RD-063

Examination of the Collection and Concentration Method of the Loach Viscous Liquid

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[Introduction]

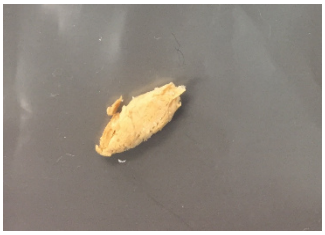
The dojo (pond loach) is a popular freshwater fish in East Asia. Majority loaches are active and hardy fish that sometimes secretes viscous liquid including mucin in an aquarium. Their secreting liquid is known to pollute the breeding water, on the other hand, the mucin is very useful in a pharmaceutical or in a nutritional for becoming smooth the joints in the body. In this research, we used a loach and tried collection (and concentration) of the mucin.

[Materials and Methods]

Some loaches (*Misgurnus anguillicaudatus*, *Madojo*, edible fish) are used for study. Rearing tank was retained under constant condition more than 3 months. The collection of the viscous liquid used the breeding water 24 hours later. The breeding water measured by Cannon-Fenske viscometer, pycnometer and rheological property (capillary tube).

Mucin is known to have a low thermoresistance (<35℃). Therefore, the breeding water was concentrated using rotary evaporator. The liquid after the concentration performed freeze drying overnight.

[Results]



The right figure of is viscous liquid after concentration and freeze drying. The color of this photo was slightly yellow and was not a powdery state. This study provided a dry sample of approximately 0.06 [g/L] per one loach. This sample include a large quantity of N-source (coloring component). It is necessary to examine purification method.

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RD-064

Callus Induction from Leaf Explants of Flowering Dogwood (*Cornus florida*)

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Flowering dogwood (*Cornus florida*), called 'HANAMIZUKI' in Japanese, is a popular flower tree that is used as trees lining a street tree and as garden trees. In some municipalities in Japan, it is designated as a city tree or flower. Every season, flowering dogwood appears its different beauties such as flowers, fresh spring greenery, red fruits, and autumn leaves.

Callus is a mass of dedifferentiated, unorganized cells. In plant tissue or cell cultures, calli can be obtained by culturing various plant tissues, and can regenerate whole plants, according to culture conditions with culture media and plant hormonal treatments. To establish suitable culture conditions for callus induction and plant regeneration is an essential basis for plant breeding in each plant species.

It has been reported that calli could be derived from embryos and that whole plant regeneration was not successful in flowering dogwood [1]. However, the method for callus induction was also difficult, because embryo are within stony endocarp of flowering dogwood fruits and it is hard to put embryos out of endocarp without injury. Therefore, a more simple method for callus induction is required in flowering dogwood.

In this study, successful callus induction from leaf explants was established in flowering dogwood. Leaves are easily available and soft and easy to cut and there is no need complicated manipulation for preparation of experimental materials. The leaf explants were cultured on Murashige-Skoog (MS) medium supplemented with plant hormones. The combination of 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin and kinetin as cytokinin was used for callus induction. We examined the effect on the concentrations of 2,4-D and kinetin on callus formation. The results showed that the combination of 2 ppm 2,4-D and 0.4 ppm of kinetin was effective for the highest callus induction efficiency. In addition, it was suggested that the sampling time or age of leaf explants had significant influence on callus formation.

A method for successful plant regeneration from calli was remained unestablished, we will start to study for it.

ACKNOWLEDGEMENT

This research was financially supported by a NUT grant for collaborative research with Colleges of Technology.

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RD-065

Development of Cell Density Measurement Method in Water-in-oil Emulsion Droplet

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Microorganisms are widely used to generate valuable products such as food, biofuel, and antibiotics. In order to use a microbial resources, it is necessary to cultivate microorganisms, so various cultivation methods have been developed, for example, flasks, dishes, tubes, plates and reactors [1]. Recently, water-in-oil emulsion that aqueous droplets are dispersed in a continuous oil phase has been expected major advances for the cultivation and screening of microorganisms. By encapsulating and cultivation of microorganisms in emulsion droplets, for example, it is possible to screen mutant strains and co-cultivation to mimic natural communities of interacting microorganisms [2][3]. It has been found that various microorganisms can be cultivated in emulsion droplets, so quantification of cell density is necessary for screening based on growth of microorganisms. However, it is difficult to measure a cell density in emulsion droplets.

Here, we introduce the measurement method of cell density in water-in-oil emulsion droplets. This time, droplets encapsulating *Escherichia coli* as represent microorganism were captured using confocal microscopy, and cell density was quantified (OD_{600} as a indicator) by "ImageJ". Firstly, the calibration curve was prepared expressing occupancy rate of cells in droplet and OD_{600}. Secondly, *E. coli* was cultivated over time by emulsion droplets, and OD_{600} at each cultivation time was determined from the calibration curve. Hence, we succeeded in drawing a growth curve of *E. coli* in emulsion droplets.

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RD-066

Epigenetic Mutants Causing Defects in Plant Leaf Development

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DNA methylation is one of chemical modifications associated with gene expression. In the eukaryotes, methyl group is added on the 5th position of cytosine. The pattern of DNA methylation is maintained during DNA replication by the action of DNA methyltransferases. In plants, the change of DNA methylation pattern is inherited to the next generation in contrast to mammals in which DNA methylation is erased and reprogrammed at the beginning of the development. Change of DNA methylation pattern is called epi-mutation since it alters gene function by changing its expression level without any base sequence change. Epi-mutations are reported to cause various defects in environmental responses and development in *Arabidopsis thaliana*.

