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YU FUJII

MOLECULAR TOOLS FOR FOCUSED PROTEOMIC ANALYSIS OF BACTERIAL CELL FACTORIES

Master of Science thesis

Examiner: Assistant Prof. Ville Santala and Postdoctoral Researcher Suvi Santala
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ABSTRACT

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Cell factories are important and new tools to produce valuable products without depending on depleting fossil fuels. As there are successive cell factory constructions, there are emerging needs to develop better analysis tools for them. The conventional analysis methods such as 2-dimentional electrophoresis and mass spectrometry are certainly eligible for cell factory analysis, but they are not necessarily the most reasonable methods because of the need for special equipment. Therefore, new tools for cell factory analysis are needed.

Aldehyde-deformylating oxygenase (ADO) and bacterial luciferase (LuxAB) are interesting target proteins whose bacterial productions were analyzed here: ADO is an enzyme involved in fatty alkanes or alkenes generation in bacteria, and LuxAB is an enzyme catalyzing the bioluminescence production. Fatty alkanes and alkenes production by bacteria is drawing attention since it could replace the traditional chemical production of traffic fuels. Bioluminescence is also notable because it can be used as a real-time monitoring tool for certain bacterial production.

Therefore, in this study, immunoassay was studied as a new method for cell factory analysis. Especially, the immunoassay systems for ADO and LuxAB were constructed. The best working scFvs for the immunoassays were successfully selected, and the standard curves were also drawn for both of them. Thus, enzyme immunoassay using alkaline phosphatase (phoA) for ADO and a new immunoassay for LuxAB were established in this study. In the immunoassay for LuxAB, LuxAB generates the signals to be measured using exogenously added long-chain aldehyde as substrate, thus no tracer scFv or labels are needed. The systems were confirmed to work with ADO and LuxAB produced by *Escherichia coli* but not by *Acinetobacter baylyi* ADP1. The possible reason could be that *A. baylyi* ADP1 strains did not produce enough amounts of the proteins for the immunoassays. Thus, further research is still needed, but this study offers a meaningful foundation for the development of the immunoassays.

PREFACE

This research was conducted in the research laboratory of Industrial and Environmental Biotechnology at Tampere University of Technology.

I am very grateful to Assistant Professor Ville Santala and Postdoctoral Researcher Suvi Santala who supervised me during the whole study kindly and patiently. They have spent so much time on teaching me, and they have given me a lot of knowledge about biotechnology. I would also like to thank Professor Matti Karp for giving me the chance to do research in his research group in the beginning. He has also given many advices on my study. I am thankful to all the members of the laboratory for helping me and giving me advices. I also enjoyed our conversations with them.

Finally, I would like to express my gratitude to my family. My parents have supported me very much from all the aspects. I thank Anssi so much for listening to me and helping me all the time during my whole study in Finland. I could not achieve this without him.

Japan, 18.2.2017

YU FUJII

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

A. baylyi ADP1	*Acinetobacter baylyi* ADP1
scFv(s)	Single-chain variable fragment(s)
ADO	Aldehyde-deformylating oxygenase
LuxAB	Bacterial luciferase
E. coli	*Escherichia coli*
SBOL	Synthetic Biology Open Language
IEF	Isoelectric focusing
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
MS	Mass spectrometry
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionization
ESI	Electrospray ionization
TOFMS	Time-of-flight mass spectrometry
FTICR	Fourier-transform ion cyclotron resonance
BSA	Bovine serum albumin
C_H	Constant heavy chain
C_L	Constant light chain
V_H	Variable heavy chain
V_L	Variable light chain
BCCP	Biotin carboxyl carrier protein
CARD	Catalyzed reporter deposition
GOX	Glucose oxidase
phoA	Alkaline phosphatase
HRP	Horseradish peroxidase
pNPP	*p*-nitrophenyl phosphate
TMB	3,3′,5,5′-tetratmethylbenzidine
ONPG	o-Nitrophenyl-,3-D-galactopyranoside
LB medium	Luria-Bertani (LB) medium
LA medium	Luria agar medium
SB medium	Super Broth medium
PCR	Polymerase chain reaction
IPTG	Isopropyl β-D-1-thiogalactopyranoside

1. INTRODUCTION

Recombination of bacterial cells has become exceedingly common in the engineering world today. Especially, the research of cell factories is conducted intensively because they offer a way to produce valuable products from raw materials more efficiently than what is possible with the existing chemical production methods. Conventional chemical production of the valuable products is less environmental friendly and efficient as biotechnological production with bacterial cells. It is because chemical production very often requires petroleum or expensive resources, such as in the production of nylon, alkanes or alkenes and wax ester. (Singh, Bhadani and Singh, 2007; Sato, Aoki and Noyori, 1998; Mabuza, 2011) There are numbers of bacterial cells which are able to produce valuable products naturally, but the production volume does not satisfy industrial needs. Cell factories are the genetically modified cells to produce the targeted products in better efficiency or to obtain the ability to produce the targeted products if the cell does not possess the pathway for the production yet.

The more common the construction of the cell factories becomes, the more important the analysis of them becomes. Especially, proteomic analysis of the cell factories is arising to be more important for the development of the technology of cell factories today. There are many different methods for the proteomic analysis of the cell factories nowadays, but it is always vital to select the most suitable method for the target. The tools which have been used in research in other fields, for example, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or mass spectrometry (MS) could be applied to proteomic analysis (Raymond, 1964; Guy and Fenaille, 2006). However, these methods are not necessarily reasonable for the cell factory analysis in the point of implementation. Thus, there are obviously needs to invent easier proteomic analysis tools for bacterial cell factories. The goal of this thesis is to provide those analysis tools.

Immunoassay is an easy and simple method for the proteomic analysis compared to the methods mentioned above. There are numerous types of immunoassay systems, but enzyme immunoassay is one of the easiest and the safest assays (Yolken , 1986). In immunoassay, antibody-antigen interaction is utilized to detect and quantify the amount of the target protein. The target antigen binds to the immobilized antibody in the assay system, and the antigen is labelled with a substance which generates signals, or different antibody specific to the same antigen is labelled and added to the system after antigen. The labels generate signals, for example, colour change or luminescence. Enzyme immunoassay does not require any special certificates or treatments of the wastes as radioimmunoassay requires (Sancho, Amal and Garcia-Fayos, 2013). The procedure consists

of only addition of the samples, incubation, washing of the plates, and the measurement of the signals, which is moderately simple.

This study suggests enzyme immunoassay as a proteomic analysis tool for wax ester producing cells, recombinant *Acinetobacter baylyi* ADP1 cells, by constructing the suitable immunoassay system for them. In this study, the most suitable single-chain variable fragments (scFv) for aldehyde-deformylating oxygenase (ADO) and bacterial luciferase (LuxAB) produced by the modified *A. baylyi* ADP1 were selected by testing the immunoassay systems. Since these *A.baylyi* ADP1 strains were relatively new strains, the systems were initially tested with ADO and LuxAB produced by recombinant *Escherichia coli* XL1 for the scFv selection to confirm that the systems work properly. For LuxAB immunoassay, a new type of enzyme immunoassay in which the antigen generates the signal as luminescence without any labels is established. In the end, the confirmed immunoassay systems were applied with ADO and LuxAB produced by *A. baylyi* ADP1, and the results were analysed.

Construction of the immunoassay system for *A. baylyi* ADP1 producing ADO or LuxAB will be useful for developing biochemical alkanes, alkenes or wax ester production with bacterial cells. It is a more eco-friendly method for the production of them than the chemical production, contributing to reduce the negative effects on environment.

2. CELL FACTORIES

Today, cell factories are very important in biotechnology as mentioned in introduction. Cell factories are the microbial cells which are engineered to produce valuable products from raw ingredients. They have the potentials to replace the chemical production which are not environmental-friendly once appropriate processes with cell factories are developed. To obtain environmental-friendly world with using cell factories, technologies for their designs and analysis have to be developed.

2.1 Cell Factory Design and Synthetic Biology

To design desired cell factories, metabolic engineering has been playing a very important role for a long time. In metabolic engineering, the metabolic fluxes of microbial cells are analysed and improved by, for example, adding continuation to specific metabolic fluxes or deleting undesired fluxes. It has been leading the biotechnological research, but synthetic biotechnology is rapidly developing as a tool for cell factory design these days in addition to metabolic engineering. In synthetic biotechnology, genetic circuits are created and introduced to the cells to obtain desired products. The term synthetic biology was already invented in 1912 by Stephane Leduc, but it started to be noticed only recently (de Lorenzo and Danchin, 2008). As shown below in Figure 1, it has been developing since the start of 21st century very quickly (Fong, 2014).

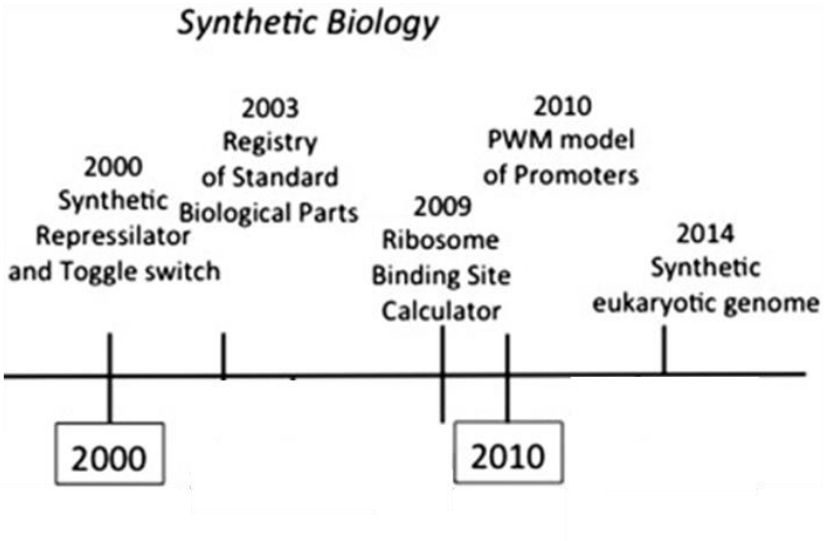

Figure 1. *Development of synthetic biology. Synthetic biology has rapidly developed since 2000. Modified from (Fong, 2014).*

In synthetic biology, it is important to have the proper and suitable data sources, such as scientific literature, bioinformatics databases, and *in silico* design tools as shown in Figure 2. Having reliable sources is important because accurate data of DNA sequences and characterization and experimental data are essential in designing production pathways for the cell factories. To design effective and feasible cell factories, it is always necessary to have the reliable and accurate information of the Bioparts to be used. For example, Synthetic Biology Open Language (SBOL) is one of the tools for exchanging the characteristics of Bioparts between different systems. The selection of the data sources should be conducted properly, and also the obtained data should be analysed for the design if needed (Gibson et al., 2010).

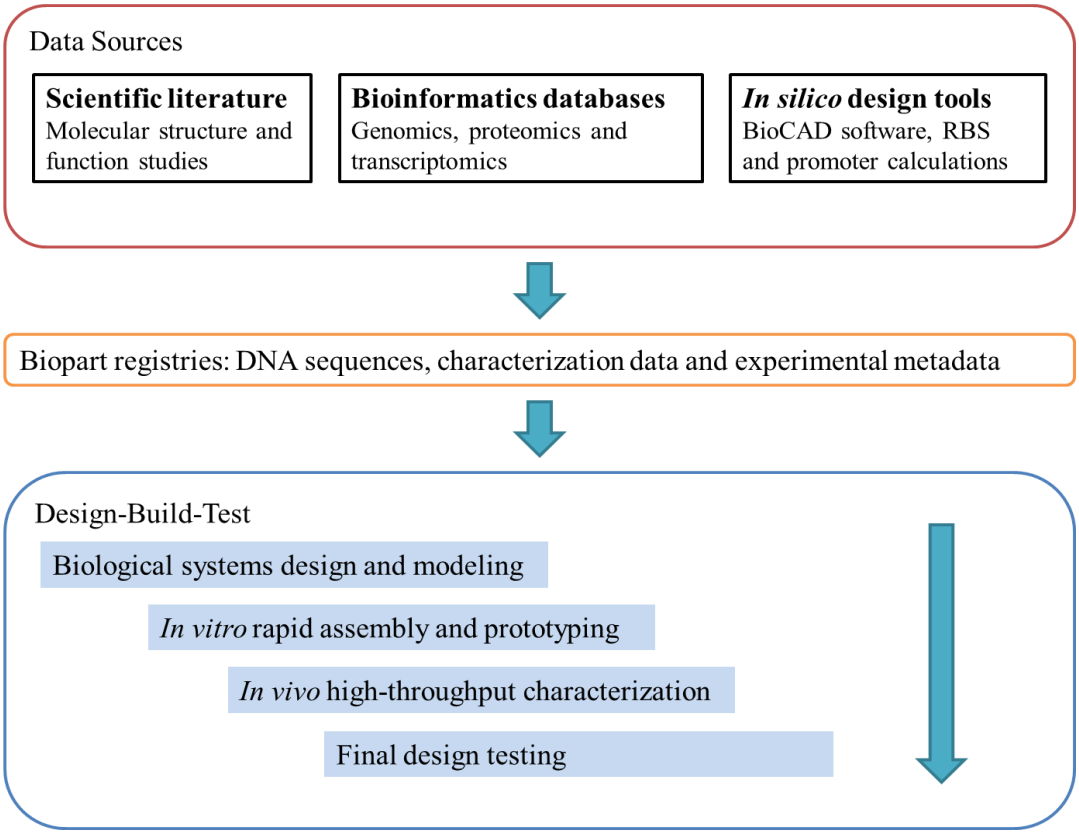

Figure 2. *Description of synthetic biology process. In synthesis of cells, it is required to have suitable data sources in the beginning. After that, required Bioparts are selected, and the designing, assembly, and the test of the cells are conducted . These three steps are conducted repeatedly until the ideal results are obtained. Modified from (Kelwick et al., 2014).*

Finally, the Bioparts are designed, and the parts are assembled. One of the most important elements to be discussed about the assembly of Bioparts is the standard biological parts, often referred as Biobrick parts. Biobrick parts allowed researchers to focus on their research without caring about the restriction enzymes to be used. Figure 3 shows the example of assembly of Biobrick parts. In this example, since *SpeI* and *XbaI* have the same restriction end, the restricted sites of Part A and Part B can be ligated together.

As it can be seen from this example, Biobrick parts have the standardized restriction ends, *SpeI*, *XbaI*, *PstI* and *EcoRI*, making it possible for us to simply use those parts without having trouble with looking for suitable restriction sites every time. (Wang et al., 2011; Shetty, Endy and Knight Jr., 2008) Using this technology, the creation of new gene circuits has become much easier and less troublesome, enabling the cell factory design with synthetic biology.

