Visualization of Transgenes in Transgenic Rice Plants Using Fluorescence In Situ Hybridization

Background

Rice (*Oryza sativa*) is the model plant for monocotyledons/ cereals (Collard et al. 2008) which has been extensively studied, since production of transgenic plants in rice is relatively easy compared to that of other major cereals (Shimamoto and Kyozuka 2002). Due to its importance as a food commodity, a lot of research and studies has been done to improve the desirable traits in the rice. Resistance to drought, resistance to saline water (Zhou et al. 2007), higher yields of product (Zhang 2007) or even the β-carotene producing rice (golden rice) (Schaub et al. 2005) is some of the traits that had been developed throughout the last century. To facilitate the construction of transgenic plant with enhanced desirable traits or to introduce a new characteristic into the plants, appropriate method to transform foreign DNA into the plant cells is indispensable. Typical rice plant transformation method such as *Agrobacterium* mediated transformation (Hiei Y et al. 1997) and particle bombardment (Christou 1997) are widely known and used but there are some limitations in these methods to deliver large DNA fragments effectively to the plant cell (Bhalla 2006).

Wada et al. (2009) proposed the advantage of using bioactive beads to facilitate the transformation of large DNA fragments to the rice plant cell. A set of *Aegilops tauschii* hardness genes (ca. 100kb) were introduced to rice and the presence of the inserted transgenes in the second generation (T2) of the plant had been confirmed with PCR and FISH analysis.

In making a new transgenic plant, it is important to determine of whether the transgenes are stable in the plant genome or not. One of the ways to confirm this is by the use of Fluorescence In Situ Hybridization (FISH). FISH is a powerful tool for the detection of specific DNA fragments of interest, especially for large DNA fragments. For the purpose of analyzing the presence of transgenes in plant chromosomes, FISH offers the advantages such as the ability to visualize the location of the transgenes and providing the information of whether the inserted DNA is homozygous or heterozygous. The presence of the transgenes in the offspring would confirm that the genes are stable and that the offspring will inherit the transgenes in its genome.

Purpose

This experiment was conducted to confirm the presence of the inserted transgenes in T2 and T3 of the transgenic rice plant.

Result

Probe for detection

pBI-BAC tagged with fluorophores (digoxigenin and biotin) by nick translation were used as the probe for FISH analysis of the transgenes. Figure 1 shows the obtained tagged probes and figure 2 shows the dot blot result of the probes.

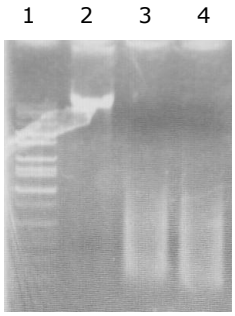


Figure 1. Gel electrophoresis of the tagged probe. *lane* 1: DNA marker, *lane* 2: unnicked pBI-BAC, *lane* 3: DIG-tagged probe, *lane* 4: biotin-tagged probe.

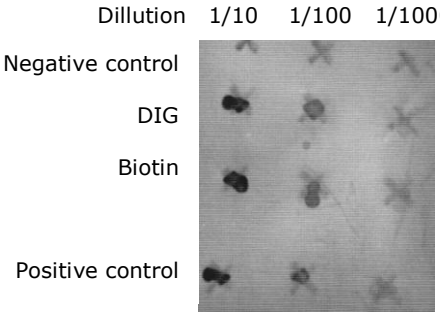


Figure 2. Dot blot analysis of the tagged pBI-BAC probe diluted to several dilution.

FISH analysis

The plant chromosome specimens of wild-type, T2 and T3 of the transgenic rice plants were prepared from the tip of the roots growing from the rice kernels of each plant (Fig. 1).

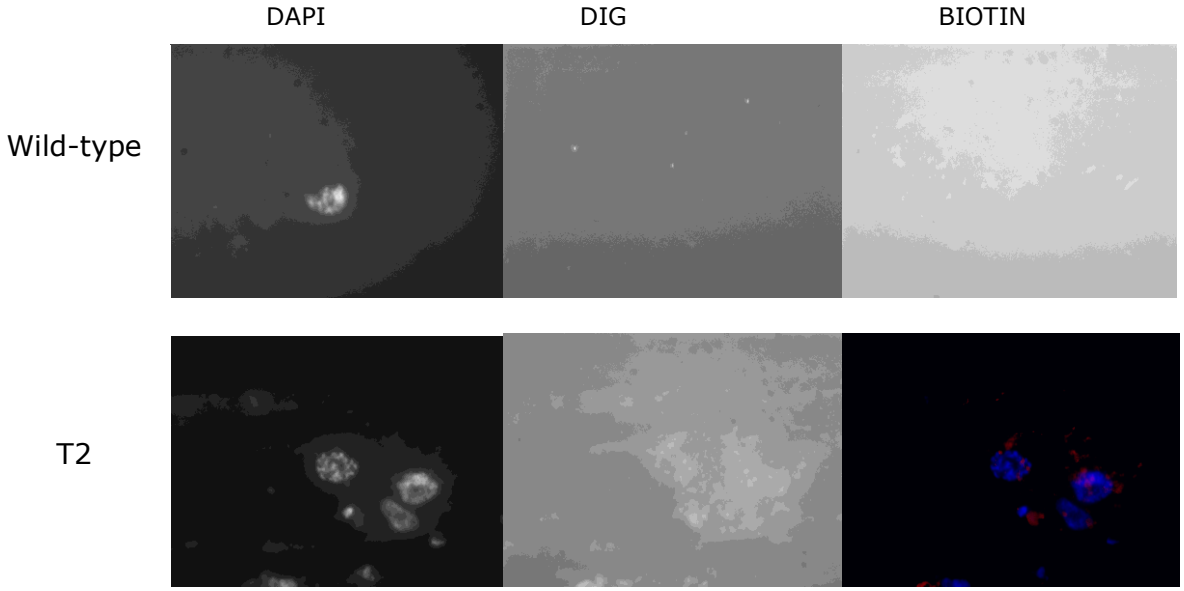


Figure 3. FISH analysis of the Wild-type and T2 chromosome obtain from the root tip grown from the rice kernels.

As expected, no signal was detected from wild-type plant chromosome (Figure 3). Unfortunately, due to the poor preparation of the chromosome specimen, the shape of the chromosome cannot be seen clearly either in the wild-type specimen or in the T2 specimen. Even though the T3 specimen had also been made, unfortunately it turned out to be it unusable. Thus, there are no data at all regarding the FISH analysis of T3.

Figure 4 shows a closer look to the chromosomes of T2 and what might be signal from the transgenes. However, due to unclear visualization of the chromosome specimen and the high signal noise present in the sample, it cannot be concluded that two of the small red dots present in the left side of the picture are the signal.

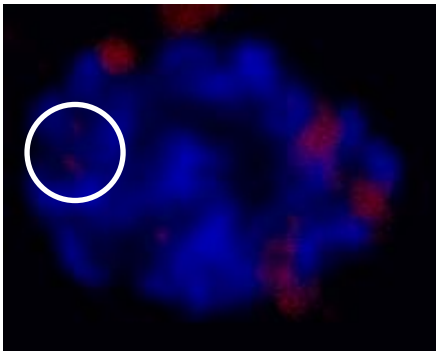


Figure 4. FISH analysis of the T2 chromosomes, showing what might be the two paired signal (shown as red dots inside the white circle) in one of the chromosome.

Discussion

Preparation of chromosome specimen in the slide is apparently an important step to obtain a good visualization of the chromosome. A clear visualization of the chromosome will give a more in-depth analysis on FISH result, in which the morphology of the chromosome can also be observed. Unfortunately, in this experiment a clear visualization of the chromosome were not obtained. As shown in figure 4, the shapes of the chromosome were unclear and rather look fuzzy.

In his paper, Wada et al. (2009) reported that the transgenes were detected to be in the telomeric region of chromosome 2 or 3, where two pairs of green signal were observed by FISH analysis. However, in this experiment, only a pair of what might be signal (Figure 3, shown in red) was observable. Due to the fuzziness of the chromosome, it is unclear to determine the region of where the transgenes are located in the chromosome. Furthermore, due to the poor preparation during the FISH hybridization and detection, there is a high noise in the fluorescence signaling.

High noise in the fluorescence signaling shows that there are a lot of nonspecific attachment between the probe and the nontarget DNA in the chromosome. Therefore, to reduce the noise means to prevent or to decrease the amount of nonspecific attachment. Some ways to reduce the noise in the signaling are decreasing the concentration of the SSC and adding more washing steps during the preparation for the detection.

Since the chromosome specimen for the T3 was unobservable, this experiment was only confirming the presence of transgenes in T2 plants. However, this work had already been done and the result has already been published in Wada, et al. (2009). Even though the result in this experiment was not satisfying, further confirmation of the result might not be necessary.

Acknowledgements

I am grateful for the warm welcome and kind gestures given to me from the lab members of Fukui Laboratory during my visit for the Project-based learning. Special gratitude goes to Mr. Naoki Wada for his kind guidance, helpful assistance and his patience in teaching a student who has never done any experiment with chromosome nor FISH analysis before.

References

Wada N, Kajiyama S, Akiyama Y, Kawakami S, No D, Uchiyama S, Otani M, Shimada T, Nose N, Suzuki G, Mukai Y, Fukui K (2009) Bioactive beads-mediated transformation of rice with large DNA fragments containing Aegilops tauschii genes. Plant Cell Rep. doi: 10.1007/s00299-009-0678-2.

Collard BCY, Vera Cruz CM, McNally KL, Virk PS, Mackill DJ (2008) Rice molecular breeding laboratories in the genomics era: current status and future considerations. Int J Plant Genomics 2008:524847. doi:10.1155/2008/524847

Zhang Q (2007) Strategies for developing green super rice. Proc Natl Acad Sci USA 104(42): 16402–16409. doi: 10.1073/pnas.0708013104.

Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong LZ, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol 63:591–608. doi:10.1007/s11103-006-9111-1

Bhalla PL (2006) Genetic engineering of wheat-current challenges and opportunities. Trends Biotechnol 24: 305-311. doi: 10.1016/j.tibtech.2006.04.008

Schaub P, Al-Babili S, Drake R, Beyer P (2005) Why is golden rice golden (yellow) instead of red? Plant Physiol 138(1): 441–450. doi: 10.1104/pp.104.057927.

Shimamoto K, Kyozuka J (2002) Rice as a model for comparative genomics of plants. Annu Rev Plant Biol 53:399–419. doi: 10.1146/annurev.arplant.53.092401.134447.

Christou P (1997) Rice transformation: bombardment. Plant Mol Biol 35(1-2):197-203.

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by Agrobacterium tumefaciens. Plant Mol Biol **35:** 205–218.

2009 年 3 月 21 日
280995 Project-based Training Course
Extracelluar overproduction, purification and crystallization of PML.
Eric Lindberg (28A08093)
Supervisor: Dr Clement Angkawidjaja (Kanaya 研)

In order to induce secretory over expression of PML in *E. coli* to a dilute overnight culture of *E. coli* DH5/pUC180-PML/pYBCD20 in 1 L fresh Luria broth was added 50 mg/L ampicillin and 30 mg/L chloramphenicol. The solution was incubated at 30°C for 20 hours. The culture was centrifuged at 8000 rpm for 40 min. The supernatant was collected and 100 mL of 1 M Tris-HCl pH 8.0 was added. PML was precipitated out of solution by 80% ammonium sulfate. Following centrifugation (8000 rpm, 40 min), the protein pellet was resuspended in 50 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, and 5% glycerol and dialyzed overnight against the same buffer solution. The dialysate was collected via centrifugation (15000 rpm, 20 min), filtered (0.22 um) and, applied onto a HiTrapQ HP anion-exchange column, equilibrated with 50 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, and 5% glycerol. The flowthrough was collected and concentrated to 1-2 ml by centrifugation (5000 rpm, 20 mins) using an Amicon membrane ultrafiltration device. The sample was loaded onto a gel-filtration column, equilibrated with 5 mM Tris-HCl pH 8.0, 0.02% Trition X-100. The protein was eluted from the column using the AKTA Prime system at 0.5 ml/min using the same buffer used for equilibration. Protein concentration was determined from UV absorption using a cell with optical length of 1 cm ($\varepsilon_{0.1\%}$; A_{280} = 0.91; 1.0 mg ml^{-1} as previously reported[1]). Fractions 33-42 were collected and analyzed by 15% SDS-Page and stained by Coomassie Brilliant Blue to assess purity of protein、>90% pure protein at ~ 65 kDa (Figure 1.). The GF fractions were concentrated to 8 mg $ml^{-1}$and then used for crystallization. Total protein content was estimated to approximately 3.5 mg. Crystallization was performed using the sitting-drop vapor diffusion technique. 4μl of the protein solution and 4μl of reservoir solution were mixed vapor equilibrated against reservoir solution. Small crystals were observed after 2 weeks (Figure 2.). The tray was left for another 2 weeks.

1[1]

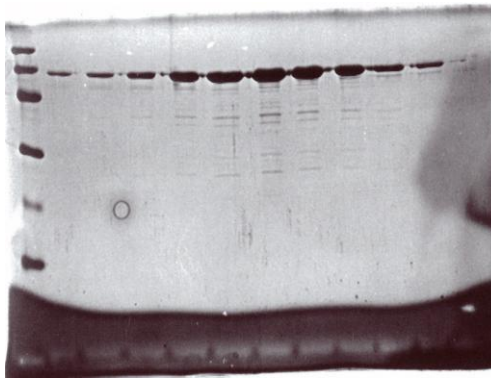


Fig 1. 15% SDS-Page stained with CBB.

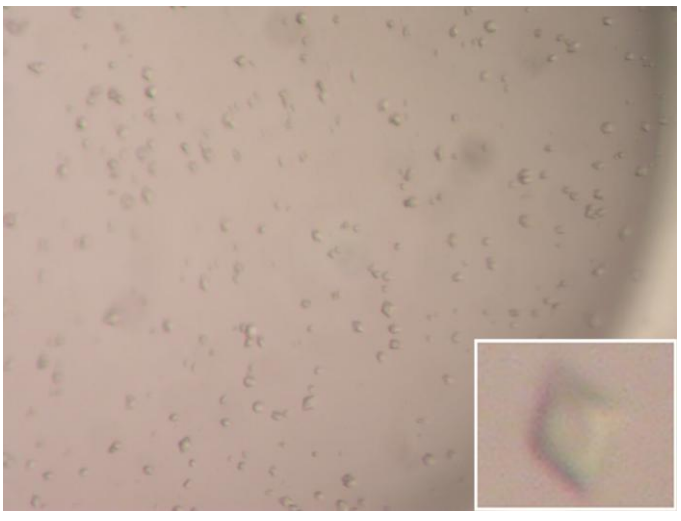


Fig 2. Observed crystals after 2 weeks.

2009 年 3 月 21 日
280995 Project-based Training Course
Extracelluar overproduction, purification and crystallization of PML.
Eric Lindberg (28A08093)
Supervisor: Dr Clement Angkawidjaja (Kanaya 研)

(1) Angkawidjaja, C., You, D-J., Matsumura, H., Koga, Y., Takano, K., Kanaya, S. *Acta Crystallographica Section F* **2007**, *63*, 187-189.

Name: Ahmad Mohamad haredy
Number: 90

Report for Project training Course

Aim: Identification of bacterial isolates using 16S rDNA technique
Trainer: Dr. Joong Jae KIM
Lab: Prof. Kanya Laboratory

Introduction

The main objective of microbial classification is to identify an isolated microorganism up to species level. However, discriminating or typing the different strains or genotypes of a species is gaining increasing importance from an industrial point of view. One of the most widely used techniques is the 16S rDNA identification which has been used as a project for this course.

Day 1:

- An introduction was given by Dr. Kim about the theoretical basis of the technique and introduction about bacterial classification.
- Preparation and sterilization of liquid LB media
- Inoculation of the isolates and incubation at 60 ℃for the second day in a shaker incubator 180 rpm overnight.

Day 2:

- Estimation of bacterial concentration using Normal OD measurement.
- Preparation of chromosomal DNA extraction from the isolated strains using the following protocol (extraction-purification)
    - Separation of cell by centrifugation at 8000g for 10 min
    - Decantation of the media and re-suspension of the pellet in 500ul of 1X TE buffer pH 8.0 containing 1mg lysozyme (roughly added)
    - Incubation of the previous solution at 37℃for 15 min
    - Add 50ul of 20%SDS (lysis) and stir gently for 15 sec. by inversion of the tube.
    - Add of 550ul phenol and mix then centrifuge at 14,000 rpm at 4℃from 10 to 30 min.
    - Carefully transfer the supernatant to a fresh tube without taking any part from the lipid floating layer.
    - Add equal volume of previously prepared phenol:Choloroform: Isoamylalcohol (25:24:1) to the transferred supernatant and mix gently
    - Centrifuge the mixture at 14,000 rpm 4℃for 10-30 min and carefully transfer the supernatant to a new tube
    - Repeat the phenol:choloroform: isoamyl alcohol step with centrifugation and separation of the supernatant 1 more time (remove proteins and other macromolecules)

- Add 25 ul of 3M sodium acetate(pH 5.2) and 0.7 volume of the supernatant (approx. 300ul) of ice cold isopropanol and store at -20℃one hour (for enchancement of pptn.)
- Centrifuge at 14,000 rpm at 4℃for 30 min and discard the supernatant. (keep in mind the direction of the tube)
- Rinse the pellet from the reverse side gently with 1ml of 70% ethanol (washing step)
- Remove the rinsing solution with the aspirator and dry the pellet using speed-vac for 30-min (centrifugal concentrator)
- Resuspend the pellet with 400ul of 1x TE-buffer(pH8.0)
- Add 2ul of RNase (10mg/ml) and incubate at 37℃for 30 min (to get rid of all RNA and purify the DNA)
- Add 400 ul of phenol and Mix (to remove the RNA lysate) and centrifuge at 14,000 rpm at 4℃for 20 min and transfer the supernatant carefully to a fresh tube.
- Add 20ul of 3M sodium acetate (pH5.2) and 800ul of ice-cold absolute ethanol and store overnight at -20℃. (Enhancement of DNA pptn.)

Day 3:

- purification of DNA, amplification of DNA, and preparation for sequencing
- The next protocol was used
  - Centrifugation of the tubes that were kept one day before at -20 ℃at 14,000 rpm 4℃for 30 min (keep in mind the direction of the tube in the centrifuge)
  - Rinse the pellet with 500ul of the ice cold 70% ethanol two times (keep in mind to rinse in the reverse direction of the pellet)
  - Remove the rinsed solution and dry for 10 min then resuspend with 40 ul of sterilized milli-Q water.
  - Take 3ul from every tube and mix with loading buffer and run on 0.8% agarose ge then stain with ethidium bromide to check for DNA existence in the tubes. Pic (1) against the DNA marker
  - Amplification of the 16s rDNA from the isolated DNA using universal forward and reverse primer as follows:
    - F1: 20 mer

    5`-AGA-GTT-TGA-TCC-TGG-CTC-AG-3`
    - R13: 20 mer

    5`-AGA-AAG-GAG-GTG-ATC-CAG-3`
  - Amplification of the 16s rDNA using the following protocol
    - **PCR mixture**
    - Template (DNA from Isolates) 1ul (may increase as needed)
    - 10 pmole primer 1 (F1) 2ul
    - 10 pmole primer 2 (R13) 2ul
    - dNTP 2.5ul
    - 10X buffer 5ul
    - Ex-taq polymerase 0.5ul
    - dWater up to 50ul

- **Reaction condition**

Temp	time (min)	cycle
94℃	5:00	1 cycle
94℃	1:30	
35℃	5:00	4 cycle
72℃	1:30	
94℃	1:00	
55℃	1:00	35cycles
72℃	1:00	
72℃	10:00	1 cycle
4℃	infinity	

- Load the amplified 16S rDNA for gel electrophoresis as before (pic.2)
- Extraction and purification of the amplified 16s rDNA from the gel using Promega Kit protocol
- Preparation of the sample for sequencing
  - **PCR mixture**
  - **Ready reaction mix** **1ul**
  - **5x seq. buffer** **3.5ul**
  - **1.6 pmole primer** **1ul**
  - **Template DNA** **max 4ul**
  - **dwater** **up to 17.5ul**
- Primers used for sequencing as follows
  - F1: 20 mer (8-27)

  5`-AGA-GTT-TGA-TCC-TGG-CTC-AG-3`
  - R13: 20 mer (1524-1544)

  5`-AGA-AAG-GAG-GTG-ATC-CAG-3`
  - F 340: 20 mer (338-351)

  5`-ACT-CCT-ACG-GGA-GGC-AGC-AG-3`
  - R-8: 16 mer (1100-1115)

  5`-AGG-GTT-GCG-CTC-GTT-G-3`
- PCR cycle

Temperature	time	cycle
96℃	5:00 min	1 cycle
96℃	10 sec	
50℃	5 sec	25 cycle
60℃	4:00 min	
10℃	infinity	

- Purification after pcr for Sequencing
  - Add 17.5ul of sample plus 82.5ul of water (100 ul final volume)
  - Add 10ul of 3M sodium acetate, pH 5.2

- 250ul of 100% ethanol (room temperature) and store at -70℃ for 15 min and then centrifuge 15,000 rpm 4℃ for 30 min
- Discard supernatant
- Wash with 500ul 70% ethanol , discard the rinse and dry under vaccum for 10-30 min (try to keep away from light as much as possible as the product is light sensitive)
- Re-suspend the product in 15 ul of tsr Buffer or (25ul Hi-dye buffer)
- Denaturate the product at 95℃ for 2 min
- Transfer to sequencing
- Because that there is no place in the sequencer we can store the plate over night in the cold room.