Since epi-mutation is supposed to be a reversible mutation unlike genetic mutation which occurs in the base sequence, we supposed to be able to develop new kind of breeding techniques by applying epi-mutation. Most of epi-mutations which have been reported, however, are identified in nature or by chance in laboratory. In order to identify new epi-mutations, we used epigenetic recombinant inbred lines (epiRILs). EpiRILs are produced by crossing wild-type with a mutant deficient in DNA methyltransferase to maintain DNA methylation on genome, then select of wild-type homozygous plant in the second-generation and repeating self-fertilization. The resultant lines are expected to have an almost identical genome sequence to wild-type one, but have different DNA methylation pattern on each position of a chromosomal [1]. By screening of epiRILs, we isolated several epi-mutations causing interesting phenotypes, and are trying to identify genes with a change of DNA methylation responsible for the phenotypes.

In the poster presentation, we will present about epi-mutations showing defects in leaf development. Leaf is one of important organs as a place for photosynthesis. In the epi- mutations, leaves show hyponastic growth under the normal growth condition. In the results of genetic analysis, one of the epi-mutations is dominant and would be associated with at least three gene loci. Now we are trying to specify the candidate genes by genome analysis approaches.

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RD-067

Investigation of the Role of Signal-like Sequence of Organophosphorus Hydrolase from *Sphingobium* sp. TCM1

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The chlorinated organophosphate triester, tris(2-chloroethyl) phosphate (TCEP), has been widely used as a flame retardant and a plasticizer. However, recently, carcinogenicity, refractory and mutagenicity of TCEP has been reported and thus raises concern about its harmful effect on organisms including human. In our laboratory, we isolated a TCEP-degrading bacterium, *Sphingobium* sp. TCM1, using TCEP as the sole phosphorus source [1], and the TCEP-degrading enzyme (TCM1 haloalkylphosphorus hydrolase; cHAD) was purified, characterized [2]. Although, it was suggested that cHAD contains signal-like sequence, the function of the signal-like sequence are not clear. In this study, we investigated the role of signal-like sequence of cHAD.

A vector for expression of mutated cHAD was constructed using pAK405. The vector was introduced into the TCM1, and transformants were obtained by growth on a selective medium. The deletion of signal-like sequence region of had gene was confirmed by colony PCR. The wild-type strain was able to grow on TCEP as sole phosphorus source, but the mutant strain was unable to grow on the same medium. Furthermore, the resting-cells of the mutant strain did not degrade TCEP, although the that of wild-type strain showed TCEP degradation activity. These results revealed that this signal-like sequence is essential for HAD expression or activity.

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RD-068

The Effect of Carbon Source on Oleaginous Yeast *Rhodosporidium toruloides* and *Lipomyces starkeyi*

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Oleaginous yeast *Rhodosporidium toruloides* and *Lipomyces starkeyi* can accumulate high amount of lipid as Triacylglycerol (TAG) in the cell. TAG is constituted by one molecule of glycerol and three molecules of fatty acid. The fatty acid composition of the lipid made by these yeasts is similar to that made by oleaginous plants. Therefore, these yeasts can be applied for edible oil production. However, they still have some problems that the cultivation technology on large scale has not been developed and that the costs of carbon source and lipid extraction are high. As a solution to these problems, a process of producing lipid using the sugar derived from unutilized-cellulosic biomass has been considering in order to reduce the cost. Cellulosic biomass is the most abundant resource on Earth. The main components of this biomass are cellulose and hemicellulose. Some microorganisms can convert them into sugar by secretion of the cellulase, hemicellulose and another enzyme. The sugar derived from cellulosic biomass contains some kinds of saccharide. Therefore, it is necessary to investigate the effect of carbon source for the growth and the lipid production.

R. toruloides and *L. starkeyi* were cultivated on glucose, xylose, galactose and cellobiose which were sugar obtaining by saccharification of cellulosic biomass. For the growth and the lipid production, *R. toruloides* grew on sugars except cellobiose and accumulated the highest amount of lipid on glucose cultivation. This result suggested that *R. toruloides* could not metabolize cellobiose. On the other hand, *L. starkeyi* grew on all four sugars and accumulated the highest amount of lipid on xylose cultivation. This result suggested that *L. starkeyi* could use xylose more effectively than glucose for the growth and the lipid production. Then, the fatty acid composition is analyzing because fatty acid which structured by hydrocarbon chain is expected for affecting by carbon source. In addition, since the carbon source used for culturing was difference, it was predicted that the flux of metabolic system not only glycolysis system but also lipid production system was changed. Therefore, the gene expression analysis is also in progress.

RD-069

Generation of Carotenoid Hyper-producing Mutants by Mutagenesis Using DNA-cleaving Antibiotics

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Carotenoids are recently valuable molecules as additives in foods, animal forages, and cosmetics. Efficient production of carotenoids has been therefore required. Because the yeast *Rhodotorula gracilis* is known to produce a high level of carotenoids, more than 70% of its dry weight, biotechnological carotenoids production using this yeast is currently attracting great attention. However, further production of carotenoids by the yeast is required for commercial applications. In the course of the yeast transformation study, we observed the yeast mutants showing a higher intensity of red color, which is derived from carotenoids, on an agar plate containing DNA-cleaving antibiotics, such as zeocin and phleomycin. We therefore predicted that carotenoid hyper-producing mutants of the yeast could be obtained by mutagenesis using DNA-cleaving antibiotics. To date, genome mutagenesis using DNA-cleaving antibiotics has not yet been reported. In this study, we thus aimed to develop a novel mutagenesis method of the yeast *R. gracilis* using DNA-cleaving antibiotics and to obtain carotenoid hyper-producing mutants using this method.