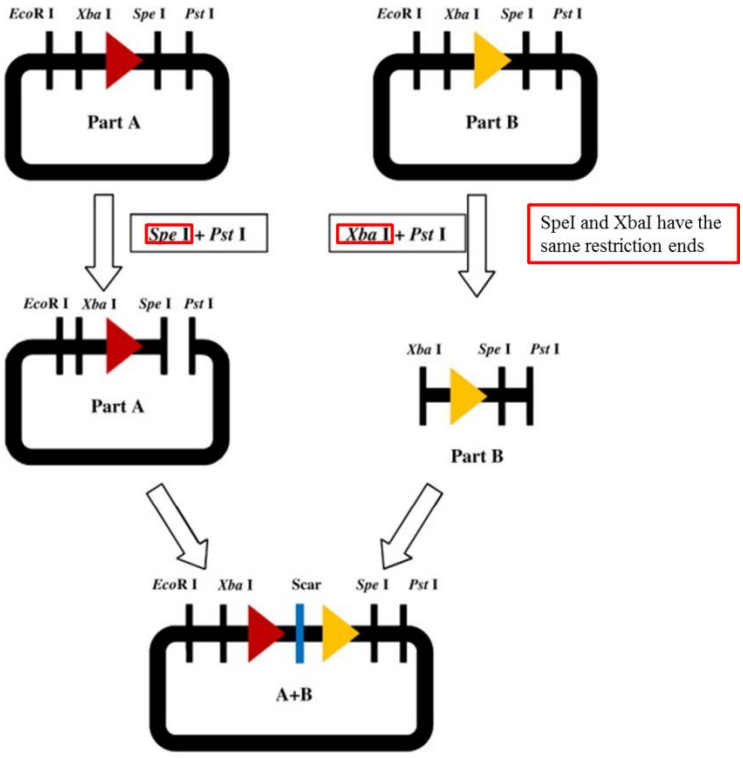

Figure 3. *Assembly of Biobrick parts. PartA has SpeI and PstI restriction sites, and Part B has XbaI and PstI restriction sites in this figure. The ligation of them can be conducted easily because SpeI and XbaI have the same restriction sites. Modified from (Wang et al., 2011).*

In the end, the synthesized cells are tested for the eligibility, and the synthesis of cell factories is accomplished completely (Figure 2).

2.2 Examples of Cell Factory Design with Synthetic Biology

A good example of a cell factory design with synthetic biology has been conducted by Santala (2014). Santala (2014) succeeded in reconstructing a wax ester producing flux with *A. baylyi* ADP1 strain. *A. baylyi* ADP1 possesses the pathway to produce wax ester naturally. However, the natural pathway was knocked out in the research, and a new pathway was introduced to make the wax ester production easier to control. In this case, fatty-acyl coenzyme A (acyl-CoA) gene, *acr1*, was knocked out, and *luxCDE* was introduced with Arabinose promoter (Figure 4). LuxC, D, and E are fatty acid reductase, acyl transferase, and acyl-protein synthetase, respectively. As mentioned above, arabi-

nose promoter was also introduced to the strain, which enables the regulation of the wax ester production. (Santala et al., 2014) This novel study shows the importance of synthetic biology in the production of valuable products by replacing the natural pathway with synthetic pathway.

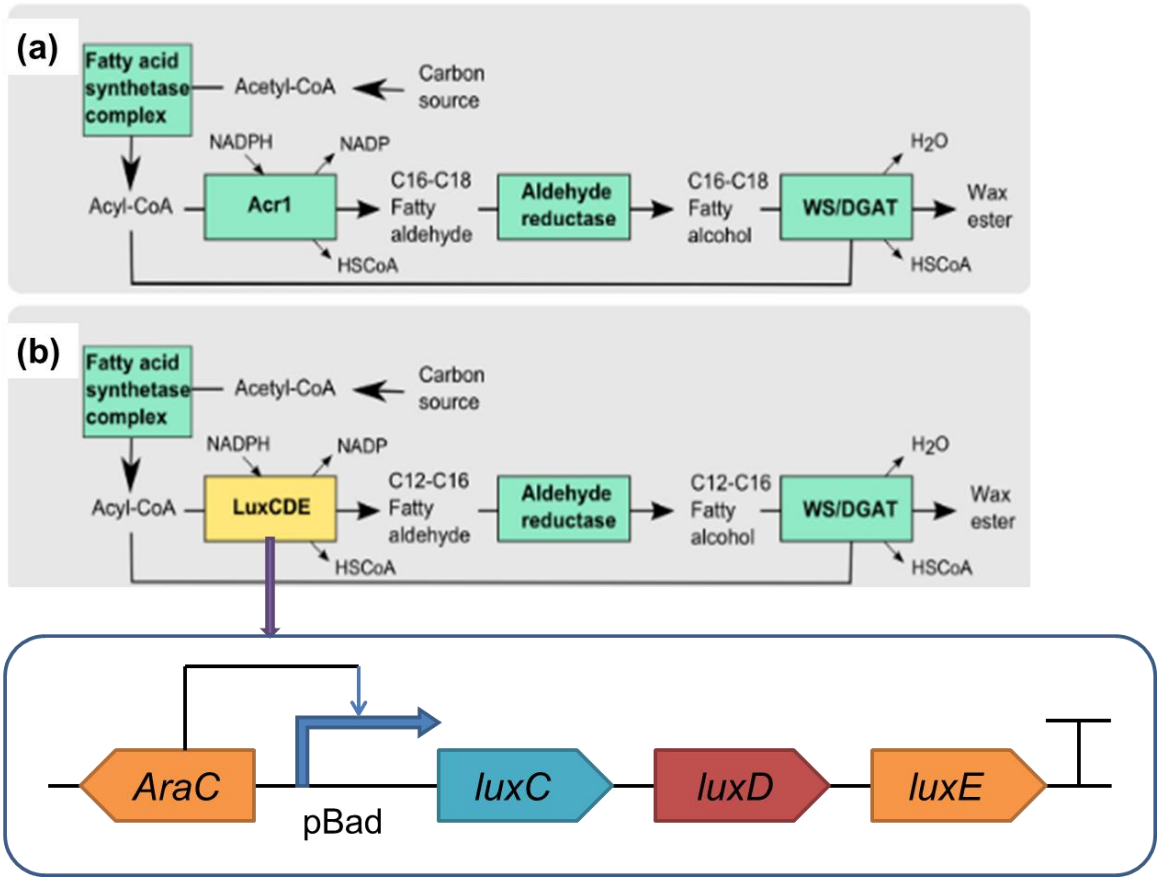

Figure 4. *Metabolic flux of engineered Acinobacter baylyi ADP1. It produces wax ester through synthesized pathway. The fatty-acyl coenzyme A (acyl-CoA) gene in (a) is knocked out and LuxCDE gene is introduced in (b). The synthesized cassette LuxCDE is shown in the bottom of the figure in detail. Modified from (Santala et al., 2014).*

As mentioned earlier, cell factory design could be directly useful in productions of novel products, but it has also been used for biosensor. Santala (2011) has succeeded in monitoring the production of wax ester by *A. baylyi* ADP1 strain with bioluminescence. Luminescence-producing enzyme, LuxAB, was introduced into *A. baylyi* ADP1 strain using synthetic cassette, and the luminescence was observed during the cultivation since long-chain aldehyde is produced by the strain during wax ester production. (Santala et al., 2011) This method was employed as an analytical tool for the above-mentioned study by Santala (2014).

There is also research which has succeeded in producing alkanes or alkenes using synthetic biology. Kallio (2014) was able to produce propane from glucose by engineered *E. coli* which was modified to express aldehyde-deformylating oxygenase (ADO).

(Kallio et al., 2014) ADO is an important protein in producing alkane and alkenes. This protein is explained in more details in the next section. Crépin (2016) succeeded in producing alkanes and alkenes by expressing acyl-ACP and ADO in *Cupriavidus necator* which does not possess the pathway to produce alkanes or alkenes naturally. In their research, they were also able to use CO_2 as the only carbon source for fatty alkanes and alkenes production. (Crépin , Lombard and Guillouet, 2016)

From these examples, it can be said that the use of synthetic biology is not limited, increasing the possibilities for the cell factory design. Furthermore, it also implies the necessity of inventing and improving the analytical tools for the cell factories.

2.3 Proteins useful in cell factory constructions

Examples of the proteins drawing attention in cell factory research today are ADO and Bacterial Luciferase (LuxAB). ADO is an enzyme which catalyzes the conversion of fatty aldehydes to fatty alkanes or alkenes. Fatty alkanes and alkenes are necessary in this world since they are the main constituents of fossil fuels (Mabuza, 2011; Howard et al., 2013). However, because the depletion of the fossil fuels is becoming more and more serious today, it is important to develop other methods to produce them. Biosynthesis is one of those new methods, and it is considered as a promising system to solve this problem. That is why ADO is an essential enzyme to be researched. Its function and mechanism of the catalysis of fatty alkanes or alkenes production has been recently investigated. It has been discovered that cyanobacteria have this pathway to produce fatty alkanes or alkenes using ADO naturally. In cyanobacteria, fatty acids store the energy obtained from the sunlight, and the fatty acids are converted into fatty aldehydes by acyl-acyl carrier protein (acyl-ACP). These fatty aldehydes are then converted into fatty alkanes or alkenes by ADO. (Rajakovich et al., 2015) For this reaction, O_2 molecule and four electrons are required for with fatty aldehyde, and the products of this reaction are fatty alkane or alkene and formate (Warui et al., 2011; Li et al., 2011). However, though some examples are introduced in the previous section, there is not yet much research which has succeeded in producing the fatty alkanes or alkenes by expressing ADO in bacteria not able to produce them naturally.

LuxAB is an enzyme existing in luminous bacteria which is able to catalyze the generation of luminescence as mentioned earlier. There are mainly three types of bioluminescent organisms, *Vibrio*, *Photobacterium*, and *Photorhabdus* (*Xenorhabdus*). The *lux* operon includes *luxA, luxB, luxC, luxD, luxE,* and *luxG,* and *luxA* and *luxB* encode for the bacterial luciferase. It is well known that long-chain aldehydes, $FMNH_2$, and O_2 are required for bioluminescence production for LuxAB. When the bacteria possess the genes *luxD, luxC* and *luxE* which encode enzymes LuxD, LuxC, and LuxE, they catalyze the reaction to generate long-chain aldehyde from fatty acyl group. LuxD, LuxC, and LuxE are transferase, reductase and synthetase, respectively. In addition to that, if the gene *luxG* is also included, LuxG which is encoded by *luxG* catalyze generation of

$FMNH_2$. (Figure 5) As the bioluminescence research has been conducted intensively, it is possible to express *luxA* and *luxB* in bacteria not naturally producing luminescence, such as in *E. coli*. In this kind of case, the aldehyde needs to be provided exogenously. (Close et al., 2012; Tinikul and Chaiyen, 2016; Close, Ripp and Sayler, 2009) The reaction of bioluminescent generation with lux operon is described in Figure 5. Luciferase catalyzes the oxidation of $FMNH_2$ and long-chain aldehyde, so FMN, carboxylic acid, H_2O, and luminescence are obtained. The obtained light is 490 nm whose color is blue to green. (Tinikul and Chaiyen, 2016)

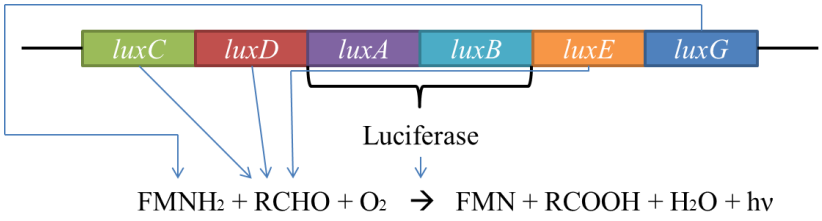

Figure 5. *Reaction of bioluminescent generation with lux operon.luxA and luxB encode for bacterial luciferase, and luxC, luxD, and luxE encode for reductasse, transferase, and synthetase which are related to the long-chain aldehyde production. luxG encodes for the enzyme which catalyze the reaction of $FMNH_2$ production. Modified from (Tinikul and Chaiyen, 2016).*

LuxAB is researched intensively in bioengineering because it can be utilized in biosensor systems. A typical biosensor system using LuxAB is the determination of the toxic compounds levels of, for example, wastewater and soil. In this kind of system, *luxAB* is employed in microorganisms, and they are exposed to toxic compounds during the cultivation. When the microorganisms are exposed to the toxicity, the bioluminescence generation should decrease. The toxic compounds could be, for example, cadmium, mercury, or nano-Copper oxide, depending on the microorganism. As a result of the measurement of toxicity, EC_{50} of each toxic compound is compared. EC_{50} is the concentration of toxicity which caused the 50 percent loss of the luminescent. (Park, Hwang and Kim, 2002; Anjum et al., 2015) Microtox is a representative tool which is used to test the toxicity using this principle. (Johnson, 2005) In addition to the toxicity test, LuxAB is employed as a real-time monitoring tool of the cell factories today, indicating that it can be also used as a cell factory analysis tool. As explained in 2.2 as an example, Santala (2011) has succeeded in constructing a real-time monitoring system of wax ester production of *A. baylyi* ADP1. LuxAB was expressed by *A. baylyi* ADP1 producing wax ester using the long-chain aldehyde which was an intermediate of wax ester production. Thus, it was possible to monitor the growth of the cells and the wax ester production by measuring the level of luminescence. (Santala et al., 2011) This is a novel and new approach of the use of LuxAB in the cell factory analysis, so it could be said that there are still a lot of potentials to utilize LuxAB as a monitoring tool into other cells as well.

3. PROTEOMIC ANALYSIS

Proteomic analysis, also called proteomics, is a relatively new concept in biotechnological research which studies the proteome. After this concept had been acknowledged in some means by researchers, the term "proteomics" was introduced by Wilkins (1995), establishing it as a concrete field of research (Wilkins et al., 1996). The term "proteome" was created from "the entire PROTein complement expressed by genOME, or by a cell or tissue type", according to Wilkins (1995). Since the introduction of the concept, numbers of proteomic tools have been developed, such as 2D-PAGE, MS, and protein arrays. The principles of them are explained in the following sections.

3.1 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE was one of the first technologies used for proteomic analysis, and it is a combination of isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). There were numbers of works conducted on two-dimensional electrophoresis (2D) already in 1960's. 2D utilizes two types of electrophoresis, which makes it useful in separating the samples containing many different proteins in biotechnological experiments. Among the works conducted on 2D, one of the most remarkable researches was conducted by Kenrick and Margolis who succeeded in protein separation by 2D technique combining native IEF and SDS-PAGE in 1970. This was named as 2D-PAGE. (Raymond, 1964; Orrick, Olson and Busch, 1973; James, 1997) As illustrated in Figure 6, in 2D-PAGE, the first electrophoresis, isoelectric focusing, separates the proteins in one dimension, and then the second electrophoresis, SDS-PAGE, separate them to another dimension. The two separations are done using two different parameters, and it was possible to obtain higher resolution with this method.

