DEVELOPMENT OF SERUM-FREE MEDIUM FOR *SPODOPTERA FRUGIPERDA* (SF9) INSECT CELL CULTIVATION AND RECOMBINANT PROTEIN PRODUCTION

SUREEWAN SAISUD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN APPLIED BIOLOGY

FACULTY OF SCIENCE AND TECHNOLOGY

RAJAMANGALA UNIVERSITY OF TECHNOLOGY THANYABURI

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Name-Surname	Miss Sureewan Saisud
Program	Applied Biology
Thesis Advisor	Assistant Professor Jantima Teeka, Ph.D.
Academic Year	2023

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ABSTRACT

This research aimed to: 1) develop a serum-free medium (SFM) for *Spodoptera frugiperda* (Sf9) insect cell culture, 2) study the effect of microfiltration and ultrafiltration of soytone and yeast extract on the growth of Sf9 cells, and 3) examine recombinant protein production in Sf9 insect cells using a developed serum-free medium (OSF9- SFM).

Sf9 cells were cultured in Grace's insect cell culture media supplemented with different concentrations of chemically defined lipid concentrate (CDLC), soytone (ST), and yeast extract (YE), using the Central Composite Design method. Low-medium-high levels of substances were assigned as follows: CDLC at 0.5, 0.75 and 1 % (v/v); ST at 3, 7 and 11 g/L; and YE at 3, 7 and 11 g/L. Microfiltration and ultrafiltration of ST and YE fractions were done with 10 and 3 kDa molecular weight cut-off membranes. Fraction #1 (crude ST or YE), fraction #2 ($\leq$ 10 kDa ST or YE), and fraction #3 ($\leq$ 3 kDa ST or YE) were formulated for OSF9-SFM. Sf9 cells were transfected with plasmid pEGFP-N1 encoding recombinant green fluorescent protein (GFP) and infected with recombinant-baculoviruses (r-baculoviruses) generated by recombinant-Bacmid (r-Bacmid) containing anti-hepatitis B virus surface antigen (anti-HBsAg). The effect of OSF9-SFM on recombinant protein production in Sf9 cells was determined using immunofluorescence assay (IFA).

The optimum concentrations of CDLC, YE, and ST in OSF9-SFM were 0.5% (v/v), 11 g/L and 3 g/L, respectively, in which 10-kDa ultrafiltration fractions of YE and ST were used. The maximum cell density obtained from adherent culture was 7.17 x 10^5 cells/mL, whereas cell numbers from suspension culture reached 2.17 x 10^6 cells/mL with a specific growth rate of 0.027 h^{-1}. The average GFP cells in the medium

supplemented with serum peaked at 34 cells/well, while SF900 II and OSF9-SFM fluorescence was only 13 and 2 cells/well, respectively. Recombinant-Bacmid was unable to transfect Sf9 cells in OSF9-SFM, possibly due to the hydrolysate present in the media inhibiting transfection of r-Bacmid. However, Sf9 cells grown in OSF9-SFM could be effectively infected with passage 2 of r-baculoviruses derived from SF900 II culture, and an expression of anti-HBsAg fluorescence signals was clearly detected by IFA. The development of in-house OSF9-SFM will continue to increase both cell numbers and protein expression in the next step.

Keywords: chemically defined lipid concentrate, serum-free medium, *Spodoptera frugiperda* (Sf9), soytone, yeast extract

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Sureewan Saisud

Table of Contents

Page

Table of Contents (Continued)

Page

List of Tables

List of Figures

List of Figures (Continued)

List of Figures (Continued)

List of Abbreviations

CDLC	Chemically defined lipid concentrates
FBS	Fetal bovine serum
GFP	Green fluorescent protein
IFA	Immunofluorescence assay
OSF9-SFM	The developed medium for Sf9 insect cells (Optimized Sf9-Serum Free Media)
SC	Grace's insect cell culture medium contained 10% FBS
ST	Soytone
YE	Yeast extract

CHAPTER 1
INTRODUCTION

1.1 Background and Problem Description

Serum is a key raw material for cell culture in research laboratories and industry. Serum-containing media creates an artificial environment for cell growth. Additionally, serum is a source of energy and nutrients necessary for cell growth making cells able to grow well. There are many types of serum used in anima cell culture such as calf serum, fetal calf, adult horse, and human serum. At the same time, serum has several disadvantages, such as expensive prices, ethical concerns, risk of mycoplasma contamination and variations in FBS can affect the reproducibility of experiments [1].

However, over the past 15 years, serum-free medium has been increasingly used. Serum-free medium has an appropriate nutritional and hormonal composition that allows for cell culture without using animal serum. Serum-free medium has several advantages such as easier downstream protein purification, better control over physiological culture conditions, more consistent performance, reduced cost of purchasing serum and reduced the impact of the disadvantages of serum [2].

Therefore, this research project focuses on the development of serum-free medium for *Spodoptera frugiperda* (Sf9) insect cell cultivation and recombinant protein production for Sf9 cell growth based on the DoE method. The fractions obtained from the microfiltration and ultrafiltration of two peptones, yeast extract and soytone, on Sf9 cell growth were investigated. This medium was also used for transfection of Sf9 cells with recombinant green fluorescent protein (GFP) and anti-hepatitis B virus surface antigen (anti-HBsAg) to examine the effect of medium on protein production.

1.2 Purpose of the Study

The three objectives of this thesis are as follows:

1.2.1 To develop serum-free medium for *Spodoptera frugiperda* (Sf9) insect cell cultivation.

1.2.2 To study the effect of the combination of microfiltration and ultrafiltration of soytone and yeast extract on the growth of Sf9 cells.

1.2.3 To examine recombinant protein production in Sf9 insect cells using a serum-free medium.

1.3 Research Questions and Hypothesis

Currently, large amounts of serum and products from animals and humans are imported for use in insect cell cultures for research and development in medical science, which requires large investments. The researcher, therefore, has an idea to reduce the import of serum and products obtained from animals and humans. Therefore, a study was conducted to find suitable conditions for Sf9 insect cell culture, with the following factors: CDLC, yeast extract, and soytone being studied to replace serum in the culture medium. The hypothesis of this research can be divided into three parts. The first part is the development of a serum-free medium (SFM) for culturing Sf9 cells. The second part is to investigate the effect of the combination of microfiltration and ultrafiltration fractions on Sf9 cells growth. Third, serum-free medium (SFM) is used to examine recombinant protein production in Sf9 cells.

1.4 Theoretical Perspective

In this thesis we apply several basic theories related to the research of insect Sf9 cell culture media and recombinant protein production. The related theories include:

1.4.1 Serum-free medium development.

1.4.2 Recombinant protein production.

1.5 Delimitations and Limitations of the Study

The limitations of this thesis study are:

1.5.1 Development of serum-free medium for Sf9 insect cell culture by varying a concentration of three factors; CDLC, yeast extract, and soytone using DoE method.

1.5.2 Study the effect of combination between microfiltration and ultrafiltration at 10 kDa and 3 kDa fractions of yeast extract and soytone in serum-free medium on Sf9 growth.

1.5.3 Production of recombinant green fluorescent protein (GFP) and anti-hepatitis B virus surface antigen (anti-HBsAg) in Sf9 cells using developed serum-free medium.

1.6 Significance of the Study

The significance of this research are;

1.6.1 The optimum concentration of CDLC, yeast extract, and soytone in the developed serum-free medium for *Spodoptera frugiperda* (Sf9) insect cell cultivation will be known.

1.6.2 The effect of microfiltration and ultrafiltration combination of soytone and yeast extract on Sf9 growth will be known.

1.6.3 The effect of developed serum-free medium for *Spodoptera frugiperda* (Sf9) insect cell on recombinant protein production will be known.

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 Serum-free media overview

Serum-free media (SFM) are culture media used in cell culture and biotechnology applications that do not contain serum, specifically fetal bovine serum (FBS) or other animal-derived sera. Traditional cell culture often involves the use of FBS because it provides hormones, various types of nutrients, growth factors, and other elements that support growth and propagation of many cell types. However, serum-free media are formulated to provide these necessary nutrients and factors without relying on serum from animals. The serum-free development challenge is finding serum substitutes that provide support for cell growth. Some cells are sensitive to the nutrients used for their growth. Therefore, a variety of serum-free formulas must be designed to suit the growth of every cell. For this reason, a variety of serum-free formulas have been developed.

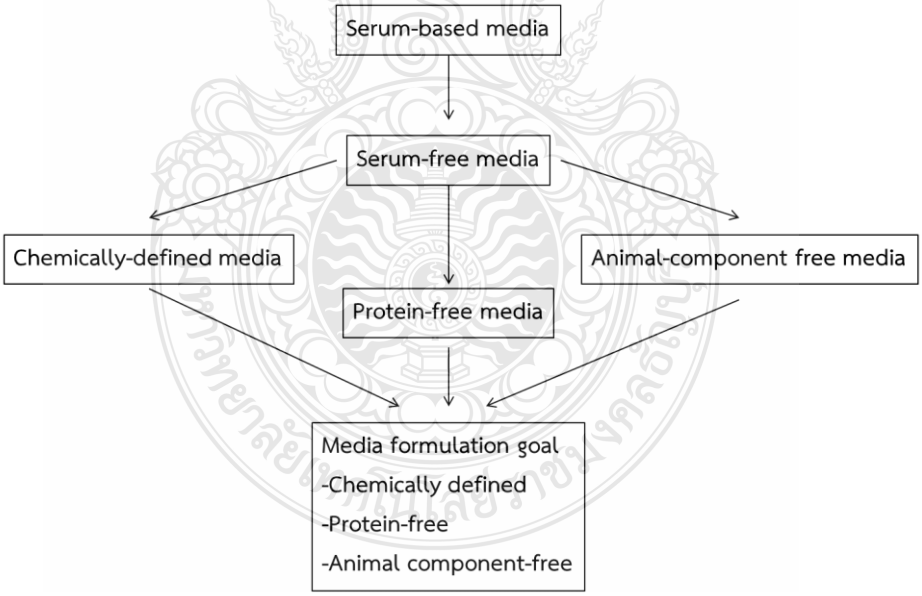

Figure 2.1 Evolution of serum-free media [3]

The evolution of serum-free media formulation is shown in Figure 2.1. Various components supplemented into serum-free media such as chemically defined media are known through distinct molecular characteristics and may consist of proteins, Protein-free media does not comprise of large proteins and cannot define their exact chemical compositions, and animal-free media is media that does not contain ingredients derived from animal sources [3].

2.1.1 Factors influencing the development of cell culture media

Serum-free media for Sf9 has been continuously developed and is commercially available to avoid using serum and animal-derived components. It is critical to develop formulas that require the addition of other substances to the basal medium. The new formulas developed must support cell survival and growth of the cell. The factors that influenced to the development of new serum-free media included ;

1. Hormones - Some cell types require hormones like insulin, glucocorticoids (dexamethasone and hydrocortisone), and triiodothyronine (T3) for proper growth and function. These can be added to serum-free media as needed.

2. Growth factors - It was added to the basal medium to stimulate activity and increase cells.

3. Protease inhibitors - FBS, α_1-antitrypsin and α_2-macroglobulin were added to the media to inhibit trypsinization and to inhibit lysosomal peptidase that may be released occasionally during cell turnover.

4. Protein hydrolysates - It is not required for cell culture. This is because protein hydrolysates are unable to make cells grow and a high concentration of hydrolysate will cause the cell growth rate to decrease and has an impact on the repeatability of the experiment.

5. Shear force protectors - Shear force protectors are not required for normal cell culture, but they are necessary for growing cells at the reactor level. This is because it will help reduce shear force to prevent cells from being damaged.

6. Proteins - Most proteins act as carriers for small molecules and help in cell adhesion to the culture surface.

7. Vitamins - Vitamins are obtained from basic media. It has been found to contain at least 7 types of vitamins that are essential for the growth and increase of cells,

including riboflavin, choline, thiamine, nicotinamide, pyridoxal, folic acid, and pantothenate.

8. Amino acids - In animal cell culture, there are 13 indispensable amino acids necessary, namely Cys, Val, Arg, Phe, Ile, Tyr His, Lys, Gln, Trp, Met, Leu, and Thr, while Ser, Glu, Asn, Pro, Asp, Gly, and Ala are not required for cell culture.

9. Glutamine - It is an important reactant for protein biosynthesis and ribonucleotides. However, unstable glutamine breakdown results in ammonia buildup, which is toxic to the cells due to it is not absorbed, serum-free media has been developed to use other nutrients instead of glutamine in the culture medium, such as using glutamate instead of glutamine in cell culture.

10. Lipid - Lipids act as energy stores and components of cell membranes. This is necessary for serum-free media.

11. Trace elements - These trace elements are normally included in basic cell culture media or specialized formulations. This depends on the specific requirements of the cells being cultured. The concentration of these elements must be carefully controlled to ensure proper cell growth and function. At the same time, toxicity is avoided. Researchers often tailor the elemental composition of cell culture media according to the needs of the experiment and the characteristics of the desired cell lines.

12. Antibiotics - Antibiotics are commonly used in cell culture to prevent bacterial and fungal contamination, which can compromise the health and integrity of cultured cells. When working with cell cultures, antibiotics are typically added to the growth medium to create a sterile environment and protect the cells from potential microbial contaminants.

13. Attachment factors - Attachment factors in cell culture refer to substrates or surface coatings used to promote the attachment and adhesion of cultured cells to the culture vessel. Cells require a suitable surface to adhere to, as this is essential for their growth and proliferation.

14. Osmolality - The optimal osmolality for animal cell cultures is between 270-330 mO/L. In addition, when developing new formulas for serum-free media, their osmolality should be regularly monitored [4].

2.1.2 Biological products from serum free media

Due to the increasing demand for biological products nowadays. Biological products such as cytokines, antibodies, clotting factors, interferons, hormones, vaccines, which are mainly used in humans for treatment and prevention. Therefore, it has been continuously developed. Biological products produced in animal cells are biological because they are similar in specificity to their natural structure.

Animal cells undergo a process called post-translational modification (such as glycosylation). This process is found only in higher animal cells. While lower animal cells or other microorganisms cannot do this. Animal cell culture combined with genetic engineering or recombinant DNA technology is now widely accepted. Animal cell culture has gradually changed from serum supplementation to no serum because of the safety and control of the final product. This is because the serum contains dangerous contaminants such as mycoplasma, fungi, prions, viruses, and bacteria that cause BSE (mad cow disease) or mad cow spongiform encephalopathy. The use of serum in animal cell cultures has more disadvantages. In addition, media containing 10% serum may have a serum protein concentration of at least 4-5 g/L, which causes problems. Serious in the protein purification process to avoid using serum and animal-derived components in cell culture media. Therefore, at least 4 types of cell culture media have been developed and available in the market: 1) chemically defined media (CDM) 2) protein-free media (PFM) 3) animals - receive media free of components. (ADCFM) and 4) serum-free medium (SFM). The type of medium required depends primarily on the cells being manipulated and the complications of cell physiology, but most commercially available media are quite expensive. In addition, the content of the media is undisclosed and is owned by the licensee company. These serum-free media clearly contribute to their limited use in routine cell culture due to their high cost.

Moreover, if the desired protein is to be produced in large quantities, The cost of investing in serum-free media can lead to project failure. As a result, most manufacturers develop their own media to keep production costs down. Factors required for developing media for cell culture that do not contain serum or animal ingredients are shown in Figure 2.2. The starting point for developing most cell culture media is basic media, which contains

essential components such as vitamins, indicators pH (phenol red), glucose, amino acids, sodium bicarbonate. and inorganic salts [4]

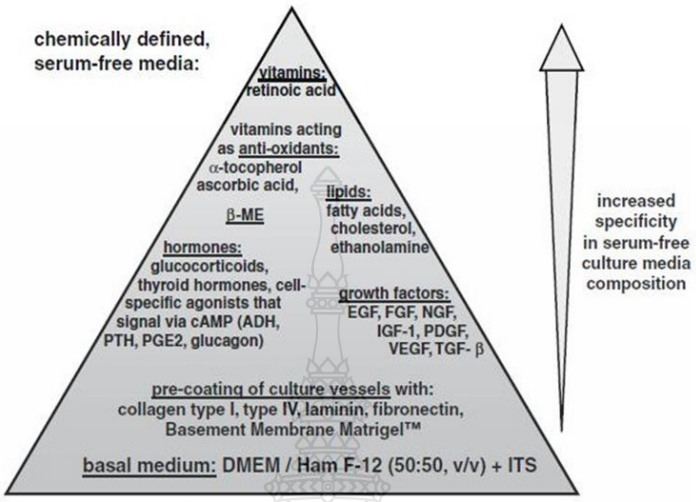

Figure 2.2 Method for developing cell culture media without serum or animal-derived components [4].

2.1.3 Advantage and disadvantage of serum free media

Serum-free media (SFM) offer several advantages in cell culture and research settings, which have contributed to their increasing popularity. Some of the key advantages of serum-free media include:

- Increased productivity.
- Does not contain cell growth inhibitors such as chalone, glucocorticoids, and transforming growth factor (TGF).
- Reduces batch-to-batch variation as compared to serum.
- There is little chance of contamination from bacteria, viruses, and mycoplasma.
- Decreased protein levels, this results in fewer problems in downstream processing [5].

Serum-free media (SFM) offer several advantages in cell culture and research settings, such as reducing batch-to-batch variability, minimizing the risk of contamination, and eliminating ethical concerns related to serum collection. However, there are also some disadvantages associated with serum-free media:

- Multiplicity of media – Advantage to labs maintaining specific cell types but difficult for maintenance.
- Selectivity – Cells have different media requirements.
- Reagent purity – Degree of purity in reagents, water and cleanliness of apparatus needs to be high.
- Increase of cells – Cells grow slowly in serum-free media.
- Availability - Serum-free media are more expensive than normal cell media and therefore serum-containing media is more commonly used.

Despite these disadvantages, serum-free media have become increasingly popular in research and biotechnology due to their benefits in terms of reproducibility, safety, and ethical considerations. Researchers often weigh these disadvantages against the advantages when deciding whether to use serum-free media in their experiments [6].

2.2 General information of Sf9

Spodoptera frugiperda, commonly known as the fall armyworm, is a pest that mainly eats crops, especially maize (corn), but can also attack other important crops like rice, millet, sugarcane, cotton, and other vegetables. The fall armyworm is native to the Americas but has spread to different parts of the world, causing significant agricultural damage [7]. Sf9 is an insect cell line, derived from a fall armyworm, a good host for baculovirus expression systems to generate recombinant proteins (Figure 2.3 and Table 2.1).

Figure 2.3 Morphology of Sf9

Table 2.1 Detail classification of *Spodoptera frugiperda* [7]

Scientific classification	
Domain:	Eukaryota
Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Lepidoptera
Superfamily:	Noctuoidea
Family:	Noctuidae
Genus:	*Spodoptera*
Species:	*S. frugiperda*

2.3 Baculovirus

Baculoviruses are a family of large, double-stranded DNA viruses (Figure 2.4) that infect insects, particularly members of the order Lepidoptera (moths and butterflies). These viruses have a complex and unique structure that allows them to effectively infect and replicate within their insect hosts. Baculoviruses are commonly used in biotechnology applications, such as the development of insecticides for pest control and the production of recombinant proteins due can produce large amounts of protein [8].

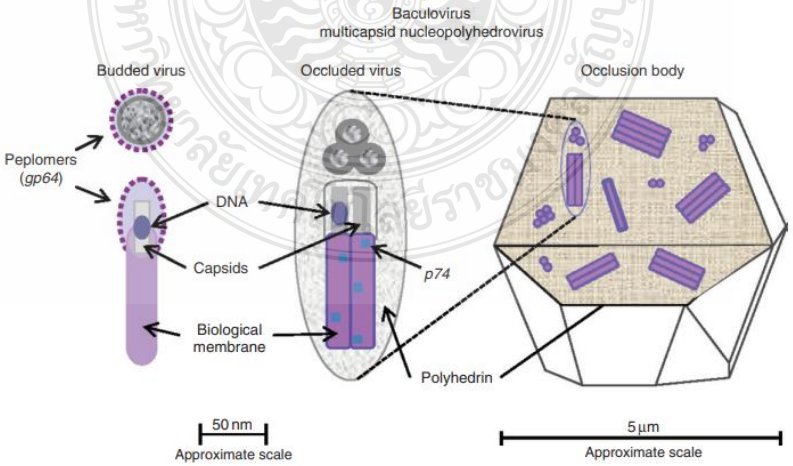

Figure 2.4 Diagram of virions of a nucleopolyhedrovirus [8].

2.4 Chemically Defined Lipid Concentrate (CDLC)

A Chemically Defined Lipid Concentrate (CDLC) is a concentrated lipid emulsion used in cell culture media to either replace or reduce fetal bovine serum. CDLC is a mixture of various types of lipids as shown in Table 2.2. CDLC is used to support the growth of cells, function of cells, and produce monoclonal antibodies by hybridomas and virus expression in insect cells [9].

Table 2.2 Components of CDLC [9].

Components	Molecular Weight	Concentration (mg/L)	mM
Other Components	304.74	2.0	0.006562972
Arachidonic Acid	386.65	220.0	0.56899005
Cholesterol	386.65	70.0	0.14807294
DL-alpha-Tocopherol Acetate	472.74	Confidential	N/A
thyl Alcohol 100%		10.0	0.035656977
Linoleic Acid	278.44	10.0	0.03591438
Linolenic Acid	228.38	10.0	0.04378667
Oleic Acid	282.47	10.0	0.03540199
Palmitic Acid	256.43	10.0	0.038997
Palmitoleic Acid	254.41	10.0	0.03930663
Pluronic F-68	7680.0	90000.0	11.71875
Stearic Acid	284.48	10.0	0.035151854
Tween 80®	1310.0	2200.0	1.6793894

2.5 Soytone

Soytone is derived from digestion of soybean with high content of vitamins and carbohydrates and low endotoxin. Soytone is a nutrient source for bacterial growth and mammalian cell culture. Therefore, it can be used in the fermentation process, cell culture media, vaccines, antibody production, and BioPharma processes [10]. Currently, peptone is being developed to be used as a substitute for fetal bovine serum for insect cell culture. This is due to concerns about infectious substances that may be present in animal peptones. Therefore, non-animal peptones have begun to be developed as another option for replacing fetal bovine serum for cell culture [11].

2.6 Yeast extract

Yeast extract is an important component of many media including serum-free media because it contains amino acids, vitamins, minerals, nucleotides, and other important substances which are essential nutrients and affects the growth of various microorganisms, including bacteria, yeast, fungi, and animal cells. Yeast extract should be ultrafiltrate through a 10 kDa membrane cut-off to remove large molecules that may have proteolytic activities or infest the purification process of the recombinant protein production [12].

2.7 Fractionation composition by microfiltration and ultrafiltration technique

Microfiltration and ultrafiltration are membrane fractionation techniques that use semipermeable membranes with different pore sizes to separate components from fluids based on size and molecular weight. Microfiltration is suitable for the removal of larger particles and solids, while ultrafiltration is used for the separation and concentration of macromolecules and solutes.

Recovery of the compound of interest requires at least one fractionation step. This typically used UF followed by a concentration or purification step. As shown in Figure 2.5, UF is a process driven by pressure used to separate protein solutions using molecular weight cutoff (MWCO) which is suitable for the desired protein size range. Downstream processing of protein hydrolysate by appropriately designed UF cascades and recycling loops can possibly improve peptide purity and their biological activity.