Day 4: Sequencing and Sequence analysis

- This day Dr. Kim give me a complete lecture and taught me how to use the sequencing machine, how to prepare and insert the sample and use the software
- After the sequence is finished Dr. Kim Started a lecture one more time about how to analyze and edit the sequence using gene doc and then make one sequence using DNA star program, them how to use this sequence and make a phylogenic tree. Using Mega 4 program.
- Homework was given to identify a strain and edit a sequence by me.
- The Homewark strain was with a very similar homogeneity to Themus-thermophilus.with 99% similarity.
- Another Homework was given to write a report about other molecular techniques for identification of micro-organisms.

Other Molecular techniques for identification of micro-organism

As with prokaryotes *classification*, identification of *bacteria* is increasingly using *molecular methods*. Diagnostics using such DNA-based tools, such as polymerase chain reaction, 16-s RNA, and other methods are used because rather than the classical classification methods. It decreases the human error and depends much more on the machine.

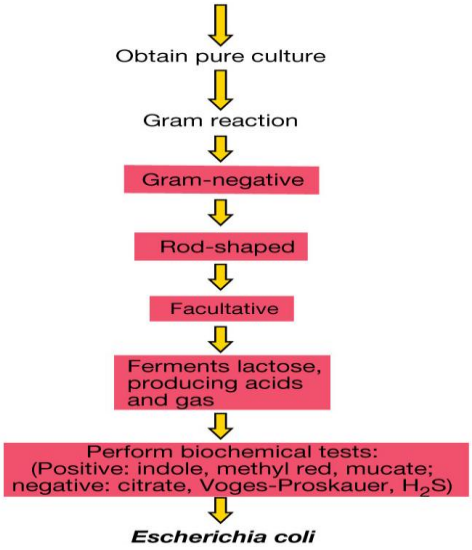


Fig shows classical old way of classification

The classical old way of identification is as Follows

1- Complete morphological and cytological description of the microorganism
2- Search using the key proposed by Bergey`s Manual of systematic bacteriology for for the phyla characteresticis , then genus characterestics. This is to search where it can belong to and complete the identification following the keys regulation as of biochemical tests and other required regulation.

Nowadays, a lot of techniques are used for molecular identification of microorganisms. It is important to be able to determine how well each technique can discriminate among strains and to know how many species or strains are involved in the various processes. Depending on the polymorphism degree obtained with the different molecular markers, those are more suitable either for inter-specific or for intra-specific discrimination. In this sense I am going describe some of the molecular techniques used according to those that can discriminate up to species level and those that can discriminate up to strain level.

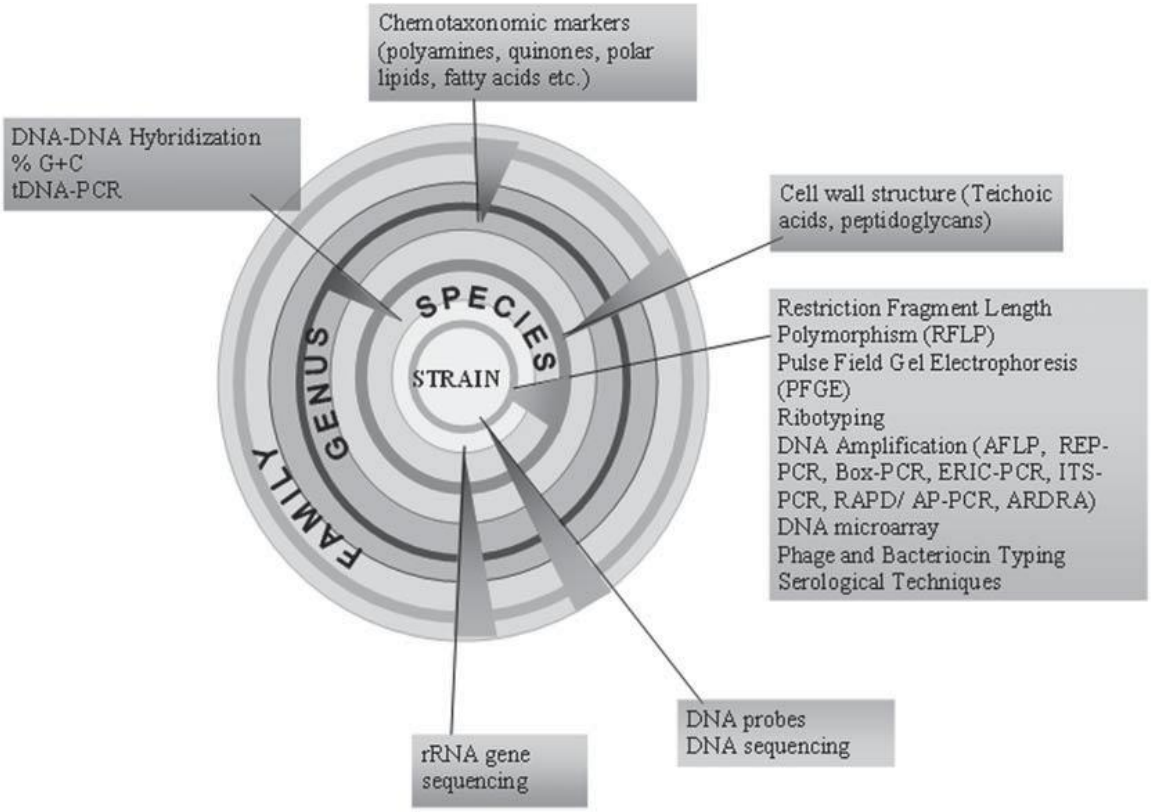


Figure from **O. Prakash et al. 2007 showing the different molecular techniques for classification of bacterial.**

1- Species level:

- **PCR-RFLP of the rDNA 16S :**

It is appropriate for differentiating and characterizing microorganisms on the basis of their phylogenetic relationships, The technique consists on the amplification of the 16S rDNA region followed by the digestion of the amplified fragment with a restriction enzyme. The DNA fragments obtained are separated by electrophoresis.

The resulting patterns are characteristic of every species and make it possible to characterize almost all the species.

- **PCR-RFLP of the 16S-23S rDNA Internally Transcribed Spacer (ITS):**

The 16S-23S ITS region have a great variation and length difference among the species, and conserved sequences with functional roles such as tRNA genes and antitermination sequences. This technique consists in the amplification of a region of the ITS (this one spanning between the 16S and 23S rRNA genes) followed by the digestion of the amplified products with one restriction endonuclease. The result is DNA patterns that are characteristic of every species. Intergenic sequences are known

to have higher variability than functional sequences, and they make it possible to distinguish below the species level.

- **Denaturing gradient gel electrophoresis (DGGE):**

  DGGE separation of bacterial DNA amplicons is a common method used to characterize microbial communities from specific environmental niches.This technique has been used to characterize all of the microorganisms in wine (yeasts, lactic acid bacteria..etc.).It does not require the microorganisms to be isolated. The most commonly used genes for the DGGE method are the 16S and 23S rDNA genes because they present bacterialspecificity.

  The technique consists on the amplification of a region of the genome. The amplicons obtained are resolved in a polyacrylamide electrophoresis in denaturing conditions created by using urea and formamide in the running buffer. The DNA fragments are separated because of their low electrophoretic motility in these denaturing conditions. The 5' primer used in the amplification has a poly GC tale of about 40 bp. During the electrophoretic process, the DNA remains doubled stranded until the gel zone in which the denaturing conditions are the same as its melting temperature (Tm). At that point, the DNA double strand is partially denatured and its motility reduced. Molecules with different base sequences will have different denaturing behaviours and, therefore, different migration rates in the polyacrylamide gel. DGGE makes it possible to separate DNA fragments that are of equal lengths but which have different base sequences. The band pattern obtained is indicative of the number of different species present in a sample.

- **Fluorescence in situ hybridisation (FISH):**

This technique has been used by to detect *Ga. sacchari* and other wine-related microorganisms such as lactic acid bacteria. FISH allows the direct identification and quantification of bacterial species at microscopic level without previous cultivation. It consists of DNA fluorescent labelled probes that will specifically hybridise each of the species or genera.

- **Real Time PCR:**

This technique identifies and enumerates bacterial species without having to culture. It was used to identify species of *Vibrio vulnificus*, and also to detect other bacterial species such as the fecal bacteria, *Bacillus cereus*. It is a fast and reliable method for identification and enumeration. Real-time PCR determines the initial template concentration by continuously measuring the product throughout the reaction and the initial number of cells can be accurately estimated by comparing it to a standard curve.

- **DNA-DNA hybridization**

According to the International Committee for Systematic Bacteriology was convened for a Workshop on Reconciliation of Approaches to Bacterial Systematics at the Institut Pasteur, **Paris,** on 14 to **16** May **1987.**It was approved that bacterial species generally would include the strain with 70% or greater DNA-DNA hybridization values with 5°C or less ΔTm values and both the values must be considered. However, it must be noted that this technique gives the relative % of similarity but not the actual sequence identity.

- **GC mol percent Analysis**

DNA G+C ratio or mole percent of guanosineand cytosine is one of the classical genotyping methods in the bacterial systematics. The variation in the percent GC content is not more than 3% within a well-defined species and not more than 10% within a well-defined genus and it varies from 24 to 76% in the bacterial world

2- Strain level:

- **Random amplified polymorphic DNA-PCR (RAPD-PCR):**

RAPD fingerprint is based on the amplification of the genomic DNA with a single primer of arbitrary sequence, of 9 or 10 bases of length, which hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures so that they can be use to initiate the amplification of bacterial genome regions. The amplification is followed by agarose gel electrophoresis, which yields a band pattern that should be characteristic of the particular bacterial strain.The technique has already been used to characterize rice vinegar acetic acid bacteria. It discriminates among acetic acid bacterial strains and the patterns yielded between 7 and 8 DNA fragments.

- **Amplified fragment length polymorphism (AFLP):**

It has been used for bacterial typing. It is a genome fingerprint based on a selective amplification of DNA fragments that are generated by restriction enzyme digestion. The bacterial DNA is extracted and digested with two different restriction enzymes (*Eco*RI and *Mse*I). The restriction fragments are then ligated to linkers containing each restriction site and a sequence homologous to a PCR primer binding site. These primers contain DNA sequences that are homologous to the linker and have one or two selective bases at their 3' ends. These selective nucleotides, then, make it possible to amplify only a subset of the genomic restriction fragments. To visualize the patterns, one of the primers contains either a radioactive or a fluorescent label, and these patterns can be alternatively resolved in agarose gels. This technique is laborious and costs more than the others because it uses labelled primers.

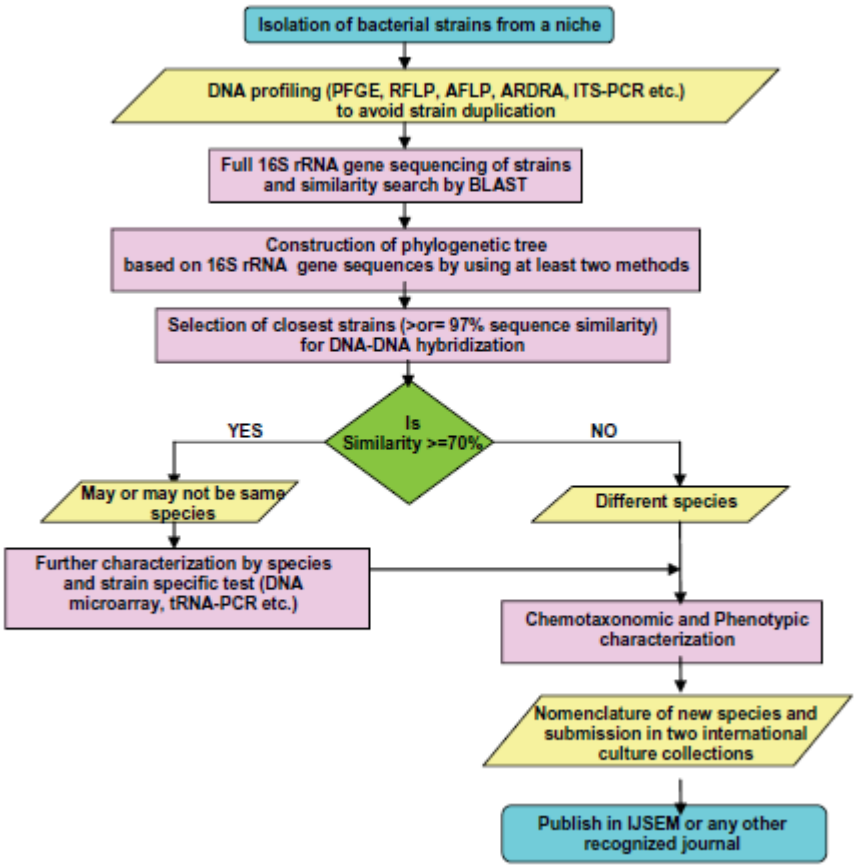
- **Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR):**

  Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR): ERIC and REP elements have been described as consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria. However, these sequences seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between these repetitive elements has generated DNA fingerprints of several gram-negative and gram-positive species.

- **DNA- Microarray:**

  Another method used by Martien Broekhuijsen 2003 DNA Microarray Analysis of Francisella tularensis Strainsthat is to overcome the shortcomings of DNA-DNA hybridization is DNA microarray. It too involves hybridization of DNA, but instead of whole genomic DNA, fragmented DNA is used. Numerous DNA fragments can be hybridized on a single microarray and generates portable data. This method givesResolution up to strain level and has been used in detecting virulence among the strains of pathogenic bacteria by identifying the strain-specific unique regions40

Before concluding the report ,We should also mention here a flow diagram for identifying a new strain



This Schematic Digram describes how to identify the new species, adopted from O.prakash et el. 2007

The benefits that I got from the Project Based training course:

1- Using Advanced molecular techniques
2- Revision of Methodologies for gel-electrophoresis and PCR
3- Sequencing and sequence analysis
4- How to make a phylogenic tree and to use Websites based programs to analyse the and to find the nearest strain with similarity
5- Reading about the new methodologies for Identifying the microorganisms.
6- Something Important to be noted that I already am using now the sequencing technique in the laboratory without any problem

Report in Project-based Training Course 2009

Juthaporn Sangwallek

28A08087

The operation of Consolidated Continuous Solid-State Fermentation System (CCSSF System) for ethanol production

Introduction

The demand of non-renewable energy resources like oil and natural gas, nowadays, keeps increasing; therefore the investigation of new alternative fuel has attracted worldwide attention. The ethanol production from renewable agricultural wastes is considerable interest; however, the development of its production process has still ongoing. The collection, delignification, saccharification, fermentation and product recovery have necessary to be considered. For this study, the improvement of the saccharification, fermentation and product recovery steps were focused. Accordingly, the conventional process consists of 80-90% of water; thus, the requirement of size of reactor tank and energy-intensive waste water treatment increase, thereby resulting in increasing of production cost. Consequently, Consolidated Continuous Solid-State Fermentation System (CCSSF System) has been adopted for the improvement of ethanol production system in order to overcome the previous problems. The most pivotal advantages of CCSSF System is minimizing of water content in the fermentation system, thereby reducing in size of reactor and decreasing cost to provide for waste water treatments. The operation of CCSSF System by using starch as a substrate model was performed in this study in order to indicate the potential of using this system for yielding the highest amount of ethanol continuously.

Objective

To elucidate the performance of Consolidated Continuous Solid-State Fermentation System (CCSSF System)

Materials and methods

TJ14 preparation

0.45 µl frozen stock of TJ14 was inoculated in 200 ml of YNB including supplementary media with 20g/L of glucose for 2 flasks and incubated at 37°C, 300 rpm for 12 hours as a seed culture. Then, the seed culture was transferred into 5L fermentor containing 2L of YNB including supplementary media with 20g/L of glucose under the condition at 37°C, 300 rpm and 1 VVM of air flow rate. After glucose concentration was nearly all used up, 100 g/L glucose with double concentration of YNB including supplementary media was fed and the growth rate was controlled at 0.25 h^{-1}. TJ14 seed

culture was washed by 0.85% water saline and then collected at -20°C before the fermentation step.

YNB + supplementary media

Yeast Nitrogen Base without amino acids	6.7 g/L	
Sodium dinitrogen citrate	2.74 g/L	YNB buffer
Aspartic acid	2.5 g/L	
Palmitic acid (dissolved in 99% hot ethanol)	0.12 g/L	Supplementary
$ZnSO_4.7H_2O$	80 mg/L	
Biotin	2 mg/L	
pH 5.2-5.3		

Consolidated Continuous Solid-State Fermentation

30 g-wet of TJ14 was inoculated into YP-starch media preparing for bench-scale CCSSF System. pH, amount of starch, glucose and ethanol were observed during the ongoing ethanol production.

YP-starch media for CCSSF System

YP media		200 ml
- Yeast extract	10 g/L	
- Polypeptone	20 g/L	
- Potassium disulfite	0.5 g/L	
Raw corn starch		50 g
Glucoamylase from *Rizopus* sp.		2000 U
α-amylase from *Bacillus* sp.		2000 U
28% ammonium solution (for controlling pH)		
Yeast extract (Nutritional addition)		

Measurement of the amount of starch, glucose and ethanol

0.2 ml of culture sample was harvested and centrifuged 15,000 rpm for 5 minutes in order to separate pellet and supernatant. The pellet was utilized for measuring the amount of starch content by using Huskin's tube method whereas the supernatant was utilized for measuring the amount of glucose by using Glucose CII test (Wako®) and the amount of ethanol by using Gas Chromatography (GC)

Result and discussion

TJ14 preparation

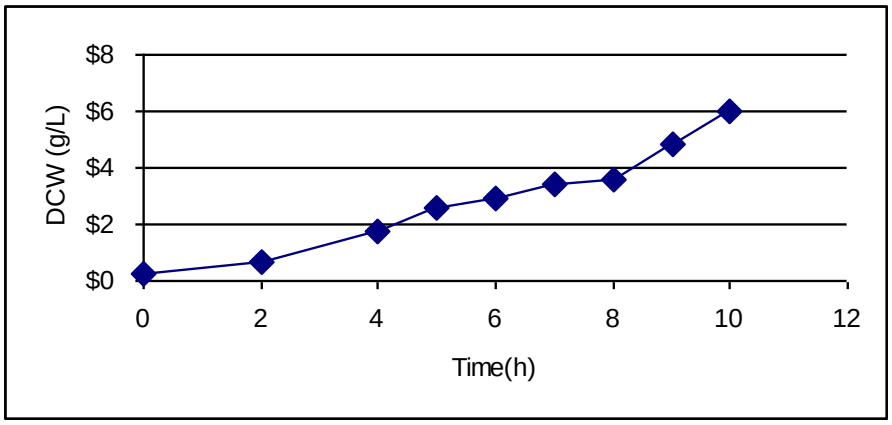


Figure 1 show the dry cell weight of TJ14 preparation

TJ14 was prepared via the batch culture (0h to 4h) and following with fed-batch culture (5h to 10h) after the amount of glucose was nearly all used up. The figure exposes that the amount of cells increase rapidly during the batch culture and slow down when the amount of glucose was nearly all used up. Switching to the fed-batch culture, the cells were controlled to achieve the steady quantity (μ=0.25 h^{-1}) by adjusting the flow rate; therefore, the amount of cells increase gradually. The total amount of cells was prepared for Consolidated Continuous Solid-State Fermentation (CCSSF) was 6 g/L at the end of process.