We first examined zeocin concentration for efficiently obtaining carotenoid hyper-producing mutants of *R. gracilis* strain ATCC 26217. The wild-type strain was cultured in YPD liquid medium until the OD_{600} reached approximately 1.0 (30°C, 200 rpm), and then 1.0×10^7 cells were spread onto YPD agar containing various concentrations of zeocin (0 – 1.5 μg/ml). The higher ratio (approximately 90%) of colonies showing higher or lower red color intensity than the wild-type strain to total colonies grown on the plates was obtained at 1.0 μg/ml zeocin or more (Fig. 1). We therefore selected 1.0 μg/ml zeocin for screening hyper-carotenoid producing mutants and isolated some mutants. We now analyze the carotenoid production of the isolated mutants.

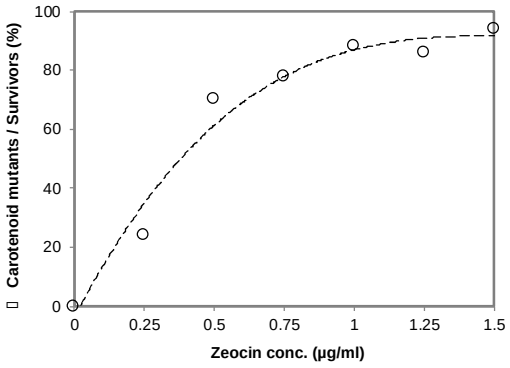

Fig. 1 Effect of zeocin concentration on the generation of colonies showing higher or lower red color intensity.

RD-070

Development of D-aspartic Acid-biosensor Element

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D-Amino acids were formerly considered to be absent in animals. However, various kinds of D-amino acids are recently found in the free form in a wide range of animals, in which they are suggested to play a role in various important physiological processes. For example, D-aspartate (D-Asp) is shown to be involved in the regulation of hormone secretion, developmental processes, and steroidogenesis. Also, a line of evidence suggests that D-Asp functions as an agonist of *N*-methyl-D-Asp receptor and is involved in neurotransmission and schizophrenia. D-Asp is therefore regarded as an important target for detection and quantification. D-Aspartate oxidase (ChDDO) of the yeast *Cryptococcus humicola* strain UJ1 found in this laboratory has high specificity and catalytic activity against D-Asp and is useful for the detection and the quantification of D-Asp. In this study, we aimed to develop a biosensor element using ChDDO for the detection and the quantification of D-Asp.

A D-Asp biosensor element was prepared by immobilizing the mixture of ChDDO and glutaraldehyde on the surface of Au electrode. Phosphate buffer containing a substrate and a mediator, ferrocenecarboxylic acid (Fc-COOH), was used as a measurement solution. Current response caused by the enzymatic reaction was measured by cyclic voltammetry or chronoamperometry.

Current response was observed in a measurement solution containing D-Asp using D-Asp biosensor element immobilized with ChDDO. This current response was higher than that in a measurement solution without D-Asp, suggesting that the current response against D-Asp was generated by the reaction between the biosensor and D-Asp. D-Asp could be therefore detected electrochemically using the biosensor element. We further improved the biosensor element by optimizing the amount of immobilized enzyme, immobilization time, and immobilization method. The optimal temperature and pH of the biosensor element were similar to those of free ChDDO. The current response against the second substrate of ChDDO, D-glutamate (D-Glu), was about 20% of that against D-Asp. Because the reactivity of the free enzyme against D-Glu is reported to be about 1% of that against D-Asp, this result suggests that the substrate specificity of ChDDO was changed by the immobilization.

RD-071

Thalidomide Reduces FABP7 Expression in Human Induced Pluripotent Stem Cell

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Thalidomide was used as a medicine which stops morning sickness in 1950's. However, it left from the market because of teratogenicity to human embryo. Recently, it has been attracting attention because it is effective in treating serious diseases such as leprosy. However, the mechanism of thalidomide's teratogenicity is still unknown. Generally, a rodent such as a mouse is used to confirm the toxicity, but thalidomide is not toxic to rodents. Moreover, since it is ethically and technically impossible to administer thalidomide to human embryo, investigation of mechanism of thalidomide's teratogenicity is difficult.

Here we used human induced pluripotent stem cells (hiPSCs), with properties equivalent to epiblast as a replacement for the fetus, to investigate the effects of thalidomide [1] [2]. In a previous study in our laboratory, qRT-PCR array showed that thalidomide exposure reduces the expression of *FABP7* in undifferentiated hiPSCs. To confirm this result using different methods, I investigated the expression of *FABP7* in undifferentiated hiPSCs after thalidomide exposure by immunostaining, flow cytometry and qPCR.

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of human induced pluripotent stem cells

RD-072

Effect of Thalidomide on Neuroectoderm Human iPS Cells

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Thalidomide was once administered to pregnant women as a mild sedative. However, it was subsequently shown to be strongly teratogenic. Recently, there has been renewed interest in thalidomide because of its curative effects against intractable diseases. However, the teratogenicity of thalidomide is manifested in various ways and is still not fully understood. In the present study, we evaluated the effects of thalidomide on early endodermal differentiation by examining the differentiation of human induced pluripotent stem cells (hiPSCs).

The most common symptom of thalidomide teratogenicity is limb abnormality, which led us to hypothesize that thalidomide prevents early endodermal differentiation. However, the effect of thalidomide on ectoderm is unknown. Therefore, this experiment investigated the effect of thalidomide on ectoderm. Therefore, endodermal differentiation of hiPSCs was induced over a 6-d period. Thalidomide exposure was initiated at the same time as endodermal differentiation. After 4 d of thalidomide exposure, the hiPSCs began expressing a endodermal marker; and, the number of viable cells increased significantly as compared to that of control cells.

Our findings may help elucidate the mechanism underlying thalidomide teratogenicity and bring us closer to the safe use of this drug.