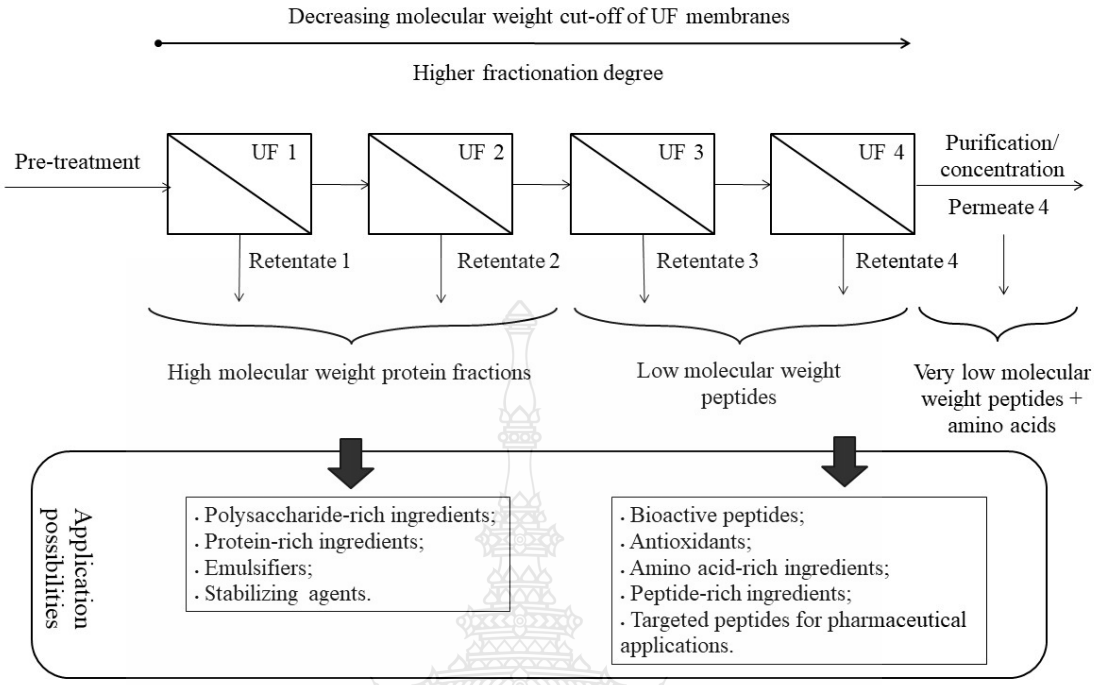

Figure 2.5 Ultrafiltration (UF) is the filtration of mixtures containing various proteins and peptides using a membrane molecular weight cut-off of 500–1 kg mol-1 [13].

Figure 2.6 shows that certain membrane technologies can be used to recover rich-protein fractions and peptides from yeast protein hydrolysates. Yeast protein hydrolysate is a very complicated matrix after interruption and enzyme hydrolysis. Most high molecular weight compounds that may interfere with protein and peptide separation efficiency include cellular debris, non-hydrolyzed proteins and other non-protein components are generally removed by operation of the stabilization unit or pre-treatment [13].

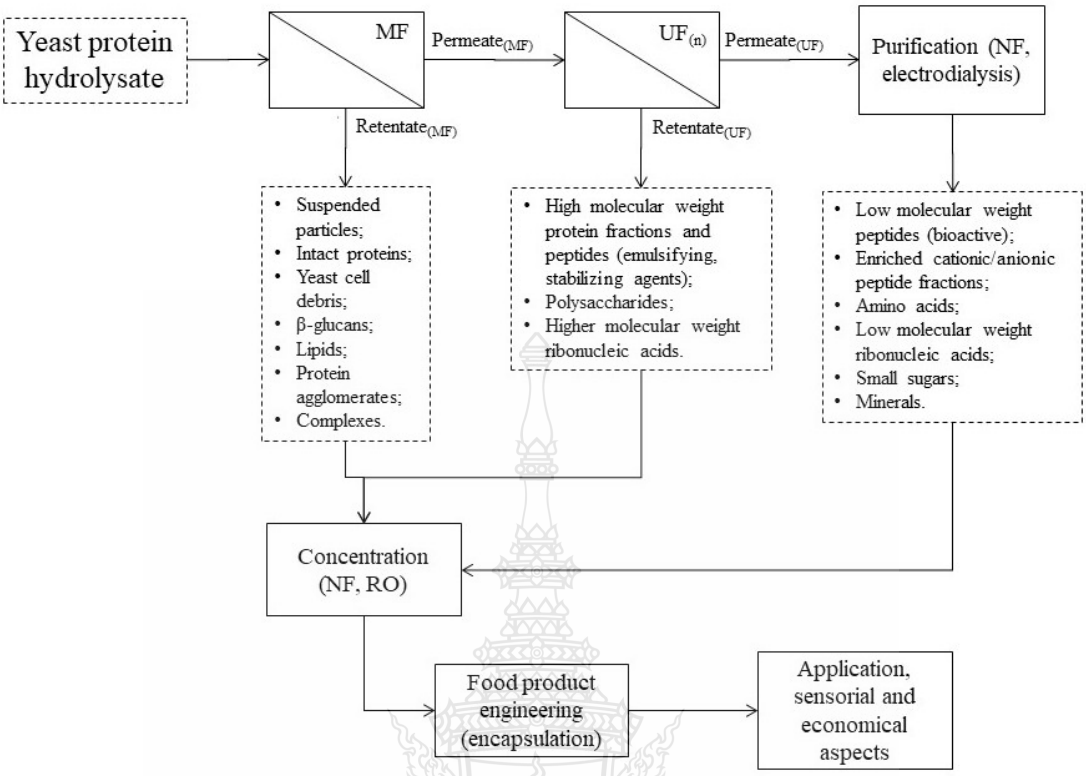

Figure 2.6 Membrane separation technologies was used to recover rich-protein bioactive ingredients and peptides from yeast protein hydrolysate Including the main compounds recovered in fractions [13].

2.8 Hepatitis B Virus

2.8.1 Structure of Hepatitis B virus

Hepatitis B virus is a member of the Hepadnavirus family. The virus is structured as a Dane particle, comprising an outer lipid envelope and an icosahedral nucleocapsid core primarily composed of protein. Inside the nucleocapsid, viral DNA and a DNA polymerase with reverse transcriptase activity are found, as illustrated in Figure 2.7. The outer envelope incorporates proteins that aid in the virus's attachment to and entry into susceptible cells. Notably, the Hepatitis B virus is one of the smallest enveloped animal viruses, with virions measuring 42 nm in diameter. These particles do not cause infection and consist of lipid and protein components present on the virion's surface, known as the surface antigen (HBsAg), and are overproduced during the virus's life cycle [14].

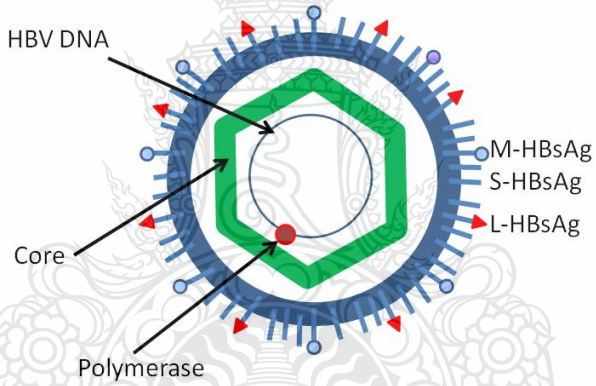

Figure 2.7 The hepatitis B virus structure [14].

2.8.2 Recombinant bacmid of Anti-HBsAg

A recombinant bacmid is a specific type of DNA construct used in the Baculovirus Expression Vector System (BEVS) for the produce of recombinant proteins, including the hepatitis B surface antigen (HBsAg) in this case. HBsAg is a key component of the hepatitis B vaccine. Bac-to-Bac® performs site-specific transposition in *E. coli* to create recombinant baculovirus instead of homologous recombination occurring in insect cells (Figure 2.8). Clone the desired gene into a pFastBac™ vector and transform it into DH10Bac™ competent *E. coli*. DH10Bac™ is a parental bacmid containing a *lac*Z-mini-*att*Tn7 fusion. Transposition occurs between the pFastBac™ vector element and the

parental bacmid with a helper plasmid-derived transport protein. The expression cassette disrupts the *lac*Z gene upon successful transposition. As a result, the newly expressed bacmid can be visualized as white bacterial colonies. High molecular weight DNA was isolated and the transfection reagent, Cellfectin®, was used to transfect Sf9 or Sf21 cells after two days. High titers of purified recombinant baculovirus can be isolated for amplification and expression. Using a virus stock, cells are infected and protein expression can be detected within 24-48 h [15].

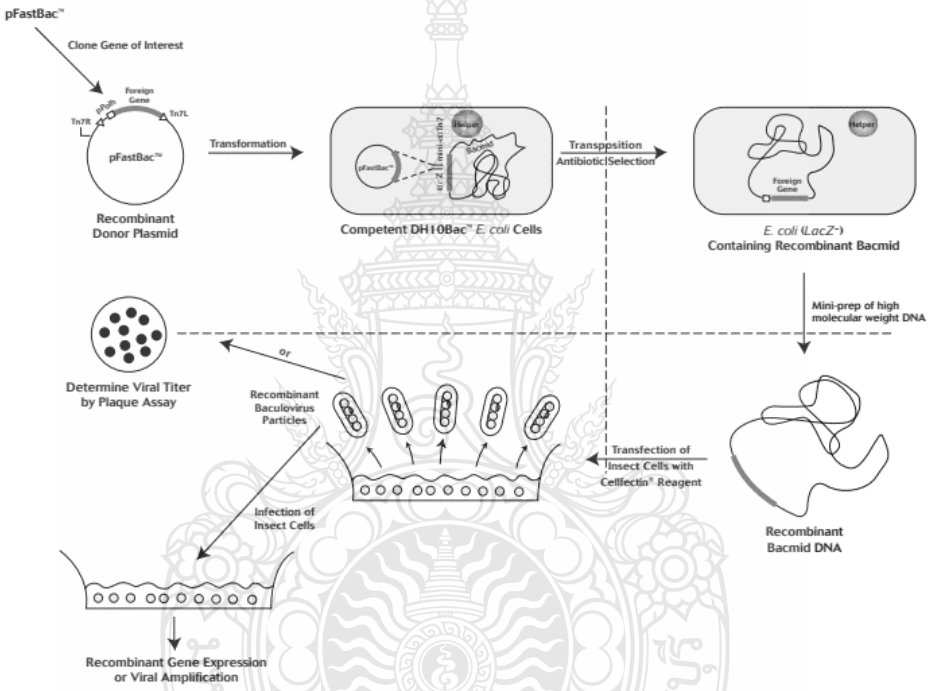

Figure 2.8 The Bac-to-Bac® Baculovirus Expression System for the creation of recombinant baculovirus and expression of the desired gene. [15].

2.9 Green fluorescence protein (GFP)

Green fluorescent protein (GFP) isolation and characterization from the jellyfish *Aequorea victoria*. Cloning and sequencing of the wild type gfp gene and integration into *Autographa californica* multi nucleopolyhedrovirus (AcMNPV) providing a system for compound expression. In a wide range of in vivo and in vitro studies, no substrate or exogenous cofactor are needed.

Recombinant baculovirus expression of the gfp gene can aid in determining the viral host range by more easily distinguishing whether infection is successful and cell line replication has occurred, especially in a semi-permitted cell line where cytopathological effects or occlusion body (OB) production may be difficult to detect (in the case of the gfp gene controlled by a promoter other than the polyhedrin promoter). Monitoring recombinant baculovirus gfp genes in other cells may provide additional information about specific cells' ability to express foreign proteins, which could aid operators in selecting appropriate cell strains [16].

Fang *et al*. (2007) transfected Sf9 cells with Bacmid's recombinant AcGCRVs8/-eGFP to generate recombinant baculovirus. GCRV VP6 and eGFP were observed under an inverse phase fluorescence microscope three days after transfection (Figure at 2.9 A, B). After 2 days, more than half of the cells fluoresced green (Figure 2.9 C, D). By infecting Sf9 cells and multiplying the regenerating virus, the clear P1 virus stock was used to generate a high-titer P2 bacterial stock. More than 80% of Sf9 cells fluoresced green under UV light 48-72 h after infection (Figure 2.9 F), which shows a high concentration of P2 recombinant bacteria (vAc-GCRV/eGFP). When a control empty bacmid was exposed, no green fluorescence was observed in Sf9 cells (Figure 2.9 E) [17].

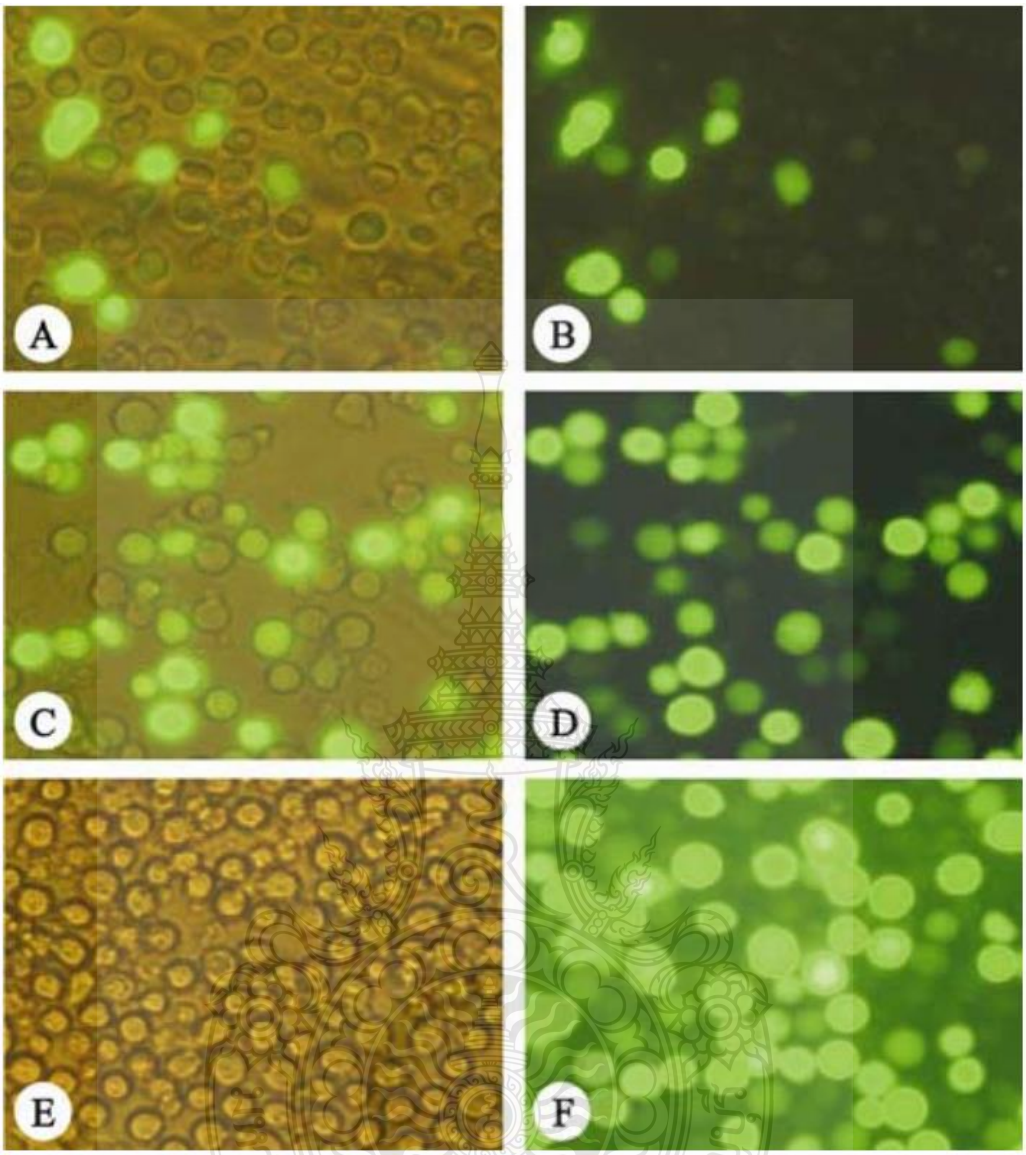

Figure 2.9 Recombinant vAcGCRVs8/GFP virus that is expressed in Sf9. A and C, transfected Sf9 cells were observed 3 and 5 days after transfection under visible light and UV light. B and D, consistent to A and C, visible only under UV light. E: bacmid cells that were transfected under normal light (Control). F: expanded P2 virus stock observed under UV light [17].

2.10 pEGFP-N1

pEGFP-N1 is a widely used plasmid vector commonly used in molecular biology and biotechnology research. It is part of the Enhanced Green Fluorescent Protein (EGFP) series of plasmids developed by Clontech (now part of Takara Bio). These vectors are designed for the expression of genes fused to the green fluorescent protein (GFP) or its variants, allowing for the visualization and tracking of protein expression and localization in living cells.

The pEGFP-N1 plasmid contains a modified version of wild-type GFP that is optimized for enhanced brightness and increased expression in mammalian cells. This variant has a maximum excitation wavelength of 488 nm and a maximum emission wavelength of 507 nm. Specifically, pEGFP-N1 encodes the GFPmut1 variant, which is characterized by the double-amino-acid substitutions of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene with more than 190 silent base changes, aligning with the preferred codon usage patterns in humans. This was done to enhance the efficiency of translation in eukaryotic cells by modifying the flanking EGFP sequences to the Kozak consensus translation initiation site. Within the pEGFP-N1 plasmid, the multiple cloning sites (MCS) are positioned between the CMV immediate early promoter (P CMV IE) and the EGFP coding sequences, as depicted in Figure 2.10. When genes are inserted into the MCS in the same reading frame as EGFP, with no intervening stop codons, they will be expressed as fused to the N-terminus of EGFP. Downstream of the EGFP gene, SV40 polyadenylation signals direct proper processing of the 3' end of the EGFP mRNA. In addition, the vector backbone incorporates an SV40 origin to enable replication within mammalian cells that express the SV40 T antigen. The SV40 early promoter, the Tn5 neomycin/kanamycin resistance gene, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene comprising a neomycin-resistance cassette (Neor) facilitates the selection of stably transfected eukaryotic cells using G418. In E. coli, a bacterial promoter located upstream of this cassette is responsible for expressing kanamycin resistance. The pEGFP-N1 vector backbone also includes a pUC replication origin for E. coli propagation and an f1 origin for single-stranded DNA production [18].

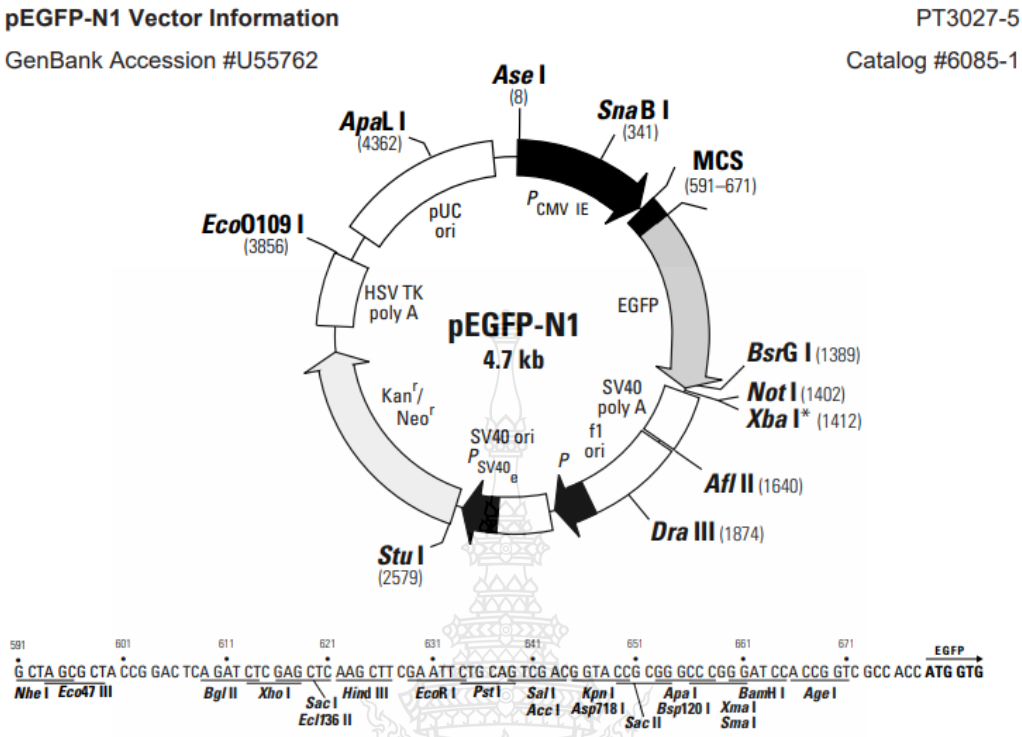

Figure 2.10 Map showing constraints and the multiple cloning sites (MCS) of the pEGFP-N1 vector [18].

2.11 Cellfectin®II Reagent

Cellfectin® II Reagent is a positively charged lipid formulation specifically engineered for effective transfection of insect cells. Cellfectin® II Reagent is an improved version of the original Cellfectin® Reagent. Cellfectin® II Reagent is produced to confident lot-to-lot consistency and has been optimized for application in a more rapid protocol. The BaculoDirect™ and InsectSelect™ Expression Systems, transfected with Cellfectin® II Reagent affect dependable and effective transfection of Sf9, Sf21, and High Five™ cells consistently. Additionally, Cellfectin® II Reagent can serve as a transfection tool for both adherent and suspended mammalian cells, whether they are cultivated in serum-containing or serum-free growth media.

2.11.1 Crucial Recommendations for Transfection

- Cellfectin® II is a lipid-based suspension that has the potential to settle over time. Before using, thoroughly mix by inverting the tube 5 - 10 times.
- When using Cellfectin® II, the time required to form a DNA: lipid complex is 15 - 30 minutes.
- To achieve the best results, it is essential to execute transfection following the correct protocol tailored to the specific system and cell type in use.
- Antibiotics should not be added to the media during transfection because they cause cell death.
- Serum-free medium is required for the formation of transfection complexes.
- Certain serum-free formulations could potentially impede cationic lipid-assisted transfection. Therefore, it is advisable to assess the compatibility of serum-free media with Cellfectin® II reagent [19].

2.11.2 Transfection methods by using Cellfectin®II reagent

Lipotransfection with Cellfectin® II Reagent is a specialized technique for delivering exogenous nucleic acids, including DNA or RNA, into eukaryotic cells. Cellfectin® II Reagent is a commercially available lipid-based transfection reagent developed by Thermo Fisher Scientific. This reagent is known for its high transfection efficiency and is widely used in molecular biology and cell biology research. Two doses of DNA tagging (0.3 µg and 0.03 µg of DNA) were used for transfection and flow cytometry was used to analyze the transfection efficiencies at 72 h post transfection. The high DNA tagging reached the significant transfection efficiencies than lower DNA tagging (Figure 2.11) [20].

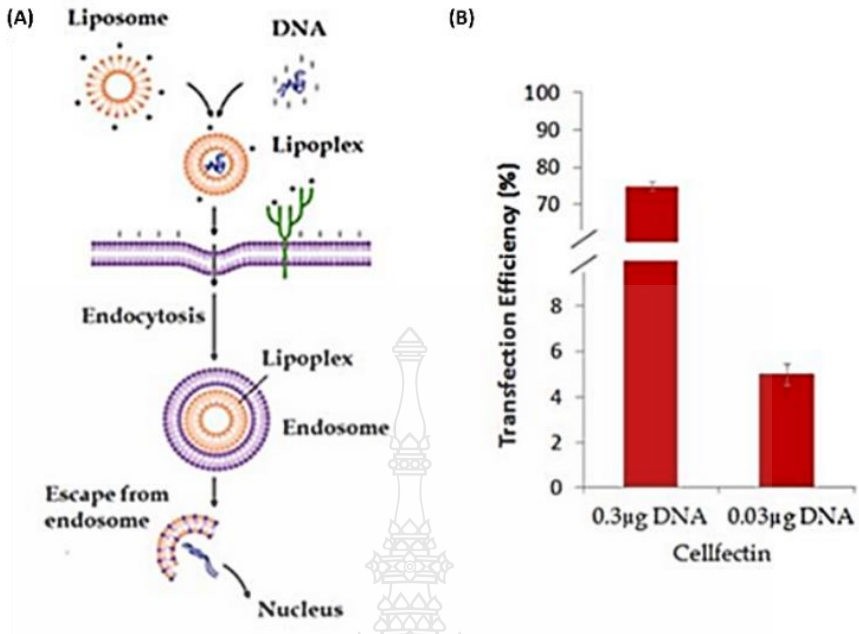

Figure 2.11 (A) Lipotransfection method schematic; (B) Transfection efficiencies obtained from each amount of DNA concentration used. The error bars represent standard deviation (n=3) [20].

2.12 Minitab statistical software

Minitab Statistical Software® is a package of software for statistical analysis that is easy to use, convenient, and fast. Especially the quality improvement work using statistical principles, such as SPC (Statistical Process Control), which is popularly applied to both the manufacturing industry and service work, experimental research work (Design of Experiment-DOE), or organizations that carry out quality programs such as six sigma or operation excellence, including standard work for the automotive industry using AIAG Core Tools, SPC and MSA volumes, or food industry group medicines and medical devices that follow the FDA (Food and Drug Administration).