Consolidated Continuous Solid-State Fermentation

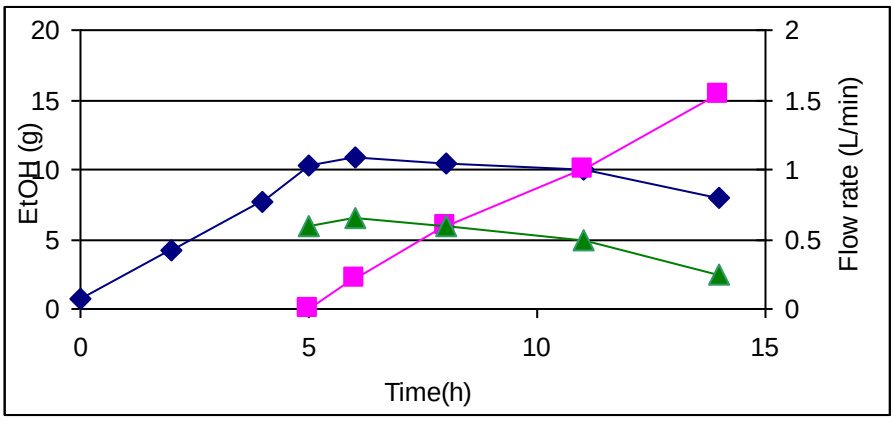


Figure 2 show the amount of ethanol production in the fermentor (blue) ethanol production in the recovery system (pink) and air flow rate (green)

Before starting the air flow, the figure demonstrates that the ethanol was rapidly produced. The specific production rate of ethanol was 0.32 g-ethanol/g-DCW/h. After

starting the air flow, the ethanol in the fermentor becomes decline whereas the ethanol in the recovery system increases, thereby the increase of total amount of ethanol production from the system as indicated in figure 3 below.

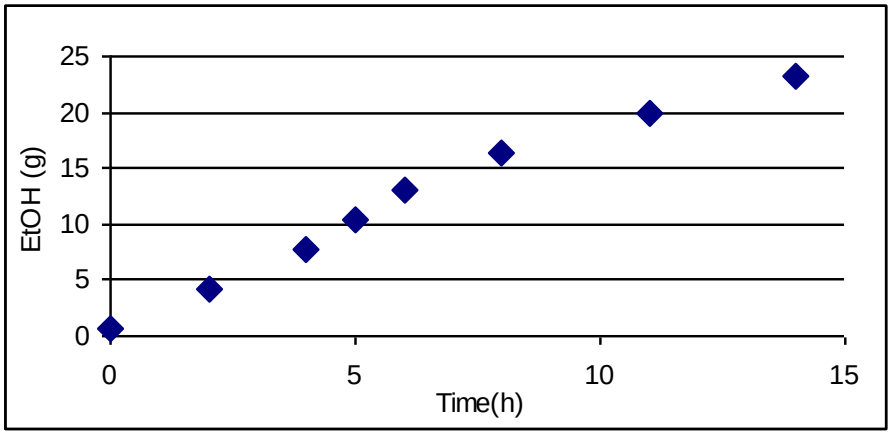


Figure 3 show the amount of total ethanol production from CCSSF system

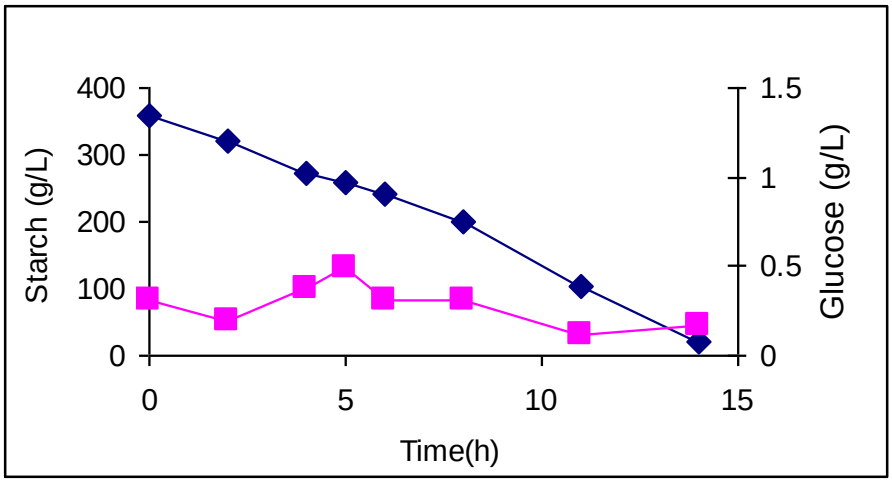


Figure 4 show the amount of remaining starch (blue) and the amount of glucose (pink) in CCSSF system

According to the starch served as substrate for this CCSSF system, the figure can be described that the amount of starch remaining in the system became gradually decrease since the starch was saccharifigated by enzymes in order to achieve the ethanol production. Additionally, during the saccharification of starch, the accumulation of remaining glucose in the system did not exist. This can perform that after saccharification of starch, glucose was efficiently used for ethanol production.

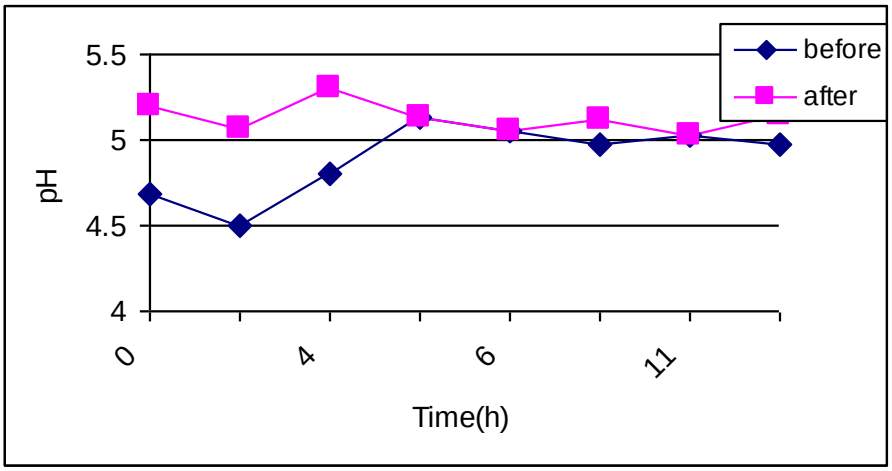


Figure 5 show the pH measurement during Consolidated Continuous Solid-State Fermentation (CCSSF)

Due to the reduction of pH during the fermentation, the mixture in CCSSF system was controlled in 5.0-5.5 by adding the appropriate amount of ammonium solution. The figure indicated that at the beginning of the fermentation, pH in the mixture rapidly declined in compare with the further period as a result of the reduction of cell activity in ethanol production.

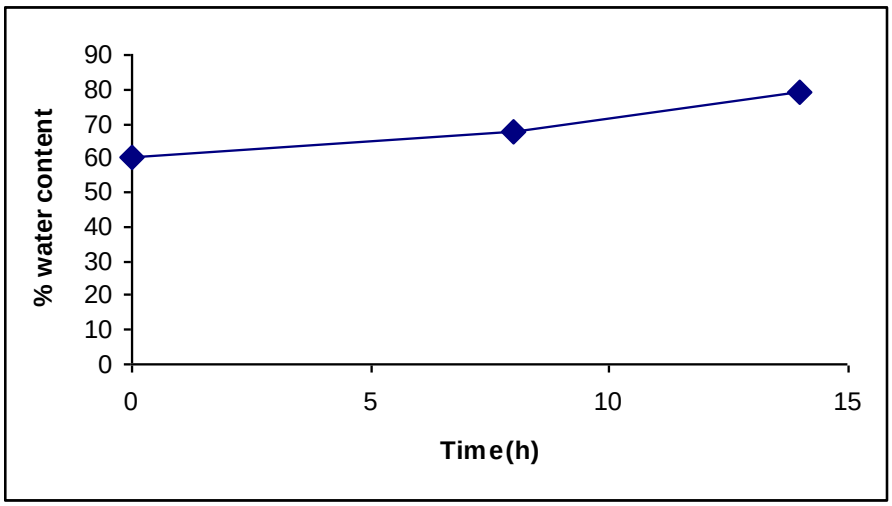


Figure 6 show the percentage of water content in CCSSF system

The percentage of water content increased after the fermentation proceeded. This can display that solid mixture in CCSSF system was utilized for ethanol production, thereby the reduction of water content in the system.

SOLID AND LIQUID PHASE PEPTIDE SYNTHESIS

(Report of Project-based Training Course)

Mustafa SUPUR

Solid phase synthesis is a process by which chemical transformations can be carried out on solid support in order to prepare a wide range of synthetic compounds. This idea was first developed by Bruce Merrifield to synthesize polypeptides and earned him the Nobel Prize in1984.

In solid phase peptide synthesis (SPPS) small solid beads, insoluble yet porous, are treated with functional units on which peptide chains can be built. The peptide will remain covalently attached to the bead until cleaved from it by a reagent such as trifluoroacetic acid (TFA). The peptide is thus detached from the solid-phase and can be retained during a filtration process, while liquid-phase reagents and by-products of synthesis are flushed away.

SPPS offers many advantages over conventional synthesis in terms of efficiency as well as convenient work-up and purification procedures. SPPS provides quickness during overall procedure comparing with other traditional methods. Purification is also easy since reagents can be simply washed away at each step. In addition, synthetic intermediates do not have to be isolated. Another advantage related with intermediates is no solubility problems with them during the process. Moreover, excess reagents are used which drives reactions to completion. Mechanical loss of the material is avoided because the series of reaction steps are taken place in the same reaction vessel. Last but not least, SPPS enables automation so that instruments can perform peptide synthesis automatically.

The disadvantages of SPPS can be listed as below,

- Reaction cannot be monitored by the standard method.
- Incomplete reaction may lead to deletion sequences.
- Growing peptide chain and the polymer support are incompatible.
- During the deportation step, at least 1% of the resin-peptide bond is also cleaved, resulting in an overall decrease in yield.

- Cleavage of the peptide sequence from the resin requires strong acid such as TFA, leading to nitrile formation, which disables HPLC analysis.

Liquid-phase or solution phase peptide synthesis is another approach to peptide synthesis. Although it has been replaced in most laboratories by SPPS, it retains usefulness in large-scale production of peptides for industrial purposes. It requires no expensive machinery. Additionally, peptides with unusual chemical features such as C-terminus aldehydes can be readily produced. In this method reaction tracking is also available.

However, in solution phase peptide synthesis, particularly in longer sequences, the repetition of coupling and deprotection cycles can become very labor intensive and require the isolation of all peptide intermediates. Purification is time-consuming step compared to that of SPPS.

Borimas Krutsakorn

M1 28A08086

Abstract

ATP is the major compound to give energy to the cell. The energy level it carries is just the right amount for most biological reactions. To study the affect of the changing of medium between DMEM+(D)-glucose and DMEM+(D)-galactose on ATP concentration, HeLa cell was used for studying by infecting with plasmid fluorescence ATP probe and imaging the fluorescent cell. The probe contained CFP, YFP protein and ε subunit of ATP synthase as the censor. The FRET phenomenon between CFP and YFP can be detected by fluorescence microscope. The proportional of YFP/CFP called average intensity showed the concentration of ATP in the cell. The changes in medium conditions was carried out by continuous-flow medium into the cell, i.e. switching medium from DMEM+(D)-glucose to DMEM+(D)-galactose and vice versa. The average intensity for ATP concentration decreased when starting flow of DMEM+(D)-glucose or DMEM+(D)-galactose medium. However, when switching media the ATP concentration was stable. Overall, there are many factors involved and therefore concrete conclusions cannot be made as results did not demonstrate that DMEM+(D)galactose had no affect on ATP concentration.

Objective

To study the affect of nutrient condition ((D)-glucose and (D)-galactose) on the concentration of ATP

Introduction

Every living organism can live by consuming food and producing substance needed for growing, breeding and so on. All mechanisms that take place in cell need energy for working. As far as known, all organisms from the simplest bacteria to humans use ATP as their primary energy currency. ATP is composed of a base (adenosine), a sugar (ribose) and three phosphate groups. The chemical bonds between the phosphate groups contain the energy stored in this molecule, and it is the breaking of these bonds (as ATP is converted into ADP or adenosine diphosphate) that provides the energy. A major role of ATP is in chemical work, supplying the needed energy to synthesize the multi-thousands of types of macromolecules that the cell needs to exist. Moreover, the ATP is used for transporting work that moves substances across cell membranes. ATP is also used as an on-off switch both to control chemical reactions and to send messages. Therefore, the concentration of ATP in cell is important to observe for understanding biological reactions occurring in cell.

All life produces ATP by three basic chemical methods only: oxidative phosphorylation, photophosphorylation, and substrate-level phosphorylation (Lim, 1998, p. 149). Most commonly the cells use ADP as a precursor molecule and then add phosphorus to it. In prokaryotes ATP is produced both in the cell wall and in the cytosol by glycolysis. In eukaryotes most ATP is produced in chloroplasts (for plants), or in mitochondria (for both plants and animals).

In the case of eukaryotic animals, the energy comes from food which is converted to pyruvate in cytosol. Then, pyruvate is converted to acetyl-CoA and CO_2 within the mitochondria. Acetyl CoA then enters the Krebs cycle which produces ATP and some compounds that enter to electron transport chain. The electron transport chain takes place in the mitochondrion's inner membranes by accumulating hydrogen ions in the space between the inner and outer membrane, it provides an electrical potential that releases its energy by causing a flow of hydrogen ions across the inner membrane into the inner chamber. The energy causes ATP synthase complexes to be attached to ADP which catalyzes the addition of third phosphorus to form ATP.

To aim of this study, the HeLa cell was used for observing ATP concentration. HeLa cell is an immortalized cell line and adherent cell (they stick to surfaces). The FRET phenomenon method and fluorescence microscope are applied for displaying the ATP concentration and imaging the cell when switch the medium. The FRET (Fluorescence resonance energy transfer) is a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor). In this report, CFP and YFP were used as the donor and the acceptor respectively. Moreover, ε subunit in ATP synthase will be changed the conformation when ATP appears in cell by decreasing distance between N-C terminals. Hence, ε subunit was used as the censor.

Construction of plasmid probe shows below.

Figure 1: Absence of ATP

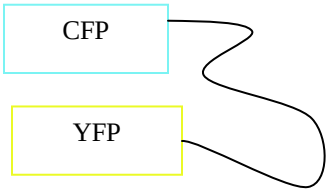


Figure 2: Appearance of ATP

Appearance of ATP makes the shorter distance between CFP and YFP so the energy is easily to transfer from CFP to YFP. For this reason, the proportional of YFP/CFP called average intensity can indicate the change of ATP concentration in the cell the more average intensity the more ATP concentration. To study how change the ATP concentration by switching nutrient condition, continuous medium flowing system was set up for imaging cell.

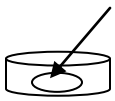
Material

- DMEM medium
- HeLa cell
- Trypsin-EDTA
- D-PBS
- Small glass cover dish
- Cell-mix
- OPTI-MEM
- FuGENE 6
- L-Glutamine
- HEPES
- Sodium bicarbonate
- Micro filter
- Sodium pyruvate
- D-glucose
- D-galactose
- Fluorescence microscope

Method

1) HeLa cell passaging

1. Warm up DMEM medium and Trypsin-EDTA at 37°C
2. Sterilize pipet on fire
3. Remove old medium
4. Wash old medium with D-PBS 5 ml remove D-PBS by pipet
5. Sterilize Trypsin-EDTA on fire, add 2 ml and remove some of them (for detaching cell from surface)
6. Incubate 37°C (10-15 minutes)
7. Add 5 µl of cell-mix in 5 small cover glass dishes and spread around glass area



8. Add D-PBS 0.5 ml each dish, incubate 1 minute and remove D-PBS by pipet
9. Repeat number 8.
10. Add 2 ml of DMEM medium
11. Cap incubated cell and resuspend with 4 ml DMEM medium
12. Disperse cell and transfer cell 150 µl to prepared dish
13. gently shake ↔ and ↕ direction
14. Incubate 37°C

2) Medium preparation

1. Dissolve 900 ml milli Q water to Dulbecco's Modified Eagle's Medium (DMEM)
2. Add L-glutamine 0.584 g
3. Add HEPES 3.57 g and Sodium bicarbonate 3.7 g
4. Adjust pH equal 7.4
5. Adjust volume by milli Q water for 1 liter
6. Sterilize by micro filter (for heat sensitive compound)
7. Pipet 100 ml of medium to 2 new bottles
8. Add 100 mM sodium pyruvate 1 ml to both bottle(final concentration 1mM)
9. Add 45% D-glucose 1 ml to bottle and 15% D-galactose 3 ml

3) Tranfection

1. Preparation of solution (for 1 dish)
    - Pipet 100 µl OPTI-MEM to 1.5 ml micro tube
    - Add 3 µl FuGENE 6
    - Short vortex and incubate room temp 5 minutes
    - Add 1 µg plasmid ATP probe
    - Short vortex and incubate room temp 20-40 minutes
2. Add 100 µl solution to each dish
3. Incubate at 37°

4) Set up medium flow continuous system

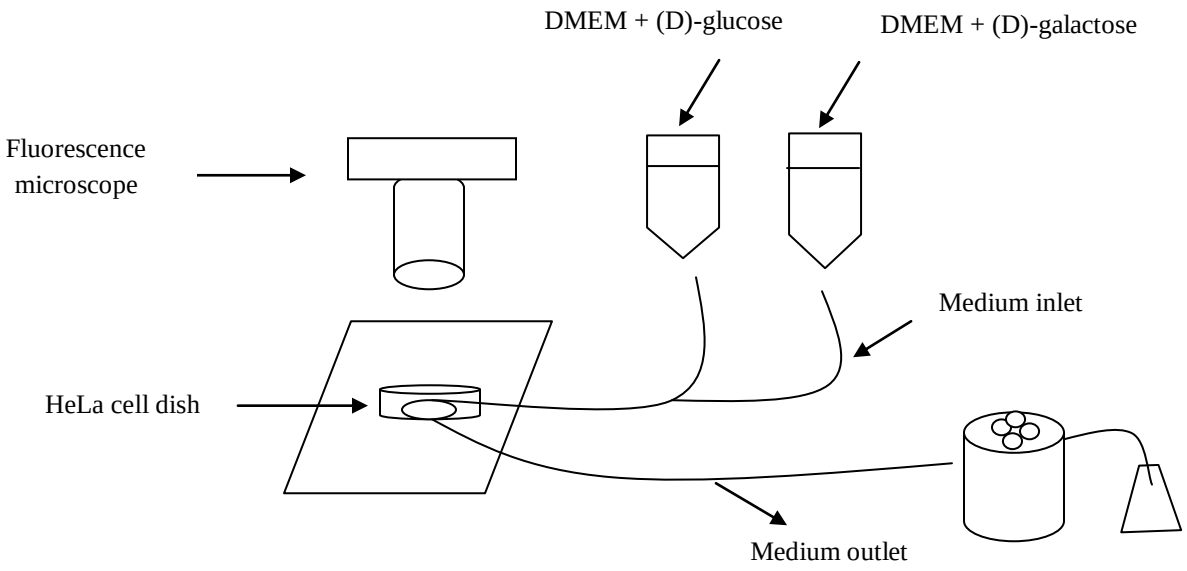


Figure 3: Medium flow continuous system

After finished set up system, the HeLa cell's medium was changed into DMEM + (D)-glucose and imaged by fluorescence microscope for 30 minutes. Then, starting to flow DMEM + (D)-glucose in to the dish for 30 minutes. Subsequently, switching flow medium from DMEM + (D)-glucose to DMEM + (D)-galactose for 45 minutes. Afterward, stop flowing medium.

On the contrary, the experiment started from DMEM + (D)-galactose medium first at 30 minute starting to flow DMEM + (D)-galactose. Then, switching flow medium from DMEM + (D)-galactose to DMEM + (D)-glucose at 60 minute. Later, the medium was stopped flowing at 90 minute.

