Towards a Chemically Defined Medium for Sf-9 Cell Culture: Micronutrients Reduce Dependence on Yeast Extract

by

Scott Joseph Boegel

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Applied Science

in

Chemical Engineering

Waterloo, Ontario, Canada, 2019

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Spodoptera frugiperda clonal isolate 9 (Sf-9) insect cells in conjunction with recombinant baculovirus are an industrially relevant system for producing biologics. Sf-9 cells are capable of robust high-density growth in single cell suspension. However, unlike many other continuous cell lines, Sf-9 cell culture media remains undefined. Typically, the growth medium requires undefined hydrolysate supplementation (most often yeast extract) in order to support cell proliferation. The lack of chemical definition makes medium and process optimization difficult, leads to batch-to-batch variability, and potentially affects downstream processing. This work aims to combine available information on the composition of yeast extract and the composition of media for other cell lines to reduce the concentration of undefined components (yeast extract) in the medium and elucidate the effects of micronutrient compounds.

Utilizing an in-house medium based on the classic IPL-41 medium with yeast extract as the only undefined component, several steps were taken towards chemical definition. Through fortifying the trace metal and vitamin content in the medium and the addition of 11 micronutrients, the yeast extract content was successfully reduced 10-fold (from 4 g/L to 0.4 g/L). Without medium fortification and micronutrient addition, the cells were incapable of growth at low yeast extract concentration. Sf-9 cells adapted to this new medium were capable of long-term consistent growth. Micronutrients of key importance in this medium were identified as glycine betaine, ascorbic acid, and the polyamine putrescine. The presence of glycine betaine (1 mM), ascorbic acid (10 μM), and putrescine (10 μM) improved maximum cell density by 32%, 41%, and 28% respectively in the low yeast

extract medium. The role of these micronutrients could be properly investigated only after medium enhancement and yeast extract reduction. Further, this medium was found to be cost-effective compared to commercially available alternatives and the potential for added cost-savings related to lipid supplementation was identified.

This enhanced low yeast extract medium could allow for micronutrient and other component investigation with less convolution and is particularly applicable to designed compound screening experiments (e.g. Plackett-Burman). Identification and supplementation of additional required components provided solely by the yeast extract could lead to a chemically defined medium.

Acknowledgements

First, I would like to thank my supervisor, Professor Marc Aucoin. Thank you for the opportunity to pursue this degree in your lab, and for steering me in the right direction while allowing me the freedom to explore where the results led me.

Next, I want to thank all of my lab mates from throughout my time here. You have all contributed to making the Aucoin Lab truly a great environment. In particular, I would like to thank Mark Bruder for his depth of knowledge, experimental advice, and for his constant willingness to discuss and formulate ideas. Megan Logan for her advice and guidance in general, and for sharing her cell culture media expertise. Marco Quattrociocchi for setting me down this path and for providing the preliminary data from which much of this thesis stems. Sadru Waliji for the much-needed sports and miscellaneous discussions.

Finally, I want to thank my friends and family who are always there for me. I apologize for the countless "quick" trips to the lab.

Dedication

For my parents, their support unwavering.

Table of Contents

List of Tables

List of Figures

Nomenclature

AcMNPV *Autographa californica* nuclear polyhedrosis virus

ALIM Aucoin lab insect cell culture media

DMSO Dimethyl sulfoxide

F.A. Feed additive

FBS Fetal bovine serum

IC-BEVS Insect cell-baculovirus expression vector system

NMR Nuclear magnetic resonance

PBS Phosphate-buffered saline

pGICs Potential growth inducing compounds

Sf-9 *Spodoptera frugiperda* clonal isolate 9 cell line

YX Yeast extract

Chapter 1

Introduction

1.1 Introduction

Continuously dividing cell lines have wide application in the production of viral vaccines and other biologics. Insect cell culture in particular is of interest in these applications due to its relative safety and robustness. Recombinant baculovirus allows for the exploitation of insect cell culture, specifically *Spodoptera frugiperda* clonal isolate 9 (Sf-9), to produce proteins of interest. This platform is known as the Insect Cell-Baculovirus Expression Vector System (IC-BEVS). Recent developments in this field have seen an increased applicability to commercially approved products [1].

Insect cell culture growth media utilizes a basal medium containing a mixture of carbohydrates, amino acids, salts, trace metals and vitamins. Historically, this basal medium has been supplemented with undefined components and is otherwise incapable of support-

ing cell proliferation. Undefined supplements have progressed from insect hemolymph [2] to animal sera [3] to non-animal derived plant hydrolysates and yeast extract [4, 5]. Yeast extract is often the most successful complex media additive in insect cell culture and is most prominently utilized. Presently, the most popular commercially available Sf-9 insect cell media are proprietary in formulation and still contain undefined hydrolysates or yeast extract (e.g. Gibco^{TM} Sf-900III). However, the use of undefined components results in the potential for batch-to-batch variability and difficulties in medium optimization and metabolic understanding.

Replacement of yeast extract with known components would result in a chemically defined medium. In contrast to many mammalian cell lines, a chemically defined medium for Sf-9 insect cell culture has been slow to develop. Only recently (2018), has a chemically defined Sf-9 medium become available (Gibco^{TM} ExpiSf^{TM} CD). However, this medium is proprietary in formulation and requires the use of an Sf-9 derivative cell line, ExpiSf9^{TM} to guarantee results. A known chemically defined formulation would allow for batch-to-batch consistency and for the ability to study the effects of compounds without convolution from undefined components. This would allow for a greater understanding of Sf-9 insect cell metabolism and would enable optimization of the medium for specific processes. Additionally, a chemically defined medium could simplify the purification of products of interest produced via IC-BEVS.

1.1.1 Hypothesis and Objectives

This work utilizes an in-house medium ("ALIM + YX"), which is a simplified version of the classic published IPL-41 medium [3], containing yeast extract as the only undefined component. It is theorized that by studying available partial yeast extract characterization, particularly characterization performed previously in this lab on the utilized yeast extract products [6], and by studying chemically defined medium formulations for other cell lines, the necessary components to replace yeast extract can be elucidated. It is hypothesized that the effects of simply adding compounds of interest to a typical medium are likely to be convoluted by their presence or the presence of similar compounds in the yeast extract. As such, a more robust platform for compound screening must be developed, where live cell density may be used to quantify medium component effects.

The primary objective of this work is to develop an Sf-9 cell culture medium platform where components of interest can be tested and their effects determined with more certainty and less convolution. To achieve this, the yeast extract concentration will be reduced via media alterations and cell adaptation. The secondary objective is to utilize this low yeast extract medium in designed screening experiments in order to determine necessary compounds in a step-wise fashion. This process should eventually lead to a fully chemically defined medium. The economic benefits of the in-house medium will also be analyzed and optimized.

1.2 Approach to Objectives

The experimental approach and structure of this work are as follows:

1. Investigate in-house medium and feed additives as they compare to commercially available products (Chapter 4).

2. Optimize and fortify in-house medium utilizing information gained from preliminary experimentation (Chapter 4).

3. Alleviate some of the limiting compounds to chemically defined growth based on other medium formulations and partial yeast extract characterization. Through micronutrient addition, minimize the concentration of undefined yeast extract in the medium (Chapter 4).

4. Utilize the developed enhanced low yeast extract medium for designed compound screening experiments (Chapter 5).

5. Investigate the economic viability of the in-house medium and potential for further improvement (Chapter 6).

Chapter 2

Literature Review

2.1 Insect Cell Culture

2.1.1 Continuous Cell Culture

Cell culture is widely used for the production of biologics, such as therapeutic proteins and viral vaccines. Animal cells are ideal for such production as they naturally have the ability to create and modify proteins in complex ways [7]. Primary cell culture is when cells are taken directly from an animal and then utilized. These cells may also be cultured into a cell line, where they can be grown and passaged but only for a finite amount of time. After dividing a certain number of times the cells will no longer propagate, and such cell lines are considered secondary cell culture. A cell line that does not stop dividing is known as an immortal or continuous cell line. Continuous cell lines offer advantages over primary cell lines in that they can be grown to higher densities and are capable of secreting

more product [8]. Continuous cell lines can be propagated to a certain passage number and frozen as cell banks. This process allows for the creation of an almost unlimited supply of consistent cells and results in lower cost and higher availability [9]. Additionally, such cell lines can be screened to ensure that they do not contain adventitious pathogens, which is an inherent risk with animal derived cells and cell lines that have been exposed to animal serum [10]. These advantages mean that continuous cell lines are desirable for the consistent and cost-effective production of biologics, although they must go through strict regulatory procedures before being approved. These procedures include testing for tumourigenicity and viral contamination [8]. In addition to creating products, animal cell lines are also useful in that they allow for virus replication and propagation, and as a result are highly desirable for human and animal viral vaccine production.

2.1.2 Insect Cell Lines

Insect cell lines are of particular interest due to their ability to be grown readily in suspension and serum free cultures, and to high cell densities [11, 12, 5]. Additionally, insect cells possess the ability to perform post-translational processing of proteins in a similar manner to mammalian cells [13, 14, 15]. The combination of these factors results in a relatively simple, safe, and cost-effective system for the production of biologics. A large number of insect cell lines from a variety of orders have been developed [16]. Of particular note are cell lines developed from Lepidoptera [17] due to their susceptibility to baculovirus infection and replication. One of the most useful and popular of these Lepidopteran cell lines comes from the ovarian tissue of the fall army worm (*Spodoptera frugiperda*), which

was initially developed by Vaughn et al as IPLB-SF-21 [18]; adaptation of this cell line to a less complex, hemolymph-free medium resulted in IPLB-SF-21-AE [19]. *Spodoptera frugiperda* clonal isolate 9 (Sf-9) was subsequently isolated from this cell line. Sf-9 is one of the most commonly used insect cell lines and its popularity stems from the versatility and robustness (e.g. suspension, serum-free and high density growth) of the cells, and further, due to their high susceptibility to baculovirus infection [20].

2.1.3 Baculovirus

The driving force behind academic and industrial interest in insect cell culture is its susceptibility to baculovirus infection, particularly by *Autographa californica* nuclear polyhedrosis virus (AcMNPV). AcMNPV was originally isolated from the Alfalfa Looper (*Autographa californica*) and demonstrated high infectivity [21]. AcMNPV is capable of replication in Lepidopteran species, including the Sf-9 cell line. The virus encodes for polyhedrin protein under the control of a very strong, very late promoter (polh). However, it was determined that polyhedrin is not required for viral replication in cell culture [22], and this can be exploited to recombinantly express a protein of interest, as first demonstrated by Smith et al [23]. Likewise, the baculovirus P10 protein has also been shown to be unnecessary for viral replication, and its very strong, very late promoter (p10) has been similarly exploited [24]. The use of recombinant AcMNPV in insect cell culture (e.g. Sf-9) for protein expression is known as the insect cell-baculovirus expression vector system (IC-BEVS). Since its initial development, IC-BEVS has been utilized in the production of thousands of recombinant protein products [24, 25], and recently has seen an increase in products

approved for both veterinary and human use [1].

2.2 Insect Cell Media

In the IC-BEVS system, the cell is responsible for the actual production of the protein of interest. It follows that cell metabolic requirements are important when designing an IC-BEVS process, both prior to and after baculovirus infection. Currently, these metabolic requirements are still not fully understood. This section will address the present understanding of insect cell metabolism as it relates to growth media design, with specific focus on Sf-9 cell culture.

2.2.1 Historical Media

Early success in insect culture media development came by means of mimicking properties of insect hemolymph in basal media, and supplementing that media with hemolymph of the species of interest. Wyatt pioneered this strategy in culturing the Lepidopteran *Bombyx mori* [26]. Grace improved on this medium through the addition of several vitamins and by means of matching osmotic pressure, pH and ion ratios of his medium to that of the Lepidopteran *Antheraea eucalypti*, which resulted in the first continuous insect cell lines [2]. Multiple improvements were made to Grace's medium in the following years, notably eliminating the need for insect hemolymph by utilizing a variety of animal-derived complex components (most prominently fetal bovine serum (FBS)) [27, 19, 28]. Undefined supplements such as FBS were more desirable for these early media formulations

than insect hemolymph due to their ease of availability. These formulations were further improved by Weiss et al in the development of IPL-41 growth medium for *Spodoptera frugiperda* [3], which incorporated higher concentrations of most amino acids and vitamins and utilized 10% FBS. Most subsequent and current serum-free insect culture media are based on IPL-41, as are the formulations utilized in this study.