Some of the statistical analyses and procedures that Minitab offers include descriptive statistics, regression analysis, experimental design, hypothesis testing, time series analysis, statistical process control, reliability analysis, quality tools, multivariate analysis, survival analysis, power and sample size calculations, nonparametric tests, statistical reporting, and graphical visualization [21].

2.13 Review of the literature

Lui *et al*. (2018) according to the study conducted by the assessed the five supplements through a two-level fractional factorial experiment, utilizing SDM medium as the reference or baseline medium. After 72 h of culture, cell concentrations and antibody production results revealed that ferric citrate and trace elements could improve 10% at a significant level of cell growth. Antibody formation can be significantly increased by peptone, glutathione, and ferric citrate (*p*-value = 0.05) [22].

Butler *et al.* (2000) studied vero cell expansion in DMEM supplemented plus 10% FBS and M-VSFM (Formula without serum) in microcarrier culture. The cells were cultivated in 100 mL spinner flasks that were warmed at 37 °C under 10 % CO_2. A 2.0 g/L concentration of cytodex-1 microcarriers was added with the initial concentration of vero cell at 2.0 x 10^5 cells/mL. M-VSFM demonstrated its capacity to sustain vero cell development. After being infused into M-VSFM for 4 days, vero cells attained their highest density of 1.0 x 10^6 cells/mL. There was no indication of cell aggregation, and the cell-surface attachment persisted throughout the cultures [23].

Posung *et al*. (2016) studied six serum substitutes, namely Bacto™ soytone, Bacto™ yeast extract, vitamin C, vitamin B12, SITE liquid media supplement, and recombinant human epidermal growth factor to develop a customized serum-free medium denoted as SFM01-M, tailored for Vero cell cultures. So, a 2^{6-2} fractional factorial design was employed, with Eagle's minimal essential medium (MEM) as the foundational medium. The study's findings revealed that the optimum concentrations for vitamin C, vitamin B12, and recombinant human epidermal growth factor were 9.72 mg/L, 0.173 mg/L, and 0.058 mg/L, respectively [24].

Rourou *et al*. (2017) studied 10 factors (Hypep 1510, Hypep 4601, Hypep 4605, Hypep 5603, Hypep 7401, Basal medium, Ethanolamine, Insulin, and Transferrin) to develop an animal-derived component-free medium (IPT-AFM) that helps Vero cell growth. The result showed that the optimal formulations of medium experiment no. 10 (Medium 10) were Hypep 1510 at 5 g/L, Hypeps 4601 at 2 g/L, and Hypep 4605 at 2 g/L. The basal medium M199 plus medium 10 could target cell yield achievement (8 x 10^6 cells/T-25) [25].

Batista *et al.* (2008) studied the development of an animal protein-free medium intended for *Drosophila melanogaster* S2 cells (S2AcGPV2), which were genetically engineered to produce the rabies virus G glycoprotein. The result showed that S2AcGPV2 cells displayed full growth capability when cultured in a serum-free supplemented IPL-41 medium. This medium consisted of 6 g/L yeastolate ultrafiltrate, 10 g/L glucose, 3.5 g/L glutamine, 0.5 g/L fructose, 2 g/L lactose, 0.6 g/L tyrosine, 1.48 g/L methionine, and 1% (v/v) lipid emulsion, resulting in a cell density of 19 x 10^6 cells/mL. The maximum specific growth rate was measured at 0.025 h^{-1}, and the cell productivity reached 0.57 x 10^5 cells/mL h^{-1} [26].

Cleuziat *et al*. (2018) studied the formulation of an animal-component-free insect medium using the rational culture media design^{TM} approach for Sf9 cells. In the investigation, a range of yeast hydrolysate sources were screened with the aid of multifactor design of experiment software. This screening encompassed various nutrient categories, such as amino acids, vitamins, and metals. The findings revealed that the group of metal components had the most significant impact on both cell growth and productivity. Subsequently, the concentrations of these metal components were further fine-tuned. As a result, the developed animal-component-free insect medium exhibited enhanced growth performance and productivity that could be favorably compared to a widely utilized commercially available animal-derived medium [27].

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals, Apparatus and Equipments

		Company / Brand
3.1.1	Analytical Balance	Mettler Teledo, Switzerland
3.1.2	Autoclave	Hirayama, Japan
3.1.3	Beaker	Schott Duran, Germany
3.1.4	Biochemistry Analyzer	YSI 2900, Xylem, USA
3.1.5	Biosafety cabinet class II	Nuaire, USA
3.1.6	Cell Culture Flasks	Thermo Fisher Scientific, China
3.1.7	Centrifuge	Hettich, Germany
3.1.8	Automated cell counter	Countess™ II FL, Invitrogen by Thermo Fisher Scientific, USA
3.1.9	Osmometer	OSMOMAT® 030, Gonotec, Germany
3.1.10	Duran	Schott Duran, Germany
3.1.11	Hemacytometer	Boeco, Germany
3.1.12	Incubator	Innova® 42, Eppendorf, Germany
3.1.13	Inverted microscope	Nikon, Japan
3.1.14	Micro pipette	Eppendorf, Germany
3.1.15	Microtube	Axygen, China
3.1.16	Scraper	SPL Life Sciences, Korea
3.1.17	NanoDrop Spectrophotometer	Thermo Scientific, USA
3.1.18	Spinner flask	Techne, UK
3.1.19	Water bath	Yamato, Japan
3.1.20	6-well plate	Thermo Fisher Scientific, China
3.1.21	96-well plate	Thermo Fisher Scientific, China
3.1.22	Ultrafiltration	Sartorius, Germany

		Company / Brand
3.1.23	Microfiltration	Sartorius, Germany
3.1.24	Chemically defined lipid concentrate (CDLC)	Gibco, USA
3.1.25	Cellfectin^{TM} II Reagent	Gibco, Canada
3.1.26	D-glucose	Sigma, USA
3.1.27	Fetal bovine serum	Gibco, UK
3.1.28	Fluorescence microscope	Nikon, Japan
3.1.29	Grace's insect cell culture medium (powder)	Gibco, USA
3.1.30	Sf-900^{TM} II SFM (1X)	Gibco, USA
3.1.31	L-glutamine	Gibco, Brazil
3.1.32	MEM vitamin solution	Gibco, USA
3.1.33	Methyl cellulose	Sigma, USA
3.1.34	Sodium hydrogen carbonate	Merck, Germany
3.1.35	Soytone	BD, USA
3.1.36	Trypan blue solution 0.4%	Invitrogen by Thermo Fisher Scientific, USA
3.1.37	Vitamin B12	Sigma, USA
3.1.38	Lactalbumin	Himedia, India
3.1.39	Yeast extract	BD, USA
3.1.40	Rabbit Anti-Mouse IgG (H+L)-FITC	SouthernBiotech, USA

3.2 Insect cell line and recombinant protein plasmid

3.2.1 Recombinant-Bacmid (r-Bacmid) containing anti-hepatitis B virus surface antigen (anti-HBsAg)

3.2.2 pEGFP-N1 encoding recombinant green fluorescent protein (GFP)

3.2.3 *Spodoptera frugiperda*

3.3 Development of Serum-Free medium (OSF9-SFM) for Sf9 insect cell culture

3.3.1 According to the study of relevant documents, three factors, chemically defined lipid concentrate (CDLC), yeast extract (YE), and soytone (ST) [28], [29], [30], [31], [12], [26], [23], were studied by Central Composite Design. Grace's insect cell culture media plus 9 g/L D-Glucose [32], 6 mM L-Glutamine [28], [23], 0.15 mg/L Vitamin B12 [33], [34], and 0.01% Methylcellulose [28] were used as the basal medium. The designed experiment was shown in Table 3.1 using the Minitab® 16.2.1 program.

Table 3.1 Central Composite design for development of OSF9-SFM

Std Order	Pt Type	Block	YE (g/L)	ST (g/L)	CDLC (% (v/v)
1	1	1	3	3	0.5
2	1	1	11	3	0.5
3	1	1	3	11	0.5
4	1	1	11	11	0.5
5	1	1	3	3	1
6	1	1	11	3	1
7	1	1	3	11	1
8	1	1	11	11	1
9	-1	1	0.27	7	0.75
10	-1	1	13.7	7	0.75
11	-1	1	7	0.27	0.75
12	-1	1	7	13.7	0.75
13	-1	1	7	7	0.33
14	-1	1	7	7	1.17
15	0	1	7	7	0.75
16	0	1	7	7	0.75
17	0	1	7	7	0.75

3.3.2 The cell destiny of Sf9 starter cells were 4-6 x 10^4 cells/cm^2 grown in T175 flasks containing 20 mL Grace's insect cell culture medium supplemented with 10% FBS (this medium was named "SC"), then incubate at 28 ^{o}C for 3-4 days. After that spent media was discarded and 20 mL of new SC was added, then the cells was scraped using cell scraper. Aggregated cells were resuspended several times using pipette to break up the aggregated cells. A small volume of cell samples was taken and then mixed with 0.4% trypan blue solution. The viable cells were counted using a hemacytometer under a microscope.

3.3.3 Initial cells obtained from 3.3.2 were scraped and washed with phosphate-buffered saline (Ca2+. Mg2+-PBS without Mg2, pH 7.4) three times by centrifugation at 110 x g for 10 min. Phosphate buffer saline was used to resuspended Sf9 cells making the cell concentration in the range of 4-7 x 10^6 cells/mL. For the experiment, the 6-well plate was used. The initial cell density was 4 x 10^5 cells/well containing 3 ml of test medium, incubated at 28 ^{o}C for 5 days, with three replicates. Take sample 1 mL, and then the cell sample was mixed with 0.4% trypan blue solution. The concentration of viable cells was counted by using a hemacytometer.

3.3.4 The growth curves of cells obtained from the 17-tested media were constructed by using the average value of triplicate experiments of each tested medium. The growth curves of cells during the exponential phase or log phase were selected and the probability test for each day was created and the *p*-value and Anderson-Darling (AD) values of their residues were selected. To estimate other factors involved in the data analysis, the estimated regression coefficients, and the analysis of variance (ANOVA) were also analyzed using the software. Contour/surface plot analysis and the response optimizer function for predicting the target (cell yield) were also performed. Statistical analysis of CDLC, YE, and ST were expressed as the mathematical model afterward.

3.3.5 The developed medium for Sf9 insect cells, now named "OSF9-SFM", was further subjected to the verification study to confirm the results of the study. For the experiment, the starter cells were grown as described in the section of 3.3.2 and prepared for the experiment as described in the section of 3.3.3. The cell density of 4 x 10^4 cells/cm^2 (4 x 10^5 cells/well) was placed in 1.5 mL microtube (3 tubes) and centrifuge at 110 x g for 10 min, then spent medium was discarded and washed again with

OSF9-SFM. The cell pellet was resuspended in 1 mL of new OSF9-SFM and then the cells were transferred to each well of the 6-well plate (3 replicates). The plate was incubated at 28 ºC for 4 days (as the same day of data selected for the analysis). After culturing for 4 days, the subculture was carried out by cell scraper and then pipetting up and down several times to dislodge cell clumps. A small volume of cell samples was taken and mixed with 0.4% trypan blue solution. The concentration of viable cells was counted as described in the previous section. To continue the next culture or passage, the cell density as mentioned above was placed in 3 of the 6-well plate, and the OSF9-SFM was, in each well, adjusted to 3 mL. The plate was incubated at the same condition as mentioned above. For the first culture, the cells obtained were named P0. Subculture was continuously performed to complete 5 Passages (P1 to P5). During the subculture, the cells number obtained from P0, P1, P2, P3, P4, and P5 were compared analysis by using Minitab®16.2.1.

3.3.6 In this study, the optimum cell density of Sf9 insect cells for adherent culture, using the initial cell densities of 2 x 10^5, 3 x 10^5, 4 x 10^5, and 5 x 10^5 cells/mL [34] containing 5 mL of OSF9-SFM incubate 28 ºC for 4 days, after that the cells were scraped and well resuspended before counting the viable cell yields. The maximum cell yield from each cell density was compared and selected for further experiments.

3.4 The combined effect of microfiltration and ultrafiltration of ST and YE on Sf9 cell growth

3.4.1 Prepare stock solutions of YE and ST with a concentration of 110 g/L in distilled water. Then filter YE and ST through 0.22 μm of microfiltration (labelled "Fraction #1 ST" and "Fraction #1 YE"). Fraction #1 was then filtered through ultrafiltration with a molecular weight cut-off of 10 kDa (labelled "Fraction #2 ST" and "Fraction #2 YE") and fraction #2 was then filtered through ultrafiltration with a molecular weight cut-off of 3 kDa (labelled "Fraction #3 ST" and "Fraction #3 YE"). The total protein concentrations of each filtrated fraction of YE and ST were measured using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientifc, USA). The final concentration of YE at 11 g/L and ST at 3 g/L were used for the combination study. Fractions #1, #2 and #3 were stored at 2-8 ºC until use. YE and ST were mixed in the OSF9-SFM as designated in Table 3.2.

3.4.2 Starting cells with a cell density of 4 x 10^4 cells/cm^2 were used in OSF9-SFM with a volume of 5 mL of each mixture incubated at 28 ºC for 4 days (three replicates). The concentration of viable cells was counted by using a hemacytometer.

Table 3.2 Design of an experiment examining the combined impact of different filtrated ST and YE

Run no.	Combination effect
1	Fraction #1ST + Fraction #1YE
2	Fraction #1ST + Fraction #3YE (3kDa)
3	Fraction #1ST + Fraction #2YE (10kDa)
4	Fraction #3ST (3kDa) + Fraction #1YE
5	Fraction #3ST (3kDa) + Fraction #3YE (3kDa)
6	Fraction #3ST (3kDa) + Fraction #2YE (10kDa)
7	Fraction #2ST (10kDa) + Fraction #1YE
8	Fraction #2ST (10kDa)+ Fraction #3YE (3kDa)
9	Fraction #2ST (10kDa)+ Fraction #2YE (10kDa)

3.5 Adaptation of Sf9 cells to grow in suspension

3.5.1 The initial viable cell concentrations at 4 x 10^4 cells/cm^2 were grown in T175 cm^2 tissue culture flasks containing culture medium 30 mL OSF9-SFM and incubated at 28 °C for 4 days.

3.5.2 A cell density of 3 x 10^5 cells/mL was inoculated into 250 mL spinner flask containing 50 mL of culture medium and stirred at 50 rpm at a temperature of 28 °C.

3.5.3 The subcultivation was conducted when the viable cells concentration reached 1 - 2 x 10^6 cells/mL by transferring the viable cell density of 3 x 10^5 cells/mL in a 50 mL fresh medium in spinner flasks. The cultivation was carried out for 5-7 days at

28 °C. The suspension culture was done for 2 batches. The viable cell number of each batch was counted and morphology of Sf9 cells was photographed.

3.6 Transfection of Sf9 cells with plasmid pEGFP-N1 of recombinant green fluorescent protein (GFP)

3.6.1 Sf9 cells were cultured in three mediums (Grace's insect medium plus 10% FBS, SF-900II and OSF9-SFM) using an initial cell density of 5 x 10^5 – 1 x 10^6 cells/mL in a T75 cm^2 tissue culture flasks at a temperature of 28 ^oC for 3 - 4 days and then, counted viable cells using a cell counter.

3.6.2 The cells were subculture in each medium for 3 to 5 passages. A portion of 100 µL of cell suspensions from each medium culture were seeded into a 96-well plate at a density of 2 x 10^4 cells/well.

3.6.3 Cellfectin^{TM} II reagents at 0.2, 1, and 2 µL were added separately and mixed into a microtube containing 10 µL of Grace's insect medium.

3.6.4 Prepared of plasmid pEGFP-N1 150 ng was separately added into a microtube containing 10 µL of Grace's insect medium, and mixed.

3.6.5 The plasmid pEGFP-N1 (3.6.4) was added to the diluted Cellfectin^{TM} II reagent tube (3.6.3) and incubated for 15 - 30 min at room temperature.

3.6.6 The mixture of 20 µL from 3.6.5 was added into each well, and incubated at a temperature of 28 ^oC for 72 h.

3.6.7 At 72 h post-transfection, the expression of a gene encoding a green fluorescent protein (GFP) was monitored daily under a fluorescence microscope and counted using a countess^{TM}II FL automated cell counter. The residual amount of glucose in each medium was measured using a biochemistry analyzer.

3.7 Transfection of Sf9 cells with recombinant Bacmid of Anti-HBsAg

3.7.1 Preparation of Sf9 cells

Sf9 insect cells were cultured with an initial cell concentration of 5 x 10^5 - 1 x 10^6 cells/mL in T75 cm^2 tissue culture flasks in three mediums (Grace's insect medium plus 10% FBS, Sf-900 II, and OSF9-SFM). The SF9 cells were incubated at a temperature of 28 °C for 3-4 days. The cells were scraped, stained with trypan blue, and counted using a

countess^{TM}II FL automated cell counter and cells were subcultured at least 3 to 5 passages before transfection.

3.7.2 Transfection of Sf9 cells methodology I

3.7.2.1 Sf9 cells were prepared in three mediums (Grace's insect medium plus 10% FBS, Sf-900 II, and OSF9-SFM) with an initial cell concentration of 8 x 10^5 cells/well in 6 well plates and incubated at room temperature for 15 min. In Grace's insect medium + 10% FBS, discarded the medium and added 2.5 mL of plating medium (medium without serum). While Sf-900 II and OSF9-SFM, did not discard the medium.

3.7.2.2 Prepared Cellfectin^{TM} II reagent 8 µL in each 100 µL of three mediums (Grace's insect medium, Sf-900 II, and OSF9-SFM) and mixed.

3.7.2.3 Prepared r-Bacmid (anti-HBsAg) 3 µg in each 100 µL of three mediums (Grace's insect medium, Sf-900 II, and OSF9-SFM) and mixed.

3.7.2.4 Cellfectin^{TM} II reagent (3.7.2.2) and r-Bacmid (anti-HBsAg) (3.7.2.3) were mixed and incubated at room temperature for 15 - 30 min.

3.7.2.5 The transfection mixture of 210 µL was dropped into each 6 well plates and incubated at 28 °C for 5 h.

3.7.2.6 In Grace's insect medium plus 10% FBS, discarded the transfection mixture and then added 4 mL of Grace's insect medium plus 10% FBS. In Sf-900 II and OSF9-SFM medium, did not discard the transfection mixture and added more 2 mL of each Sf-900 II and OSF9-SFM and incubated at 28 °C for 120 h.

3.7.2.7 Take samples 1 mL and centrifuged at 500 x g at 4 °C for 5 min and using cell for immunofluorescence (IFA) determination.

3.7.3 Determination of the expression of recombinant antibodies to HBsAg by Immunofluorescence (IFA) method [35]

3.7.3.1 The Sf9 insect cells obtained from step 3.7.2.7 were washed with 500 µL of 1xPBS and centrifuged at 500 x g for 5 min.

3.7.3.2 Discarded the supernatant and added 100 µL of 1xPBS, mixed and dropped samples 2 µL on the slide and left to dry.

3.7.3.3 The slides were washed with 40% methanol in acetone, incubated at -20 °C for 16-18 h and left to dry.

3.7.3.4 Rabbit anti-mouse IgG (H+L)-FITC was diluted at a 1:250 ratio in 1xPBS containing 0.1 % BSA and dropped on the slide, incubated at room temperature for 1 h.

3.7.3.5 Washed the slides three times with 1xPBS-T (0.05%) for 5 min and left to dry. Then, drop the oil on the slide.

3.7.3.6 The slides were examined for fluorescence under an inverted fluorescent microscope.

3.7.4 Transfection of Sf9 cells methodology II

3.7.4.1 Sf9 cells were prepared in two mediums (Sf-900 II and OSF9-SFM) with an initial cell concentration of 8 x 10^5 cells/well in 6 well plates and incubated at room temperature for 15 min.

3.7.4.2 Prepared Cellfectin™ II reagent 8 µL in each 100 µL of two mediums (Sf-900 II, and OSF9-SFM) and mixed.

3.7.4.3 Prepared r-Bacmid (anti-HBsAg) 3 µg and 5 µg in each 100 µL of two mediums (Sf-900 II, and OSF9-SFM) and mixed.

3.7.4.4 Cellfectin™ II reagent (3.7.4.2) and r-Bacmid (anti-HBsAg) (3.7.4.3) were mixed and incubated at room temperature for 15 - 30 min.

3.7.4.5 The transfection mixture of 210 µL was dropped into each 6 well plates and incubated at 28 °C for 5 h.

3.7.4.6 Added 2 mL of each Sf-900 II and OSF9-SFM and incubated at 28 °C for 4 days.

3.7.2.7 Take 1 mL of each sample and centrifuged at 500 x g at 4 °C for 5 min and collect the supernatant as a P1 viral stock. Keep at 4 °C until use.

3.7.2.8 Use P1 viral stock to transfect into Sf9 cultured in two mediums (Sf-900 II and OSF9-SFM) and incubated at 28 °C for 4 days.

3.7.2.8 Take 1 mL of each sample and centrifuged at 500 x g at 4 °C for 5 min and collect the supernatant as a P2 viral stock. Keep at 4 °C until use.

3.7.2.9 Use P2 viral stock to transfect into Sf9 cultured in OSF9-SFM and incubated at 28 °C for 4 days.

3.7.2.9 Take 1 mL sample and centrifuged at 500 x g at 4 °C for 5 min. Cells were used for immunofluorescence (IFA) according to method in 3.7.3. The flow chart of transfection method II in Sf9 was shown in Figure 3.1.

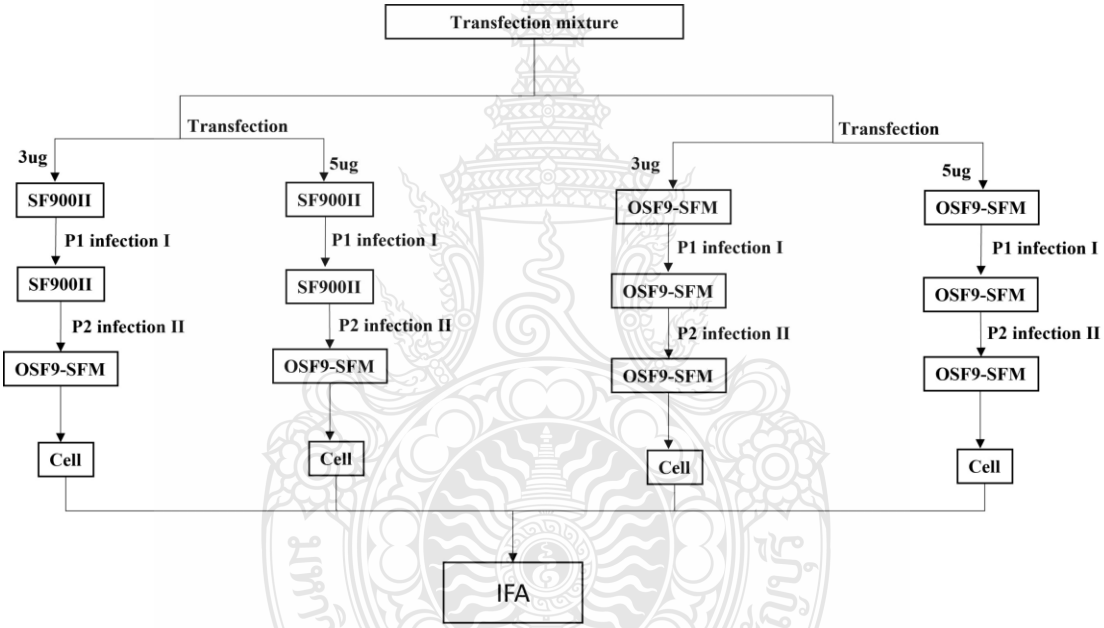

Figure 3.1 The flow chart of transfection method II

CHAPTER 4
RESULT AND DISCUSSION

4.1 Development of Serum-Free medium (OSF9-SFM) for Sf9 insect cell culture

4.1.1 Development of formulated serum-free medium for Sf9 insect cell culture

Optimal conditions for Sf9 cell growth were determined using a central composite design, which included a total of three factors (chemically determined lipid concentration (CDLC), yeast extract (YE), and soytone (ST)) from Table 3.1. It was found that the maximum cell yields were peak, 1.75 x 10^5 cells/mL on day 3^{rd}, in RUN9 compared to others RUN (Table 4.1). This indicated that Sf9 cell cultures in different media conditions affect the growth of Sf9. However, the average cell yields on the 4^{th} day of every RUN had better growth than the other days. This revealed that almost of the Sf9 cells in each RUN reached the maximum growth on the 4^{th} day (Figure 4.1). Therefore, the experimental data on the 4^{th} day was taken for statistical analysis in the next step.