5) Data analysis

- Metamorph software was used for intensity measuring

Result and discussion

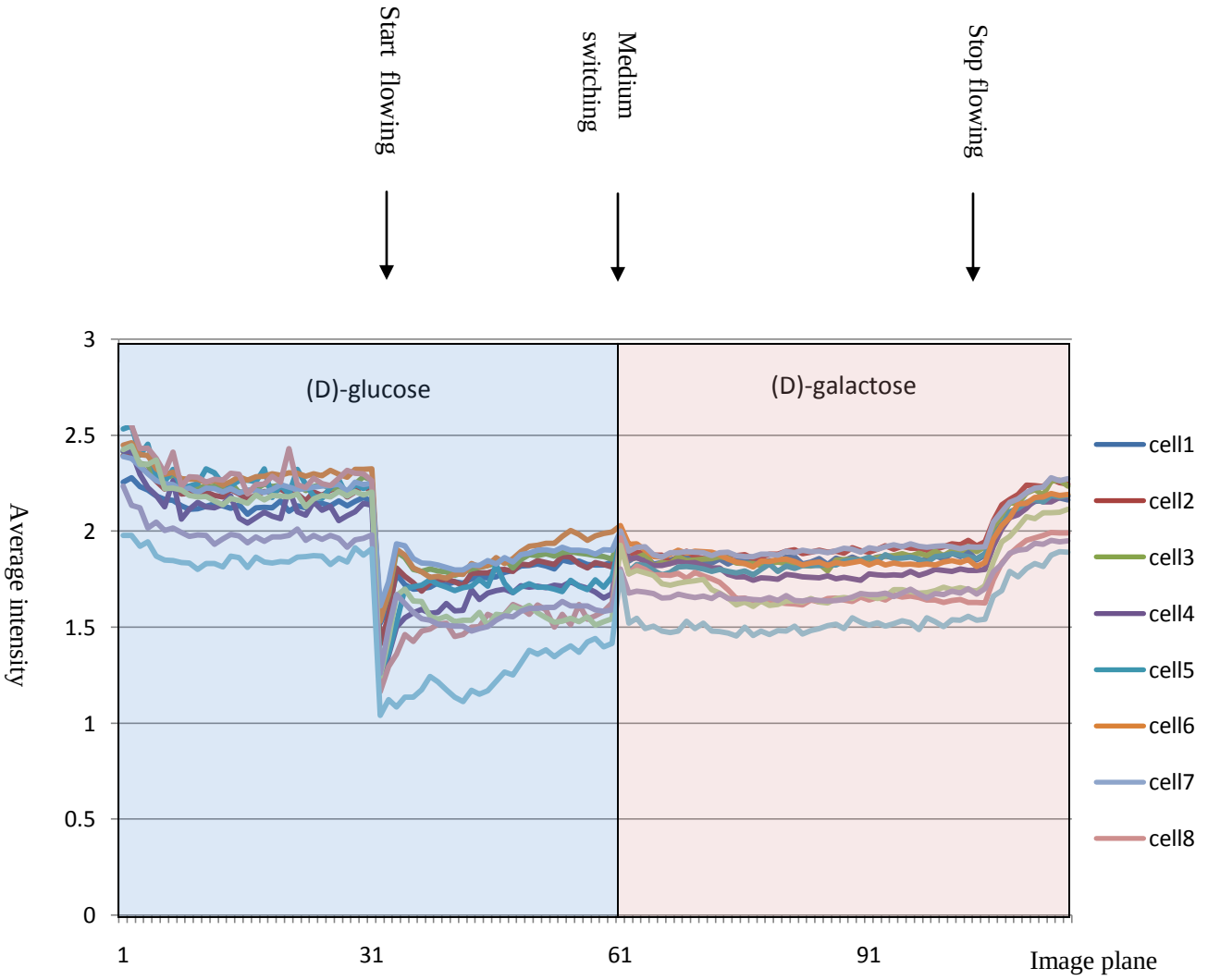


Figure 4: Graph shows average intensity and image plane. At 30 minute, starting to flow DMEM + (D)-glucose in to the dish. At 60 minute, switching the medium to DMEM + (D)-glalactose. At 105 minute, the flow of medium was stopped.

From figure 4, at 30 minute when media started flowing, the average intensity started decreasing which mean that the proportional of YFP/CFP decreased. Therefore, the ATP concentration in the cell also decreased. Due to absence of ATP, the fluorescence probe would change conformation: CFP and YFP would be far away from each other (Figure 1). As a result, the energy transfer from CFP to YFP also reduced. At 60 minute, media started converting from DMEM + (D)-glucose to DMEM + (D)-galactose and resulting in the changing of the average intensity slightly. So, the ATP concentration in the cell didn't change or insignificantly change. Then after 105 minutes, media was stopped flowing and the average intensity increased.

From this result refer to the increasing of the proportional of YFP/CFP as the ATP concentration also increased (when ATP appearance the probe censor change the conformation into Figure 2 so that it's easily to transfer the energy between CFP and YFP).

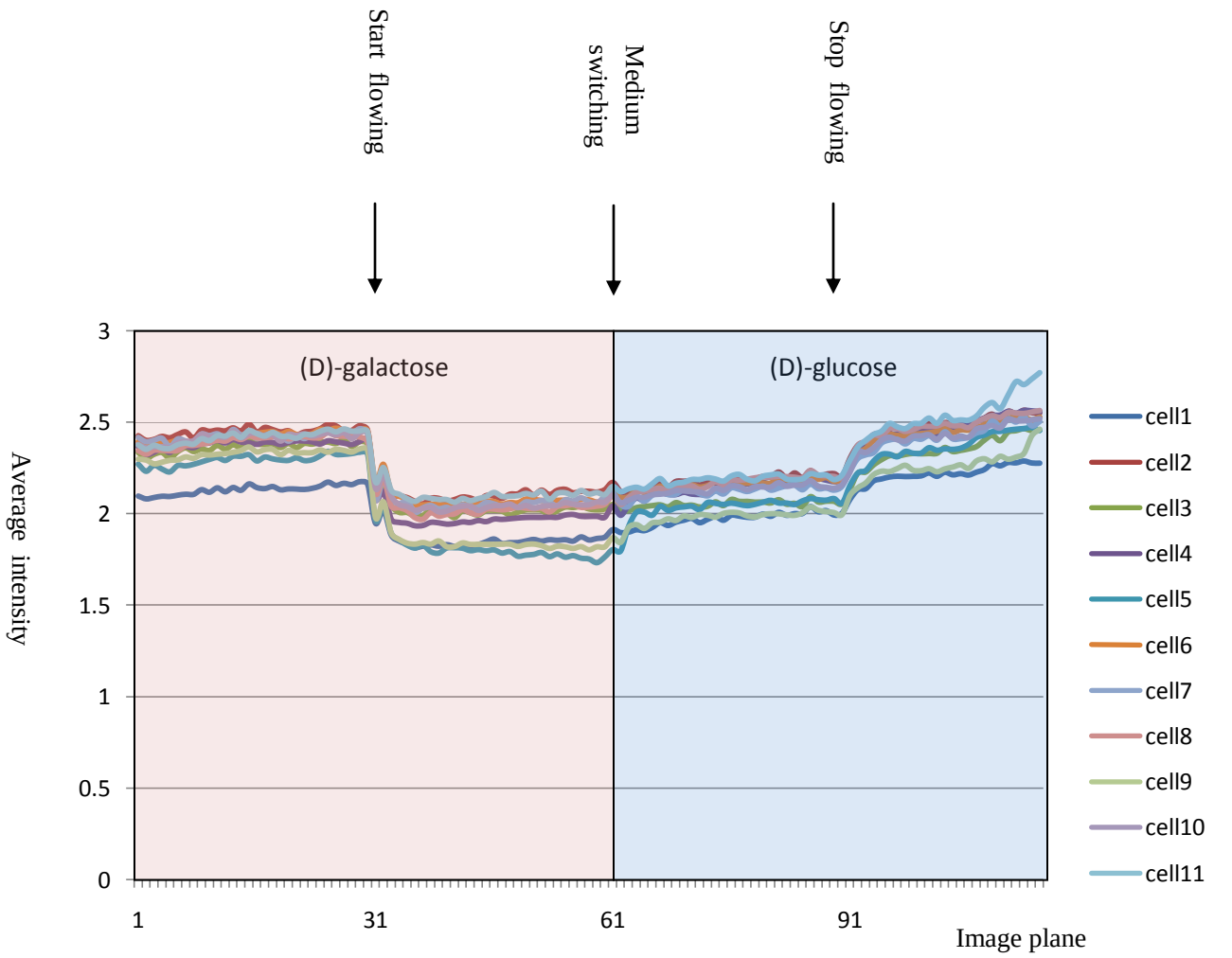


Figure 5: Graph shows average intensity and image plane. At 30 minute, starting to flow DMEM + (D)-galactose in to the dish. At 60 minute, switching the medium to DMEM + (D)-glucose. At 90 minute, the flow of medium was stopped.

From figure 5, at 30 minute when media started flowing, the average intensity also decreased so the ATP in the cell decreased. At 60 minute, switching medium to DMEM + (D)-glucose also cannot change the average intensity. Hence, the ATP concentration was stable. At 90 minute, when media stopped flowing, the average intensity increased so that the flowing of medium certainly reduced the ATP concentration.

Conclusion

In conclusion, all this results showed that flowing of media no matter what DMEM + (D)-glucose or DMEM + (D)-galactose reducing the ATP concentration in the cell. Moreover, converting of DMEM + (D)-glucose to DMEM + (D)-galactose has no effect on ATP synthesis from this data analysis even if in there is some nutrition that could generate ATP such as piruvate and another amino acid. Further investigation is needed in order to determine whether galactose has no affect on ATP synthesis by modifying suitable medium condition.

Reference

A. Lerant, B. Kanyics and Marc E. Freeman (2001). Nuclear translocation of STAT5 and increased expression of Fos related antigens (FRAs) in hypothalamic dopaminergic neurons after prolactin administration. Brain research. *904*, 259-269.

J. Bergman (1999). ATP: The Perfect Energy Currency for the Cell. Creation Research Society. *36*.

R. B. Sekar and A. Periasamy (2003). Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J Cell Biol. *160*(5), 629–633.

http://edoc.hu-berlin.de/dissertationen/xie-jing-2003-12-15/HTML/chapter1.html

http://www.elmhurst.edu/~chm/vchembook/640reviewmetab.html

http://www.wormbook.org/chapters/www_obesity/obesity.html

http://pathmicro.med.sc.edu/lecture/trypanosomiasis.htm

http://www.cptips.com/bscphys.htm

http://www.microbiologybytes.com/video/culture.html

http://glycolysis.co.uk/

http://www.studentsguide.in/microbiology/microbial-photosynthesis/glycolytic-pathway-glycolysis.html

http://www.cellml.org/examples/repository/qualitative/metabolic_models_doc.html

http://www.lamondlab.com/pdf/FRET.pdf

Project base training course – Nihira Lab
REPORT

I. Extraction and Detection of Thaxtomin

Streptomyces scabies, a soilborne actinomycete, is considered the principal causal agent of potato scab. Phytotoxins called thaxtomins are produced by this pathogen. Thaxtomins are unique 4-nitroindol-3-yl-containing 2,5-dioxopiperazines. These toxins induce the development of necrotic lesions on aseptically cultured potato minitubers

1. Procedure

Medium : Oatmeal medium [50 ml in 500 ml baffled flask]

- 10 g oatmeal / 1 liter distilled water, Autoclave medium 1st time then remove debris by centrifugation, adjust pH = 7. Autoclave again (2nd)

Cultivation condition

- Inoculated 10^6 spores / 50 ml medium in reciprocating shaker 120 rpm, cultivated for 48 hours

Thaxtomin extraction

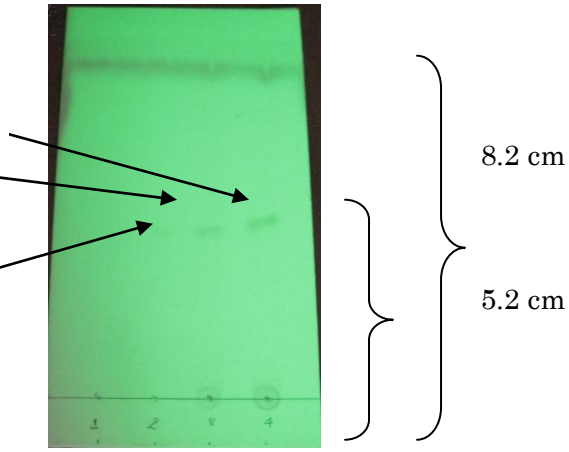
- Centrifuged the culture broth by using falcon tube; 3,500 rpm for 10 min.
- Extracted thaxtomin in 20 ml culture broth with equal volume of *n*-Butanol by hand shaking. Centrifuge to separate butanol layer.
- Evaporated the butanol layer in 40^{o}C, washed the extract with methanol and evaporate. Finally, dissolved in 500 µl methanol.

Detection of thaxtomin by TLC

- Prepared 50 ml of mobile phase (methanol: chloroform = 1:9).
- Cut the TLC plate in proper size according to the number of samples.
- 1. Methanol 5 µl 2. Load standard thaxtomin 1 µl
- 3. Load the extract 5 µl 4. Load the extract 10 µl

- After loading all samples, dried the plate, Put TLC plate into the chamber contained mobile phase, vertically.
- After mobile phase reached almost the top of TLC place (approx. 30 min), took the plate out from the chamber and dried with drier.
- Detected compound by UV light.

2. Result:



The Retention factor (Rf)

Rf of Thaxtomin = 8.2/5.2

II. Bioassay and HPLC Analysis of Virginiamycin

The Virginiamycin family, has been used successfully as a performance promoter in animal husbandry 1), used as an antibiotic mixed in animal feed. The family constitutes a unique class of antibiotics consisting of two groups of compounds: type A, exemplified by VM1, and type B, exemplified by virginiamycin S (VS). Although the two types have quite different structures, they act synergistically to provide greatly enhanced levels of antibacterial activity.

1. Procedure

Inoculated pre-culture of *Streptomyces virginiae* into 70 ml of f-media at the final OD_{600} of 0.075 for 24-h at 28^{o}C and 120 spm with a reciprocating shaker. The 1 ml of culture was centrifuged at 14.000 rpm in 10 minute. The supernatant was collected for bioassay test and HPLC.

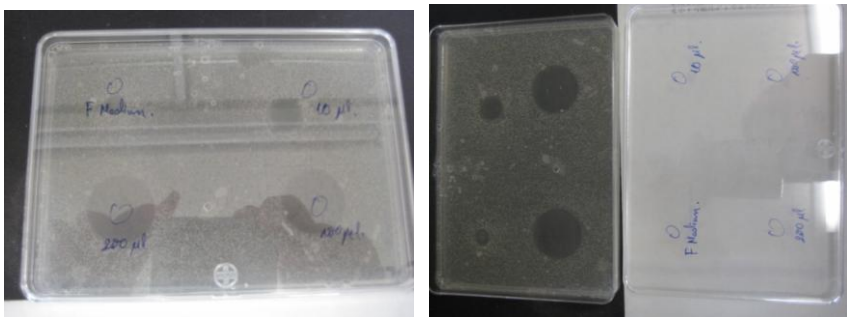
*** Bioassay against *Bacillus subtilis*.**

→ Poured out 50 ml of agar media into small square plate with inoculation of spore suspension of *B. subtilis*. (1% Inoculation by using *B. subtilis* spore suspension diluted to OD_{600}=0.8)

→ Put 10, 100 and 200 µl of culture supernatant and 200 µl of F medium as negative trial into penicillin cup.

→ Incubated plate at 30°C for 14 hours.

The result of Bioassay as follow:



As the picture, the clear circles appeared, that mean *Bacillus subtilis* inside these circles were killed by Virginiamycin. The position of 200µl of culture supernatant have the biggest clear circle (diameter of the circle = 2.6 cm), next is 100(l of culture supernatant (diameter = 2.4 cm), and 10(l of culture supernatant (diameter = 1.1cm). *

HPLC. Assay:

- Start the HPLC, set the condition (flow rate – 0.75 ml/min, UV detection 305 nm)

HPLC program – 20% Acetonitrile (0–5 min)

20–80% Acetonitrile (5–35 min)

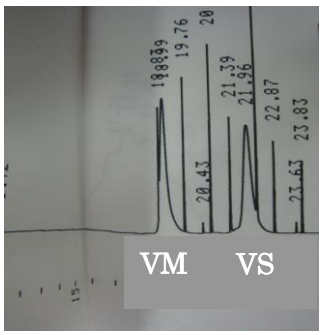
80% Acetonitrile (35–45 min)

80-20% Acetonitrile (45-55 min)

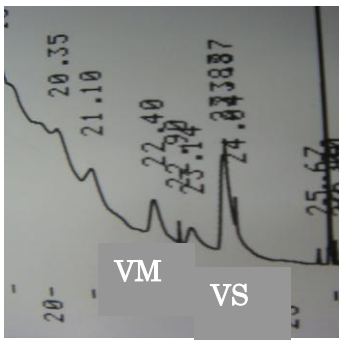
The column is Cosmosil $5C_{18}$ (4.6 × 100 mm)

- Injected 10 µl of Virginiamycin standard with concentration 0.4 µg/µl,
- Injected 130 µl of filtrated culture supernatant.
- Injected 200 µl of filtrated culture supernatant

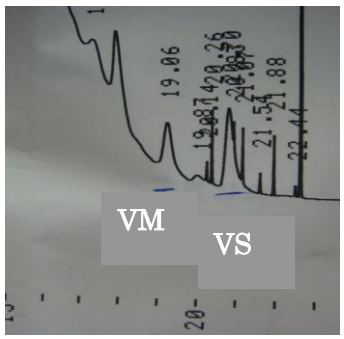
2. Result



10 µl of Virginiamycin standard



130 µl culture supernatant



200 µl culture supernatant

Calculation the concentration of Virginiamycin in culture broth

	Area		
	Standard	200µl culture supernatant	130µl culture supernatant
VS	141290	25837	18122
VM	119232	22671	13533
	260522	48508	31655

*** Injected 130 µl of filtrated culture supernatant**

$$\text{The concentration of Virginiamycin} = \frac{31655 \times 4}{260522 \times 0.13} = 3.74 \text{ µg/ml culture medium}$$

*** Injected 200 µl of filtrated culture supernatant**

$$\text{The concentration of Virginiamycin} = \frac{48508 \times 4}{260522 \times 0.2} = 3.75 \text{ µg/ml culture medium}$$

The average concentration of virginiamycin produced by *Streptomyces virginia*e is around 70 µg/ml culture medium. My result is quite low in comparison with that (3.75 µg/ml vs 70 µg/ml culture medium) .

References:

Cocito, C. 1979. Antibiotics of the virginiamycin family, inhibitors which contain synergistic compounds. Microbiol. Rev. **43:**145–198.

King, R. R., C. H. Lawrence, and M. C. Clark. 1991. Correlation of phytotoxin production with pathogenicity of *Streptomyces scabies* isolates from scab infected potato tubers. Am. Potato J. **68:**675–680.

Lambert, D. H., and R. Loria. 1989. *Streptomyces scabies* sp. nov. Int. J. Syst. Bacteriol. **39:**387–392.

Lawrence, C. H., M. C. Clark, and R. R. King. 1990. Induction of common scab symptoms in aseptically cultured potato tubers by the vivotoxin, thaxtomin. Phytopathology **80:**606–608.

Locci, R. 1994. Actinomycetes as plant pathogens. Eur. J. Plant Pathol. **100:**179–200.

Nattika P. **S. Kitani, T. Nihira**., 2007. Characterization of biosynthetic gene cluster for the production of virginiamycin M, a streptogramin type A antibiotic, in Streptomyces virginiae. Gene 393 (2007) 31–42.

Topic: **Analyze gene expression noise by double fluorescence report system**

Goal of learning: understanding how to use the Flow Cytometer instrument and apply it to measure the double fluorescence system for noise analysis.

1. Flow Cytometry

1.1. Overview

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics *(relative size, relative granularity or internal complexity, and relative fluorescence intensity)* of single particles (usually cells), as they flow in a fluid stream through a beam of light.

A **flow cytometer** is made up of three main systems: **fluidics** (transporting particles in a stream to the laser beam), **optics** (illuminating the particles by lasers and directing the light signals to detectors), and **electronics** (converting the detected light signals into electronic signals that can be processed by the computer).

1.2. Data collection and display

A particular particle (or cell) can be analyzed based on the **Forward-scatterd light** (FS) data, **Side-scattered light** (SC) data and **Fluorescent light** (FL) data.

- **Forward-scattered light:** proportional to cell-surface area or size
- **Side-scattered light**: proportional to cell granularity or internal complexity
- **Fluorescent light:** proportional to the number of fluorochrome molecules on the cell *(more than one fluorochrome can be used simultaneously if the peak emission wavelengths are not extremely close to each other)*

Once a data file has been saved, cell populations can be displayed in several different formats.

