Molecular Microbiology and Biotechnology Group

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Development of a Biorefinery Technology that Contributes to Carbon Neutrality

1. Introduction

The global trend toward decarbonization is accelerating, and each country is actively implementing support for green research and development activities and support for the introduction of advanced green technologies. In October 2020, the Japanese government declared that Japan would be "Carbon Neutral by 2050," and therefore aims to reduce greenhouse gas emissions to net zero by 2050. Because bioprocess manufacturing proceeds under normal temperature and pressure conditions, CO_2 emissions are expected to be lower than for comparable chemical processes, which are manufactured under high temperature and high pressure conditions. In addition, unlike chemical processes, "bio-manufacturing" generally involves synthetic processes involving multi-step reactions within cells. Moreover, bioproduction of complex chemical structures containing a large number of carbon atoms are more competitive than alternative methods of generating these chemicals. At the 4th Conference for the Realization of New Capitalism in March 2022, Prime Minister Kishida positioned "bio-manufacturing" as one of the five fields of science, technology, and innovation that will be target for intensive investment in the future. At the conference, it was also stated that "'bio-manufacturing' will be promoted as a research field that can pursue both solutions to the problem of global warming and economic growth."

Rapid progress has been made in recent years, in biotechnology, synthetic biology, genome editing technology, and in similar scientific fields. Furthermore, technological innovation in “bio-digital technology,” a fusion of biotechnology with (digital) information technologies (i.e., internet of things, IoT, and artificial intelligence, AI) are also developing at a rapid pace. The anticipated "bio-digital technology" will accelerate the social implementation of "bio-manufacturing" that can convert a wide range of raw materials, including petroleum, biomass resources, and even atmospheric CO_2. It is therefore expected to make a significant contribution to achieving carbon-neutrality and/or carbon-negativity.

Given this background, and with the aim of helping the global environment and the economy to coexist, our group has been developing biorefineries. A biorefinery is a biofuel and green chemical production technology that uses renewable resources (e.g., biomass) as a raw material for processing by microorganisms. We have observed that coryneform bacteria (a typical industrial microorganism) show suppressed growth under reducing (anaerobic) conditions, but they maintain normal metabolic function. Under such conditions, these bacteria can metabolize saccharides to produce organic acids and related compounds efficiently. We therefore developed a growth-arrested bioprocess called the "RITE Bioprocess." In addition, we have established the essential technologies for industrial implementation of this process, as shown in the "complete simultaneous use of mixed sugar derived from non-food biomass" and "high resistance to fermentation inhibitors" sections (see Chapter 2).

Using these technologies, we seek to develop the world's most highly efficient process for producing biofuel products, including ethanol, isobutanol, and biohydrogens, among others, and green chemicals, including lactic acid, succinic acid, alanine, valine, tryptophan, shikimic acid, protocatechuic acid, 4-aminobenzoic acid, and 4-hydroxybenzoic acid. Currently, we focus on the development of production technologies for aromatic compounds, which are used as raw materials for higher value-added fragrances, cosmetics, pharmaceuticals, fibers, and polymers. (see Chapter 3).

In addition, to develop new technologies—and to integrate biotechnology and digital technology—we participated in the New Energy and Industrial Technology Development Organization (NEDO) "Smart Cell" project, the Cross-ministerial Strategic Innovation Promotion Program (SIP), and the NEDO "Bio-Manufacturing" project. We also promote research and development, for example, by using "Smart Cell Creation technologies" to improve the efficiency of biosynthesis and the production processes for highly functional chemicals that are difficult to produce using conventional synthesis methods. In July 2022, a project to demonstrate and commercialize the production of fragrances and carotenoids in collaboration with private companies using "Smart Cell Creation technologies" was adopted as a NEDO bio-manufacturing demonstration project. We are also participating in the NEDO Moonshot Project and are working on research and development of marine-degradable multi-lock biopolymers produced using non-food biomass (see Chapter 4).

In this overview, we first explain our core technology, i.e., the "RITE Bioprocess," as well as our new core technologies, i.e., "Smart Cell Creation technologies." Next, we introduce the development results we will target using these. We then describe national projects based on innovation in "bio-digital technologies," which have made remarkable progress in recent years. Finally, commercialization efforts are introduced.

2. The core technologies of RITE

2.1 "RITE Bioprocess"

RITE Bioprocess, developed by our group, is a proprietary technology that enables highly efficient production of biofuels and green chemicals such as amino acids and aromatic compounds (Fig. 1). The three features of RITE Bioprocess are described below (for details, see RITE Today 2022).