Lui *et al*. (2018) studied the development of medium and the optimum conditions to produce antibodies in spinner flask. In this research, they used SDM based medium to evaluate the effect of five components, ferric citrate, ascorbic acid, peptone, glutathione, and trace elements, on cell yield and IgG production by using the two-level fractional factor experiments. After 72 h, ferric citrate and trace elements showed positive effect on cell growth at significant level (p-value < 0.10). For IgG production, ferric citrate and peptone had a strong positive effect on protein production at significant level ($p<0.05$) [36].

Table 4.1 Cell yield of Sf9 insect cells grown in OSF9-SFM development obtained using the Central Composite Design experiment.

Run No.	1st day	2nd day	3rd day	4th day	5th day
Run1	35000	70000	105000	70000	90000
Run2	15000	15000	65000	20000	15000
Run3	25000	50000	125000	155000	105000
Run4	60000	30000	50000	30000	30000
Run5	20000	30000	80000	80000	45000
Run6	15000	20000	20000	25000	47500
Run7	15000	65000	70000	130000	155000
Run8	15000	20000	20000	10000	5000
Run9	15000	10000	175000	135000	125000
Run10	5000	5000	5000	15000	5000
Run11	45000	40000	55000	30000	35000
Run12	5000	25000	10000	35000	25000
Run13	5000	20000	40000	105000	57500
Run14	10000	30000	30000	45000	60000
Run15	5000	10000	30000	55000	25000
Run16	0	10000	30000	50000	60000
Run17	0	10000	25000	25000	40000

Remark : unit in cells/mL

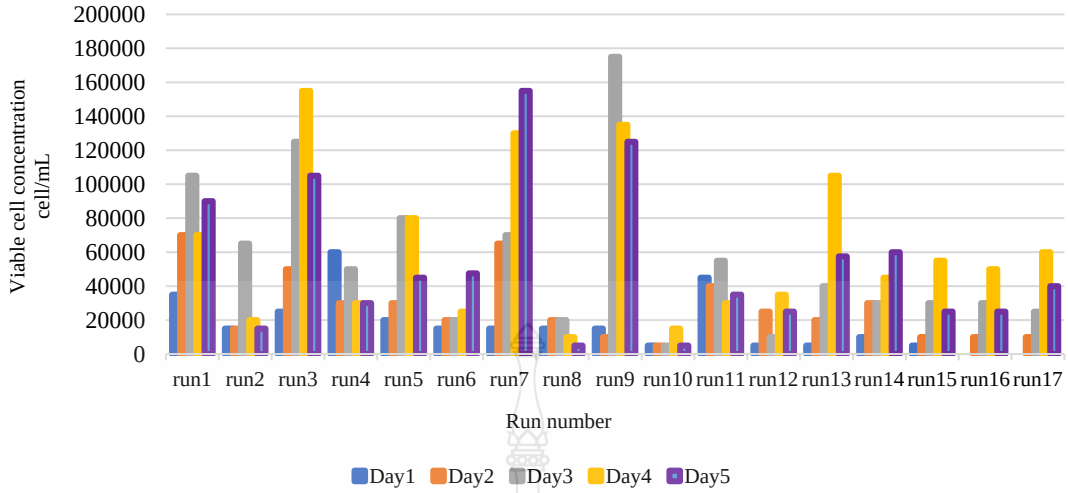

Figure 4.1 The experimental results of determining the optimum conditions for cell culture**.**

The data obtained from the experiment were plotted into the Minitab program to test the distribution of the data to see whether there is a normal distribution. If there is an abnormal distribution, it will affect further analysis. From the picture, it can be explained that the data has a normal distribution, most of them were in a straight line of 45 degrees. In addition, the *p*-value was 0.055, which was greater than 0.050 using the Anderson - Darling normal distribution method, indicating that the distribution of this data was fragmented normal. Therefore, it was suitable data for further analysis (Figure 4.2).

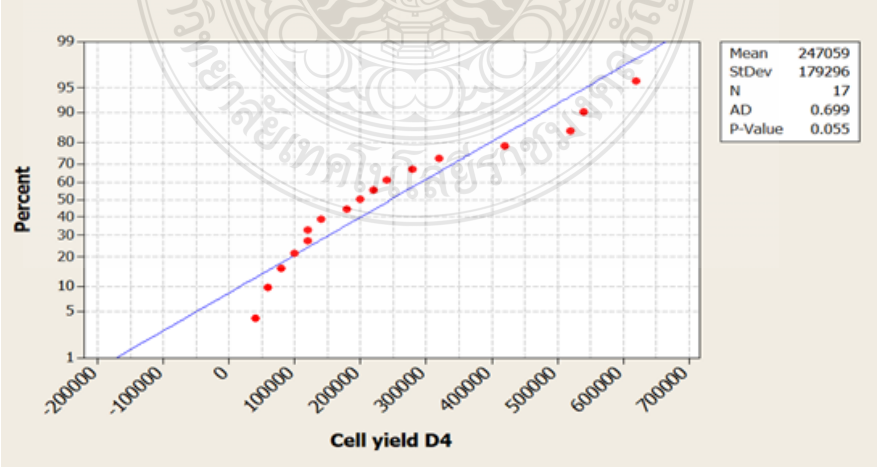

Figure 4.2 Probability plot of cell yield day 4th

4.1.2 Regression analysis of the selected model

The optimum development conditions of Serum-Free medium (OSF9-SFM) for Sf9 insect cell culture include three factors: CDLC (X1), yeast extract (X2), and soytone (X3). The experimental results revealed the viable cell yield on day 4 of culturing demonstrated that this model was significantly effective (*p*-value 0.05). The *p*-value of X1 (CDLC) = 0.000, X2 (yeast extract) = 0.047, and X1 x X2 = 0.016 indicated that they were all significantly effective on viable cell yield (Table 4.2).

According to the *t*-value, CDLC (X1:-9.568) had the most negative effect on viable cell concentration, YE (X2: 2.400) had a slightly positive effect, and ST (X3: -2.270) had a slightly negative effect. These indicated that increasing CDLC and ST concentration in medium can cause cell death while YE concentration increment pushing cell growth. The CDLC and YE pairwise interaction had the greatest negative effect on viable cell concentrations (X1 x X2: -3.172), demonstrating that combination of CDLC and YE can also cause cell death. All squares (X1 x X1: 1.703, X2 x X2: -1.529, X3 x X3: 1.703) did not significantly affect viable cell concentrations with *p*-value greater than 0.05 (Table 4.2). The full-squared model's coefficient of assignment, or R^2, was 94.70%, while the (adjusted) R^2 was also high at 87.88%, indicating that the model could explain the correlation of all variables and responses. Furthermore, the *p*-value for lack of fit was relatively high at 0.072 (Table 4.3), indicating that the model was adequate for assessing response.

The model construction was arranged in a series by using all coefficients with symbols of all variables in terms of linear, squares, and interactions. The mathematical equation of full quadratic model was shown in Equation 1.

$$Y = 73006 - 53874X1 + 13513X2 - 12781X3 + 10557 (X1 \times X1) - 9478$$
$$(X2 \times X2) + 10557 (X3 \times X3) - 23333 (X1 \times X2) - 0.1 (X1xX3) - 10000$$
$$(X2 \times X3) \quad \text{----------} \rightarrow \quad \text{(Eq.1)}$$

Table 4.2 Estimated regression coefficients for the data of cell concentrations on day 4 of culturing

Term	Coefficients	SE coefficients	*t*-value	*p*-value
Constant	73006	11990	6.089	0.000
X1 (CDLC)	-53874	5631	-9.568	0.000
X2 (YE)	13513	5631	2.400	0.047
X3 (ST)	-12781	5631	-2.270	0.057
X1xX1	10557	6197	1.703	0.132
X2xX2	-9478	6197	-1.529	0.170
X3xX3	10557	6197	1.703	0.132
X1xX2	-23333	7357	-3.172	0.016
X1xX3	-0.1	7357	-0.000	1.000
X2xX3	-10000	7357	-1.359	0.216

S = 20807.6 PRESS = 22595653257 R^2 = 94.70%
R^2 (predicted) = 60.46% R^2 (adjusted) = 87.88%

Table 4.3 Analysis of variance (ANOVA) for the data of cell concentrations on day 4 of culturing

Source	DF	Sums of Squares	Mean Squares	*F*- values	*p*-value
Regression	9	5.41E+10	6.01E+9	13.89	0.001
Linear	3	4.43E+10	1 47E+10	34.15	0.000
X1	1	3.96E+10	3.96E+10	91.55	0.000
X2	1	2.49E+9	2.49E+9	5.76	0.047
X3	1	2.23E+9	2.23E+9	5.15	0.057
Square	3	4.60E+9	1.53E+9	3.54	0.076
X1xX1	1	1.38E+9	1.25E+9	2.90	0.132
X2xX2	1	1.95E+9	1.01E+9	2.34	0.170
X3xX3	1	1.25E+9	1.25E+9	2.90	0.132
Interaction	3	5.15E+9	1.718E+9	3.97	0.061
X1xX2	1	4.35E+9	4.35E+9	10.06	0.016
X1xX3	1	0	0	0.00	1.000
X2xX3	1	8.00E+8	8.00E+8	1.85	0.216
Residual Error	7	3.03E+9	4.32E+8		
Lack-of-Fit	5	2.94E+9	5.88E+8	13.24	0.072
Pure Error	2	8.88E+7	4.44E+7		
Total	16	5.71E+10			

The Response Optimizer function of Minitab R16 software was used to analyze the optimal point of X1 (CDLC), X2 (YE), and X3 (ST). The retrieved results were CDLC at 0.5% (v/v), YE at 11 g/L, and ST at 3 g/L. At the optimal concentration of these three independent variable, the empirical mathematical model estimated a cell concentration will reach 1.98 x 10^5 cells/mL. However, a cell concentration in the range of 1.68 x 10^5 - 2.27 x 10^5 cells/mL, ±20% from the estimated data from the model, will be acceptable for experimental conducting [37].

The 3D response surface and contour plot (Figure 4.3A-4.3C) illustrated the relationship between the three variables (X1=CDLC, X2=YE, and X3=ST) and cell growth. The interaction between CDLC+YE (Figure 4.3A) was found to have a significant effect on cell growth. Cell yield of this combination reached a highest peak when the concentration of CDLC was 0.5% (v/v) and YE was 11 g/L. Increasing CDLC concentration from 0.5 to 1% pushed cell viability deceased, while increasing YE could promote better growth (p-value$\leq$ 0.05). Interaction between CDLC+ST (Figure 4.3B) showed the highest peak when CDLC and ST were at the lowest concentration, 0.5% (v/v) and 3 g/L, respectively. Both independent factors showed negative effect on cell yield when increasing each concentration in the medium. Interaction between YE+ST (Figure 4.3C) in the experiment indicated that the combination between YE and ST had a little effect on Sf9 growth (p-value $\geq$ 0.05). This results quite different from the interaction between CDLC+YE and CDLC+ST, they showed clear trend of positive or negative effect when increasing or decreasing concentration of each factor. Whereas interaction between YE+ST, in the range of tested concentration, cell yield was not different (p-value $\geq$ 0.05). This might be CDLC was the most effective factor on Sf9 cells growth.

ST and YE have been used extensively as medium substitutes as sources of vitamins, amino acids, peptides, and carbohydrates in serum free medium to improve cell density and productivity [37]. It has been reported that suspension cultures, like CHO cell, may require a protein source higher than that of adherent cell cultures [38]. Shen *et al*. (2007) concluded that yeastolate fraction could improve growth of Sf9 from 32 up to 80% compared to the control group [39]. Andreassen *et al*. concluded that YE alone could restore cell growth and replace FBS during bovine muscle cell cultivation in serum-free

media [40]. However, there is no information that which elements in yeast extract are responsible for the growth-promoting effects [41]. Keratinocyte cells culture with a low dose (1 g/L) of ST could stimulate the growth up to 201%, while increasing ST in medium resulted in growth deceased [42]. Utilization of amino acids inside animal cells could turn into ammonia by-products accumulation in cells. Hence, feeding high amount of amino acids in medium can cause the inhibition of cells due to ammonia toxic to cells [43]. In this research, YE was able to support Sf9 growth than ST might be because of the protein composition of the two hydrolysates was different. Up to 62% of protein in YE are small proteins with molecular weight less than 250 daltons while only 40% of protein in ST are small protein subunits [44]. Most proteins in ST are globulin, which has a high molecular weight protein-containing protease inhibitor that may not be suitable for the growth of Sf9 [45]. In our model, it was found that increasing ST from 3 g/L to 11 g/L causing cell yield decreased. The component in ST might cause this inhibition. Lipids and related components are necessary for various biological functions: components of cell membranes, storing and transporting of nutrients, and signal transduction [46]. To develop a serum-free medium, lipids at a concentration in the range of 10–100 µg/L are necessary to include in the medium for supporting animal cell growth. Specialty, insect cell requires higher lipid for their growth up to 1,000 µg/L [47]. For Sf9, cholesterol in CDLC is an important element for serum-free media development because Sf9 cannot synthesize cholesterol by itself [48]. The results from analyzed model revealed that low CDLC (0.5% v/v) had a higher viable cell yield than higher CDLC (0.75 – 1% v/v). This was probably due to the high hydrophobicity of CDLC that contact to Sf9 cells and resulting in growth reduction of Sf9 [26].

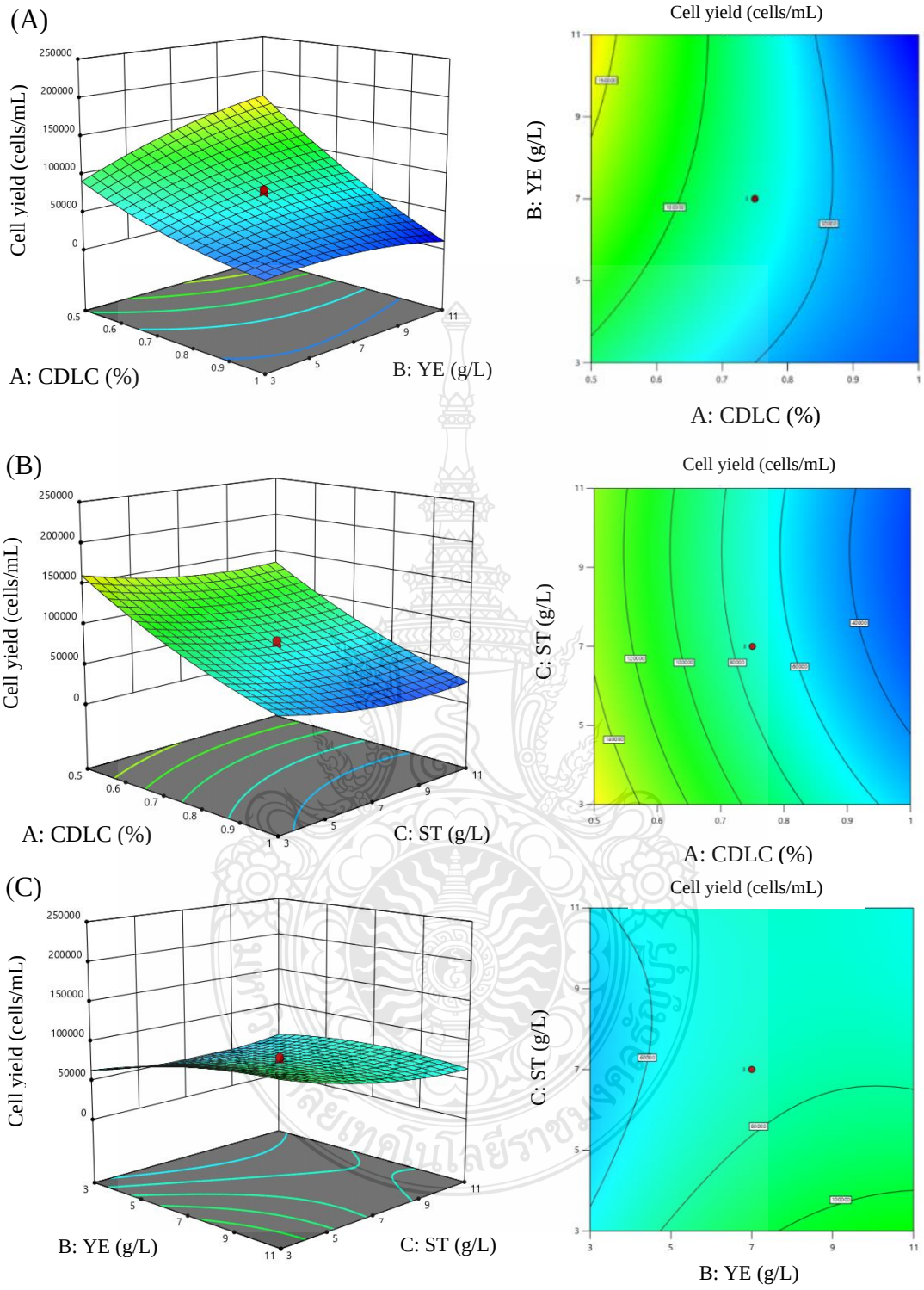

Figure 4.3 Effects of CDLC and YE concentrations on viable cells in the 3D response surface (left) and contour plot (right): (A) Effects of CDLC and YE concentrations; (B) Effects of CDLC and ST concentrations; and (C) Effects of YE and ST concentrations.

4.1.3 Verification study

Verification experiments were performed using a base medium supplemented with three independent variables at optimal concentrations: CDLC 0.5% (v/v), YE 11 g/L and ST 3 g/L. Figure 4.4 showed the viable cell concentration of Sf9 cells grown in OSF9-SFM that were subculture for 5 passages (P1 to P5). In the first passage, Sf9 inoculum was subcultured from a serum-containing medium in which residual serum proteins in media may stimulate cell growth after replacement with OSF9-SFM. The maximum viability cells from P2-P4 were 1.91 ± 0.15 - 1.93 ± 0.15 x 10^5 cells/mL with a relative cell number greater than 107% of P0. Afterward, the viable cells number decreased rapidly to 1.20 ± 0.10 x 10^5 cells/mL with a relative cell count of 67% of P0. In the last P5 passage, percentage of cell viability was still high enough (97% ± 2) to be used for long-term subcultivation. Overall, the average cells concentration obtained from P1-P5 was 1.71 ± 0.14 x 10^5 cells/mL with an average cell viability percentage of 96.8% ± 1.0. The maximum cell number calculated from empirical mathematical model was 1.98 x 10^5 cells/mL, whereas the obtained average viable cell yields under optimal conditions were in the accepted range of 1.68 x 10^5 - 2.27 x 10^5 cells/mL, indicating the suitability of this model [37]. OSF9-SFM had an osmolality of 511 ± 3 mOsm/kg (n = 3) which was significantly higher than that of commercial serum-free media which had an osmolality of 345-359 mOsm/kg [49]. However, the medium osmolality for insect cells could vary between 250 and 500 mOsm/kg [50]. For *Trichoplusia ni* BTI-TN-5B1-4 (High-Five™) cultures, it was found that increasing the osmotic pressure from 350 to 500 mOsm/kg did not cause a significant difference of proliferation rate during log phase [51]. Grace's medium was supplemented with 2.7 g/L glucose, 8 g/L YE, 0.1 % (w/v) Pluronic F-68, 1 % (w/v) milk whey ultrafiltrate (MWU), and 3 % (v/v) FBS had high osmolality of 438 mOsm/kg, but it could support 5 fold of Sf9 cell growth (4.7 x 10^6 cells/mL) compared to Grace's medium with 10 % (v/v) FBS (9.5 x 10^5 cells/mL) [50]. Moreover, IPL-41 supplemented with 6 g/L yeastolate ultrafiltrate, 0.6 g/L tyrosine, 3.5 g/L glutamine, 2 g/L lactose, 0.5 g/L fructose, 10 g/L glucose, 1.48 g/L methionine, and 1% (v/v) fat emulsion had an osmolarity as high as 460 mOsm/kg, but it was able to support the growth of *Drosophila melanogaster* S2 (S2AcGPV) up to a maximum cell concentration of 19 x 10^6 cells/mL [26].

Basically, the modified Grace's insect medium used as a basal medium had an osmolality of 413 ± 2 mOsm/kg (n = 3). It is likely that the addition of YE 11 g/L and ST 3 g/L were able to significantly increase the osmolality of OSF9-SFM. In this circumstance, high osmolality of OSF9-SFM did not affect Sf9 growth because it could still grow in P1-P5. However, lower cells yield in serum-free medium is common due to insufficient nutrients when compared to serum-containing medium.

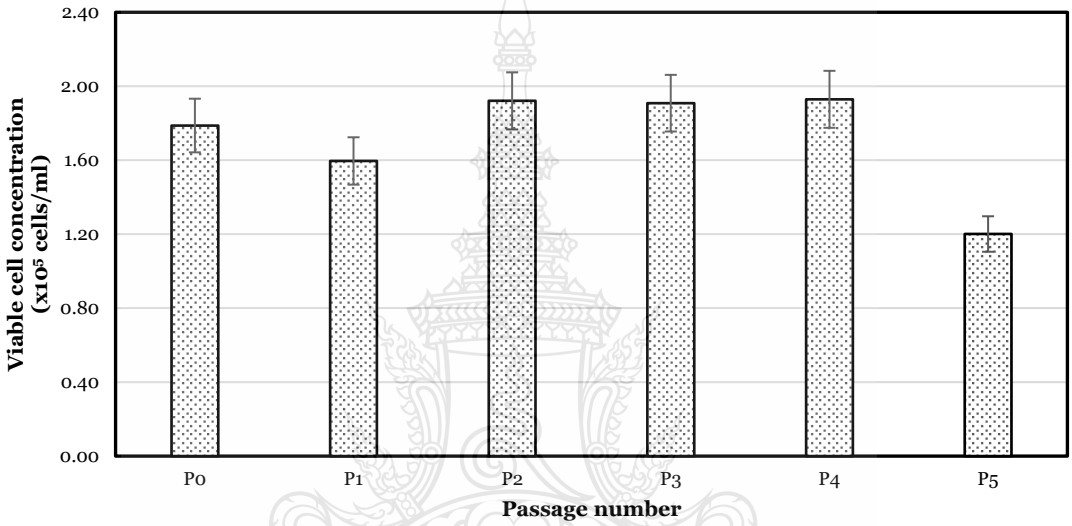

Figure 4.4 The viable cell concentration of Sf9 cells obtained from a continuous sub-cultivation for 5 passages in OSF9-SFM. Data are represented as mean ± standard deviation (SD) bar.

4.1.4 Study of the optimum cell density in the static culture

Optimal initial cell density in static culture was tested in the range of 2 - 5 × 10^5 cells/mL in OSF9-SFM. The use of a low cell concentration (2 × 10^5 cells/mL) was not able to reach high cell concentration after 5 days of culture (Figure 4.5A). However, when the initial cell concentration was higher than 2 x 10^5 cells/mL, the final cell concentration was noticeably higher. The initial cell concentration of 3 - 5× 10^5 cells/mL, results in nearly identical the final cell concentrations. However, an initial cell concentration of 3 x 10^5 cells/mL could give the maximum multiplication ratio of 6.69 ± 0.15, while an initial cell concentration of 2 x 10^5, 4×10^5, and 5×10^5 cells/mL were 1.340 ± 0.07, 5.32 ± 0.17, and 4.05 ± 0.09, respectively (Figure 4.5B). Parizi *et al.* (2017) concluded that the optimum initial cell density of BHK1 cells in suspension culture was 3× 10^5 cells/mL with the highest multiple ratios of 3.7. High cell density causes competition for obtaining limited nutrients and influences the pH of the medium. Meanwhile, some signals produced from cells can stimulate the growth of other cells [56]. Spens (2006) suggested that increasing the amount of serum or specific growth factors in the medium may reduce the critical initial cell density (cID). Inoculation of initial cell number below cID may cause the viable cell density to decrease before it begins to multiply [54]. The optimal starting cell density will vary between the cell line and medium used. For Sf9 cultivation in OSF9-SFM, the best initial cell concentration was 3 x 10^5 cells/mL.