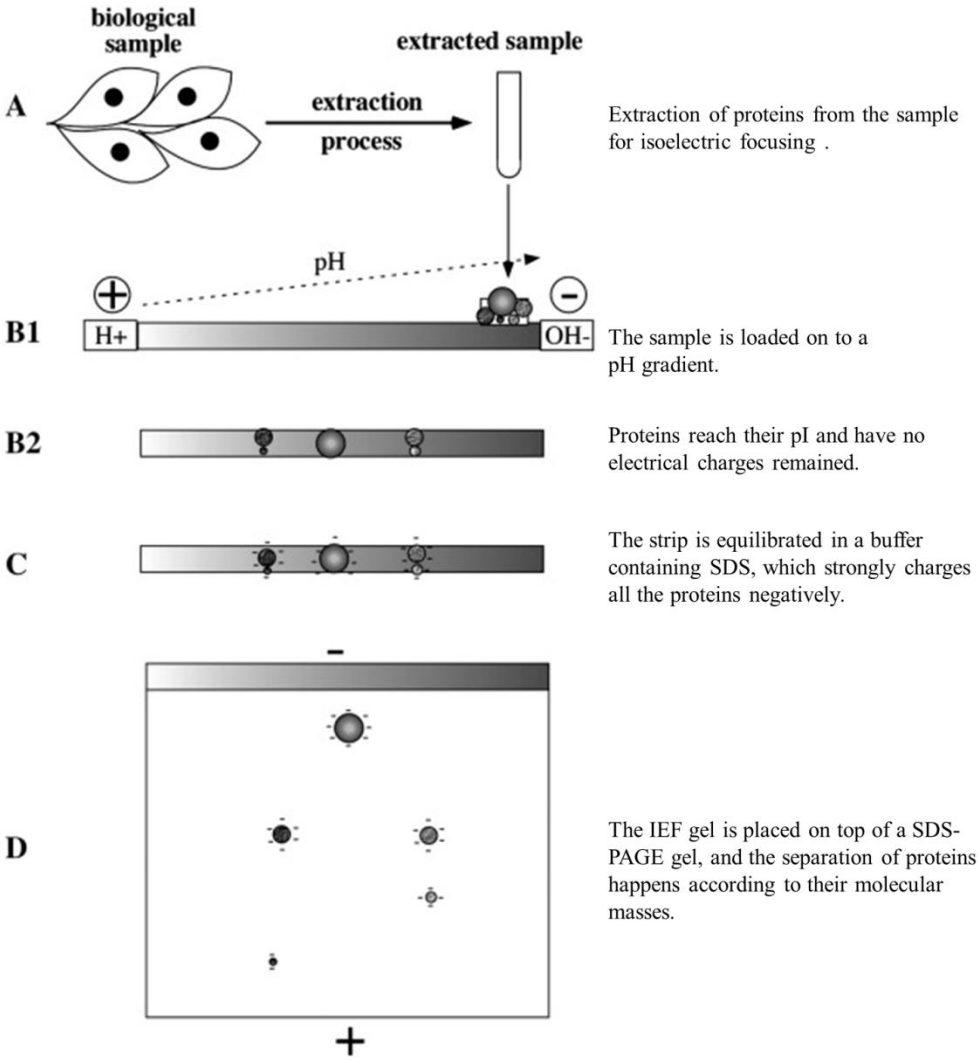

Figure 6. *Schematic chart of principle of 2D gel electrophoresis. In 2D gel electrophoresis, firstly, the extracted proteins are separated to one dimension by isoelectric focusing according to their pIs.. After that, they are separated to another dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the molecular masses of the proteins. Modified from (Rabilloud and Lelong, 2011).*

However, five years later, O'Farrel (1975) and Klose (1975) invented new methods using denaturing IEF, which are one of the most typical 2D-PAGE techniques nowadays (O'Farrell, 1975; Klose, 1975). The resolution and sensitivity have been improved even more with this method, which opened up the potentiality of 2D-PAGE to be used in proteomic analysis. 2D-PAGE technique has been developed even further since this innovative event, for example, by changing the gels for IEF to ultrathin polyacrylamide gels or replacing IEF with Blue-Native-PAGE (Görg, Postel and Westermeier, 1978; Camacho-Carvajal et al., 2004). These allowed to obtaining more improved resolution. However, it was still possible only to quantify the proteins by staining the 2D-PAGE gels after separation, but not to identify them. Therefore, analysis tools which are able to identify the proteins started to appear for proteomic analysis.

3.2 Mass spectrometry (MS)

Proteomic analysis using MS could be mentioned as an essential method in this field of research today. It enabled the identification in addition to the quantification of proteins which was not possible by using only 2D-PAGE. The history of MS starts from the time when Joseph John Thomson succeeded in determining the mass-to-charge ration of electrons in 1897 (Gottfried, 2013). Since its advent, MS has been playing an important role in variety of analysis, for example, clinical analysis and food analysis (Vogeser, 2010; Guy and Fenaille, 2006).

Mass to charge ratio (m/z) can be measured with MS by ionizing the targeted analyte. As described in Figure 7, mass spectrometer consists of ionization source, mass analyzer, and detector.

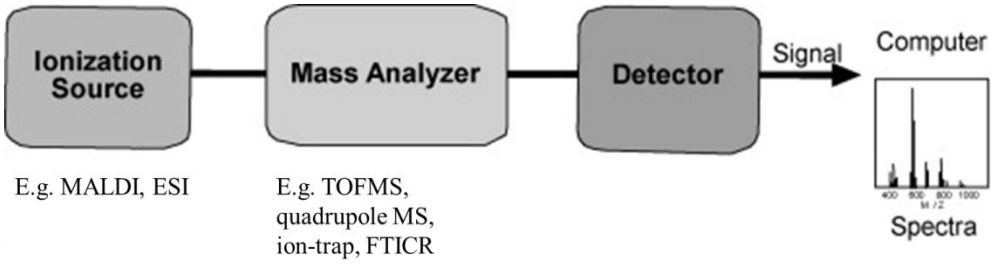

Figure 7. *Schematic chart of MS. MS consists of ionization source, mass analyzer, and detector. The signals are the in the end analyzed by computer. Modified from (Kicman, Parkin and Iles, 2007).*

The ionization of the analyte is usually conducted with matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) since their appearance in 1990's (Whitelegge et al., 1999; Liu et al., 2011; Susnea et al., 2013). These methods are called "soft ionization" because the fragmentation which happens by them is small. As a mass analyzer, time-of-flight mass spectrometry (TOFMS) is frequently used with MALDI, and quadrupole MS is very often combined with ESI.

The principle of MALDI-TOFMS is described below in Figure 8. Firstly, the analyte is mixed with UV-absorbing matrix, for example, sinapinic acid or gentisic acid, and this mixture becomes crystalized on MALDI plate. The matrix absorbs the energy from the laser light produced by MALDI and isolates each protein molecules when big molar excess exists in solid matrix (Chait and Kent, 1992). The crystalized mixture is then exposed to the laser light, and it is ionized and desorbed. Then, the proton is transferred from the matrix to the analyte. After that, the protonated molecules are accelerated by a potential difference and enter into the flight tube (mass analyzer). The m/z of the analyte is measured based on the time which took for the protonated molecules to fly through the tube and reach the detector (time-of-flight). (Kicman, Parkin and Iles, 2007; Marvin, Robrets and Fay, 2003)

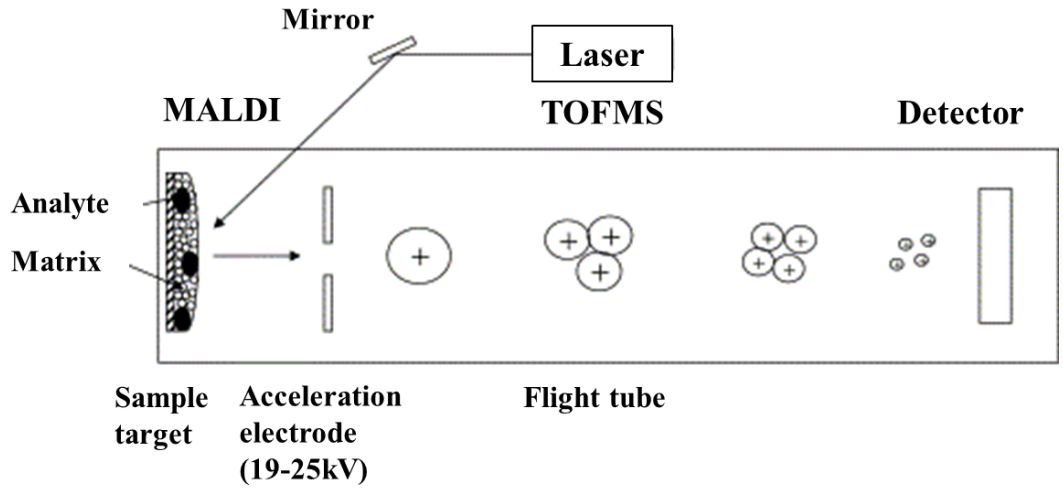

Figure 8. *Schematic chart of MALDI-TOFMS. The analyte is mixed with UV-absorbing matrix, and this mixture is crystalized on the MALDI plate. After they have been exposed to laser light, the protonated molecules fly into the mass analyzer by the potential difference. Time-of-flight, the time which took for the protonated molecules to fly through the mass analyzer, is measured, and m/z of the analyte can be calculated. Modified from (Marvin, Robrets and Fay, 2003).*

ESI-quadrupole mass analyzer's principle is shown in Figure 9 (a). In the first part, ESI, liquid sample is flown through a capillary where potential gradient is applied from the tip. There is also gas provided in small capillaries attached to the capillary, which sprays out the analyte. By this process, charged droplets are created. After this, the droplets become smaller by solvent evaporation, and they repulse each other because of their electrical charges. This makes the diameters of droplets even smaller, and the small droplets pass through the counter and skimmer electrodes. The analyte molecules finally enter into the mass analyzer with high vacuum after this process. (Gaskell, 1997; Zhang et al., 2013)

Quadrupole mass analyzer consists of four electrically charged rods as shown in Figure 9 (b). They are placed so that the two rods with the same charge are on the opposite side. The electrical charge applied is created by radio frequency which is 180 degrees different on the opposite rods. The analyte ions enter into the quadrupole mass analyzer and fly through the space between the rods by the electrical field. However, by adjusting the DC and radiofrequency voltages, the ions which do not have certain m/z cannot continue drawing the stable trajectory, therefore this electrical field works as a filter. In the end, only the ions which were able to fly through the analyzer reach the detector. (Yinon, 2009; Kicman, Parkin and Iles, 2007)

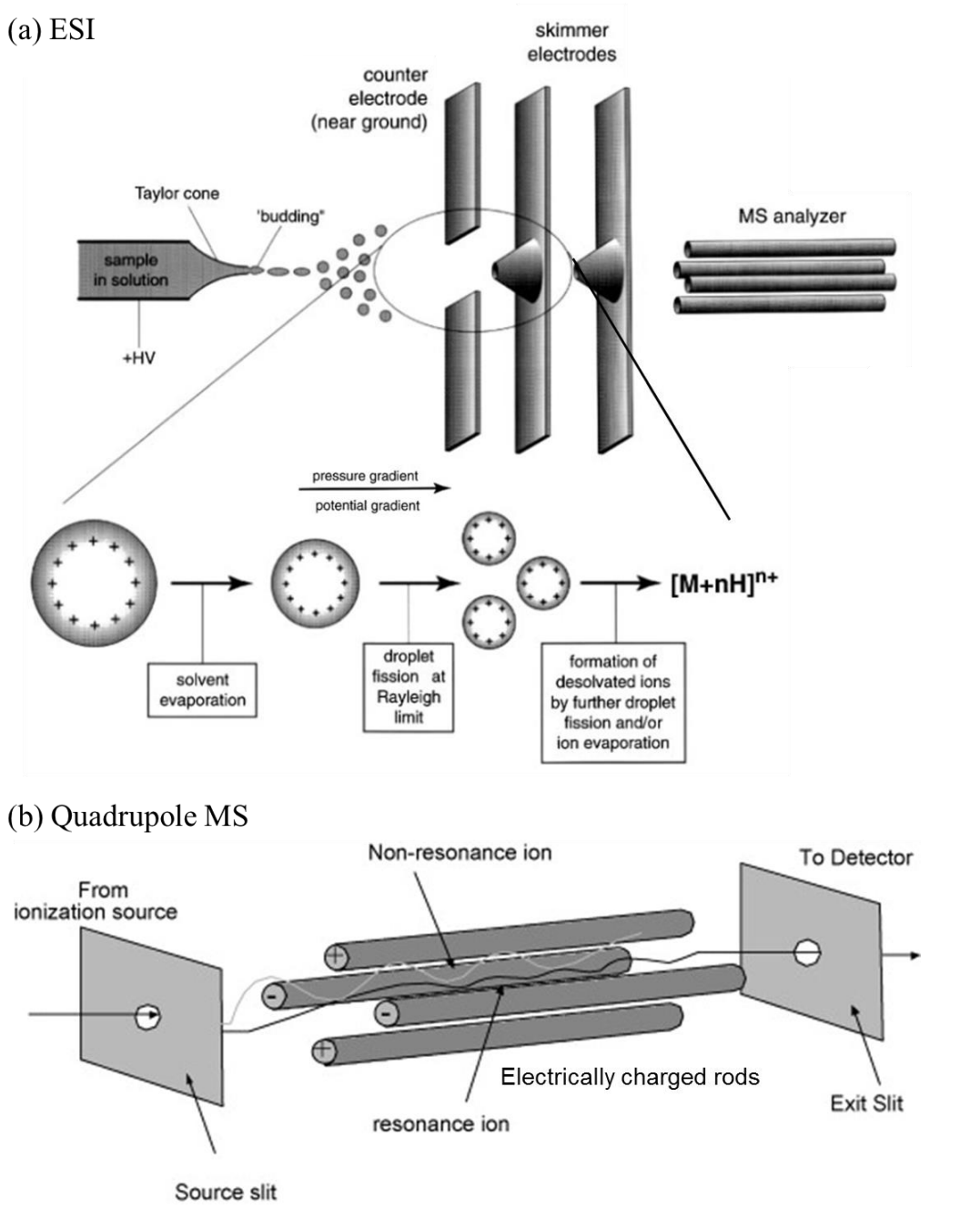

Figure 9. *Schematic chart of (a) ESI and (b) quadrupole MS. (a) In ESI, liquid sample is flown through the capillary by potential gradient. The analyte is sprayed out by gas and charged droplets are generated. The droplets repeat the fission and the small droplets goes through the electrodes, and they reach the analyzer. (b) In ESI-quadrupole MS, the analyte which flew through ESI reaches quadrupole, and the ions fly to the space created by the electrically charged rods. When the DC and radiofrequency voltages are adjusted, ions which do not have certain m/z cannot stably fly anymore. As a result, only the ions with certain m/z can reach the detector. Modified from (Gaskell, 1997; Kicman, Parkin and Iles, 2007).*

There are also ion-trap mass analyzer and Fourier-transform ion cyclotron resonance (FTICR) mass analyzer which can be combined with MALDI or ESI (Figure 10). Ion-trap mass analyzer utilizes similar principle as quadrupole. The difference is that it traps

the ions in the region between the electrodes, but the analyzer does not work as a filter as in quadrupole mass analyzer (Sleeman and Carter, 2005). The trapped ions in ion-trap mass analyzer are screened by radiofrequency voltage. Only the stable ions stay in the analyzer when certain voltage is applied. In FTICR, ions are trapped by magnetic field. Then, the signals from ions are detected as frequency from which m/z can be calculated. By FTICR, very high resolution can be achieved. (Scigelova et al., 2011)

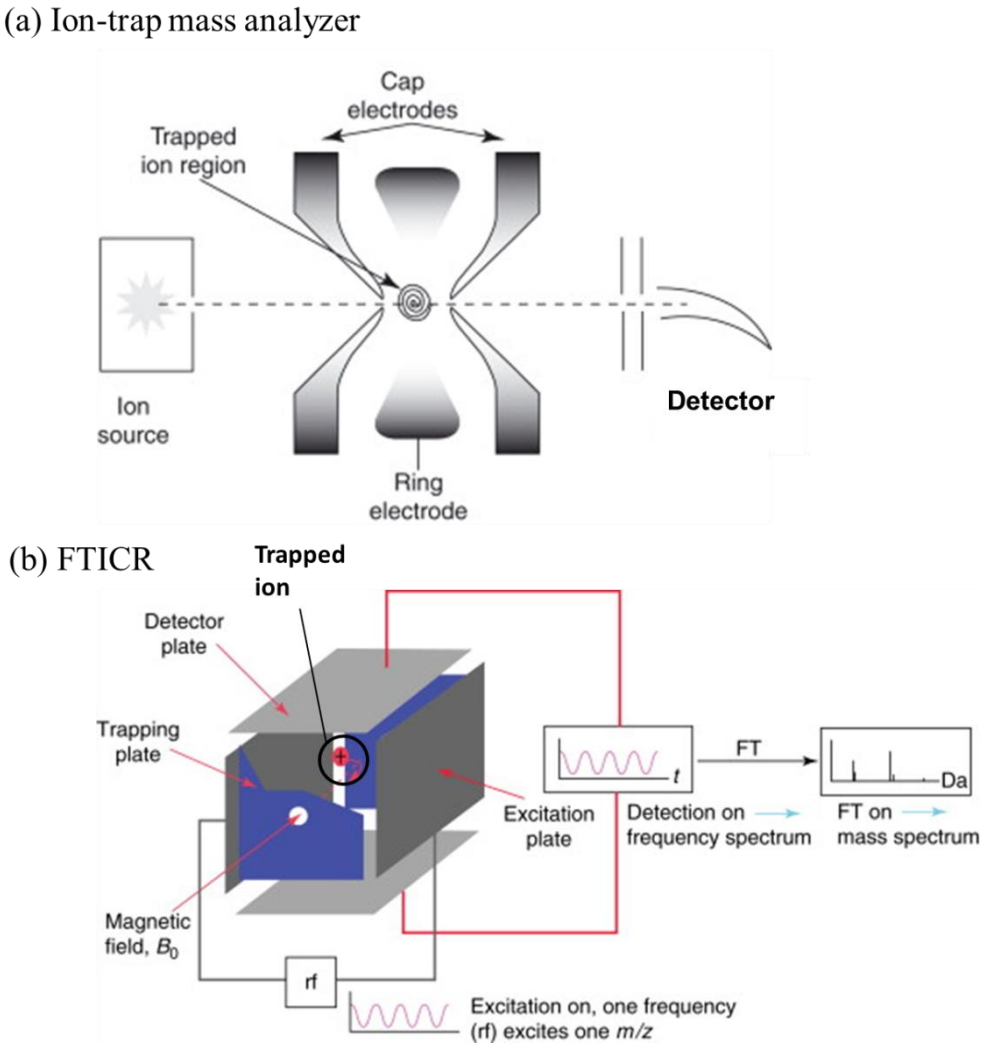

Figure 10. *Descriptions of (a) Ion-trap mass analyzer and (b) FTICR. In (a), ions are trapped between the electrodes, and they are screened by radiofrequency voltage. Only stable ones can stay in the analyzer when certain voltage is applied. In (b), ions are trapped by magnetic field, and m/z of them is calculated from the obtained frequency. Modified from (Sleeman and Carter, 2005; Twyman, 2005).*

These are the methods to identify proteins by measuring m/z. As described above, when the sample is in solid phase, MALDI-TOFMS should be used, and when it is in liquid phase, ESI-quadrupole is suitable. However, there are also other approaches to the identifications of proteins.