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RD-073

Elucidation of Substrate Recognition Mechanism of Dipeptidyl Aminopeptidase IV from Gram-negative Bacteria *Pseudoxanthomonas mexicana* WO24

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Dipeptidyl peptidase (DPP) is a type of exopeptidase possessing the activity of releasing a dipeptide from the N-terminal of a peptide chain. DPP4 is a serine peptidase showing specificity to Pro / Ala of substrate P1 and has a spatial structure (S2-ex) opened to the N terminal side than S2 subsite. DPP4 has been reported that there are orthologs in many organisms, especially human DPP4 (HsDPP4) which is actively studied and reported to play a role in various biological reactions. On the other hand, there is little known about bacterial DPP4. Recently it is suggested that various pathogenic bacteria possess DPP4 orthologs that is an enzyme involved in growth of them. Therefore, a specific inhibitor of bacterial DPP4 can be an antibacterial agent having a novel mechanism of action. DPP4 from *Porphyromonas gingivalis* (periodontal pathogen bacteria) and *Stenotrophomonas maltophilia* (multidrug resistant opportunistic infection bacteria) had been analyzed. However, their substrate recognition mechanism has not been revealed, since co-crystal structure with ligand was not obtained. In this study, to develop bacterial DPP4 specific inhibitors, we performed enzymatic analysis of DPP4 (PmDAP IV) from aerobic Gram-negative bacteria *Pseudoxanthomonas mexicana* WO24 and investigated the difference in substrate recognition mechanism with HsDPP4.

Here, we report the co-crystal structure of PmDAP IV with dipeptide and nonpeptide inhibitor. Structure analysis and site-directed mutagenesis analysis indicated that side chain of Arg106 residues in PmDAP IV participate in fixing substrate. Furthermore, depending on the presence or absence of Arg106 residue and its surrounding structure, Pro-specific DPP can be roughly divided into two types, type of HsDPP4 or type of human DPP8/9. PmDAP IV is classified as HsDPP8/9 type and this result will contribute to drug development targeting not only DPP4 but DPP8/9.

RD-074

SUMO E3 Ligase Homologues Function in Transcriptional Gene Silencing in *Arabidopsis thaliana*

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Expression of foreign genes introduced into plants are often suppressed by DNA methylation. This phenomenon is called Transcriptional Gene Silencing (TGS). The molecular mechanism how DNA methylation represses gene expression is less known. In *Arabidopsis thaliana, mom1* mutation releases TGS of hypermethylated genes without major changes of their DNA methylation [1]. This suggests that MOM1 protein acts downstream or independently of DNA methylation in TGS, although the molecular mechanism is still unknown. We identified PIAL1 (*PROTEIN INHIBITOR OF ACTIVATED STAT-LIKE1*) and PIAL2 proteins which are homologues of SUMO E3 Ligase [2], as interacting partners of MOM1 by co-immunoprecipitation experiment. Loss of function mutants of PIAL1 and PIAL2 showed defects in TGS similar to those of *mom1* mutant which consistent with a report by Han et al. [3]. We established *mom1 pial1 pial2* triple mutant and revealed that the resultant triple mutant did not show additive phenotype. These results suggest that MOM1 protein, and PIAL1/2 proteins act in the same pathway in TGS. In order to investigate functional relationship between MOM1 protein and PIAL proteins, we have established a plant expressing PIAL2-GFP fusion protein in *mom1* mutant background and observed localization of PIAL2-GFP protein. We will present the results of cytological analyses of PIAL2 protein.

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RD-075

Planarian Cell Adhesion on Extra Cellular Matrix

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The final goal of our experiment is whole body regeneration from a single cell. To achieve this goal, we focused on planaria. A planarian can regenerate itself even if it loses more than 90 percent of its body. From this planarian powerful regenerative ability, we expected that planaria could produce one individual from a single cell. Although isolation and short-term cultivation of Planarian cell have already been reported, the cells can not proliferate[1,2].

Here we searched two conditions for planarian cell culture. The one was sterilization and the other was adhesion on the culture dish. Many kinds of cells do not proliferate without adhesion. We carried out sterilization operation on planaria and the dissociated cells. The planarian was rinsed every day with antibiotics mixture (penicillin, streptomycin, and amphotericin) containing water for 7 days and then the planarian was dissociated into single cells. Dissociated cells were cultured for 2 days. As a result, although many living planarian cells were observed, bacteria were not observed, suggesting that we succeed in culturing planarian cells without bacteria contamination. We also perform adhesion assay on four extra cellular matrices (Fibronectin, Vitronectin, Gelatin, Laminin).

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RD-076

Inhibition of Bacterial Catabolism of Lignin-derived Aromatics in the Presence of High Concentration of Methionine

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Sphingobium sp. strain SYK-6 is a bacterium which is able to grow on various lignin-derived biaryls and monoaryls. In the SYK-6 cells, these compounds are catabolized to vanillate (VA) or syringate (SA), and then VA and SA are converted into protocatechuate and 3-*O*-methylgallate (3MGA)/gallate, respectively, by tetrahydrofolate (THF)-dependent *O*-demethylases LigM and DesA. In these O demethylation steps, the methyl moiety of VA and SA/3MGA is transferred to THF to produce 5-methyl-THF. 5-methyl-THF functions as a methyl group donor essential for methionine (Met) biosynthesis. It has been shown that the growth of SYK-6 on lignin-derived aromatics, including VA and SA is inhibited in the presence of high concentration of Met (> 300 μM). This inhibition is thought to be caused by inhibition of O demethylation of VA and SA/3MGA. The objective of this study was to elucidate the mechanism of this inhibition.

Total RNAs were isolated from the SYK-6 cells grown on 5 mM VA in the presence or absence of 500 μM Met. Using the cDNA obtained by reverse transcription (RT), the transcript amounts of the genes involved in the Met recycling system and catabolism of VA and SA (*desA*, *ligM*, and *desB*) were measured by quantitative RT-PCR analysis. The amounts of *desA*, *ligM*, and *desB* transcripts from the cells grown in the presence of Met were 3 to 22 times lower than those from the cells grown in the absence of Met. On the other hand, no significant changes in the transcript levels of the genes responsible for the Met recycling system were observed. These results strongly suggest that the inhibition of growth of SYK-6 on lignin-derived aromatics in the presence of high concentration of Met is due to transcriptional repression of the VA and SA catabolic enzyme genes.