2.2.2 Traditional Media Composition and Cell Metabolism

2.2.2.1 Sugars

Early media formulations utilized multiple sugars as potential energy sources for the cell, typically the disaccharides sucrose and maltose, as well as glucose [26, 2, 27, 28]. However, it was shown early on that Sf cells could subsist and proliferate with glucose as the only sugar source [19]. While Sf-9 cells have been shown to be capable of utilizing disaccharides and other sugars (e.g. fructose and lactate) as carbon and energy sources [29, 30], it is established that glucose is the most important carbohydrate energy and carbon source, and in fact, the only sugar necessary to include in media formulations [29, 30, 31]. Excess glucose in growth media results in the formation of alanine as a byproduct [32, 33]. Alanine formation represents energetic inefficiency; however, alanine does not accumulate to inhibitory levels [34], which is likely why glucose feed limitation strategies do not result in growth improvement [31], despite successfully limiting alanine formation [33]. It is clear that glucose can be used as the sole sugar source in Sf-9 cell culture media, and that concentration is not of extreme importance as long as it is sufficient to maintain exponential growth and achieve high cell densities. Glucose should be provided at levels sufficient to

maintain high growth, but excess should be kept to a minimum to avoid energetic inefficiency.

2.2.2.2 Amino Acids

Insect hemolymph contains a significant amount of free amino acids [35], and as a result early insect cell culture media had high amino acid concentrations. The development of IPL-41 basal media increased these free amino acid concentrations even further [3]. However, such media has shown to be inefficient, utilizing only 26% of the available amino acids during typical culturing, with high cell densities still achievable when significantly reducing the amino acid content [31].

The cell utilizes amino acids for energy, growth, compound synthesis and protein production. Some amino acids are essential for Sf-9 cells (i.e. cannot be synthesized by the cell) and must be supplied (e.g. glycine [36]), while others are supplied in excess to reduce the metabolic burden on the cell and enhance growth. Typical insect cell media contains 19-20 amino acids (alanine is included in early media formulations but not required). All amino acids are consumed to varying degrees during Sf-9 cell culture (except alanine which is produced), with the most significant consumption usually being of glutamine [31, 29]. Glutamine can function as an energy source (via the TCA cycle) as well as a significant carbon and nitrogen source for Sf-9 cells, and its role is well studied [30, 33, 37]. Notably, Ohman et al demonstrated that Sf-9 cells could be grown in a glutamine-free medium, although the inclusion of ammonium for glutamine biosynthesis was necessary [38]. However, a glutamine-free medium is likely simply increasing the metabolic burden on the cells

by forcing them to produce glutamine. The utilization of most amino acids by Sf-9 cells is likely for cell biomass proteins, although several amino acids are utilized for energetic purposes (e.g. aspartate, asparagine, glutamate, glutamine and serine [31, 34], as well as arginine and methionine [34]).

While amino acids are likely not at optimal concentrations in current media, optimization would be laborious due to their complex metabolic interactions with one another and with other media components. There are likely different optimal concentrations for each cell line (e.g. [39]). Further, hydrolysates are providing an undefined and variable source of amino acids in addition to those provided in basal medium [40]. If a chemically defined medium were developed it would then be prudent to optimize amino acid concentrations for growth and/or protein production. Mathematical modeling approaches would be useful in this scenario, having been employed in similar ways for insect cell feed additive design [41, 42]. During development of chemically defined media, current amino acid composition is adequate for all amino acids, and likely not of extreme importance.

2.2.2.3 Trace Metals

Trace metals or inorganic trace elements undoubtedly play a significant role in eukaryotic cells, largely as co-factors [43, 44, 45]. Historically, such metal ions have been introduced to media as impurities resulting from water or component contamination [46], a problem which may persist today [47]. Additionally, insect hemolymph [35], animal sera [48], hydrolysates [49] and other undefined media components provide a variety of trace elements to the medium. Previous work in this laboratory identified several trace metals present

in yeast extract [6]. Although trace element requirements of Sf-9, or even insect cells in general have not been well investigated, they are likely a required component provided in modern serum-free media by undefined hydrolysates. IPL-41 provides several trace elements which were not previously contained in insect cell media (e.g. Co, Cu, Fe, Mn, Mo and Zn) [3]. In contrast to the inorganic trace elements provided by IPL-41, defined media for other animal cell lines [50, 51], various media patents [52, 53, 54], and several commercially available products (e.g. Corning™ Trace Elements A, B and C from Fisher Scientific, Pittsurgh, USA) contain significantly more trace element compounds. These additional trace metals, if required by Sf-9 insect cells, must be provided by the undefined hydrolysates utilized in conjunction with IPL-41 basal media. When attempting to remove undefined components towards a chemically defined media, careful consideration should be given to trace metals added.

2.2.2.4 Vitamins

Vitamins are organic compounds, some of which are necessary in cell culture. Vitamins play a variety of cellular roles, most prominently as enzyme co-factors [55]. Grace incorporated the water-soluble B vitamins (thiamine, riboflavin, niacin, pantothenate, pyridoxine, biotin and folate) and B vitamin-like compounds (4-aminobenzoic acid, inositol and choline) into early successful insect cell culture media [2]. These have remained similar throughout the development of insect cell media, with IPL-41 only differing in its incorporation of Vitamin B12 (cyanocobalamin) and increased concentrations of all B vitamins [3]. The lipid soluble Vitamin E (alpha tocopherol acetate) played a key role in the development of a complex lipid solution for serum replacement by Inlow et al [5], where it acts as an antioxidant.

There are several vitamins not typically included in insect cell culture that are worth consideration in media development. Vitamin C (ascorbic acid) is a known antioxidant in insect cells [56] and may have other roles in cell culture [57]. It is apparent that ascorbic acid may be an important additive in chemically defined insect cell media [58]. The role, if any, of the fat-soluble vitamin classes A, D and K have not been investigated in insect cell culture.

Serum provides proteins that stabilize the vitamins in media. With the move towards chemically defined media, care must be taken with respect to the stability of the vitamins in solution. Stability of the B vitamins in terms of: chemical interaction, pH, light exposure, oxidation and temperature is well reviewed [59] and of particular interest when eliminating serum and hydrolysates from insect cell culture media. Previous work in this laboratory has shown high concentrations of B vitamins are contained in yeast extract [6], and it follows that replacement of the yeast extract likely requires increasing basal medium B vitamin concentrations.

2.2.2.5 Lipids

Lipids are well known as integral components in continuous cell culture and play a variety of roles (e.g. membrane structure, cell signaling, energy) [60]. However, lipid composition differs significantly between mammalian and Sf-9 cells [61]. At least dietarily, insects cannot synthesize sterols [62], and early media provided these and other lipid components by means of hemolymph addition [63]. The replacement of hemolymph with animal sera maintained the supplementation of necessary lipids [60].

The importance of lipids in Sf-9 culture media was demonstrated by the necessity of a complex lipid supplement when replacing animal serum [5, 4]. This supplement was composed of: cholesterol, alpha tocopherol acetate (as an antioxidant), Tween-80 and undefined cod liver oil fatty acid methyl esters. These components were dissolved in ethanol and solubilized utilizing the polyol Pluronic^{TM} F-68. In the 30 years since the development of this lipid supplement, very little has changed, and similar supplements are widely used today. A current popular lipid media supplement, chemically defined *Lipid Mixture 1* (Sigma) has improved on this early lipid supplement only slightly, replacing the cod liver oil fatty acid methyl esters with a defined combination of non-animal derived fatty acids (arachidonic, linoleic, linolenic, myristic, oleic, palmitic and stearic).

Gilbert et al studied the variation of these lipid mixture components in High-Five^{TM} insect cells [64]. While minimal growth effects were seen in the absence of the supplement's lipid components, cholesterol and Tween-80 were shown to be critical for protein production. It is useful to note that these experiments were carried out in a yeast extract containing medium. More careful study of the lipids and their related compounds (e.g. choline, ethanolamine), and their necessity and availability in Sf-9 cell media is likely of importance when developing and optimizing a chemically defined medium. This is of particular interest from a cost perspective, as currently available lipid concentrates are a relatively expensive component in insect cell media.

2.2.2.6 Inorganic Ion Ratios

Inorganic salts are always included in cell culture media, with early insect media mimicking the hemolymph of that particular species. Particular attention is paid to the ratio of Na^+ to K^+ ions which varies considerably amongst insect species. Lepidopteran media is typically already tailored in this respect to these species. However, it has been established that insect cells can tolerate a wide range of ionic ratios (e.g. no effect on growth rate was observed when altering *Spodoptera* media from a physiological Na^+:K^+ of 0.67 to 1.38 [63]). As such there is likely little room for improvement in this aspect of media development.

2.2.2.7 Other Components

Organic Acids Insect hemolymph contains relatively high concentrations of TCA cycle intermediate organic acids [65] and as a result, insect cell media typically include these compounds (e.g. IPL-41 contains malic, α-ketoglutaric, succinic and fumaric acids [3]). However, some insect cell media has been shown to support growth in the absence of these organic acids [19], and so their necessity in media is unclear. The inclusion of these organic acids is likely an artifact of early hemolymph analysis as Sf-9 cells are capable of synthesizing the organic acids through their fully functioning TCA cycle [66]. However, such compounds may be of use to relieve metabolic burdens, particularly during protein production.

Antioxidants Cell culture is a highly oxidative environment and protective or "antioxidant" compounds are critical for cell health; however, they act in a complex and often

convoluted manner [67]. Examples of antioxidants utilized in cell culture include: alpha tocopherol acetate (Vitamin E; prevents lipid oxidation), ascorbic acid, cysteine, glutathione and selenium (acts in conjunction with other antioxidants; seleno-enzymes) [56, 68]. Oxidative stress has been shown to increase in Sf-9 culture after baculovirus infection, and as such is of interest in IC-BEVs systems [69]. Many of these antioxidants were provided in insect cell media previously by animal sera and presently by yeast extract or plant hydrolysates. A move towards chemically defined media would require a greater understanding of antioxidant roles and requirements in Sf-9 cells.

Chelators When trace metals are introduced to cell culture in complex solutions (e.g. animal sera or hydrolysates) they are often in chelated form. This reduces their toxicity and allows them to become accessible to the cell. Metal chelating agents are likely an important aspect of a chemically defined media [70]. Very little information is available on metal chelation specifically in insect cell media, however such compounds will be of interest when moving away from yeast extract supplementation.

2.2.2.8 Byproducts

As outlined previously, alanine represents the most significant byproduct in typical Sf-9 culture but does not accumulate to inhibitory levels. It is of interest to look at lactate and ammonia, which are the classic mammalian cell culture byproducts, and which often inhibit growth in these systems.

Unlike in mammalian cell culture, lactate does not accumulate during Sf-9 cell culture under normal conditions [71, 34]. Likewise, ammonia does not accumulate [34], except un-

der glucose-limiting conditions [32, 33]. Further, Bedard et al demonstrated that the addition of 10 mM of ammonia to Sf-9 cultures did not inhibit cell growth [29]. Very high (40-80 mM) ammonia and lactate concentrations have been shown to reduce β-galactosidase protein production in IC-BEVS [72].

Since metabolic byproducts do not appear to be inhibitory in typical Sf-9 culture, they are mostly of interest in terms of: metabolic efficiency, protein production, and specialty operating conditions that allow for greater accumulation (i.e. fed batch systems). In such cases, glucose and glutamine limited feeding strategies may be beneficial towards reducing overall byproduct accumulation [32].

2.2.3 Other Media Considerations

2.2.3.1 Osmotic Pressure

The osmotic pressure of a solution or medium is determined by the overall concentration of solutes, or the medium osmolality/osmolarity. The balance between osmotic pressure of the medium and intracellular osmotic pressure is important for cell health. Hyperosmotic stress occurs when medium osmolality is higher than intracellular osmolality, and conversely hypoosmotic stress occurs when medium osmolality is lower. These stresses negatively affect the cells ability to maintain homeostasis, interfering with water flux, and other cellular processes [73].

A study of 22 insect species found a hemolymph osmolality range of 319-421 mOsm/kg [74]. Insect hemolymph osmolality is notably higher than that of mammalian serum. Insect cell

culture media are typically kept within an osmolality range of 340-390 mOsm/kg [65], with IPL-41 calling for an osmolality between 360-375 mOsm/kg [3]. Similarly, Zhang et al showed that the optimal osmolality range for *Bombyx mori* insect cell growth was between 350-385 mOsm/kg, with maximum growth rate occurring at 370 mOsm/kg [75]. Generally, insect cells are considered to be less sensitive to osmolality changes than mammalian cells [76].