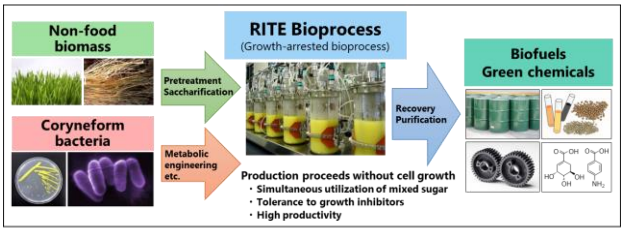

Fig. 1 Biorefinery concept using the "RITE Bioprocess"

2.1.1. Feature 1: Growth-arrested bioprocess

Anaerobic conditions and the removal of factors essential for proliferation allow the desired series of reactions to take place even when cell proliferation is arrested (Fig. 2). That is, nutrients and energy previously used for propagation can now be used for the production of the target substance. This has made it possible to use microbial cells extremely efficiently like a chemical catalyst, realizing a bioprocess with high productivity equal to or greater than that of ordinary chemical processes.

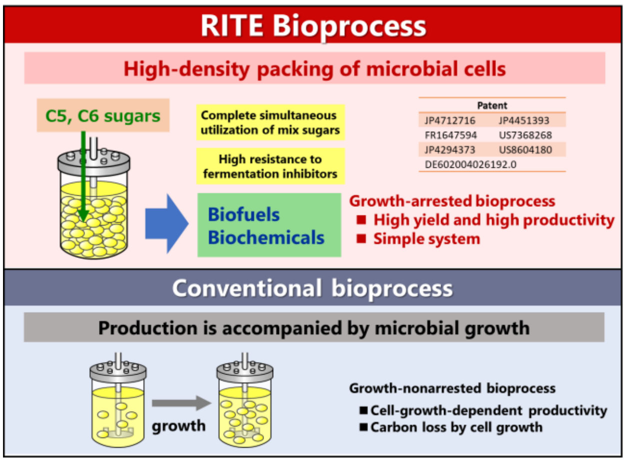

Fig. 2 Feature 1 of the "RITE Bioprocess"
(Growth-arrested bioprocess)

2.1.2. Feature 2: Complete simultaneous use of C5 and C6 mixed sugars

Most non-food biomass (cellulosic biomass) is composed of a mixture of C5 sugars such as xylose and arabinose and C6 sugars such as glucose.

Our group has succeeded in increasing the utilization rate of C5 sugars to that of C6 sugars by introducing a C5 sugar transporter gene in addition to a C5 sugar metabolism gene (Fig. 3). This has enabled full simultaneous utilization of C5 & C6 sugars and efficient utilization of cellulosic (non-food biomass) materials.

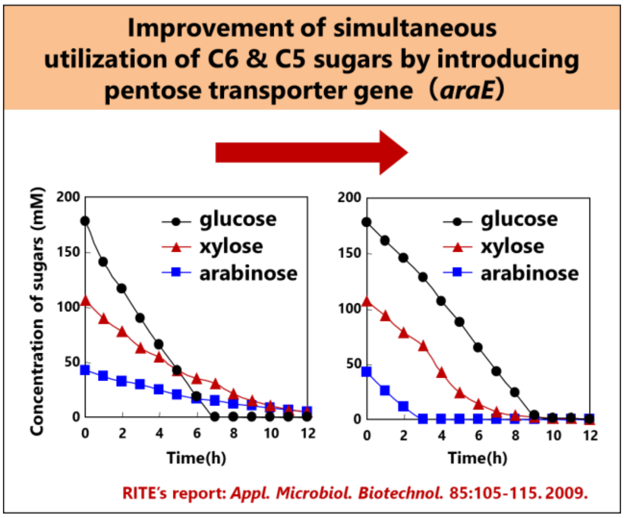

Fig. 3 Feature 2 of the "RITE Bioprocess"
(Simultaneous usage of mixed sugars)

2.1.3. Feature 3: High tolerance to fermentation inhibitors

RITE Bioprocess has demonstrated high resistance to fermentation inhibitors because the microorganisms do not proliferate as described above (Fig. 4). Therefore, it can be applied to the use of saccharification solutions containing various fermentation inhibitors, and even to the production of fermentation inhibitors.

