manner.

(2) Development of highly productive microorganism design system

Based on the data obtained, this theme aims to develop a "highly productive microorganism design system," which is a system for designing gene sequences that lead to the realization of the production cell models. This system consists of a metabolism model, a gene expression control model, and an integrated model (these are collectively called "production cell models"), which are designed to dramatically increase productivity to produce useful materials and to overwhelmingly increase feasibility.

(3) Verification of effectiveness of information analysis system that contributes to the creation of highly productive microorganisms

This theme aims to expand the applicability of the information analysis system not only to conventional host microorganisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, but also to industrial microorganisms, in order to verify its effectiveness using specific production materials targeted by private companies and develop technologies for practical use. Moreover, this theme also aims to create a new industrial form in the bio-based material production using microorganisms, by building a foundry (biofoundry) that incorporates basic technologies.

参考文献

1）スマートセルインダストリー -微生物細胞を用いた物質生産の展望-（バイオテクノロジーシリーズ）, シーエムシー出版 (2018)

2）Hillson, N. *et al.*, Nature Communications., 10, 2040 (2019)

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Project Summary

THEME

Summary of Design-Learn (DL) Domain

Sachiyo ABURATANI
Deputy Director
Computational Bio Big-Data Open Innovation Laboratory,
National Institute of Advanced Industrial Science and Technology (AIST)

What We Can Do in This Domain

The DBTL cycle in this project means Design (cell design) → Build (host construction) → Test (productivity evaluation) → Learn (learning of results). Of these, the Design (cell design) and Learn (learning of results) steps utilize information analysis technologies.

DLAbout This Domain

In the field of business, a method called the PDCA cycle is becoming widely used. The PDCA cycle is a strategic method for continuously improving various operations in business management, by repeatedly running the cycle of Plan (planning) → Do (execution) → Check (evaluation) → Action (improvement). This cyclic improvement method is also being developed as a DBTL cycle in this project, which aims at efficient material production by microorganisms. Of the DBTL cycle in this project [Design (cell design) → Build (host construction) → Test (productivity evaluation) → Learn (learning of results)], the Design (cell design) and Learn (learning of results) steps use information analysis technologies.

Japan has a long history in the field of fermentation and production, and has been ahead of other countries in breeding and modification technology for host microorganisms, etc. However, the field of "bio-based production" using synthetic biology lags behind Europe and the United States. The "bio-based production" conducted in Europe and the United States consists of the acquisition of large-scale data using robotics, the identification of candidate genes for modification using AI technology, such as machine learning and deep learning, and more efficient construction of material production strains through the genetic modification of host cells. In this project, while incorporating the advantages of these concepts but not just simply following other countries' lead, we are developing a smart cell design system as a new information analysis technology to strengthen global competitiveness and activate the "manufacturing industry" in Japan.

The foundation for the information science technology used in host cell modifications, led by Europe and the United States, is machine learning. The advantages of this technology are that a large amount of data (tens of thousands to hundreds of thousands of samples) enables highly accurate rule extraction, and that the accuracy improves as the number of data increases over time. On the other hand, in order to obtain a certain level of accuracy, data as large as about ten-thousands of samples are required for each host cell and production material, raising concern about a greater economic burden. At present, to compete internationally on an equal footing with these leading countries in this field where Japan has fallen behind, it is essential for Japan to overcome the two disadvantages of using machine learning only, namely, "mass data" and "host-dependence." Therefore, in this project, to construct "smart cells, i.e., living cells having highly optimized and artificially designed capabilities to produce highly functional materials," a smart cell design system has been developed, which can be used with [1] a realistic number of data samples (a minimum of about 100 samples), [2] more accurately, and [3] host-independently, by integrating Japan's unique information analysis technologies and superior bioinformatic technologies.

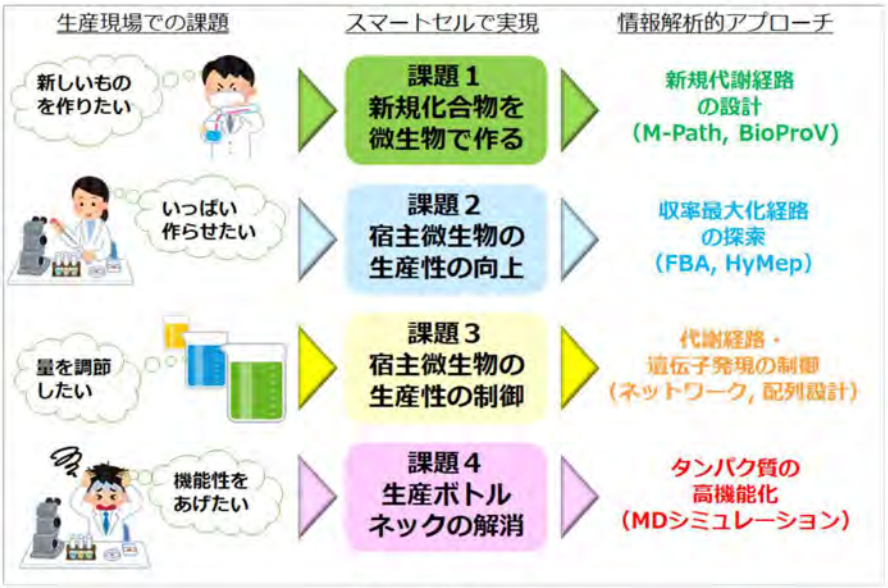

図1. 現場課題を情報解析へ

Figure 1 shows the relationship between challenges occurring at the production site and information analysis-based solutions and approaches in the development of this system. Each challenge occurring at the production site is listed on the left. First, in order to realize these challenges with a new concept of living cells called smart cells, the challenges were roughly classified into 4 groups. To overcome these 4 challenges, we are developing the following information analysis technologies.

≫Technology of Knowledge Extraction and Learning from Literature, etc.（Proposing Enzymes Utilizing Machine Learning・Knowledge Base Supporting Smart Cell Designing）
≫Metabolic Design and Optimization Technology
≫Gene Regulatory Network Modeling Technology
≫Gene Sequence Design Technology
≫Enzyme Modification Design Technology

The above information analysis technologies, the theoretical basis for which has already been published by the project participants, have been established as a theoretical system (References 1, 2, 3, and 4). In this project, whose objective is to put into practical use such various, theoretically backed up technologies, we are collaborating with a task demonstration group that has various challenges at the production site. By improving and modifying our developed theoretical base for designing smart cells in each demonstration task and by feeding back the obtained results to the material production site, we have been developing information analysis technologies that can be used more practically.

Finally, Fig. 2 shows the overall configuration of the smart cell design system being developed in this project. This system is structured by setting the above-mentioned database, which has been constructed in this project as the base of the various information analysis technologies, as the hub. In this system, respective information analysis technologies can be applied to data stored in the database. The database, located at the core of the system, stores data for learning, including various data measured in the project and data required to design smart cells obtained from existing databases. By using these publicly known data and our original data in combination, the information analysis technologies listed in the middle circle are applied. By applying the information analysis technologies, various "models" listed in the outer circle are constructed. The "model" derived here means a schematic diagram of a system that simply shows living activities expressed by graphs, pathways, or simulations that represent the functions of living cells. By interpreting the various models constructed in this way, the system has realized the provision of proposals on candidate genes for modification that could not be predicted in the conventional breeding methods, the design of gene sequences to be introduced to host cells, and the provision of proposals on novel biosynthetic pathways that living cells did not originally have.

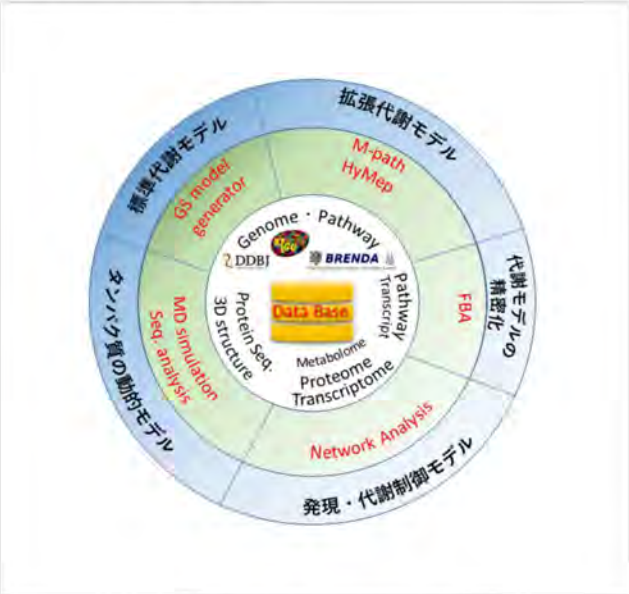

図2．スマートセル設計システム全体像

References

1)Araki, M., *et al.*, Bioinformatics, 31(6), 905-911, 2015

2)Shirai, T., *et al.*,Microbial Cell Factories, 15(13), 1-6, 2016

3)Aburatani, S., Gene regulation and systems biology, 5, 75-88, 2011

4)Kameda, T., *et al.*,Proc. Natl. Acad. Sci., 103 (47), 17765-17770, 2006

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Project Summary

THEME

Build-Test (BT) Domain

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Director and Professor
Engineering Biology Research Center, Kobe University

What We Can Do in This Domain

In basic technologies for the Build and Test steps of the DBTL cycle, automation technology has made remarkable progress in recent years. In conventional microbial breeding, after DNA to be introduced into microorganisms is designed and constructing the designed DNA, the processes of plasmid construction, transformation (genetic recombination), examination of culture conditions, and productivity evaluation are performed manually. Currently, on the other hand, robots have been developed that semi-automatically perform the processes from plasmid construction to simple productivity evaluation.

BTAbout This Domain

In general, in microbial breeding using genetic recombination, it is necessary to enhance or reduce the expression level of a specific gene to a specific level at a specific time. To achieve this, it is necessary to have at hand molecules involved in the gene expression regulation as available parts. These parts include promoters, repressors, RBS, and terminators, and are collectively called "DNA parts." In the current molecular biological knowledge, DNA parts are not clearly specified, and the expression level of genes varies depending on their combinations. Therefore, trial and error through experiments is required. Under these circumstances, business ventures in the United States and other countries have already created a lab automation system that automatically performs bio-operations using catalogued DNA parts and robotics.

In the NEDO Smartcell Project, we have been developing our original high-throughput technologies, such as high-throughput long-chain DNA synthesis technology with the highest DNA integration accuracy in the world, a technology for constructing diverse microorganisms in a short time using long-chain DNA, and a technology for obtaining productivity data and omics data with high accuracy and high throughput. In particular, we are working on the following research and development themes.

Build steps:

1. Development of a long-chain DNA synthesis and analysis technology
2. Development of a high-throughput microbial construction and evaluation technology
3. Construction of a platform for searching for compound efflux transporters

Test steps

4. Development of metabolite sensors
5. Development of an autofluorescence microscope
6. Development of a transcriptome analysis technology
7. Development of a technology for high-accuracy, quantitative, targeted proteomic analysis
8. Development of a metabolomic analysis technology
9. Networking of the evaluation system

Long-chain DNA is extremely useful for high-speed breeding of microorganisms because it can enhance and reduce the expression levels of many genes by one recombination operation. It also leads to the realization of new metabolic pathways designed by information analysis in a short period of time. The main achievements so far are as follows.

- Establishment of a technology to synthesize long-chain DNA of more than 30 kb in size accurately (mutation rate 0.1% or less) at a low cost (5 yen per base) in a period one-quarter that conventionally taken (about 2 weeks).
- Establishment of a semi-automatic high-throughput transformation technology using a 96-well plate format
- Development of a platform for searching for compound efflux transporters
- Development of a technology for high-throughput evaluation of the target material productivity by image analysis, etc.
- Development of a microbial proteomic analysis technology capable of quantitating proteins
- Construction of a high-throughput, high-accuracy, comprehensive metabolomic analysis system through the development of preprocessing robots, etc.

In this project, we are working on the incorporation of basic technologies into the Platforms Creating Smart Cells (Fig. 1). In addition, we are systematically obtaining and managing data by further improving the accuracy of our original high-throughput evaluation technology and strengthening the coordination with information analysis technology.

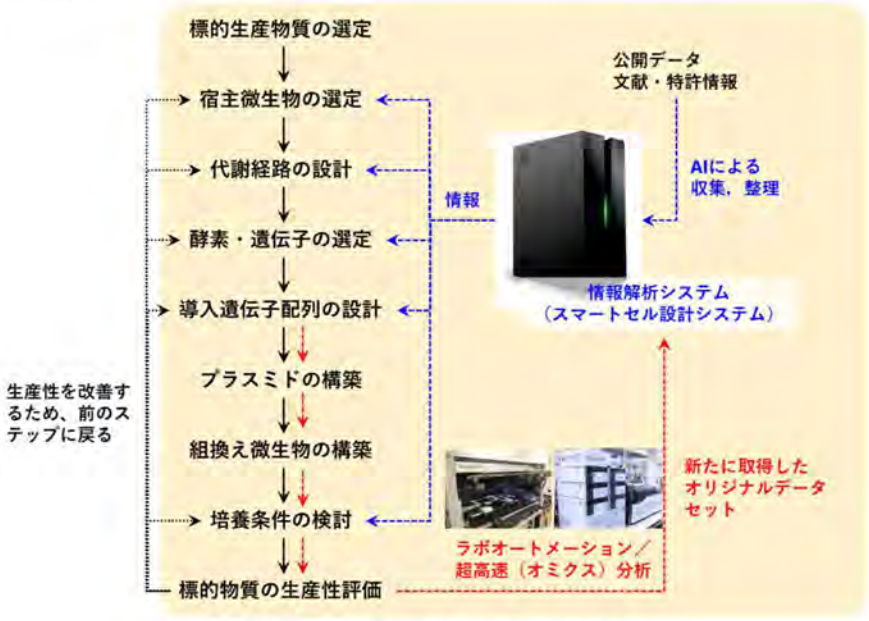

図1．スマートセル創出プラットフォームを利用したワークフローの例

Many of the useful materials required for bioproduction are biosynthesized in cells from common precursors. These precursors are defined as "hub compounds," and strains with a high productivity to produce the hub compounds are defined as "chassis strains." Once a chassis strain is generated, it becomes possible to shorten the breeding period of the target production strain, using the chassis strain as a host. Therefore, chassis strains have high industrial demand.

Conventionally, an enormous amount of time has been required to isolate and breed useful hosts. However, through this project, the development period of chassis strains and practical strains can be shortened, by utilizing a highly efficient multiple gene transfer and destruction technology using long-chain DNA, a technology for optimizing expression levels of multiple genes, semi-automatic high-throughput transformation technology, and a high-throughput evaluation technology.

We are verifying the effectiveness of the Platforms Creating Smart Cells, using specific production materials targeted by private companies, by expanding the scope of its application not only to general-purpose microorganisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, but also to industrial microorganisms.

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Technology Introduction

THEME

Long-chain DNA Synthesis Technology

Kenji TSUGE
Specially-Appointed Associate Professor
Graduate School of Science,
Technology and Innovation, Kobe University

What This Technology Enables

It enables accurate construction of designed long-chain DNA in a short period of time at a low cost.

Introduction to This Technology

To accelerate the development of smart cells, how fast the DBTL cycle can be run is the key. Looking at each step, the Build step, where bacterial strains with sequences designed at the Design step are actually created, takes the longest time. Within the step, the synthesis of long-chain DNA with designed sequences has required a long period of time, specifically several months.

This technology uses the ordered gene assembly in *Bacillus subtilis* (OGAB) method, which we developed to construct long-chain DNA (Fig. 1).[1,2] The OGAB method is a method for assembling multiple DNA fragments using the plasmid transformation system of *B. subtilis*. Utilizing the specificity of 3 to 4 bases at the end of a DNA fragment, the method enables one-step assembly of a maximum of more than 50 DNA fragments. In order to construct long-chain DNA with arbitrary sequence, it is necessary to prepare a double-strand DNA fragment having a size of several hundreds to several thousands of base pairs (bp), as required by the OGAB method, using chemically synthesized single-strand DNA as a starting material. So far, this synthesis has been outsourced to a contract DNA synthesis company. When ordering the synthesis of 50 DNA fragments at a time, however, cases such as that where the synthesis of some fragments is rejected or where the synthesis is attempted but fails and is eventually rejected occur almost invariably although many DNA fragments are successfully synthesized. In such cases, the synthesis is outsourced once again to another contract DNA synthesis company or the design of the DNA fragments is modified, and sometimes it takes as long as 2 months to finally prepare all the necessary DNA fragments.

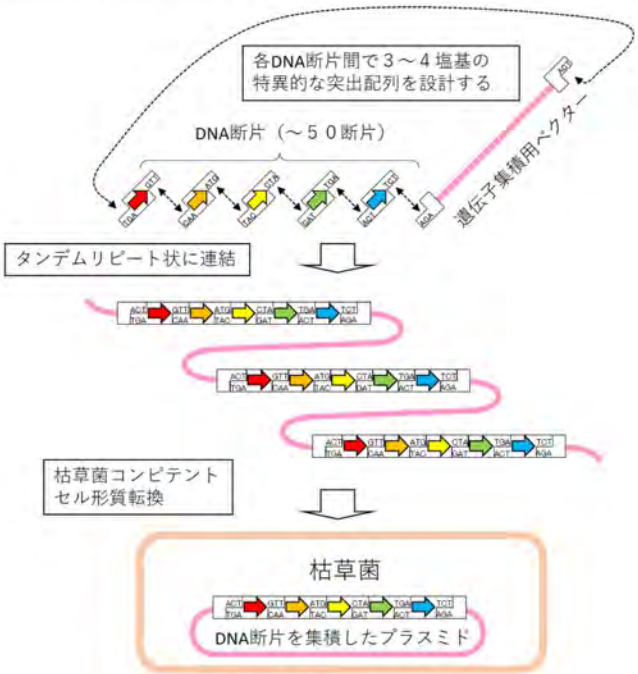

図1．OGAB法による遺伝子集積の概要

Therefore, in order to shorten the time taken for DNA fragment preparation, a total system capable of mass preparation of long-chain DNA constructed through chemical synthesis was established in Kobe University, and technologies were developed from a comprehensive perspective (Fig. 2). Individually, through joint research with Nihon Techno Service Co., Ltd., we have developed a DNA chemical synthesizer specializing in the synthesis of long-chain DNA, which synthesizes 96 single-stranded DNAs of 200 bases in about 1 day at a low cost (Fig. 2, left). In addition, we have also developed a new PCR method for annealing and elongating chemically synthesized DNA using DNA complementarity. This made it possible to prepare double-stranded DNAs of any sequence in about 3 days. In addition, the process in which this double-stranded DNA fragment is cloned in E. coli and only the selected clones having the correct base sequence are used as a material in the OGAB method was substantially automated by a liquid dispensing robot (Fig. 2, center). As a result

of these research and development activities, it has become possible to produce long-chain DNA of about 30 kb in a short period of about 2 weeks at a cost of several yen per base. Thus, we have achieved a significant reduction in the time and financial costs required by conventional methods.

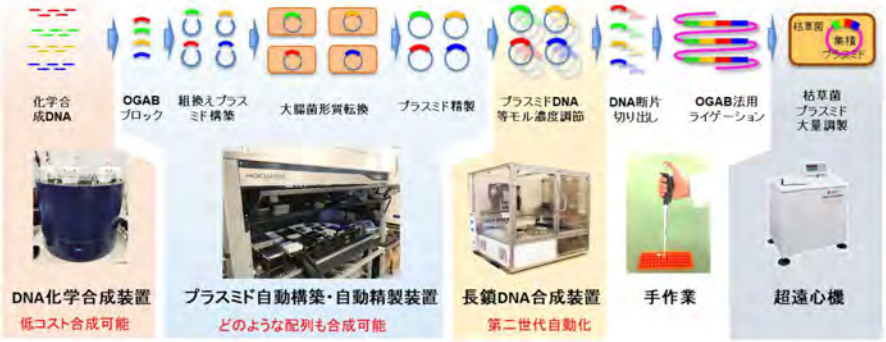

図2. 神戸大学に構築した長鎖DNA合成トータルシステム

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Related Patents

Japanese Patent No. 4479199 "Method for producing plasmid containing insertion DNA unit"
Japanese Patent No. 6440636 "Method for preparing DNA unit composition and method for creating DNA assemblies"

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Technology Introduction

THEME

Metabolite Sensors Construction Technology

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Graduate School of Engineering, Chiba University

What This Technology Enables

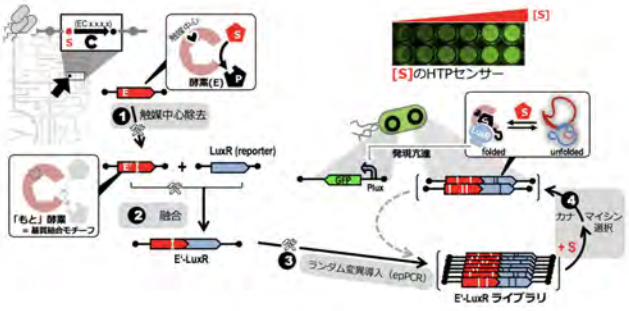
It enables the development of sensors for any metabolite using evolutionary engineering methods and the development and improvement of biosynthetic pathways and smart cells using those sensors.