When producing media, osmolality is a good measure to maintain batch to batch consistency. Often, pH adjustment may result in high osmolality due to the addition of either HCl or NaOH to the medium. As such, fed batch operation may run in to the issue of high osmolality. In this case, there are a number of compounds that may act as "osmoprotectants" (e.g. glycine betaine, sarcosine, glycine). Such osmoprotection has been demonstrated in certain hybridoma cell lines [77, 78], but is poorly studied with respect to insect cell culture and may not apply.

2.2.3.2 pH

Much like with osmotic pressure, insect cell media pH is based on observations of insect hemolymph. Insect cell media pH is typically more acidic than that of mammalian cells, ranging from 6.2-6.9 [65], with IPL-41 calling for pH of 6.2 +/- 0.01 [3]. Consistent batch to batch media pH is important for consistent growth.

2.2.3.3 Shear Protection

Since Sf-9 cells are grown in suspension culture, mixing is required. This leads to shear stress, especially during large-scale operation, from which the cells must be protected. Care must be taken to ensure adequate oxygen is being supplied to the medium, while not exceeding critical shear force [79]. In early media, animal sera supplied adequate shear protection [80], however with the move toward replacing sera with hydrolysates, shear protectants were required. By far the most popular shear protectant media additive is the polyol Pluronic^{TM} F-68 (Polaxamer 188). Pluronic^{TM} F-68 has been shown to adequately protect insect cell culture from shear forces in the absence of serum [5, 81], and further to be necessary in such formulations [64].

2.2.4 Undefined Media Components

As described previously, early insect cell culture media utilized hemolymph from the insect of interest as a complex medium component. As large quantities of hemolymph were difficult to obtain and not readily available, it was desirable to replace it with complex animal derived components. Examples of these components include: whole egg ultrafiltrate, lactalbumin hydrolysate, and various vertebrate sera (turkey, chicken, fetal bovine). These complex animal components were able to successfully take the place of insect hemolymph in insect cell media, and eventually FBS was used as the sole complex medium component (e.g. IPL-41). However, there are various drawbacks associated with the use of FBS in cell culture. These drawbacks include: high cost and low availability, undefined and inconsistent composition, protein purification difficulty, and the potential presence of adventitious

agents or other contaminants [65, 82]. Potential for adventitious agents is of particular concern when producing biologics for human or animal use [83, 84].

The most notable breakthrough in replacing serum, and as such animal derived components in Sf-9 cell culture media was made by Maiorella and Inlow who replaced FBS in IPL-41 based media with a combination of 4 g/L yeast extract (also known as yeastolate or yeast hydrolysate) and a complex lipid emulsion [4, 5]. Yeast extract replaces the growth factors, trace elements and undefined micronutrients required for cell growth that were previously provided by FBS. The lipid emulsion provides necessary lipids which are likely not present in the yeast extract and further, utilizes Pluronic^{TM} F-68 as both a means to solubilize the lipids and as a necessary shear protectant in culture. The resultant insect serum-free media (ISFM) forms the basis for commercial media available today and for the media used in this study (particularly the starting point of 4 g/L yeast extract and the use of lipid emulsion). Simplified major early steps in insect cell media are outlined in Figure 2.1:

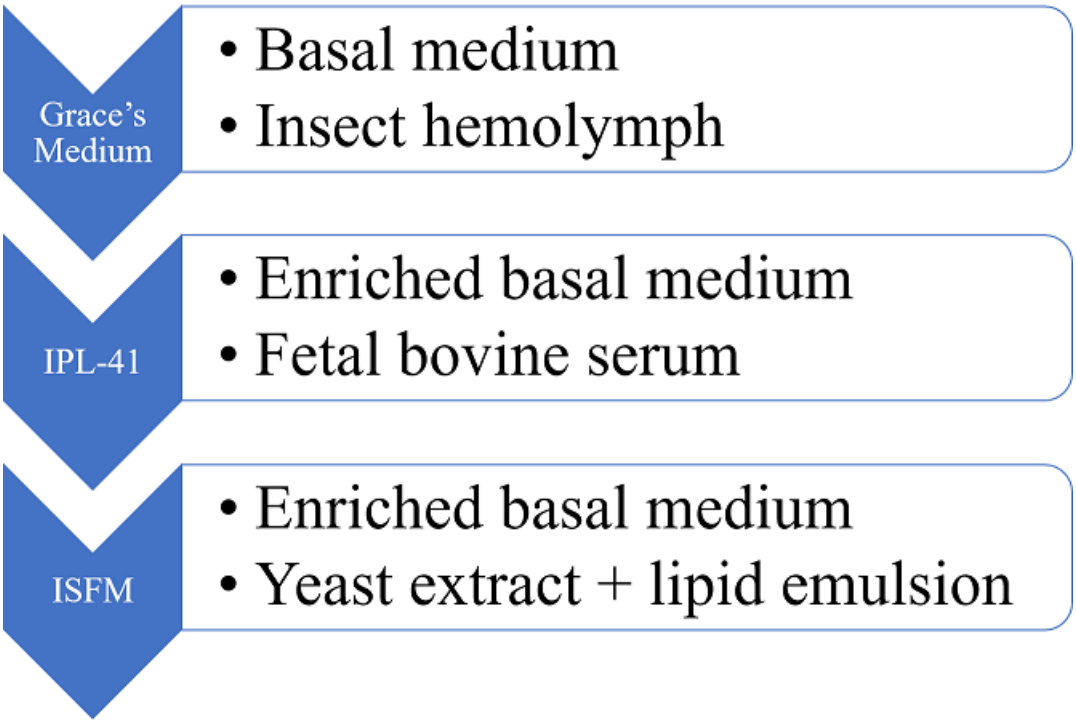

Figure 2.1: Simplistic outline of major milestones in animal component free insect media development. Grace's (Grace 1962); IPL-41 (Weiss et al 1981); ISFM (Maiorella et al 1988, Inlow et al 1989)

A variety of animal derived (e.g. lactalbumin, casein, primatone RL) [85, 86] and plant derived (e.g. wheat, soy, rice, pea) [87] hydrolysates have been tested in various combinations in insect and mammalian cell lines. Soy hydrolysate, in particular, has been used in conjunction with yeast extract in insect cell culture [88]. However, animal derived products are not ideal as discussed previously, and often yeast extract proves to be most effective supplement [85, 89].

2.3 Yeast Extract and Replacements

2.3.1 Properties of Yeast Extract

2.3.1.1 Basic Composition

Yeast extract (also referred to as yeast autolysate or yeastolate) is a complex undefined mixture of amino acids, carbohydrates, vitamins, trace metals, nucleic material and other micronutrients. A portion of yeast extract components are contained in complex forms such as oligopeptides and oligonucleotides [49, 90, 91]. BD Biosciences (Becton Dickinson, New Jersey, USA) has provided basic composition analysis of 6 of their yeast extract products. These compositions are summarized and compared in Table 2.1 and Figure 2.2 [49].

Table 2.1: Yeast extract general composition properties. Data selected from 6 products and adapted from BD Bionutrients Technical Manual 3rd edition. "%" refers to mass percentage.

Property	**Low Value**	**High Value**	**% Difference**
Total Nitrogen (%)	10.6	11.4	7%
Amino Nitrogen (%)	6.0	6.9	14%
AN/TN	0.54	0.61	12%
Total Carbohydrate (%)	6.8	16.3	82%
Inorganic Ash (%)	10.0	18.2	58%

Component (ug/g)	**Low Value**	**High Value**	**% Difference**
Calcium	130	320	84%
Iron	32	74	79%
Magnesium	250	799	105%
Potassium	31950	60940	62%
Sodium	760	8190	166%

Component (%)	**Low Value**	**High Value**	**% Difference**
Chloride	0.07	0.52	153%
Sulfate	0.09	1.02	168%
Phosphate	1.10	3.73	109%

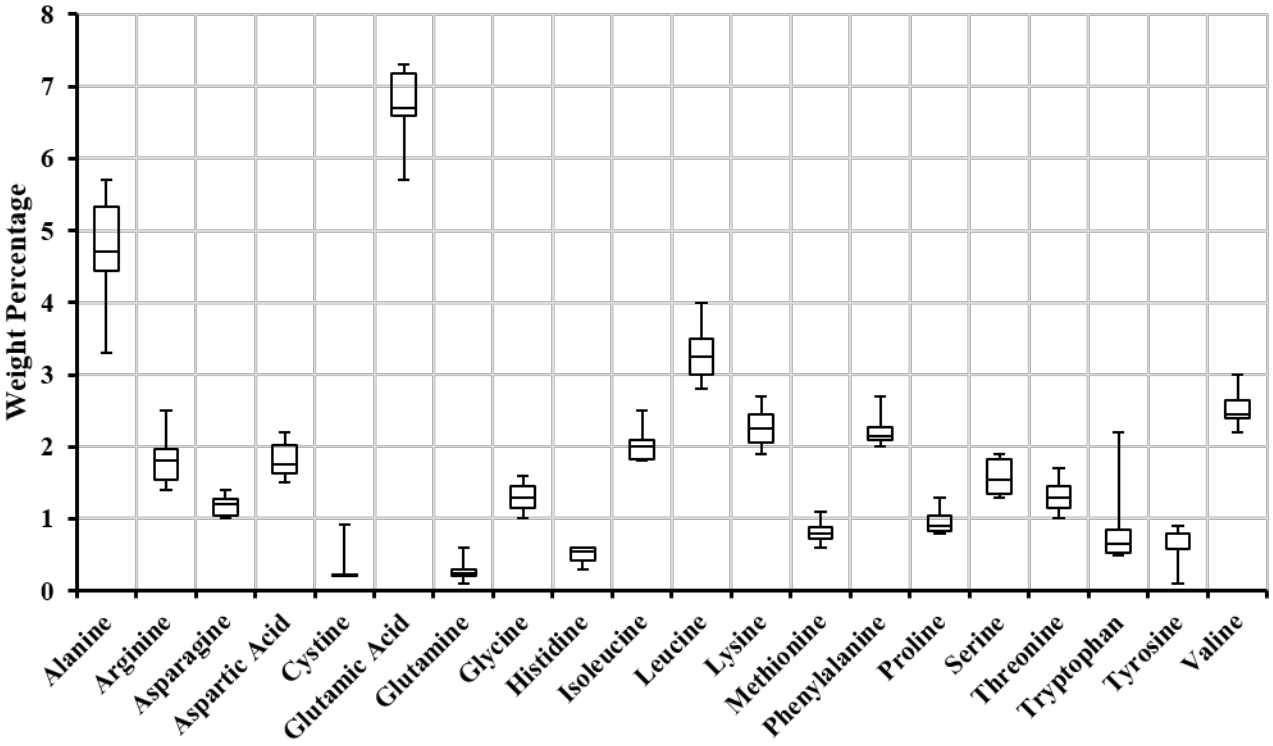

Figure 2.2: Free amino acid composition and variation in 6 different yeast extract products. Raw data provided in BD Bionutrients Technical Manual 3rd edition.

2.3.1.2 Variability

Commercial yeast extract production typically involves the culturing of *Saccharomyces cerevisiae* to high density. The cells are then killed by moderate temperature increase or osmotic pressure increase. Protein and other macromolecules are broken down by the yeasts own enzymes, making this an autolytic process. The water soluble portion is then processed via centrifugation and filtration (to varying degrees), often followed by spray

drying to obtain the final product [49, 92]. Autolysis is the most common method of producing yeast extract products [90, 93]. A variety of factors contribute to the variable composition of yeast extract, such as: yeast culture conditions, autolysis temperature, enzyme variation and addition, as well as downstream processing [93, 94, 95, 96]. Such variability is demonstrated in Table 2.1 and Figure 2.2. Lobo-Alfonso et al demonstrated insect cell growth variability both due to different yeast extract providers as well as lot-to-lot variability from the same provider [92].

2.3.2 Chemically Defined Media

Due to the undefined nature and inherent variability of yeast extract, it is desirable to replace it in insect cell culture media. Successfully replacing yeast extract with defined components would result in a *chemically defined* medium.