- **Single-parameter histogram:** the x-axis represent the parameter's signal value in channel numbers (FS, SS or FLs), while the y-axis represents the number of events per channel number.
- **Two-parameter histogram:** one parameter is displayed on the x-axis and the other parameter is displayed on the y-axis (ex: FL1 and SS, FL1 and FS, FL1 and FL2…)
- **Three-dimensional histogram:** x- and y-axes represent parameters and the z-axis displays the number of events per channel.

1.3. Operation the flow cytometer instrument (ex: EPICS XL instrument)

- Start-up: checking the sheath fluid level, checking the emptiness of waste tank, make sure the room temperature is suitable for the air-cooled lasers

- Quality control: injecting the quality control material (ex: Flow-Check solution) and checking if the data (ex: the CV value…) shows that the instrument is operated well

- Setting protocol: selecting parameters to be digitized by the ADC, defining the histograms and regions drawn on histograms

- Loading samples: samples should be prepared with suitable particle (or cell) concentration before injecting into the instrument

- Cleaning sample line: injecting cleaning agent and rinsing several times by distilled water

2. Analyze gene expression noise by double fluorescence report system

Basic idea: Using two identical copies of the genes were present in the same cell → their protein products were measured simultaneously.

→ They will have the same value of the extrinsic variables because both are present in a single cell (same intracellular environment)

→ They will have different values of the intrinsic variables

Method: to measure the level of gene expression (protein products), using genes encoding the green fluorescence protein (*gfp*) and red fluorescence protein (*rfp*). Level of expression of the GFP and RFP will be proportional to the GFP and RFP light emission intensity which can be measured by flow cytometry method.

- Target gene expression: expression by gene promoters involved in the lysine biosynthesis pathway of *E. coli* cells

- Promoter for comparison and analyzing the noise: strong promoter P_L and P_R

- Report system: *gfp* gene constructed downstream of the target promoters and *rfp* gene constructed downstream of P_L and P_R promoter

- Negative control: both *gfp* gene and *rfp* gene constructed downstream of two P_L and P_R promoters

Project Based Training Course Report

Yeast Transformation and Preparation of Yeast Genomic DNA

Yohanes Novi Kurniawan
28A08092

Goal(s)

In this project based training course, the first goal was to learn techniques of how to do transformation in yeast using two different types of transformation, which are integration type and plasmid based type transformation. The second goal was to ampifly conditional centromere 14 (CEN-14) by PCR.

Strain(s) and Plasmid:

-Yeast Strain 3A
-Yeast Strain W3617-1B
-Plasmid BYP 5166 (YCp50-HO)

Method(s)

1.Primer design

Summary of the designed primers:
i.Forward
Name: FwdCEN14
Seq:CTGATGGACTCCGTAGAGAGCAATGG
Length:26 nt
GC content:53.8%
Tm: 61.1^{o}C
Annealing position/chromosomal coordinates:710-735/628469-628494
ii. Reverse
Name: RevCEN14
Seq: TCATCGATAGGGCTGTTGGTGCTC
Length: 24-mer
GC content:54%
Tm: 61.1 ^{o}C
Annealing position/Chromosomal Coordinates: 1939-1962/629698-629721

2.Inoculation

-For preparation of yeast genomic DNA, strain 3A and strain W were inoculated to 5 ml YPAD liquid media and incubated at 30^{o}C overnight.

3.Genomic DNA extraction

Yeast Genomic DNA extraction method is based on the protocol described in Methods in Yeast Genetics, A Cold Spring Harbor Laboratory Course Manual.

4. Determination of concentration of yeast genomic DNA

Concentration of yeast genomic DNA can be calculated by measuring the OD value in 260nm using the spectrophotometer.

5. Plasmid digestion

Reagent(s)	**ul**
*Not*I	1
pCEN-01 UG(0.39ug/ul)	10
buffer 10xH	5
BSA	5
Triton	5
dH_2O	24
Total	50

Sample was incubated at 37^{o}C for 1 hour and run on gel agarose (0.8%) electrophoresis.

6. Transformation

i. Competent Cells

Competent cells was prepared in accordance to protocol describe in : Gietz,R.D. and R.A. Woods (2002), Transformation of yeast by the LiAc/SS carrier DNA/PEG method. Methods in Enzymology, **350**:87-96 for high efficiency transformation.

Transformation mix

Reagents	(ul)
PEG 3500 50% w/v	240
LiAc 1.0 M	36
Boiled SS-carrier DNA*	50
DNA(BYP5166/CEN01)**	34

*Salmon DNA (10ug/ul)

**BYP5166 (0.3ug), CEN01 (3.9ug)

7.PCR

i. PCR for amplification of CEN14

Reagents	(ul)
Premix*	25
DNA(W/3A)	1
FwdCEN14(10uM)	2.5
RevCEN14(10uM)	2.5
dH_20	19
Total	50

*dNTP, ExTag buffer, ExTag polymerase

PCR (Hot Start) condition:

94°C 4 min

94°C 30s
55°C 30s
72°C 5 min
} 30 cycles

ii. Colony PCR for mating type (a/α) determination

PCR master mix (for 16 reactions):

Reagent(s)	ul
10x Tag buffer	34
dNTP	34
MAT primer	34
a primer	34
α primer	34
Tag polymerase	3.4
dH_20	166.6
Total	340

*20ul for each tube, add yeast colony/DNA into each tube.

DATA

1. Genomic DNA concentration

	3A	W
$OD\text{-}_{230nm}$	1.473	3.582
$OD\text{-}_{260nm}$	1.850	3.234
$OD\text{-}_{280nm}$	1.015	1.872
$A_{260/280}$	1.82	1.73
$A_{260/230}$	1.25	0.90
C (ng/ul)	92.5	161.7

2. Plate Count

	10^{-0} (undiluted)	10^{-1} fold dilution
3A (BYP5166)	1278 colonies	122 colonies
3A(CEN-01)		
Plate 1	160 colonies	
Plate 2	297 colonies	
Total	457 colonies	

3. Transformation efficiency
   - plasmid based type (BYP 5166) transformation:
     Transformation efficiency=2.1×10^4 transformants/ ug plasmid
   - integration type (CEN-01) transformation:
     Transformation efficiency=117 transformants/ug DNA

Result and Discussion

Transformation of 3A with CEN01 (Integration type)

In general, there are two types of yeast transformation, integration type and plasmid based transformation. In integration type transformation, naked DNA fragment is introduced to the competent cells. Naked DNA will integrate into the host genome by homologous recombination phenomenon. Yeast is known actively integrates naked DNA by homologous recombination in a relatively high rate if to be compared with other organisms. Transformation efficiency of this type is relatively low if to be compared with transformation efficiency using plasmid based type. In this short project based course, I conducted integration type and plasmid based type transformation. For the integration type, I introduced yeast centromere number 01 that has been modified and named as conditional centromere 01 (CEN-01). CEN01 is liberated from the plasmid pCEN01 by digestion with *Not*I restriction endonuclease. CEN01 then mixed with another reagent to make transformation mix. Transformation mix was mixed with yeast competent cells and followed by heat shock at 42^{o}C for 40 min to introduce the DNA into the cells. Cells suspension was plated to appropriate selection medium (SC) and incubated for minimum of 2 days at 30^{o}C. After two days of incubation, a numbers of transformant could be seen. Total of 457 visible transformants can be counted. The calculated transformation efficiency of this type of transformation is 117 transformants/ug DNA.

For the plasmid based type transformation, I introduced plasmid BYP 5166 to strain 3A. Strain 3A is categorized as heterothallic strain with mating type a. There are two types of strain based on the stability of the mating type gene, which are homothallic and heterothallic strain. Heterothallic strain is a strain that has a stable mating type. In homothallic strain, the mating type of the

progeny cells can be switched from a to α and vice versa. This kind of switching is because of the work of HO gene, a kind of endonuclease. Yeast has two different mating type, mating type a and mating type α based on the specific gene in mating locus (MAT). In heterothallic strain, HO gene is recessive, resulted the same and stable mating type between the mother cells and its offsprings. Plasmid BYPP 5166 contained an active copy of HO gene. Strain 3A and W used in this experiment are haploid cells (n). By introducing this plasmid to 3A strain, the HO gene will be actively transcribed and change the mating type of 3A to **α** mating type. This **α** mating type will be mating with the **a** type via conjugation and become heterozygous diploid cells (**a**/ **α**). The changing of mating type can be assessed rapidly by colony PCR using three different primers, each specific for MAT locus, **a** specific, and **α** specific primer. When these three oligonucleotides are used in a single PCR reaction, sample with DNA from strain 3A (a type) generates a 544bp products, sample with DNA from strain W(α type) generates a 404 bp products, and for transformant (**a**/ **α)** generates two products. I did colony PCR for 12 transformants and 2 control (W and 3A). All the transformants generates two products. From this result, it can be concluded that the transformation was successful, because the mating type of transformants are now mixed between a and α type or (**a**/ **α)**. Transformants cell are diploid cells. Transformation efficiency of integration type of transformation is $2x10^4$ transformants/ug plasmid. If we compared transformation efficiency between integration type and plasmid based type, we can see very clear that the plasmid based type transformation efficiency is much higher than integration type. This is because the phenomena of homologous recombination in yeast (although relatively high if to be compared to other organisms) is very low and not efficient.

PCR

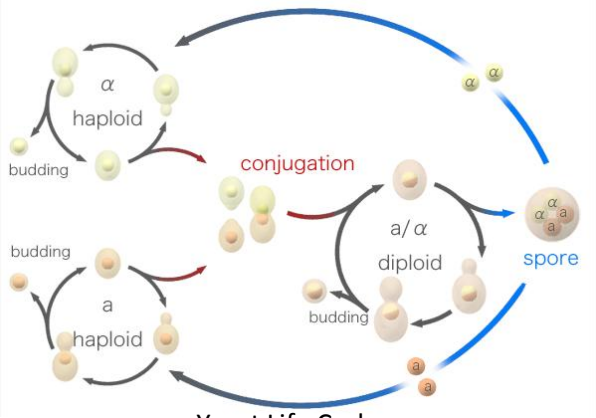
Yeast has 16 different chromosomes. Robert et al has successfully constructed 15 plasmid that contain conditional centromeres of chromosome (CEN01-13,CEN15-16) out of 16 chromosomes. Conditional centromere strain can be used to rapidly map genetic traits to specific chromosomes in yeast. These strains produce chromosome specific 2n-1 monosomy and concomitant loss of heterozygosity (LOH). Conditional LOH is a useful method to map recessive and/or dominant marker in the yeast chromosome. Conditional centromere 14 (CEN14) was not successfully constructed via plasmid based. It was constructed via PCR fusion. I designed primer in a way so the whole length of CEN14 can be amplified by single PCR reaction. The expected size of PCR product is 3.5 kb, 1.2 kb from the original size of centromere 14 and its flanking region and about 2.3 kb from the GAL1 and URA3 marker. However, the first attempt of PCR was not successfully amplified the conditional centromere 14 (CEN14). But centromere 14 and its flanking region from wild type strain (3A) was amplified successfully because of the presence of single thick band with size about 1.2kb on the stained gel. This is true regarding the theoretical size of centromere 14 and its flanking region is 1.2kb. The condition of PCR needs to be optimized further, probably by changing the type of polymerase or by changing the conditions and/or number of cycles. Primers are

already suitable for the PCR, because it was successfully amplified centromere 14 from wild type strain (3A).

Question(s)

1. **Please describe yeast life cycle**

The life cycle of yeast is alternating between haploid (n) and diploid cells (2n). These two phases are able to divide mitotically as buds that will eventually segregate from the mother cells when it grows bigger in size. The mating type of yeast haploid cells can be classified into two types, the a type and α type, based on the type at mating locus (MAT) on chromosome III. Both of mating types can undergo vegetative life cycle as stable haploid cells. They also can engage in sexual reproduction via conjugation mechanism between the two mating types. These two mating types use pheromone like molecule to interact with one another. It induces fusion between the two mating types and resulted in diploid cells with mix mating type a/α. This diploid cells also can undergo stable vegetative life cycle by mitotic division.



Yeast Life Cycle

Diploid cells can be differentiated from haploid cells by simple observation under microscope. Diploid cells appear as ellipsoidal cells and tend to appear individually, while haploid cells appear as round shape and tend to form budding. In condition of nutrient starvation, yeast cells are induced to do meiosis division and followed by sporulation. Diploid nucleus reduces to 4 haploids nucleus in meiosis division and followed by encapsulation of each haploid nucleus. Each of encapsulated haploid nucleus can undergo germination to form budding and divide mitotically when the environment condition is desirable. In homothallic strain, mating type of the daughter cells can switch to the opposite mating type and mate back with a cells of opposite mating type. In heterothallic strain, mating type of the daughter cells will be the same as the mother cells. This switching mechanism of mating type is due to the role of HO gene. Homothallic phenotypes arises from a dominant, functional HO allele at the HO locus on chromosome IV, while heterothallism result from recessive allele at this same locus.

2. **Yeast Genome Structure and Organization**

Yeast genome has a size of more than 12 Mb packaged in 16 chromosomes with total of 6000-6500 genes,5800 of them were predicated to correspond to actual protein-coding genes. The size of the chromosome is ranging from 200-2200 kb. Yeast genome also has a relatively low frequency of introns and low frequency of processed pseudogenes. Yeast genes are not clustered according to function or organized like bacterial operon.

Ty elements

Yeast genome has five different types of Ty elements that exhibit substantial homology to retroviruses and retrotransposons from plants and animals. These Ty elements are named: Ty1-5. Ty1, Ty2, and Ty4 belong to the 'copia' class of retrotransposons, while Ty3 is a member of the 'gypsy' family. Ty5 found in chromosome III.

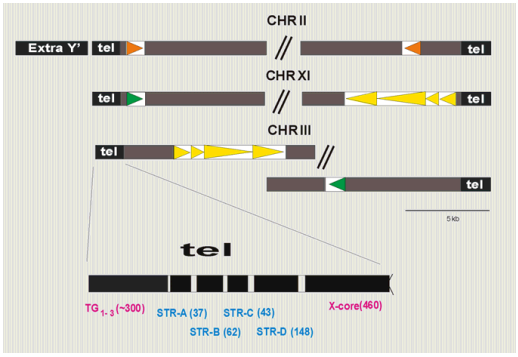
Centromere

Centromeres are the sites of kinetochore formation and chromosome attachment to mitotic and meiotic spindles. Centromere ensures faithfull segregation to the offsprings. The centromeric DNA in yeast consist of only around 200bp, and they share a common structure with the others centromere. The centromeric DNA sequences in all yeast chromosomes share a common substructure, which extends over only some 200 bp. The centromere sequences from *S.cerevisiae* can be subdivided into three distinct regions, which differ in base composition. During mitotic cell division, it will be normally replicated once and the two copies are segregated between mother and daughter cells in a 1:1 ratio. In meiotic division, the four copies will be segregated at a ratio 2:2.

Telomere

Telomeres function to protect chromosome termini from fusion and promote chromosomal end replication. Because of their 'open' structure, telomeres have to be stabilized and replicated by a specialized **telomerase system.**

All yeast chromosomes share characteristic **telomeric and subtelomeric structures.** Telomeric (TG1-3 or C1-3A) repeats, some 300 nucleotides in length, are found at all telomere ends.



Yeast Telomere Structure

Yeast Ribosomal Genes

The **ribosomal RNA genes** are located in yeast chromosome XII, where they form an array ofapproximately 100 tandemly arranged transcriptional units, each comprising the sequences for 28S, 5.8S, and 18S rRNA. Number of the units may vary slightly between strains. The ribosomal protein genes are scattered throughout the genome. In most instances, each of these genes has an identical duplicate somewhere else in the genome. Nearly all of these genes contain an intron sequence near their 5'-end. The DNA sequence revealed that yeast contains 274 tRNA genes, of which 80 have introns. By several criteria, the 274 yeast tRNA genes can be considered active genes and grouped into 42 **families** of distinct codon specificity.

Reference(s)

http://biochemie.web.med.uni-muenchen.de/Yeast_Biol/05%20The%20Yeast%20Genome.pdf

http://dbb.urmc.rochester.edu/labs/Sherman_f/yeast/13.html

Name: Huynh Nhat Kim Phuong
Student ID: 28A08082

Report for Project based Training course

Project topic: RNAi knockdown analysis of a human chromosome protein RRS1.

1. Introduction and purpose:

RNA interference (RNAi) is a system within living cells that helps to control which genes are active and how active they are. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference. RNAi can bind to specific other RNAs and either increase or decrease their activity, for example by preventing a messenger RNA from producing a protein. Gene knockdown utilizes RNAi mechanism to drastically decrease the expression of a target gene and studying the effects of this decrease can show the physiological role of the interested gene product.

Regulator of Ribosome Synthesis 1 (RRS1) is a nuclear protein that is conserved in eukaryotes. RRS1 locates on chromosomes and is suggested to have relationship with chromosome dynamic. In order to confirm how RRS1 affects to the attachment of the two pair of sister chromatids to centromere during metaphase, we conducted the project named: "RNAi knockdown analysis of human chromosome protein RRS1".

2. Results:

- Control sample: Hela cells without siRNA transfection
- siRNA sample: Hela cells were transfected with siRNA.

After siRNA transfection, control sample and siRNA sample were incubated at 37^{O}C for 3 days. Then, Western blotting was done to check the expression of RRS1 protein and Hela's chromosomes were observed under fluorescent microscope.

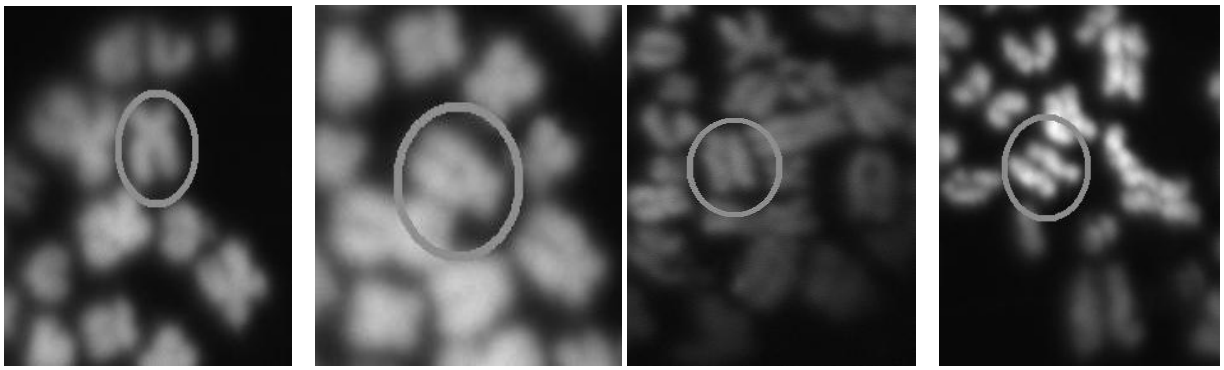
a. *Result of Western blotting:*

Western blotting was performed with control and siRNA samples, then, α-tubulin and RRS1 protein were detected. The result of Western blotting shows that RRS1 was knockdown in the siRNA-transfected sample (data attached)

b. *Results of chromosome spread:*

As in Western blotting experiment, we performed chromosome spread with 2 samples: control sample and siRNA sample.

There were 4 states of chromosomes observed: opened arms, closed arms, mild separation and complete separation.



Open arms Close arms Mild separation Complete separation

Figure 1. The 4 states of chromosomes (in circle)

Following graphs show the percentage of mitotic cells with different states of chromosomes of the control and siRNA samples.