Introduction to This Technology

To construct "smart cells" that maximize the material production capabilities of cells, it is necessary to prepare and test many host libraries by systematically introducing mutations into the host genome. In addition, for the biosynthetic pathway to be introduced, trial and error are important to determine, for example, the expression level of each gene and the type of gene used. If the accumulation level of a specific metabolite can be investigated for each of the huge specimens obtained by combining the host libraries and the biosynthetic libraries, the smart cell construction for each goal can be dramatically accelerated. By combining two items, namely, (1) high-throughput evolutionary engineering platform for biosensors[1,2] and (2) methods for using biosynthetic enzymes as molecular recognition elements of biosensors,[3] we aim to develop a technology to construct metabolite sensors for various metabolites.[4]

A biosensor basically consists of reporter genes, such as fluorescent proteins, arranged under the control of transcription factors. By setting gene expression levels as the output, evolutionary engineering methods can be simplified. We have developed a fast and highly efficient evolutionary engineering method for our unique transcription factors.[1-3] The feature of this method is that either of the on or off state of the output can be selected only through liquid manipulation in a short period of time. This allows the development of many biosensors to be conducted simultaneously using multi-well plates.

The Smartcell Project has various development goals, and each has a different target metabolite. It has been difficult to prepare sensors for all the innumerable metabolites that exist in nature. In this research, we have developed a sensor design technology that can utilize biosynthetic enzymes as sensor elements. This technology has enabled the production of sensors targeting various metabolic intermediates, which could not previously be produced.



図．メタボライトに対するバイオセンサの開発サイクル

In this project, we are pursuing the on-demand development of biosensors for intermediates of various biosynthetic pathways reconstructed in host cells. Using the sensors produced by this technology, it has become possible to select and obtain excellent mutants in a very high-throughput manner from various host and biosynthesis libraries created in the smart cell development cycle.

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Related Patents

Japanese Patent No. 5959127
Japanese Patent No. 5904494 (US-9315816)
Japanese Patent No. 5757608
Japanese Patent Application No. 2018-057314

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Technology Introduction

THEME

High-accuracy Metabolomic Analysis Technology

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What This Technology Enables

It enables the identification of culture conditions and cells suitable for material production and also the identification of bottleneck reactions in metabolic biosynthetic pathways by highly reproducible multi-sample processing and comprehensive detection of water-soluble metabolites using robots. This clarifies the causal relationship between genetic modifications and the material production capability of cells.

Example of Application: Useful aromatic compounds, ω-3 polyunsaturated fatty acid-containing lipids

Introduction to This Technology

Metabolomic analysis is a technology that can simultaneously identify the pool sizes of various metabolites present in cells. As the obtained metabolomic data reflect the growth environment and genetic background of cells, it becomes possible to identify culture conditions and cells suitable for material production by performing metabolomic analysis.[1]

In other words, measuring the isotope labeling ratio of metabolites over time after a stable isotope-labeled carbon (13C) or nitrogen (15N) source is added to the culture medium allows the comprehensive identification of metabolite turnover rates. The present method (dynamic metabolomic analysis) also allows identifying bottleneck reactions (rate-limiting reactions) in metabolic biosynthetic pathways, and if the rate-limiting reactions are identified, the cellular productivity can be improved by eliminating the rate-limiting reactions through genetic engineering.[2]

In recent microbial breeding, the following DBTL cycle is being established: (1) Designing the metabolism, etc. of recombinant microorganisms in a computer using biological information; (2) Building various types of microorganisms using standardized DNA parts and robotics; (3) Testing productivity to produce the target material in a short time, and (4) Learning the difference between the test outcome and the initial design computationally, which leads to the next design.[1]

When the metabolomic analysis is incorporated into the Test of the DBTL cycle, it can mimic a mechanism for improving productivity. Therefore, the quality of the Design is expected be improved and the number of cycles to be reduced (i.e., shortening of the period of breeding microorganisms). However, the metabolomic analysis has problems in terms of throughput and data accuracy. More specifically, [1] the pretreatment process for extracting metabolites from cells involves complicated and time-consuming manual operations, with a low reproducibility; [2] the ion-pairing agent added to the metabolite detection system (LC-MS/MS) reduces the detection sensitivity and accuracy; and [3] since the number of metabolites that can be quantitated simultaneously is limited to about 100, important trace metabolites cannot be determined. The incorporation of metabolomic analysis into the DBTL cycle has not been achieved anywhere in the world.

Therefore, we first developed a robot that automatically performs pretreatment (Fig. 1). This automated pretreatment system consists of a sampling unit and an extraction unit. The sampling unit samples cell suspensions from a 12-chamber bioreactor at regular intervals, and then immediately stops the metabolic reaction. After collecting the cells, the extraction unit removes the medium and extracts metabolites.

When the performance of the extraction unit was evaluated using *Saccharomyces* cerevisiae as an experimental material, the process that took 3 hours for a skilled operator was shortened to about 1 hour. Since it can be operated overnight, it can achieve a processing speed (288 samples per day) that is 10 times faster than that achieved by humans.

図1．メタボローム解析用自動前処理ロボット

The automation has improved not only processing speed but also accuracy. The conventional pretreatment requires attention to many aspects, such as stopping the metabolic reaction, preventing leakage of metabolites immediately after the stoppage, removing the culture medium, efficiently extracting metabolites, removing lipid or peptide contaminants, and preventing degeneration of the extracted metabolites, and this has complicated the procedure and led to low reproducibility. However, our automated system has overcome these problems. In addition, the incorporation of a barcode reader has facilitated the management of the vast amounts of samples and data.

For the analysis after pretreatment, the S/N ratio has been improved by constructing an LC-MS/MS system that requires no ion-pairing agents. In addition, the method has enabled the isolation and detection of 158 water-soluble metabolites required for designing microbial smart cells.

In the metabolomic analysis, in which the identification and relative quantitation of various metabolites are performed for each sample, the amount of data processing is enormous. Therefore, we are constructing an information analysis system (Fig. 2) that assists in peak picking from chromatograms and projects the analysis results on a metabolic map.

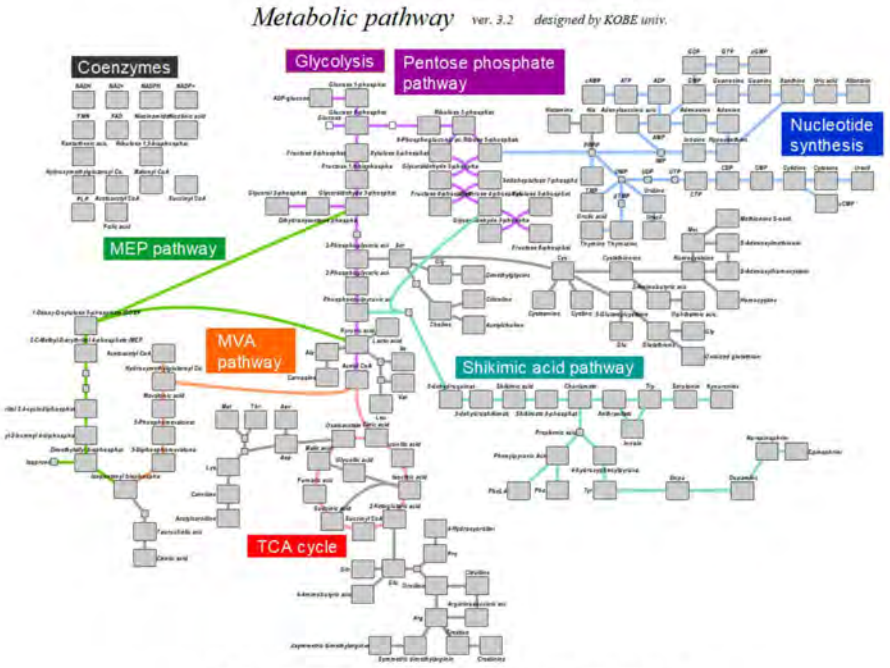

図2．開発したシステムにより測定可能な水溶性一次代謝物の代謝マップへの投影

Recently, with an increased interest in biotechnology-based material production processes, the evaluation of bioproduction has become more important. In microbial breeding so far, even though multiple metabolic modification strategies could be proposed, an optimal solution could not be derived due to a lack of adequate knowledge about the metabolic control mechanism.

We intend to elucidate the metabolic control mechanism, which has been a black box, by obtaining a large amount of highly reproducible metabolomic data through the development of this technology, and to develop a DBTL cycle that incorporates the mechanism analysis. The cycle is expected to create groundbreaking production strains (smart cells) successively in a short period of time.

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Related Patents

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Japanese Patent Application No. 2018-134174
Japanese Patent Application No. 2018-134169
Japanese Patent Application No. 2018-134177
Japanese Patent Application No. 2018-134179
Japanese Patent Application No. 2018-207920

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Technology Introduction

THEME

Quantitative Targeted Proteomic Analysis Technology

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Osaka University

What This Technology Enables

Highly sensitive simultaneous quantitation of the expression levels of dozens of proteins.

Example of Application: Useful aromatic compounds, ω-3 polyunsaturated fatty acid-containing lipids

Introduction to This Technology

The Smartcell Project requires rational modifications of metabolic pathways in order to maximize the material production capability of cells. To modify metabolic pathways, the expression levels of enzyme proteins are increased or decreased by artificially modifying the genome of the host microorganism. Since the protein amount in cells involve various factors associated with transcription, translation and proteolysis, it is necessary to have a measurement method for rapidly evaluating whether the amount of enzyme protein has been adjusted as designed. Under these circumstances, we focused on the high-accuracy quantitative targeted proteomic analysis as an optimal method for the evaluation of smart cells. In this method, a trypsin-digested peptide mixture is separated by liquid chromatography and analyzed in the multiple reaction monitoring (MRM) mode of a triple quadrupole mass spectrometer. Currently, we are developing a targeted proteomic analysis system authentically made in Japan, in collaboration with Shimadzu Corporation (Fig. 1).

図1．定量プロテオミクス法の概要

The key points of the targeted proteomic analysis are establishing a sample pretreatment method and a quantitative analysis method (MRM assay method). First, for sample pretreatment, a crude protein extract of about 100 µL containing 50 µg of total proteins was used as a starting material. After a reductive alkylation of this extract, trypsin digestion is performed overnight, and the resulting trypsin-digested peptides are desalted by solid-phase extraction. This pretreatment method for *Saccharomyces cerevisiae* was effective against various useful microorganisms, such as oleaginous yeasts, *Escherichia coli* and *Corynebacterium glutamicum*.

Data were obtained by nano-LC-MS/MS (LCMS^{TM}-8060, Shimadzu Corporation). This system, in which the number of MRM channels per second can be increased up to 500, has performance suitable for quantitative proteomic analysis. The current system, in which nano-LC is used for emphasizing sensitivity and the processing time is set to be 1.5 hours per analysis, has achieved a throughput of a sample size of 20 to 30, which is the size of a general analysis project, in about 2 days.

The multiple reaction monitoring (MRM) assay method selects 3 to 4 quantitative peptides that can be detected with high sensitivity by LC-MS from a large number of trypsin-digested peptides produced from the protein to be measured, and further selects 4 MRM sequences optimal for each peptide. Since the MRM assay method depends on the amino acid sequences of the protein, it must be prepared for each protein. To promote efficiency in establishing an MRM assay method, the number of reactions to be monitored needs to be reduced. Therefore, we have developed a new method to predict peptides suitable for quantitation from peptide sequences. This method, using a different approach from the conventional method, searches for hopeless peptides that are unlikely to be adopted in the MRM assay method. While minimizing the overlooking of suitable peptides, 30% to 40% of all peptides can be successfully removed as hopeless peptides.[1] We will continue to promote the efficiency in constructing the MRM assay method, and this is expected to lead to the rapid construction of an MRM method and eventually to a rapid development of smart cells.

Using these methods, we have completed the construction of MRM assay methods for main metabolic enzyme proteins, such as *E. coli*, *S. cerevisiae*, *C. glutamicum* and oleaginous yeasts. The MRM assay methods we constructed are compatible with mass spectrometers of different manufacturers. Therefore, currently, we are building a database of MRM assay methods constructed in the Smartcell Project, so as to establish a platform that can be customized as required (Fig. 2).

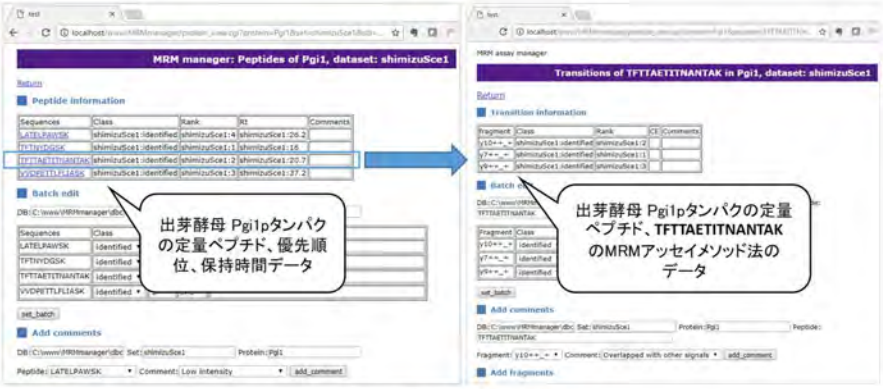

図2．MRMアッセイ法データベース

References

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Technology Introduction

THEME

Sample Non-destructive Cell Evaluation Technology

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What This Technology Enables

It enables the non-destructive identification of cell types at the resolution of a single cell, using a cellular autofluorescence pattern as the index, and the estimation of the metabolic state of the cell.

Introduction to This Technology

1. Current status and issues of cell evaluation technology

"Cell evaluation technology" is a core technology used for cell breeding technologies, differentiation induction technologies from stem cells to target cells, and technologies for the synthesis of artificial cells using a synthetic biological approach. However, it requires a lot of time and labor. For example, in order to evaluate the phenotype of useful material-producing bacteria, the useful material-producing bacteria that have been bred need to be batch-cultured, and the useful materials produced by the bacteria are extracted and quantitated. Therefore, the complicated work associated with cell culture time and quantitation is the rate-limiting factor for breeding. In addition, the existing cell evaluation technologies include fluorescent protein expression, antibody staining, etc. The majority of these methods require a process that modifies or destructs cells. There are few methods that evaluate the properties of living cells in a non-destructive and comprehensive manner. Moreover, the existing cell evaluation methods evaluate the average phenotype of a cell population. Accordingly, the phenotype of cells with some different traits that appear in association with proliferation and differentiation is overlooked. In order to select the target cells only from a heterogeneous cell population, a method that can evaluate the phenotype of each cell is required.

2. Development of a non-destructive method for cell evaluation at the single cell level using autofluorescence

Therefore, we have recently developed an innovative cell evaluation technology, the confocal reflection microscopy-assisted single-cell innate fluorescence (CRIF) method, which can identify cells "non-destructively" at the "single cell level" and estimate the cell's metabolic status, using a cellular autofluorescence pattern as the index.[1]
Intracellular proteins and metabolites emit autofluorescence of various wavelengths and intensities. The autofluorescence pattern, which is formed by the synthesis of respective autofluorescence, functions as a "fingerprint" that expresses the properties of each cell. In the CRIF method, information on cell position and morphology is obtained by reflection microscopy, and cellular autofluorescence information is obtained by confocal laser microscopy.[2,3] Then, by performing image analysis for each cell, the CRIF systematically and comprehensively extracts autofluorescence information for each cell, and reconstructs the information as an autofluorescence pattern. Thus, a "cell's fingerprint" that identifies each cell can be obtained. Moreover, it has been clarified that the application of the "cells' fingerprints" to various types of machine learning enables the construction of a machine learning model that reflects each cell's underlying characteristics in the autofluorescence pattern, and also enables the high-accuracy identification of cell types and the prediction of the metabolic state (Fig. 1). Previous studies allowed the identification of cells at different growth stages and the discrimination of *Pseudomonas aeruginosa* and *Escherichia coli* cells with a single gene mutation (Fig. 2).

3. Future prospects

The CRIF method, which can identify "cells' fingerprints" having almost infinite variations, has a potential to identify dozens (even hundreds, in principle) of cell types and metabolic status. In addition, since this is a simple method that can analyze the properties of intact living cells at the single cell level, it can be the key technology that improves the efficiency of the cell property evaluation, which is essential for cell breeding technologies, stem cell differentiation induction technologies, and technologies for the synthesis of artificial cells in various fields. At present, we are developing a technology for efficiently separating target cells after evaluating the properties of cells by the CRIF method, aiming at practical application of the CRIF method in screening. In addition, in cooperation with a Japanese microscope manufacturer, we are also developing a high throughput screening system that can perform a series of processes in the CRIF method (i.e., microscopic observation, extraction of cells' fingerprints, and evaluation of cell properties by machine learning). We aim to establish the CRIF method as a highly versatile cell evaluation technology so that it can be utilized in a wide variety of fields from basic research on, for example, microbial, plant and animal cell breeding, to applied research on, for example, regenerative medicine, in the future.

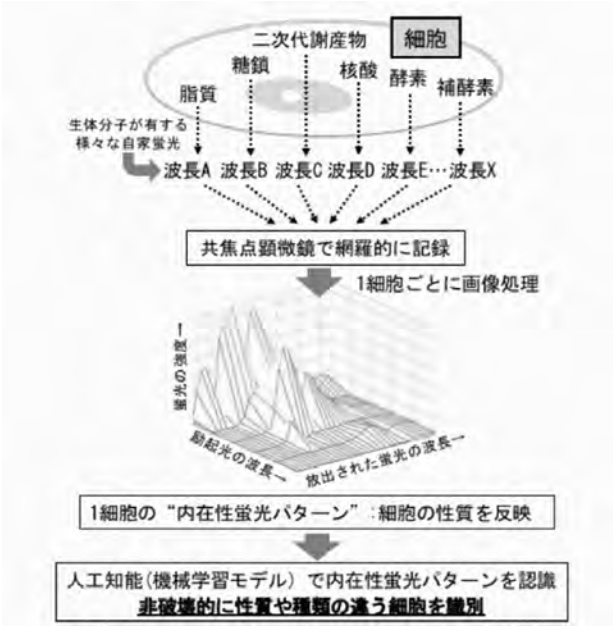

図1．CRIF（Confocal Reflection microscopy-assisted single-cell Innate Fluorescence）法の概念図

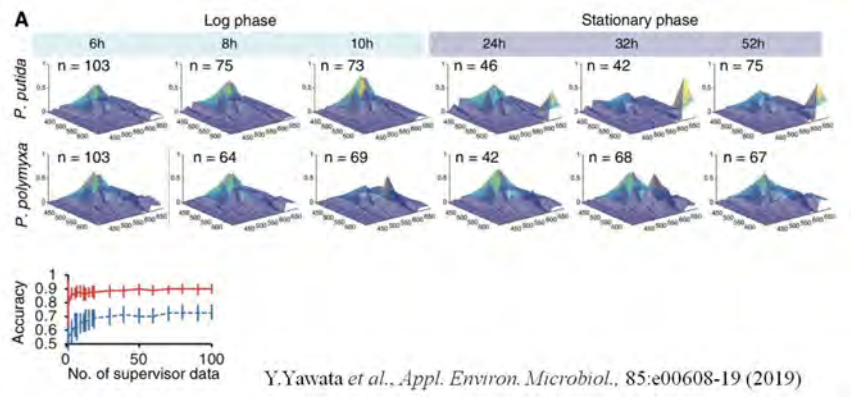

図2．*P.putida*および*P.polymyxa*の各生育段階における平均自家蛍光パターン（上段）、ニューラルネットワークによる対数増殖期と定常期の細胞の分類（下段　赤：*P.putida*、青：*P.polymyxa*）

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2）Y. Yawata, K. Toda, E. Setoyama, J. Fukuda, H. Suzuki, H. Uchiyama, and N. Nomura : Monitoring biofilm development in a microfluidic device using modified confocal reflection microscopy, *J. Biosci. Bioeng.*, 110, 377~380 (2010)

3）Y. Yawata, N. Nomura, and H. Uchiyama : Development of a novel biofilm continuous culture method for simultaneous assessment of architecture and gaseous metabolite production, Appl. Environ. Microbiol., 74, 5429~5435 (2008)

Related Patents

Japanese Patent No. 6422616 "Data creation method and data usage method"

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Technology Introduction

THEME

Metabolic Pathway Design Technology

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What This Technology Enables

It enables designing artificial metabolic reaction pathways, and further, designing intracellular metabolism for achieving high productivity to produce target compounds. In addition, based on the blueprints, candidate genetic modifications (deletions and enhancements) can be extracted and proposed.