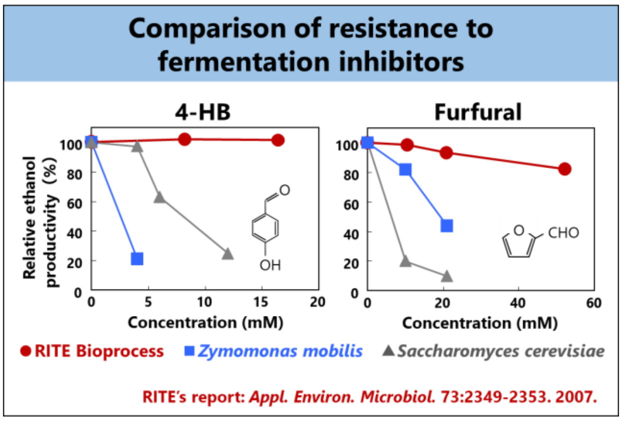

Fig. 4 Feature 3 of the "RITE Bioprocess"
(High tolerance to fermentation inhibitors)

2.2. New core technologies of RITE

In recent years, biotechnology has become more sophisticated, and new applications of IT and AI technology are becoming more common. In multiple national projects, we have collaborated with universities, research institutes, and companies to design and breed "biological cells with highly functionally designed and controlled functional expression" so-called smart cells (see Chapter 4). Some of these new and useful technologies are introduced below.

2.2.1. Smart Cell Creation technologies

In general, a "Smart Cell" is a finely designed cell in which protein and RNA expression is controlled by genetic modifications. More specifically, our group refers to cells whose functions have been designed using cutting-edge information analysis technology and biotechnology to maximize the production of target materials as "Smart Cells."

The technologies that efficiently create smart cells are called "smart cell creation technologies" or "smart cell design systems" (Fig. 5). Our group has participated in the NEDO Smart Cell Project (2016-2020) and the NEDO Bio-Manufacturing Project (2020-ongoing) and has developed these technologies. We have also demonstrated the effectiveness of these technologies by developing highly productive *C. glutamicum* strains.

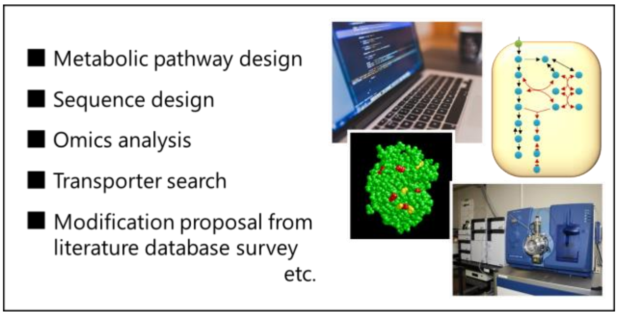

Fig. 5 Smart Cell Creation technologies

2.2.2. Continuous reaction system

Our group has developed biotechnological processes for the production of various compounds. During this development, we found that production can cease due to the cytotoxicity caused by the accumulation of the target compound itself in the reaction solution. For example, the production of catechol, which is under development in both the NEDO Smart Cell Project and the NEDO Bio-Manufacturing Project, generated sufficient cytotoxicity to cease production when using a conventional batch method. Therefore, in order to avoid cytotoxicity and maximize high production, we constructed a continuous reaction system that can selectively remove and recover the target compound from the reaction system. For example, by constructing a continuous reaction system that combines resin adsorption and membrane separation (shown in Fig. 6). When we applied it to catechol production, we achieved a very high catechol yield.

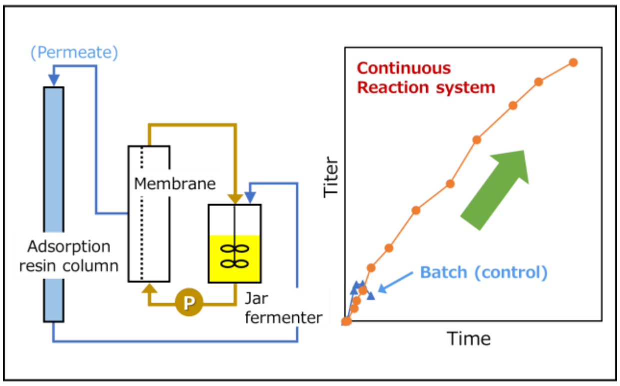

Fig. 6 A continuous reaction system using membrane separation and resin adsorption

2.3. Substances produced by the "RITE Bioprocess"

Fig. 7 shows some of the substances that are currently produced in high quantities by our group. As mentioned above, this process has resulted in highest recorded production yields for many materials. We are expanding from ethanol and biohydrogen to butanol and high-performance bio-jet fuel. We have also expanded from L-lactic acid, D-lactic acid, and amino acids to high-performance chemicals, including green chemicals that contain aromatic compounds.