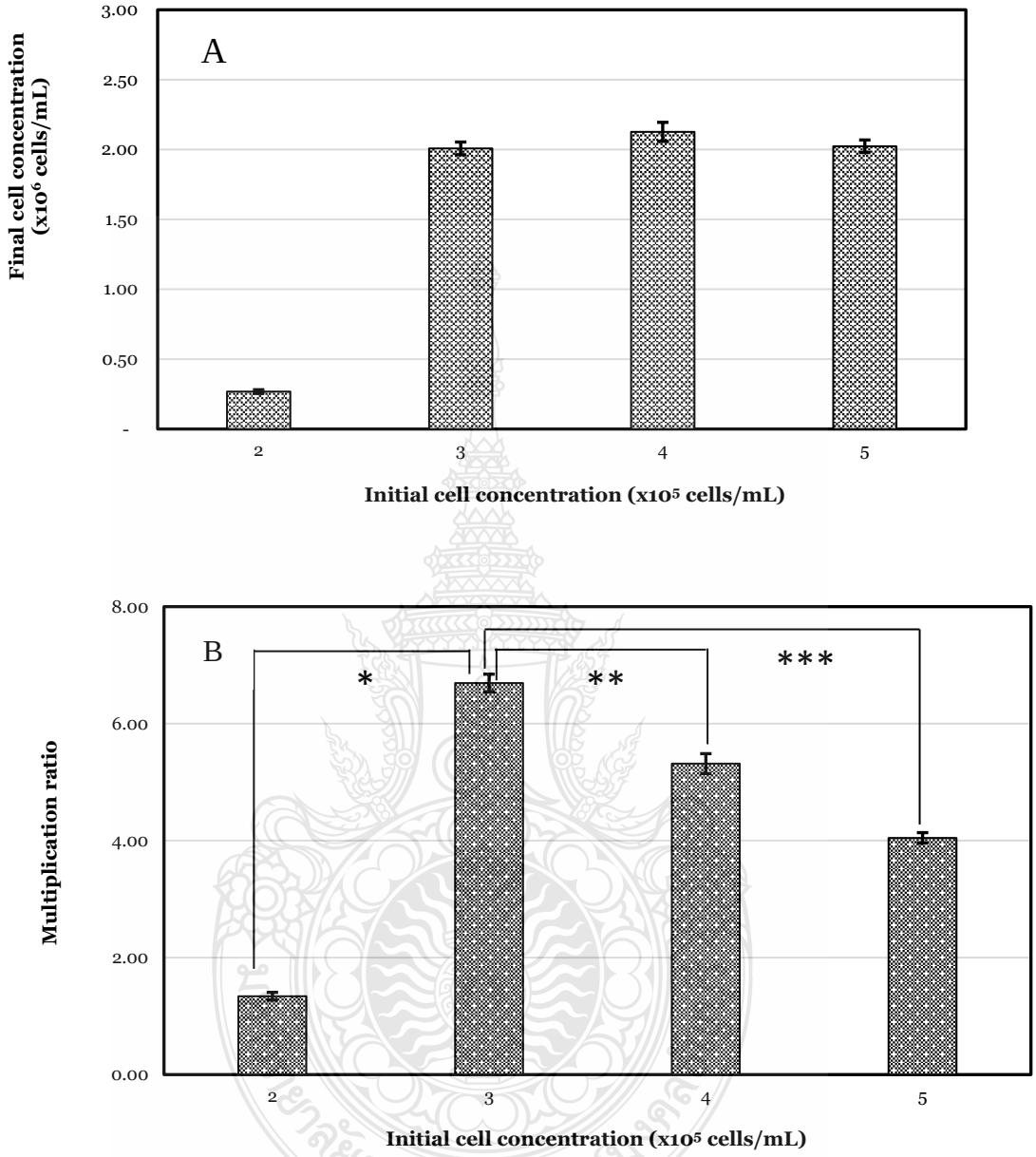

Figure 4.5 The final cell concentration (4.5A) and the multiplication ratio (4.5B) were expressed as a mean value ± standard deviation (*error bar*) obtained from a triplicated experiment. A paired samples Student's *t* test was carried out and *p*-value was as follows: * *p*-value = 0.010, ** *p*-value = 0.011, *** *p*-value = 0.015. Data are represented as mean ± standard deviation (SD).

4.2 The combined effect of microfiltration and ultrafiltration of YE and ST on Sf9 cell growth

Microfiltration and ultrafiltration of ST and YE were used to study the effect of the protein fraction on Sf9 growth by combining the individual fractions as shown in Table 3.2. Fraction #1 contained crude yeast extract and crude soytone, fraction #2 contained proteins with a molecular weight less than 10 kDa and fraction #3 contained proteins with a molecular weight less than 3 kDa. The concentration of viable cells obtained from the combination of microfiltration and ultrafiltration of ST and YE at Run 8, Run 6, and Run 9 was 5.98 ± 0.13 x 10^5, 6.27 ± 0.07 x 10^5, and 7.17 ± 0.08 x 10^5 cells/mL, respectively, which higher than the others (Figure 4.6). Interestingly, it was found that only combination of fraction#2 and fraction#3 of ST and YE was able to stimulate better growth of Sf9 cells, namely Run 8: Fraction #2ST + Fraction #3YE, Run 6: Fraction #3ST + Fraction #2YE, and Run 9: Fraction #2ST + Fraction #2YE. Notably, the combination with high molecular weight YE and ST (Run 1) was unable to support Sf9 good growth compared to others.

Moreover, Run 5 in which contained #3 ST and #3 YE had the lowest concentration of viable cells. Fraction #3 contains all free amino acids with molecular weights from 75 - 204 daltons, including some oligopeptides [55]. However, some carbohydrates may be removed from this fraction resulting in Sf9 unable to grow in this Run due to insufficient nutrients. Run 1, 2, and 4 had similar concentrations of viable cells. These Run had fraction #1 and lacked fraction #2. Run 3 and 7, which both contained fraction #1 and fraction #2, had better growth than Run 1, 2, and 4. Run 6, 8, and 9, all contained fraction #2. The high molecular weight of YE and ST in fraction #1 may contain some inhibition factor for Sf9, while 10 kDa of both YE and ST in fraction #2 may be suitable for growth. However, the scientific basis for such a synergistic effect remains to be elucidated. A paired sample of Student's t-test revealed that the final cell concentrations of Run 9 were significantly different from other Run.

Actually, ST and YE fraction used in Run 9 were the same as the supplements used in OSF9-SFM. The low molecular weight (LMW) YE (less than 30 kDa) had more positive effect on the growth of Sf9 cells than high molecular weight (HMW) [29]. Sf9

culture using sequential ethanol precipitation of yeastolate ultrafiltrate (YUF1) combined with other YUF showed a synergistic effect to enhance the growth of Sf9 [39].

Moreover, Chou (2013) noticed that yeastolate with molecular weights less than 10 - 14.2 kDa were able to fully support the growth of IPLBSf-21AE (Sf 21) [57]. Our finding revealed that ultrafiltration (10 kDa cut-off) of YE and ST can enhance Sf9 growth, which is consistent with previous research. However, the synergistic effect of ST on insect cells was not clear. However, integrating the ultrafiltration step in the preparation process can eliminate the hydrolysate lot variability and guarantees reproducible growth of insect cells [28], [30].

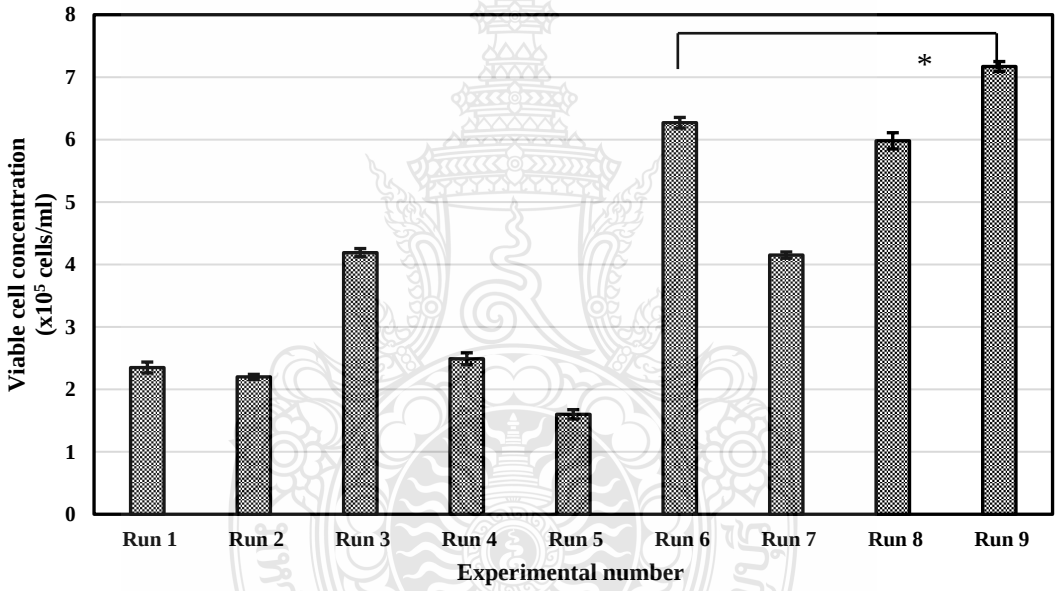

Figure 4.6 The influence of YE and ST microfiltration and ultrafiltration components on Sf9 cell viability. The paired sample t-test was carried out, and the *p*-value was calculated as follows: **p*-value < 0.05. Data are represented as mean ± standard deviation (SD).

4.3 Adaptation of Sf9 cells to grow in suspension

Sf9 cells were adapted to grow in suspension culture in 250 mL spinner flasks containing OSF9-SFM with initial cell concentration of 3 x 10^5 cells/mL. Sf9 cell growth was examined in two separate batches. It was found that maximum viable cell concentrations in batches 1 and 2 were 1.79 x 10^6 and 2.17 x 10^6 cells/mL, respectively (Figure 4.7). The maximum multiplication ratios of batches 1 and 2 were 4.96 on day 5 and 6.23 on day 4, respectively. Hensler *et al.* (1994) concluded that the lag phase is related to population density. To overcome the long culture lag phase of Sf9 growth in serum-free medium, they suggested to use seeding densities greater than 6 x 10^5 cells/mL [59]. Because of the cell numbers between batch 1 and 2 were different, the kinetic parameters (number of generations of cell growth, doubling time, and specific growth rate) were calculated as follows: batch 1, 1.72, 28 h, and 0.025 h^{-1}, and batch 2, 1.90, 26 h, and 0.027 h^{-1}, respectively. This result suggested that Sf9 cells culture in two batches of OSF9-SFM could grow in a similar manner. Sf9 culture in OSF9-SFM had doubling time and specific growth rate similar to Sf9 culture in EX-CELL 420 at 26 h and 0.026 h^{-1}, respectively [60]. The morphology of Sf9 taken from batch no. 1 revealed that cells aggregated as a clumping with an average diameter of 12.8 μm (Figure 4.8). Then, dextran sulfate (0.25 g/L) was added to batch no. 2 to reduce cell clumping. Dextran sulfate successfully reduces cell aggregation, that might be the reason of the higher countable cell number in batch no. 2. Sf9 adaptation to grow in suspension culture could reach a higher maximum cell number (2.17 x 10^6 cells/mL) than adherent culture (7.17 x 10^5 cells/mL) about 3 times. Based on the results, Sf9 cells could be adapted to grow and maintained in OSF9-SFM.

Wu *et al*. (1990) studied the adaptation of *Spodoptera frugiperda* and *Trichoplusia ni* cells to suspension culture in shake flasks. The results showed that the growth of the suspension in the shake flask through seven passages consecutively subcultures resulted in a final maximum cell density of 3 × 10^6 cells/mL, which was higher than the initial 5 × 10^5 cells/mL [56]. Beas-Catena *et al.* (2013) studied the culture of *Spodoptera exigua* Se301 cells in serum-free medium (SFM; Ex-Cell 420 and Serum-Free Insect Medium1) in suspension culture. They found that cell concentrations reached 1.5×10^6 cell/mL and viability of cell was more than 90 % [58].

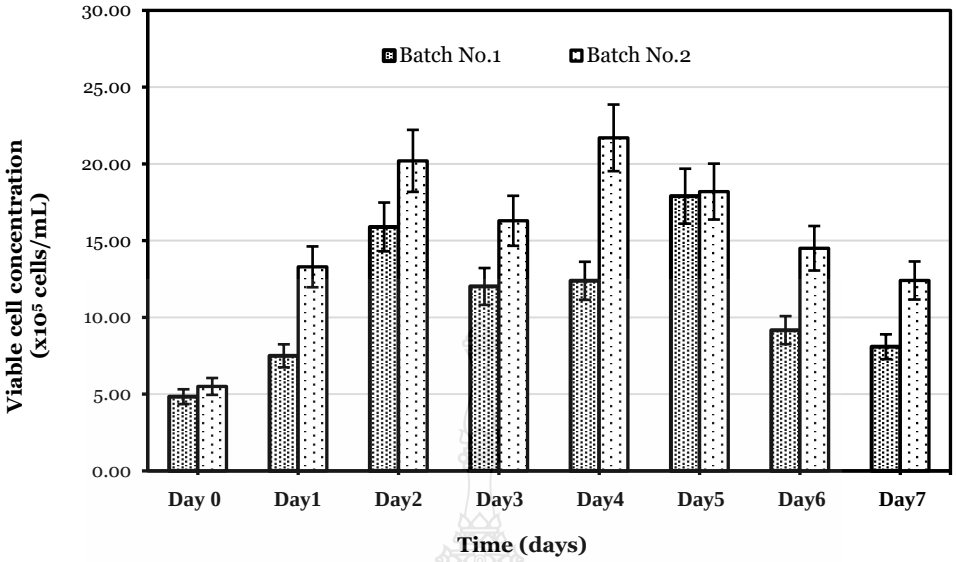

Figure 4.7 Initial viable Sf9 cell concentration of 5.00 x 10^5 cells/mL were grown in 50 mL of OSF9-SFM in a 250 mL spinner flask. Cultures were grown at 28 °C and a stirring rate of 50 rpm. The mean value of viable cell concentration from triplicated experiments of two batch cultured was compared. Data are represented as mean ± standard deviation (SD).

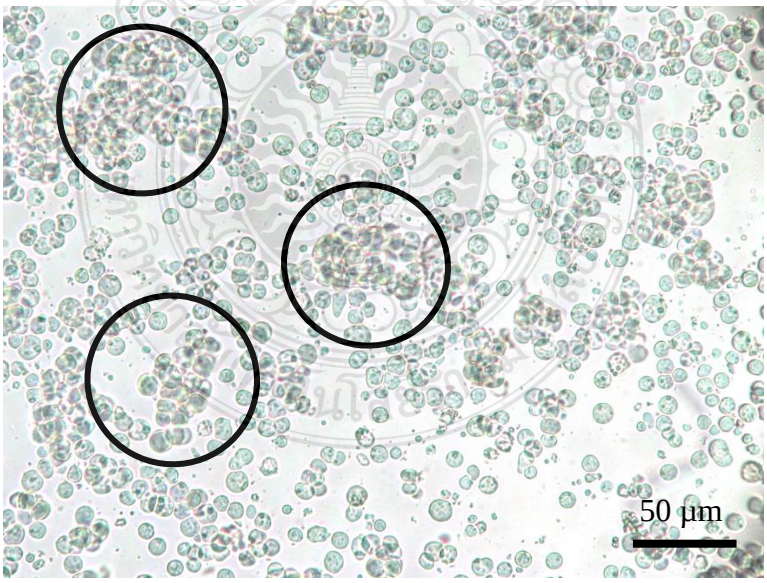

Figure 4.8 The morphology of Sf9 cells on day 2 culture growing in OSF9-SFM in 250 mL spinner flasks containing 50 mL of Run9 medium. Cell aggregation was indicated by the black circles. The black line was 50 µm scale bar.

4.4 Green fluorescent protein expression of Sf9 in OSF9-SFM

For transfection of Sf9 cells, the pEGFP-N1 plasmid containing the CMV-IE promoter, which is optimal for protein expression in mammalian cells, was used to examine protein production in Sf9. After 72 h of culture, uninfected Sf9 cells cultured in Grace's insect medium plus 10% FBS, SF-900II, and OSF9-SFM had cell number of 1.21 x 10^6, 2.82 x 10^5, and 2.25 x 10^5, respectively. The number of cells in medium containing serum was 4.29 and 5.38 times higher than in SF-900II and OSF9-SFM, respectively. Sf9 growth in OSF9-SFM was only 1.25 times lower than in SF-900II while the cost of OSF9-SFM was more than 50% cheaper. The size of uninfected Sf9 cells were 15.53 and 14.02 µm in SF-900II and OSF9-SFM respectively, whereas serum-containing cells was approximately 17.55 µm (Figure 4.9).

In Grace's medium plus 10% FBS, the maximum number of GFP fluorescent cells per well was 34 cells/well while the total cell count was 4.57 x 10^5 cells/mL (4.57 x 10^4 cells/well). SF-900II and OSF9-SFM produced very low GFP fluorescent cells with an average GFP fluorescent cell count of less than 13 cells/well in SF-900II and less than 2 cells/well in OSF9-SFM (Figure 4.10). This result indicated that serum may provide good support for Sf9 transfection in Grace's insect medium plus 10% FBS.

Higher volume of Cellfectin™ II reagent were toxic to Sf9 cells, resulting in lower cell numbers than uninfected cells 2.62- 3.77 times. Dead cells in SF-900II and OSF9-SFM infected with 2 µL Cellfectin™ II reagent were 85.35% and 94.30%, respectively. The CMV-IE promoter in pEGFP-N1 plasmid was unappropriated for gene expression in insect cell. This may explain why GFP fluorescent cells are so scarce in these three culture mediums.

Cheng *et al.* (2016) constructed the pEGFP-N1-hPer2 expression vector by inserting the human period 2 (hPer2) gene into pEGFP-N1 plasmid and determined the protein expression by transfection in human osteosarcoma MG63 cell line. After 48 h post-transfection, the expression of EGFP was clearly seen under fluorescence microscopy in the pEGFP-N1-hPer2, but not in the control group. The results revealed that in the pEGFP-N1-hPer2, EGFP was expressed in about 70% of total cells, indicating that pEGFP-N1-hPer2 successfully transfected into MG63 cell line, resulting in a high level of EGFP expression [59].

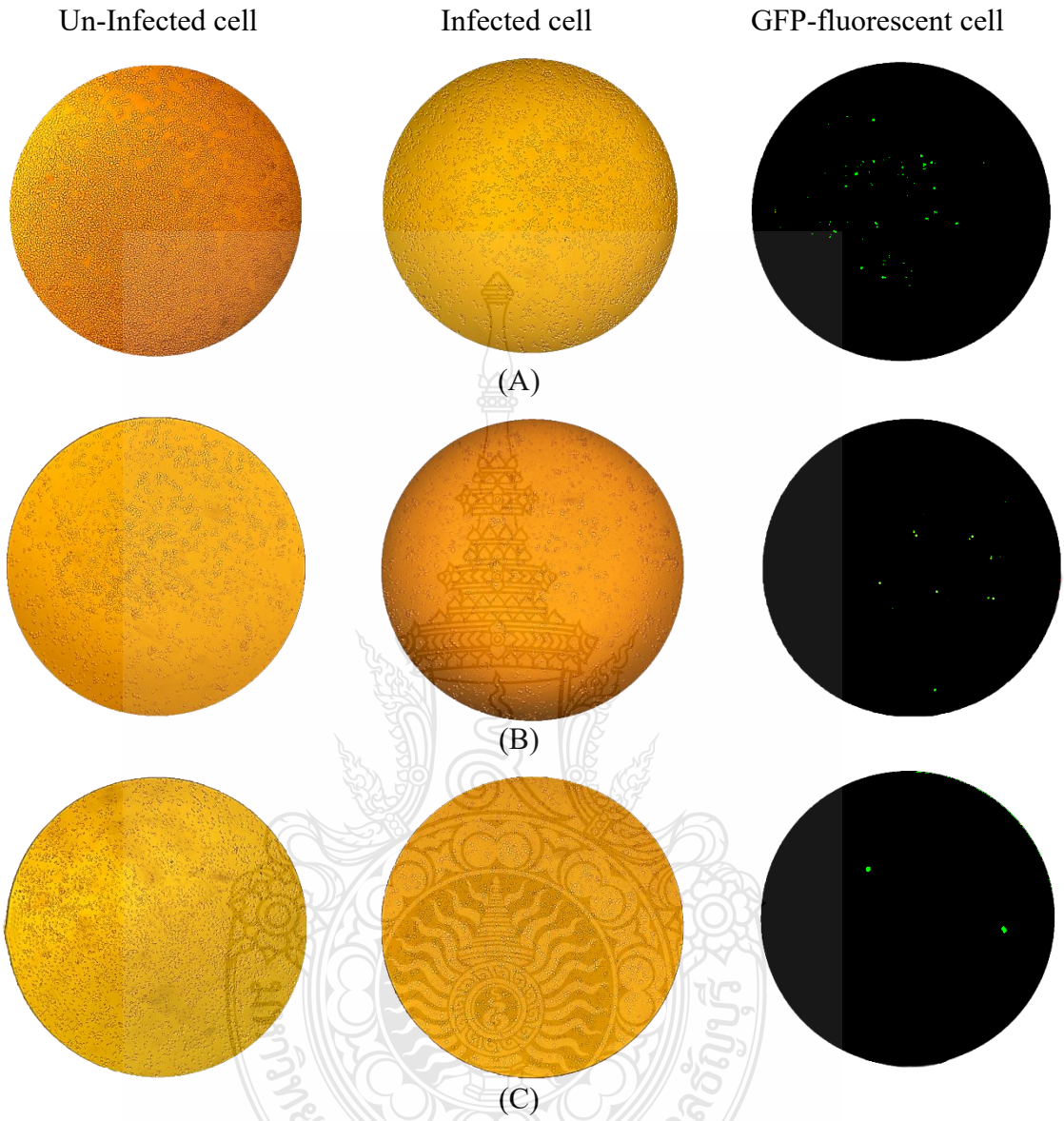

Figure 4.9 Sf9 cells in Grace's insect medium plus 10% FBS (A), SF-900II (B), and OSF9-SFM (C).

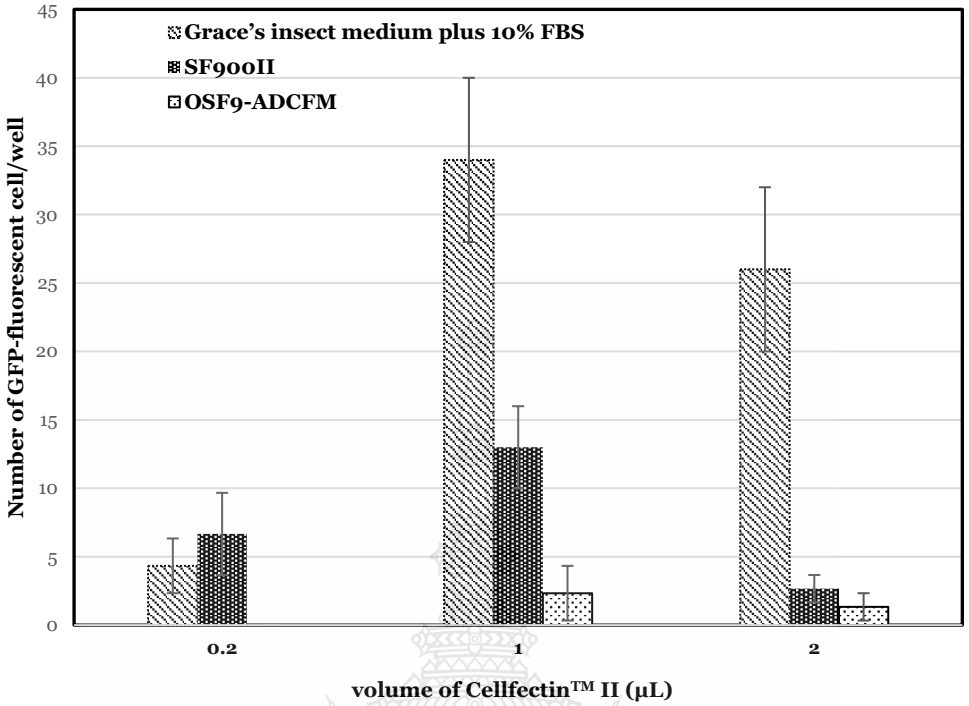

Figure 4.10 Cell number of GFP-fluorescent cells in Grace's insect medium plus 10% FBS, SF-900II, and OSF9-SFM with 0.2, 1, and 2 µL of Cellfectin™ II reagent.