3.3 Analytical Protein Microarray - Immunoassay

Protein microarray has surface to which certain proteins are attached. These proteins are exposed to the analyte, and certain proteins are captured. Because protein microarray has many wells on one plate, it makes parallel analysis possible and requires less time compared to 2D-PAGE or MS. It is also more suitable for identifying low-abundant analyte. (Rigbolt and Blagoev, 2012) For the reasons above, this technology has been progressively researched for proteomic analysis today. Protein microarray can be mainly classified into two main types; analytical protein microarray and functional protein microarray.

Immunoassay is an analytical tool which can be applied in microarray format, and it utilizes the antibody-antigen interaction. Its history dates back to 1959 when Yalow and Berson succeeded for the first time in using radioimmunoassay for bioanalysis. (Yalow and Berson, 1959) Since then, immunoassay has been applied increasingly, and it has become one of the most important tools in proteomic analysis recently. In protein microarray, protein which detects the analyte can be attached to the bottom of each well of the microtiter plates. For immunoassay, microtiter plates are very often used, so the samples could be added as liquid to each well. The proteins to detect the analyte are antibodies in immunoassay because of their high specificity and sensitivity towards the antigens, enabling accurate measurements.

3.3.1 Types of immunoassays and their principles

Immunoassays can be divided into competitive immunoassay and non-competitive immunoassay. Competitive immunoassay is shown in Figure 11. In this type of immunoassay, firstly, antibody is immobilized to the wells of microplate. Then, the blocking agent is added to the wells. This blocking agent is added to block the unspecific binding of the antigens to be added later to the surface of the wells. Bovine serum albumin (BSA) is often used as the blocking agent in the wash buffer for the assay system. The plates are washed, so that the excess amount of antibodies or antigens does not disturb the whole system. After that, the target antigen and the known amount of labeled antigen are added to the wells. The labeled antigens are attached, for example, with enzymes or radioisotopes. Therefore, when the antigens are bound to the antibodies and the substrates are added, measurable signal could be observed, such as absorbance or radioactivity. Since known amount of the labeled antigens are used, the amount of non-labeled antigens can be calculated. (Yu, Jiang and Cui, 2014)

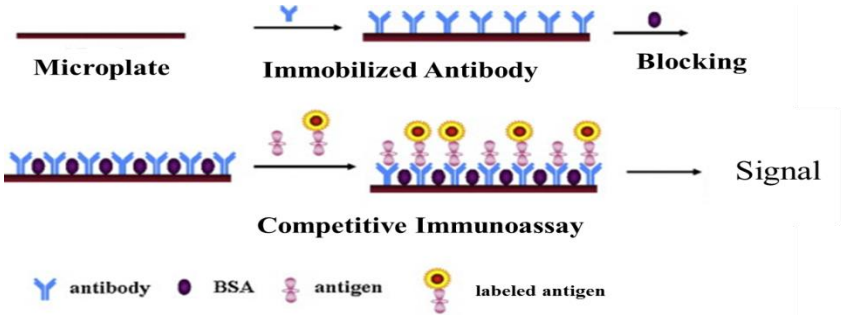

Figure 11. *Schematic picture of competitive immunoassay. Antibody is immobilized to the wells of the plate, and the blocking agent is also added to the wells to prevent unspecific binding. Target antigen and enzyme-labeled antigen are added, and the signals are measured. Modified from (Yu, Jiang and Cui, 2014).*

The non-competitive immunoassay, on the other hand, works in a slightly different way as described in Figure 12. It is also called as sandwich immunoassay. In Figure 12, enzyme non-competitive immunoassay is described as an example. In non-competitive immunoassay, there are antibodies adhered to the microplate as in step 1 (capture antibodies) and also the ones adhere to the antigen as in step 3 (tracer antibodies). The tracer antibodies are labeled, and the signals are obtained. The signal is absorbance in this case. (Yolken , 1986) This type of immunoassay is effective especially when antigen has two or more epitopes (Wisdom, 1976). There are no competitions between labeled antigens and not labeled antigens in this type of immunoassay compared to the competitive immunoassay, which is why it is called non-competitive immunoassay.

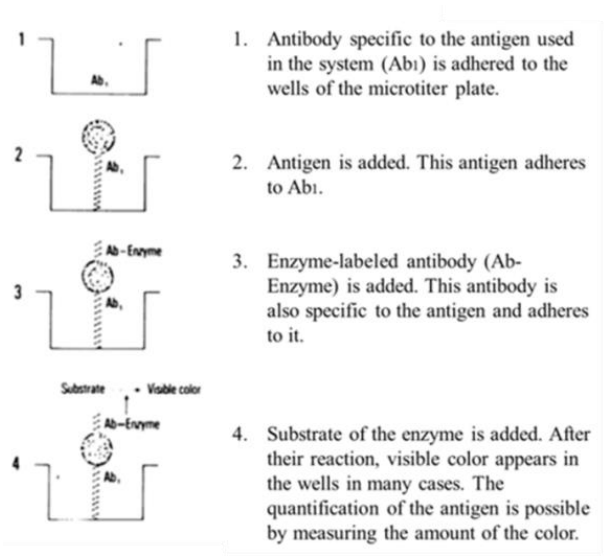

Figure 12. *Principle of enzyme sandwich immunoassay. 1. Antibody specific to the target antigen (AB_1) is adhered to the surface of the plate. 2. Antigen binds to AB_1. 3. Enzyme-labeled antibody is added, and it attaches to the antigen. 4. Substrate of the enzyme is added, and the signal is produced by the reaction of enzyme and its substrate. Modified from (Yolken , 1986).*

3.3.2 Substitutes for antibodies

For the immunoassay, capturing and tracing of the antigen does not always have to be done with the whole antibodies. Aptamers have been rising in to be a substitute for the antibodies during these 20 years. Aptamers are the single-stranded DNA or RNA which bind to specific ligands. They consist of 20 to 60 nucleotides and form three-dimensional conformations for the specific binding. (O'Sullivan, 2002; Chen and Yang, 2015; Xu et al., 2015) There have been research proving that aptamers are able to bind to small molecules too, for example, nucleoside, antibiotics, and fluorescent dyes (Haller and Sarnow, 1997; Song et al., 2012; Wilson and W Szostak, 1998). Aptamers are able to bind to their ligands in a similar way as antibodies, but they have many advantages over antibodies such as; the target could be non-immunogenic compounds too, the selection could be done more easily in vitro, lower cost chemical synthesis is possible, not sensitive for redox, pH or temperature, and the difference among the batches are smaller. For these reasons, aptamers are suitable candidates for the substitutes for antibodies in immunoassay. (Chen and Yang, 2015)

Single-chain variable fragments (scFvs) are also used for immunoassays instead of antibodies. As shown in Figure 13, scFv is a recombinant protein consisting of light chain of variable region (V_L) and heavy chain of variable region (V_H) domains of antibody domains linked together by a short peptide. The sizes of scFvs are usually 25kDa. This means that they are approximately six times smaller than antibodies, but they still maintain the ability to bind to the specific target of the original antibody. (Reddy and Robinson, 2010; De Meyer, Muyldermans and Depicker, 2014; Kierny, Cunningham and Kay, 2012) Other advantages of scFvs are the easiness for the gene manipulation for the improvement of their functions and the possibility of isolation by phage display from naïve libraries enabling rapid generation of them. (Cois, Hudson and Irving, 2001; El-Magd et al., 2016; Buckler et al., 2008) Due to the reasons above, the substitutes for antibodies are used more extensively nowadays.

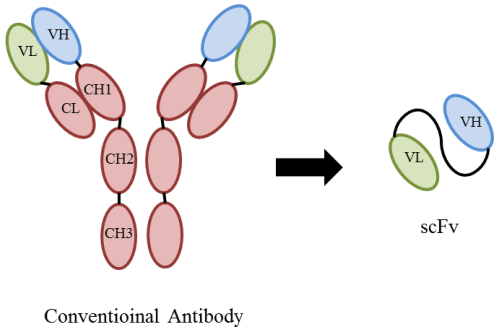

Figure 13. *Schematic description of conventional antibody and scFv. scFv consists of variable light and heavy chain bound with peptide chain. Modified from (De Meyer, Muyldermans and Depicker, 2014). Abbreviations; CH heavy chain (constant), CL light chain (constant), VH heavy chain (variable), VL light chain (variable).*

3.3.3 Immobilizations of antibodies and their substitutes

There are several methods to immobilize the antibodies to the wells for immunoassay. The easiest method for the immobilization is physical adsorption caused by hydrophobicity. The stableness of the adsorption depends highly on the materials of the surface or the characteristics of the samples. As mentioned above, it is the easiest method, but it has been proved that adsorption could cause conformation change to antibodies since it is not possible to adjust the orientation of the antibodies (Butler et al., 1992). Therefore, other methods have also been developed for antibody immobilization to overcome those problems.

Covalent attachment is one of these methods. By using covalent linking, the strength and the orientation of the attachment can be improved. An often-used technique of covalent immobilization is the covalent bond built by benzoquinone function and peptides containing cyclopentadiene. This is one of the most representative combinations in testing the activity of protein kinase. (Houseman et al., 2002)

There are also non-adsorptive or covalent methods for the immobilization. The most famous methods of them are the ones with streptavidin-biotin interaction which is known for the high affinity between them. To utilize this interaction, the wells of microtiter plate can be coated with streptavidin, and the protein (e. g. antibodies) to be immobilized can be biotinylated. Zhu (2014), for example, has succeeded in conducting a sandwich immunoassay utilizing this interaction into the analysis of virus for clinical use. Traditionally, biotinylation has been conducted with the chemical methods. However, with chemical methods, it is difficult to control to which sites of the antibodies biotin attach. Therefore, Santala (2004) described a new method to attach biotin to scFv. In this method, scFv is fused with biotin carboxyl carrier protein (BCCP) which binds to biotin inside the cells (Santala and Lammimmäki, 2004). The design of the scFv-BCCP fusion is possible with this technique, thus the orientation of the scFvs attached to biotin could be homogenous in the system.

3.3.4 Selection of the antibodies for immunoassay

To conduct immunoassays, specific proteins which bind to the target have to be selected. Phage display and biopanning are one of the techniques for the selection, and they are commonly used together sequentially. In phage display, proteins, peptides or antibodies, are displayed on bacteriophages. Phage display technology enables us to select specific binder proteins from a diverse protein library. Ff *E. coli* phages (f1, fd, M13), filamentous phages which replicate episomally, are often used for phage display. For phage display, the genes of the target molecules to be displayed are fused to the phage genes of the coat proteins. There are two coat proteins mainly used for phage display; major coat protein pVIII and minor coat protein pIII. (Figure 14) (Rakonjac et al., 2011) Major protein pVIII possesses α-helix structure, and its number of copies is 2700, making it

the most abundant. But, when the length of peptides is more than six, it copies in smaller numbers because of the structural problem. (Hoess, 2001) On the other hand, the number of copies of pIII is only five, but it does not have strict limitation for the length of the peptide to be fused with. pIII is composed with three domains; N1, N2 and C domains. These three domains are linked by glycine linkers. (Bazan, Calkosinski and Gamian, 2012) This coat protein plays important roles in the infection of phage (Figure 14). Firstly, N2 domain of pIII binds to the receptor, tip of the F-pilus. This triggers the conformational change of N2 domain, and the N1 domain is released. N1 domain then binds to the TolA-III. TolA works as a co-receptor in the infection of *E. coli*, and TolA-III is the C-terminal domain of its three domains. (Riechmann and Holliger, 1997) After that, C-domain of pIII coat protein opens up, and the coat proteins enter to the inner membrane. The complex of TolA, TolQ, and TolR mediates the process of the integration of the coat proteins pIII and pVIII to cytoplasmic membrane and entry of ssDNA into the cytoplasm. (Karlsson, 2004; Rakonjac et al., 2011; Grönwall and Stahl, 2009) However, the steps which N2 domain of pIII binds to TolA-III and C-domain of pIII opens up are still speculative. Thus, there needs to be more research to elucidate the phage infection mechanism.