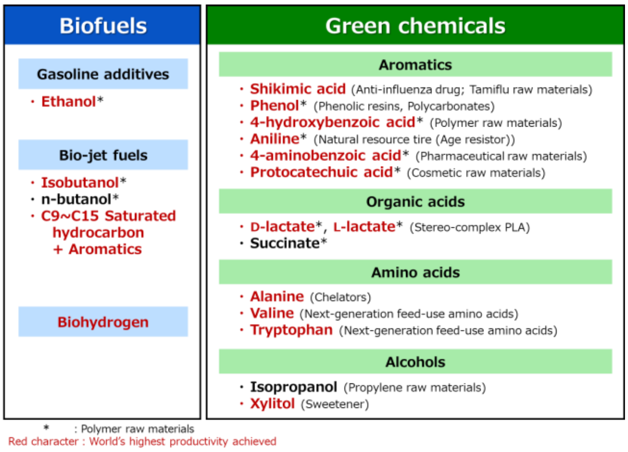

Fig. 7 Substances produced using the "RITE Bioprocess"

3. Target product development

3.1. Bio-jet fuels

Starting from ethanol (C2) or butanol (C4), jet fuel (C9-C15) can be produced via chemical conversion. Given the importance of replacing crude oil products with plant-derived raw materials for reducing CO_2 emissions from aircraft, industry groups are accelerating efforts to produce bio-jet fuels from plant-derived bioethanol or biobutanol. Jet fuel made from bioethanol or biobutanol is called ATJ ("Alcohol to Jet") fuel. Such fuels have been approved by the American Society for Testing and Materials (ASTM) and can be used for commercial flights.

Our group has developed a highly efficient bioethanol and biobutanol production process using the RITE Bioprocess. Moreover, this process is the world's most productive for the bioproduction of ethanol and isobutanol.

In the future, we will further optimize the RITE Bioprocess technology, integrate it with other elemental technologies, and utilize various non-edible raw materials. Ultimately, we aim to commercially produce jet fuel from bioethanol and biobutanol.

3.2. Biohydrogen

Hydrogen is a key energy carrier for realizing carbon neutrality because (i) its combustion generates only water; (ii) it can be produced from diverse energy sources, including renewables; (iii) it can be stored in large quantities for long periods; and (iv) it can be distributed and used for power generation, transportation, and industrial processes. However, current-generation hydrogen production processes use fossil fuels as a feedstock and therefore present problematic levels of CO_2 emissions. Therefore, a Basic Hydrogen Strategy was drawn up at the Ministerial Council meeting on Renewable Energy, Hydrogen, and Related Issues in 2017. This conference noted the importance of developing innovative technologies for CO_2-free hydrogen production to build a hydrogen society over the medium to long term (i.e., by 2050). Moreover, hydrogen strategies have been developed in many countries around the world. For example, the US Department of Energy launched the "Energy Earthshots Initiative," in which the first "Hydrogen Shot" was set as a challenging goal, i.e., reducing the cost of CO_2-free hydrogen by 80% within one decade. Regarding the promising CO_2-free hydrogen production pathways, fermentative hydrogen production from biomass/waste has been under development, as has large-scale hydrogen production in combination with CCUS from fossil resources and water electrolysis using renewables.

Fermentative hydrogen production using microbial functions (biohydrogen production) can be a sustainable CO_2-free hydrogen production technology of the future, but a great increase in productivity is needed to establish an economically feasible biohydrogen production technology. The key to achieving this is the creation of highly efficient hydrogen-producing microorganisms through advanced biotechnology. Our group has developed a biohydrogen production process with a very high production rate (i.e., max 300 L H_2/h/L). This process uses the formate-mediated dark fermentative hydrogen production pathway. Building on this achievement, our group is now working on improving hydrogen yield from biomass-derived sugars by integration with photofermentation (Fig. 8). We have enhanced a heterologously expressed hydrogen production pathway that can utilize the excess reducing power not utilized in the formate-mediated dark fermentative hydrogen production pathway, and have also succeeded in establishing a photofermentative hydrogen producing microorganism by engineering regulators of hydrogen metabolism, carbon storage, and acetate metabolism.