4.5 Anti-hepatitis B virus surface antigen (anti-HBsAg) expression of Sf9 in OSF9-SFM

Sf9 insect cells were grown in three media: Grace's insect medium plus 10% FBS, Sf-900 II and OSF9-SFM. Recombinant bacmid (r-Bacmid) containing anti-hepatitis B virus surface antigen (anti-HBsAg) gene was introduced into Sf9 cells by transfection method. Immunofluorescence (IFA) method was used to observe the fluorescence under an inverted fluorescent microscope.

In transfection method I, Sf9 cells were transfected in each medium using 3 µg of r-Bacmid (anti-HBsAg). After 120 h post-transfection, the control cells and transfected cells were stained with Rabbit anti-mouse IgG (H+L)-FITC and observed under fluorescent microscope. The results revealed that only Grace's insect medium plus 10% FBS and Sf-900 II SFM showed fluorescence signal of FITC (Figure 4.11B and 4.11D). Sf 9 cells grown in the developed OSF9-SFM cell culture medium were not fluorescent observed inside the cells, as shown in Figure 4.11F. These results indicated that OSF9-SFM was not suitable for transfection of r-Bacmid into Sf9. The fluorescence of Sf9 cells transfected in SF-900 II showed less fluorescence cells than in Grace's insect medium plus 10% FBS might be because of the efficiency of transfection in SF-900 II was lower and virus particle might be low in the first transfection.

Transfection method II was adjusted to increase the viral particle number before infection in developed OSF9-SFM. In this method, r-Bacmid was transfected into Sf9 cultured in SF-900 II and OSF9-SFM. After transfection, P1 viral stock was collected and infected into Sf9 in each medium (as shown in Figure 3.1). Afterward, P2 viral stock was collected and then infected into Sf9 in OSF9-SFM. Cells collected from each condition were stained with Rabbit anti-mouse IgG (H+L)-FITC and observed under fluorescent microscope. The results revealed that at the first step of transfection, only Sf9 transfected in SF-900II showed fluorescent signal (Figure 4.12 A and 4.12 B). While Sf9 first transfected in OSF9-SFM had no fluorescence signal (Figure 4.12 C and 4.12 D). The number of fluorescent cells in 5 µg of r-Bacmid observed under microscope was higher than in 3 µg of r-Bacmid indicated the higher transfection efficiency into Sf9 (Figure 4.12 A and 4.12 B). The fluorescence signal of Figure 4.12 A and B were stronger than in

Figure 4.11 D because the viral particle number was higher in the P2 viral stock than in the transfection method I.

In summary, both experiments showed that OSF9-SFM was not suitable for transfection, but it can be used for infection and production of recombinant protein by recombinant virus particle producing from transfection step under appropriated medium. There might be some components in OSF9-SFM that inhibit or obstruct the transfection process. Manoch et al. (2021) used the lower amount of yeast extract at 7 g/L and soytone at 0.1 g/L in serum-free media development for Vero cell [37]. While in this research, the optimal amount for Sf9 growth from CCD model were yeast extract at 11 g/L and soytone at 3 g/L. Therefore, OSF9-SFM may contain too much hydrolysate in the media, which would cause a negative effect on transfection. OSF9-SFM medium requires additional medium development to be used for Sf9 cells growth and protein production.

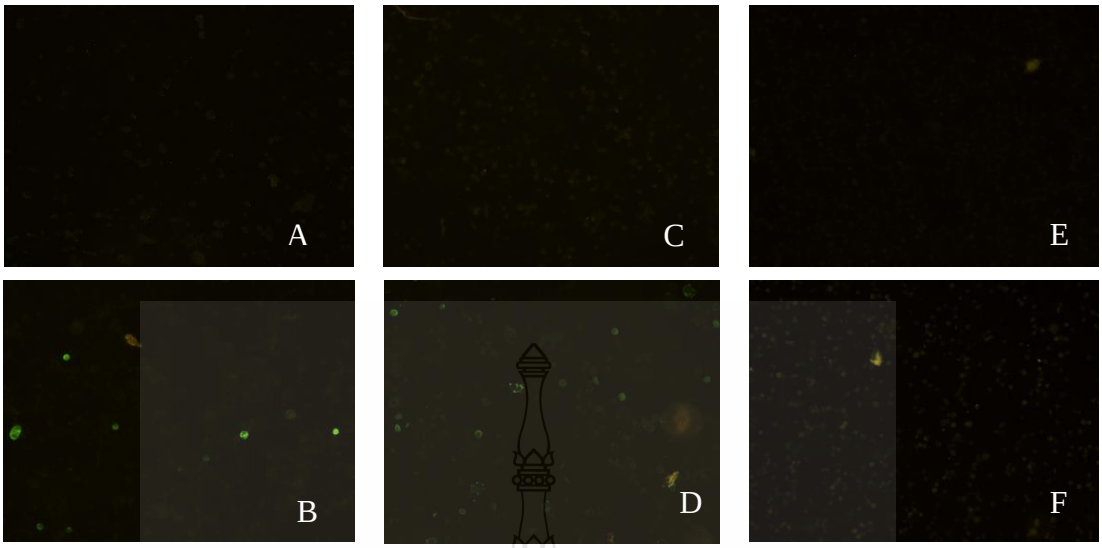

Figure 4.11. Fluorescence of Sf9 cells transfected with r-Bacmid containing Anti-HBsAg

(A) Control SF9 grown in Grace's insect medium plus 10% FBS

(B) r-Bacmid inoculated Sf9 cells cultured in Grace's insect medium plus 10%FBS

(C) Control SF9 grown in Sf-900 II

(D) r-Bacmid-inoculated Sf9 cells cultured in Sf-900 II

(E) Control SF9 grown in OSF9-SFM

(F) r-Bacmid-inoculated Sf9 cells cultured in OSF9-SFM

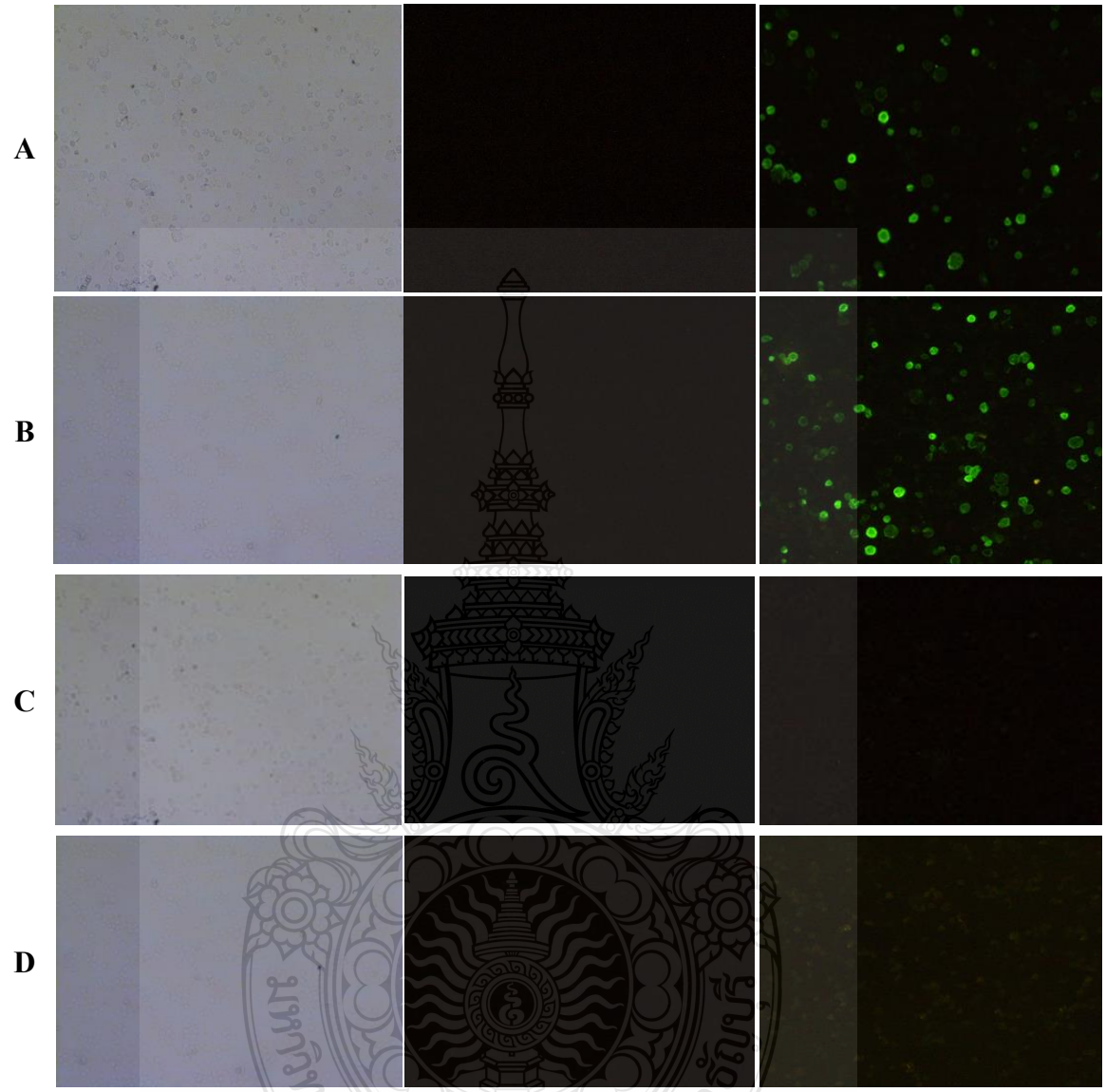

Figure 4.12 Fluorescence of Sf9 cells infected with P2 of r-Bacmid in SF-900II and OSF9-SFM medium

(A) 3µg of r-Bacmid (Anti-HBsAg) in SF-900II

(B) 5µg of r-Bacmid (Anti-HBsAg) in SF-900II

(C) 3µg of r-Bacmid (Anti-HBsAg) in OSF9-SFM

(D) 5µg of r-Bacmid (Anti-HBsAg) in OSF9-SFM

CHAPTER 5
CONCLUSION AND SUGGESTION

5.1 Conclusion

5.1.1 Development of serum-free media by CCD was found that CDLC had a strong negative effect, YE had a slight positive effect, and ST had a slight negative effect influence on the yield of live cells. The combination of CDLC and YE was found to be significantly effective on the yield of live cells. The optimum point of CDLC, YE, and ST of OSF9-SFM consisted of 0.5% (v/v) CDLC, 11 g/L YE, and 3 g/L ST with maximum cell of $1.98x10^5$ cell/mL. Additionally, fraction of 10 kDa of both YE and ST were suitability for insect cell growth.

5.1.2 The average viable cell concentration in the verification study of Sf9 in OSF9-SFM continuously in 5 passages (P1 to P5) was $1.71\pm 0.14 \times 10^5$ cells/mL, which was in the range of 1.68×10^5 - 2.27×10^5 cells/mL as calculated from the empirical mathematical model, indicating the suitability of this model. The best starting cell concentration was 3×10^5 cells/mL.

5.1.3 Two batches of suspension cultured Sf9 cells in OSF9-SFM demonstrated that it could adapt to grow in suspension culture with doubling time and specific growth rate of 28 h and 0.025 h^{-1} in batch no. 1 and 26 h and 0.027 h^{-1} in batch no. 2. Batch no. 1 and no. 2 yielded maximum viable cell concentrations of 1.79×10^6 and 2.17×10^6 cells/mL, respectively. According to the findings, in-house OSF9-SFM could be employed for Sf9 culture in both static and suspension cultures.

5.1.4 The transfection of Sf9 cells with plasmid pEGFP-N1 encoding green fluorescent recombinant protein (GFP) revealed that uninfected Sf9 cells had a cell count of 1.21×10^6, 2.82×10^5, and 2.25×10^5 cells/mL in Grace's insect medium with 10% FBS, SF-900II, and OSF9-SFM, respectively. The growth of Sf9 in OSF9-SFM was 1.25 times lower than in SF-900II, while the cost of OSF9-SFM was cheaper than SF-900II up to 50%. The average number of GFP-fluorescent cells in Grace's insect medium plus 10% FBS, SF-900II, and OSF9-SFM were 34, 13 and 2 cells/well, respectively. The low expression of GFP in all mediums might be caused from inappropriate promoter of plasmid in Sf9 expression system.

5.1.5 Transfection of Sf9 cells with r-Bacmid (Anti-HBsAg) found that r-Bacmid was unable to directly transfect Sf9 in OSF9-SFM. However, P2 baculoviruses, produced from r-Bacmid transfect Sf9 in SF-900 II medium, could infect Sf9 in OSF9-SFM medium. The overall finding of this research indicated that inhouse OSF9-SFM could be used for Sf9 cultivation and protein production.

5.2 Suggestion

5.2.1 OSF9-SFM should be further optimized or adding an appropriate reagent to reduce cell aggregation.

5.2.2 Minimization of hydrolysates such as yeast extract should be considered to avoid the reduced transfection efficiency.

5.2.3 Optimization of transfection of r-Bacmid in OSF9-SFM should be further study.

5.2.4 Increasing protein production in OSF9-SFM should be further study.

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APPENDIX

APPENDIX A
Grace's insect cell culture medium

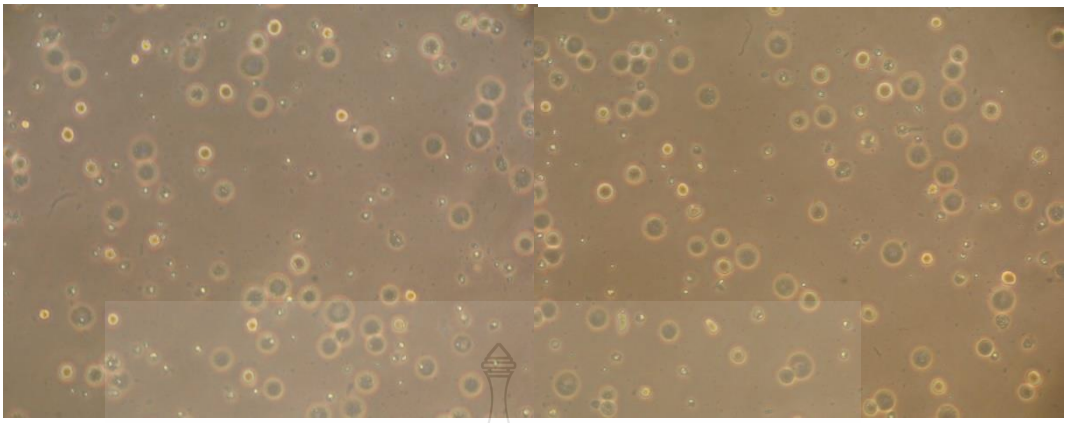
Grace's insect cell culture medium

Composition:

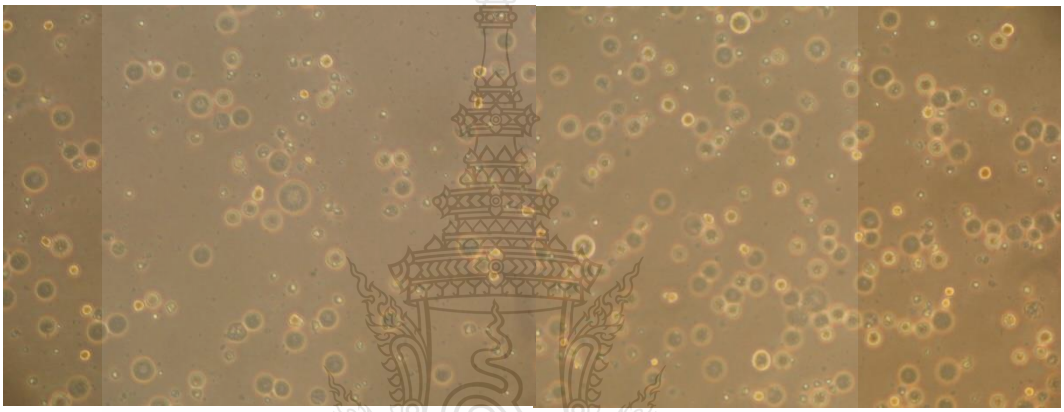
Components	**mM**
Inorganic salts	
Calcium chloride dihydrate	6.75
Magnesium chloride anhydrous	11.26
Magnesium sulphate anhydrous	11.31
Potassium chloride	54.66
Sodium phosphate monobasic monohydrate	7.34
Amino acids	
DL-Serine	5.23
Glycine	8.66
L-Alanine	2.52
L-Arginine hydrochloride	3.31
L-Asparagine	2.65
L-Aspartic acid	2.63
L-Cystine 2HCl	0.09
L-Glutamic acid	4.08
L-Glutamine	4.10
L-Histidine hydrochloride monohydrate	16.12
L-Isoleucine	0.38
L-Leucine	0.57
L-Lysine hydrochloride	3.41
L-Methionine	0.33
L-Phenylalanine	0.90
L-Proline	3.04
L-Threonine	1.47
L-Tryptophan	0.49
L-Tyrosine disodium salt	0.27
L-Valine	0.85

ß-Alanine	2.24
Vitamins	
Choline chloride	0.001
D-Biotin	4.09
D-Ca-Pantothenate	4.19
Folic acid	4.53
Niacin	1.62
Pyridoxine hydrochloride	9.70
Riboflavin	5.31
Thiamine hydrochloride	5.93
myo-Inositol	1.11
p-Amino benzoic acid (PABA)	1.45
Others	
Alpha-Ketoglutaric acid	2.53
D (+) Glucose	3.88
D-Fructose	2.22
Fumaric acid, free acid	0.47
L-Malic acid, free acid	5.0
Succinic acid	0.50
Sucrose	78.01

APPENDIX B
The growth of SF9



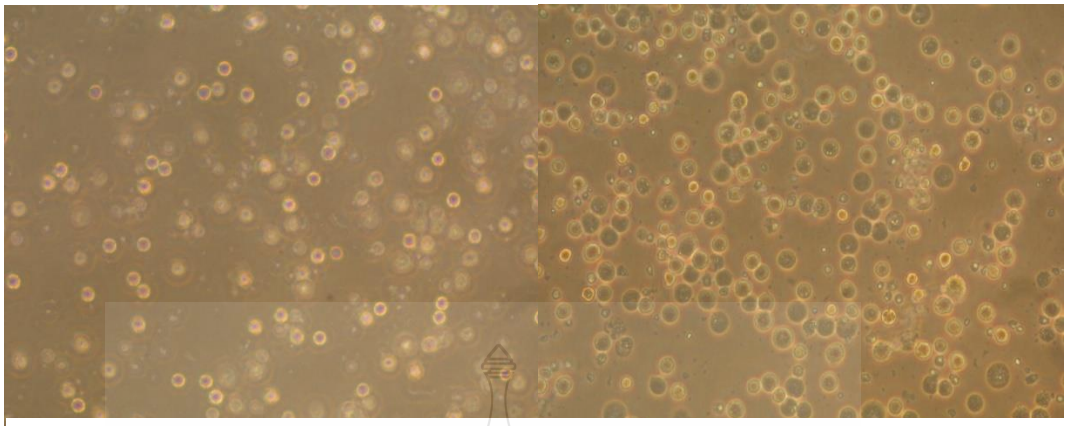
Run 1 **Run 2**



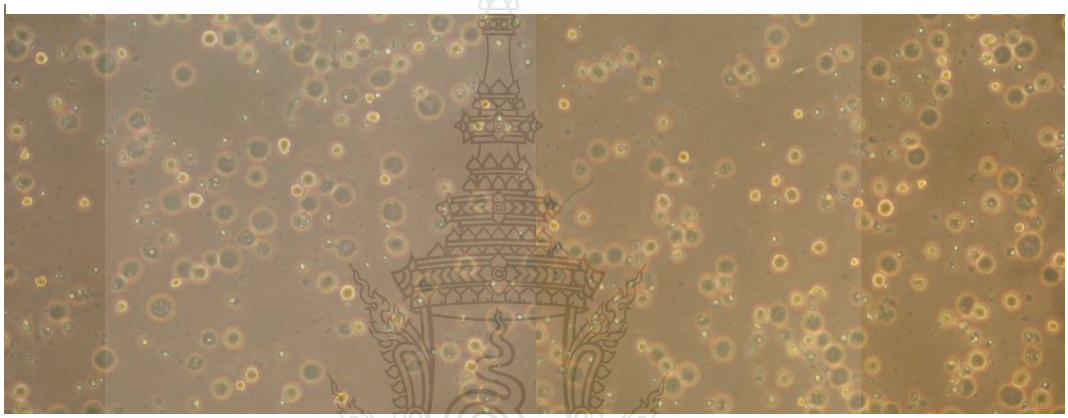
Run 3 **Run 4**

Run 5 **Run 6**

Figure 1 Cell growth of day 1



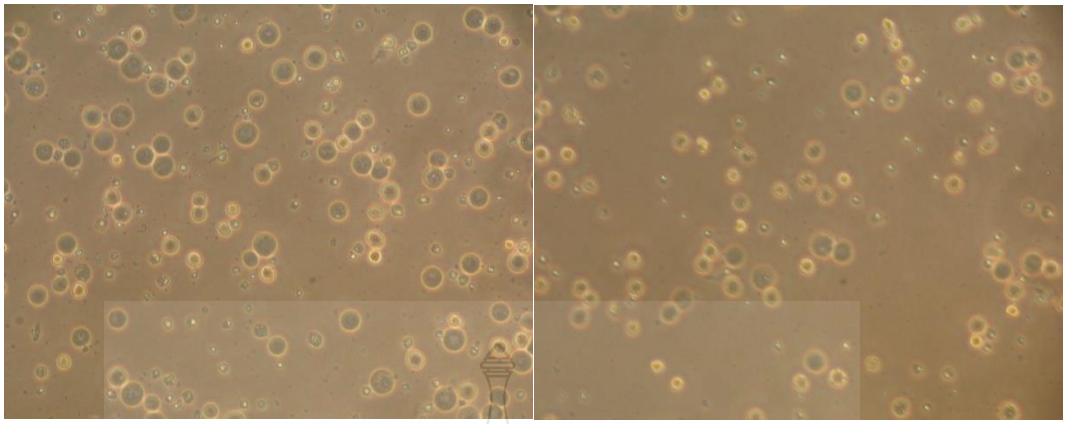
Run 7 **Run 8**



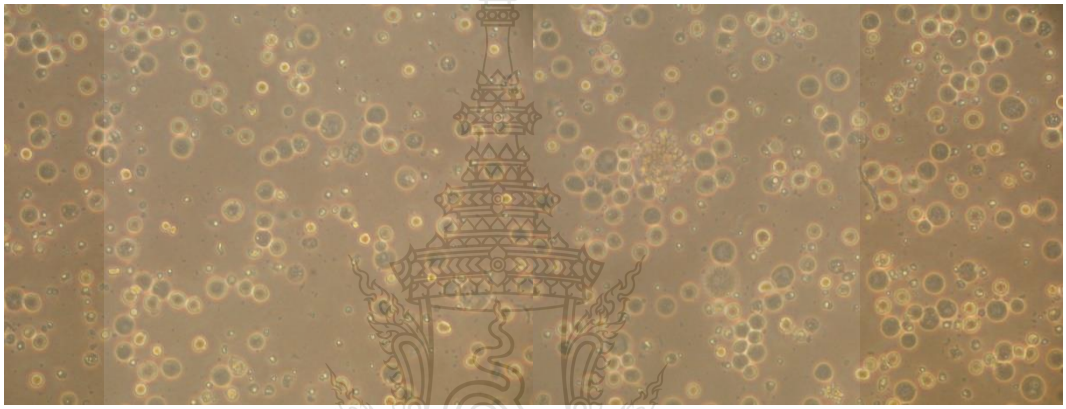
Run 9 **Run 10**

Run 11 **Run 12**

Figure 2 Cell growth of day 1 (Continued)



Run 13 **Run 14**



Run 15 **Run 16**

Run 17 **Run 18 (Control)**

Figure 3 Cell growth of day 1 (Continued)