2.3.2.1 Mammalian Cell Lines

As with insect cell lines, early mammalian cell culture required undefined supplements (most commonly serums, e.g. FBS). van der Valk et al have reviewed the movement away from FBS supplementation towards serum-free and chemically defined media [97]. This review outlines the fact that different cell lines have different nutritional requirements and indicates compounds often necessary in the development of chemically defined mammalian cell media. These compounds include: growth factors, hormones, lipids, antioxidants, vitamins and Insulin-Transferrin-Selenium (ITS) supplement. Presently, several serum-free mammalian cell culture media are commercially available, e.g. Gibco^{TM} CD CHO for

Chinese Hamster Ovary (CHO) cell lines (Thermo Fisher Scientific, Massachusetts, USA), EX-CELL^{TM} CD Hybridoma Medium for hybridoma cell lines (Sigma-Aldrich, Missouri, USA) and Gibco^{TM} CD 293 for human embryonic kidney 293 (HEK 293) cells (Thermo Fisher Scientific).

2.3.2.2 Insect Cell Lines

In contrast to mammalian cell lines, there has been much less success in the development of chemically defined media for insect cell lines. Wilkie et al reported the first chemically defined insect cell medium in 1980, for *S. frugiperda* cells [98], however it has not been successfully utilized since then. On the contrary, Wilkie's medium has been demonstrated to not work in a variety of insect cell lines [66, 99], and specifically in Sf cell lines (without the use of yeast extract) [31]. Mitsuhashi successfully developed a chemically defined medium (MTCM-1520) capable of sustaining the flesh fly (*Sarcophaga peregrina*) cell line, NIH-SaPe-4, for more than 200 passages [99]. This medium was poorly optimized, containing 105 compounds, which is significantly more than in previously discussed media. Further experimentation by Mitsuhashi allowed for elimination of many of these compounds and elucidated the importance of polyamines for cell proliferation [100]. However, cell growth of NIH-SaPe-4 in this medium is dictated by a strong density dependence, requiring passaging at a ratio of 1:2. Further, MTCM-1520 has not been shown to sustain defined growth for non-Dipteran cell lines. More recently, Burnette et al utilized a small molecule screening technique to develop a chemically defined medium for *Drosophila* cells [101, 58]. This medium was successful in sustaining long term growth of the adherent cell line Clone 8 (Cl.8) over the course of at least 95 passages while maintaining consistent growth rates.

However, the medium was unable to sustain growth of suspension cell line Schneider 2 (S2) for more than 4 passages. This study provides more evidence towards the importance of the polyamines in chemically defined insect cell growth, identifying spermidine as a key growth inducing component.

Presently, the most popular commercially available Sf-9 insect cell media, Gibco^{TM} Sf-900II and Sf-900III (Thermo Fisher Scientific) still utilize undefined hydrolysates. Recently (2018), Thermo Fisher Scientific released the first commercial chemically defined medium for insect cells, Gibco^{TM} ExpiSf^{TM} CD. This medium is designed for Sf-9 cells and is advertised to achieve very high cell densities (2×10^7 cells/mL) and allow for high protein yield for over 20 passages. However, its formulation is proprietary and the promised results are utilizing Thermo Fisher's Sf-9 cell line derivative, ExpiSf9^{TM}. They do not guarantee that adapting other Sf-9 cells to the medium will achieve the same results, even after the recommended slow acclimatization of 25-30 passages. This difference points towards a metabolic difference between typical Sf-9 cells and the ExpiSf9^{TM} derivative.

2.3.2.3 Compounds of Importance

In order to develop a successful chemically defined growth medium for Sf-9 cells, it is important to consider defined media for other cell lines as well as yeast extract composition. Different cell lines often require different media and different nutrients or nutrient levels. However, there are many similarities between different media, and chemically defined media for other cell lines, particularly insect cell lines, will likely provide clues as to what is required in Sf-9 cell culture. Since it is well established that Sf-9 insect cells are capable of

strong growth in a medium where the only undefined component is yeast extract, it follows that the composition of yeast extract is of key importance in the development of a chemically defined medium. Although yeast extract is very complex and not fully characterized, it is worthwhile to look to existing characterization work to find components that may be of interest in its replacement. Likewise, plant hydrolysates have shown similar growth promoting properties and their characterization is of interest in determining compounds that may be needed in a chemically defined medium. Low molecular weight fractions (<3kDa) of yeast extract and other hydrolysates have been demonstrated to provide the majority of growth promoting abilities in both Sf insect cell lines [85, 102, 103] and in mammalian cell lines [104, 105, 106]. This effect is likely indicative of free components and short chain peptides being of primary importance to growth rather than large macromolecules and proteins. Compounds of potential interest in the development of a chemically defined medium for Sf-9 insect cells based on other defined media and yeast extract characterization are summarized:

- B vitamins: Already provided in typical Sf-9 media, however yeast extract is also a significant supplier of B vitamins [90, 107, 6]. Supplementation may be required when eliminating yeast extract.

- Nucleic material: Yeast extract contains a significant amount of nucleic material [91, 108], mostly from RNA [6, 109]. However, basal insect cell media does not typically contain nucleic material. This lack of nucleic material is in contrast to many mammalian media formulations (e.g. Medium 199, Ham's F-12 [110]). It is worth investigating whether supplementation of nucleic material might aid in the transition

away from yeast extract in insect cell culture, likely by easing the metabolic burden necessary for the cells to synthesize these compounds.

- Oligopeptides: It is well established that yeast extract is a rich source of short chain peptides. However, simply replacing these peptides with their equivalent free amino acids may not be adequate when eliminating yeast extract. There is evidence that short chain peptides play roles (e.g. cell signalling) beyond simply providing amino acids to cell culture, as reviewed by Franek [111]. Although chemically defined cell culture media does not typically contain synthetic peptides, it may be a useful avenue of investigation in Sf-9 cell culture yeast extract replacement.

- Other micronutrients: Both polyamines [106] and betaine [6] are present in yeast extract. These compounds have been described earlier, with polyamines being shown necessary in many chemically defined media and betaine playing an important osmoprotective role in some mammalian cell culture.

The compounds summarized in Table 2.1 and Figure 2.2 are all provided in basal media. These compounds are therefore likely not essential in yeast extract replacement. However, the basal medium may need to be fortified with such compounds in order to restore their ideal concentrations.

Chapter 3

Materials and Methods

3.1 Materials

All compounds used in cell culture media were purchased from Sigma-Aldrich (St. Louis, Missouri, United States) unless otherwise stated. These compounds were mostly "Bioreagant" grade, and if not were either "BioXtra" or "BioUltra" grade. Yeast extract used in "ALIM" media was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, United States). Yeast extract used for all experiments in this study was Product BP-1422, Lot 171921. Sf-900III media was also purchased from Thermo Fisher Scientific. Water used for production of cell culture media was of Ultrapure quality (in-house purification system), and was ensured to have a resistivity of greater than 17 $M\Omega \cdot cm$ at room temperature.

3.2 Cell Culture

Spodoptera frugiperda clonal isolate (Sf-9) cells were grown in screw-capped glass Erlenmeyer shake flasks. Flasks were incubated at 27°C and on an orbital shaker set to 130 rpm. Screw caps were not completely closed as to allow for adequate air circulation. Cells were typically grown to densities of 2.5 - 4 $\times 10^6$ cells/mL before being passaged into fresh medium at 0.5 - 0.6 $\times 10^6$ cells/mL. For low yeast extract adaptation experiments, seeding density was often higher, at 0.7 - 0.8 $\times 10^6$ cells/mL. Cells were cultured in either 50 mL, 125 mL, 250 mL or 1 L flasks at working volumes of 13 mL, 30 mL, 60 mL and 250 mL, respectively. Ramping up for experiments was done typically in 250 mL flasks and occasionally in 1 L flasks. Experiments were typically carried out in 125 mL flasks and occasionally in 50 mL flasks (due to media or space restrictions).

3.2.1 Cell Counting

Cell counting was performed using the Countess^{TM} II Automated Cell Counter (Life Technologies; Thermo Fisher Scientific). Cell samples of 0.5-1 mL were taken directly from flasks and counted immediately so as to avoid additionally variability due to cell settling. Samples were diluted 1:1 with a 0.1% trypan blue in phosphate buffered saline (PBS) solution (Sigma-Aldrich). It was ensured that the cells were evenly distributed prior to selecting "count". Experimental counts were repeated and the average used for analysis. To ensure accurate results and stay within the recommended measurement range, cell densities of greater than 1×10^7 cells/mL were further diluted with PBS prior to counting. Cell density, viability, and size were measured with the Countess^{TM} II Automated Cell

Counter.

3.3 Media Production

All media formulations may be found in Appendix A. Aucoin Lab Insect Cell basal media (basal "ALIM"; 1 L batch in this case) was produced by dissolving the appropriate amount of dry amino acids, sugars, and salts into approximately 600 mL of fresh UltraPure water. The components were then mixed in a beaker with a magnetic stir bar and allowed to fully dissolve at room temperature. Next, 1 mL of 1000x concentrated trace metal solution and 1 mL of 1000x concentrated vitamin solution were fully thawed from frozen at -80°C, mixed well and added to the solution. All concentrated stock solutions were previously produced in-house by dissolving at room temperature before being aliquoted into 1 mL volumes and frozen. Next, 13.3 mL of 75x concentrated L-cystine and L-tyrosine stock was thawed and also added to the solution. This amino acid concentrate was produced using 1 M hydrochloric acid due to solubility issues in water. After ensuring all previously added components were fully dissolved, 30 mL of chemically defined *Lipid Mixture 1* (Sigma-Aldrich) was added to the solution. UltraPure water was then used to bring the mixture to an approximate volume of 900 mL. After allowing time for adequate mixing, the pH of the solution was raised to 6.2 by adding the required volume of 3 M sodium hydroxide. During pH balance, care was taken to not overshoot 6.2, as having to reduce the pH after overshooting would result in a higher osmolality and therefore an inconsistent media. The solution was subsequently topped up to 1 L in a volumetric flask and sterile filtered (0.20μm polyethersulfone (PES) membrane; VWR International (Mississauga, Canada))

into a sterilized bottle. All media were stored at 4°C.

The concentrations of compounds mentioned in this section are formulation dependent, i.e. "enhanced basal ALIM" utilized larger volumes of trace metal and vitamin concentrates. Additional components for experiments were either built in to the media during production, or added via concentrate spike. Such concentrate spikes were produced in UltraPure water at a high enough concentration such that the volume added to culture would have a negligible effect on the greater media composition; controls were spiked with corresponding volumes of UltraPure water. All component and water spikes were sterile filtered prior to use in cell culture.

3.4 Osmolality

All osmolality measurements were performed using The Advanced^{TM} Micro-Osmometer (Model 3300; Advanced Instruments Inc., Massachusetts, United States) utilizing freezing-point osmometry. Media samples were aliquoted and analyzed immediately. Supernatant samples were gathered by centrifugation of cell culture samples and subsequent collecting of the supernatant from the top of the sample, ensuring no significant cell debris remained. The instrument was properly calibrated, and a standard was tested prior to each use. The instrument was properly cleaned between samples and all tested solutions were run in at least triplicate. Duplicate samples were run non-consecutively to ensure consistency of measurements. Osmolality measurements were routinely taken to ensure batch-to-batch consistency of media produced in-house.

Chapter 4

Media Platforms for Compound Testing and Optimization

4.1 Premise

Fully chemically defined growth medium is desirable as it allows for the system to be more precisely monitored and efficiently fed/adjusted. Furthermore, a fully chemically defined medium allows for the testing of compounds such that their effects can be fully seen and not convoluted by the presence of complex hydrolysates. This enables the use and effect determination of micronutrients as one can be sure of the exact amounts which may or may not be present in the growth media.

Commercial media (e.g. Gibco Sf-900III) is not desirable as a platform for media optimization and component testing as the formulations are proprietary and further, contain

hydrolysate. In addition to the presence of hydrolysate, there are many factors which may affect growth and production, and which may confound other results, such as pH, osmolality, Na:K ratios, etc. Simply adding compounds to finished commercial media products will alter the product in ways that are not consistent from compound to compound. Incorporating components desired for testing into the medium during production allows for pH, osmolality, and ion ratios to be consistent from batch to batch and from experiment to experiment for whatever compound is being analyzed. It is useful, then, to utilize an in-house growth medium such that more controllable alterations may be made and such that there is more information as to what is already present in the medium.