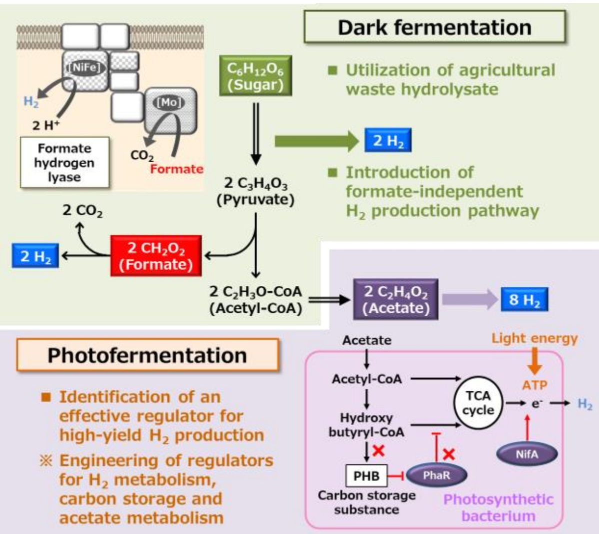

Fig. 8 Metabolic engineering of dark fermentative and photofermentative hydrogen-producing microorganisms

3.3. Green-aromatic compounds

Aromatic compounds are essential industrial chemicals for synthesizing polymers and various value-added chemicals for use in the pharmaceutical, nutraceutical, flavor, cosmetic, and food industries. Although they are currently derived from petroleum or natural plant resources, environmentally friendly biotechnological production from renewable feedstocks is desirable to create a sustainable society that is no longer dependent on petroleum resources and but maintains highly efficient production processes. Bacterial cells can synthesize various aromatic compounds, including amino acids (i.e., phenylalanine, tyrosine, and tryptophan), folate (vitamin B_9), and coenzyme Q, all of which are derived from the shikimate pathway. Therefore, by employing the metabolically engineered *C. glutamicum*, our group successfully established a highly efficient bioprocess for producing the aromatic compounds from non-food feedstocks. These include shikimate, an essential building block of the anti-influenza drug Tamiflu; 4-aminobenzoate, the building block of a potentially useful functional polymer; and aromatic hydroxy acids, having potential applications in the polymer, pharmaceutical, cosmetic, adhesive material, and flavor (vanillin) industries.

Since 2022, our group has also participated in the NEDO “Bio-Manufacturing” project with Takasago International Corporation. We are driving development of an "industrial smart cell" that can produce rose fragrance and a bioproduction system that can avoid microbial inhibition derived from fragrance materials (Fig. 9).

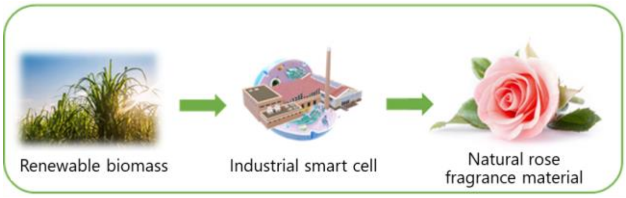

Fig. 9 Production of rose fragrance material

4. Fundamental technology development (national projects)

4.1. NEDO Bio-Manufacturing Project

We are developing Next-generation production technologies for the foundation of the bio-manufacturing industry in the "Development of bio-derived product production technology that accelerates the realization of carbon recycling" (NEDO Bio-Manufacturing Project).

Bio-based material production is expected to save energy relative to conventional chemical processes. In addition, the use of biomass as a raw material instead of fossil resources can greatly contribute to the realization of a carbon recycling society.

During the predecessor of this project, "Development of Production Techniques for Highly Functional Biomaterials Using Plants and Other Organisms" (NEDO Smart Cell Project), our group—in collaboration with universities, research institutes and companies—developed technologies to design and construct Smart Cells. We then demonstrated the effectiveness of these technologies by constructing hyper producer cells in a short period of time. To further promote the social implementation of Smart Cells, the Bio-Manufacturing Project will develop production process technologies including upscaling and purification.

Our group has been participating in this project, which started in 2020, from the first year, and is developing new technologies to solve the problems associated with the practical application of biomanufacturing technologies (Fig. 10).

In FY2022, we promoted the development of new technologies to help overcome product toxicity. In addition, we developed information analysis technologies to determine optimal components of culture media. This year, we will develop other useful technologies and verify their effectiveness. We aim to accelerate the social implementation of bio-derived products through the development of these technologies.