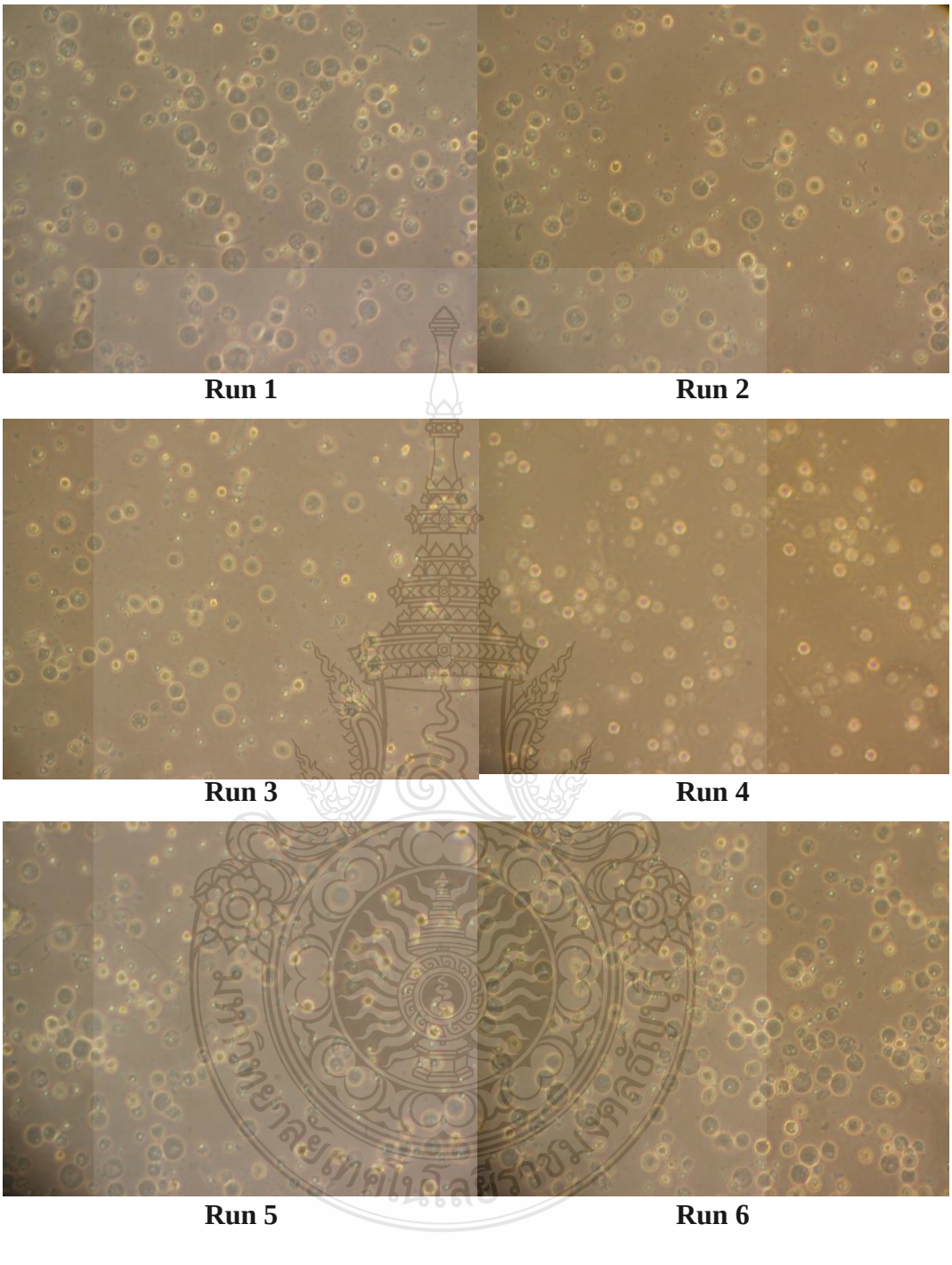


Figure 4 Cell growth of day 2

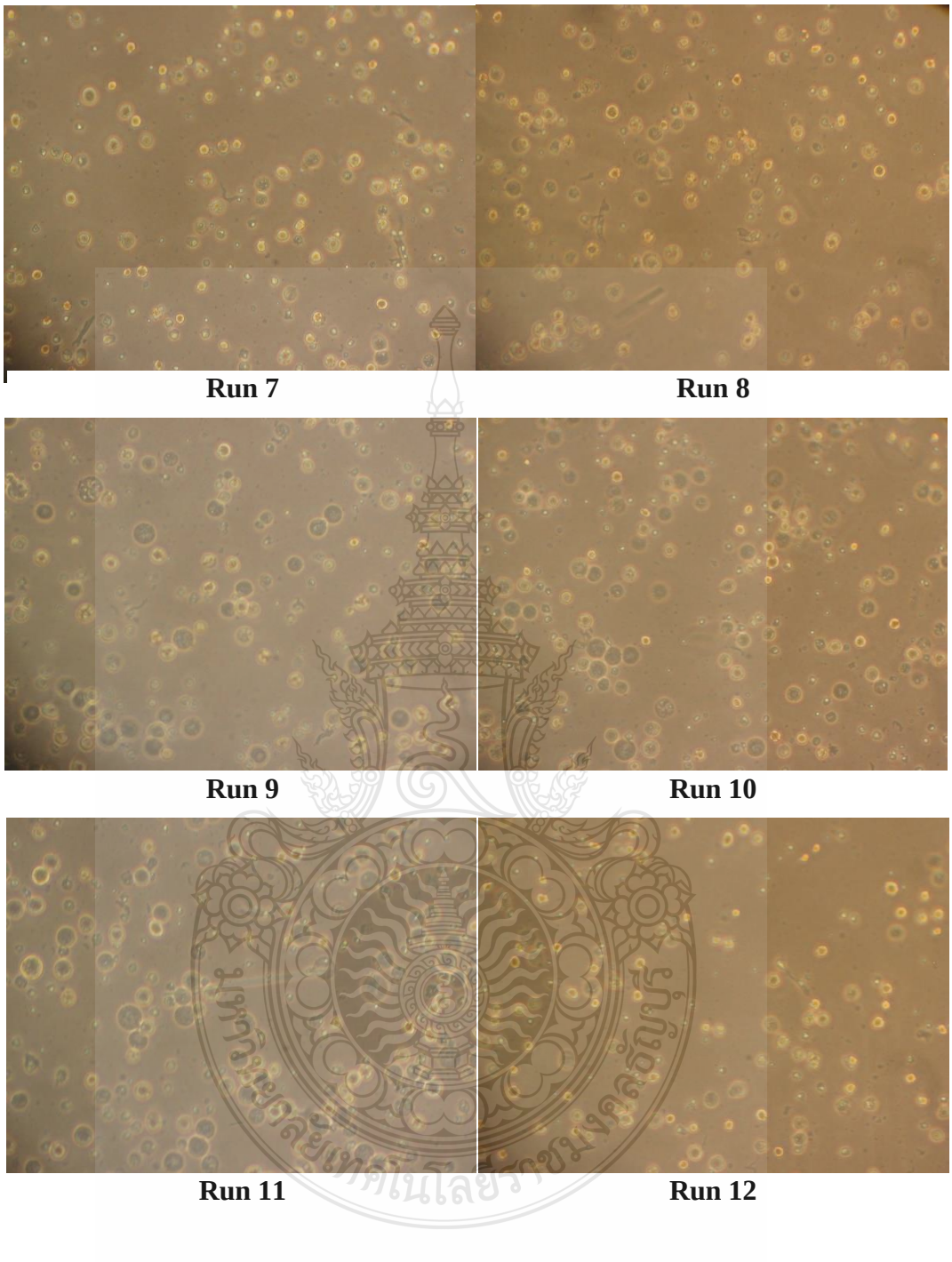
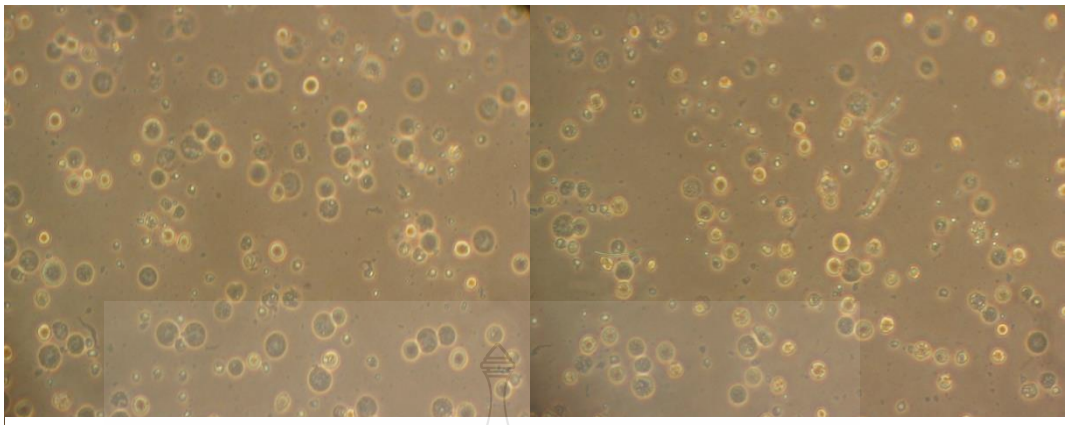
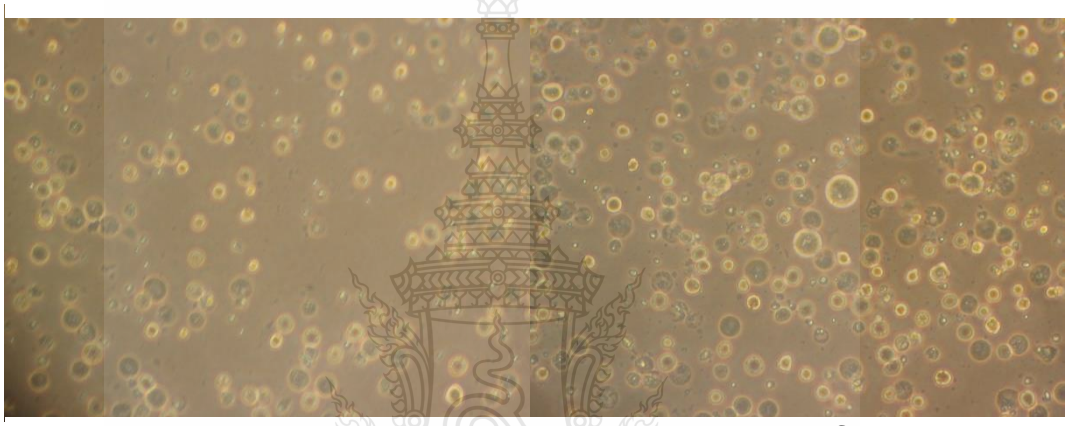


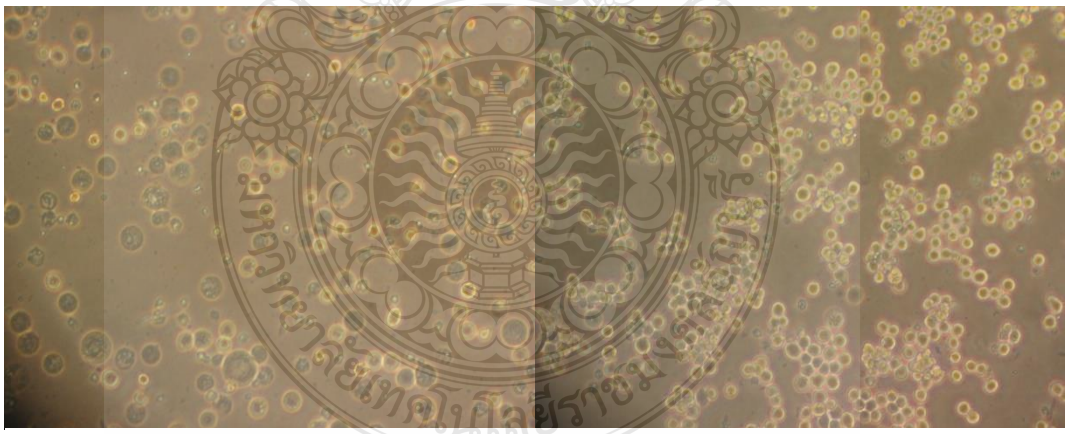
Figure 5 Cell growth of day 2 (Continued)



Run 13 **Run 14**



Run 15 **Run 16**



Run 17 **Run 18 (Control)**

Figure 6 Cell growth of day 2 (Continued)

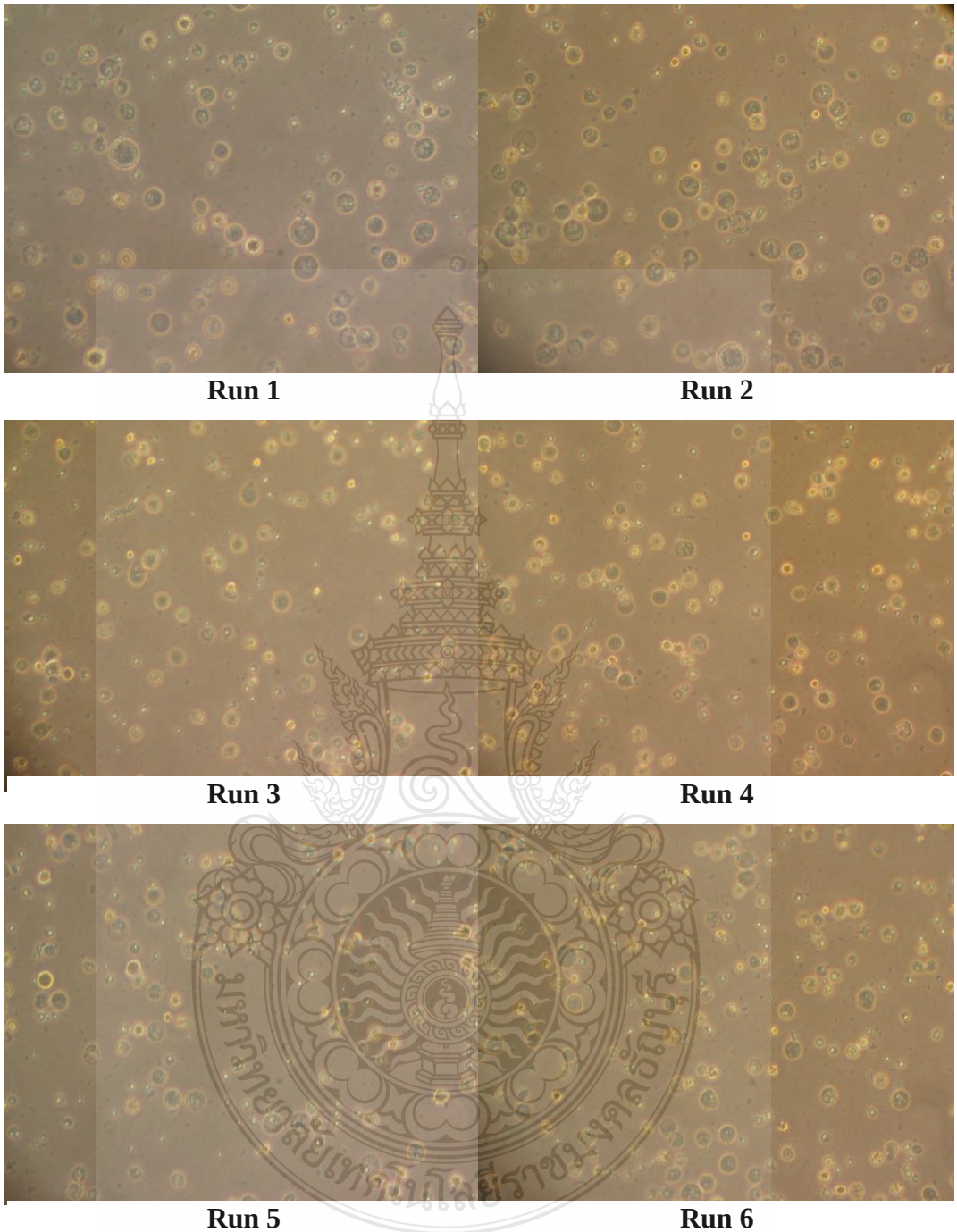


Figure 7 Cell growth of day 3

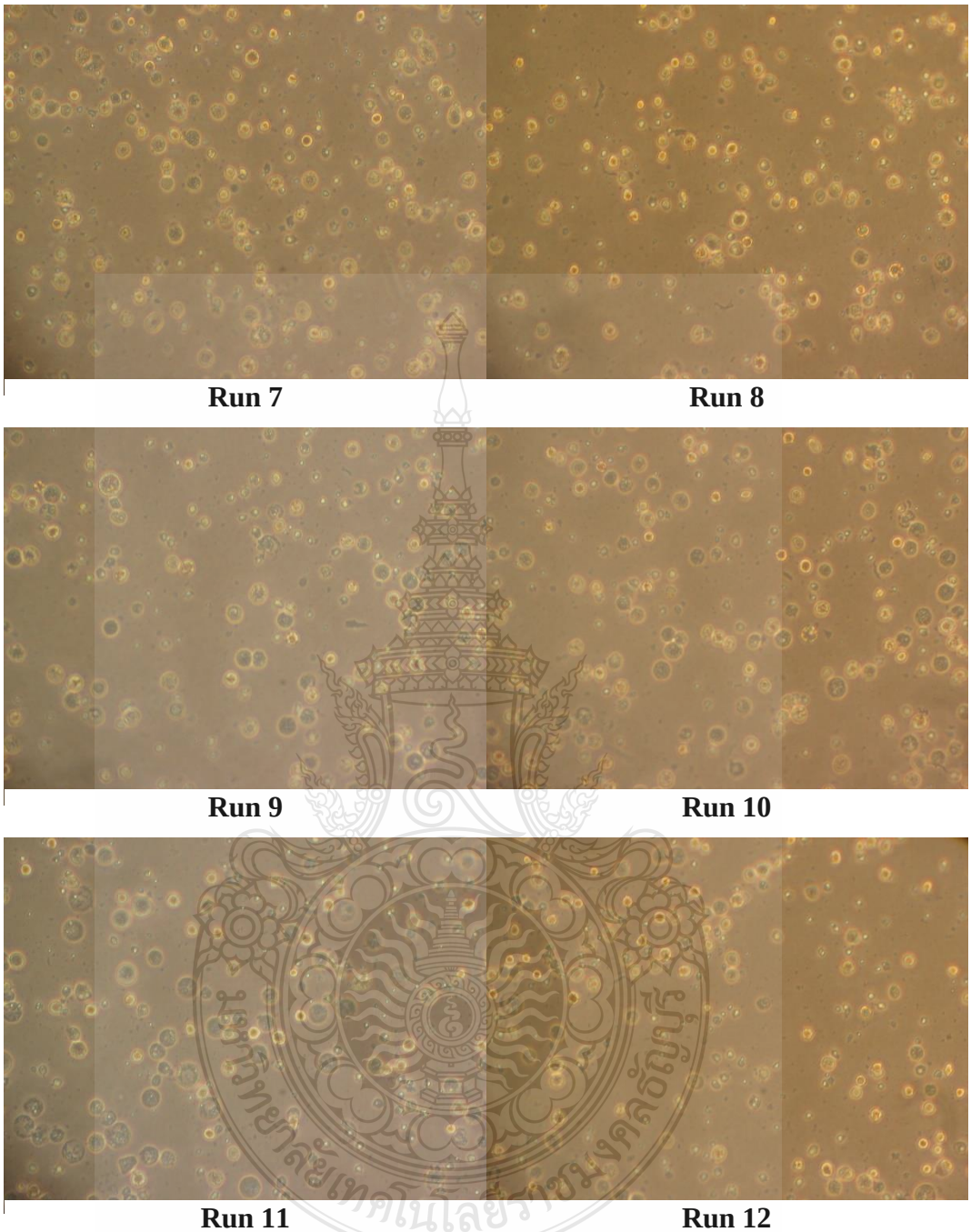
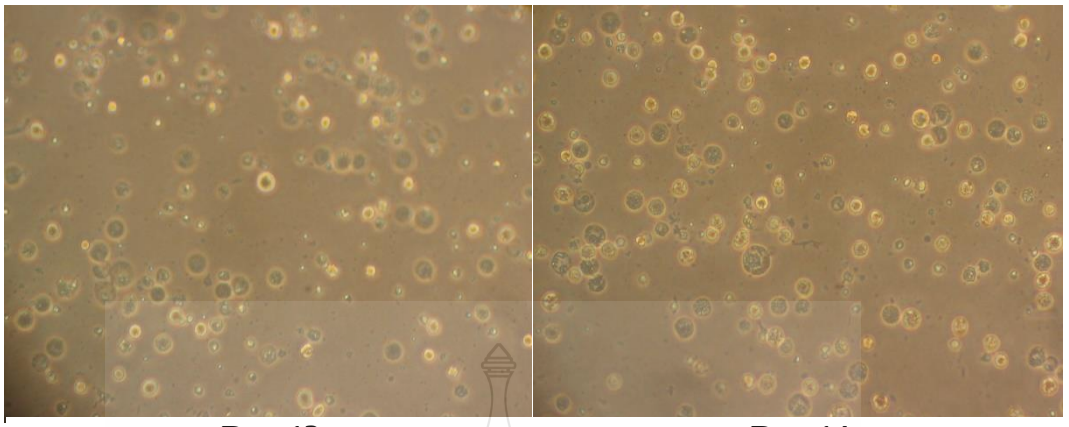
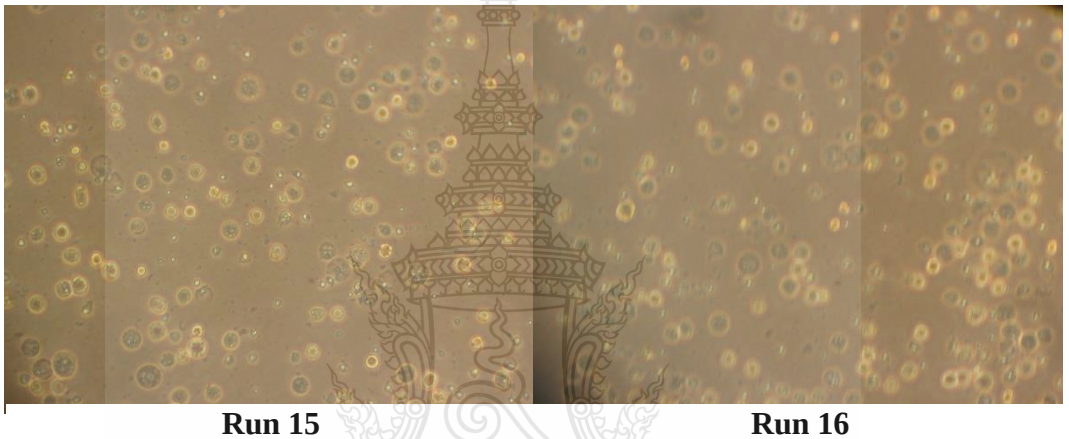


Figure 8 Cell growth of day 3 (Continued)



Run 13 **Run 14**



Run 15 **Run 16**

Run 17 **Run 18 (Control)**

Figure 9 Cell growth of day 3 (Continued)