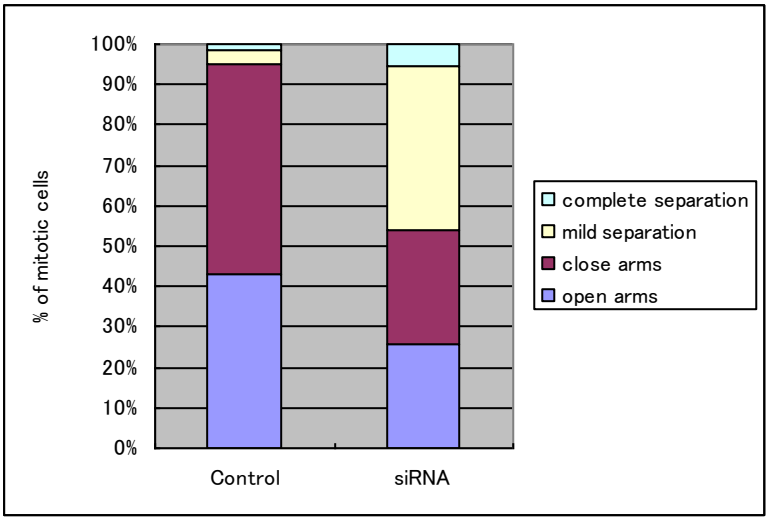


Figure 2. Percentage of mitotic cells with different states of chromosomes (data on 2009/03/10)

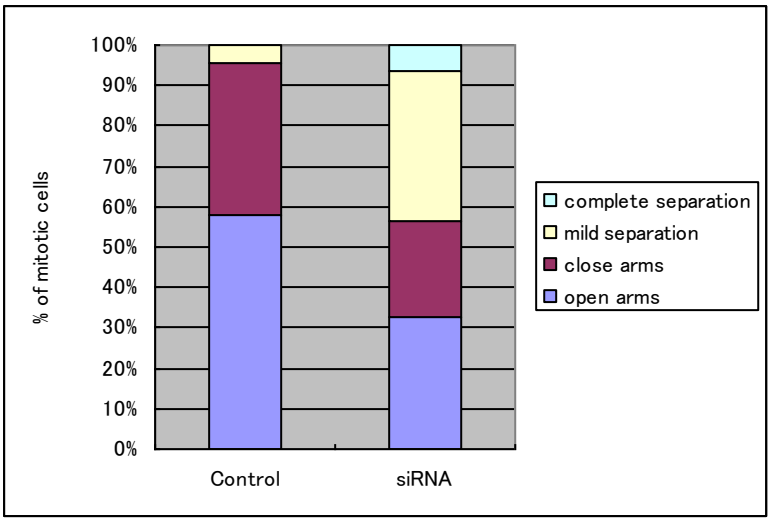


Figure 3. Percentage of mitotic cells with different states of chromosomes (data on 2009/03/13)

3. Discussion:

The mild separation state and complete separation state are considered as abnormal states of chromosomes. Figure 2 shows that the percentage of cells with mild separation and complete separation chromosomes in control sample are about 3.5% and 1.5%, respectively whereas the percentage of those in siRNA are about 40% and 6%, respectively.

Data from figure 3 also shows similar phenomenon. The percentage of cells with mild separation and complete separation chromosomes in control sample are about 4% and 0.2%, respectively while the percentage of those in siRNA are about 37% and 6.5%, respectively.

From the above data, the percentage of cells with mild separation chromosomes increased about 10 times in siRNA sample compare with control sample. In other hand, comparing with control sample, percentage of cells with complete separation chromosomes in siRNA sample increased 4 times in data collected on Mar. 10 and increased 32.5 times in data collected on Mar. 13. The reason why percentage of cells with complete separation chromosomes in siRNA sample of data on Mar.10 only increased 4 times but this of data on Mar.13 increased 32.5 times is unknown, it requires more experiments to obtain an accurate data. However, in general, the two data indicate that RRS1 knockdown increasing munber of cells with abnormal states of chromosomes. This result suggested that RRS1 protein may have an important role in the attachment of the two pair of sister chromatids to centromere in metaphase of mitosis.

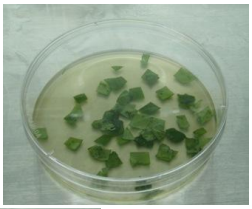
Project- based training
Fujiyama lab (09.02.20)

Atefeh Alipour
Fukui lab

Infection of Agrobacterium into Tobacco leaf

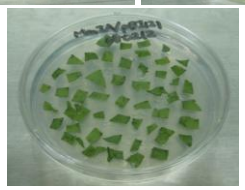
1st day_ Culture a single colony of *A. tumefaciens* harboring plasmid to 3mL of 2xYT medium supplemented with the appropriate antibiotics (Kanamycin 100 Mg/ml, Hygromycin 20Mg/ml), 50ul/ml Rifampicin, and 100ul/ml Streptomycin. Culture the cells at 28°C with shaking for overnight.

(OD600=0.8-1.3)



2nd day_ Cut one of nice tobacco leaf and prepare 5mm square disks using surgical knife and then put adaxial side of leaves up onto MS-B5 medium (without any Antibiotics) for keeping fresh them .

Soak the leaf paces (50) to Agrobacterium solution for 20 min, and then put them on the sterile filter paper for remove the extra solution. Put the leaves paces adaxial side up onto MS-B5 medium for callus formation. Then co-cultivate the explants at 25C, in dark place for 3 days.



5th day_ Wash the leaves by liquid MS medium. (Add 250Mg/ml carbenicillin to medium, when you want to use) Put them on filter paper and then on the MS-B5 medium for callusing. (adaxial side up and ~20 paces per plate)

Incubate the plates at 28C, under 18-h light, for 2 weeks.

After every 2 weeks transfer them to fresh MS-B5 medium for shoot formation.

After getting shoot

Cut shoots individually, which has at least 3 mm length, circle leaf and strong green color.

Plant them on the rooting medium, at first time in plate and then for plantlet development transfer to rooting medium in the plastic jar. Incubate at 25C and 16-h light.

Hygromycin has blocking effect for rooting, so we didn't add on the rooting medium.

I used 3 strain of Agro. ;

PBI121/SR1 , PBI121/DSRed ⟶ which have resistance to Kanamycin
PGPTV/HPT/GFP/KDEL ⟶ which has resistance to hygromycin

All experiment was done Sucrose and maltose plates at the same time, and I used Tobacco (Nicotiana benthamiana) and Jatropha leaf for infection.

Medium for washing, callusing, shooting and rooting

_MS medium for washing leaf

Murashige and Skoog Plant Salt Mixture (Wako)	4.3g
Sucrose	30g
KH2PO4 (x500)	2ml
pH 5.8 by 0.1N KOH (about 2ml)	1L

Plate: Gellan Gum 3g/L

After autoclave add;

*1ml Vitamin (x1000)

*250ug/ml carbenicillin

_MS-B5 medium

Murashige and Skoog Plant Salt Mixture (Wako)	4.3g
Sucrose	30g
MES	0.5 g
pH 5.8 by 0.1N KOH (about 10ml)	1L

Plate: Agar (BA-30, INA) 8g/L

After autoclave add;

*1ml Vitamin (x1000)
*250ug/ml carbenicillin
*appropriate antibiotics
Kana;100Mg/ml
Hygro;20Mg/ml

Hormone for callus formation;

NAA, final conc. 2ug/ml
BA, final conc. 0.2ug/ml

Hormone for shoot formation;

NAA, final conc. 0.1ug/ml
BA, final conc. 1ug/ml

_Rooting medium

Murashige and Skoog Plant Salt Mixture (Wako)	2.15g
Sucrose	15g
MES	0.5 g
pH 5.8 by 0.1N KOH (about 10ml)	1L

Gellan gum (0.2%) 2g/L

Add after autoclave;

*1ml Vitamin (x1000)
*250ug/ml carbenicillin
*IBA(200ug/ml) 100ul
*Just Kana;100Mg/ml

Result:

I got the shoot after 1 month in Hygromycin, as we know Hygromycin is one of strongest Antibiotics ,so transformant plant can't easily growth on it, but we get very nice shoot in Maltose plate.

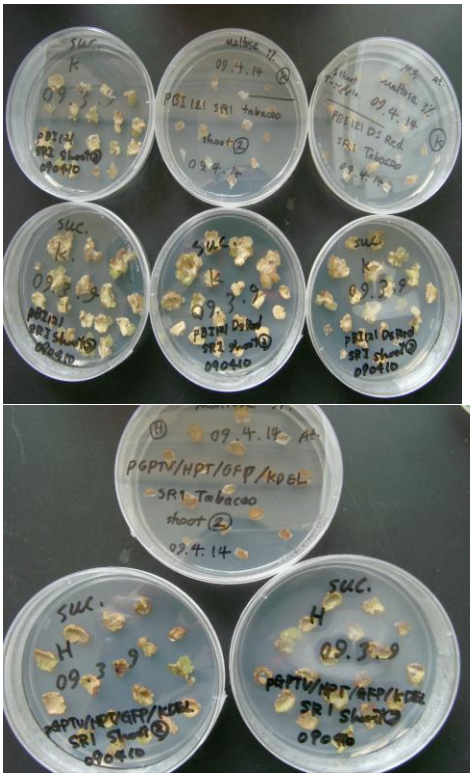
That shown Maltose instead of Sucrose for shoot formation has best result.

Also in case of Kanamycin, Maltose plate for shooting showed significant result.

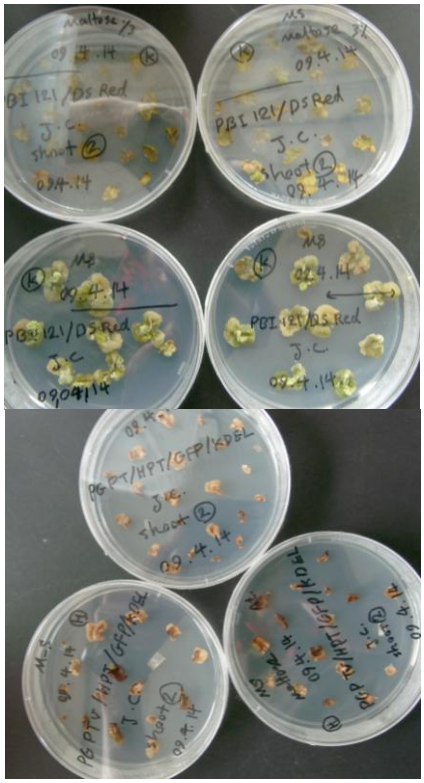
I transferred the shoot to rooting plat and I'm still waiting for root regeneration.

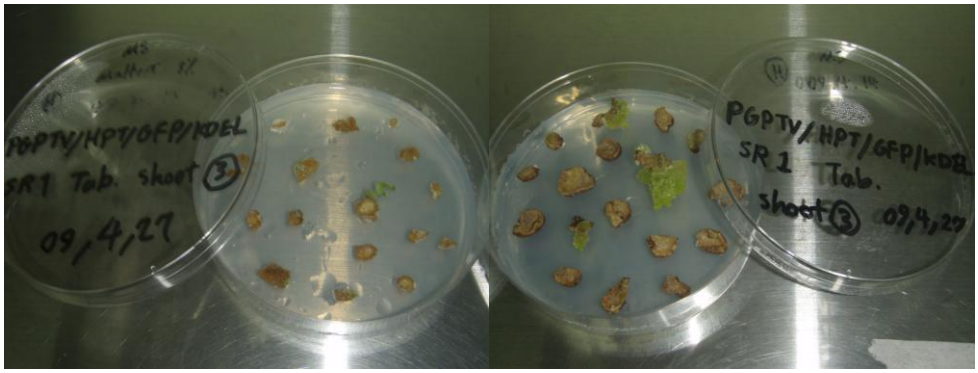
About Jatropha I didn't get any good result by this method until now.

_Tobacco



_Jatropha





Topic: Metabolic fingerprinting of commercial *Angelica acutiloba* (toki) roots by GC-TOF-MS

Introduction

Angelica acutiloba (Dong Dang Gui in Chinese and Toki in Japanese) is one of Traditional Chinese Medicine(TCM) that have been used for its pharmaceutical benefits in treating women's gynecological disorders such as painful menstruation, recovery from childbirth and menopause [1]. Moreover, its pharmaceutical benefit possesses potent immunotherapeutic properties in prevention and treatment of cancer; increase the effectiveness of conventional cancer treatments. At the present many kind of TCM are widely commercialized. Toki is commercialized in Japan. For classifying the quality of toki, scent and appearance of dried toki roots are used. These methods are inconsistency and time-consuming for industry.

Metabolic fingerprinting is a strategy of classification of samples on the basis of their biological statues origin, using high throughput methods, usually spectroscopy. It is involved in sorting datasets into categories so that conclusions can be drawn about classification of individual samples [2]. The advantages of this method are: observation in the pattern changes in all metabolites without previous knowledge and metabolite identification is not necessary. Therefore the aim of this work is to classify the quality of toki roots by using metabolic fingerprinting.

Materials and Methods

Samples

Dried toki roots provided by Fukuda-shoten. There are five sample classified by traditional method.

Sample	Origin (Cultivation area)	Ranking
YNA	Nara	A
YNB	Nara	B
YNC	Nara	C
YHC	Hokkaido	C
YCE	Southern China	E
HHE	Hokkaido	E

Remark:

A, B = Good quality

C = Moderate quality

E = Bad quality

Sample preparation

The overall methodology of sample preparation for GC-TOF-MS (Gas Chromatography Time of Flight Mass Spectrometry) was shown in Figure 1. There are composed two main processes sample extraction and derivatization process. The detail of extraction process was shown in red box and derivatization was shown in gray box.

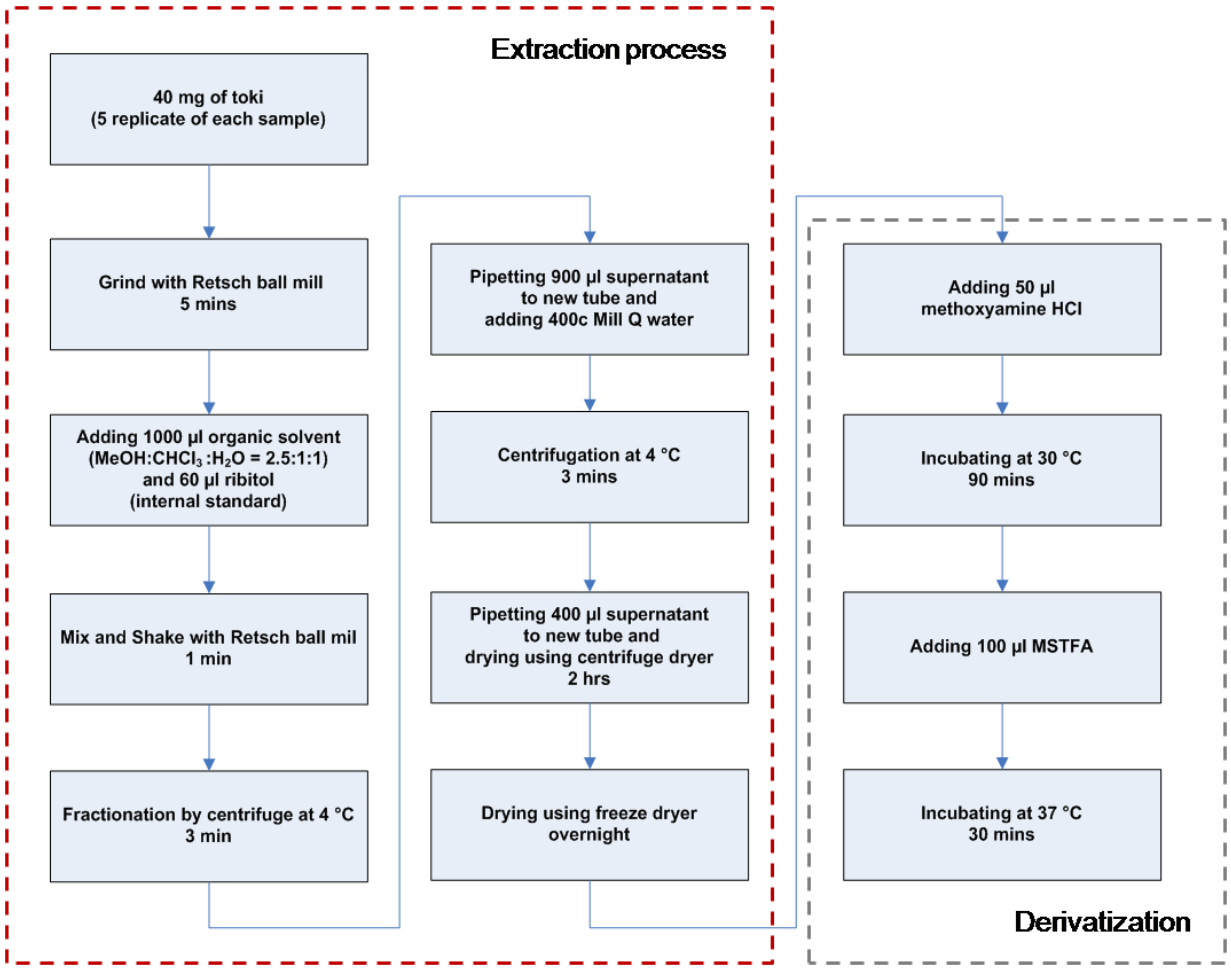


Figure 1: Overall methodology of sample preparation for GC-TOF-MS

GC-TOF-MS analysis

One microliter of each derivatized sample was injected into Agilent 6890N Gas Chromatograph and separated by a 30 m x 0.25 mm I.D. x 0.25 µm WCOT fused silica capillary column. The injector temperature was set at 230 °C and gas flow rate was set at 1 ml/minute. The column temperature maintained at 80 °C for 2 minutes, and then increased to 330 °C with the increasing rate 15 °C/minute. The column effluent was later introduced to mass spectrometer (LECO® Pegasus III time-of-flight mass spectrometer) with transfer line and ion source temperatures were set at 250 °C and 200 °C respectively. The detector mass range was 85-500 m/z, while the detector voltage was set at 1600 volts.

Data preprocessing and metabolite identification

Raw data from GC-TOF-MS were processed by ChromaTOF in which automatic peak detection and metabolite identification were performed with references to an in-house library, Max-Planck Institute of molecular plant physiology library, and to the main library. After that peak alignment was done by Line up software for attaining the highest similarity. Then based line correction and data normalization was done by PiroTran software.

Multivariate analysis

Principle Component Analysis (PCA) is a multivariate analysis method applied to these data done by Pirouette software. This software created two dimensions of score plots for visualizing the difference between samples. The detail of discrimination between samples was shown in loading plot corresponding principle component or factor.

Results and discussion

Multivariate analysis especially Principle Component Analysis (PCA) was applied to GC-TOF-MS data. PCA is the way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. Since patterns in data can be hard to find in high dimensional data, where graphical representation is not available, PCA is a powerful tool for analyzing data. Moreover, the advantage of PCA is that once the patterns were found and the dimension of data was reduced without much loss of information. This technique helps to find the important factor for discrimination the quality of toki roots.

PCA analysis of toki samples (YNA, YNB, YNC, YHC, YCE, and HHE) was shown in figure 2. The score plot show that factor 1 (Principle component 1:PC1) is the significant factor that can discriminate toki samples in two groups which are bad and good moderate quality. The good moderate quality group composed of YNA, YNB, YNC, and YHC. The bad quality group composed of YCE and HHE. Therefore the factor 1 represents the quality of toki sample. For detail of the factor 1, loading plot was shown in figure 3. From the loading plot, the good moderate quality group of toki sample had high concentration of citric acid and fructose. In contrast, the bad quality group of toki sample had lower concentration of these two compounds, but high concentration of proline, malic acid, glucose, and inositol were found.

As the result of all samples including HHE different species from others this sample may effect to clustering, therefore in order to classify sample by cultivation area HHE sample was remove and then PCA was applied to others as shown in figure 4. From this result, it show that cultivation region of toki samples was influenced the toki quality by factor 1. Loading plot of factor 1 was shown in figure 5; it shows that YCE have high concentration glucose than others.

The toki cultivation in Japan was observed and the result shown in figure 6. It show that factor 1 discriminated toki by cultivation prefecture Nara and Hokkaido. Additionally toki quality corresponded to the cultivation prefecture. In Hokkaido (YHC) has high concentration of glucose than toki sample from Nara as shown in loading plot (figure 7).

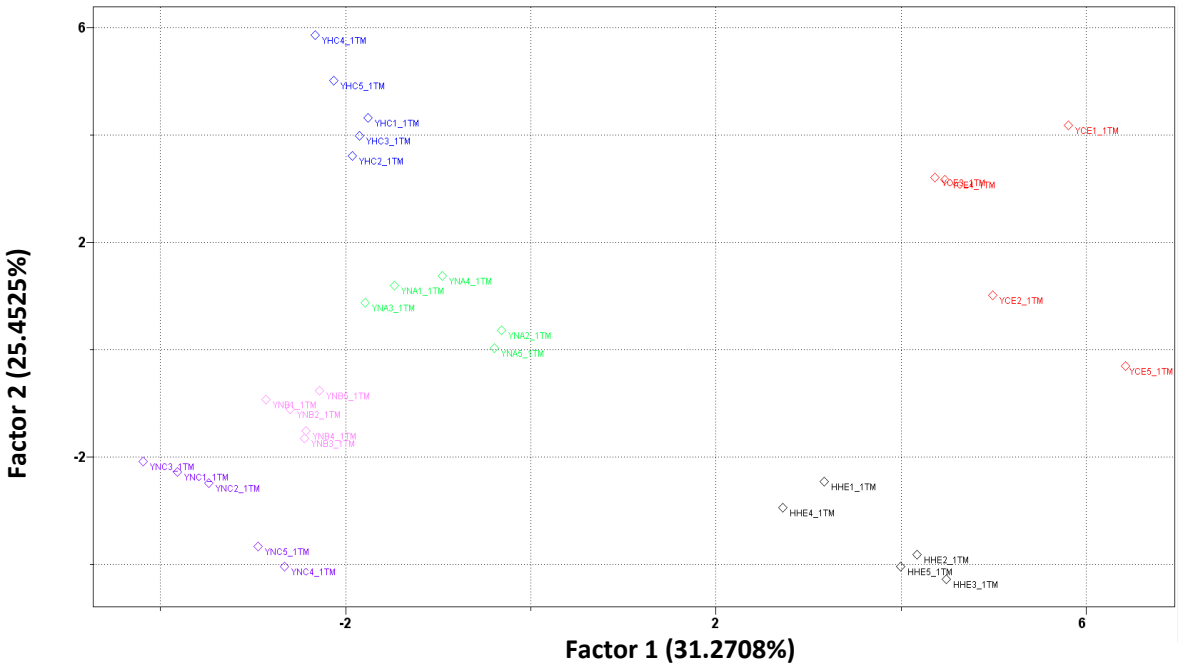


Figure 2: PCA score plot with factor 1 and factor 2 discriminating the toki samples.