4.2 In-house Medium

4.2.1 Media with Hydrolysate

In order to develop a platform for medium optimization testing with the overall goal of eliminating the need for hydrolysates, Sf-9 insect cells were adapted to an in-house medium, "ALIM", containing 4 g/L yeast extract ("ALIM + YX"). This in-house medium is based on and remains similar to IPL-41. See Table A.1 for initial "ALIM + YX" formulation. Cells frozen at low passage number (p16) in a medium composed of 80% Sf-900III, 10% Fetal Bovine Serum (FBS) and 10% dimethyl sulfoxide (DMSO) were thawed into 100% Sf-900III growth medium. The cells were then allowed to return to their normal growth rates in Sf-900III over the course of approximately 4 passages (during which time any residual FBS and DMSO were eliminated from the system). Cells were subsequently adapted to

"ALIM + YX" media by halving the amount of Sf-900III and replacing with "ALIM + YX" each passage until Sf-900III was eliminated altogether (approximately 5 passages). Figure 4.1 illustrates this simple adaptation process. As expected, after this adaptation, the cells experienced a reduced growth rate compared to that of cells in Sf-900III. Figure 4.2 compares exponential cell growth in Sf-900III medium to that of low passage/early adapted "ALIM + YX".

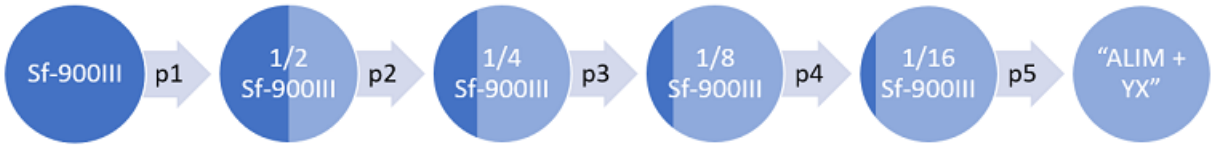

Figure 4.1: Adaptation process for transitioning Sf-9 cells from Sf-900III commercial medium to "ALIM + YX" in-house medium. Fraction indicates amount of Sf-900III in fresh medium.

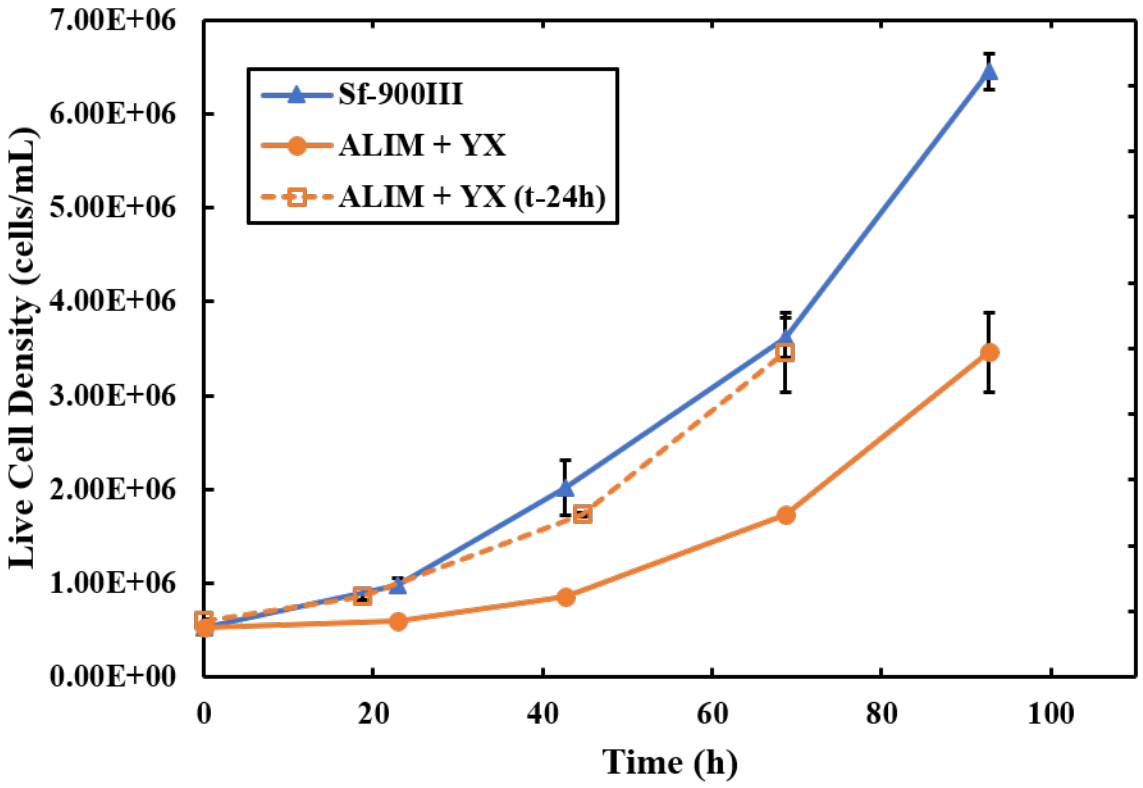

Figure 4.2: Exponential growth profile comparison of Sf-9 cells in Sf-900III medium and post-adaptation to "ALIM + YX" medium. Error bars represent ± standard deviation (n=3) of independent cultures.

As can be seen, most of the growth reduction is due to an increased lag phase (approximately one day). This lag phase is illustrated in Figure 4.2 by the inclusion of the "ALIM + YX" growth curve shifted back 24 hours (dashed line), and may be largely overcome by simply seeding new flasks at a higher cell density (e.g. 6 - 8 $\times$ 10^5 cells/mL). Regardless, this growth rate reduction does not inhibit the medium's usefulness as a platform for optimization as it still affords the ability to control and compare compounds. Maximum cell density is comparable to the commercial medium (13 - 14 $\times$ 10^6 cells/mL; data not shown)

and is beyond what is necessary for even "high density" infections and protein production using the IC-BEVs. Over many passages, the cell growth rate in "ALIM + YX" was observed to steadily improve and even approach the growth rate in the commercial Sf-900III medium (data not shown).

4.2.2 Basal Media

With the cells fully adapted to, and growing well in "ALIM + YX", it was then desirable to attempt to eliminate the yeast extract entirely. Passaging directly from "ALIM + YX" into "ALIM" without yeast extract ('Basal ALIM'), yields slow growth to a maximum of approximately 1-2 cell doublings (see Figure 4.3 comparing typical first passage Basal "ALIM" growth to that of "ALIM + YX"). However, subsequent passage attempts (from Basal "ALIM" into Basal "ALIM") show no growth whatsoever, as expected. The small amount of cell proliferation in the first passage into Basal "ALIM" is due to residual yeast extract. Initially, it seemed as though cells passaged into Basal "ALIM" from "ALIM + YX" were ideal candidates for growth inducing compound/yeastolate replacement testing. However, as the minor growth seen is entirely due to residual yeast extract, inconsistencies arise. These inconsistencies are due to seed volume and therefore seeding flask density, and further due to the degradation level (or 'freshness') of the yeast extract being carried over from the previous culture during passaging. Figure 4.4 demonstrates this inconsistency with examples of observed first passage Basal "ALIM" growth profiles.

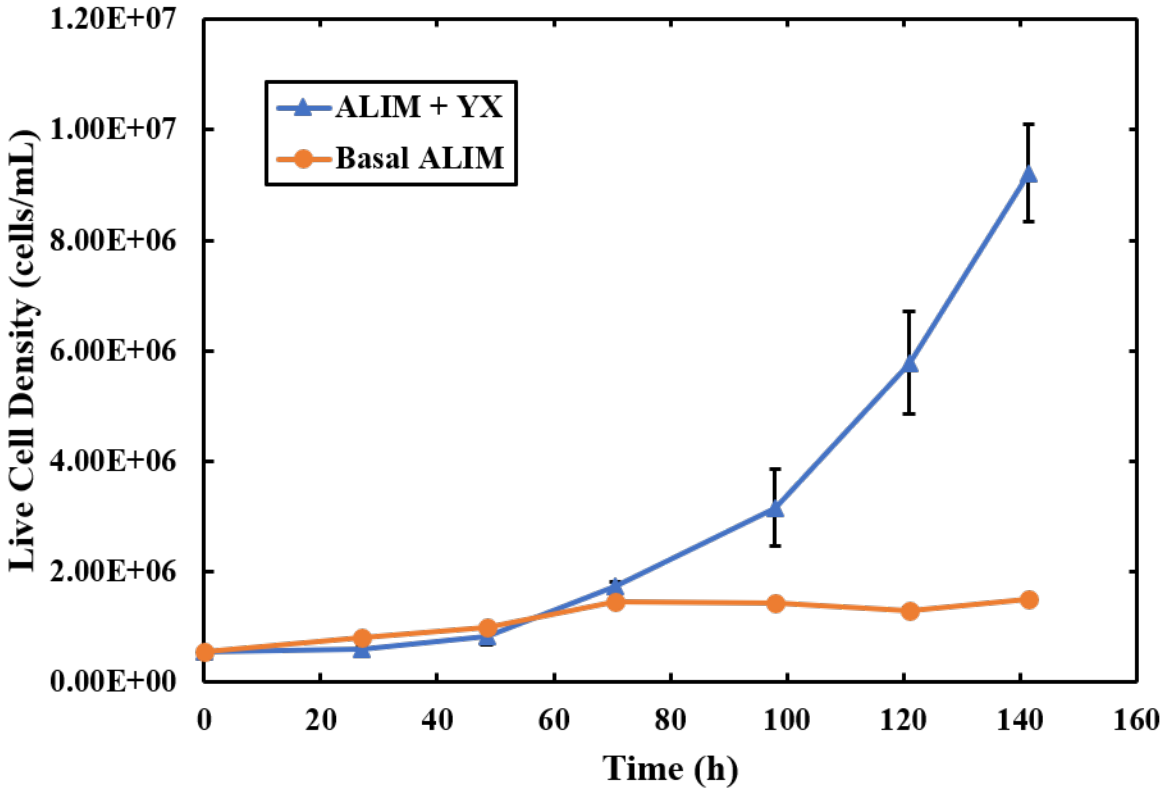

Figure 4.3: Growth profile comparison of Sf-9 cells in "ALIM + YX" medium to first passage growth in Basal "ALIM" medium. Error bars represent the range of duplicate cultures.

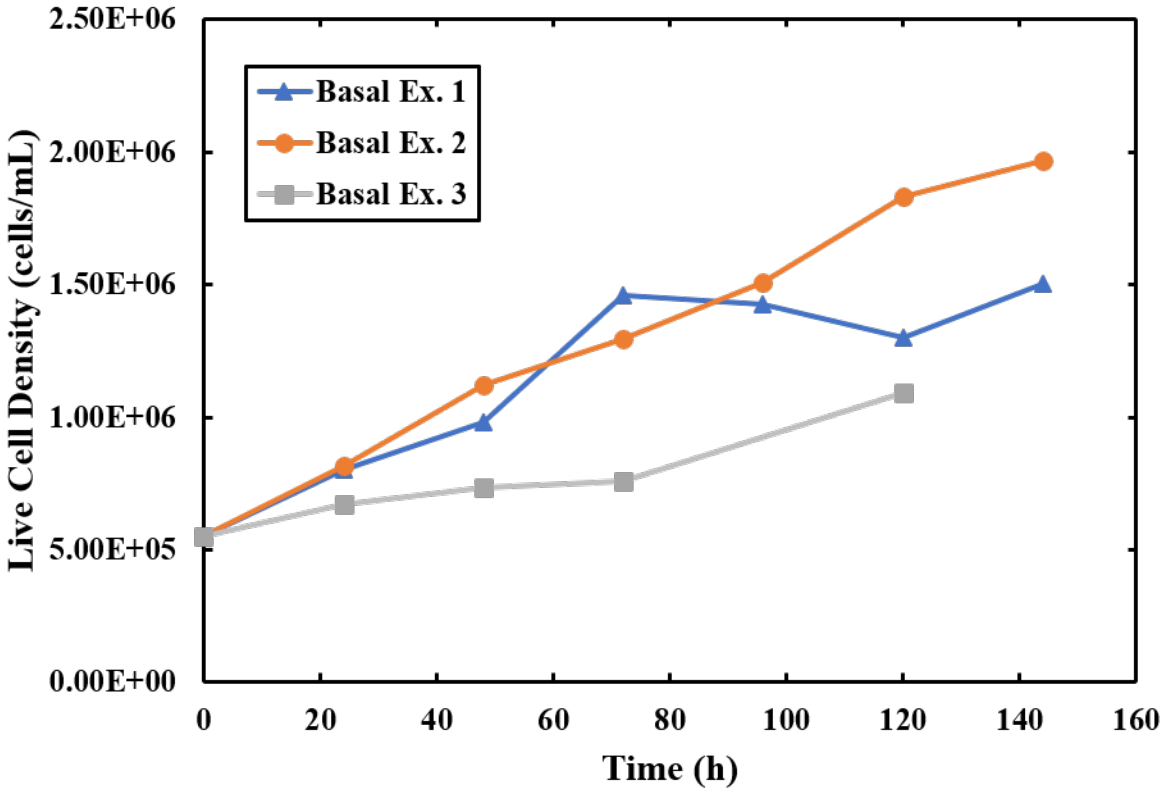

Figure 4.4: Three examples of Sf-9 cell growth profiles during first passage into Basal "ALIM" medium from "ALIM + YX" medium.