Example of Application: Useful aromatic compounds; ω-3 polyunsaturated fatty acid-containing lipids

Introduction to This Technology

In the biotechnology industry, many useful compounds have been conventionally produced by utilizing microbial "fermentation." In recent years, the technology for producing useful compounds by utilizing microbial "fermentation" is used as a technology for utilizing non-fossil raw materials. This technology, where sugars, etc. that are produced through carbon dioxide fixation by plants and photosynthetic microorganisms are utilized as a carbon source, is used to have genetically modified microorganisms produce target useful compounds. Europe and the United States are leading the way in microbial production technology using this "synthetic biology," and cataloging of the production technologies using fermentative production is already in progress for many general-purpose chemicals. Concrete examples of the general-purpose chemicals that have been cataloged are general-purpose polymer raw materials, such as bioethanol, which replaces fossil fuels in automobiles, lactic acid, which is a raw material for polylactic acid, 1,3-propanediol, γ-aminobutyric acid and 4-aminocinnamic acid. Furthermore, as an example of manufacturing industrial application, a US bio-venture Genomatica and BASF (Germany) have succeeded in jointly producing more than 50,000 tons per year of 1,4-butanediol (BDO), which is a core general-purpose compound.

For the microbial production of useful compounds, it is essential to have a technology that optimally designs "metabolism" including not only the intracellular carbon flow but also the balances between energy production and consumption and between oxidation and reduction. This is because such a technology allows the understanding of the intracellular phenotype and the information obtained can be applied to the metabolism design of the target cell and to subsequent breeding. However, there is a limit to the human brain's capacity to think about more than 1,000 metabolic reactions occurring in a cell. Therefore, the computing power of computers is indispensable. Especially in recent years, due to the acceleration of the annotation process by the innovations in the genome sequencing technology and information processing technology, it has become possible to describe all metabolic reactions on a computer at the genome scale level. In other words, a technology for predicting the metabolic behavior of microbial cells in a certain environment has been established (genome scale model: GSM). Currently, research is being actively conducted by systematically performing the process from cell metabolism design using GSMs to verification by actual experiments, in order to improve the target compound productivity in a high-throughput manner. However, the existing GSMs cannot predict or design biosynthetic pathways for non-natural compounds. In addition, design using metabolic reactions that occur in non-host cells is difficult. We are developing 2 metabolic pathway design tools that can be a technology to solve these problems.

(1) BioProV: An artificial metabolic pathway design tool

An overview of this tool is provided below (Fig. 1).

1. From the databases containing metabolic and enzyme reactions, such as KEGG (http://www.genome.jp/kegg/) and BRENDA (http://www.brenda-enzymes.org/), the concept of individual enzymes was uncoupled, and only the chemical reaction patterns were described. Then, similar chemical reaction patterns were reclassified as one chemical reaction and the computer was made to learn the reclassified patterns (Fig. 1 (a)).
2. In the learning process, the precursor and product in each reaction were described using a notation called SMILES, and then the reaction mechanism was described entirely using a notation called SMIRKS (Fig. 1 (b)).
3. In the actual simulation, the target compound is described using the SMILES, and the description is used as input data. Then, based on this, the precursors are retro-synthesized randomly and comprehensively. The simulation is successful if a compound known to exist in vivo is present in the retro-synthesized precursors. In other words, if the designed artificial metabolic reaction takes place using the known biological compound as a starting material, the target compound can be biosynthesized (Fig. 1 (c)).

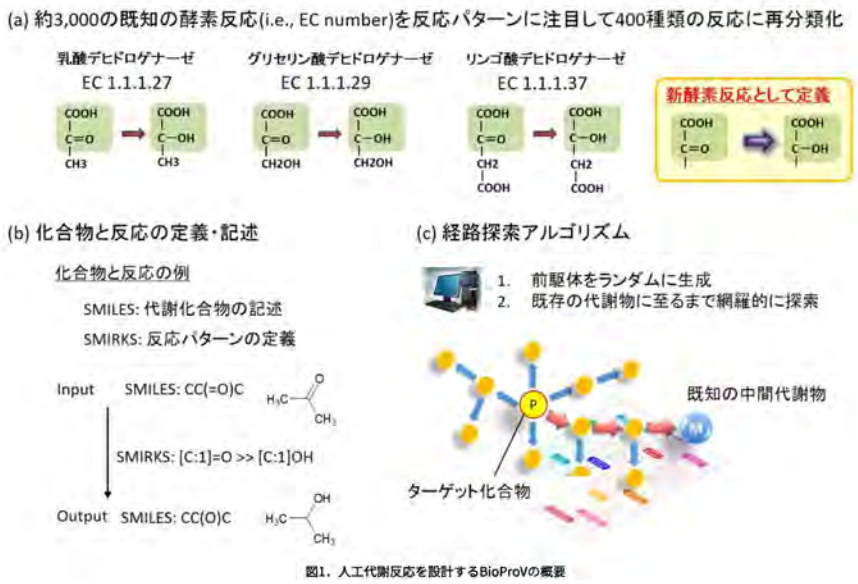

図1．人工代謝反応を設計するBioProVの概要

M-Path[1] developed by Michihiro Araki, Professor, at Kyoto University has a different algorithm but the same concept, and is known as a tool for designing artificial metabolic pathways.

(2) HyMeP: A hybrid metabolic pathway design tool

The existing GSMs allow metabolic pathway design only within the scope of metabolic reactions that occur in the host cell. Therefore, we have developed a tool that enables hybrid metabolic pathway design in order to efficiently produce target compounds, by comprehensively adding metabolic reactions that occur in organisms other than host cells.[2] The outline of this tool is as follows.

1. From all species' metabolic reactions contained in the KEGG database (http://www.genome.jp/kegg/), metabolic reactions that occur in the host cell to be used were excluded, and the remaining reactions were stored in the database.
2. From the database prepared, the reaction pathways that connect to the GSMs of the host cell were selected to construct a hybrid metabolic pathway (HyMeP) (Fig. 2).
3. Using the constructed HyMeP, we have designed an efficient metabolic pathway that can achieve the maximum production of target compounds. Drawing an ideal metabolic pathway blueprint using the HyMeP enables rapid and rational metabolic pathway design.

- **2つの外来反応を追加する場合**

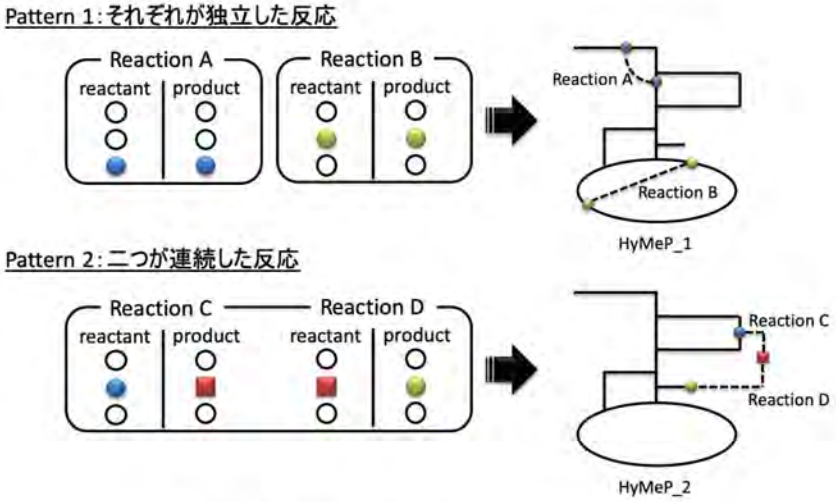

図2．2つの外来反応を宿主の代謝モデルに付加しHyMePを構築するスキーム

In order to efficiently produce useful compounds using microbial cells, it is indispensable to design an optimal intracellular metabolic pathway by computation. We have developed a tool BioProV, which proposes new metabolic pathways, and a tool HyMeP, which enables the efficient production of useful compounds by hybrid metabolic pathway design. By combining these 2 tools, an optimal metabolic pathway can be designed, for example, to have microbial cells produce petroleum-derived non-natural useful compounds. In the future, it will be important to highly activate the metabolic reaction that takes place in the designed metabolic pathway.

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Technology Introduction

THEME

Enzyme Modification Design Technology

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What This Technology Enables

It enables proteins to have high functionality, specifically, a highly enhanced enzymatic activity, by modifying genetic sequences through a molecular dynamics simulation-based analysis.

Introduction to This Technology

The Smartcell Project aims to construct "smart cells" that maximize the material production capability of cells, and to create useful materials that are difficult to produce by conventional synthesis methods, as well as to achieve cost reduction and energy saving in the production processes. We focused on enzymes, which are actually responsible for material production in cells, and developed a method for predicting the enzyme modification site that improves their functions. An enzyme binds properly to a substrate that is used as a raw material for the material production and forms an enzyme-substrate complex to produce the target product (main product). However, depending on the shapes of the enzyme and the substrate, the reaction efficiency may be significantly reduced, or the purity of the main product may be lowered due to the formation of unintended products (by-products). Therefore, we aimed to maximize the material production capacity of enzymes, by making some enzymatic modifications to modify their structures to suit the main product formation.

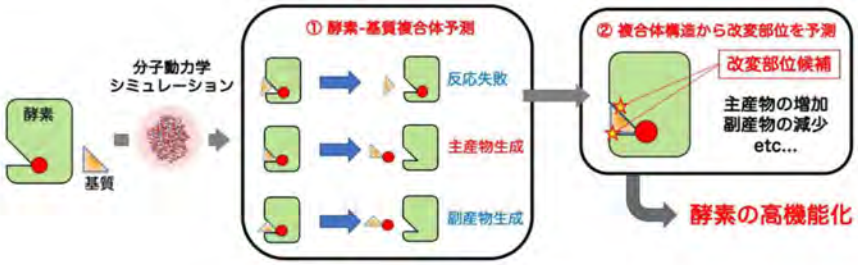

図1. 酵素改変部位予測法の２つのステップ

This method for predicting enzyme modification sites can achieve high enzyme functionality by 2 steps (Fig. 1). In the first step, the adaptive lambda square dynamics (ALSD) method, which is a molecular dynamics (MD) simulation method developed by us,[1,2] is used to exhaustively search for all possible enzyme-substrate complex structures at the atomic level. In the second step, from the information obtained by the ALSD method on the atomic level-structures of the complexes, the patterns of enzyme-substrate interactions are analyzed in detail, and then potential enzyme modification sites where the amount of the main product can be increased or the amounts of by-products can be decreased are proposed.

A major breakthrough in achieving high enzyme functionality was the use of the ALSD method,[1,2] which is a powerful simulation method. In the MD, the structures of enzyme-substrate complexes are searched, by reproducing the environment of the enzymes, substrates and the solvents surrounding them, such as water molecules and ions, in the computer in order to track the time-series changes similar to reality. However, the conventional MD method requires a lot of computation time and computational resources to search the complex structures, and therefore it was very difficult to apply the method to actual enzyme modification research. On the other hand, the ALSD method is a modified MD method that can significantly promote structural changes at specific sites (such as substrate binding region) arbitrarily selected. In the currently ongoing research, various complex structures occurring during the formation of the main product or by-products are exhaustively searched, by promoting structural changes of the substrates inside the enzyme's pocket, and the patterns of enzyme-substrate interactions are analyzed in detail. As a result, it has become possible to predict the enzyme modification site at a simulation time of several days to about 1 week.

An actual application example is presented in Fig. 2. Although this enzyme has high reaction efficiency, it produces numerous by-products, resulting in low yield of the target compound, which accounts for only 13% of all the reaction products. By using this method, however, we succeeded in increasing the yield to about 70%. In addition, we succeeded in increasing the amount of the target compound produced up to about 6 times.

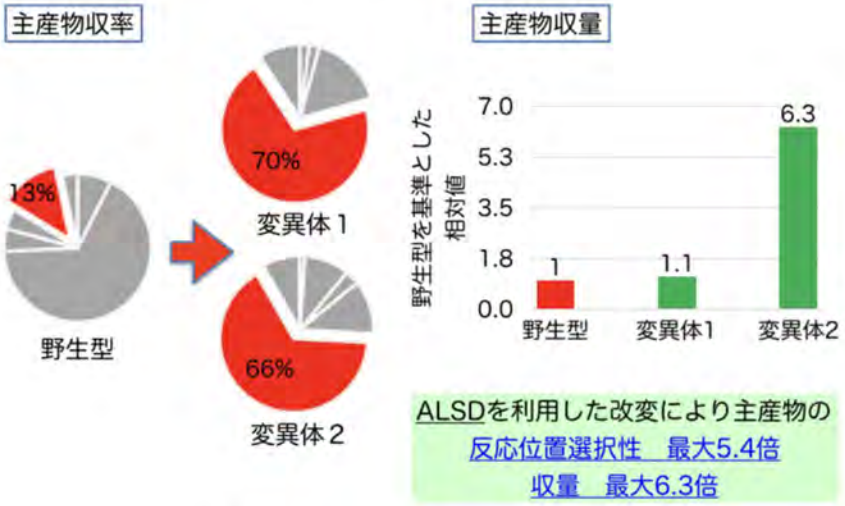

図2．酵素改変部位予測法によって提案された変異体の主産物収率、収量例

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— Technology Introduction

THEME

Transgene Sequence Design Technology

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What This Technology Enables

It enables controlling (increasing or decreasing) protein expression levels by modifying genetic sequences.

Example of Application | Useful aromatic compounds

Introduction to This Technology

In bio-based material production using microorganisms, it is sometimes performed to introduce heterologous genes into the target microorganism in order to have the microorganism produce proteins it does not naturally possess. In that case, to improve the production level of the target protein, the process of properly designing the DNA sequence of the transgene (codon optimization) is important. Conventional research on codon optimization is conducted on microorganisms used for research purposes, which are easy to handle in experiments, such as E. coli. For microorganisms for industrial purposes, such as actinomycetes, there has been no established method for codon optimization. We have developed a new codon optimization method by extracting rules through information analyses, of large-scale protein production experiment data owned by the National Institute of Advanced Industrial Science and Technology (AIST), and demonstrated its effectiveness in *Rhodococcus* sp.[1] This method can be applied to material production in various hosts other than actinomycetes, and we have actually confirmed its effectiveness. In addition, since designed genetic sequences contain mutations only at the head of the original sequence, they can be synthesized at a low experimental cost (Fig. 1).

In this study,[1] we analyzed information on protein production experimental data in *Rhodococcus* sp. owned by AIST. The data contained information on the gene sequences of 204 genes, with a link to their protein production levels. By calculating various sequence feature values from the genetic sequences, the correlation between protein expression levels and sequence feature values was evaluated. As a result, the stability of mRNA secondary structures at the head of the sequence and the sequence feature values used as the Codon Adaptation Index (CAI) showed a high correlation with protein production levels. Based on this result, we have developed a new codon optimization method that designs sequences to prevent the formation of secondary structures and also to have high CAI values. In this method, first, for the original genetic sequence, all combinations of DNA sequences in which sequences for amino acids of the protein are identical and only codon usage patterns are modified are computationally generated. Then, for each sequence generated, the stability of the mRNA secondary structure at the head of the sequence and the CAI are calculated. Using the obtained values, the optimum sequence, which has the CAI greater than the specified threshold and is least likely to form a secondary structure, is sought. In general, there are a huge number of DNA sequences with a modified codon usage pattern for a certain genetic sequence. Therefore, such a search task requires a very long calculation time and is not practical. This time, the information analysis of the data owned by AIST has revealed that the head of genetic sequences is particularly important for protein expression levels. Based on this finding, we were able to accelerate the calculation by narrowing the search range to only the head of sequences and to realize this method. Thus, we have succeeded in developing a novel codon optimization method, by closely linking experimental data acquisition and information analysis.

In order to verify the effectiveness of this method, we designed the sequences of 12 genes, and introduced the designed sequences into *Rhodococci* to evaluate protein production levels. For each gene, 6 types of sequences that improve protein production levels and 3 types of sequences that decrease protein production levels were designed by variously changing the CAI threshold setting, and these sequences were compared with 9 wild-type sequences. Each comparison was repeated three times in a large-scale verification (12 genes × 10 sequences × 3 times = 360 experiments). As a result, we succeeded in improving the production levels of 9 genes (75%) in comparison with the wild-type sequences, and also identified the optimum threshold setting for the CAI (Fig. 2). In particular, regarding 5 genes whose wild-type sequences lead to low protein production levels, an improvement in production levels by the sequence design was observed in all genes (100%) (Fig. 3). This result indicates that this method is particularly effective for proteins that are difficult to produce. Regarding the sequences considered to decrease protein production levels, the production level decreased in all the 12 genes, as expected. In addition, possessing mutations only at the head of the designed sequences compared to the wild type has another advantage of this method (Fig. 1). This allows designed sequences to be synthesized not by a high-cost method, such as full-length gene synthesis, but by a very simple and inexpensive method, using only PCR with primers carrying mutation. Furthermore, this method can be applied to various microorganisms other than *Rhodococcus* sp. by adjusting the CAI to that of the target microorganism, and we have actually confirmed its effectiveness.

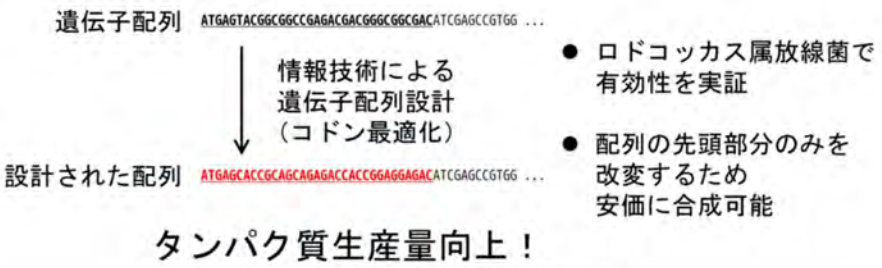
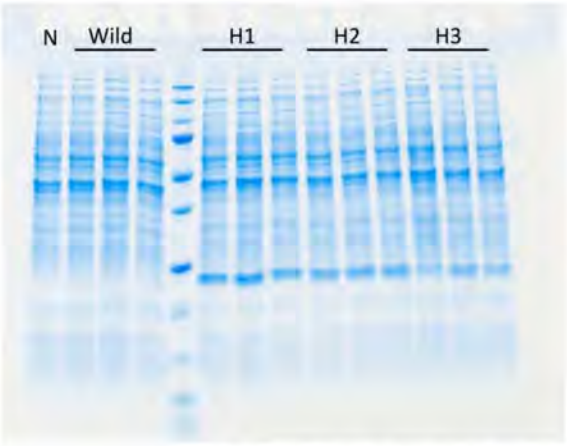


図1．情報技術による遺伝子配列設計でタンパク質生産量を向上

野生型配列での タンパク質生産量	配列設計の有効性を 検証した遺伝子数	タンパク質生産量の 向上した遺伝子数	成功率（%）
小	5	5	100
中	4	3	75
大	3	1	33
合計	12	9	75

図2．遺伝子配列設計によるタンパク質生産量向上の有効性検証



N: ネガティブコントロール
Wild: 野性型
H1: 配列設計型1
H2: 配列設計型2
H3: 配列設計型3

図3．野生型配列でのタンパク質生産量が少ない遺伝子の配列設計による改善例

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Technology Introduction

THEME

Expression Regulation Network Construction Technology

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What This Technology Enables

It enables us to understand phenomena occurring in cells as one system, by expressing interactions between genes from gene expression data as a network model, and to propose candidate genes to be modified for artificial regulation.