RD-077

Study on Protease Biosynthesis of *Bacillus pumilus Meyer and Gottheil* in Single Protein-Supplemented Media

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Protease is an enzyme hydrolyzing proteins into smaller molecules and is utilized as industrial catalysts because of highly selective catalyst activity [1]. Moreover, in the deproteinization process of shells of crustacean such as crab and shrimp, chitin and chitosan have enzymatically produced by hydrolysis process. Unlike chemical method conducted using strong bases under high temperatures. Such enzymatic method reduces waste collection, disposal, and environmental problems. However, the production of protease in the biosynthesis by microorganisms is relatively low.

In the present study, biosynthesis of protease was studied using alkaline protease producer like *Bacillus pumilus Meyer and Gottheil* (*B. pumilus*). The bacteria were incubated in a single protein medium containing yeast extract or peptone. These culture media were compared with LB medium containing 5% (w/v) of yeast extract and 10% (w/v) of peptone at pH 7. Growth of bacteria was examined in terms of colonies forming units (CFU) per mL of cell suspension. The highest number of bacterial cells was 4.5×10^{11} CFU/ml yielded after culturing for 2 days in the LB medium. After culturing in peptone or yeast extract medium for 2 days, the maximal growth was also observed as 4×10^{10} and 1.6×10^{11} CFU/ml, respectively. For protease biosynthesis, yeast extract medium had protease activity of 356-452 U/ml during 1-7 days of the incubation. This was higher than incubated in peptone and LB media (Fig. 1). When the cultivation was conducted at pH 5.5 of the media, the protease activity was lower. The protease activities of culturing *B. pumilus* in LB, peptone and yeast extract media were 74-80, 47-60, and 217-299 U/ml, respectively, for 1-3 days. This was resulted from the effect of pH. Cultivation at pH 7 supported the growth of *B. pumilus* and protease activity than cultivation under more acidic condition at pH 5.5. And the protease activity in YE medium was improved markedly.

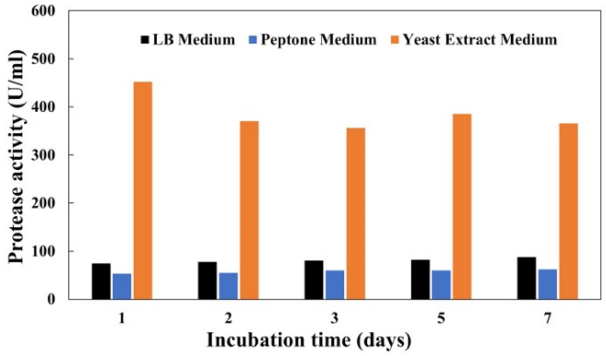

Fig.1 Activity of protease biosynthesized by *B. pumilus* in Luria–Bertani (LB), peptone and yeast extract media during 1-7 days.

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RD-078

Expression and Purification of Phosphodiesterase from *Sphingobium* sp. strain TCM1.

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The chlorinated organophosphorus triesters, tris(2-chloroethyl) phosphate (TCEP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are widely used as flame retardants mainly in a range of plastic foams, resins, and latexes and in the production of liquid unsaturated polyester resins, respectively. Their widespread use has led to contamination of various environments. These chemicals are physicochemically and microbiologically stable in the environment and are also reported to be toxic. We have previously isolated a TCEP and TDCPP-degrading bacterium, *Sphingobium* sp. strain TCM1[1], and then a phosphodiesterase catalyzing the second step of TCEP and TDCPP degradation (phosphodiesterase; PDE) was purified and cloned[2]. In the future, it is necessary to clarify the structure and reaction mechanism of PDE to develop more efficient degradation technique. In this study, we have constructed *E. coli* expression system for PDE from *Sphingobium* sp. strain TCM1.

The genes encoding PDE (*pde*) and His-tagged PDE (*pdehis*) were amplified by PCR using the primers and genomic DNA of *Sphingobium* sp. strain TCM1 as a template. The resulting PCR products was ligated into the pET25b, and expressed in *E. coli* BL21 (DE3). Crude extracts of *E. coli* cells harboring pET25b-*pde* showed hydrolase activities of 7.01 μmol min^{-1} mg^{-1} for B*p*Npp and 1.97 μmol min^{-1} mg^{-1} for *p*Npp. On the other hand, Crude extracts of *E. coli* cells expressed His-tagged PDE did not show the activity, although high expression of the enzyme was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These results suggest that the His-tag affects the structure of substrate-binding or metal-binding site of PDE. We now purify wild-type PDE from *E. coli* BL21 (DE3).

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RD-079

Identification and Characterization of the Glutathione *S*-transferase Genes Involved in Catabolism of arylglycerol-β-aryl Ether

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The β-aryl ether linkage is the most abundant linkage in lignin (approximately 50%). Accordingly, decomposition of this linkage is considered a crucial step in lignin biodegradation. In *Sphingobium* sp. strain SYK-6 cells, four stereoisomers of a β-aryl ether-type biaryl, guaiacylglycerol-β-guaiacyl ether are converted into two enantiomers of α-glutathionyl-β-hydroxypropiovanillone (GS-HPV) via stereospecific Cα oxidation and ether cleavage [1, 2]. It was suggested that (β*R*)-GS-HPV was converted into HPV by the reaction catalyzed by a glutathione *S*-transferases (GST), LigG. However, the GST genes actually involved in the conversion of both GS-HPV enantiomers in SYK-6 have not been identified. In this study, we identified and characterized the GST genes responsible for the conversion of GS-HPV enantiomers.