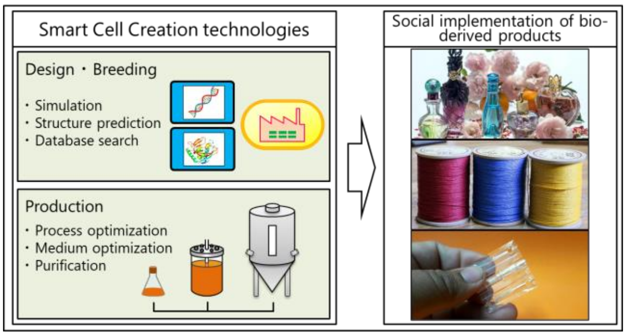

Fig. 10 Social implementation of bio-derived products using Smart Cell Creation technologies

4.2. NEDO Moonshot-type R&D Project（Development of Multi-lock Biopolymers Degradable in Ocean from Non-food Biomasses）

The project is carrying out research and development to introduce a "multi-lock mechanism" for plastic degradation. In other words, multiple stimuli such as light, heat, oxygen, water, enzymes, microorganisms, and catalysts should be required to trigger for start degradation, but prevent degradation and maintain durability and toughness when in use, and when accidentally dispersed in the marine environment, the multi-lock mechanism is unlocked to enable fast on-demand degradation.

Products targeted for practical application in this project include tires and textiles, which generate secondary fine debris when used, as well as plastic bottles, fishing nets and fishing tackle that contribute to ghost fishing, all of which have a negative impact on the environment due to runoff into the ocean.

In FY2022, our research group successfully achieved high production of several monomers requested by some companies by designing artificial metabolic pathways, constructing high production strains, and testing production conditions. In the future, we aim to further increase of the production titer through modification of the artificial metabolic pathway. In FY2022, we succeeded in improving the production concentration of plastic-degrading enzymes by introducing mutations, and achieved remarkable improvement of functionality. In the future, we aim to conduct research and development, including the development of technology to artificial control for the timing of the starting point of degradation of multi-locked plastics (development of new technology utilizing degradation enzymes).

(The HP of the project can be found at: http://www.moonshot.k.u-tokyo.ac.jp/en/index.html).

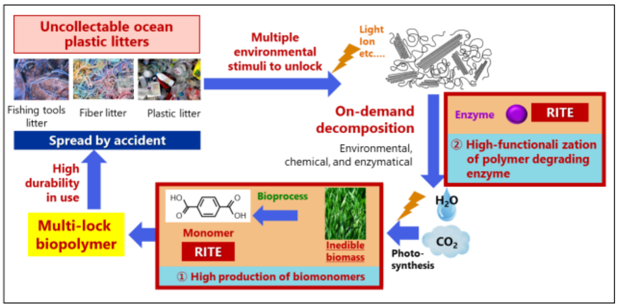

Fig. 11 Marine-degradable multi-lock biopolymers fabricated from non-food biomass

4.3. The Cross-ministerial strategic innovation promotion program (SIP)

(Development of biomonomers for high-performance plastics)

In bio-based material production, production of the target compound is made possible by a successfully constructing a specific biosynthetic pathway for that compound. Biosynthetic pathways are constructed using multiple enzymes, and the starting compound or substrate is converted via enzymatic reaction to a bio-compound, which in turn becomes the substrate for the next enzyme and undergoes conversion. More than 8000 enzymatic reactions are currently known, and by combining these in specific ways, biosynthetic pathways are improved and expanded.

RITE is participating in the theme “Technologies for Smart Bio-industry and Agriculture” of the SIP, a joint program between industry, academia, and the government designed to apply findings from basic research for practical and industrial applications beyond the scope of government ministries and traditional disciplines. The goal of this project is to realize a sustainably growing society and is made possible by manufacturing technologies that employ bio-capabilities developed by fusing biotechnology and digital resources.