Example of Application: Useful aromatic compounds, ω-3 polyunsaturated fatty acid-containing lipids

Introduction to This Technology

In order to achieve efficient material production by microorganisms, it is necessary to understand the underlying mechanisms of phenomena occurring in microbial cells during material production and regulate the phenomena as an operating system. For understanding and utilizing this complex "system" in living cells, a network model of gene expression regulations is useful. By constructing the regulatory relationship between genes as a network model, we were able to express the process occurring in living cells in a causal graph. This has led us to the development of a technology that allows the search for modification sites necessary for the bottleneck search in the process and for promoting efficiency.

First, we developed a technology for estimating gene groups that contribute to the material productivity. It is estimated that, in living cells, about 10% to 20% of genes are required for intracellular phenomena, such as material production. Therefore, it is necessary to identify gene groups that contribute to material production. In this study, we have developed a work flow that allows the gene groups related to material production to be extracted with higher accuracy, by combining statistical testing, correlation analysis and logical operations (Fig. 1).

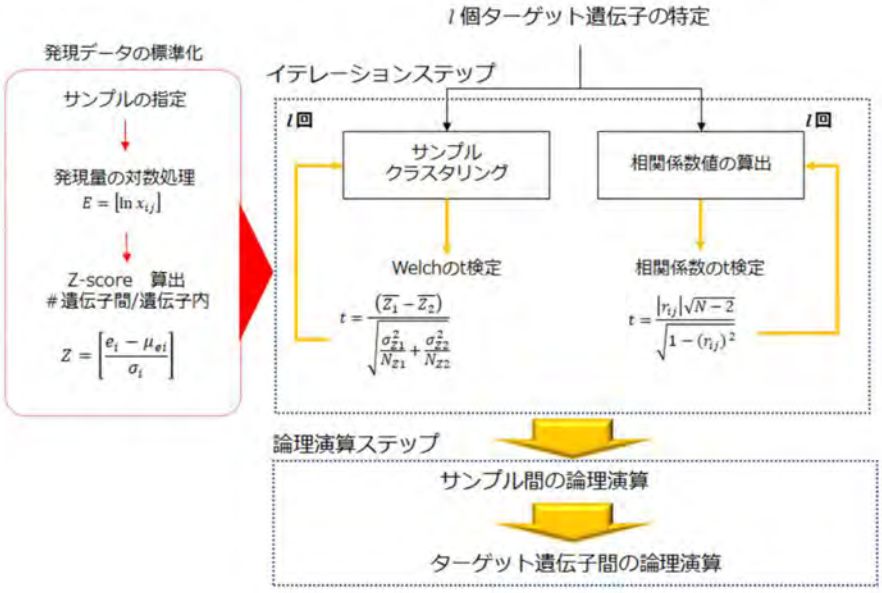

図1．論理演算を組み合わせた遺伝子選択フロー

Secondly, we developed a technology for network modeling of the regulatory relationships between genes. The gene groups selected in the previous section are likely to correlate with the production level of the target material, and there is no directionality in the relationship between the selected gene groups and the target materials. Therefore, it is necessary to identify the genes that are located upstream of the target material and can be regulatory factors to control the production level of a target material in the material production process, from the selected gene groups, using the network model. In this study, we are working on the high-accuracy estimation of network structures, by combining Bayesian networks[1] and the structure equation modeling[2,3] developed by the National Institute of Advanced Industrial Science and Technology (AIST) (Fig. 2).

Technology Introduction

THEME

Knowledge Extraction and Learning from Literature, etc.

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What This Technology Enables

This technology allows the extraction of useful knowledge for microbial development from literature and public data on smart cells, which enables the proposal of the next step to improve the current design, such as promising gene modifications or enzyme genes for increasing productivity.

Example of Application: Useful aromatic compounds, ω-3 polyunsaturated fatty acid-containing lipids

Introduction to This Technology

The "Learn" process in the Design-Build-Test-Learn (DBTL) cycle of the smart cell development, i.e., the interpretation of various data/models, and the following process of creating the design hypothesis based on them, relies on the background of personal knowledge and on manual literature/database searches and surveys. Such a knowledge acquisition process dependent on individual skills is a rate limiting factor in the smart cell development, which interferes with systematic accumulation/discovery/reuse of knowledge, and poses a major issue to be resolved technically. For example, the metabolic pathway design, metabolism model construction and optimization, and search for enzyme genes and modification candidate genes largely depend on knowledge extraction from the existing literature and database information. In many cases, existing databases on metabolic pathways and enzyme reactions do not contain sufficient information necessary for smart cell development. Therefore, there was plenty of room for reconsideration regarding the extraction of literature knowledge for smart cell development. In addition, with the recent development of machine learning and artificial intelligence (AI) technologies, it has become possible to extract new knowledge and patterns from existing data, and such technologies are expected to become applicable. Under these circumstances, this project works on the development of a knowledge base from the literature that supports metabolism and enzyme design proposals and the development of a machine learning technology focusing on enzyme gene search.

(1) Knowledge base development

In order to organize the individually acquired knowledge on microbial design and systematize it in a reusable form, we developed a technology to organize and accumulate the design history of microbial strains and the genetic modification content of each strain associated with the design history, in terms of information on purpose, means, and rationale. The "visualization" of the accumulated knowledge in a tree form along the modification history allows an overview of the entire design data to date and utilization of the data to inspire new hypotheses. In addition, based on the accumulated and systematized design history, the system calls up the associated knowledge extraction technologies, and presents useful information that leads to design improvement.

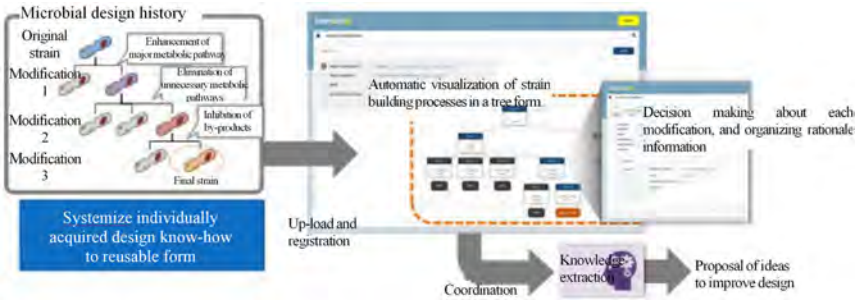

Figure 1 Knowledge base for supporting smart cell design

The knowledge extraction technology consists of **smart cell literature automatic collection technology** and **promising gene recommendation technology**. The former technology identifies characteristics of literature related to metabolism design and gene modification by natural language processing and literature search technology, and widely collects useful literature information for smart cell design. The latter technology extracts and proposes genetic modifications deeply related to the previous metabolism design/gene modifications from the collected literature information. These technologies allow the collection of literature information related to the design from existing strain designs accumulated in the knowledge base, and the proposal of gene modifications to be added to the current design. As a result, it can be expected to run the DBTL cycle more efficiently and reduce the number of processes required to create the desired smart cell.

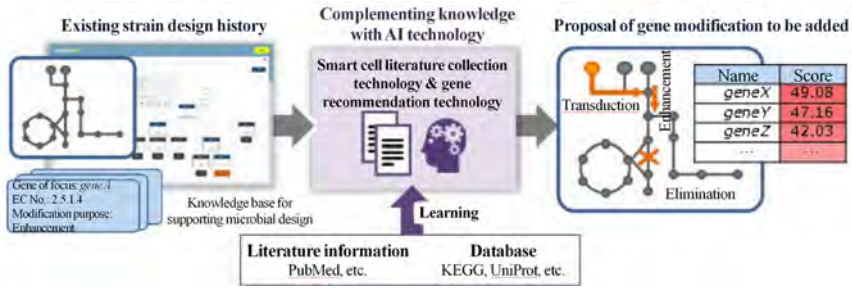

Figure 2 Gene modification proposals from literature information based on design history

(2) Enzymatic reaction data learning and activity estimation model

In each metabolic pathway created through a metabolism design process, multiple estimated enzyme gene candidates appear, regardless of whether they are unknown or known. Therefore, the selection of enzyme genes is an important challenge for actually constructing a metabolic pathway. This technology works as follows: First, it digitalizes the chemical structure and enzyme amino acid sequence of a substrate/product, and determines whether the enzyme regulates the reaction positively or negatively; then, it gives a score to test data having a new combination of a substrate/product and enzyme amino acid sequence, in order to determine whether it is positive or negative. Along with the conventional procedures for the machine learning and deep learning base, we also work on improving models by incorporating substantiation and verification data.

For example, we have developed a technology that provides guidance for enzyme gene selection by comparing the distribution of results judged by the machine learning method (Fig. 3). We also developed a technology[2] for searching new metabolic pathways by predicting chemical structures possibly occurring after known and unknown enzymatic reactions, using a deep generative model that has learned the chemical structures of substrates/products (Fig. 4).

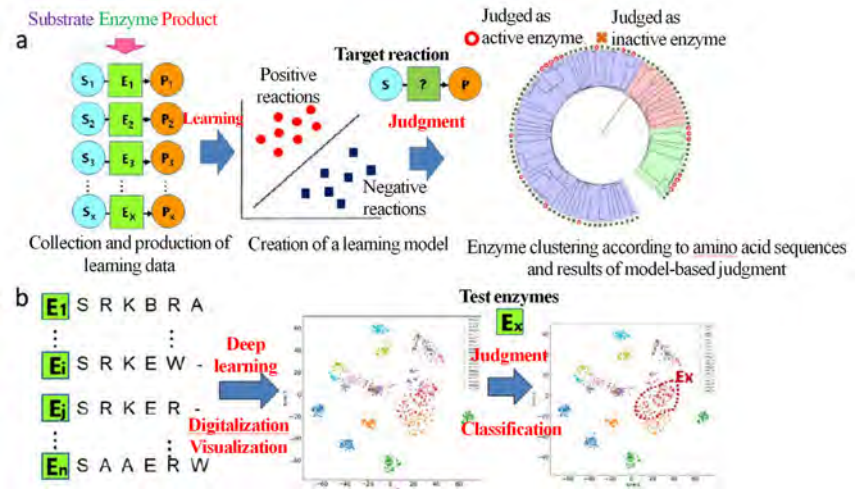

Figure 3 Learning based on enzyme reaction data and development of activity estimation model

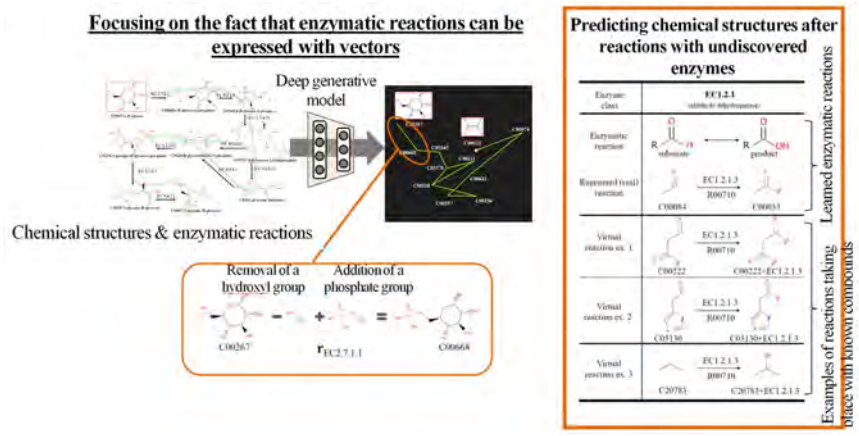

Figure 4 Search technology for new metabolic pathways using deep generative models

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Technology Introduction

THEME

Transporter Search Technology

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What This Technology Enables

It enables the search of transporters that discharge target compounds out of cells.

Example of Application: Useful aromatic compounds

Introduction to This Technology

When a compound is produced using a microorganism, etc., particularly when a membrane-impermeable compound is targeted, the discharge of the product out of the host cell affects the efficiency of production. If the extracellular discharge of the product is delayed, the product accumulates inside the cell, causing negative feedback, leading to the inhibition of the biosynthetic reaction. In such cases, the solution is to express a "transporter," which properly transports the target compound out of the cell, on the cell membrane.[1] Recent advances in genomic analysis have revealed the presence of genes encoding 300 to 1,000 transporters on the genome of living organisms. On the other hand, since transporters are localized in the membranes, it is necessary to solubilize the cell membranes using a detergent to obtain transporters during purification. In addition, to analyze enzymatic functions, complicated technologies, such as an artificial membrane vesicle (liposome) reconstruction method, are required. Therefore, the functions of many transporter genes remain unknown. Actually, even though the search for the efflux transporter of the target compound is attempted based on literature information and genomic information, obtaining the information on the transporter of the target is extremely difficult. In this project, by integrating the information analysis technology owned by the National Institute of Advanced Industrial Science and Technology (AIST) and the transporter search technology developed by Tohoku University, we have developed a technology for searching for transporters of the target compounds while shortening the time required with the conventional transporter search method.

The research group led by Aburatani et al. at the Biotechnology Research Institute for Drug Discovery of AIST has developed a technology for extracting transporter genes, contributes to the production of target compounds, based on genomic information and gene transcriptional analysis data. Using this technology, they narrowed down the candidate transporter genes from the transporter genes encoded in the genome. Next, at Tohoku University, by applying the transporter search technology[2] developed by them independently, they conducted the search for transporters of target compounds, through experimental investigation of the functions of transporter genes extracted by Aburatani et al. at AIST, using the technologies such as mass spectrometry. In this project, we succeeded in searching for transporters of amino acids and organic acids (kojic acid, etc.) in a shorter time, which was one-third to one-fifth of the time required with the conventional search method. Furthermore, we have verified that the introduction of the searched transporter genes can promote efficiency in the production of the target compound.

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Related Patents

特願2018-087700　所定の化合物に対する膜タンパク質のスクリーニング方法及び所定の化合物の生産方法, 2018年4月27日, 発明者　七谷圭, 阿部敬悦, 新谷尚弘, 米山裕, 中山真由美, 出願人　国立大学法人　東北大学

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Technology Introduction

THEME

High-throughput (HTP) Transcriptome Analysis Technology

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Bioproduction Research Institute/Biomedical Research Institute,
National Institute of Advanced Industrial Science and Technology (AIST)

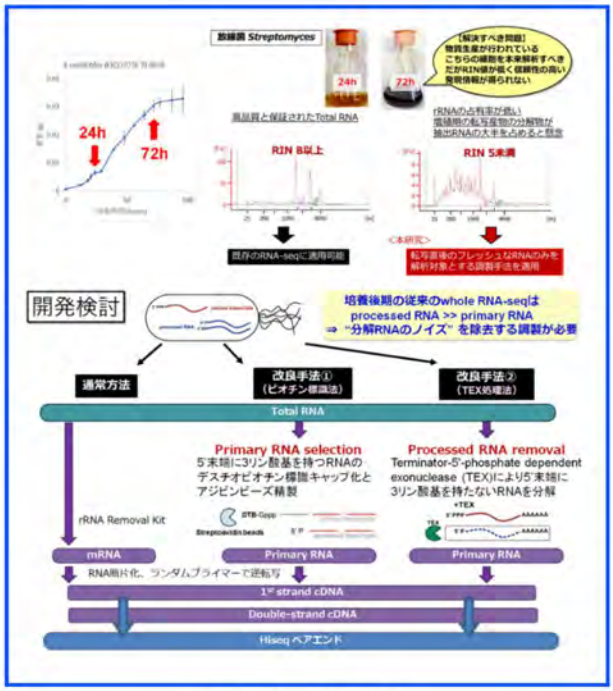
What This Technology Enables

It aims to establish a highly reliable transcriptome analysis technology.

Introduction to This Technology

In order to establish a highly reliable transcriptome analysis technology that is applicable to various industrial microorganisms, we will develop nucleic acid reference standards for spike-in control and verify their utilization strategies. In addition, for that purpose, we will establish a simple method for the valuation of the nucleic acid reference standards.
In some industrial microorganisms, the production level of a targeted useful material increases especially at the end of the culture period. However, it is difficult to extract RNA in good condition from such samples. Therefore, we are developing a technology that allows the transcriptome analysis, which has been difficult to perform so far, by establishing a technology for selectively analyzing only non-degraded RNAs.

スパイクイン用核酸標準物質の開発と検証



未分解RNA選択的調整技術の適用

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Application Examples

TITLE

List of Application Examples

Verification of Effectiveness

Aiming to create smart cells, we have developed various basic technologies necessary to run the DBTL cycle.
Here are examples showing how these technologies were utilized in the creation of bacteria that produce useful compounds.

Example01

Regulation of simultaneous production of useful proteins in filamentous fungi

Example02

Improvement in productivity to produce useful aromatic compounds using Corynebacteria

Example03

Improvement in productivity to produce ω-3 polyunsaturated fatty acid-containing lipids

Example04

High production of alkaloids in Escherichia coli based on metabolic pathway design, enzyme design and metabolomic analysis

Example05

Development of a highly-productive smart cell that produces the rare amino acid ergothioneine

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— Application Examples

THEME

Regulation of simultaneous production of useful proteins in filamentous fungi

Technologies Used

Expression Regulation Network Construction Technology

Participating Institutions

Nagaoka University of Technology, Kao Corporation, Japan Bioindustry Association, Kyushu University, and National Institute of Advanced Industrial Science and Technology (AIST)

Research and Developmental Goals

To establish a technology platform for constructing strains producing optimal enzyme preparations for saccharification of plant biomass of various components, by controlling the production ratio of each plant biomass saccharifying enzyme excreted by filamentous fungi.

Objectives

Plant biomass is a sustainable source of energy, existing abundantly on the earth. The sugar obtained by hydrolyzing (saccharifying) the polysaccharides that make up the plant cell wall, which is a non-edible part of plants, is not only used as a raw material for biofuels, but also usable as a carbon source for the production of high value-added products in microorganisms. The technology for saccharifying plant cell walls is essential also for creating a bioeconomy society based on material production using microorganisms. Since filamentous fungi produce an enzyme "cellulase" derived from a microorganism capable of efficiently saccharifying plant cell wall polysaccharides, research and development aiming at enzymatic saccharification of plant biomass using cellulase are underway. In Japan, development using a filamentous fungus *Trichoderma reesei*, which has a high cellulase productivity, has been promoted since the 1980s. Initially, capability to produce enzymes was improved by inducing mutations, and the finally acquired PC-3-7 strain is still used as the base strain for the development of industrial enzyme production strains. In addition, the production of saccharifying enzyme-producing strains that have the world's top level plant biomass saccharification capacity was succeeded through the production of an enzyme that plays an important role in saccharification in the PC-3-7 strain using genetic recombination technology. However, since the composition of the constituents of the cell wall polysaccharide differs depending on the type of plant biomass, the creation of a "tailor-made saccharifying enzyme," which consists of the optimal enzyme components for the plant biomass, is currently being required (Fig. 1).

Strain developments so far have been based on previously published papers and findings obtained in the laboratory. In such developments, although it was possible to control the total production level of enzymes, controlling only the production levels of specific enzymes was difficult. However, the identification of factors that control multiple genes is difficult with the conventional "wet" approach that relies on the experience and intuition of researchers. Therefore, in the Smartcell Project, we aim to clarify which gene regulates which gene and how the network of regulation is structured and also to identify the factors regulating the production ratio in the group of glycoside hydrolases, by preparing a large amount of expression data on all the *T. reesei* genes and making the best use of the network model construction technology of the smart cell design system.