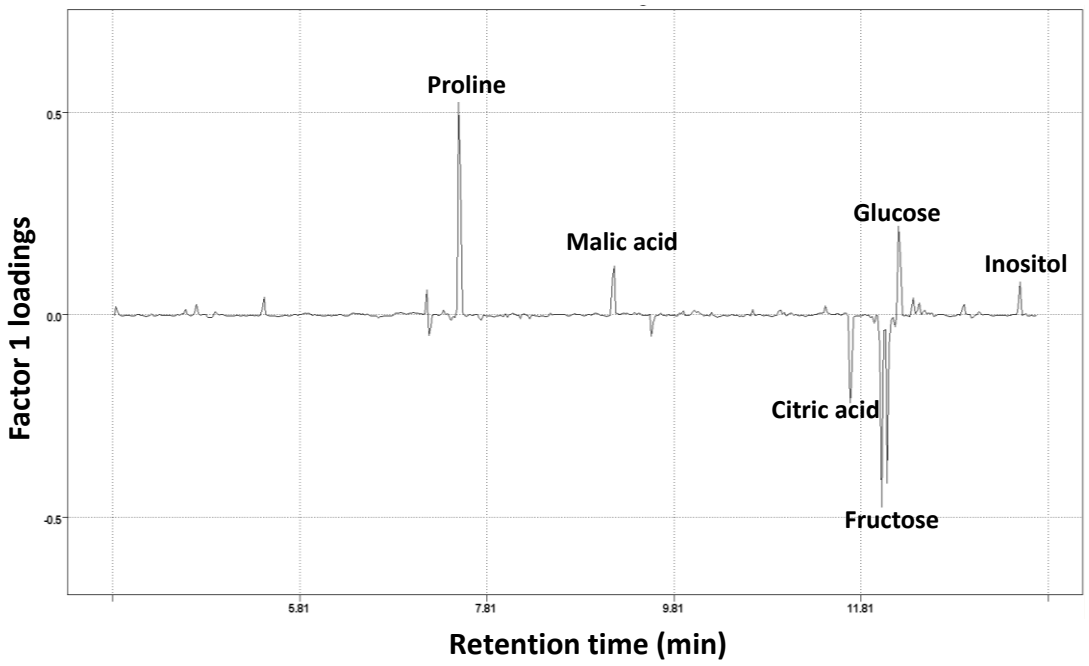


Figure 3: Loading plot of factor 1 showing significant metabolite that discriminate between all toki samples.

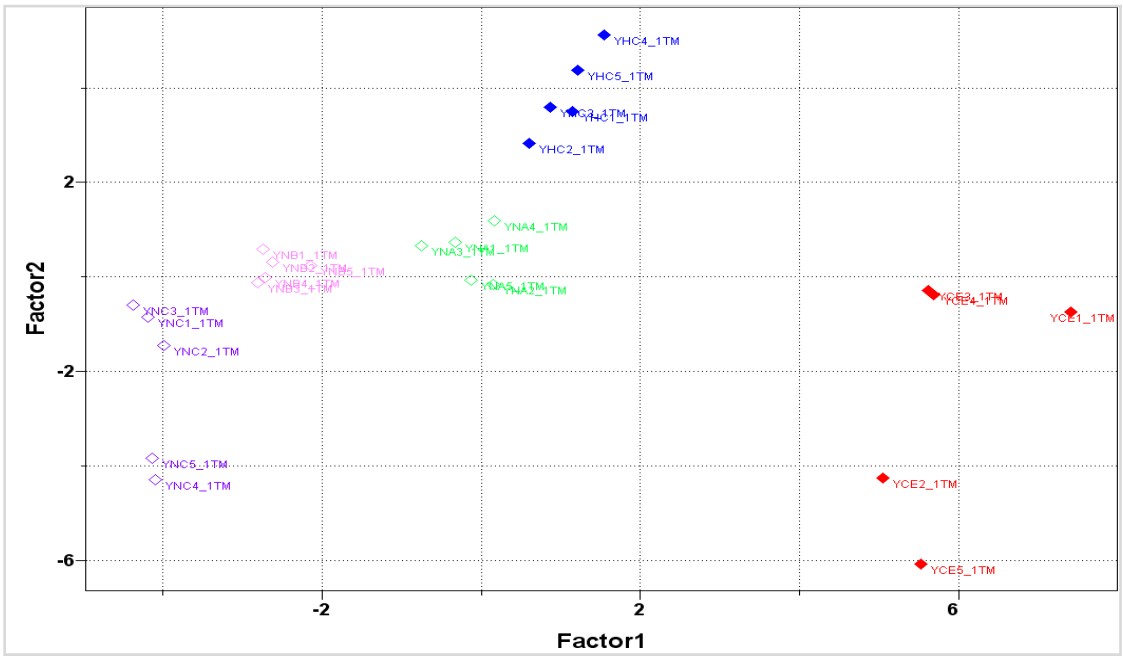


Figure 4: PCA score plot with factor 1 and factor 2 discriminating the same species of toki samples

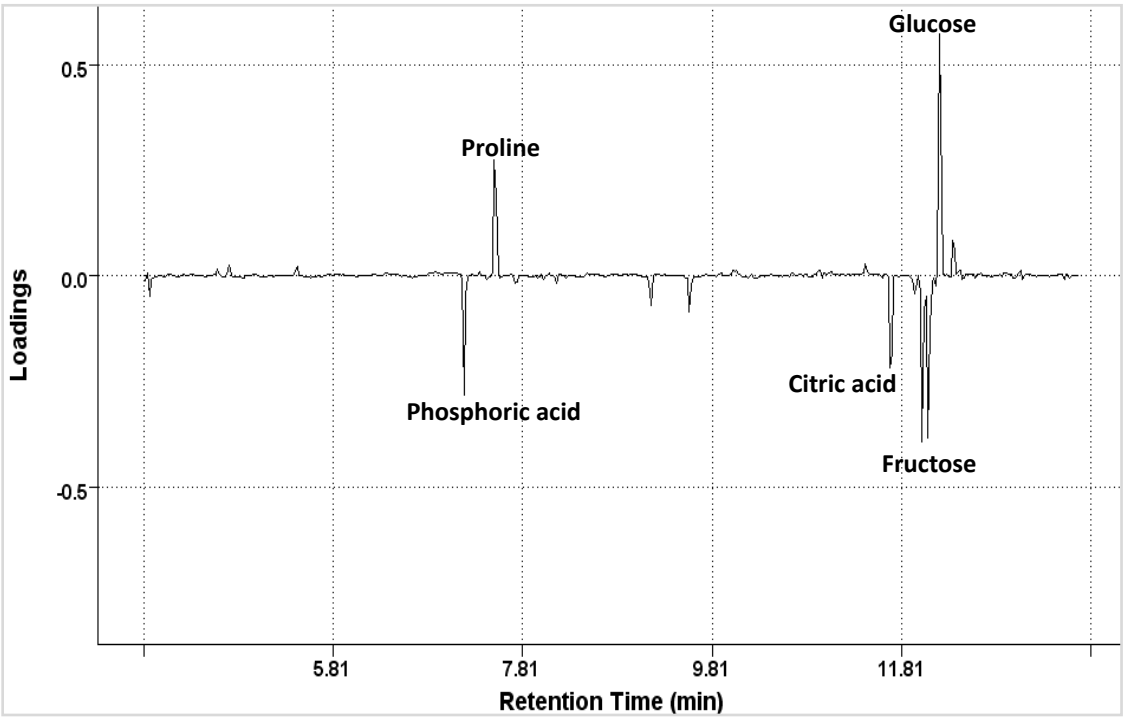


Figure 5: Loading plot of factor 1 showing significant metabolite that discriminate between the same species of toki samples.

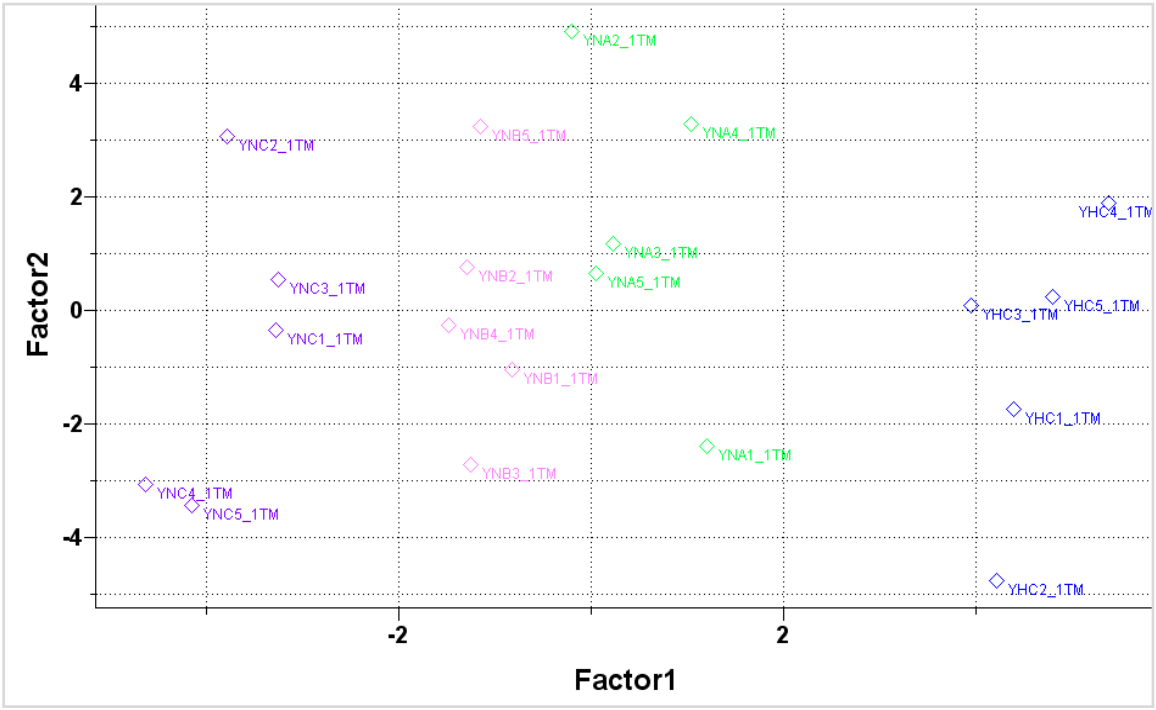


Figure 6: PCA score plot with factor 1 and factor 2 discriminating Japan toki samples.

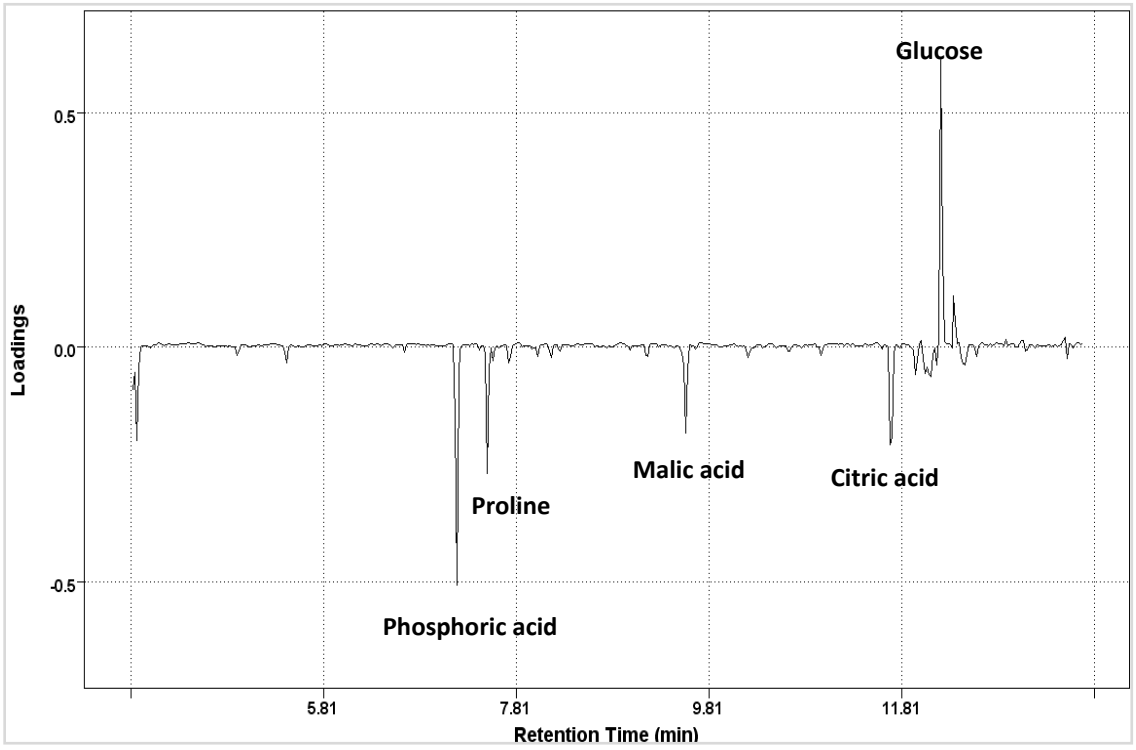


Figure 7: Loading plot of factor 1 showing significant metabolite that discriminate Japan toki samples.

The toki sample in Nara prefecture was observed and the result show that toki samples were not clearly separated by quality as shown in figure 8. Loading plot of toki samples from Nara (figure 9) show that glucose and fructose concentration was not clearly identifying even though fine peak alignment.

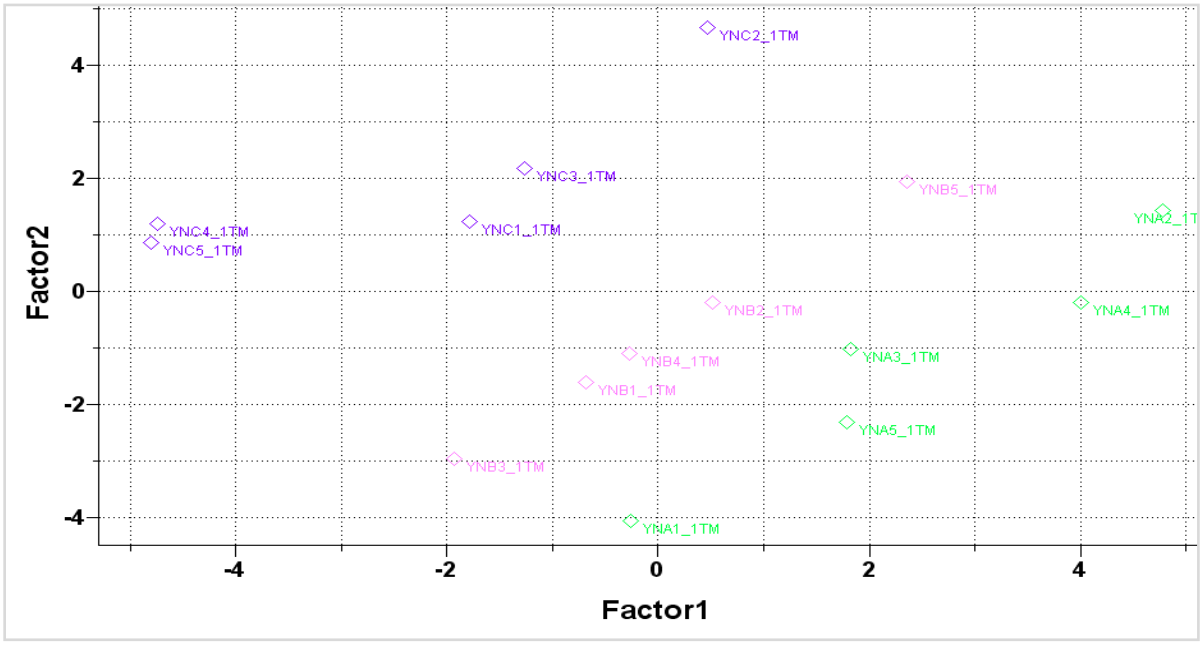


Figure 8: PCA score plot with factor 1 and factor 2 discriminating toki samples cultivation origin from Nara prefecture.

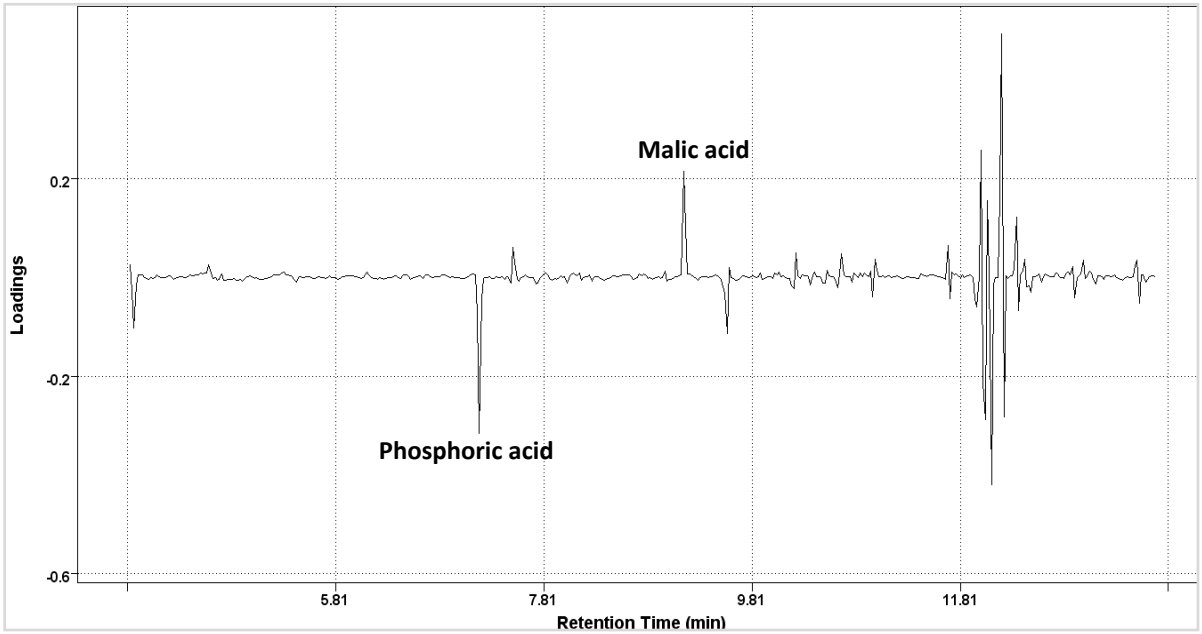


Figure 9: Loading plot of factor 1 showing significant metabolite that discriminate toki samples cultivation origin from Nara prefecture.

In bad quality group between two species (YCE and HHE samples) was observed result in figure 10. It is clearly separated between these samples even though same quality toki root. Moreover loading plot was observed and showed that in YCE sample has high concentration of glucose, but low concentration of phosphoric acid and malic acid. As the result the significant might be from specie or cultivation (different country). For finding what is the main factor another toki sample from Hokkaido should be added for this analysis.