Figure 4.4 displays three examples of first passage Basal "ALIM" growth profiles. Seeding flask conditions for examples 1 and 2 do not differ in any appreciable way, while example 3 was passaged from a slightly higher density and one day older flask. More significant growth has also been observed when passaging from flasks which were seeded high (e.g. $>1 \times 10^6$ cells/mL) and passaged after less days of growth, but from the same seeding flask density as examples 1 and 2 (i.e. more 'fresh' yeast extract). As can be seen in Figure 4.4, these factors play a significant role in growth observed in Basal "ALIM" and

cannot be precisely controlled in a practical manner. This makes consistency from experiment to experiment very difficult and as such defeats the whole purpose behind utilizing a controllable in-house medium. Additionally, the one passage limitation of growth in Basal "ALIM" does not allow for thorough investigation of the effects of tested compounds.

4.2.3 Preliminary Low Yeast Extract Media

Next, it was desirable to attempt to adapt these Sf-9 "ALIM + YX" cells to growth in a low yeast extract environment. This was done in an effort to achieve consistent growth and maintain the ability to passage cells continuously, in contrast to Basal "ALIM". The major benefit of a low yeast extract medium to this work is that it provides a jumping off point for the testing of potential growth inducing compounds in a way that their effects are not convoluted by either their presence, or the presence of other unknown compounds, in yeast extract. Results may still be confounded by the presence of even small amounts of yeast extract, but lower concentrations and consequent improved consistency allows for insight into the actual effects of the compound being tested. First, to determine a baseline for the effects of yeast extract levels on cell growth/proliferation, the standard "ALIM + YX" (4 grams of yeast extract per litre) was simultaneously compared to 2g/L, 1g/L, 0.1g/L, and Basal "ALIM". The results of this may be seen in Figure 4.5.

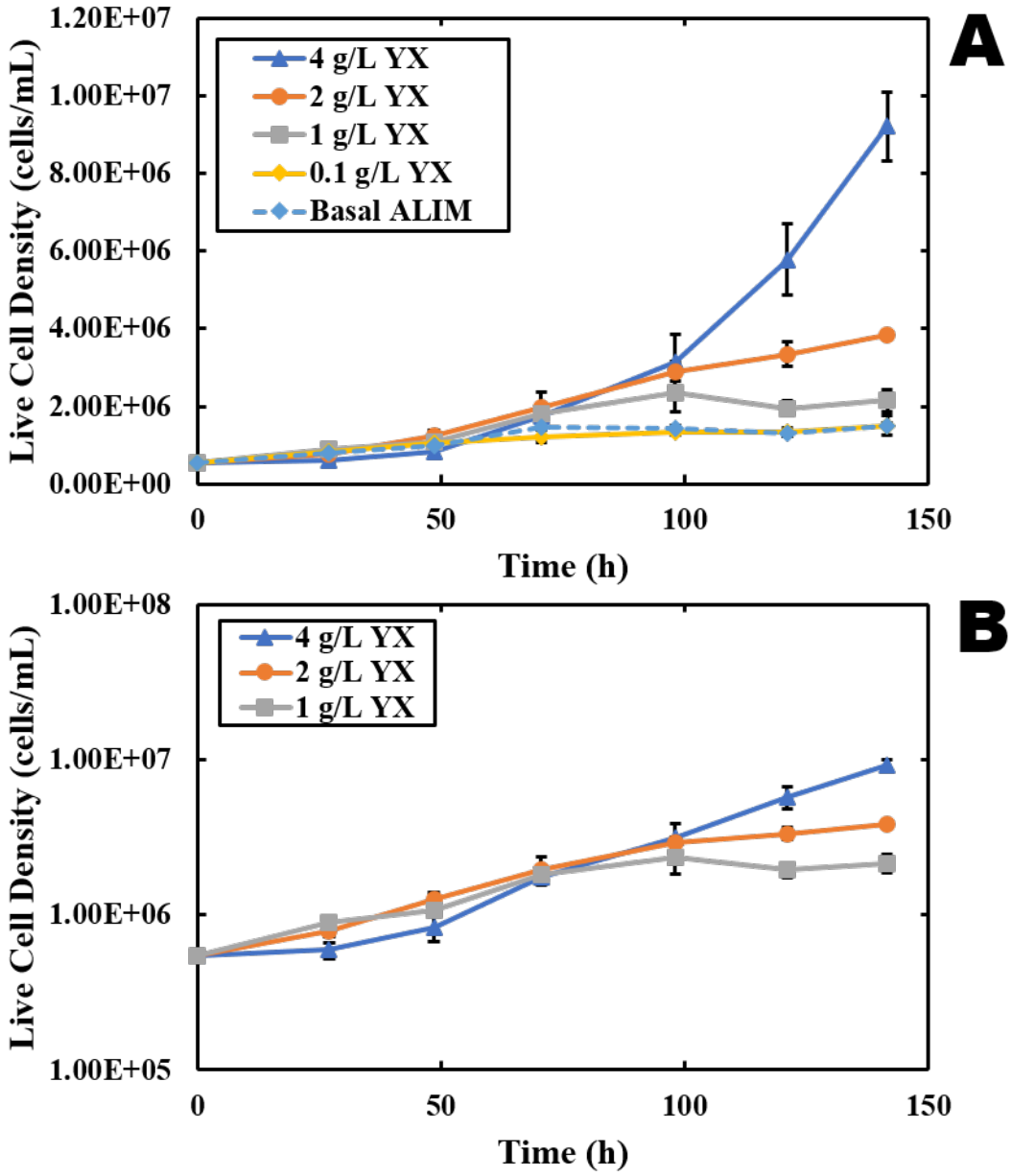

Figure 4.5: A) Growth profile comparison of Sf-9 cells in "ALIM" media with YX levels varying from 4 g/L ("ALIM + YX") to 0 g/L (Basal "ALIM"). B) Semi-log exponential growth comparison of 4, 2, and 1 g/L YX "ALIM". Error bars represent the range of duplicate cultures.

Clearly, reduction of yeast extract has a detrimental effect on cell growth, inhibiting it in an almost linear fashion as yeast extract is reduced. Maximum cell density is also adversely affected. Yeast extract likely contains multiple components necessary for cell growth that are present at different levels. As can be seen in Figure 4.5, when yeast extract is reduced to 50% and 25% (i.e. 2g/L and 1g/L), growth begins normally, but then falters as the cells begin to reach a density of 2-3 $\times 10^6$ cells/mL. Exponential growth rates are similar for 4g/L YX ($0.024h^{-1}$), 2g/L YX ($0.021h^{-1}$), and 1g/L YX ($0.024h^{-1}$), however exponential growth phase continues significantly longer with 4g/L YX. This could be explained by a co-factor (likely a trace metal) that is present in the yeast extract at levels high enough to only maintain exponential growth for a limited number of cells when yeast extract is at reduced levels. In this case, inhibition would not be realized immediately, but only when the cell density requires more of the co-factor than is available. Overcoming this hypothesized low yeast extract co-factor limitation would be required such that other limitations (i.e. micronutrients) may be realized and addressed.

4.3 Feed Additives

4.3.1 Preliminary Work

Based on the co-factor limitation hypothesis from the previous experiment, it was thought that the trace metal content should be increased. Initially, and for maximum flexibility, this was performed/tested by utilizing a concentrated feed additive (F.A.) at approximately 5% culture volume. In this work, 'feed additives' may be used both as a medium enhancement

(i.e. added to standard media at the start of culture) or as a nutrient spike (i.e. added at certain time or at cell density points during the culture). Feed additives are a good way to boost nutrients provided to the cells (particularly trace metals and vitamins in this case) and to ensure that none of the 'defined' components already provided to the cells in the medium are limiting to growth. This allows for focus on compounds that are not provided in the medium or that are solely or largely provided by the yeast extract.

Preliminary work with feed additives was done in order to ensure their effectiveness and gauge the effects of increasing certain components in the medium. The initially tested feed additive is described by Bédard et al [112]. A version of this feed additive excluding yeast extract was also tested. Figures 4.6, 4.7 and 4.8 show results from baseline feed additive experiments.

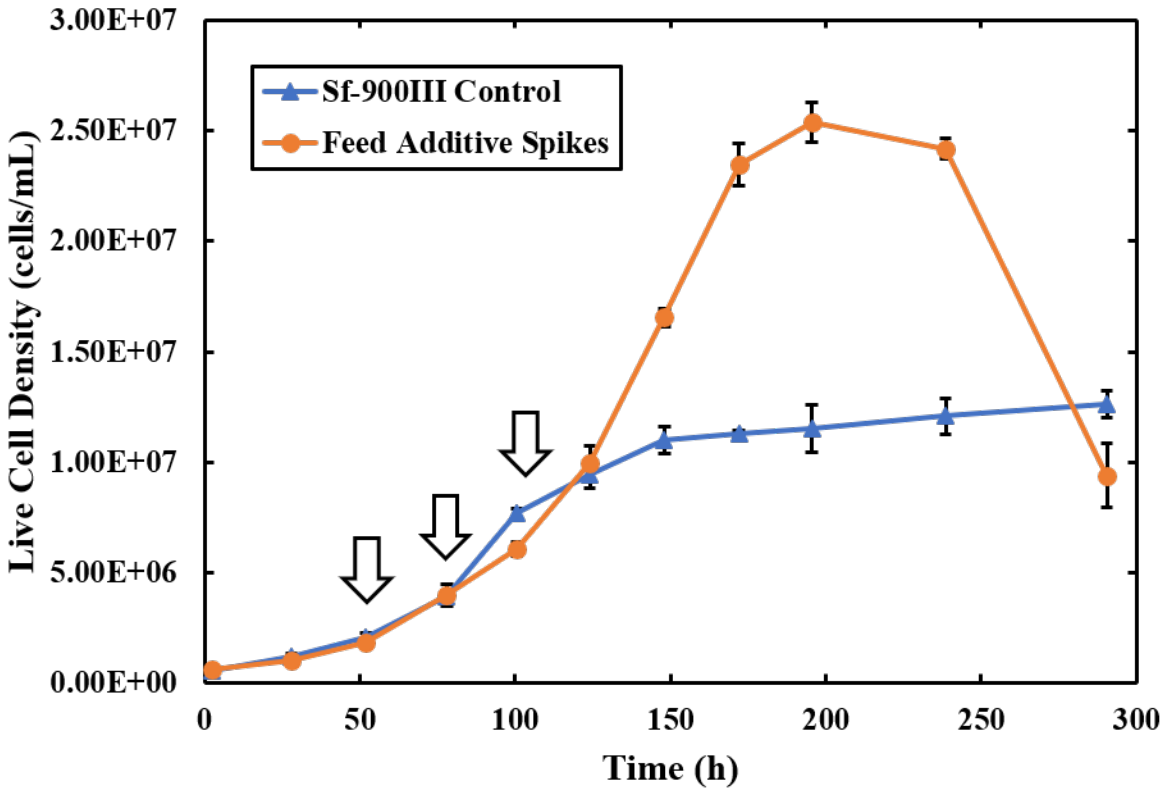

Figure 4.6: Growth profile comparison of Sf-9 cells in Sf-900III medium with and without the use of a feed additive spike regimen. Arrows indicate feed additive spikes. Error bars represent the range of duplicate cultures.