Four GST genes found in SYK-6 were expressed in *E. coli*, and the ability of the gene products to convert both (β*R*)-GS-HPV and (β*S*)-GS-HPV was examined. As a result, the gene products of *ligG* and *ligQ* converted both enantiomers into HPV. In order to examine whether *ligG* and *ligQ* are involved in the conversion of GS-HPV enantiomers in SYK-6, *ligG* and *ligQ* mutants were created. The *ligG* mutant showed GS-HPV conversion capability equivalent to that of SYK-6. On the other hand, the ability of the *ligQ* mutant to convert (β*S*)-GS-HPV was almost lacked, and its conversion rate for (β*R*)-GS-HPV was significantly reduced. Furthermore, the *ligG ligQ* double mutant no longer converted both GS-HPV enantiomers. Purified LigG exhibited approximately 1000-fold higher activity for (β*R*)-GS-HPV than for (β*S*)-GS-HPV. In contrast, purified LigQ showed similar activity toward both GS-HPV enantiomers. All these results suggest that *ligG* and *ligQ* are responsible for the conversion of GS-HPV enantiomers in SYK-6, and *ligQ* plays a major role in their conversion.

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RD-080

Subcellular Localization of Putative Transceptor Crt1 in Filamentous Fungus *Trichoderma reesei*

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Trichoderma reesei is one of the most studied cellulolytic fungus that produces a wide range of cellulose degrading enzymes called as cellulase. When cellulose is used as a sole carbon source, *T. reesei* dramatically upregulates expression and secretion of genes encoding cellulases. However, how *T. reesei* senses the presence of cellulose in the environment remains to be revealed. It has been proposed that certain cellulases expressed at a basal level degrade celluloce into cello-oligosaccharides and they act as inducer molecules. Therefore, it is possible that membrane proteins that recognize or transport low molecular weight compounds play the important role for cellulase expression. Transporters belonging to the major facilitator superfamily (MFS) have been shown to play important roles in sugar uptake in filamentous fungi. Recently, it has reported characteristically transporter that involves in transportation and signaling, called as transceptor. In *T. reesei*, MFS transporter Crt1 was reported as putative transceptor and was necessary for cellulase production by cellulose.

In this study, to analyze function of Crt1 on cellulase production condition, we constructed GFP-fused Crt1(Crt1cGFP) expression strain and analyzed for the localization of Crt1cGFP in the cell. As a result, fluorescence of GFP was observed at surface and specific organella of the cell. From colocalization analysis with confocal laser scanning microscope and super-resolution microscope, it was revealed that those localizations were plasma membrane and ER. Moreover, fluorescence was transition from plasma membrane to ER. Consequently, it was supported that those localization might be related signaling of Crt1 which it is necessary for cellulase production.

RD-081

Microsatellite Capture Sequencing as a Massive Simple Sequence Repeat (SSR) Detection Approach for DNA Marker Development and Polymorphic Analysis

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Microsatellite (simple sequence repeat, SSR) regions have efficacy as one of robust assays for various molecular phylogenetic and population genetic researches. Although non-model organisms are often-used in these research fields, some SSR regions need to be detected as prior information to utilize SSR marker. General SSR detection techniques utilize colony hybridization and/or microsatellite enrichment based on the biotin-streptavidin interaction and a dual-suppression-PCR technique. However, these approaches are low-throughput since they depend on capillary sequencing. Here, we developed a new approach using DNA fragmentation technique by restriction enzyme or sonication, target capture sequencing with a combination of microsatellite enrichment and high-throughput DNA sequencer, and *in silico* polymorphic detection [1].

For Myrtaceae plants, DNA library with the insert size of 400-600 bp was constructed after DNA fragmentation by restriction enzymes or sonication. Then, the DNA library containing SSR region was hybridized to the biotinated SSR probe and captured using streptavidin. The enriched DNA library was sequenced by paired-end 600 cycles of Illumina MiSeq. Preprocessed paired-reads were integrated using FLASh. Then, the integrated reads with same sequence were clustered using CD-HIT-EST. Each sequence data were mapped to the merged clustered sequences as a reference using CLC Genomics Workbench. And then, SSR regions from consensus sequences were detected using SSRIT. A polymorphic table was constructed using the custom script.

Our microsatellite capture sequencing approach detected over ten thousands sequences with SSR region were detected in each sample. Particularly, a comparison of the fragmentation of genomic DNA revealed that the use of restriction enzymes was better than sonication for identification of the heterozygous genotype, whereas sonication was better than restriction enzymes for detecting various SSR flanking regions with both species-specific and common characteristics. Therefore, our approach can be varied depending on research purposes.

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RD-082

Elucidation of Iron Uptake Mechanism in *Sphingobium* sp. strain SYK-6

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Sphingobium sp. strain SYK-6 is a Gram-negative bacterium, which can grow on various lignin-derived aromatic compounds as a source of carbon and energy. To date, the SYK-6 catabolic pathways for lignin-derived aromatics and the pathway genes have been well characterized, and the development of biological processes for the conversion of lignin into valued substances utilizing the SYK-6 catabolic pathways are in progress. However, the uptake systems for lignin-derived aromatics and other nutrients remain largely unknown. Particularly, iron uptake is essential not only for bacterial growth but also for catabolism of lignin-derived aromatics. This is because iron is included in the active centers of various oxygenases essential for aerobic aromatic catabolism. Generally, iron uptake across the outer membrane of Gram-negative bacteria is mainly achieved by TonB-dependent receptors (TBDR). TBDRs transport substrates using energy provided by the TonB-ExbB-ExbD complex, which transduces the energy from the proton motive force. In the inner membrane, ATP-binding cassette (ABC) transporters and ferrous iron transporter (Feo) are involved in iron uptake. The transcriptional of iron uptake genes are known to be upregulated under iron limiting conditions. However, iron uptake genes and their regulation system remain unknown in Sphingomonadaceae. In this study, we identified the transporter genes involved in iron uptake in SYK-6.