Our group participates in the consortium “Development of Technologies for Functional Design and Production of Innovative Biomaterials.” This consortium aims to synthesize polymers with new functions—in response to market desire—using monomers bio-synthesized from biomass and other cheap raw materials. To date, ultra-high heat-resistant polymers and polymers used as battery materials have been successfully developed (Fig. 12). In order to design monomer biosynthetic pathways for constructing such polymers, our group, in conjunction with external research groups, has been developing and validating technologies for improving enzymatic function. We have focused on enzymes involved in the biosynthesis of monomer substrates underlying polymers of interest. In greater detail, we are developing technologies for the efficient selection of promising enzyme mutants from the activity data of many mutant enzymes using machine learning. We are also developing technologies for discovering novel enzymes possessing desired functions based on enzyme amino acid sequence data. These techniques have allowed us to improve the substrate specificity of target enzymes and enhance activity. In 2022, not only were we able to increase productivity by metabolic engineering and optimization of culture conditions, but we also produced monomers from a biomass-derived raw material provided by the collaborative consortium as a substrate. Moreover, we are verifying the effectiveness of enzyme modification technology by targeting a new enzyme involved in the biosynthetic pathway for aromatic diamine.

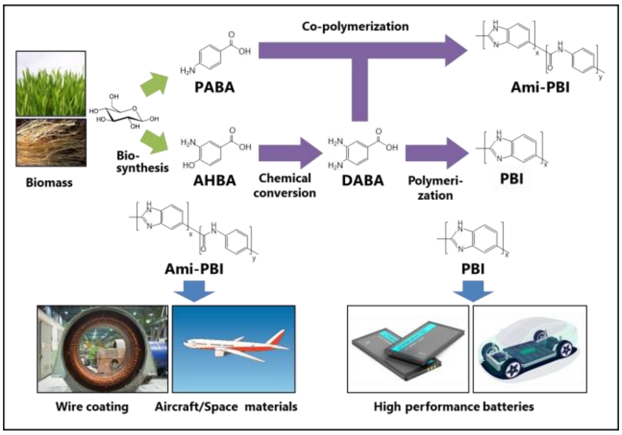

Fig. 12 Synthesis of developing monomers and polymers for polymer applications

5. Future industrialization of our technologies

5.1. Green Chemicals Co., Ltd. (GCC)

(Head Office・Laboratory: in Kyoto headquarters, RITE; Shizuoka Laboratory: in Shizuoka plant, Sumitomo Bakelite Co., Ltd.)

(Click here for GCC)

In February 2010, The Research Institute of Innovative Technology for the Earth (RITE) established the "Green Phenol and High Performance Phenolic Resin Production Technology Research Association" (GP Association) with and Sumitomo Bakelite Co., Ltd. to develop fundamental technologies related to phenol production and phenolic resin production through the application of bioprocesses that use cellulosic raw materials (non-food biomass).

In May 2014, the GP Association reorganized as "Green Phenol Development Co., Ltd." (GPD). This was the first example of demutualization of a technology research association.

In April 2018, given that GPD's technology is capable of producing useful compounds in parallel with phenol production, the trade name of Green Phenol Development Corporation was changed to Green Chemicals Co., Ltd., (GCC).

Since GCC's phenol-producing technology and knowledge apply to the production of various other aromatic compounds (Fig. 13), we are developing a bioprocess for other high value-added chemicals and commercializing products that meet customer needs.

We are also making active use of non-food biomass resources. One of the candidate feedstocks we are focusing on is the squeezed residue (extra-liquid fractions) of orange juice lees. We are currently studying the potential use of various non-food biomass resources that have not yet received significant attention.

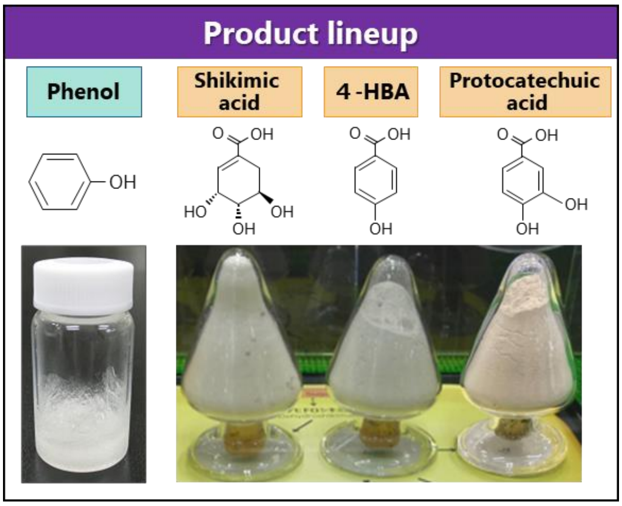

Fig. 13 Major product lineup of Green Chemicals Co., Ltd.