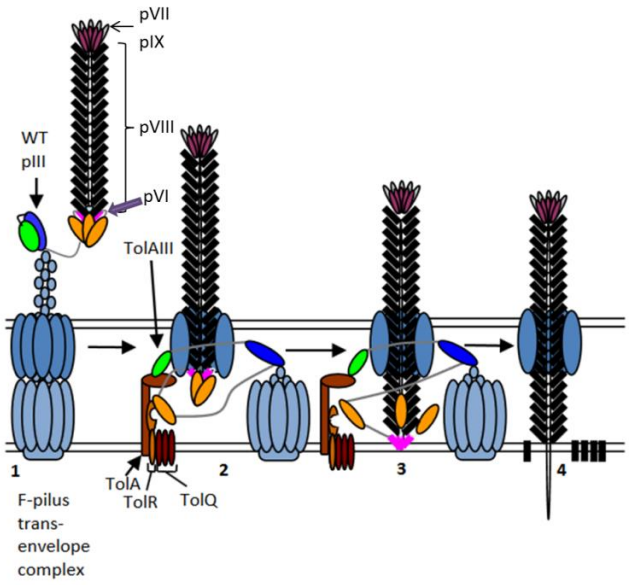

Figure 14. *Scheme of bacterial infection. Modified from (Rakonjac et al., 2011). 1. N2 domain of pIII (dark blue oval) binds to the F-pilus of the phage (light blue circles). The structure of the phage coat protein can be seen as well from this picture. 2. N1 domain of pIII (green oval) binds to TolA-III (brown oval). 3. C-domain of pIII (orange oval) opens up, and the coat proteins enters to the inner membrane. 4. The complex of TolA, TolQ (dark brown oval), and TolR (light brown oval) mediates the process of the integration of the coat proteins pIII and pVIII(black rectangles) to cytoplasmic membrane and entry of ssDNA into the cytoplasm.*

To generate the proteins, peptides, or antibodies to be displayed, phage vector or phagemid vector with helper phage are commonly used. Phage vector contains the genes of the fused pIII and the molecule to be displayed, origins of replication, and phage structural protein. Thus, phage vector contains genes of all the essential proteins for phage infection. On the other hand, phagemid vector contains only the fused genes for the coat protein pIII and the molecule to be displayed, origins of replication of *E. coli* and the phage, and antibiotics resistance. However, since it does not contain the genes for the other phage proteins, the phage cannot be constructed by phagemid plasmid alone. Therefore, after the phagemid vector is transformed to *E. coli*, helper phage is superinfected to provide those proteins. (Figure 15)

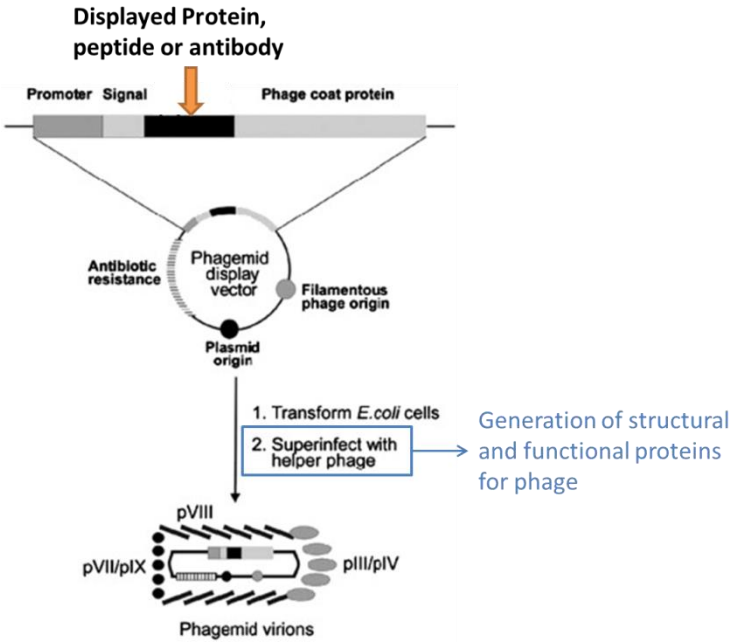

Figure 15. *Schematic description of phage infection by phagimid plasmid and helper phage. Modified from (Qi et al., 2012). The phagemid vector contains genes for promoter, signal, displayed protein, peptide or antibody, and phage coat protein. It also contains the origin of phage and plasmid. After the plasmid is transformed to the cells, the cells are superinfected with helper phage for the generation of structural and functional proteins for phage.*

Phage display of antibodies has been researched since 1990 (McCafferty et al., 1990; Rondot et al., 2001; Kugler et al., 2015). Antibodies can be displayed in the form of scFv or Fab on phage (Figure 16). When it takes scFv form, V_H and V_L genes linked by short peptide $(Gly_4Ser)_3$ are inserted into the phagemid vector. The C-terminus of V_L is attached to the gene of pIII protein. (Pansri et al., 2009). In contrast, when it takes the form of Fab, genes of C_H1 and C_L are inserted to the phagemid vector too. They are inserted to be so that the C-terminus of V_H is attached to C_H1 and the C-terminus of V_L is attached to C_L. Then, the C-terminus of C_H1 or C_L is attached to the pIII protein. (Hoogenboom et al., 1991) The advantage of Fab is that scFv tends to dimerize, but Fab does not have this tendency. When scFv takes the dimerized form or higher oligomers, it makes the selection more complicated in phage display. (Holliger, Prospero and

Winter, 1993; Kortt et al., 1994) However, it is studied that scFv expression is less toxic for the growth of the cells than Fab expression (Amdt, Muller and Pluckthun, 2001).

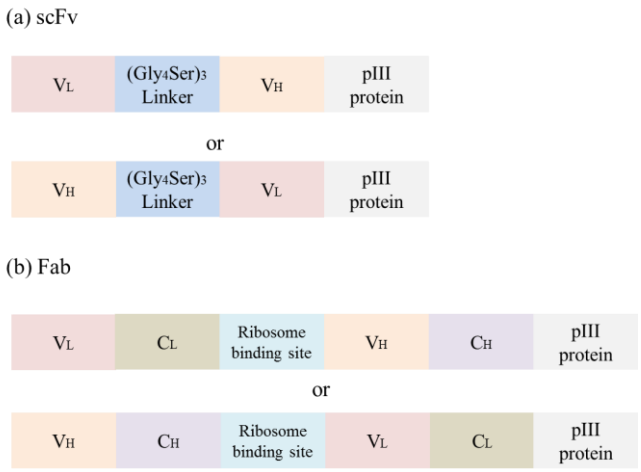

Figure 16. *The order of the genes of light and heavy chains in a phagemid vector for (a) scFv and (b) Fab form. Abbreviations; CH heavy chain (constant), CL light chain (constant), VH heavy chain (variable), VL light chain (variable). Modified from (Hoogenboom et al., 1991)*

The antibody phage library indicates the collection of those phages displaying antibody fragments. There are three types of antibody libraries; immune library, naïve library, and synthetic antibody library. Immune library is constructed with the antibody fragments isolated from lymphocyte cells of immunized animals, e. g. mouse, chicken, and rabbit (Ørum et al., 1993; Chiliza, Van Wyngaardte and Du Plessis, 2008; Popkov et al., 2003). The source could also be the patients of cancer or human which has taken the target antigen as vaccination (Wu et al., 2001; Cai and Garen, 1995). The antibody fragments, scFv or Fab, are amplified by PCR from cDNA of those lymphocyte cells. Therefore, the antibodies obtained from this library are highly specific to the antigen, enabling to isolate the antibodies possessing high-specific and high-affinity to the target from small sized library. However, the drawback of immune library is that the library needs to be specifically constructed to the targeted antigen. Naïve library is also called non-immune library, which means that its source is lymphocyte cells of non-immunized animals or healthy humans. Because the cells do not go through the immunization, the antibodies do not show as good affinity as the ones from immune library. To obtain high affinity antibodies, the size of the library needs to be large. (Schwimmer et al., 2013) While the source of immune library and naïve library is natural source, the antibody fragments are artificially designed to be rational for synthetic library. The sequences of variable chains of antibodies are amplified from template and variety of combinations are created, and then inserted into the CDR loops. (Weber et al., 2014) The templates are selected from the framework which is suitable to each case. Synthetic library is getting a lot of attention nowadays because the selection of the framework can be done according to the targets and the suitable combinations of variable chain genes

could be used for the production of functional scFvs. Hence, it is more effective and stable to express the antibodies. (Huovinen et al., 2013; Knappik et al., 2000)

The selection of the phage displaying the most desirable antibody fragment from the phage library is conducted very often with biopanning. The principle of biopanning is described in Figure 17. The antigens are commonly immobilized to the surface of a plate such as microtiter plate. This array is then exposed to the phage display, and only specific phages bind to the antigens. Then, the phages which did not bind to the antigens are washed away, and the rest is eluted by dropping pH. After that, *E. coli* is infected with these phages, and they are amplified again. The amplified phages are again panned repeating the same process. By conducting this process for several times, the phage displaying an antibody having the most affinity and the specificity can be isolated. In the end, the antibody is sequenced and identified. (Ellis et al., 2012) In biopanning, the blocking agent such as BSA is used similarly as in immunoassay to block the unspecific bindings of the phages.

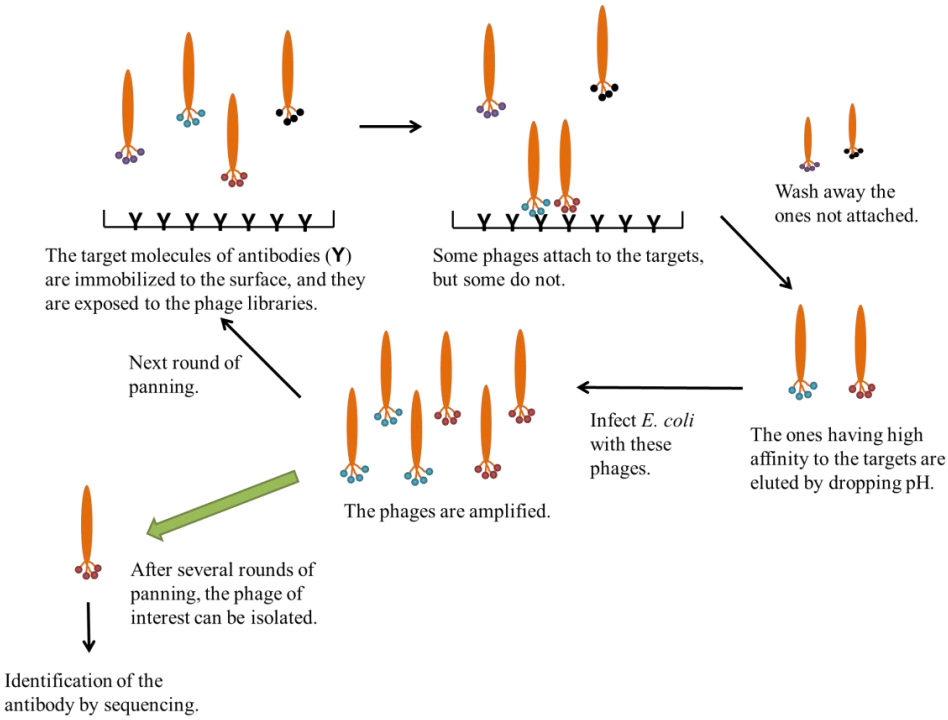

Figure 17. *Principle of biopanning. Modified from (Ellis et al., 2012) The phage display is exposed to a surface with their antigens, and the phages displaying the antibodies with high affinity to the antigens bind to them. The ones which did not attach to the antigens are washed away, and the rest are eluted. The eluted phages are used to infect E. coli, and they are amplified again. This process is repeated for several times, and then the phage of interest can be isolated. Finally, the antibody which has the highest affinity can be identified.*

The problem in biopanning is that the repetition of the amplification causing the loss of diversity (Derda et al., 2011). Therefore, it is important to reduce the number of the panning rounds. One of the methods to improve the functionality of one panning round

could be to use biotin-streptavidin interaction. This interaction can be used in biopanning too as in immunoassay. In this method, the plate is coated with streptavidin, and the phage displaying antibody is bound to biotin. For example, catalyzed reporter deposition (CARD) could be utilized into biopanning as well as introduced by Maaβ (2014). It is a method to bind the biotin to protein in which horseradish peroxidase (HRP) works as a mediator of the binding of biotin to the protein with covalent bond in CARD. In this method, the HRP is attached to the antibody, and the antigen is bound to glucose oxidase (GOX). When glucose is added, GOX produces H_2O_2 which is necessary for the HRP to mediate the binding of the biotin to the surface of phage. Therefore, only the phages attached to the target antigen are bound to the streptavidin, enabling the selection of the antibody more efficient. (Maaß et al., 2014; Bobrow et al., 1989)

3.3.5 Radioimmunoassay

Immunoassay can be also categorized in a different way than competitive or non-competitive. As mentioned above, there are many different "labels" for immunoassay, for example, radioisotope or enzyme. Furthermore, there are various types of signals to be obtained depending on the type of enzyme, such as chemiluminescense, fluorescence, electrochemiluminescense or absorbance. The antigens or the tracer antibodies can also be labeled directly with the substances which generate those signals. (Yalow and Berson, 1959; Schroeder et al., 1976; Blackburn et al., 1991; Dandliker, 2014) Radioimmunoassay is one of the types of different immunoassays.

Radioimmunoassay was invented already in 1959 and opened the way of immunoassay. The antigen or the tracer antibody is labeled with radioisotopes in this type of immunoassay. It has been leading the bioanalysis of many different substances, for example, histamine in biological fluids, hepatitis B virus-associated delta antigen, and uroterosin for a long time because of its high sensitivity (Laroche et al., 1995; Rizzetto, Shin and Gerin, 1980; Aiyar et al., 2004; Launay et al., 2010). However, the risk of using radioactive compounds has been considered as problematic, and as a consequence, the treatment of the radioactive waste becomes more complicated. It has to be treated according to the rules decided by the municipality. (Sancho, Amal and Garcia-Fayos, 2013).

In Table 1, the radioisotopes used frequently in radioimmunoassay and the important properties of them are listed. The radioisotope used the most these days is ^{125}I because of its relatively long half-life and specific activity. ^{125}I and ^{131}I are both iodine, however, ^{125}I is much more stable because its major radioactive emission does not have β. The radiation from ^{125}I does not diffuse through glass or fabrics as easily as ^{131}I, therefore it is easier to treat than ^{131}I. ^{3}H is also used especially for smaller molecules than the molecules which ^{125}I are used for since ^{125}I is moderately large. However, expensive liquid scintillation counting is required for ^{3}H since its radioactivity is very low. For these reasons, ^{125}I is still preferred for radioimmunoassay. (Lacy and O'Kennedy, 2005)

Table 1. *Radioisotopes and their properties. Modified from (Edwards, 1991; Lacy and O'Kennedy, 2005).*

Radioisotope	***Half-life ($t_{1/2}$)***	***Specific activity (Ci mmol^{-1})***	***Major radioactive emission***
^{14}C	5730 years	0.07	β
^{3}H	12.3 years	33	β
^{125}I	60 days	2560	ϒ
^{131}I	8.04 days	19250	β/ϒ

3.3.6 Enzyme immunoassay

Enzyme immunoassay is a safer immunoassay technique than radioimmunoassay. The typical types of enzymes used for enzyme immunoassay are listed in Table 2. Alkaline phosphatase (phoA), horseradish peroxidase (HRP) and β-d-galactosidase are the most common enzymes as labels. These enzymes react with their specific substrates, such as *p*-nitrophenyl phosphate (pNPP), 3,3′,5,5′-tetratmethylbenzidine(TMB), and o-Nitrophenyl-,3-D-galactopyranoside (ONPG), and the products of the reaction show different color from the reacted substrate. When alkaline phosphatase reacts with pNPP and when β-d-galactosidase reacts with ONPG, the color of the liquid changes to yellow. In the case of HRP and TMB, the color changes to blue. The change of the colors makes it possible to investigate the strength of the signals by measuring the absorbance at specific wavelength. (Giunta and Groppa, 1983; Tang, Tang and Yang, 2015; Busa et al., 2016) The same enzymes can react with chemiluminescence substrate and produce chemiluminescence too (Dai et al., 2015; Shu et al., 2016).

Table 2. *Examples of enzymes used for enzyme immunoassay and the target proteins.*

Target	Enzyme	Source
Mycotoxins	Alkaline phosphatase Horseradish peroxidase	Burmistrova (2013)
Adenosin3',5'-monophosphate	β-d-galactosidase	Yamamoto (1981)
3-amino-5-methylmorpholino-2-oxazolidinone	Alkaline phosphatase Horseradish peroxidase	Wang (2016)
Ractopamine	Alkaline phosphatase	Dong (2012)

3.4 Functional Protein Microarray

Functional protein microarray works in an almost same manner as analytical protein microarray, but does not use antibody-antigen interaction. In functional protein microarray technique, the interaction between protein and, for example, protein, peptide, DNA, small molecules, and glycan are tested. (Sutandy et al., 2013)

The aim of functional protein microarray is not to detect specific target, but to understand the properties of proteins in cells. In these cases, proteins are immobilized to the microarray, and the substances are added to bind to the proteins.