SYK-6 mutants of putative iron uptake genes were created, and their ability to grow on vanillate was examined. The growth of the mutants of *fiuA*, *tonB2*, and *feoB* encoding a putative TBDR, a putative TonB, and a putative component of the Feo system, was significantly retarded. These mutants showed more delayed growth in the presence of an iron chelator, 2,2'-dipyridyl (DIP). Furthermore, the growth of the double mutants of *fiuA* and other putative iron uptake TBDR genes (SLG_04340, SLG_04380, SLG_10860) was further retarded. The expression of these genes were upregulated at the transcriptional level under iron limiting conditions. All these results strongly suggest that the TonB system composed of FiuA, SLG_04340, SLG_04380, SLG_10860, and TonB2 are involved in iron uptake across the outer membrane, and Feo has a role in the inner membrane uptake in SYK-6.

RD-083

Elucidation of the Effect of Mutation of BGLII IN Filamentous Fungus *Trichoderma reesei*

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The filamentous fungus *Trichoderma reesei*, a potent producer of cellulase, produces the variety of cellulolytic enzyme to convert cellulose into glucose. Because this fungus has been widely used as an industrial cellulase source high cellulase producing mutant strains have developed by mutagenesis. In Japan, the high cellulase producing mutant strain PC-3-7 strain have also been obtained from the global standard strain QM9414 strain. In previous study, it was revealed that BGLII mutated in PC-3-7 from Val into Phe at 409th amino acid resulted in improvement of cellulase productivity on cellobiose [1]. BGLII is belong to GH1 enzyme and hydrolyzes cellooligosaccharide to glucose in inter cellular. In addition, BGLII causes a transglycosylation reaction to produce transglycosyl compounds. It is indicated that produced transglycosyl compounds act as inducers of cellulase. BGLII^{V409F} was remained transglycosylation activity, but not hydrolysis activity. Therefore, PC-3-7 suggested that carbon source catabolism was reduced and cellulase induction was enhanced. However, it is unknown how BGLII^{V409F} controls cellulase activity. In order to clarify the effect of the 409th mutation on BGLII, we performed saturation mutagenesis for 409th amino acid in BGLII.

Each 20 kind of *bgl2* was constructed for the expression system of *Escherichia coli*. It was successfully expressed 11 mutant BGLIIs(V409L, I, F, H, A, G, S, P, T and C). Compared to wild type BGLII, BGLII^{V409T} and BGLII^{V409S} showed about twice the hydrolytic activity. BGLII^{V409L}, BGLII^{V409A} and BGLII^{V409C} were exhibited similar activity with WT. BGLII^{V409I} showed half activity of wild-type BGLII. BGLII^{V409F}, BGLII^{V409H}, BGLII^{V409P} and BGLII^{V409G} showed quite low activity. The transglycosylation products from each mutant BGLII were investigated using HPLC. As a result, a transglycosylation product that is different from the other mutant BGLII was detected in BGLII^{V409T}.

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RD-084

Identification of Major Facilitator Superfamily Transporters Involved in the Uptake of Lignin-derived Aromatics in *Pseudomonas putida* KT2440

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Lignin, a major component of plant cell walls, is the most abundant aromatic compound on Earth. Therefore, it is expected that a large amount of industrially useful compounds can be obtained through bioconversion of lignin. For conversion of aromatic compounds into value-added chemicals, *Pseudomonas putida* KT2440 has been widely used as a host strain because of its metabolic versatility and high resistance to environmental stress. To date, although a number of KT2440 enzyme genes involved in catabolism of aromatic compounds have been identified and characterized, the transporters responsible for uptake of various aromatics into the cell remain largely unknown. In this study, we aimed to identify the genes involved in uptake of lignin-derived aromatics in KT2440.

In the KT2440 genome, there are 85 putative major facilitator superfamily (MFS) transporter genes. Phylogenetic analysis based on amino acid sequence similarity with known 24 MFS transporters for aromatic acids revealed the presence of five candidate genes that may be involved in aromatic uptake. Each gene was disrupted in KT2440, and the capacities of these mutants to grow on and convert lignin-derived aromatic acids were evaluated. Among the mutants, the *pcaK* mutant showed decreased growth on protocatechuate and 4-hydroxybenzoate, and their conversion rates were significantly reduced. In addition, the conversion rates of ferulate and vanillate by *ferK* mutant and *vanK* mutant, respectively, were significantly reduced. These results suggest that *pcaK*, *ferK*, and *vanK* are involved in uptake of protocatechuate/4-hydroxybenzoate, ferulate, and vanillate, respectively.

RD-085

Adhesion Properties of Human IPS Cells by Extracellular Matrix Coat to PDMS Surface are Different from TCPS

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INTRODUCTION

Human induced pluripotent stem (hiPS) cells are very attractive tools for modeling diseases and regenerative medicine[1]. Human iPS cells to adhere to TCPS (Tissue culture polystyrene) without serum, precoating with extracellular matrix is necessary. There was a report that protein coating and cell introduction could be performed simultaneously to TCPS using ECM called Laminin-511 (iMatrix), and it succeeded in reproducing[2]. Also, in another ECM, Vitronectin, cells did not adhere at the same time, so iMatrix was considered to be more suitable for cell adhesion. However, on the Poly-dimethyl-siloxane (PDMS), iMatrix failed to perform protein coating and cell introduction at the same time. Therefore, attention was paid to the fact that human iPS cells differed in cell adhesion characteristics between the surface of PDMS and the surface of polystyrene for tissue culture, and we searched for conditions under which cells adhere due to multiple extracellular matrices.