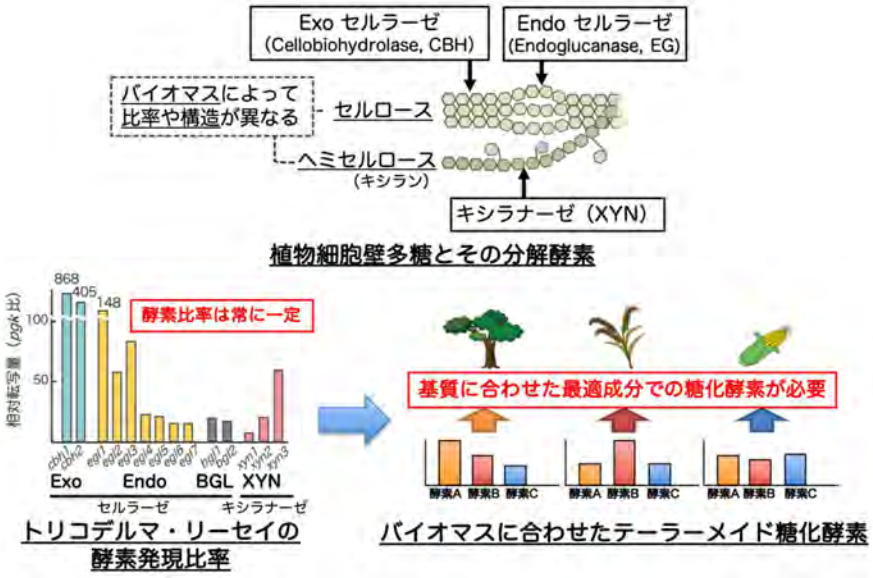

図1　本研究開発の背景

Results and Achievements

Using approximately 200 samples of all gene expression data under the cellulase production and non-production conditions, we designed a gene expression network based on the DBTL cycle shown in Fig. 2, and estimated multiple genes that may affect the gene expression of the target enzyme. By constructing knockout strains of these genes, we analyzed the cellulase productivity and the enzyme production ratio. As a result, we were able to find regulatory genes that control the production of specific cellulases. By utilizing these regulatory genes, we have succeeded in improving the saccharification capacity for a specific plant biomass. Although the constructed gene control network is a prototype, we are pursuing information analyses further to improve accuracy based on feedback from our achievements obtained so far. However, even at present, it has been found that genes whose existence was extremely difficult for researchers of *T. reesei* to predict significantly affect the production balance of enzyme components. Therefore, we expect to generate new findings by further promoting this research.

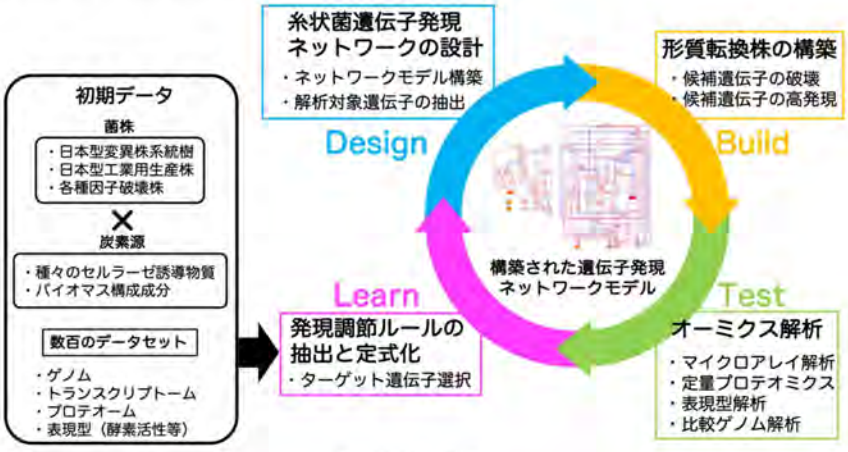

図2　本研究開発の流れ

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Application Examples

THEME

Improvement in productivity to produce useful aromatic compounds using Corynebacteria

Technologies Used

Metabolic Pathway Design, Transgene Sequence Design, Expression Regulation Network Construction, Knowledge Extraction and Learning from Literature, etc. (Proposing Enzymes Utilizing Machine Learning and Knowledge Base Supporting Smart Cell Designing), Transporter Search, High-throughput (HTP) Transcriptome Analysis, High-accuracy Metabolomic Analysis, and Quantitative Targeted Proteomic Analysis

Participating Institutions

Research Institute of Innovative Technology for the Earth (RITE), Institute of Physical and Chemical Research (RIKEN), National Institute of Advanced Industrial Science and Technology (AIST), Kobe University, Kyoto University, Osaka University, Tohoku University, and Hitachi, Ltd.

Research and Developmental Goals

To develop highly productive strains for producing materials that are difficult to produce by fermentation in a short period of time.

Objectives

We aimed to breed and develop strains producing materials that are difficult to produce by fermentation in a shorter period of time than before, by applying the smart cell design technology to Corynebacteria, which are widely used microorganisms for industrial purposes.

RITE has been working on the development of fermentation-based production technologies using *Corynebacterium glutamicum*.[1-3] *C. glutamicum* is a non-pathogenic, non-motile Gram-positive soil bacillus. It was discovered in Japan as a microorganism that excretes glutamic acid into the culture medium. RITE has independently isolated the *C. glutamicum* R strain and determined the complete genome sequences. This time, we proceeded with the development of a production strain, using this bacterium as a production host and one of the useful aromatic compounds (Compound A) as a production target.

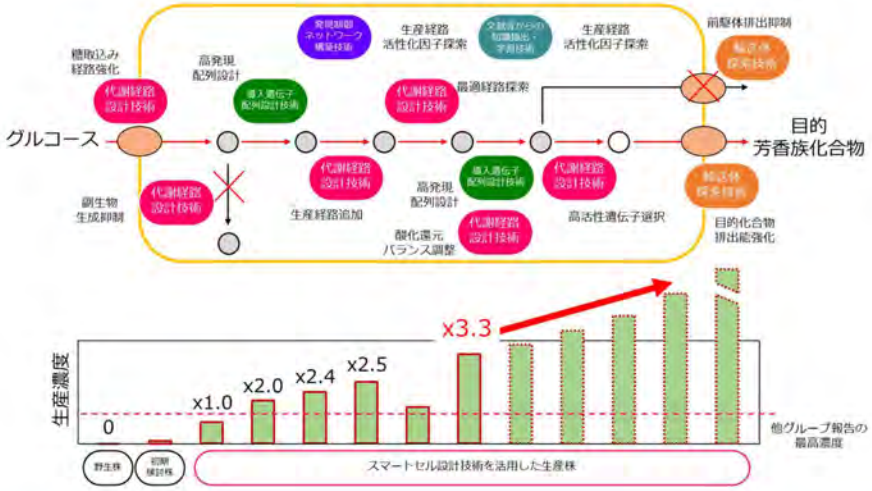
Compound A is not only used in the manufacture of electronic components, but also has a large market as a raw material for pharmaceuticals and perfumes. Although Compound A has such a high industrial utility value, it has never been produced at a high concentration using living organisms. This may be because it is strongly toxic to microorganisms in general and because the metabolic pathway from glucose involves many complicated steps. In addition, *C. glutamicum* does not originally have a pathway for the production of Compound A. Therefore, in this project, we attempted to breed highly productive strains in a short period of time, by making full use of multiple smart cell design technologies.

Results and Achievements

A soil bacterium *C. glutamicum* can degrade Compound A and use the degradation products as a nutrient source. Therefore, we first created Compound A and a knockout strain of the gene for the degradation enzyme of the precursor of Compound A. Using this as the parent strain, we developed a production strain by introducing only genes necessary for the production of Compound A. The production strain developed without using any smart cell design technologies produced Compound A at an extremely low concentration.

Thus, we discovered that Compound A could be produced in *C. glutamicum*, but the production level was extremely low. Therefore, we attempted the application of smart cell design technologies. We obtained a large amount of data from multiple production strains with different genotypes, by utilizing the "High-throughput (HTP) Transcriptome Analysis Technology," "High-accuracy Metabolomic Analysis Technology" and "Quantitative Targeted Proteomic Analysis Technology." Using these data and genomic information, we obtained multiple genetic modification proposals from the "Metabolic Pathway Design Technology" and "Transgene Sequence Design Technology." These proposals included by-product pathway genes to be knocked out, production pathway genes to be highly expressed, foreign genes to be newly introduced, and sequences that can increase expression levels. Based on these modification strategies, we pursued the breeding of *C. glutamicum*, and succeeded in significantly improving its productivity to produce Compound A. As a result, our concentration of the product exceeded the concentrations previously reported by other groups, reaching the highest concentration in the world. Although we have not applied these technologies to the production strains to date, further improvement in productivity is expected because the preliminary analysis results suggesting an improvement have been obtained in modification proposals from the "Expression Regulation Network Construction Technology," "Technology of Knowledge Extraction and Learning from Literature, etc." and "Transporter Search Technology." In addition, it should be noted that each smart cell design technology can be used in combination. Since the approach for developing each metabolic modification strategy is completely different, it is possible to accumulate the modification proposal from each technology in one production strain series in the production strain breeding process. By this property, as shown in the figure below, we have achieved gradual and significant improvement in productivity from the wild strain.

As described above, by utilizing multiple smart cell design technologies, we have achieved both the construction of production strains and a significant improvement in productivity, to produce a useful aromatic compound (Compound A), which is one of the difficult-to-produce target materials, in a short period of time.



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Application Examples

THEME

Improvement in productivity to produce ω-3 polyunsaturated fatty acid-containing lipids

Technologies Used

Expression Regulation Network Construction, Knowledge Extraction and Learning from Literature, etc.(Proposing Enzymes Utilizing Machine Learning and Knowledge Base Supporting Smart Cell Designing), Long-chain DNA Synthesis, High-accuracy Metabolomic Analysis, and Quantitative Targeted Proteomic Analysis

Participating Institutions

Niigata University of Pharmacy and Applied Life Sciences, Fuji Oil Holdings Inc., National Institute of Advanced Industrial Science and Technology (AIST), Institute of Physical and Chemical Research (RIKEN), Nagaoka University of Technology, Osaka University, Kyoto University, Kyushu University, and Kobe University

Research and Developmental Goals

To develop a new oleaginous yeast that can synthesize ω-3 fatty acids with high efficiency, for contributing to the ω-3 fatty acid market where the supply is running short of demand due to overfishing, etc.

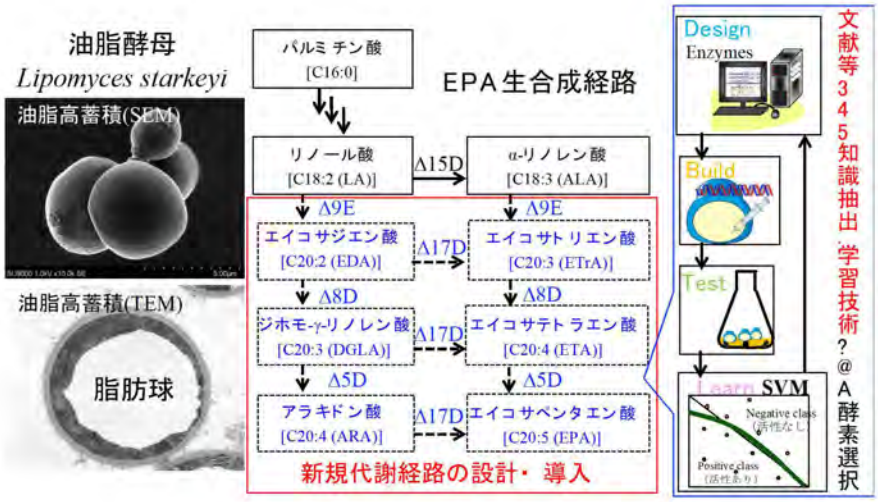
Objectives

ω-3 fatty acids, classified as polyunsaturated fatty acids, are known as essential fatty acids, which cannot be synthesized in the human body, and are vital to maintain the function of the brain and retina. Foods rich in ω-3 fatty acids are limited to some vegetable and fish oils. Therefore, we should consciously consume such foods. In addition, since α-linolenic acid, an ω-3 fatty acid, is converted to eicosapentaenoic acid (EPA), which prevents arteriosclerosis, and also to docosahexaenoic acid (DHA), which prevents dementia, their recommended daily intake has been set in many countries in the world, to ensure their consumption. Due to the rapid increase in the demand for ω-3 fatty acids, the global market is expanding, especially in the emerging countries. However, the supply of fish oil, which is the raw material for ω-3 fatty acids, continues to decline due to overfishing, and therefore the supply meeting the demand may not be secured in future. In some overseas countries, commercial production of ω-3 fatty acids in microalgae has begun. However, the production efficiency is poor, and the price is several times higher than those derived from fish oil. Therefore, the supply from the production to the market is far from adequate. Considering these circumstances, we have focused on *Lipomyces starkeyi*, an oleaginous yeast, which has a high potential to store intracellular lipids in excess of 70% of its dry cell weight, and are aiming to construct a new oleaginous yeast that can produce lipids containing high amounts of ω-3 polyunsaturated fatty acids.

Results and Achievements

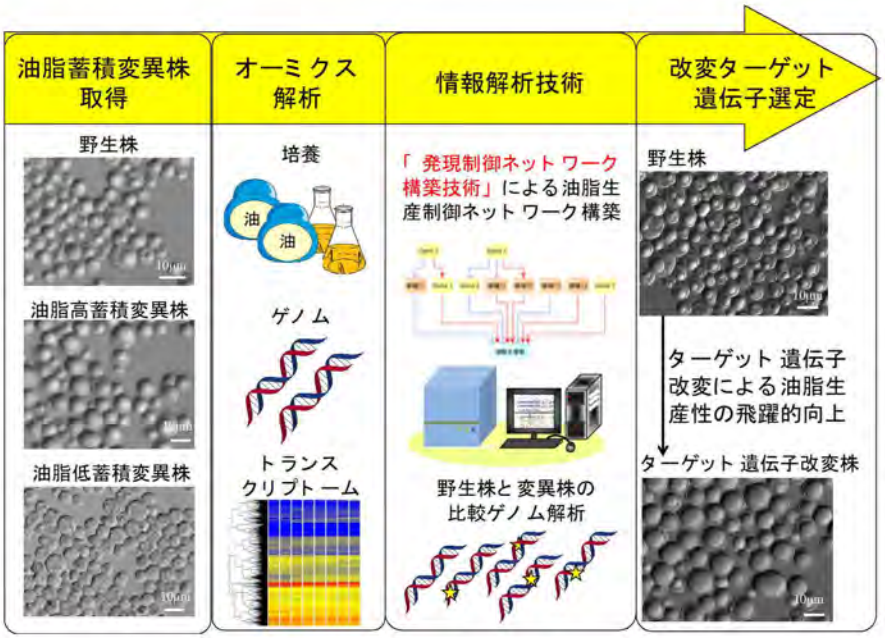
[1] Modification of fatty acid composition (improvement in the ω-3 fatty acid content)

By genomic analysis, etc., *L. starkeyi*, an oleaginous yeast, can only synthesize α-linolenic acid as an ω-3 fatty acid. In order to synthesize EPA and other ω-3 polyunsaturated fatty acids, which are more unsaturated than α-linolenic acid, in oleaginous yeasts, we first introduced the ω-3 polyunsaturated fatty acid synthetic pathway in *L. starkeyi*, using basic technologies for smart cells ("Technology of Knowledge Extraction and Learning from Literature, etc." and "Long-chain DNA Synthesis Technology"). Then, we selected and modified enzymes to improve the ω-3 fatty acid content. As a result, we succeeded in producing ω-3 polyunsaturated fatty acids in *L. starkeyi*, an oleaginous yeast, with an improved content.



[2] Improvement in lipid productivity (improvement in lipid content, and improvement in lipid yield relative to sugar)

In order to improve lipid productivity, we acquired mutant strains that accumulate large amounts of lipid,[1,2] and performed omics analysis ("High-accuracy Metabolomic Analysis Technology" and "Quantitative Targeted Proteomic Analysis Technology"). Then, by a basic technology for smart cells ("Expression Regulation Network Construction Technology") in which the obtained data were utilized, we constructed a lipid production network and have found a new factor controlling the lipid production. In addition, using the factor, we succeeded in an about 2-fold increase in the lipid productivity. Moreover, by identifying causative genes through a comparative genomic analysis of wild strains and mutant strains that accumulate lipids, we found a new lipid production controlling factor different from the above one, and by utilizing the factor, we succeeded in an about 4-fold increase in the lipid productivity.



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Related Patents

Japanese Unexamined Patent Publication No. 2019-146543

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— Application Examples

THEME

High production of alkaloids in Escherichia coli based on metabolic pathway design, enzyme design and metabolomic analysis

Technologies Used

Metabolic Pathway Design, Knowledge Extraction and Learning from Literature, etc.(Proposing Enzymes Utilizing Machine Learning and Knowledge Base Supporting Smart Cell Designing), High-accuracy Metabolomic Analysis

Participating Institutions

Kobe University, Ishikawa Prefectural University

Research and Developmental Goals

To produce benzylisoquinoline alkaloids (BIAs), which are plant-derived natural products, at high levels utilizing microorganisms

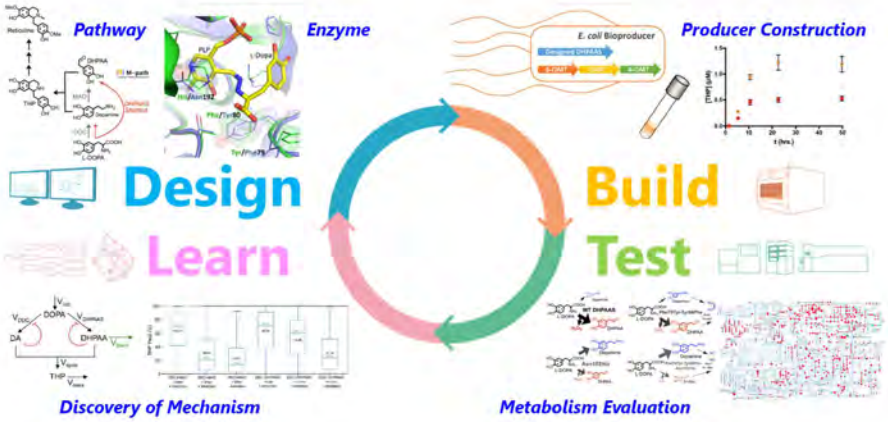
Objectives

BIAs, which are plant-derived natural products, contain many important compounds as raw materials for pharmaceuticals, including opioid analgesics. Conventionally, commercial products have been obtained by extraction from plants, but there were problems in terms of efficiency and cost. Although studies on Escherichia coli- and yeast-based production have been reported in recent years, there has been a demand to improve productivity for practical application due to their low production levels. Therefore, we aim to resolve the bottleneck of BIA production, by using the Design-Build-Test-Learn (DBTL) workflow, which combines bioinformatics and synthetic biology.

Results and Achievements

Previous studies have shown that the activity of an enzyme intracellularly producing tetrahydropapaveroline (THP), a BIA precursor, is weak. Therefore, the resolution of this bottleneck has been a critical issue. In order to create a smart cell that produces highly functional and useful materials in large quantities, it is necessary to design a metabolic pathway that increases the production level and yield, and to transduce genes realizing the designed structure into host microbial cells.

For this purpose, using "M-path," a bioinformatics-based metabolic pathway design tool developed by Dr. Araki, we have designed a new metabolic pathway capable of shortcutting the conventional metabolic pathway bottleneck to contribute to an improvement in the BIA productivity. In addition, we have also succeeded in engineering an enzyme that not only has the new pathway, but also the conventional pathway in a well-balanced manner, by discovering enzymes that constitute the new shortcut pathway from nature and modifying the amino acid sequence through the structure simulation. A verification study conducted by transducing genes related to the designed metabolic pathway and enzymes into Escherichia coli has revealed that both metabolic pathways function well within the bacterium and successfully increased the production level of THP, a metabolic intermediate in the BIA biosynthesis, more than twice the conventional level. Moreover, by metabolomic analysis of the producing bacterium, we have found a metabolism rule that leads to further improvement in productivity. We examined the culture conditions based on this rule, and succeeded in increasing the production level of reticuline, a BIA, more than 7-fold the conventional level. Thus, we are one step closer to the realization of BIA production by the microbial fermentation method.



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— Application Examples

THEME

Development of a highly-productive smart cell that produces the rare amino acid ergothioneine

Technologies Used

Metabolic Pathway Design Technology, High-accuracy Metabolomic Analysis Technology, High-throughput (HTP) Transcriptome Analysis Technology, Enzyme Modification Design Technology, Transgene Sequence Design Technology, High-throughput (HTP) Microbial Construction and Evaluation Technology, Transporter Search Technology

Participating Institutions

NAGASE & CO., LTD., Institute of Physical and Chemical Research (RIKEN), Kobe University, National Institute of Advanced Industrial Science and Technology (AIST), Nara Institute of Science and Technology, and Tohoku University

Research and Developmental Goals

Ergothioneine (EGT) is a natural amino acid contained in trace amounts in mushrooms, etc. and has excellent antioxidant properties. It is expected to be used in a variety of fields, such as food products, cosmetics, and pharmaceuticals. Existing extraction methods and organic synthesis methods involve high costs and exert a high environmental burden, which have become obstacles to promoting the use of EGT.
We aim to establish a bioprocess that can provide inexpensive and highly pure EGT, and to spread it in the market.