4. MATERIALS AND METHODS

4.1 Bacterial Strains

The bacterial strains and the plasmids used in this study are listed in Table 3. The types of the cells or plasmids and the important characteristics of them are listed. *A. baylyi* ADP1 wild type has the pathway to produce the wax ester, and long-chain aldehyde is one of the intermediates involved in the pathway. The long-chain aldehyde is used as a substrate by LuxAB and ADO.

Table 3. *Bacterial strains and the plasmids used in this study.*

	Cell or plasmid types	Important characteristics of the cells or the plasmids	Sources
Host cell			
	Escherichia coli (E. coli) Origami B	Kanamycin and tetracyclin resistance. Enables formation of disulfide bonds in cytoplasm. Improved production of scFv folded correctly. High productivity of scFv-BCCP fusion protein using pAK400cB vector.	Novagen® Jurado (2002) Santala (2003)
	E. coli KRX	Rhamnose promoter.	Promega
	E. coli XL1	Tetracyclin resistance.	Stratagen
	Acinetobacter baylyi (A. baylyi) ADP1	Wax ester production pathway.	DSMZ, Germany
	A. baylyi ADP1 *ΔpoxB::iluxAB_Cm r*	PoxB is knocked out from A. baylyi ADP1 wild type and Gene cassette *iluxAB_Cm*^{r} is integrated. Containing LuxAB, T5 promoter and chloramphenicol resistance genes.	Santala (2011)
Plasmid			
	SpT5.10/pAK400cB	SpT5.10-BCCP fusion gene, chrolamphenicol resistance, lac promoter.	Santala (2003)
	pAK400cB-ADO or LuxAB specific scFvs	ADO or LuxAB specific scFv-BCCP fusion gene, chrolamphenicol resistance, lac promoter.	This study
	pETM-14-ADO	ADO gene, kanamycin resistance gene.	Manuscript in preparation
	pETM-28-LuxAB	LuxAB gene, kanamycin resistance gene.	Manuscript in preparation
	pLK06H	PhoA gene, ampicillin resistance gene.	Huovinen (2013)
	pLK06H-ADO or LuxAB specific scFvs	ADO or LuxAB specific scFv-phoA fusion gene, chrolamphenicol resistance, lac promoter-operator, pectate lyase B coding gene, ampicillin resistance gene.	This study
	pBAV1C-ara-ado	ADO gene, arabinose promoter, chloramphenicol resistance gene.	Manuscript in preparation

4.2 Preparation of Media for Cultivation

The media used in this study are Luria-Bertani (LB) medium, Low Salt LB medium, Luria agar (LA) plates, and Super Broth (SB) medium (Table 4). LB medium consisted of 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride. Low Salt LB medium

consisted of the same concentration of tryptone and yeast extract as LB medium, but it contained 1 g/l sodium chloride instead of 5 g/l of it. LA plates contained 15 g/l agar in addition to the same composition of LB medium. SB medium consisted of 30 g/l tryptone, 20 g/l yeast extract, and 10 g/l MOPS buffer.

Table 4. *pH, components, and concentrations of the components of the media used in this study for the cultivations.*

Medium	pH	Components	Concentrations of the components (g/l)
Luria-Bertani (LB) medium	7.0	Tryptone	10
		Yeast extract	5
		Sodium chloride	5
Low Salt LB medium	7.0	Tryptone	10
		Yeast extract	5
		Sodium chloride	1
Super Broth (SB) medium	7.0	Tryptone	30
		Yeast extract	20
		MOPS buffer	10
Luria agar (LA) plates	7.0	Tryptone	10
		Yeast extract	5
		Sodium chloride	5
		Agar	15

The pH was adjusted to 7.0 for all of the cultures, and they were autoclaved after pH adjustment. LA plates were distributed to petri dishes after autoclaving.

4.3 Constructing the pAK400cB-ADO or LuxAB specific scFv plasmids

pAK400cB-ADO or LuxAB specific scFv, the plasmids containing scFv-BCCP fusion genes, were constructed for the sandwich immunoassay. pAK400cB vector was selected because it has been used for scFv-BCCP fusion protein previously in Santala (2004). Restriction, electrophoresis, gel extraction, polymerase chain reaction (PCR), and ligation were all done in the same way every time unless otherwise mentioned.

First, SpT5.10/pAK400cB plasmid was restricted with restriction enzymes *NdeI* and *EcoRI* to remove the original scFv gene *SpT5.10*. The restriction was conducted with 1µg of plasmids per 20µl of restriction sample, Buffer O (Thermo Scientific, USA), *NdeI* (Thermo Scientific, USA), *EcoRI* (Thermo Scientific, USA), and double distilled water to adjust the final volume according to the amount of each reagent. The restriction samples were incubated at 37 degrees for more than 2 hours.

After the restriction, the product was run in electrophoresis and the restricted vector was extracted from the gel. For the electrophoresis, the restriction product was mixed with 6x MassRuler DNA Loading Dye (Fermentas, USA) and run in 1% agarose gel. GeneRuler DNA Ladder Mix ready-to-use (Fermentas, USA) was used as a ladder. The extraction of the vector was done using GeneJET Gel Extraction Kit (Thermo Scientific, USA).

After that, *luxAB Vibrio fischeri* gene was amplified from plasmid BBa_K325909 (obtained from Registry of Standard Biological Parts) and restricted with restriction enzymes *NdeI* and *EcoRI*. The amplification was done using primers sa10 and sa9 (Appendix A, Table 6) by PCR with T3000 Thermocycler (Biometra, Germany). PCR was conducted with 170 ng of template plasmid, $2.5x10^{-5}$ µmol of Primers, Phusion Hot Start II (Thermo Scientific, USA) as an enzyme, 5xPhusion® HF Reaction Buffer (Thermo Scientific, USA), $1.25x10^{-4}$ mmol of dNTP, and double distilled water to adjust the final volume to 50 µl according to the amount of other reagents. Touchdown PCR was used for every PCR process.

After PCR, PCR product was purified by GeneJet PCR purification kit (Fermentas, USA). Then, restriction of the PCR products was conducted with 800 ng of PCR product instead of the plasmids. The restricted gene was run in electrophoresis and extracted from the gel. After that, this restricted LuxAB *V. fischeri* was ligated into restricted pAK400cB to construct cloning vector for the scFv-BCCP plasmids. Ligation was conducted with 50 ng of restricted vector, 40 ng of restricted PCR product, T4 DNA Ligation Buffer (Thermo Scientific, USA), T4 DNA Ligase (Thermo Fisher Scientific, USA), and double distilled water to adjust the total volume to 30 µl. The solution was kept for more than 1 hour for the ligation reaction at room temperature. Finally, pAK400cB vector containing LuxAB *V. fischeri* (cloning vector) was constructed.

Next, pLK06H-ADO or LuxAB specific scFv plasmids were amplified by PCR (Figure 18(a)). These plasmids were given as kind gifts from Urpo Lamminmäki, University of Turku. PCR and purification of the products were done in the same way as above except that the primers rm 13_2 and rm 13_1 (Appendix A, Table 6Table 6) were used. The scFv genes specific to ADO were EB34_B8, D3, D11, F3, G2, G8 and G10, and the ones specific to LuxAB were EB33_D6, D7, F10 and G4. Once they have been purified, these PCR products and the cloning vector were restricted and extracted from the gel after electrophoresis.

Finally, the scFv genes were ligated with the cloning vector. As a result, pAK400cB-ADO or LuxAB specific scFv plasmids were constructed (Figure 18(b)). The constructed plasmids were restricted with enzymes *EcoRI* and *NdeI* and run in electrophoresis for confirmation. Electrophoresis was conducted in the same way as above, but GeneRuler DNA Ladder Mix ready-to-use (Fermentas, USA) was used for the construction of plasmids containing LuxAB specific scFvs, and Generuler 100bp plus ready-to-use

(Thermo Scientific, USA) was used for the construction of plasmids containing ADO specific scFvs.

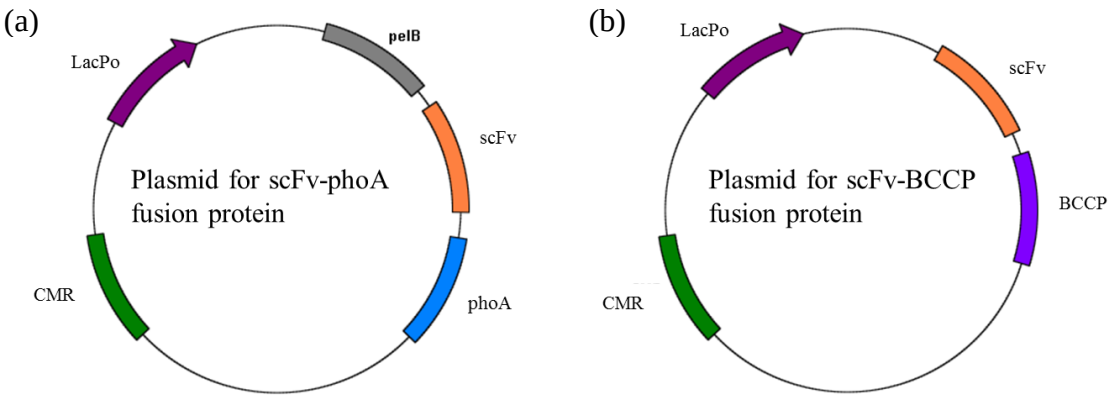

Figure 18. *Plasmids used in this study; (a) pLK06H-ADO or LuxAB specific scFv and (b) pAK400cB-ADO or LuxAB specific scFv. Abbreviations: BCCP biotin carboxyl carrier protein, CMR chloramphenicol resistance gene, LacPO Lac promoter-operator, pelB pectate lyase B coding gene, and scFv single-chain variable fragment.*

4.4 Transformation

Transformation of pLK06H-ADO or LuxAB specific scFv plasmids and pAK400cB-ADO or LuxAB specific scFv plasmids into cells was conducted for protein expression. pLK06H-ADO or LuxAB specific scFv plasmids were transformed into *E. coli* XL1 and pAK400cB-ADO or LuxAB specific scFv plasmids were transformed into *E. coli* Origami B, respectively. The transformations for the both plasmids were done using electroporation in the same way as described below.

Firstly, 2 µl of plasmid was added to 40 µl of corresponding competent cell and this mixture was moved to a BIO-RAD Gene Pulser cuvette. Then, this cuvette was placed into BIO-RAD MicroPulser^{TM} (BIO-RAD, USA), and switched on with Ec1 programme. Right after that, 1 ml of LB medium was added to the cuvette and pipetted gently. Then, the mixture including LB medium was moved to a 1.5 ml Eppendorf tube and incubated at 37 degrees for 1 hour.

After the incubation, it was spread on LA plate and incubated again at 37 degrees overnight. The colonies obtained from LA plates were picked and cultivated in appropriate media according to the cell types and the plasmid types for lysate preparation for sandwich immunoassay.

4.5 Lysate preparation

Lysates were prepared for the immunoassays using the cells harbouring plasmids and the ones without plasmids. All the cells were cultivated in suitable media. The cell types for each plasmid, the type of the media, the antibiotics added to the media and their concentrations and inducers used are listed in Table 5. Different media were used for the same type of host cells for different purposes.

Table 5. *Cell types used for each plasmid and antibiotics used during cultivation. Abbreviations: ADO aldehyde-deformylating oxygenase, LB Luria-Bertani, LuxAB bacterial luciferase, SB Super Broth, scFv single-chain variable fragment.*

Host cell	Type of medium	Plasmid contained	Antibiotics added to medium (µg/µl)	Inducer
Escherichia coli (E. coli) Origami B	SB	pAK400cB-ADO or LuxAB specific scFvs	Chloramphenicol 25 µg/µl, tetracyclin 10 µg/µl, kanamycin 30 µg/µl	Isopropyl β-D-1-thiogalactopyranoside (IPTG) 250 µM
E. coli KRX	SB	pETM-14-ADO or pETM-28-LuxAB	Kanamycin 50 µg/µl	IPTG 250 µM, rhamnose 0.1 %
E. coli KRX	Low salt LB	pETM-14-ADO	Kanamycin 50 µg/µl	IPTG 250 µM, rhamnose 0.1 %
E. coli KRX	LB	pETM-14-ADO or pETM-28-LuxAB	Kanamycin 50 µg/µl	IPTG 250 µM, rhamnose 0.1 %
E. coli XL1	SB	pLK06H-ADO or LuxAB specific scFvs	Tetracyclin 10 µg/µl, ampicillin 100 µg/µl	IPTG 250 µM
E. coli XL1	SB or LB	No plasmids	Tetracyclin 10 µg/µl	IPTG 250 µM, rhamnose 0.1 %
Acinetobacter baylyi (A. baylyi) ADP1	Low salt LB	pBAV1C-ara-ado	Chloramphenicol 25 µg/µl	Arabinose 1%
A. baylyi ADP1 *ΔpoxB::iluxAB_Cm r*	Low salt LB	No plasmids	Chloramphenicol 25 µg/µl	No inducers
A. baylyi ADP1	Low salt LB	No plasmids	No antibiotics	Arabinose 1%

4.5.1 Lysates for the immunoassays for finding the best combinations of scFvs

First, all the cells other than *A. baylyi* ADP1 were cultivated at 30 degrees and 300 rpm overnight. In addition to the antibiotics, every culture contained 250 µmol/l of isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction and 0.4% of glucose as nutrition. Rhamnose was also added to the culture of KRX cells harbouring pETM-28-LuxAB or pETM-14-ADO for the induction purpose, and the concentration was 0.1 %. KRX cells were all cultivated in SB medium for this experiment. 4 µM of biotin was added to the culture of Origami B for the biotinylation.

After cultivation, the cells were harvested by centrifugation at 6000g for 5 minutes. Then, the harvested cells were re-suspended to assay buffer in Eppendorf tubes, and about 1mg of lysozyme from chicken egg white was added to each tube. Assay buffer contained 50mM Tris-HCl (pH8), 150mM sodium chloride, 0.1% bovine serum albumin (BSA), and 0.01% Tween 20. Next, the tubes having re-suspended cells and lysozyme were incubated at 37 degrees for cell lysis for 1 hour.

After 1 hour, they were taken out from the incubator and placed into -80 degrees freezer and 37 degrees incubator alternately for 3 times also to lyse the cells. The lysed cells were centrifuged and the supernatant was aliquoted. This supernatant was the lysate to be used for the immunoassay, and they were kept in 4 degrees fridge before use.

4.5.2 Lysates for the immunoassays for standard curves and for protein expression check with *A. baylyi* ADP1

The lysate preparation for these purposes was conducted using flasks for the main cultivation. First, pre-cultivation of all the cells were conducted with 5 ml cultivation tubes with appropriate media in the same way as section 5.4.1 without the inducers. The tubes were incubated at 30 degrees and 300 rpm overnight.

After the pre-cultivation, the OD_{600} of all the cells was measured, and they were cultured into the main culture in suitable sized flasks. The starting OD_{600} of the main cultivation was adjusted to 0.01 other than Origami B and XL1 harbouring pLK06H-ADO or LuxAB specific scFv which started with OD_{600} 0.02. The main culture included the same components as the pre-culture. The cells were cultivated at 37 degrees at 300 rpm.

After the OD_{600} of the main cultures has reached from 0.5 to 1, suitable inducers were added to the culture at the appropriate concentration. For *A. baylyi* ADP1Δ*poxB*::i*luxAB_Cm*^{r}, no inducers were added because it contains T5 promoter. The culture containing Origami B strain was cultivated for 48 hours at 26 degrees at 300 rpm, but all the others were cultivated only 24 hours.