EXPERIMENTAL

Two conditions of PDMS surface and polystyrene surface, Vitronectin and iMatrix were tested under three conditions of 0.05, 0.5, 1 μg/cm^2, it was set as two conditions of coat and day coat the day before. After hydrophilization by plasma treatment, ECM coating was carried out. Cells were seeded, cultured for 4 days, and the number of adherent cells was measured by Crystal Violet staining.

RESULTS AND DISCUSSION

Vitronectin was used on the PDMS surface, cells adhered when coated at a concentration of 0.5 μg /cm^2 or more for 1 hour. iMatrix was used, cells adhered when coated at a concentration of 0.5 μg/cm^2 or more for 24 hours. From these results, the PDMS surface has different adhesion characteristics from the TCPS surface. Compared to iMatrix, Vitronectin is capable of cell adhesion on the PDMS surface at low concentration in a short time.

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RD-086

Determination of a Structural Factor Involved in the Substrate Specificity of D-amino Acid Oxidase of Thermophilic Fungi

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D-Amino acid oxidase (DAO) is a flavoenzyme that catalyzes the oxidative deamination of neutral and basic D-amino acids. This enzyme is useful for various practical applications, such as the detection of D-amino acid and the production of antibiotics. Mesophilic fungal DAOs having high catalytic activity and broad substrate specificity are usually used for the applications, but their low thermal stability limits their wide utilization. It is therefore important to obtain DAO having higher thermal stability as well as higher catalytic activity and broad substrate specificity. We recently isolated DAO of the thermophilic fungi *Rasamsonia emersonii* strain YA (ReDAO) and *Thermomyces dupontii* strain NRRL 2155 and showed that ReDAO had higher thermal stability, higher catalytic activity, and broad substrate specificity. Interestingly, ReDAO exhibited higher catalytic activity against an acidic D-amino acid, D-Glu, which is not a substrate for other DAOs. In contrast, TdDAO had narrow substrate specificity. In this study, we aimed to identify the structural factors for the difference in substrate specificity between ReDAO and TdDAO.

The amino acid sequence alignment of ReDAO and TdDAO showed an insertion of amino acid residues (Tyr57–Gly61, YVLQG) in TdDAO. The insertion was also not found in other broad substrate specificity DAOs from mesophilic fungi. In a three-dimensional structural model of TdDAO, the insertion was positioned close to the active site. These findings suggested that the insertion might be involved in the substrate specificity of TdDAO. We created a ReDAO mutant having the insertion of TdDAO at the corresponding position and expressed it in *E. coli*. The activity of the mutant and the wild-type in the cell-free extract against their respective best substrates (mutant: D-Met, wild-type: D-Ile) was comparable. However, the mutant showed higher activity against D-Met, D-Phe and D-Leu, and significantly lower activity against D-Arg and D-Lys, and D-Glu than the wild-type. These results showed that the insertion in ReDAO caused a narrow substrate specificity. We also created a TdDAO mutant lacking the insertion and analyzed its substrate specificity. In contrast to the case of the insertion in ReDAO, the deletion in TdDAO caused a broader substrate specificity.

RD-087

Development of an Enzymatic Isolation Method of Highly D-aspartic Acid-producing Lactic Acid Bacteria

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Lactic acid bacteria (LAB) are defined as bacteria that produce lactic acid by sugar metabolism. LAB contribute to human health and have been regarded as probiotics. LAB produce D-Asp by aspartate racemase and use D-Asp as a cell wall component. Recent studies showed that D-Asp also give some beneficial physiological effects on human health. Therefore, LAB that can produce a much amount of D-Asp are valuable for developing novel functional foods. In this study, we developed a simple enzymatic screening method for isolating highly D-Asp-producing LAB and screened the LAB from various samples.

D-Asp was quantified by HPLC or the colorimetric reaction using purified D-aspartate oxidase (ChDDO) of the yeast *Cryptococcus humicola* strain UJ1 and horseradish peroxidase (HRP) with 4-aminoantipyrine and phenol (phenol-4AAP method) or TOOS (TOOS-4AAP method). D-Asp dissolved in MRS medium and the supernatants of D-Asp-producing LAB (*Lactobacillus brevis* JCM 1170 and *Lb. gasseri* JCM 1131) and non-D-Asp-producing bacteria (*Staphylococcus epidermidis* JCM 2414 and *E. coli* K-12) were used as samples. LAB candidates were isolated on LBS agar or selective MRS agar containing sodium azide and cycloheximide from fermented foods, fruits, and vegetables in Niigata Prefecture by using this enzymatic method. Colonies were randomly picked up and then subjected to catalase test and halo formation test on MRS agar containing calcium carbonate.

The calibration curves of D-Asp dissolved in MRS medium showed that TOOS-4AAP method was more sensitive than phenol-4AAP method. The calibration curve generated by TOOS-4AAP method provided high linearity and R^2 value, and the detection and the quantification limits were 24 and 73 μM, respectively. Using the enzymatic method, we determined the D-Asp concentrations of the culture supernatants of *Lb. brevis* and *Lb. gasseri* were 394 and 327 μM, respectively, and those of *S. epidermidis* and *E. coli* were 180 and 197 μM, respectively. On the other hand, HPLC method determined that the respective concentrations were 219, 152, 27, and 39 μM, respectively, showing that the concentrations determined by the enzymatic method were 153 - 175 μM higher than those by HPLC method. The plot of D-Asp concentrations by the enzymatic method against those by HPLC method gave a linear relationship, showing that the concentration by the enzymatic method could be corrected by the equation of the plot. The screening of highly D-Asp-producing LAB is ongoing by using the enzymatic D-Asp determination method.