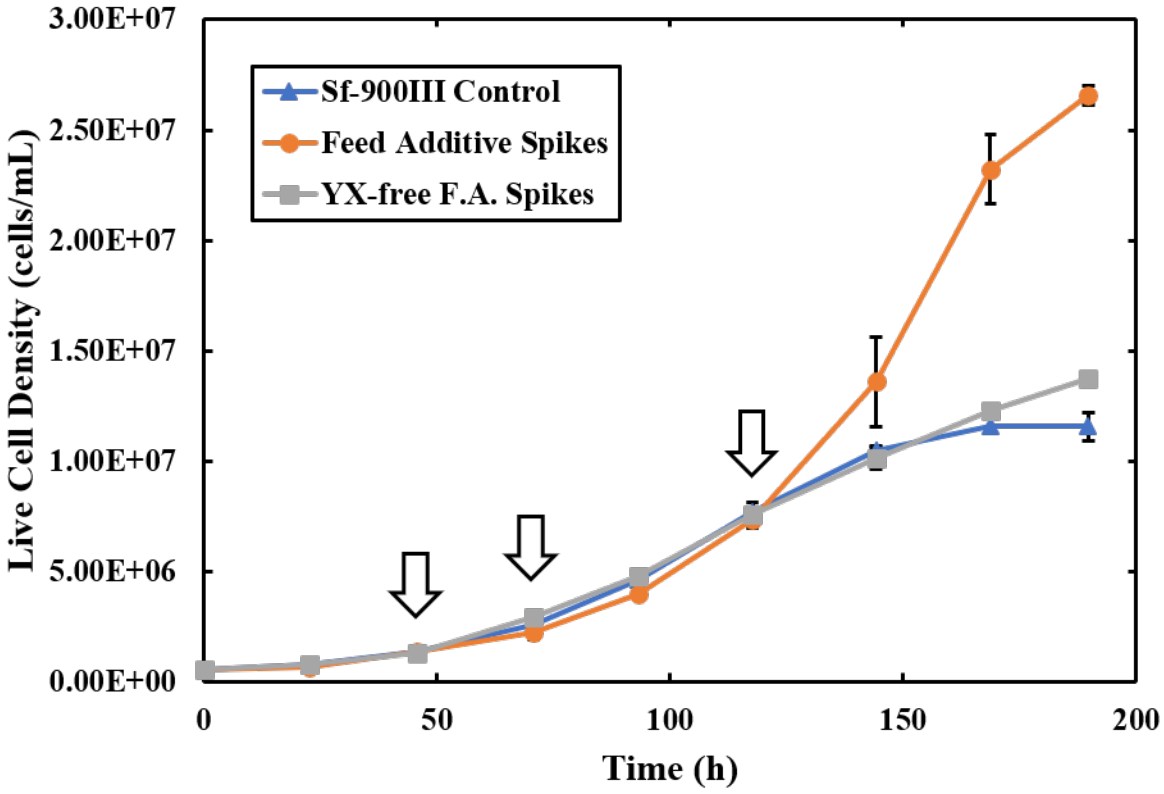

Figure 4.7: Growth profile comparison of Sf-9 cells in Sf-900III medium with and without a feed additive spike regimen. Compares original yeast extract containing feed additive to an identical feed additive excluding yeast extract ("YX-free F.A. Spikes"). Arrows indicate feed additive spikes. Error bars represent the range of duplicate cultures.

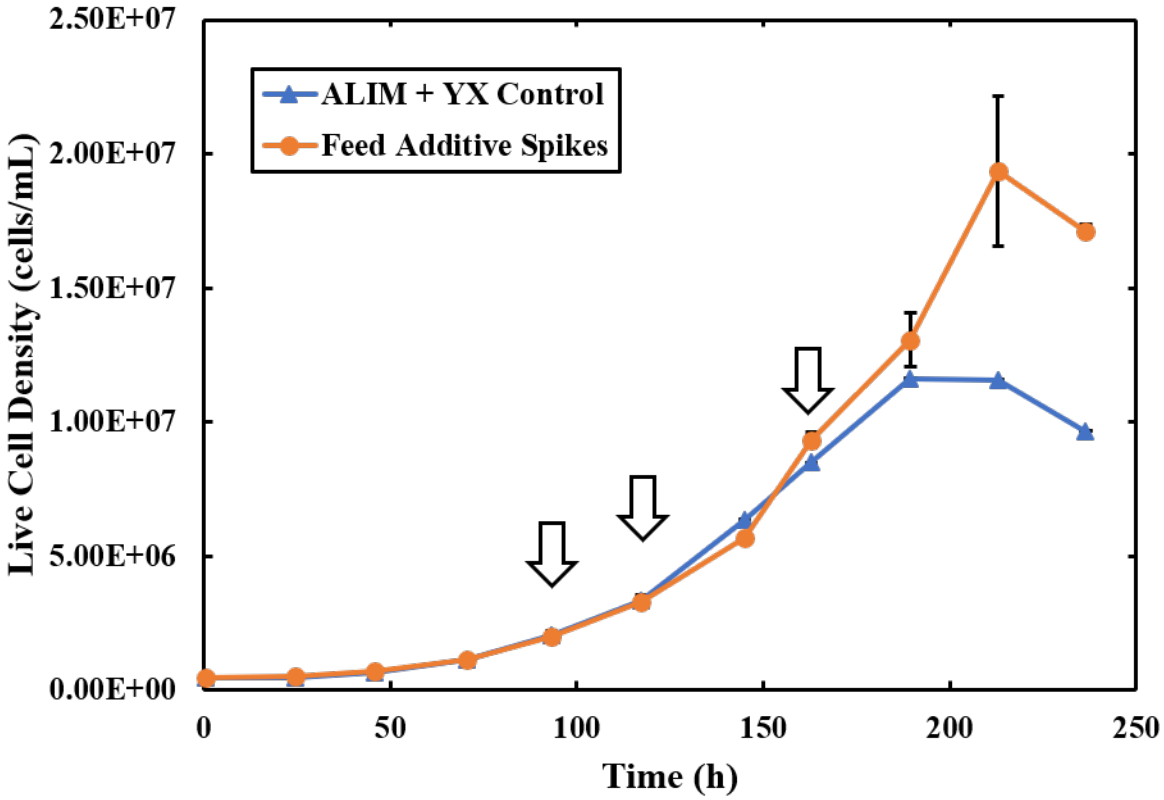

Figure 4.8: Growth profile comparison of Sf-9 cells in "ALIM + YX" medium with and without the use of a feed additive spike regimen. Arrows indicate feed additive spikes. Error bars represent the range of duplicate cultures.

In the baseline experiments, feed additives were used to supplement cultures as per the following regimen [113]: 2% culture volume feed additive spike at approximately 2×10^6 cells/mL, 4% culture volume feed additive spike at approximately 4×10^6 cells/mL, and 6% culture volume feed additive spike at approximately 8×10^6 cells/mL. Figure 4.6 demonstrates that the feed additive regimen prolongs exponential growth phase and significantly

improves maximum cell density. Figure 4.7 utilizes an identical feed additive excluding yeast extract (i.e. a chemically defined feed additive). It is apparent that the defined feed additive does not succeed in prolonging exponential growth, enforcing the idea that there are significant and essential components to growth being provided by the yeast extract, and that these components are solely provided by the yeast extract. The defined feed additive does prolong growth and improve maximum cell density, but this is likely due to replenishing macronutrients that have been depleted from the Sf-900III by that point in the culture. Figure 4.8 confirms that the feed additive (containing yeast extract) has a similar effect on "ALIM + YX" culture as was observed in Sf-900III. It follows that the feed additive can be a useful tool in conjunction with the in-house "ALIM" medium. Based on this, it was hypothesized that incorporating the use of a micronutrient boosting feed additive, while reducing the yeast extract concentration in "ALIM + YX" would be a useful approach.

4.3.2 Yeast Extract Reduction Attempt

The basis for the feed additive initially used in this study is described by Bédard et al [112], however a number of alterations were made for these purposes. Lipids were not included due to the difficulty of consistently incorporating them into the concentrated solution and due to the adequacy of lipids provided in "ALIM"; amino acids and salts were considered to be at appropriate/sufficient concentrations already and were kept consistent with their levels in "ALIM"; yeast extract was included in preliminary experiments (46 g/L in additive) but not henceforth. The feed additive formulation for initial low yeast extract "ALIM"

experimentation can be seen in Table A.2. The feed additive was used as a 5% by culture volume supplement added to the fresh "ALIM" medium prior to seeding (i.e. 1.5 mL of feed additive in a 30 mL culture). This method was chosen over the previously used spike regimen for the sake of simplicity. The most noteworthy differences between "ALIM" and "ALIM + 5% feed additive" were that the latter had nearly triple the amount of trace metals and vitamins, and approximately 30% more glucose. Essentially, use of the feed additive resulted in a richer version of "ALIM" in terms of certain media component classes. Initial experimentation with yeast extract levels and 5% feed additive may be seen in Figures 4.9 and 4.10.

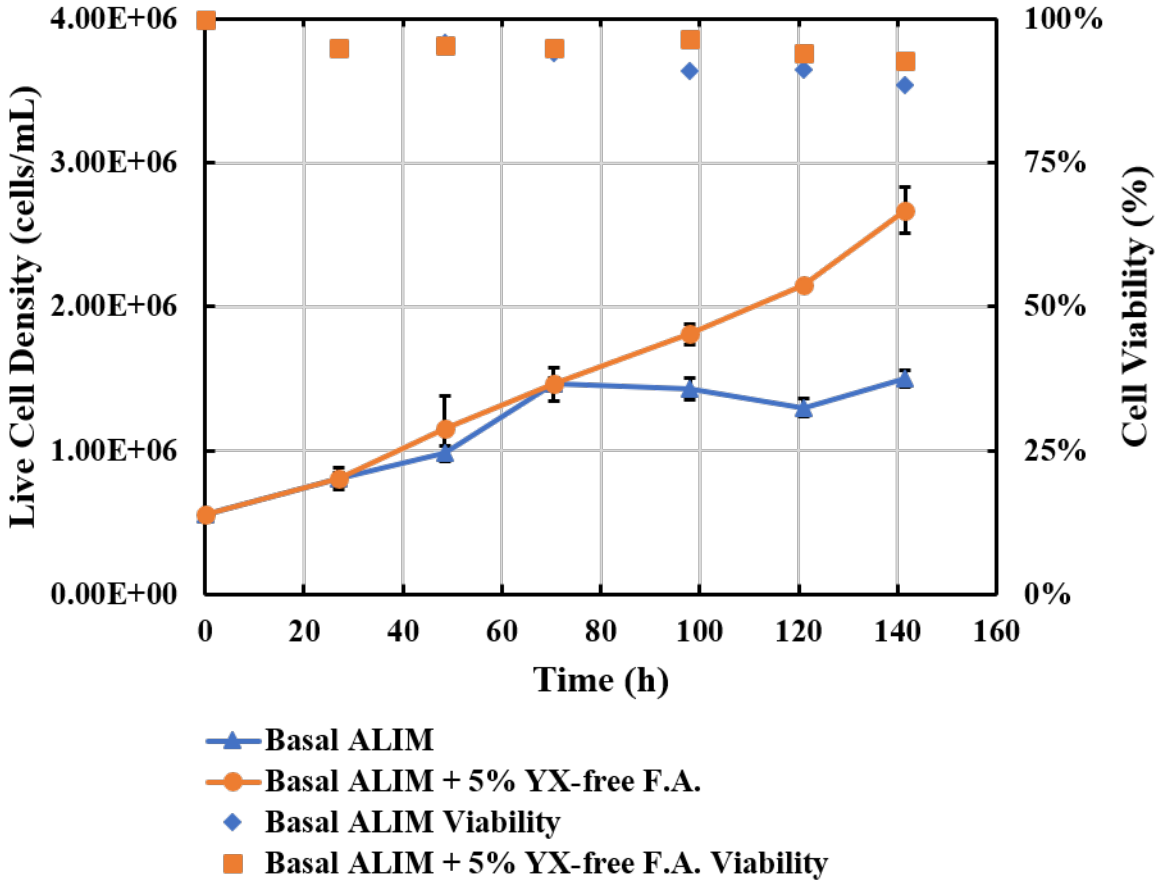

Figure 4.9: Growth profile and cell viability of first passage basal "ALIM" with and without supplemention with 5% yeast extract free feed additive by volume. Error bars represent the range of duplicate cultures.