Objectives

EGT is a natural amino acid contained in trace amounts in mushrooms, etc. and has excellent antioxidant properties. It is expected to be used in a wide range of fields, such as food products, cosmetics, and pharmaceuticals.(1) Bruce Ames, Professor Emeritus, of the University of California, Berkeley, who is a leading researcher in aging research, said, "EGT is one of the compounds called 'longevity vitamins,' and is essential to long-term health and must be absorbed through diet. Therefore, we should define these compounds as a new class of vitamins."(2) Recent studies have shown that EGT has the potential to eliminate reactive oxygen species, to prevent age-related wrinkles and weak constitutions, and to delay the onset of cognitive decline. It has also been shown that reduced ergothioneine levels in the body correlate with mild cognitive impairment, Parkinson's disease, cataracts, and cardiovascular disease.
EGT is produced only by some microorganisms, including edible mushrooms. In 2005, a mechanism (transporter) was discovered in the human body, which takes up and accumulates EGT in tissues/cells susceptible to oxidative stress and inflammation, such as the brain, skin, eyes, and liver.(3) With the discovery of the transporter and the elucidation of the above function, expectations for the use of EGT have been raised in recent years.
Extraction methods from natural products and chemical synthesis methods are the main existing EGT production methods. However, since extraction methods yield only trace amounts of EGT contained in natural products and chemical synthesis methods place a high environmental burden, an inexpensive, eco-friendly production method has not been established, which have become obstacles to promoting the use of EGT. Under these circumstances, since 2015, the Nagase R&D Center has been working on the development of an eco-friendly bio-production process that enables a stable supply of EGT by a fermentation method using microorganisms.

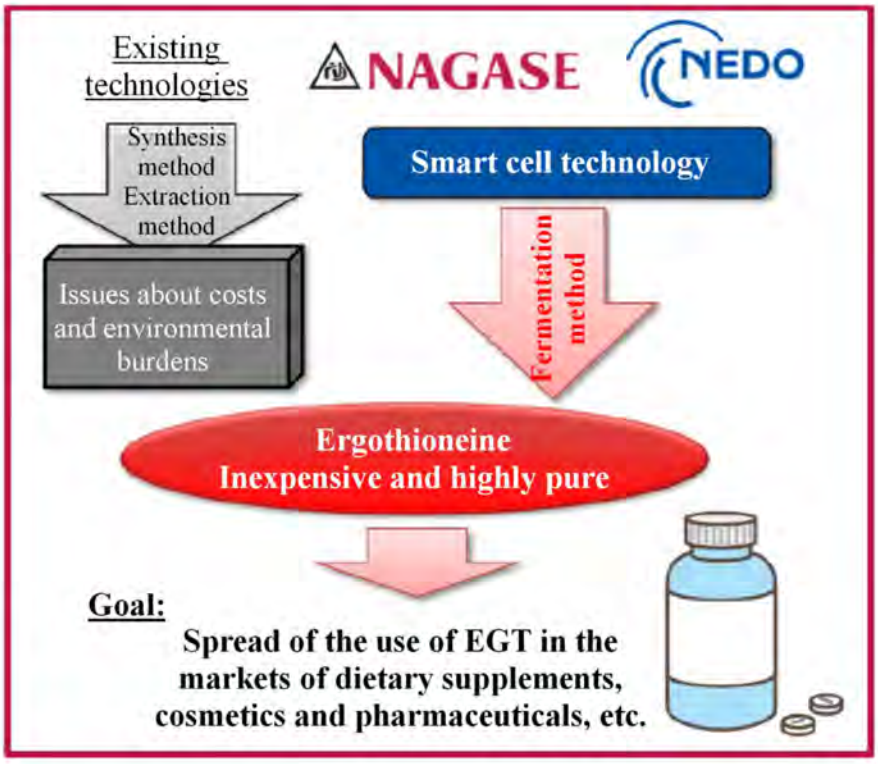

Figure 1 Objectives of this research and development

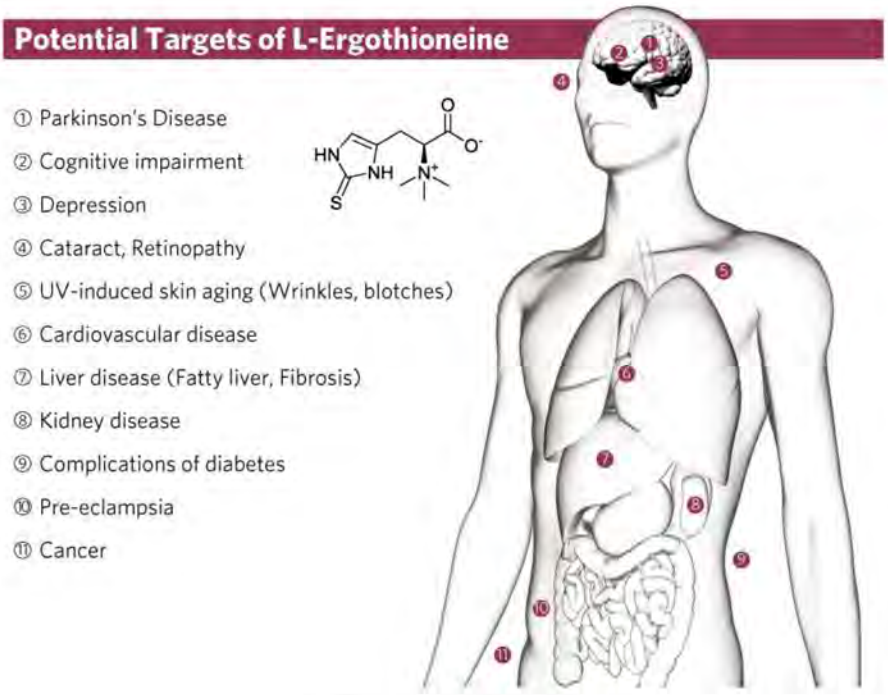

Figure 2 Potential target regions of ergothioneine

Results and Achievements

We participated in NEDO Smartcell Project in 2016. Since then, we have used 6 types of smart cell basic technologies: "Metabolic Pathway Design Technology," "High-accuracy Metabolomic Analysis Technology," "HTP Transcriptome Analysis Technology," "Enzyme Modification Design Technology," "Transgene Sequence Design Technology," "HTP Microbial Construction and Evaluation Technology," and "Transporter Search Technology." With these technologies, we have been working on the improvement of EGT production capabilities of microbial cells. So far, we have succeeded in optimizing the intracellular production reaction by utilizing each smart cell technology, leading to the realization of a dramatic increase in productivity, which is about 1000-fold that at the start of the research.

Currently, we are proceeding with the scale-up of the culture process utilizing the EGT-producing strains that we have developed. In addition, by combining with the advanced purification technology that we have independently developed so far, we are working toward early completion of a bioprocess that can supply inexpensive and highly pure EGT.

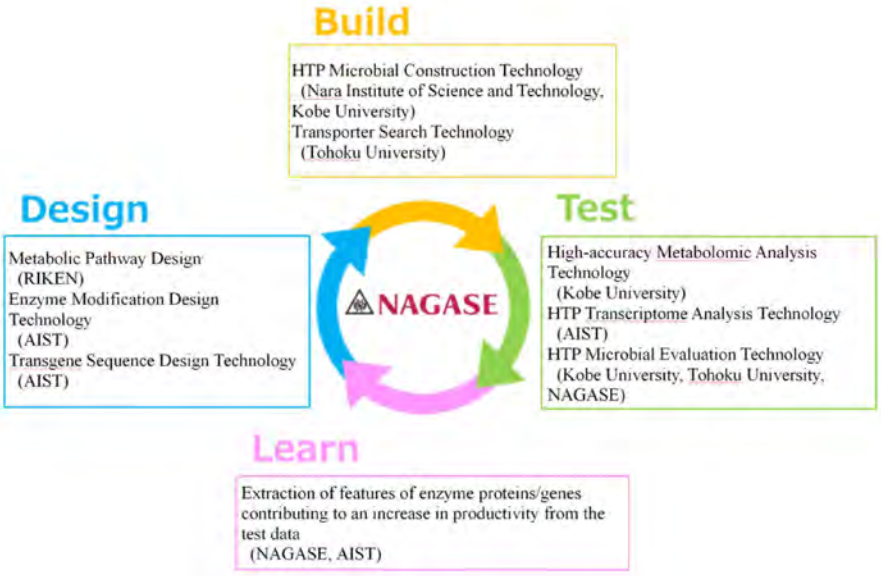

Figure 3 Flow of this research and development

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Individual Technology

TITLE

List of Individual Technologies

About Individual Technologies

In this project, we have developed various basic technologies that are useful for creating smart cells. By using these basic technologies in combination and efficiently running the DBTL cycle, it is expected to improve productivity to produce the target useful compounds.

Design	Metabolic Pathway Design Technology	Tomokazu SHIRAI Institute of Physical and Chemical Research (RIKEN)
Design	Enzyme Modification Design Technology	Tomoshi KAMEDA National Institute of Advanced Industrial Science and Technology (AIST)
Design	Transgene Sequence Design Technology	Tomoshi KAMEDA National Institute of Advanced Industrial Science and Technology (AIST)
Design	Protein Thermostabilization	Tomoshi KAMEDA National Institute of Advanced Industrial Science and Technology (AIST)
Design	Expression Regulation Network Construction Technology	Sachiyo ABURATANI National Institute of Advanced Industrial Science and Technology (AIST)
Build	Useful DNA Elements Extraction Technology	Goro TERAI Graduate School of Frontier Sciences, The University of Tokyo
Build	Chassis Strain Construction Technology	
Build	High-throughput (HTP) Microbial Construction and Evaluation Technology	
Build	Long-chain DNA Synthesis Technology	Kenji TSUGE Graduate School of Science, Technology and Innovation, Kobe University
Build	Target Gene Clone Isolation Technology	
Build	Transporter Search Technology	Keietsu ABE Graduate School of Agriculture, Tohoku University
Test	Metabolite Sensor Construction Technology	Daisuke UMENO Graduate School of Engineering, Chiba University
Test	High-throughput (HTP) Transcriptome Analysis Technology	Yasuo MITANI National Institute of Advanced Industrial Science and Technology (AIST)
Test	High-accuracy Metabolomic Analysis Technology	Tomohisa HASUNUMA Engineering Biology Research Center, Kobe University

Test

Quantitative Targeted Proteomic Analysis Technology

Fumio MATSUDA
Graduate School of Information Science and Technology,
Osaka University

Test

Sample Non-destructive Cell Evaluation Technology

Nobuhiko NOMURA
School of Life and Environmental Sciences,
University of Tsukuba

Learn

Technology of Knowledge Extraction and Learning from Literature, etc.
(Proposing Enzymes Utilizing Machine Learning)

Michihiro ARAKI
Graduate School of Medicine, Kyoto University

Learn

Technology of Knowledge Extraction and Learning from Literature, etc.
(Knowledge Base Supporting Smart Cell Designing)

Michihiro ARAKI
Graduate School of Medicine, Kyoto University

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— Individual Technology

THEME

Metabolic Pathway Design Technology

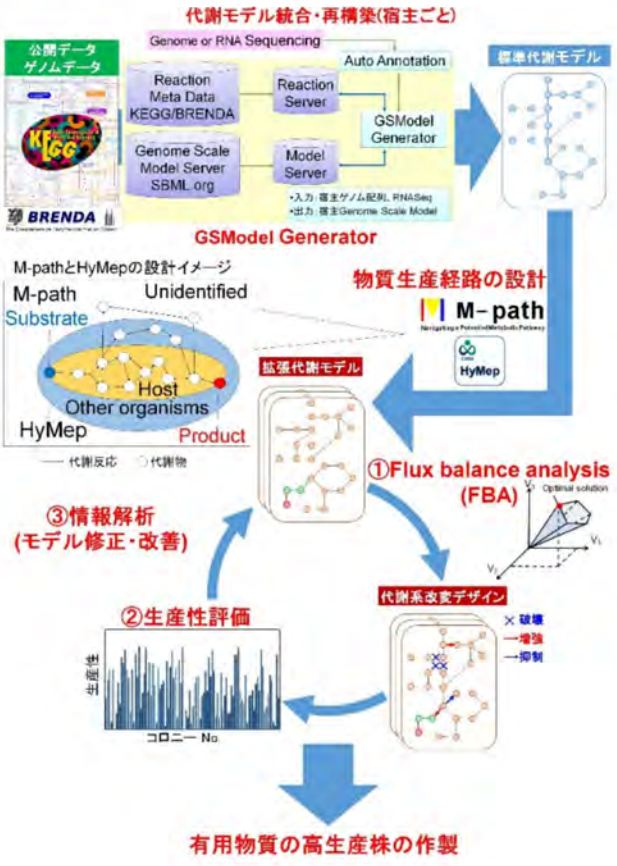
Tomokazu SHIRAI
Institute of Physical and Chemical Research (RIKEN)

Abstract

We aim to propose the design of microorganisms that produce useful compounds with high efficiency, by metabolism design using a metabolic reaction model based on genomic information (Genome Scale Model: GSM). We are working on the practical application of a highly accurate prediction tool into which omics data are introduced, by linking individual metabolic pathway design tools.

Research Contents

For the microbial production of useful compounds, it is essential to have a technology that optimally designs "metabolism" including not only the intracellular carbon flow but also the balance between energy production and consumption and the balance between oxidation and reduction. This is because such a technology allows the understanding of the intracellular phenotype and the information obtained can be applied to the metabolism design of the target cell and to subsequent breeding. However, there is a limit to the human brain's capacity to think about more than 1,000 metabolic reactions occurring in a cell. Therefore, the computing power of computers is indispensable. Especially in recent years, due to the acceleration of the annotation process by the innovations in the genome sequencing technology and information processing technology, it has become possible to describe all metabolic reactions on a computer at the genome scale level. By this improvement, a technology for predicting the metabolic behavior of microbial cells in a certain environment has been established (genome scale model: GSM). Currently, research is being actively conducted by systematically performing the processes from cell metabolism design using GSMs to verification by actual experiments, in order to improve productivity to produce the target compounds in a high-throughput manner. In this project, we have developed a system that automatically prepares metabolic reactions from genomic information. However, the existing GSMs cannot predict or design biosynthetic pathways for non-natural compounds. In addition, design using metabolic reactions that occur in non-host cells is difficult.
In this project, we have developed metabolism design tools that can serve as a technology to solve these problems, and are applying this technology to useful microorganisms.



Strengths in the Industrial Arena

We aim to develop a highly flexible metabolism design system that meets the needs of the industry and research institutions, by operating a metabolism design system that functions interactively with each metabolism design tool developed.
We aim to provide useful information on metabolism design toward the production of target compounds.

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— Individual Technology

THEME

Enzyme Modification Design Technology

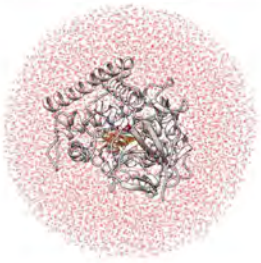
Tomoshi KAMEDA
National Institute of Advanced Industrial Science and Technology (AIST)

Abstract

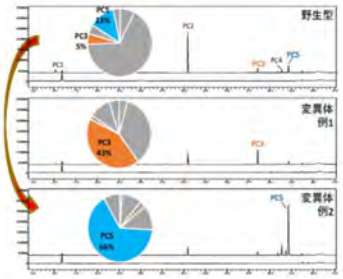
This technology estimates highly active variants through the analysis of enzymes using molecular dynamics (MD) simulations.

Research Contents

In this research, we worked on designing highly active enzyme variants using MD simulations.
By analyzing the orientation and movement of the substrate near the activation site of an enzyme by simulations, we predicted variants that are likely to be highly activated.
We have experimentally confirmed that the fraction of reaction products is improved up to 8.6 times and the reaction volume is improved up to 6.3 times, using the variants designed by this method.



蛋白質＋基質を水中で
MDシミュレーション



変異体１
目的生成物分率
～8.6倍
生成量
～4.3倍

変異体２
目的生成物分率
～5.1倍
生成量
～6.3倍

Strengths in the Industrial Arena

This method can be applied to any enzyme whose three-dimensional conformation has been determined.

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Individual Technology

THEME

Transgene Sequence Design Technology

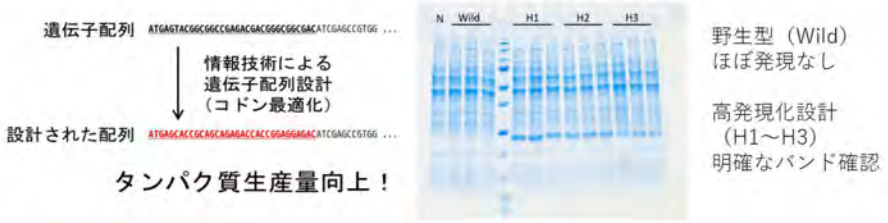
Tomoshi KAMEDA
National Institute of Advanced Industrial Science and Technology (AIST)

Abstract

This technology enables controlling (increasing or decreasing) the expression levels of microbially-produced proteins by using a gene sequence modification method focusing on the mRNA secondary structure and the frequency of codon usage.

Research Contents

In this research, we first analyzed information on protein production experimental data on *Rhodococcus* sp. owned by the National Institute of Advanced Industrial Science and Technology (AIST). The data contained information on the gene sequences of 204 genes, with a link to their protein production levels. More specifically, by calculating various sequence feature values from the genetic sequences, the correlation between protein expression levels and sequence feature values was evaluated.
As a result, the stability of the mRNA secondary structure at the head of the sequence and the frequency of codon usage (Codon Adaptation Index: CAI) were highly correlated with protein production levels.
sequences to prevent the formation of secondary structures and also to have high CAI values.
We have confirmed that this method can improve protein expression levels with a high probability, the success rate being as high as 75%.



Strengths in the Industrial Arena

Although the effectiveness of this method was verified in *Rhodococcus* sp.,[1] this method can be applied to material production in various microbial hosts other than actinomycetes. We have actually confirmed its effectiveness in *Escherichia coli*, Corynebacteria, etc.
In addition, we have succeeded in increasing the expression of target materials that are difficult to be expressed, such as membrane proteins.
Since this method modifies the sequence at only the 5'-terminal head region of a gene sequence, it also allows inexpensive synthesis.

References

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— Individual Technology

THEME

Protein Thermostabilization by MD Simulations

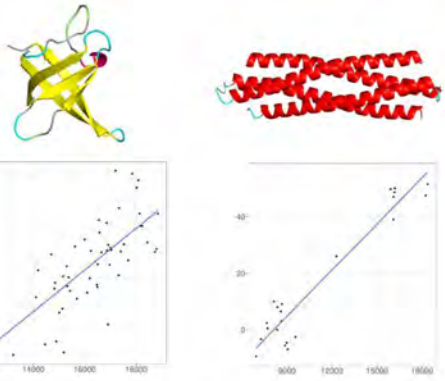
Tomoshi KAMEDA
National Institute of Advanced Industrial Science and Technology (AIST)

Abstract

We discovered that the thermal stability (degeneration temperature) of protein variants can be predicted with high accuracy by analyses using molecular dynamics (MD) simulations. We designed thermally-stable variants based on this method and verified an actual increase in stability.

Research Contents

In this research, we worked on protein thermostabilization (increase in thermal stability) using MD simulations.
Protein variants were simulated at high temperature, and the time to degeneration was estimated.
It was found that the estimated time correlated well with the degeneration temperature obtained experimentally.
Therefore, various variants were simulated to predict the variants that are likely to increase thermal stability.
Actual increase in the thermal stability of these variants was confirmed by calorimetric experiments.



Strengths in the Industrial Arena

This method can be applied to any enzyme whose three-dimensional conformation has been determined.