4.6 Immunoassays

Many different types of immunoassays have been conducted during this study. Firstly, ADO sandwich immunoassay and LuxAB capture assay were conducted to select the best-working combinations of the scFvs. After that, another ADO sandwich immunoassay and LuxAB capture assay were conducted to draw standard curves for the best combination of ADO-specific scFvs and the best-working capture LuxAB-specific scFvs. Finally, the established immunoassay systems were applied with ADO and LuxAB produced by *A. baylyi* ADP1.

All types of immunoassays were performed using KaiSA96-Lockwell Streptavidin microtiter plate (Kaivogen, Finland). Incubation and washing of the plate were conducted in the same way in all the immunoassays otherwise mentioned.

4.6.1 ADO sandwich immunoassay for selecting the best-working combination of scFvs

This sandwich immunoassay was conducted to select the best-working combination of scFvs (Figure 19). Firstly, 100 µl of each pAK400cB-ADO specific scFvs/*E. coli* Origami B lysate was added to each well of the plate. The plate was incubated at room temperature at slow shaking for 1 hour. Then, the plate was washed 4 times per each well with wash buffer with Elisa program with Hydroflex (Tecan, Switzerland). Wash buffer contained 5mM Tris-HCl (pH8), 150mM sodium chloride, and 0.01% Tween 20. The incubation and the washing of the wells were done in the same way below for the other types of immunoassays. After that, 100µl of pETM-14-ADO/KRX lysate was added as an antigen to each well. Then, the plate was incubated and washed again. Next, 100 µl of each pLK06H-ADO specific scFv/*E. coli* XL1 lysate was added to each well. After that, the plate was incubated and washed. Finally, 100 µl of alkaline phosphatase yellow (pNPP) liquid substrate system for ELISA (Sigma-Aldrich, USA) was added to each well and the plate was incubated at 37 degrees at 150 rpm for 1 hour. All the combinations of scFvs were tested as duplicate. After the final plate incubation, the absorbance at 405nm was measured for each well with Multiskan Ascent 96 plate reader (Thermo Scientific, USA).

All the combinations of scFvs were tested. In addition to that, negative controls were also tested for each antibody combination in sandwich immunoassay. *E. coli* XL1 cells without protein expression (XL1 empty) were used instead of antigen in the negative controls. The results were calculated by dividing the absorbance by the absorbance of the corresponding negative controls lacking antigen.

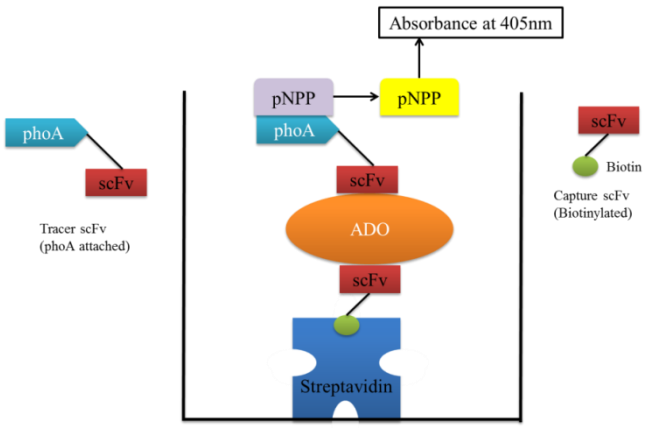

Figure 19. *Schematic picture of ADO sandwich immunoassay. The surface of the plate is coated with streptavidin, and the biotin molecule binds to it. Capture scFv is fused with biotin, and this scFv captures ADO. ADO is then again captured by tracer scFv, and phoA attached to the tracer scFv react with its substrate pNPP. The absorbance is measured in the end as a signal. Abbreviations: ADO aldehyde-deformylating oxygenase, phoA, alkaline phosphatase, pNPP alkaline phosphatase yellow, scFv single-chain variable fragment.*

4.6.2 LuxAB capture assay for selecting the best-working scFvs

This assay was conducted to select the best-working capture scFvs for LuxAB.

Firstly, the plate was washed in the same way as described in section 5.5.1. After that, 200 µl of pAK400cB-LuxAB specific scFvs/*E. coli* Origami B lysates were added to the wells, and the plate was incubated and washed. After that, 200 µl of pETM-28-LuxAB/KRX lysate was added to the wells and the plate was incubated and washed. Then, 150 µl of XL1 empty expression lysate, 45 µl of assay buffer, and 5 µl of decanal stock were added to the wells. Decanal stock consisted of 1 ml of 99.6 % ethanol and 20 µl of decanal. After the addition of decanal, luminescence was measured for each well. The ones showing the highest luminescence were selected as the best. All the types were tested as duplicate.

All the pAK400cB-LuxAB specific scFvs/*E. coli* Origami B lysates were tested, and the negative controls were also tested. For the negative controls, XL1 empty lysate was used instead of pAK400cB-LuxAB specific scFvs/*E. coli* Origami B lysate. The results were calculated by dividing the absorbance by the absorbance of the negative control.

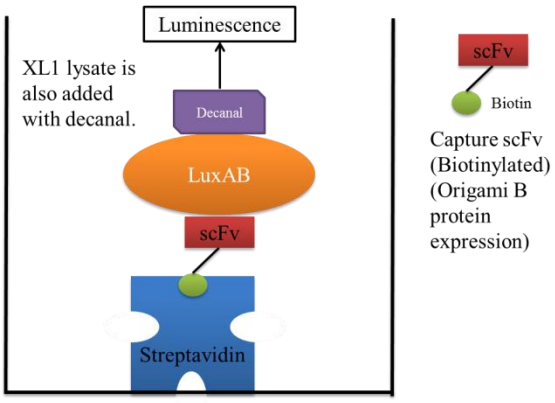

Figure 20. *Schematic picture of LuxAB capture assay. The surface of the plate is coated with streptavidin, and the biotin molecule binds to it. Capture scFv is fused with biotin, and this scFv captures LuxAB. After the LuxAB has been captured, decanal and XL1 lysate is added to the well. Then, luminescence is measured as signal. Abbreviations: LuxAB bacterial lusiferase, scFv singl-chain variable fragments.*

4.6.3 Sandwich immunoassay and LuxAB capture assay for drawing a standard curve

After finding the best combination (D11-BCCP and D3-phoA) of ADO-specific scFvs, another sandwich immunoassay was conducted to draw a standard curve with the selected combination. Only the combination of D11-BCCP and D3-phoA was tested. All the samples were tested as triplicate samples.

This sandwich immunoassay was mainly conducted in the same way as sandwich immunoassay for finding the best scFvs, but the plate was washed before the first addition of the lysate and the volume of pETM-28-ADO/KRX lysate was different. To draw the standard curve, 0 µl, 0.5 µl, 1 µl and 5 µl of the lysate was used. Negative controls were tested using XL1 empty lysate instead of D11-BCCP lysate with all different volumes of pETM-28-ADO/KRX lysate.

Similarly, after finding the best working capture scFvs (D7 and G4), another LuxAB capture assay was conducted to draw standard curves with the selected scFvs. Only D7 and G4 were tested as capture scFvs. All the samples were tested as triplicate samples.

The assay was conducted in the same way as 5.5.2 except that many different volumes of pETM-14-LuxAB lysate were used for drawing standard curves. The used volumes of the lysate were 0 µl, 10 µl, 20 µl, 30 µl and 40 µl. Negative controls were tested using XL1 empty lysate instead of D7 or G4 lysates with all different volumes of pETM-14-LuxAB lysate.

4.6.4 Sandwich immunoassay and LuxAB capture assay with *A.baylyi* ADP1

After drawing standard curve for sandwich immunoassay and capture assay with antigens expressed by *E. coli* KRX, these assay systems were tested if they could also work with ADO and LuxAB expressed by *A. baylyi* ADP1. The sandwich immunoassay and LuxAB capture assay were conducted in the same way as 5.5.3 and 5.5.2 including the negative controls, respectively, except that pBAVIC-ara-ado/*A. baylyi* ADP1 lysate and *A. baylyi* ADP1Δ*poxB*::i*luxAB_Cm*^{r} were used. For LuxAB capture assay, ADP1 empty lysate was used for the negative control. The volumes of pBAVIC-ara-ado/*A. baylyi* ADP1 lysate were 0µl, 200µl, 400µl, 600µl and 800µl. The volumes of *A. baylyi* ADP1Δ*poxB*::i*luxAB_Cm*^{r} lysate were 0µl, 50µl, 100µl, 150µl, and 200µl. All the samples were tested as triplicate samples.

5. RESULTS

5.1 Construction of pAK400cB-ADO or LuxAB specific scFvs

The plasmids, pAK400cB-ADO or LuxAB specific scFvs, were constructed in order to obtain scFvs fused with BCCP for the ADO sandwich immunoassay and LuxAB capture assay with streptavidin coated microtiter plate.

The results of the electrophoresis of restricted pAK400cB-ADO or LuxAB specific scFvs plasmids are shown in Figure 21. As it can be seen, the bands for the inserts (scFv genes) are at around 800bp for all of them other than the first G8, the first two F10 and the last two F10 (from left). Therefore, it can be said that the plasmids other than the ones mentioned are successfully constructed.

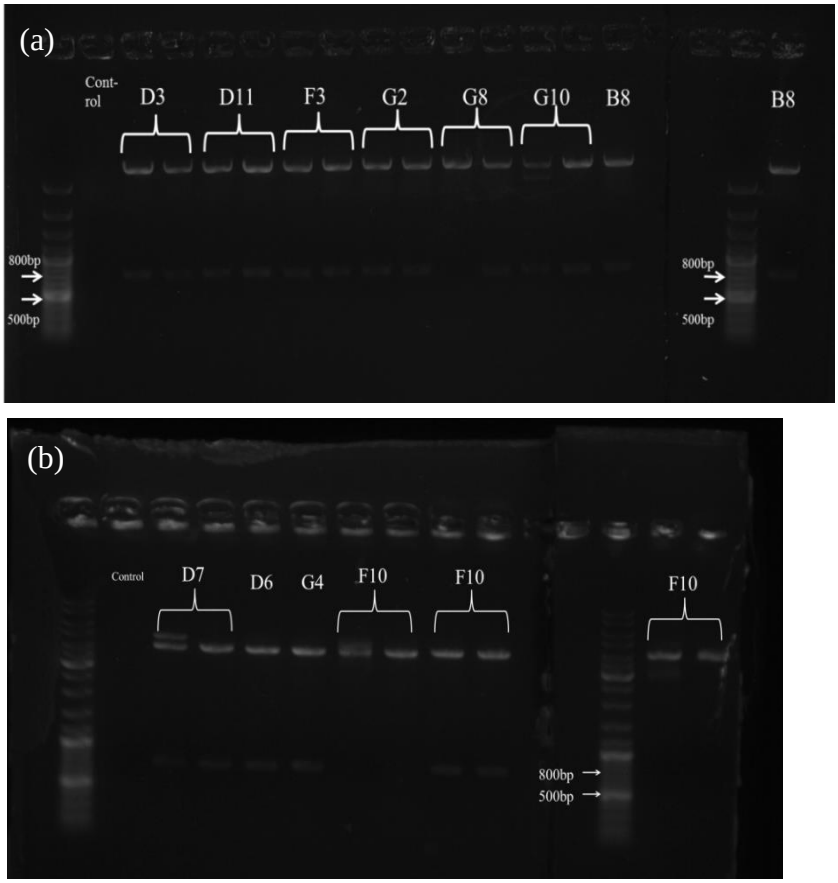

Figure 21. *Results of the electrophoresis of restricted pAK400cb-ADO or LuxAB specific scFv plasmid; (a) aldehyde-deformylating oxygenase (ADO) specific scFv containing plasmid and (b) Bacterial luciferase (LuxAB) specific scFv containing plasmid. Control was the background for ligation which had double distilled water instead of insert for the ligation.*

5.2 Immunoassays

5.2.1 Finding the best scFvs

ADO sandwich immunoassay and LuxAB capture assay were conducted to select the best-working combinations of ADO-specific antibodies and the best capture LuxAB-specific scFvs. The results for ADO sandwich immunoassay are shown in Figure 22(a). As it can be seen, the combination of D11 as a capture scFv and D3 as a tracer scFv works by far the best with this system, showing 2.56 times bigger absorbance than its negative control.

For LuxAB capture assay, the results are shown in Figure 22(b). As a result, G4 and D7 showed the best results by showing 1.61 times and 1.50 times bigger luminescence compared to the negative control, respectively. Though G4 showed a big error bar, it could still be considered as a candidate because only duplicate samples were tested.

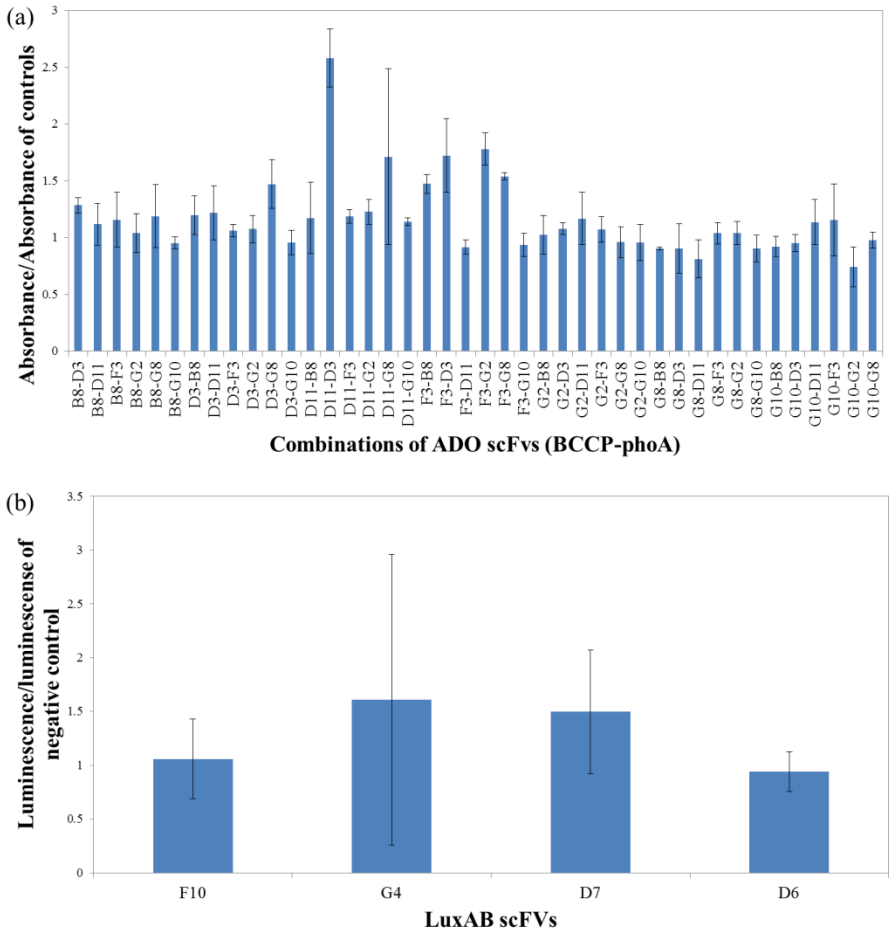

Figure 22. *Results of (a) ADO sandwich immunoassay and (b) LuxAB capture assay for the best scFv selections. In (a), the names of the scFv combinations of are shown as capture scFv-tracer scFv (e.g. B8-D11 means that the capture scFv was B8 and the tracer scFv was D11.)*

5.2.2 Standard curve

The standard curves drawn by ADO sandwich immunoassay and LuxAB capture assays with the best scFvs are shown in Figure 23 (a) and (b), respectively. As results of the assays, clear standard curves were drawn for both ADO and LuxAB assays. This indicates that the combination of the ADO-specific scFvs and the LuxAB scFvs worked in these immunoassay systems.