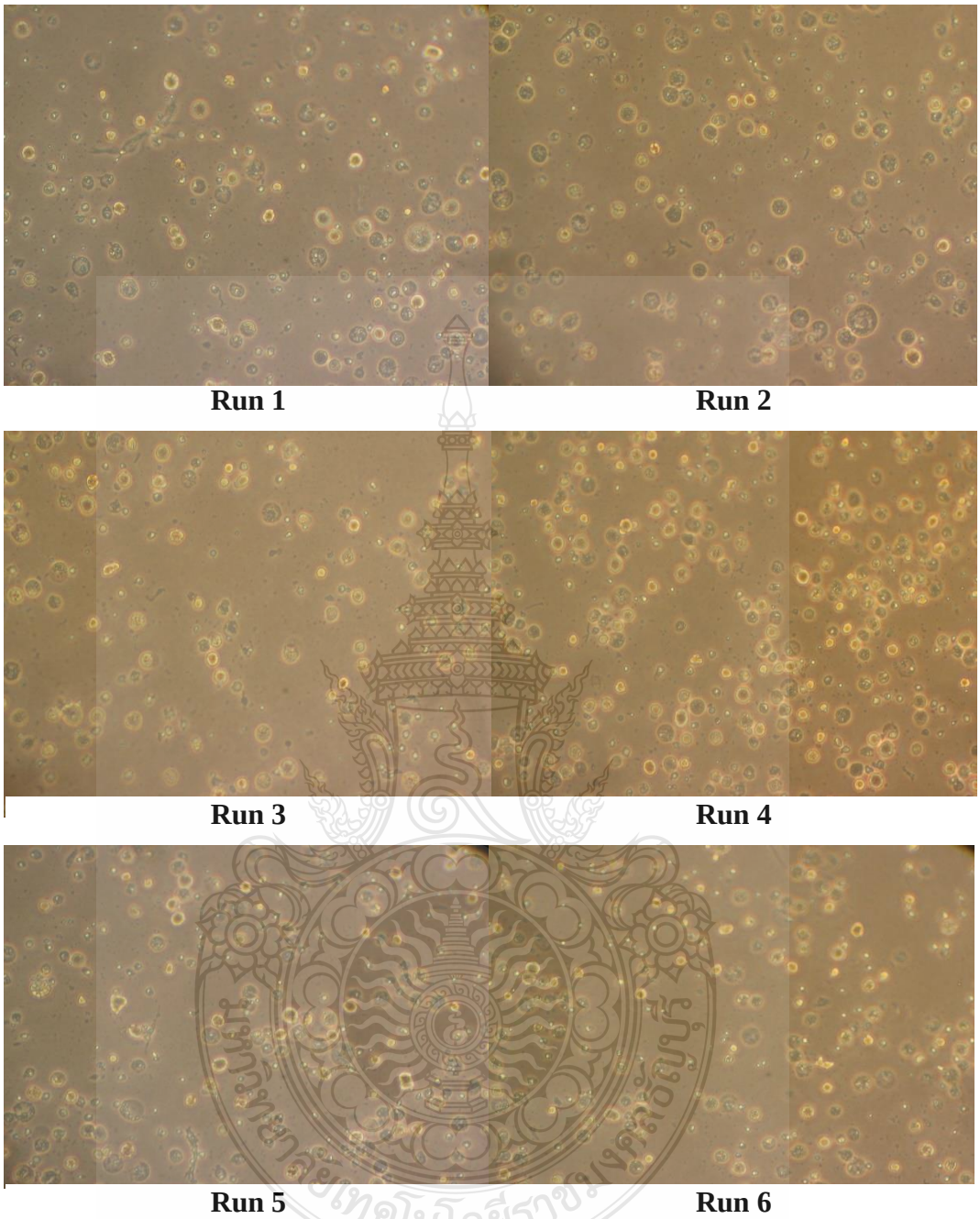
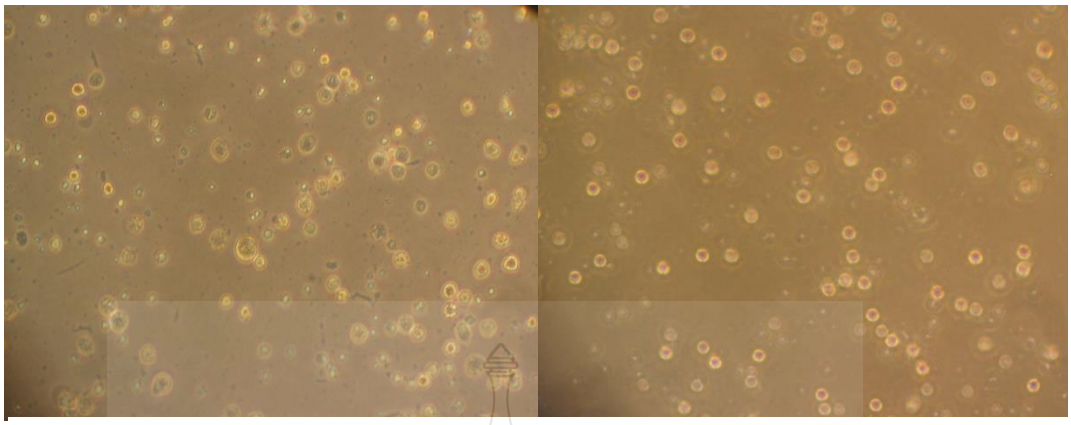
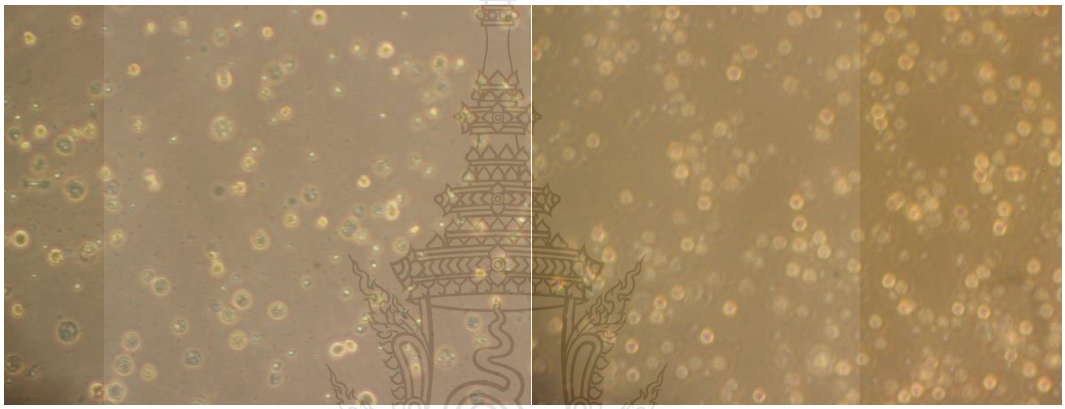


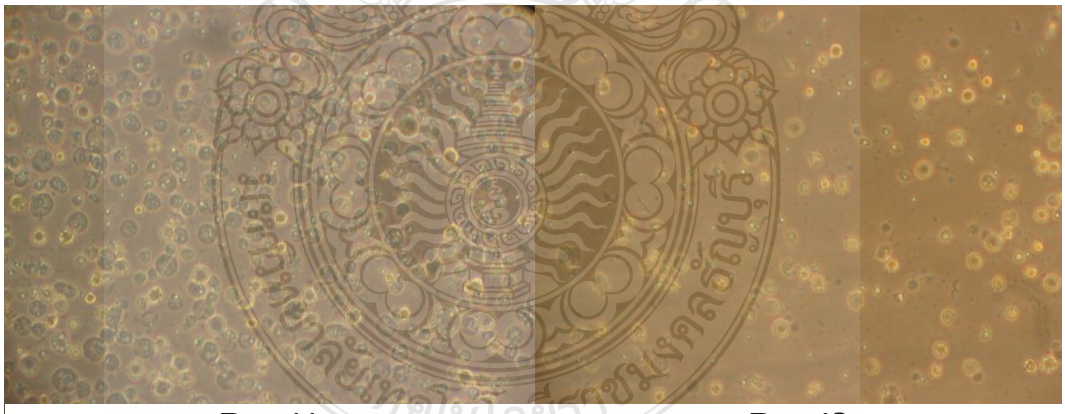
Figure 10 Cell growth of day 4



Run 7 **Run 8**

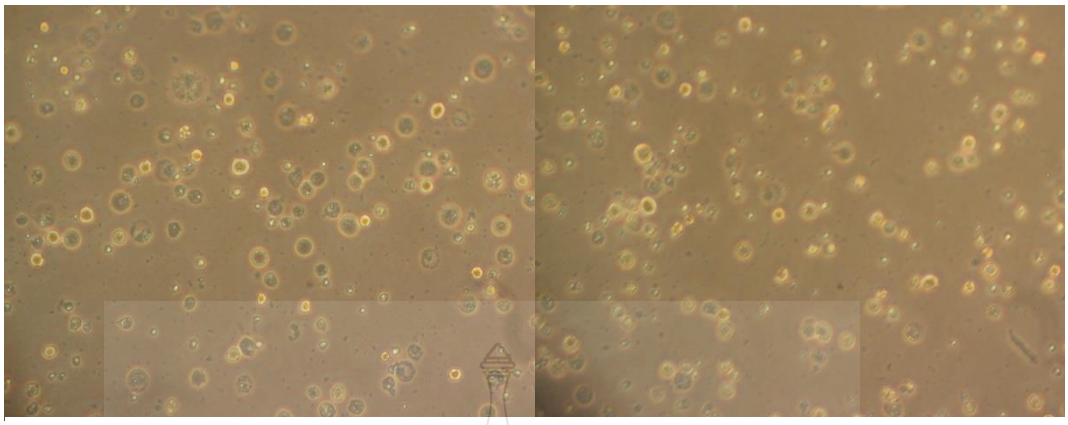


Run 9 **Run 10**

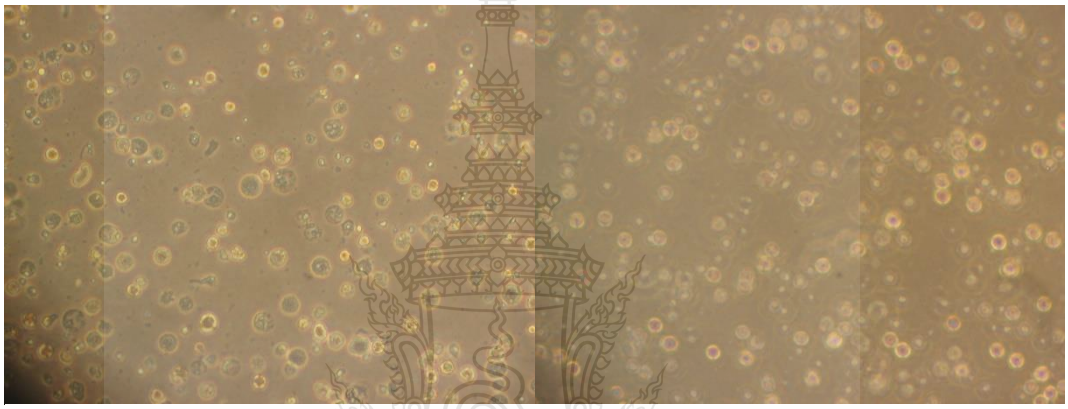


Run 11 **Run 12**

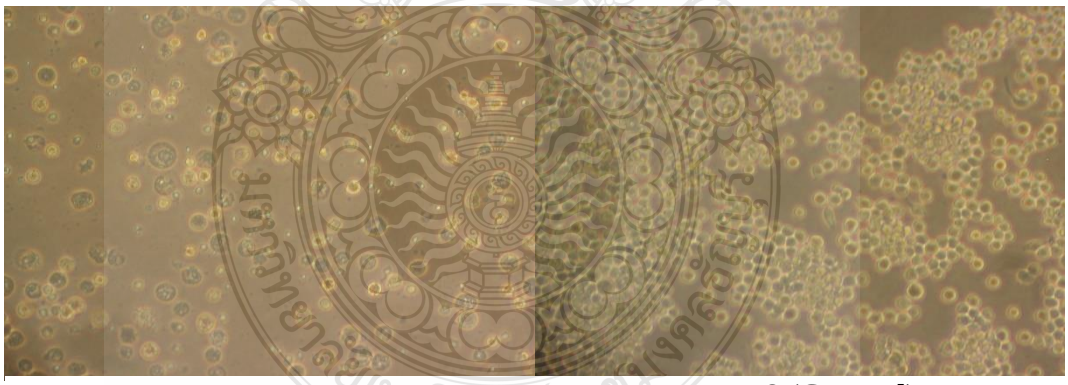
Figure 11 Cell growth of day 4 (Continued)



Run 13 **Run 14**

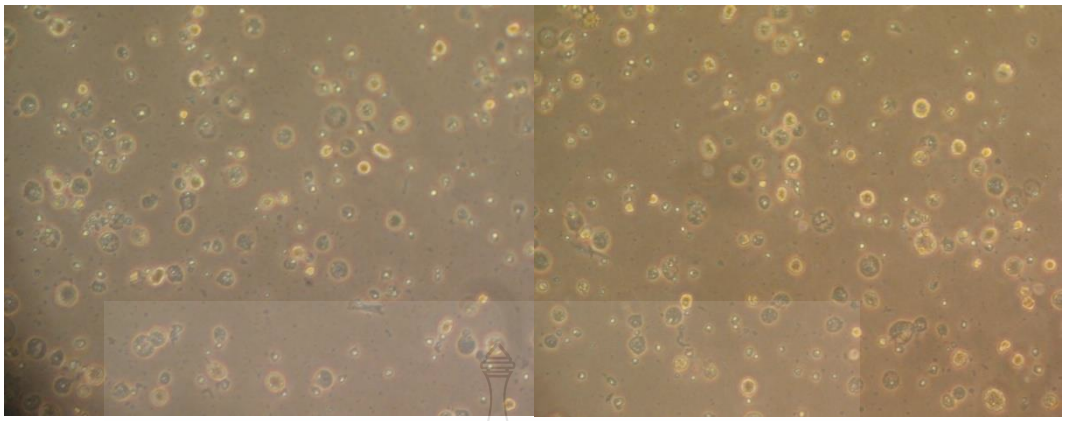


Run 15 **Run 16**

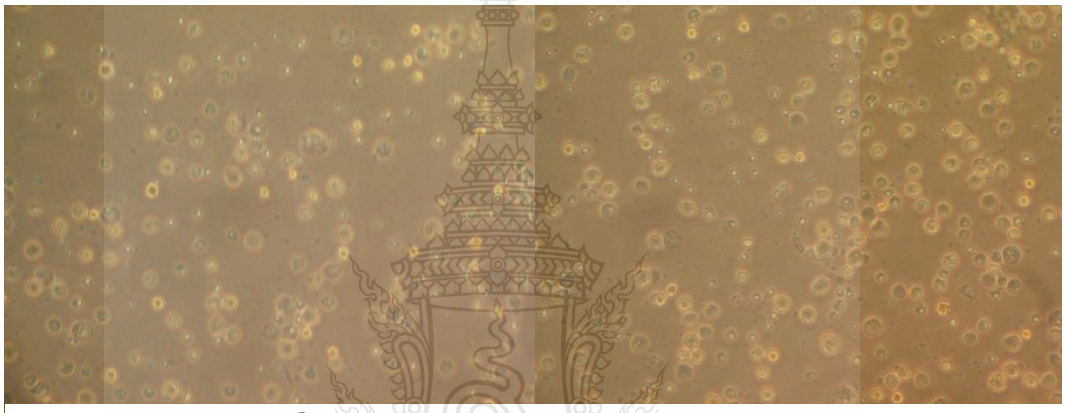


Run 17 **Run 18 (Control)**

Figure 12 Cell growth of day 4 (Continued)



Run 1 **Run 2**



Run 3 **Run 4**

Run 5 **Run 6**

Figure 13 Cell growth of day 5

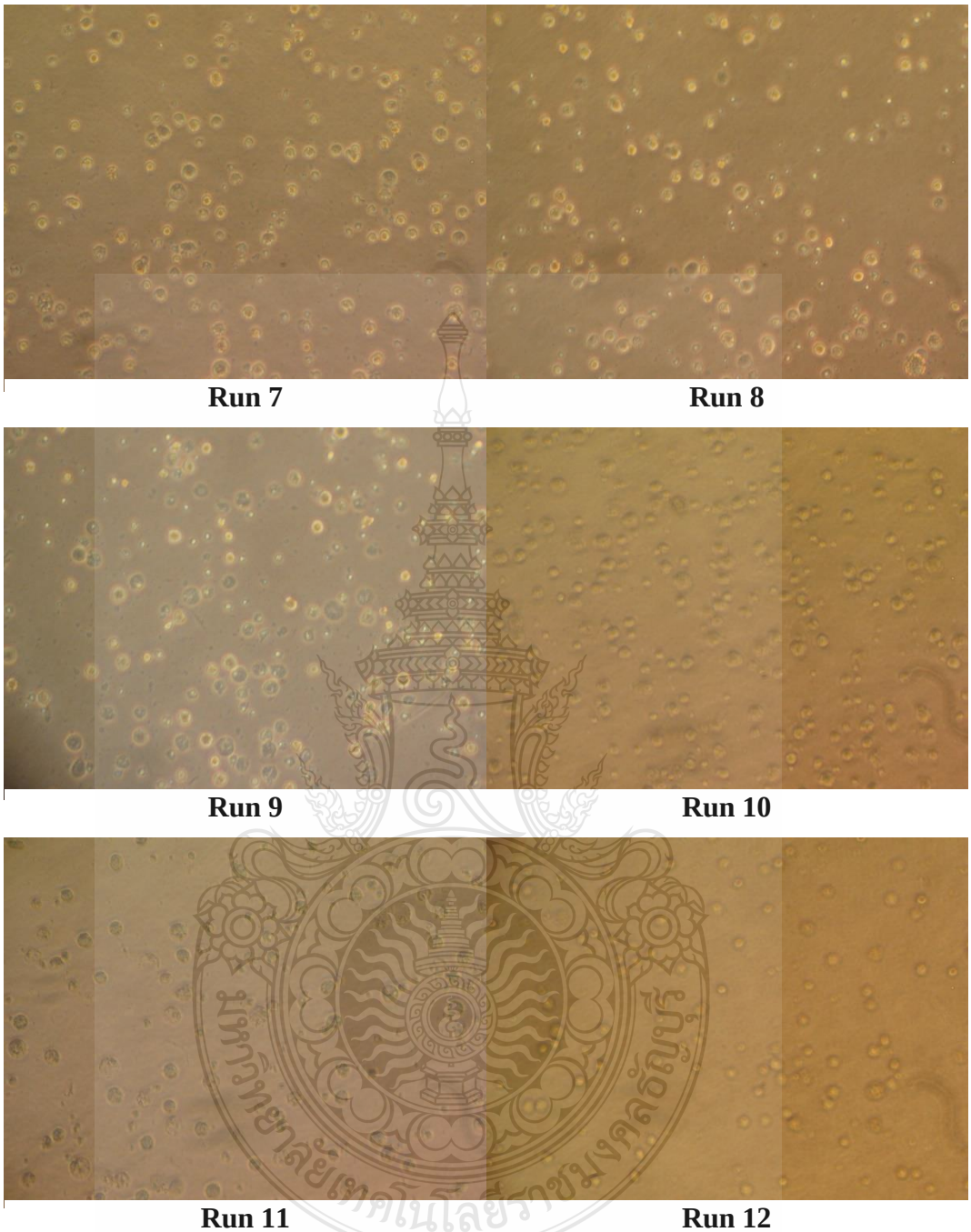
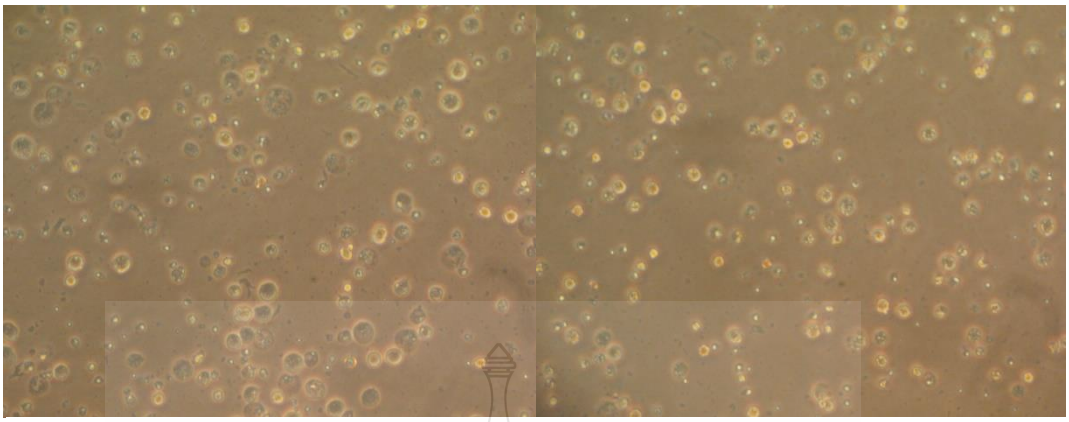
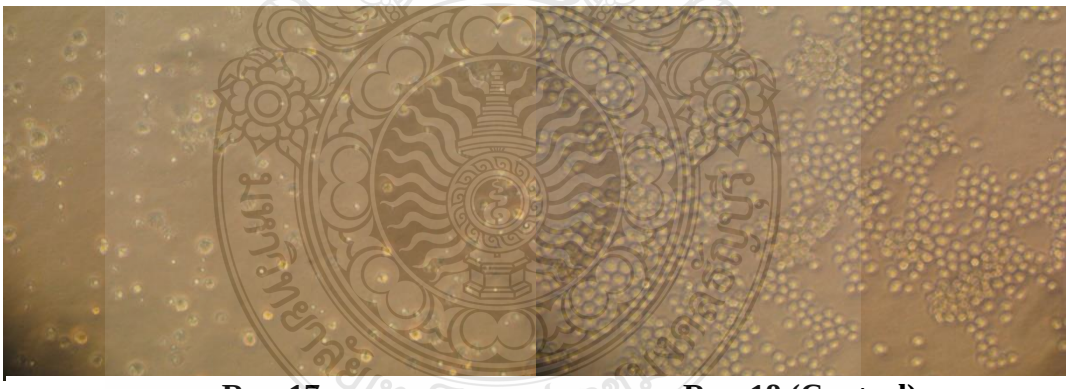


Figure 14 Cell growth of day 5 (Continued)



Run 13 **Run 14**

Run 15 **Run 16**



Run 17 **Run 18 (Control)**

Figure 15 Cell growth of day 5 (Continued)

APPENDIX C
Paired T-Test of optimal cell density studies in static culture

Paired T-Test

Paired T-Test and CI: C1, C2
Paired T for C1 - C2

	N	Mean	St Dev	SE Mean
C1	2	1.355	0.092	0.065
C2	2	6.680	0.212	0.150
Difference	2	-5.3250	0.1202	0.0850

95% CI for mean difference: (-6.4050, -4.2450)
T-Test of mean difference = 0 (vs not = 0): *t*-value = -62.65 *p*-value = 0.010

Paired T-Test and CI: C1, C3
Paired T for C1 - C3

	N	Mean	St Dev	SE Mean
C1	2	1.355	0.092	0.065
C3	2	5.255	0.177	0.125
Difference	2	-3.9000	0.0849	0.0600

95% CI for mean difference: (-4.6624, -3.1376)
T-Test of mean difference = 0 (vs not = 0): *t*-value = -65.00 *p*-value = 0.010

Paired T-Test and CI: C1, C4
Paired T for C1 - C4

	N	Mean	St Dev	SE Mean
C1	2	1.3550	0.0919	0.0650
C4	2	4.0500	0.1273	0.0900
Difference	2	-2.6950	0.0354	0.0250

95% CI for mean difference: (-3.0127, -2.3773)
T-Test of mean difference = 0 (vs not = 0): *t*-value = -107.80 *p*-value = 0.006

Paired T-Test and CI: C2, C3
Paired T for C2 - C3

	N	Mean	St Dev	SE Mean
C2	2	6.680	0.212	0.150
C3	2	5.255	0.177	0.125
Difference	2	1.4250	0.0354	0.0250

95% CI for mean difference: (1.1073, 1.7427)
T-Test of mean difference = 0 (vs not = 0): *t*-value = 57.00 *p*-value = 0.011

Paired T-Test and CI: C2, C4
Paired T for C2 - C4

	N	Mean	St Dev	SE Mean
C2	2	6.680	0.212	0.150
C4	2	4.050	0.127	0.090
Difference	2	2.6300	0.0849	0.0600

95% CI for mean difference: (1.8676, 3.3924)
T-Test of mean difference = 0 (vs not = 0): *t*-value = 43.83 *p*-value = 0.015

Paired T-Test and CI: C3, C4
Paired T for C3 - C4

	N	Mean	St Dev	SE Mean
C3	2	5.255	0.177	0.125
C4	2	4.050	0.127	0.090
Difference	2	1.2050	0.0495	0.0350

95% CI for mean difference: (0.7603, 1.6497)
T-Test of mean difference = 0 (vs not = 0): *t*-value = 34.43 *p*-value = 0.018

APPENDIX D

Comparison of prices between commercial serum-free and OSF9-SFM

Medium and supplements	Amount/volume	Price (THB/Liter)	Price (USD/Liter)
Commercial serum-free			
SF-900II		8500	246.50
OSF9-SFM			
Grace's insect powder	45.7 g/L	1027	29.78
CDLC	0.5% v/v	353.1	10.24
Yeast extract	11 g/L	51	1.48
Soytone	3 g/L	22	0.64
D-glucose	9 g/L	45	1.31
sodium hydrogen carbonate	0.35 g/L	2.2	0.06
methyl cellulose	0.1% (w/v)	47	1.36
Pluronic F68	0.1% w/v	514	14.91
vitamin B12	0.00015 g/L	2	0.06
MEM vitamin solution (100x)	0.5% (v/v)	102	2.96
L-glutamine	0.876 g/L	40	1.16
10 kDa Centrifugal Filter	1 tube	300	8.70
Total			72.65

NOTE : "1 THB = 0.029 USD" Comparison can be seen that the price difference is 173.85 USD or 70.53 %

Biography

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