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Individual Technology

THEME

Expression Regulation Network Construction Technology

Sachiyo ABURATANI
National Institute of Advanced Industrial Science and Technology (AIST)

Abstract

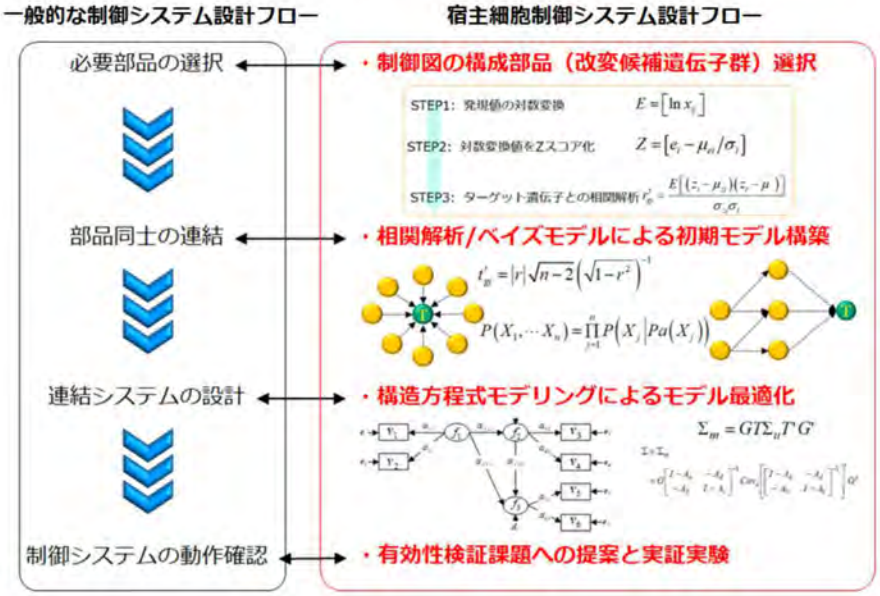
We aim to understand the underlying mechanisms of phenomena occurring in microbial cells during material production to regulate the phenomena as one operation system. For that purpose, we propose candidate genes to be modified for artificial regulation, by selecting genes contributing to material production from gene expression data and expressing their interactions as a network model.

Research Contents

In order to achieve efficient material production using microorganisms, it is necessary to understand the underlying mechanisms of phenomena occurring in microbial cells during material production and regulate the phenomena as an operation system. For understanding and utilizing this complex "system" in living cells, a network model of gene expression regulation is useful. By constructing the regulatory relationship between genes as a network model, we were able to express the process occurring in living cells in a causal graph. This has led us to the development of a technology that allows the search for modification sites necessary for the bottleneck search in the process and for promoting efficiency.

As a technology for estimating gene groups that contribute to material productivity, we have developed a work flow that allows the gene groups related to material production to be extracted with higher accuracy, by combining statistical testing, correlation analysis and logical operations.

After selecting candidate genes as parts of the regulatory system, network modeling of the regulatory relationships between genes was performed. Using the network model, we have identified the genes which can be regulatory factors to control the production level of a target material in the material production process, from the selected genes groups that are likely to correlate with the production levels of target materials. In this study, we are working on the high-accuracy estimation of network structures, by combining Bayesian networks[1] and the structure equation modeling[2,3] developed by the National Institute of Advanced Industrial Science and Technology (AIST).



Strengths in the Industrial Arena

So far, we have proposed many candidate genes to be modified that could not be searched for by the conventional breeding approach, and succeeded in about a 2- to 10-fold increase in the production level. Thus, this technology can contribute to the creation of highly productive microorganisms.

Furthermore, this technology has a wide scope of application: it is applicable not only to gene expression data, but also to any numerical data. It is a technology that can be applied not only to material production using microorganisms, but also to cases where the regulation of certain types of systems that are "black boxes" is required.

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2) S. Aburatani : Gene Reg. Sys. Biol., 5, 75-88 (2011)

3) S. Aburatani and H. Toh : Encyclopedia of Information Science and Technology, 3rd Edition, pp.458-478, IGI Global (2015)

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— Individual Technology

THEME

Useful DNA Elements Extraction Technology

Goro TERAI
Graduate School of Frontier Sciences, The University of Tokyo

Abstract

The technology for synthesizing a wide variety of long-chain DNAs at once is called the combinatorial long-chain DNA library technology. We are developing a technology for efficiently running the DBTL cycle, using the data obtained by this technology.

Research Contents

In designing the gene circuit necessary for building a smart cell, it is required not only to prepare a necessary set of genes, but also to adjust the expression level of each gene so that the genes can function in a coordinated manner. In the construction of a new metabolic pathway, 10 or more genes may be required, and there are many sites (parameters) for which the expression level needs to be adjusted. In the past, it took years to adjust the parameters, and improving its efficiency was a challenge. We are working on the development of a method to solve this problem, by utilizing the combinatorial long-chain DNA library technology based on the OGAB method.[1] For example, we have developed a technology that allows simultaneous synthesis of a combinatorial long-chain DNA library in which genes, such as A, A', and A'', whose expression levels and activities are slightly different, are randomly coded at certain sites on long-chain DNA (Fig. 1). From the production levels and the long-chain DNA sequences of the strains into which variable long-chain DNAs have been introduced, combinations of multiple genes that contribute to high production of the target material are extracted. For example, as shown in Fig. 1 (a), if the presence of the "Orange Gene A" combined with any other genes makes the strain highly productive, its rule can be easily realized by sequencing some of the long-chain DNAs of the highly productive strain. However, as shown in Fig. 1 (b), where the presence of two of the "Orange Genes U, W and Z" makes the strain highly productive, the production level is often determined by the combination of multiple genes. Therefore, we have developed a method for discovering rules even in the case of Fig. 1 (b). For a strain possessing a combination of extracted genes, the introduction of a combinatorial long-chain DNA library having another set of genes into the subsequent DBTL cycle* may make it possible to discover a strain that can produce the target material at a higher production level. By combining the construction of a strain into which a long-chain DNA library is introduced and the information analysis of the obtained data in this manner, highly productive strains can be produced with a small number of DBTL cycles.

*: A cycle of "Design → Built → Test → Learn"

(a) 単一の遺伝子が生産量に影響を与える例

(b) 複数遺伝子の組合せが生産量に影響を与える例

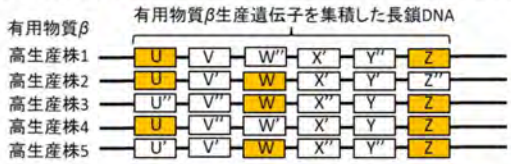

図1．長鎖DNAコンビナトリアルライブラリの概要とその解析結果のイメージ

Strengths in the Industrial Arena

We are developing and verifying a method for efficiently obtaining highly productive strains of target compounds, by combining the combinatorial long-chain DNA library technology and information analysis.

References

1) K.Tsuge *et al.*, Method of preparing an equimolar DNA mixture for one-step DNA assembly of over 50 fragments. Sci Rep. 2015 5:10655

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Individual Technology

THEME

Chassis Strain Construction Technology

Abstract

in preparation

Research Contents

in preparation

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Individual Technology

THEME

High-throughput (HTP) Microbial Construction and Evaluation Technology

Abstract

in preparation

Research Contents

in preparation

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— Individual Technology

THEME

Long-chain DNA Synthesis Technology

Kenji TSUGE
Graduate School of Science, Technology and Innovation, Kobe University

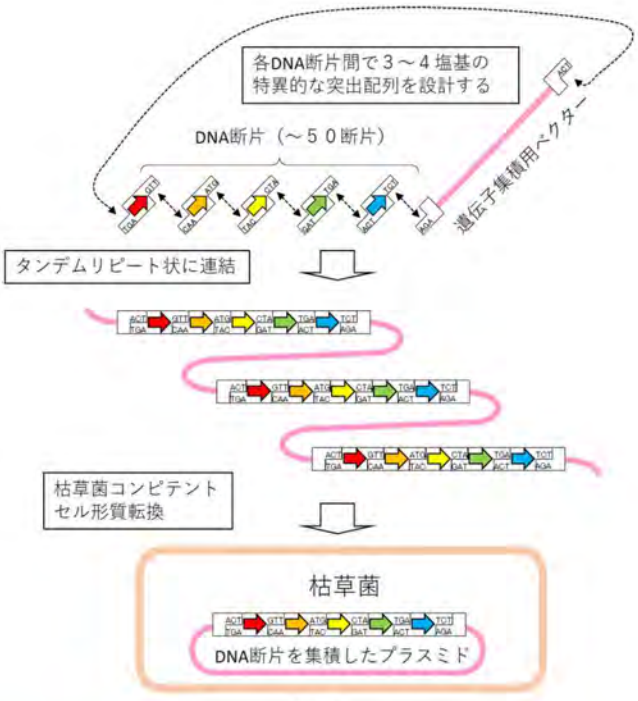
Abstract

In the development of smart cells, the construction of bacterial strains with designed long-chain DNA sequences conventionally required several months, and this has been hindering the acceleration of the development. With this series of technologies we developed this time, it has become possible to accurately construct long-chain DNA of more than 30 kb in size in a short period of about 2 weeks at a low cost.

Research Contents

For constructing long-chain DNA, the ordered gene assembly in *Bacillus subtilis* (OGAB) method, which we developed, was used.[1,2] The OGAB method is a method for assembling multiple DNA fragments using the plasmid transformation system of *B. subtilis*. Utilizing the specificity of 3 to 4 bases at the end of a DNA fragment, the method enables one-step assembly of a maximum of more than 50 DNA fragments. So far, this synthesis has been outsourced to a DNA synthesis company. When ordering the synthesis of 50 DNA fragments at a time, however, cases such as that where the synthesis of some fragments failed or was rejected occur although many DNA fragments are successfully synthesized. Accordingly, the synthesis sometimes takes as long as 2 months to finally prepare all the necessary DNA fragments.

Therefore, in order to shorten the time taken for DNA fragment preparation, a total system capable of one-stop processing, from the construction of long-chain DNA through chemical synthesis to mass preparation, was established, and technologies were developed from a comprehensive perspective. Individually, through joint research with Nihon Techno Service Co., Ltd., we have developed a high-throughput DNA chemical synthesizer specializing in the synthesis of long-chain DNA, at a low cost. In addition, we have also developed a new PCR method for annealing and elongating chemically synthesized DNA using DNA complementarity. This made it possible to prepare double-stranded DNAs of any sequence in about 3 days. In addition, the cloning process in *Escherichia coli*, in which this double-stranded DNA fragment is cloned in *E. coli* and only the selected clones having the correct base sequence are used as a material in the OGAB method, was substantially automated by a liquid dispensing robot. As a result of these research and development activities, it has become possible to produce long-chain DNA of about 30 kb in a short period of about 2 weeks at a cost of several yen per base.



OGAB法

Strengths in the Industrial Arena

The OGAB method allows inexpensive, short-time synthesis of long-chain DNA that could not be synthesized conventionally due to the long period of time required or high GC contents. The achievements from this research and development were commercialized as a long-chain DNA synthesis business by Synplogen Co., Ltd., a business venture spun off from Kobe University.

References

1) K. Tsuge *et al.* : Nucleic Acids Res., 31, e133 (2003)

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Related Patents

Japanese Patent No. 4479199 Japanese Patent No. 6440636

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Individual Technology

THEME

Target Gene Clone Isolation Technology

Nozomu YACHIE

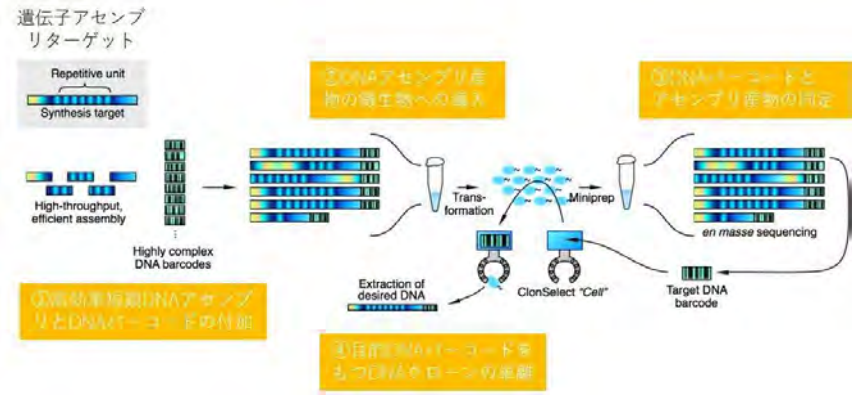
Research Center for Advanced Science and Technology, The University of Tokyo

Abstract

The efficiency of any DNA assembly reaction is incomplete. Through genome editing, we have established a technology to selectively label and isolate only microbial cell clones having the desired reaction product after DNA assembly reactions.

Research Contents

Since the efficiency of any DNA assembly reaction is incomplete, the assembly reaction products require cloning through transduction into microbial cells and the evaluation by DNA sequencing. Consequently, samples obtained from reactions with lower efficiency require a larger number of clones for the evaluation, and this process becomes a bottleneck. We have established a selective clone isolation technology, which is as follows: after molecular DNA barcoding of assembly reaction products, the reaction product pool is analyzed all at once using the en masse sequencing technology, and then cells having the target reaction product are labeled and isolated in a DNA barcode-dependent manner by genome editing. This technology accelerates the throughput of the conventional DNA assembly process by 1000-fold.



Strengths in the Industrial Arena

The construction ("Build" process) of long-chain DNA is the key component of the DBTL cycle. Without accelerating this process, the scale of useful microbial production processes cannot be expanded. Until recently, there was no method for efficiently obtaining the target DNA assembly product after DNA assembly. Due to this limitation, although efforts had been made to improve the efficiency of the DNA assembly itself, there was no method to substantially accelerate the "Build" process for various cases of DNA assembly. This technology resolves this situation.

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Individual Technology

THEME

Transporter Search Technology

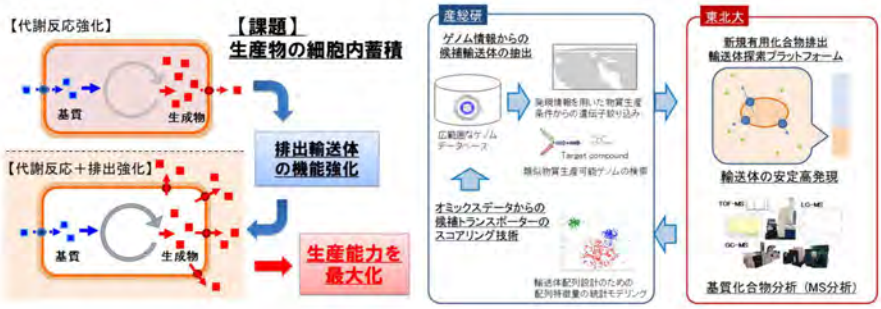
Keietsu ABE
Graduate School of Agriculture, Tohoku University

Abstract

Intracellular conversion of materials requires the uptake of substrate. In addition, the product cannot be recovered in the medium unless it is discharged out of cells. technology searches for compound transporters, which is important for promoting efficiency in material production.

Research Contents

When a compound is produced using a microorganism, etc., particularly when a membrane-impermeable compound is targeted, the discharge of the product out of the host cell affects the efficiency of production. If the extracellular discharge of the product is delayed, the product accumulates inside the cell, causing negative feedback, leading to the inhibition of the biosynthetic reaction. In such cases, the solution is to express a "transporter," which properly transports the target compound out of the cell, on the cell membrane. In this project, by integrating the information analysis technology owned by the National Institute of Advanced Industrial Science and Technology (AIST) and the transporter search technology developed by Tohoku University, we have developed a technology for searching for transporters of the target compounds while shortening the time required with the conventional transporter search method.



Strengths in the Industrial Arena

Recent advances in genomic analysis have revealed the presence of genes encoding 300 to 1,000 transporters on the genome of living organisms. On the other hand, the functions of many transporter genes remain unknown; even though the search for the efflux transporter of the target compound is attempted based on literature information and genomic information, obtaining the information on the transporter of the target is extremely difficult. This technology has simplified the search for transporters of target compounds, and is expected to improve efficiency in material production.

References

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Related Patents

Japanese Patent Application No. 2018-087700

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— Individual Technology

THEME

Metabolite Sensors

Daisuke UMENO
Graduate School of Engineering, Chiba University

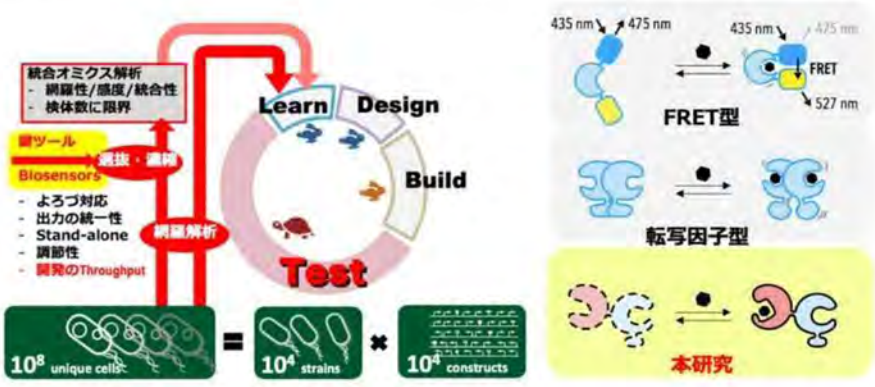
Abstract

We are developing technology for the on-demand development of sensors for various metabolites, with the reproducibility and throughput required for smart cell development. We use these sensors to determine suitable culture conditions and cells for material production and to identify bottleneck reactions in metabolic biosynthetic pathways.

Research Contents

Metabolomic analysis can simultaneously identify the accumulation levels of various metabolites present in cells, but it is difficult to expand the number of specimens. On the other hand, biosensors allow simultaneous detection and analysis of thousands to millions of specimens although they can be applied only to a limited number of metabolites. In the current situation where throughputs in "Design" and "Build" have been increased dramatically, it becomes possible to run the high-quality DBTL cycle at a high speed, by developing and organizing sensors that can detect any metabolite at any sensitivity. The use of this technology in combination with metabolomic analysis allows the identification of suitable culture conditions and cells for material production.

There are two main types of biosensors: the FRET type and the transcription factor type. Both types read out structural changes associated with binding to target metabolites. Even if evolutionary engineering technologies are used, it takes time to develop biosensors. In this research, we adopt a principle that allows high-speed development of sensors, without requiring the design of structural changes. The sensor development process consists of (1) the selection of the enzyme that uses the target metabolite as a substrate, (2) fusion with the transcription factor at the gene level, (3) the introduction of random mutations, and (4) the selection of functions. With the completion of this process, it became possible to produce a biosensor that responds to any metabolite in about one month.



メタボライトセンサの作製図

Strengths in the Industrial Arena

Through the development of this technology, various metabolite sensors can be developed with high flexibility. It is also possible to line up sensitivities and output characteristics. By preparing biosensors for various metabolites and using them in combination with the high-throughput transformation system available in this project, a high-throughput DBTL cycle can be realized. Since biosensors report intracellular metabolite levels, they can also be applied to comprehensive analysis. Therefore, the use of this technology in combination with metabolomic analysis also enables analyses of cells including transport systems.

References

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3)Y. Kimura, et al.: ACS Synth. Biol., 9, 567-75 (2020)

Related Patents

Japanese Patent No. 5959127; Japanese Patent No. 5904494 (US-9315816); Japanese Patent No. 5757608; and Japanese Patent Application No. 2018-057314

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Individual Technology

THEME

High-throughput (HTP) Transcriptome Analysis Technology

Yasuo MITANI
National Institute of Advanced Industrial Science and Technology (AIST)

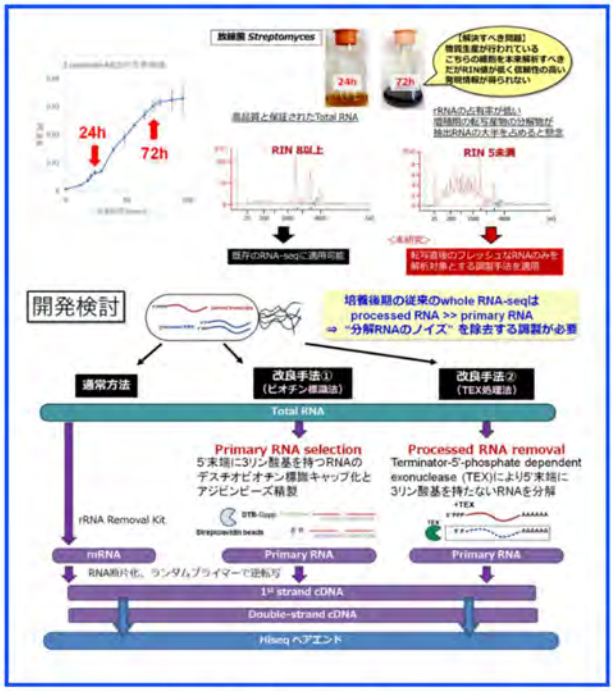
Abstract

In order to establish a highly reliable transcriptome analysis technology, we intend to develop various spike-in control materials, and to perform verification within the project first. Furthermore, we are developing various technologies aiming to apply them to industrial microorganisms.