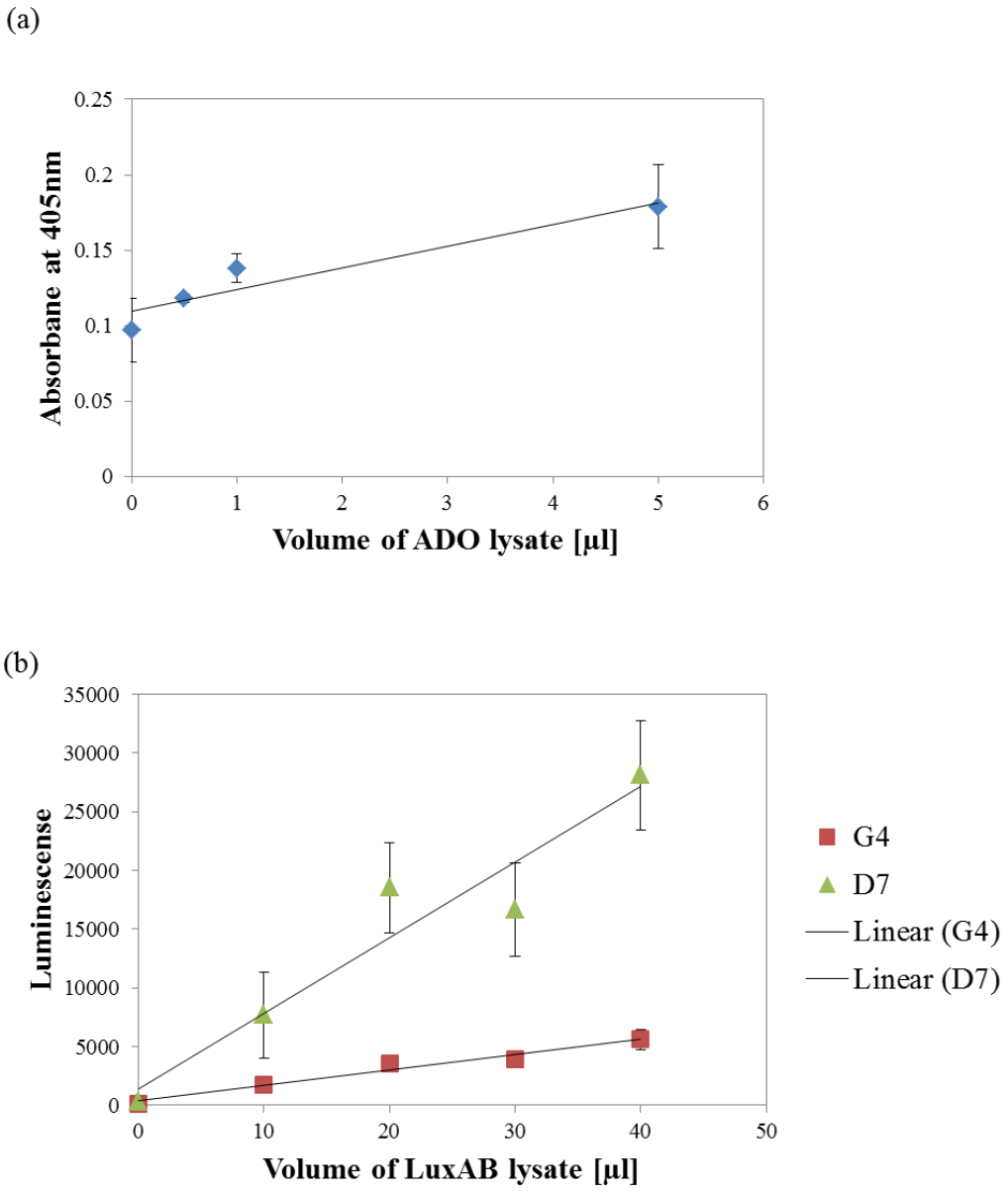

Figure 23. *Standard curves of (a) ADO sandwich immunoassay and (b) LuxAB capture assay. For ADO immunoassay, the combination of scFvs were D11 and D3. For LuxAB capture assay, scFvs G4 and D7 were used.*

5.2.3 Immunoassays with antigens expressed by *A.baylyi* ADP1

After the assay systems have been tested and confirmed by drawing the standard curve, the systems were applied into analysis of the protein expression by *A. baylyi* ADP1. As it can be seen clearly in Figure 24 (a) and (b), no co-relationships between the volume of ADO or LuxAB lysate and the signals were obtained. In addition, the signals were as small as the negative controls in both cases.

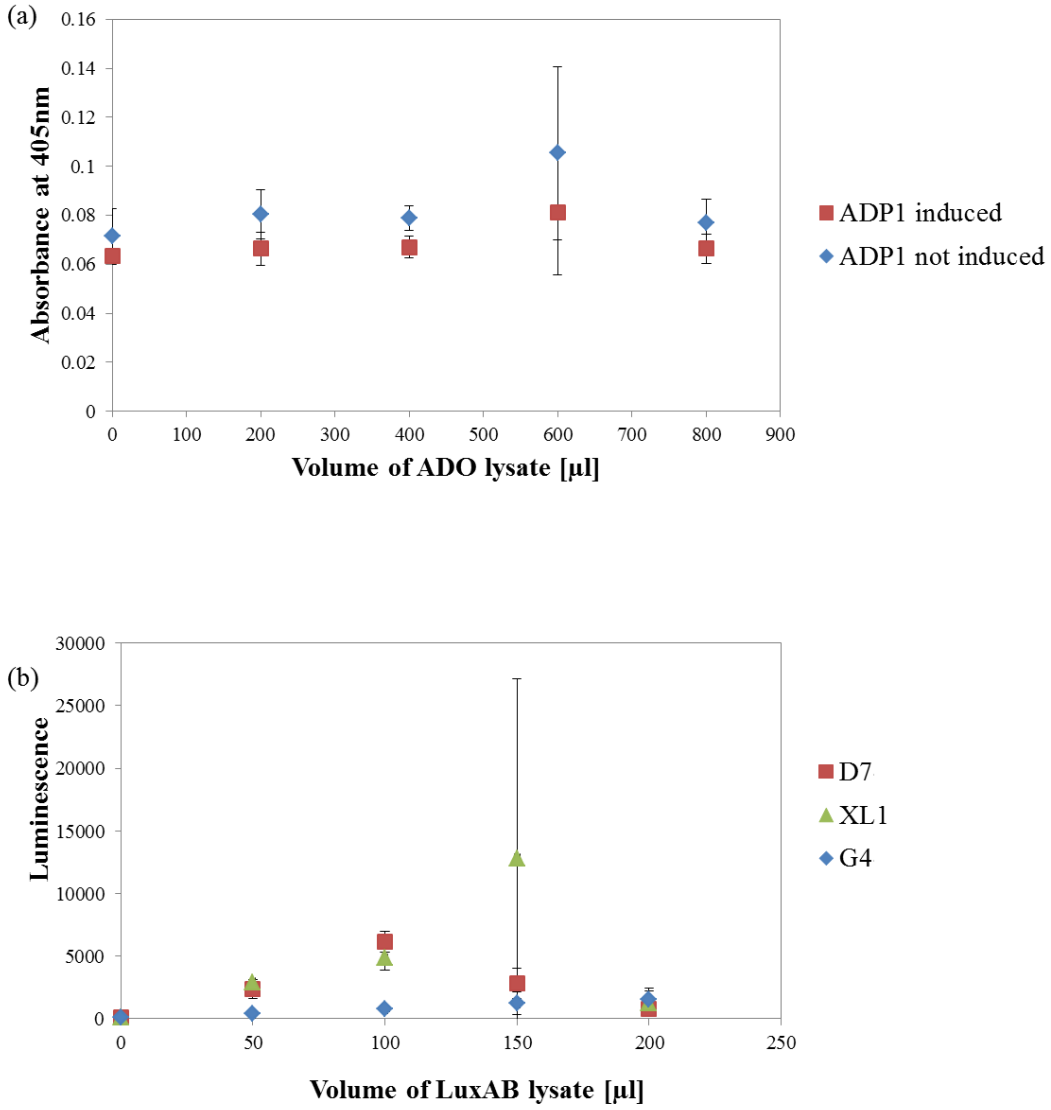

Figure 24. *The results of (a) ADO sandwich immunoassay and (b) LuxAB capture assay with the antigens produced by A. baylyi ADP1. In (a), A. baylyi ADP1 was induced by Arabinose in "ADP1 induced" and it was not induced in "ADP1 not induced". In (b), D7 and G4 are tested with D7 and G4, respectively. XL1 is the one which did not contain scFvs, but XL1 empty lysate was used instead of them (negative control).*

6. DISCUSSIONS AND CONCLUSIONS

Cell factory construction has been drawing a lot of attentions nowadays because of its potentiality to replace for the traditional production of valuable products from fossil fuels. To construct useful cell factories, there must be easy and accurate tools to analyse them. In this study, analytical tools for the ADO producing bacteria and LuxAB producing bacteria were constructed. ADO is an interesting target enzyme because it is involved in fatty alkanes or alkenes production in bacteria. Fatty alkanes and alkenes are the main components in the fuels used widely in the world (Mabuza, 2011; Howard et al., 2013). LuxAB is an enzyme which is able to produce bioluminescence, enabling it to be used as a bioreporter. As a bioreporter, it can be utilized as a real-time monitoring tool for a production of certain compounds in bacteria. Measurement of the bioluminescence produced by LuxAB during cultivation is the signal of the production of the target protein. However, the production of LuxAB itself needs to be studied more by other methods.

Here, a simple and easy method, immunoassay, was investigated as an analytical tool for ADO and LuxAB for the reasons above. Immunoassay is much simpler and cheaper compared to MS which has been used for proteomic analysis. It is because MS requires expensive equipment, such as ionization source, mass analyser and detector, and also that the capability of using those equipment is required for the researchers. On the other hand, immunoassay requires only the lysates of the cells, microtiter plates, incubators, and signal readers, for example, absorbance or luminescence measurer. Moreover, the use of the equipment does not need any special skills. Though immunoassay is a simple method, it possesses high accuracy because antibody-antigen interaction is utilized. Enzyme immunoassay is particularly important type of immunoassays in order to achieve simpler immunoassay system since no special skills are required as in radioimmunoassay. The use of streptavidin-biotin interaction also helps to improve the accuracy of the immunoassay maintaining the easiness of the system.

In this study, enzyme sandwich immunoassay for ADO (ADO sandwich immunoassay) and a new type of immunoassay for LuxAB (LuxAB capture assay) with scFvs were constructed. In LuxAB capture assay, LuxAB is captured by the capture scFvs and produces luminescence using the added decanal as a substrate. *E. coli* Origami B and *E. coli* XL1 were engineered to produce scFvs for the immunoassays, and the immunoassay systems with the best scFvs were then tested with ADO or LuxAB producing *E. coli* KRX first, and they were applied with *A. baylyi* ADP1 producing those proteins.

The plasmids pAK400cB-ADO or LuxAB specific scFvs were constructed with PCR to modify *E. coli* Origami B to be able to produce ADO or LuxAB specific scFvs, respectively. As the capture scFvs need to be fused with biotin, vector pAK400cB was selected. For the tracer scFvs, vector pLK06H was selected since the scFvs need to be fused with phoA. These plasmids were all constructed successfully and were transformed into corresponding *E. coli* strains.

After the construction of the engineered *E. coli* strains, the best scFv pairs and scFvs for ADO sandwich immunoassay and LuxAB capture assay were selected by conducting the actual immunoassays. The best scFv pairs and scFvs were defined as the ones giving the highest signal compared to the negative controls through this study. At this stage, *E. coli* KRX producing ADO or LuxAB were used to confirm the assay systems. Absorbance for ADO sandwich immunoassay and luminescence for LuxAB capture assay were measured, respectively. It was found out in this study that D11 as a capture scFv and D3 as a tracer scFv (D11-D3) is by far the best combination for ADO sandwich immunoassay. Correspondingly, D7 and G4 are the best working scFvs for LuxAB capture assay. For LuxAB, LuxAB sandwich immunoassay was also tested, but no combinations of scFvs worked (results not shown). After the selection, the assays with the best scFvs were conducted to draw standard curves. Standard curves were drawn for all the types of the assays, therefore it could be concluded that all the scFvs were able to detect the targets correctly, and the system worked properly with these scFvs. From this result, it can be also said that an immunoassay in which antigen itself generates the signal is eligible too as in LuxAB capture assay.

The immunoassay systems confirmed were finally tested with the *A. baylyi* ADP1 producing ADO or LuxAB. The strains were engineered to produce wax ester, and they generate long-chain aldehyde as an intermediate during the wax ester production (Santala et al., 2011). As a result, no relationships between the volume of the lysates and the signals were observed. Since the system was confirmed to be working in the previous experiments, it could be considered that the reasons why it did not work this time are in the strains. The possible reasons could be that the strains do not produce ADO or LuxAB or produce so small amount of them that the system could not detect them.

The strain *A. baylyi* ADP1 harbouring pBAVIC-ara-ado has been constructed in laboratory in Tampere University of Technology, but the manuscript is still in progress as described in Table 3. Therefore, the production of ADO is not analysed with any other method yet. Moreover, the sensitivity of the sandwich immunoassay constructed in this study has not been accurately tested. Therefore, it is possible that the strain does not produce any ADO, but there also remains the possibility that the strain produces too small amount of ADO for ADO sandwich immunoassay. In this study, lysate of *E. coli* containing ADO was used for the immunoassay, but the exact amount of ADO was not measured. For this reason, the sandwich immunoassay system has to be tested with

known amount of ADO to investigate the minimum and maximum amount of ADO that can be detected.

The strain *A. baylyi* ADP1 *ΔpoxB::iluxAB_Cm^r* was constructed and it was proved that luminescence was produced during the wax ester production by Santala (2011). However, the amount of LuxAB produced was not measured. Additionally, the amount of LuxAB lysate was increased only to 200 µl in this study. Considering these facts, the strain produces LuxAB, but the amount of LuxAB contained in the lysate was not enough to be detected in this system. So, LuxAB capture assay system must be tested with bigger amount of lysate. If the LuxAB cannot still be detected with bigger amount, such as 800µl, the sensitivity of this LuxAB capture immunoassay should also be tested similarly to ADO sandwich immunoassay.

The immunoassays constructed in this study give a possibility to analyse the proteomics of the cell factories engineered to produce valuable products. In this case, wax ester production and alkane or alkene production were the targets. In this study, the systems were successfully constructed, but they were not yet applicable with *A. baylyi* ADP1 strains producing the target products. Thus, it will be necessary to examine the sensitivities of the systems, and also to increase the amount of the lysates to be analysed. However, this study provides a good foundation for the novel immunoassay systems. These systems will enable researchers to analyse proteomics more cheaply and effortlessly than what is currently possible, thus helping to develop more of novel bacterial cell factories producing valuable products.

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APPENDIX A: THE PRIMERS USED IN THIS STUDY

The primers used in this study are shown in Table 6 below.

Table 6. *The description of oligo sequences of the primers used in this study.*

Name	Length (base pairs)	Description	Oligo sequence
sa10	43	PCR antisense primer, V. fischeri luxAB, EcoRI, TAA, BamHI	ATTGGGATCCTTAGAATTCTGGTAAATTCATTTCGATTTTTTG
sa9	51	PCR sense primer, V. fishceri luxAB, XbaI, RBS, NdeI	AATTTCTAGAGAAGGAGATATACATATGAAGTTTGGAAATATTTGTTTTTC
rm13_2	23	PCR antisense primer, For cloning scFvs from pAK to p1.3Tc, EcoRI	TCCGGAATTCGGCCCCCGAGGCC
rm13_1	32	PCR sense primer, For cloning scFv from pAK to pAK400c, NdeI	CAATCATATGGAAATTGTGCTGACCCAATCTC