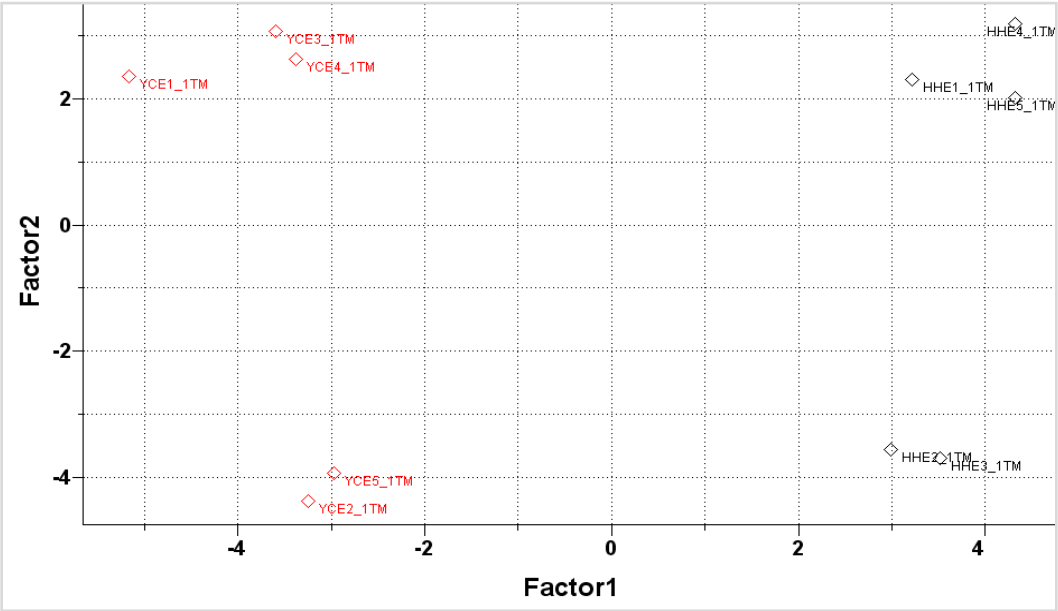


Figure 10: PCA score plot with factor 1 and factor 2 discriminating toki samples from Hokkaido and southern China.

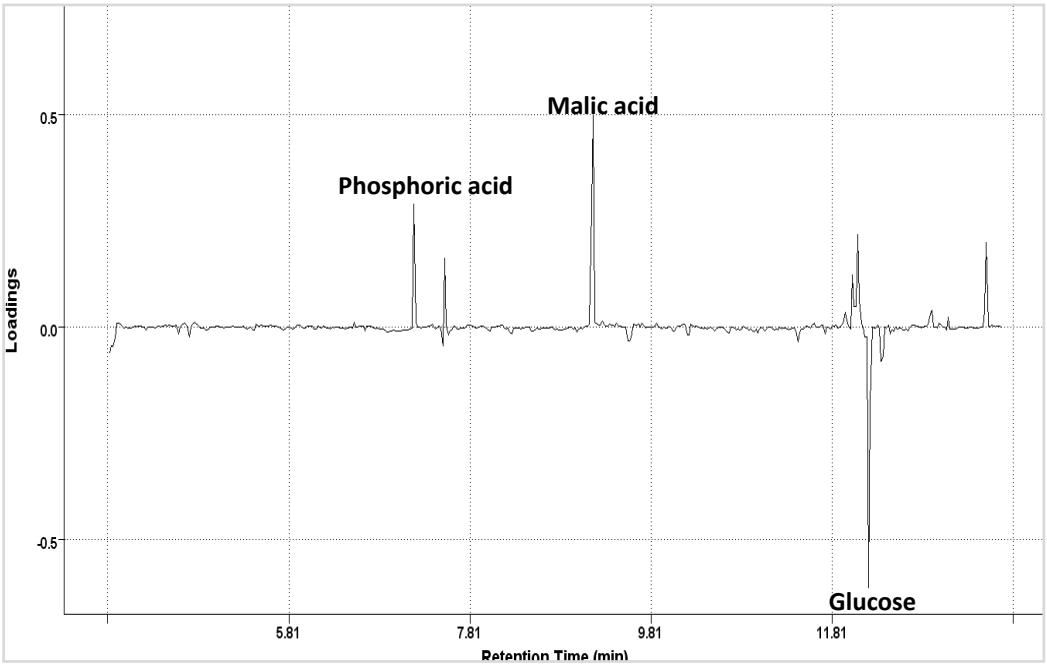


Figure 11: Loading plot of factor 1 showing significant metabolite that discriminate toki samples from Hokkaido and southern China.

Conclusion

PCA analysis is an appropriate method to categorize the toki samples, however more than one important factor may be effect to clustering. For example in this study, cultivation area and species might be the significant factor therefore result might not clearly to classify samples. In this study the result show clearly to separate good moderate quality of toki samples (YNA, YNB, YNC and YHC) from bad quality (YCE and HHE). Moreover, bad quality of toki samples has high concentration of glucose. Therefore glucose concentration may be one important metabolite that affect to quality of toki root. Furthermore cultivation prefecture is one significant factor that effect to quality of toki root and glucose concentration of toki cultivated in Hokkaido has high concentration than cultivated in Nara. From this study the result show that GC-TOF-MS and PCA analysis are the effective method for quality estimation of toki root.

References:

1. **Tianniam, S., Tarachiwin, L., Bamba, T., Kobayashi, A. and Fukusaki, E.**: Metabolic Profiling of Angelica acutiloba Roots Utilizing Gas Chromatography-Time-of-Flight-Mass Spectrometry for Quality Assessment Based on Cultivation Area and Cultivar via Multivariate Pattern Recognition. J. Biosci. Bioeng., 105, 655-659 (2008).
2. **Kell, D.B., Brown, M., Davey, H.M., Dunn W.B., Spasic, I. and Oliver, S.G.**: Metabolic footprinting and systems biology: the medium is the message. Nat. Rev., 3, 557-565 (2005).

PROJECT-BASED TRAINING REPORT

(Noji-Lab/09-13 Mar 2009)

Purification and measurement of a rotary motor protein (F1-ATPase)

I. Introduction

1. Background

Adenosin triphosphate (ATP) is one of the most important compound for living organism and serves as an energy carrier to drive many biological reactions that support the activity of life. ATP synthase, which responsible for the generation of ATP from Adenosin diphosphate (ADP) and inorganic phosphate (Pi), is using the physical rotation of its own subunit for catalysis. ATP synthase is composed of two rotary motors, namely F1 and Fo. Bacterial F1 has the subunit structure, $\alpha 3\beta 3\gamma\delta\varepsilon$. The β subunit has catalytic nucleotide-binding sites and form a ring-shape structure with α (noncatalytic). The γ subunit rotates in the $\alpha 3\beta 3$ ring, and the δ and ε subunit connect the $\alpha 3\beta 3$ and γ to Fo, respectively. The β subunit is essential because it is the catalytic part of ATPase enzyme. But which domain of the β subunit has direct role in catalytic function of β subunit hasn't been known yet. Therefore by generating a mutant of The β subunit domain (substitution of 181 Glutamic acid to 181 Aspartat) , and analyse the ATPase activity through biochemical assay and observation of its revolution by single molecule analysis, we will be able to know which domain has direct affect to ATPase catalytic activity.

2. Objective

Analyse the EF1-ATPase ($\alpha 3\beta E181D3\gamma\delta\varepsilon$) activity through biochemical assay and revolution observation by single molecule analysis.

II. Materials and methods

1. Purification of EF1-ATPase ($\alpha 3\beta E181D3\gamma\delta\varepsilon$)

E.coli cells are disrupted by sonicating the cell for 5 minutes. Cells are centrifuged for 10000 rpm for 10 minutes at 4°C to remove cell debris. The supernatant is then centrifuged again for 75000 rpm for 20 minutes at 4°C. Afterwards, supernatan is applied to histrap column that contains beads coated with NiNTA-. Wash with buffer. Followed by elution with buffer and imidazole. After treated with DTT, sample protein is separated by SDS-PAGE. Protein concentration is then measured with spectrophotometre using BSA as standard. Afterwards, Protein sample is treated with

maramide biotin with ratio 1 to 4 (protein sample to biotin respectively). Biotin is used so ATPase can interact with magnetic beads which is coated with streptavidin. Excess (unreacted) biotin is removed by gel filtration (NAP -10). The protein sample concentration is then confirmed again by specrophotmetre.

2. Measurement of EF1-ATPase (α3βE181D3γδε) activity

a. Biochemical assay (ATP hydrolysis activity) analysis

Measurement of ATPase activity is done by using ATP generating system in the tube. ATPase buffer assay consist of pyruvate kinase, Lactose dehydrogenase (LDH), Phosphoenol pyruvate (PEP), NADH, buffer (HEPES-KOH, KCl,and MgCl2) and ATP with different concentration: 1 mM; 0.1 mM; 0.01 mM, 0.001 mM.. After put the ATPase buffer assay in the spectrophotometer, 10-20 μl EF1-ATPase (α3βE181D3γδε) is then added and Followed by Measurement of ATP hydrolysis activity (NADH concentration) at absorbance 340 nm by spectrophotometer.

b. Single molecule analysis (ATP rotation rate)

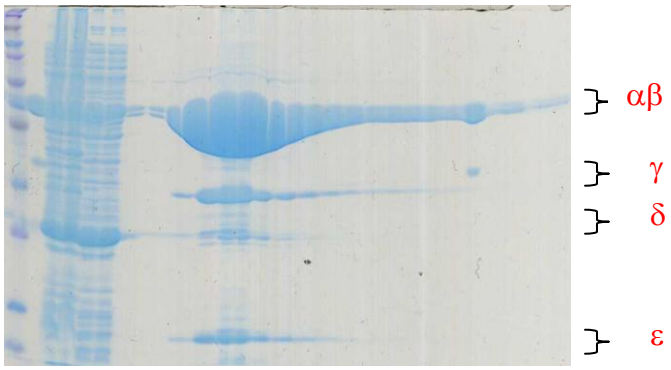
The experimental system for single-molecule observation of the rotation of F1 is as followed: The EF1-ATPase is fixed on the glass surface to suppress the lateral and rotational Brownian motion of the F1 molecule. A large probe (magnetic beads is attached to the γ subunit to visualize rotation.

Coverglass is soaked in Ni-NTA solution and wash with buffer. 10 mg/ml BSA is added. Followed by Addition of EF1-ATPase, 1mM ATP and magnetic beads without regenerating system; and EF1-ATPase, 625 nM ATP and magnetic beads with regenerating system. EF1-ATPase (α3βE181D3γδε) rotation rate is then observed with magnetic tweezer as the ATPase motor under the microscope.

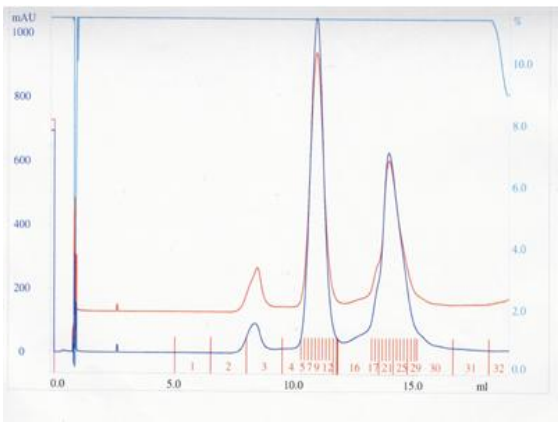
III. Result & Discussion

1. Purification of EF1-ATPase (α3βE181D3γδε)

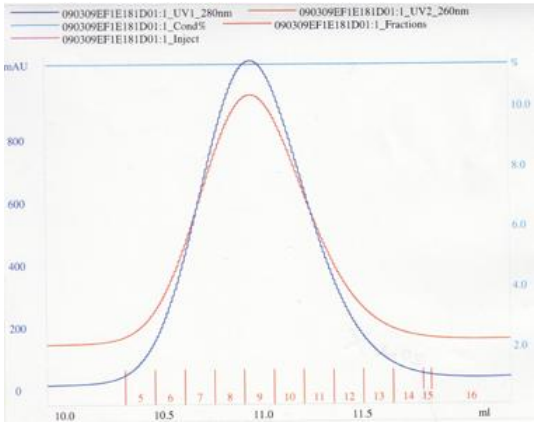
After EF1-ATPase (α3βE181D3γδε) is purified from the cell by centrifugation, EF1-ATPase (α3βE181D3γδε) subunits are then separated by SDS-PAGE. Followed by further purification of the protein by using his-trap column. The β subunit contains polyhistidine-tag which will bind to NiNTA agarose in the column. The eluted protein from his trap column will be resulted as purified EF1-ATPase (α3βE181D3γδε). Furthermore, from the gel filtration data, it informs that the most purified EF1-ATPase (α3βE181D3γδε) sample is at fraction 7-11. The result of protein concentration measured by spectrophotometre is 1.42 mg/ml.



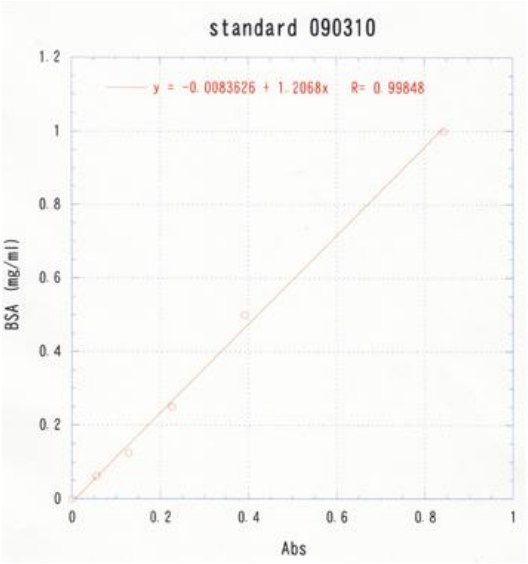
(a)



(b)



(c)



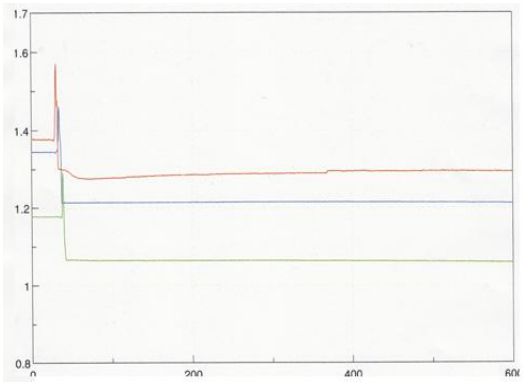
(d)

Purification of EF1-ATPase (α3βE181D3γδε) using (a) SDS-PAGE, (b) Gel filtration, and (d) Protein concentration measurement using protein standard curve

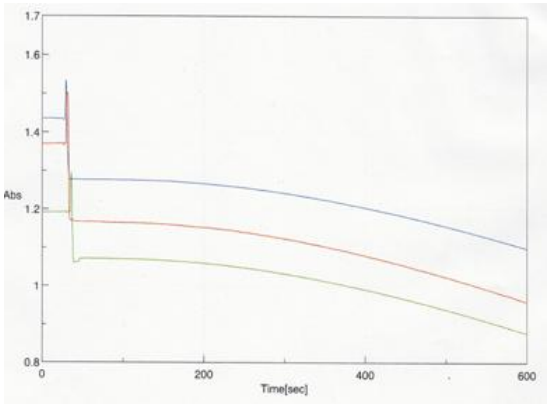
2. EF1-ATPase (α3βE181D3γδε) activity

a. Biochemical assay analysis

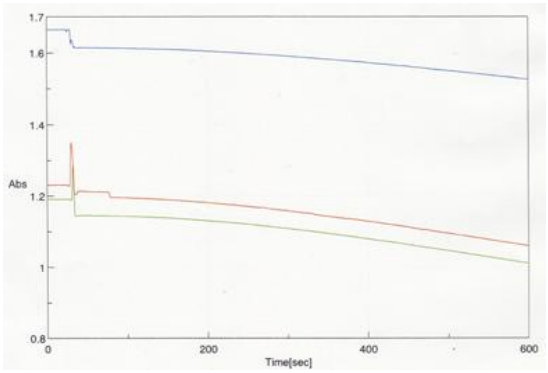
ATPase (ATP hydrolisis) activity is shown in the picture below. In ATP generating system, every 1 ATP forms, 1 NADH will be produced (ATP concentration will be constant). Concentration of NADH is then measured at absorbance 340 nm. From the result below, at 1 mM ATP concentration, the ATPase activity (hydrolisis rate) is 1.56 ATP/s. Each rotation of ATPase will produce 3 ATPs. So, 1.56 ATP/s means 0.5 rotation/s is generated in EF1-ATPase (α3βE181D3γδε). The wild type of EF1-ATPase generate 10 rotation/s to which one rotation will produce 3 ATPs.



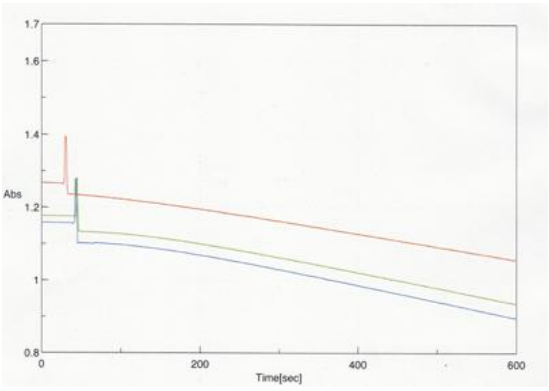
0.001 mM



0.01 mM



0.1 mM



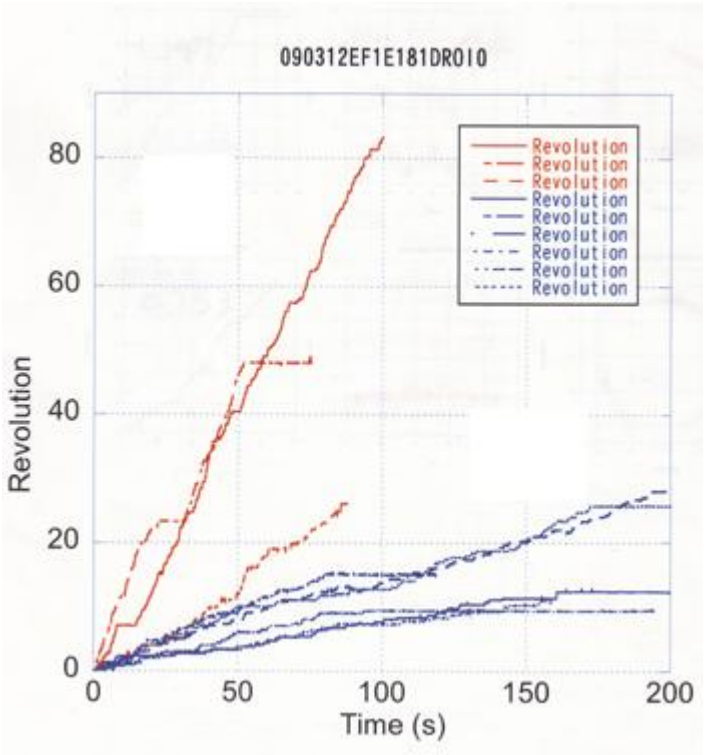
1 mM

ATP concentration (μM)	ATPaseAvg (s-1)
1000	1.5649
100	1.2067
10	0.5077
1	0.019

Average ATPase activity (ATP hydrolisis) with different ATP concentration at abs.340 nm

b. Single molecule analysis

Rotation of EF1-ATPase is visualized under the microscope by fixing EF1-ATPase on a Ni-NTA modified glass surface via a polyhistidine tag that has been introduced into the amino terminus of the β subunit. In addition to ATP, the rotation of ATPase will be generated and we will be able to viasualize it under the microscope. As the data shows, different ATP concentration will generate different ATPase rotation rate where in addition of 1 mM ATP will resulted in ATPase activity, 2.3819 ATP/s and 625 nM will generate 0.39 ATP/s. This means that in addition of 1 mM ATP, EF1-ATPase (α3βE181D3γδε) will produce 0.76 rotation/s.



ATP concentration (μM)	ATPaseavg (S-1)
1000	2.3819
0.625	0.39797

Average revolution of ATPase with ATP concentration 1 mM (red) and 625 nM (blue)

As we compare from the biochemical assay and single molecule analysis (0.5 rotation/s and 0.76 rotation/s respectively) the ATPase activity are not different significantly. Therefore This result show that EF1-ATPase (α3βE181D3γδε) has reduced activity about 20 times compare to EF1-ATPase wild type which generates 10 rotation/s. This reduced activity resulted in reduced ATP production. Therefore 181E has direct

function on the catalytic site of β subunit in generating ATP. Eventhough to conclude this, further experiment is needed to be done.

IV. Conclusion

EF1-ATPase (α3βE181D3γδε) has 20 times reduced activity compared to EF1-ATPase wild type.