Research Contents

In order to establish a highly reliable transcriptome analysis technology that is applicable to various industrial microorganisms, we will develop nucleic acid reference standards for spike-in control and verify their utilization strategies. In addition, for that purpose, we will establish a simple method for the valuation of the nucleic acid reference standards.
In some industrial microorganisms, the production level of a targeted useful material increases especially at the end of the culture period. However, it is difficult to extract RNA in good condition from such samples. Therefore, we are developing a technology that allows the transcriptome analysis, which has been difficult to perform so far, by establishing a technology for selectively analyzing only non-degraded RNAs.

スパイクイン用核酸標準物質の開発と検証



未分解RNA選択的調整技術の適用

Strengths in the Industrial Arena

Today, to improve the efficiency of material production using microorganisms, RNA-Seq transcriptome analysis is an essential technology element. Therefore, while various kits are available, it is necessary for each user to confirm the applicability of these kits, and many users tend to determine the applicability just based on their experience. In addition, also in terms of obtained data, the results may become meaningless unless a proper quantitative comparison between samples is feasible. We are developing technologies so as to make available the technologies and know-how that can serve for solving issues.

References

1) Mitani Y. *et al.*: Smart Cell Industry, CMC Publishing, (2018)

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— Individual Technology

THEME

High-accuracy Metabolomic Analysis Technology

Tomohisa HASUNUMA
Engineering Biology Research Center, Kobe University

Abstract

This technology provides metabolomic data with the reproducibility and throughput required for smart cell development. It enables the identification of culture conditions and cells suitable for material production and also the identification of bottleneck reactions in metabolic biosynthetic pathways by highly reproducible multi-sample processing and comprehensive detection of water-soluble metabolites using robots. This clarifies the causal relationship between genetic modifications and the material production capacity of cells.

Research Contents

Metabolomic analysis is a technology that can simultaneously identify the pool sizes of various metabolites present in cells. As metabolomic data reflect the growth environment and genetic background of cells, it becomes possible to identify culture conditions and cells suitable for material production by performing metabolomic analysis.

The metabolomic analysis process comprises the pretreatment process for extracting intracellular metabolites from a cell suspension, the metabolite quantitation process using LC-MS/MS, etc., and the data analysis process. Conventionally, the pretreatment process involves complicated manual operations, which has caused data variation. Therefore, we have fully robotized this process, and achieved a processing speed that is 10 times or more faster than that achieved by humans, with higher reproducibility than that achieved by experts.

For the analysis after the pretreatment, the S/N ratio has been improved by constructing an LC-MS/MS system that requires no ion-pairing agents. In addition, the method has enabled the isolation and detection of 158 water-soluble metabolites required for the designing of microbial smart cells.

In the metabolomic analysis, in which the identification and relative quantitation of various metabolites are performed for each sample, the amount of data processing is enormous. Therefore, we are constructing an information analysis system that assists in the process of peak picking from chromatograms and projects the analysis results on a metabolic map.

図1．メタボローム解析用自動前処理ロボット

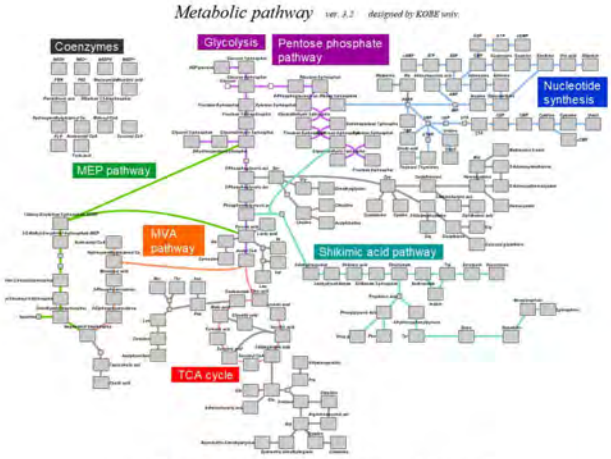

図2．測定可能な水溶性一次代謝物の代謝マップへの投影例

Strengths in the Industrial Arena

By obtaining a large amount of highly reproducible metabolomic data through the development of this technology, the metabolic control mechanism, which has been a black box, can be elucidated, and a DBTL cycle that incorporates the mechanism analysis can be developed. The elucidation of the metabolic control mechanism provides extremely useful information for acquiring intellectual property rights for groundbreaking production strains (smart cells) and the production methods. Since this method can be applied to the comprehensive analysis of metabolites secreted extracellularly, it is also effective for the evaluation of production strains during the bioproduction process (calculation of mass balance, etc.).

References

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Individual Technology

THEME

Quantitative Targeted Proteomic Analysis Technology

Fumio MATSUDA
Graduate School of Information Science and Technology, Osaka University

Abstract

We developed a method of highly sensitive simultaneous quantitation of the expression levels of dozens of proteins in smart cells. We have completed the construction of multiple reaction monitoring (MRM) assay methods for main metabolic enzyme proteins, such as E. coli, Saccharomyces cerevisiae, Corynebacterium glutamicum and oleaginous yeasts. We have built a database of MRM assay methods constructed in the Smartcell Project.

Research Contents

The Smartcell Project requires rational modifications of metabolic pathways in order to maximize the material production capability of cells. To modify metabolic pathways, the expression levels of enzyme proteins are increased or decreased by artificially modifying the genome of the host microorganism. Since the protein amount in cells involves various factors associated with transcription, translation and proteolysis, it is necessary to have a measurement method for rapidly evaluating whether the amount of enzyme protein has been adjusted as designed. Under these circumstances, we focused on the high-accuracy quantitative targeted proteomic analysis as an optimal method for the evaluation of smart cells. In this method, a trypsin-digested peptide mixture is separated by liquid chromatography and analyzed in the multiple reaction monitoring (MRM) mode of a triple quadrupole mass spectrometer. We are developing a targeted proteomic analysis system authentically made in Japan, in collaboration with Shimadzu Corporation (Fig. 1).

The sample pretreatment was started with a crude protein extract of about 100 µL containing 50 µg of total proteins. After a reductive alkylation of this extract, trypsin digestion is performed overnight, and the resulting trypsin-digested peptides are desalted by solid phase extraction. We have confirmed that the pretreatment method for *Saccharomyces cerevisiae* is effective against various useful microorganisms, such as oleaginous yeasts, *E. coli* and *Corynebacterium glutamicum.* Data were obtained by nano-LC-MS/MS (LCMS-8060, Shimadzu Corporation). This system achieved a throughput of a sample size of 20 to 30, which is the size of a general analysis project, in about 2 days.

We have completed the construction of MRM assay methods for main metabolic enzyme proteins, such as *E. coli*, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum* and oleaginous yeasts. In addition, we are building a database of the MRM assay methods constructed, so as to establish a platform that can be customized as required.

- ターゲットタンパクのトリプシン消化ペプチドをナノLC-MS/MSで定量する。
- 従来法（二次元電気泳動）より高い**選択性、定量精度、スループット**
- 各宿主、各酵素タンパク質毎に**MRMメソッドの作成**が必要

Strengths in the Industrial Arena

This technology allows highly sensitive simultaneous quantitation of the expression levels of dozens of proteins.
Antibody preparation is not required.

References

1) F. Matsuda *et al.* : Mass Spectrometry, 6(1), A0056 (2017)

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Individual Technology

THEME

Sample Non-destructive Cell Evaluation Technology

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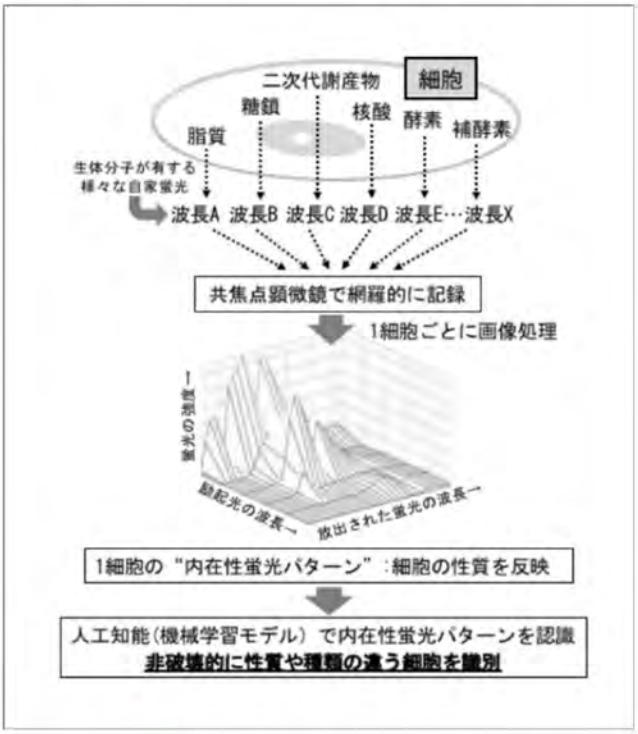
Abstract

We have developed the confocal reflection microscopy-assisted single-cell innate fluorescence analysis (CRIF) method, which is a cell evaluation technology. It enables the non-destructive identification of cell types at the resolution of a single cell, using a cellular autofluorescence pattern as the index, and the estimation of the metabolic state of the cell.

Research Contents

Intracellular proteins and metabolites emit autofluorescence of various wavelengths and intensities. The autofluorescence pattern, which is formed by the synthesis of respective autofluorescence, functions as a "fingerprint" that expresses the properties of each cell. In the CRIF method,[1] the information on cell position and morphology is obtained by reflection microscopy,[2,3] and cellular autofluorescence information is obtained by confocal laser microscopy. Then, by performing image analysis for each cell, the CRIF systematically and comprehensively extracts autofluorescence information for each cell, and reconstructs the information as an autofluorescence pattern. Thus, a "cell's fingerprint" that identifies each cell can be obtained. Moreover, it has been clarified that the application of the "cells' fingerprints" to various types of machine learning enables the construction of a machine learning model that reflects each cell's underlying characteristics in the autofluorescence pattern, and also enables high-accuracy identification of cell types and prediction of the metabolic state. As this method uses the autofluorescence emitted by cells, no special treatment, such as the introduction of fluorescent protein-expressing genes or staining, is required. In other words, the properties of intact living cells can be analyzed. Conventional methods using fluorescent protein labeling require complicated gene manipulation to track specific cells and monitor gene expression. On the other hand, the CRIF method is expected to identify cells with the targeted properties in an extremely simple manner. Moreover, due to the use of a confocal platform, this method can be applied to the analysis of a three-dimensional space, enabling the evaluation of the properties of cells that constitute three-dimensional structures, such as colonies and biofilms. Thus, by linking the information on the cell to spatial information, this method is expected to contribute to the discovery of new knowledge.



Strengths in the Industrial Arena

Since the CRIF method is a simple method that can analyze the properties of intact living cells at the single cell level, it is considered to be a key technology that improves the efficiency of the cell property evaluation, which is essential for cell breeding technologies in various fields, such as microorganisms, plants and animals, stem cell differentiation induction technologies, and technologies for the synthesis of artificial cells. By actively communicating with various research institutions and companies, we aim to establish the CRIF as a highly versatile technology so that it can be used in a wide variety of fields from basic research in various fields to applied research on, for example, regenerative medicine, in the future.

References

1) Japanese Patent No. 6422616: Data creation method and data use method; October 26, 2018; Inventor: Nobuhiko Nomura et al.; Patent holder: University of Tsukuba

2) Y. Yawata *et al.*: Appl. Environ. Microbiol., 85, e00608-19 (2019)

3) Y. Yawata *et al.*: J. Biosci. Bioeng., 110, 377-380 (2010)

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Individual Technology

THEME

Technology of Knowledge Extraction and Learning from Literature, etc. (Proposing Enzymes Utilizing Machine Learning)

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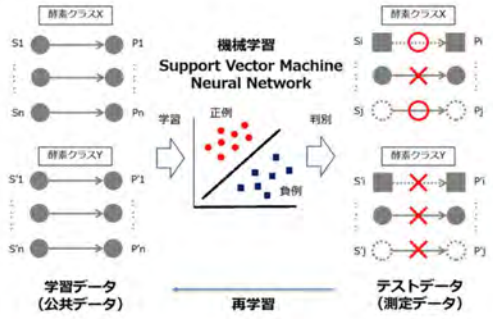
Abstract

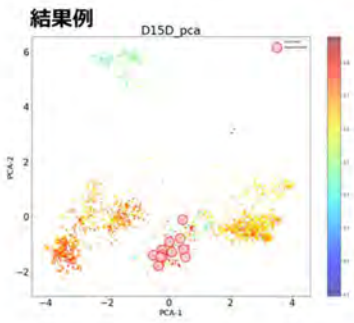
The information analysis system for designing smart cells depends on the knowledge extraction from literature and database information, during not only the processes from metabolic pathway design to the construction and optimization of the metabolism model, but also the search of enzyme genes and candidate genes to be modified. We are developing a machine learning technology focusing on enzyme gene search.

Research Contents

Estimated multiple enzyme gene candidates, either unknown or known, will appear in metabolic pathways constructed by metabolism design. Therefore, the selection of enzyme genes in the actual construction of a metabolic pathway is an important issue. However, at present, also regarding this point, each researcher manually searches the information separately contained in various enzyme reaction databases such as KEGG and BRENDA, as well as literature and patent information, and makes decisions based on his or her intuition. Under these circumstances, there is a strong demand for the development of an efficient and reliable method for selecting enzyme genes.

The machine learning method is an approach to discriminate and classify test data to be predicted, based on the learned data. It has been applied in various fields, due to an increase in data volume and an improvement in computing power in recent years. This field is no exception; if the machine learning method enables learning based on the known enzyme reaction data and leads to the discovery of new enzyme reactions, it can be a very useful method. We aim to extract features that cannot be identified by conventional sequence comparison methods and clustering methods, by developing a machine learning method that considers the combination of substrates/products and enzyme amino acid sequences and a deep learning-based method for extracting the features of an enzyme's amino acid sequences.





Strengths in the Industrial Arena

This technology can contribute to the discovery of new useful enzyme genes, in the search for enzyme genes to realize new metabolic pathways and the search for enzyme gene variants having new substrate specificities and activities in the known metabolic pathways.

References

1) Y. Saito *et al.* : Sci. Rep., 9(1), 8338 (2019)

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— Individual Technology

THEME

Technology of Knowledge Extraction and Learning from Literature, etc. (Knowledge Base Supporting Smart Cell Designing)

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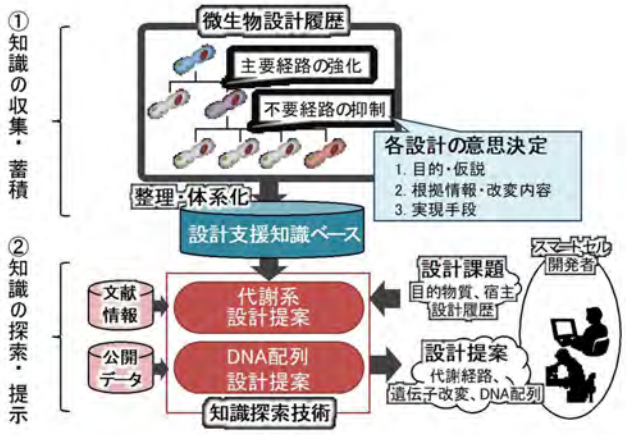
Abstract

The information analysis system for designing smart cells depends on the knowledge extraction from literature and database information, during not only the processes from metabolic pathway design to the construction and optimization of the metabolism model, but also the search of enzyme genes and candidate genes to be modified. We are developing a tool to develop a knowledge base from literature.

Research Contents

We are working on knowledge base concept design, development of elemental AI technologies, and workflow development, aiming to construct a knowledge base specialized in smart cell development and to develop AI technologies that support the knowledge base construction.
The construction of a knowledge base requires the development of two main processes (Fig. 1). That is, (1) knowledge collection and accumulation: the process of accumulating various trial-and-error results in the past smart cell development as reusable "knowledge" by organizing the relations among the events, (2) knowledge search and presentation: the process of presenting information on events, hypotheses, causal relationships, or reference literature that supports interpretation or decision-making by the user, from the accumulated knowledge, in response to the queries of the smart cell developers (users).
For Process (1), we are organizing and systematizing the history of strain modifications that have been conducted to realize the desired smart cells, namely, the design history. More specifically, contents of the decision-making in the preparation of each modified strain are normalized in terms of "purposes of the modification and hypothesis," "information that serves as a basis and contents of modification," and "means for achieving the modification (means for synthesis and verification)" and stored in the database engine.
The constructed database is utilized for cross-sectional search in terms of the "purposes of the strain modification," "modification contents," or "means for the modification" in the knowledge extraction process described later. In addition, by hierarchically organizing the derivational relations from the parent strain that is the origin of the modification, some effects, such as becoming aware of overlooked modifications or experiments and supporting the idea of new design hypotheses based on such awareness, are expected. In Process (2), we are developing a knowledge search technology that presents design proposals to the user by searching the database for the knowledge relevant to the query entered by the user through the operation terminal. More specifically, it is a technology in which, using the target material/host and the history of designs conducted so far as a query, information highly related to the contents of the query is cross-sectionally searched from the information accumulated in the database, literature, etc., and the searched knowledge, such as a metabolic pathway, gene modification, and DNA sequence, is presented to the user in an easy-to-understand format.



Strengths in the Industrial Arena

This technology allows exhaustive and comprehensive processing of a huge amount of knowledge from literature and publicly accessible data in a short time, as well as proposal of the standard design of smart cells. It is also expected to propose a "serendipitous" design principle. Thus, the technology can contribute to the acceleration of smart cell creation.

References

1) Y. Saito *et al.* : Sci. Rep., 9(1), 8338 (2019)

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Frequently Asked Questions

1) I would like to learn more about the project.

Q: I would like to learn about the technologies developed in the project. What should I do?

A: The period of this project is 5 years from 2016 to 2020. The materials for the Research Evaluation Committee held on August 29, 2018 can be downloaded at the following link.

Subcommittee Meeting of the Research Evaluation Committee for "Development of Highly Functional Materials Production Technologies Using Plants and Other Organisms" (Interim Report)
https://www.nedo.go.jp/introducing/iinkai/ZZBF_100309.html

Q: Is there any plan to hold a technology seminar?

A: We are planning to hold technology seminars in 2020, which is the final year of the project. For details, send us your questions through the "Contact Us" page on this website.

Q: Can you hold a seminar at my institution?

A: It is possible, but needs to be coordinated with the project personnel with whom you would like to attend.

Q: Are there any books about the technologies developed in the project?

A: There is a book called "Smart Cell Industry: Prospect of Bio-Based Material Production Using Microbial Cells," published by CMC Publishing Co., Ltd. on June 20, 2018 (ISBN: 978-4-7813-1334-4). In addition, those technologies are also described in the journal of the Japan Bioindustry Association (JBA), "Bioscience & Industry."

Link to the book:https://www.cmcbooks.co.jp/products/detail.php?product_id=5447

2) I would like to use the technologies developed.

Q: What should I do to use the technologies developed?

A: Please contact us from the "Contact Us" page on this website. If required, we will hold an interview to hear your request (free of charge).

Q: Is there any possibility that the details of my question will be disclosed externally?

A: We will never disclose such information externally. However, please note that we may share information with the project leader, Dr. Satoru Kuhara, Professor Emeritus at Kyushu University, and NEDO's staff members, if necessary, for example, regarding whether there is an appropriate researcher who can respond to the consultation.

Q: Tell me about expenses for joint research.

A: When conducting joint research with the project personnel, expenses may be incurred as joint research expenses, according to the rules of the organization to which the project personnel belong. The actual research expense will vary depending on the details and duration of the research. Therefore it will be determined upon consultation.

Q: What happens to the intellectual property rights that have resulted?

A: Before starting the joint research, please confirm matters relating to the intellectual property rights regarding the results obtained through the joint research with the project personnel.

3) Other matters including requests

- Please discuss the terms of the non-disclosure agreement, and specific conditions for technology provision, technology alliance, etc. between the parties.
- Please discuss thoroughly the rights and obligations in the use of technologies and joint research agreements between the parties.
- The JBA may inquire about the matching status, progress of the research, etc. We appreciate your understanding and cooperation.
- We will not disclose individual company names and the research details, but may analyze the utilization status of the technologies and use the results.

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