5.2. Green Earth Institute Co., Ltd. (GEI)

(Headquarters: Shinjuku-ku, Tokyo, Japan; Research Institute: Kisarazu City, Chiba, Japan. Mobara City, Chiba, Japan)

(Click here for GEI homepage)

GEI is a RITE-launched venture company established on September 1, 2011, to facilitate the quick commercialization of research results based on the RITE Bioprocess (described above). GEI is currently conducting joint research and commercialization activities with RITE to realize the practical uses of green chemicals and biofuel production technologies manufactured using microorganisms.

From August 2021, as a member of the core of the Greater Tokyo Biocommunity, as well as the global biotechnology community, GEI is implementing a bio foundry project (i.e., a NEDO-commissioned business) to build a biorefinery technology platform. This is in line with a cornerstone of the government's biotechnology strategy.

The company was listed on the TSE Mothers in December 2021. (Due to the revision of the Tokyo Stock Exchange's market divisions on April 4, 2022, the name of the listed financial exchange after that day will be the Tokyo Stock Exchange Growth Market.)

Currently, we are working with GEI to realize commercial production of a different type of amino acid, based on the successful commercial scale production of amino acids.

In addition to this, we are working together to develop other chemicals that can be used as raw materials for bioplastics and biodegradable resins, with the aim of realizing bio-production that contributes to sustainable development, as well as marketing for commercialization and scaling up for mass production.

5.3. Joint research with companies

In response to requests from companies, we are conducting joint research on the production of many substances other than the biofuels and green chemicals.

For example, we are conducting a joint research and development project with Harima Chemicals, Inc. as a NEDO-commissioned project regarding the development and demonstration of a mass production system for highly absorbent natural carotenoids.

We are also collaborating with Takasago International Corporation, again with support from NEDO, to demonstrate a rose fragrance production system using a continuous flow isolation method and a growth-arrested bioprocess.

We are also conducting joint research projects with many other companies, but it is not possible to introduce them all in detail here. In many of these cases, to realize early commercialization, research and development can be used to convert one of the company's products (i.e., substances currently derived from fossil resources) to a bio-derived version. In addition, we are conducting multiple medium- to long-term research and development projects to identify processes to generate bio-derived versions of the major products of these partner companies.

6. Closing remarks

In recent years, due to the development of "bio-digital technology" and accompanying research methodologies, the efficiency of smart cell development has dramatically improved, mainly related to the national projects introduced in Chapter 4. Moreover, efforts related to smart cell production demonstration have been carried out, improving their social implementation. As a result, a new industry (i.e., the smart cell industry) is being created. This is expected to have a large ripple effect not only on the energy sector but also in industrial sectors related to manufacturing (Fig. 14).

In 2023, our group will continue research and development related to the production of green chemicals including aromatic compounds and the production of biofuels such as biohydrogen. We will use the above-mentioned "RITE Bioprocess" and "Smart Cell Creation Technologies," and will participate in national projects and joint research with companies. Furthermore, we would like to contribute to the realization of carbon neutrality by developing practical production technologies at scale.

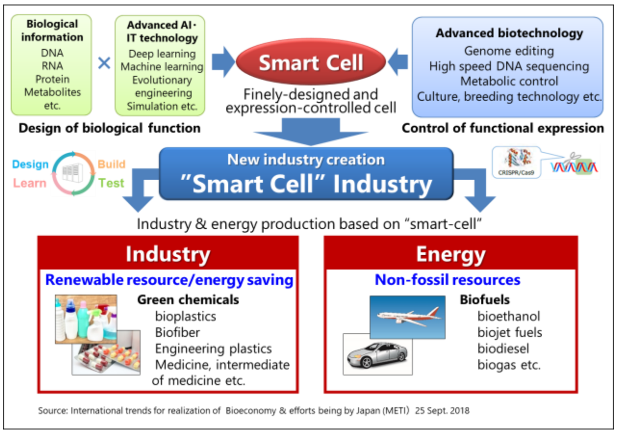

Fig. 14 Fusion of industrial/energy fields impacted by new bio and digital technologies

Since the "2050 carbon neutral" declaration in October 2020, inquiries from companies have increased, and the number of joint research projects with companies has also increased. However, our group continues to look for new research partner companies, in addition to those introduced in Chapter 5, Section 3. We also note the possibility that compounds that are difficult to produce in microorganisms can be manufactured in combination with other technologies, as in the abovementioned aromatic compound example. Therefore, if there is a compound that you want to make bio-derived, please contact us.

※ "RITE Bioprocess" is a registered trademark of RITE.