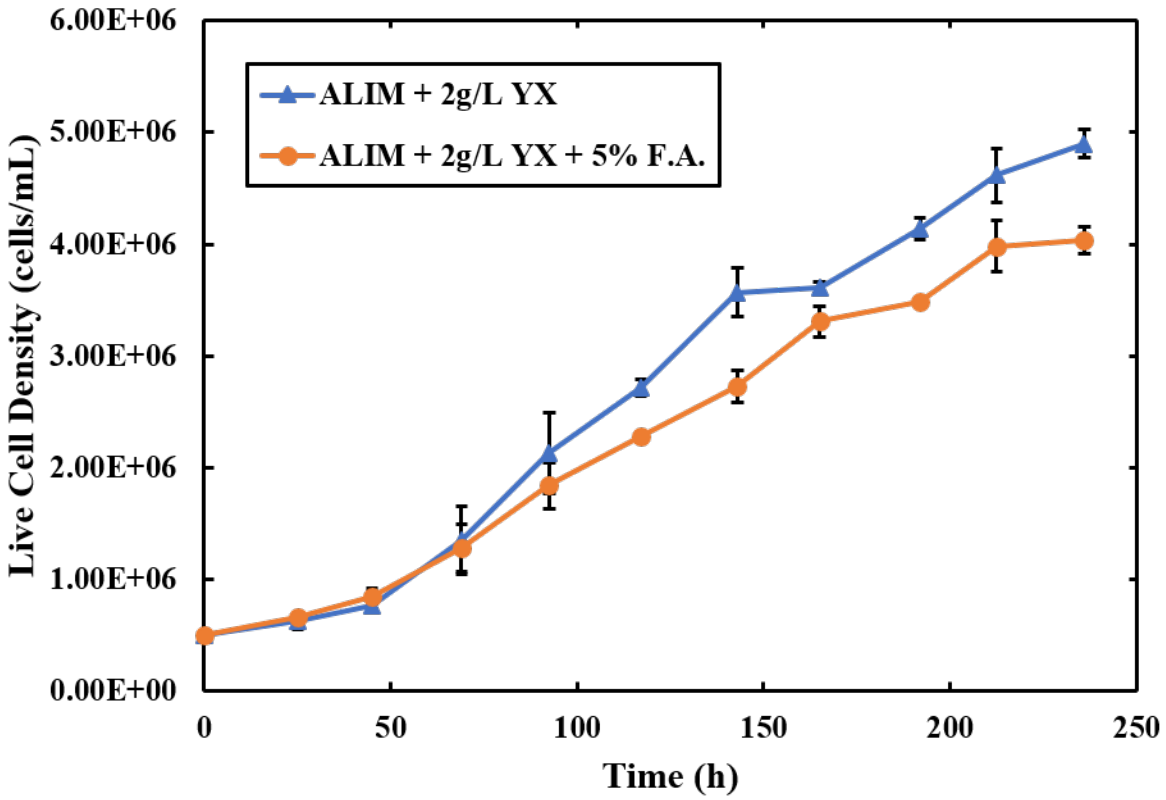

Figure 4.10: Growth profile comparison of Sf-9 cells in "ALIM + 2 g/L YX" medium and in "ALIM + 2 g/L YX" medium supplemented with 5% yeast extract free feed additive by volume. Error bars represent the range of duplicate cultures.

Figure 4.9 shows that supplementing basal "ALIM" with the defined feed additive seems to improve growth slightly, reaching a live cell density greater than 2.5×10^6 cells/mL, however subsequent passages in this medium do not yield meaningful growth (i.e. passagable cell density of 2×10^6 cells/mL is not reached). Furthermore, Figure 4.10 shows that the feed additive does not aid cell proliferation in media containing 2 g/L yeast extract (one

half the normal amount). It is apparent that the feed additive is tolerated by the cells, despite the increased osmolality, however it does not aid cell growth. This lack of improvement indicates that the growth limitation in low yeast extract media is due to a compound being provided solely by the yeast extract, and not due to some trace metal or vitamin already contained in the "ALIM" formulation. However, the somewhat promising improvement in basal "ALIM" growth is supportive of continuing to boost these micronutrients. For the feed additive to be effective, it needs to provide compounds that are not already being provided to the cells.

4.3.3 Potential Growth Inducing Compounds

In an effort to develop a low yeast extract growth medium from which to more thoroughly study yeast extract replacement, it was decided to first incorporate a variety of promising micronutrients into the feed additive. Compounds for this initial strategy were chosen based on preliminary experiments, literature, and previous yeast extract characterization work done in this laboratory (via acid digestion and nuclear magnetic resonance (NMR) targeted profiling) [6]. It was decided to simultaneously add a number of compounds at once as it is likely that multiple necessary compounds are provided by yeast extract, and to hasten the development of a platform from which compounds could be more thoroughly studied. All compounds utilized here were tested to ensure that they would not have negative effects and as such would not conceal potential positive results. This preliminary testing typically consisted of adding the compound of interest to basal "ALIM" at various concentrations and ensuring that cell viability was unaffected. The first group of compounds used in this

study are referred to as potential growth inducing compounds (pGICs). Compound choices and reasoning are described in the remainder of this section.

4.3.3.1 Betaine

Glycine betaine is a known osmoprotectant in other cell lines [77, 78]. Since the use of a feed additive increases the osmolality of the medium, and since "ALIM" is already at high osmotic pressure, it is of interest to incorporate betaine in order to combat potential negative side effects of an enriched growth medium. Preliminary experiments utilizing betaine in both feed additive spiked culture and artificially high osmolality culture (sucrose spiked) did not show any clear osmoprotective effects (Appendix B). However, those experiments were done in Sf-900III and "ALIM + YX"; since betaine has been detected in yeast extract [6], it is possible that any positive effects may have been obfuscated by the yeast extract already present in the media. These preliminary experiments did demonstrate that betaine is tolerable at high concentrations (>100mM). It is therefore appropriate to add betaine to the feed additive in order to potentially mitigate high-osmolality-related negative effects that may develop as the yeast extract content in the media is reduced.

4.3.3.2 Polyamines

One or more of the polyamines (putrescine, spermidine, spermine) and their metabolic precursor (ornithine) are widely reported to be necessary for chemically defined cell growth in multiple cell lines [100, 101, 106]. The typically accepted cellular metabolic pathway for the polyamines and their precursors is shown in Figure 4.11.

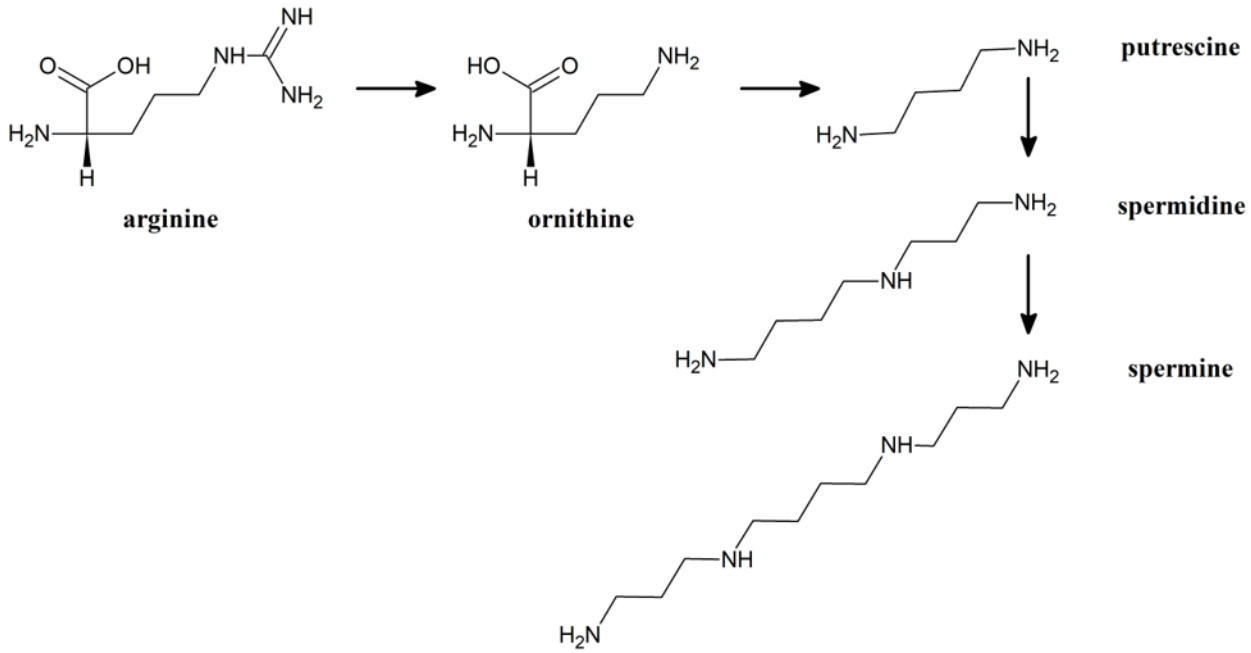

Figure 4.11: Simplistic polyamine biosynthesis pathway.

Previous work in this laboratory has demonstrated the presence of ornithine in yeast extract [6]. In the same work, the polyamines (putrescine, spermidine, spermine) were not identified, likely due to their very low concentrations and the convolution of the NMR spectra. Polyamines have been shown by others to be present in hydrolysate products [106].

Preliminary polyamine experiments (data not shown) had mixed results but allowed for the determination of polyamine levels which were tolerable to the cells. A preliminary result of note is shown in Figure 4.12.

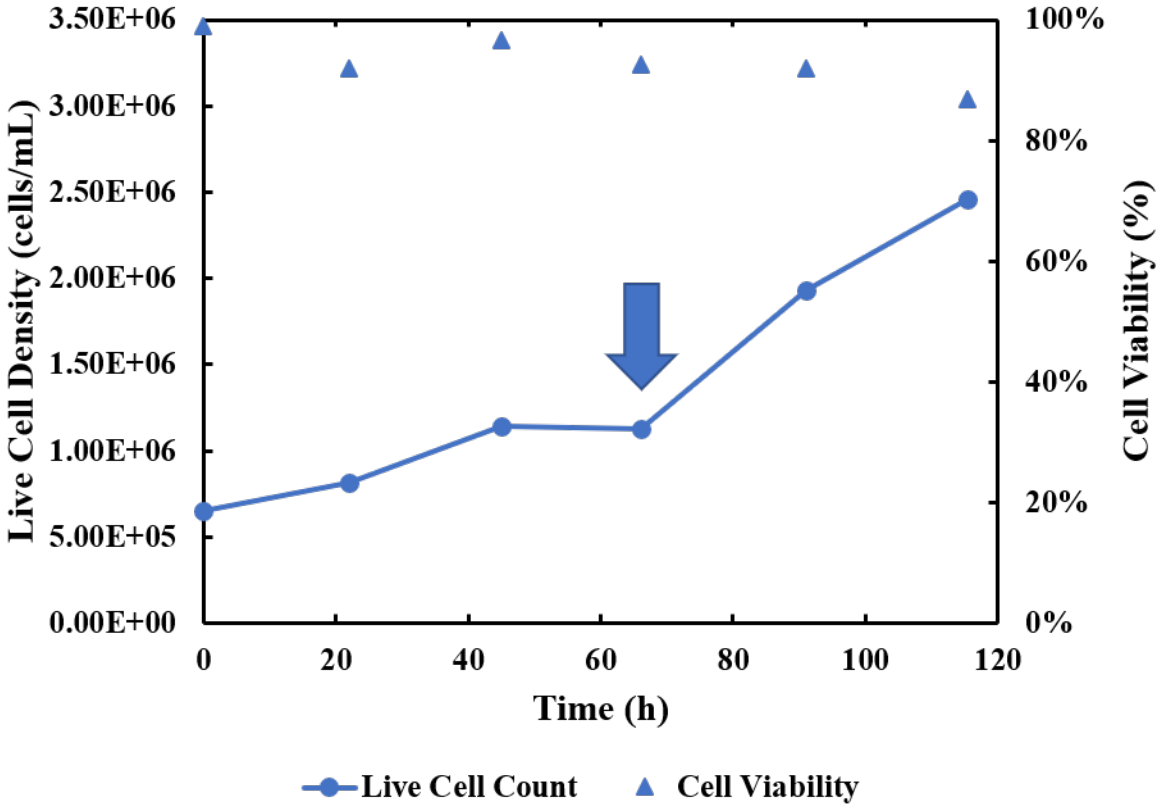

Figure 4.12: Sf-9 cell growth profile in Basal "ALIM" demonstrating polyamine-induced proliferation. Arrow indicates time of polyamine spike.

Figure 4.12 shows cell proliferation in basal "ALIM" media being induced by a polyamine concentrate spike (as indicated by the arrow; spike added after 72-hour count). This spike contained ornithine as well as the 3 polyamines. However, passaging such growth induced flasks into the same conditions (basal "ALIM" + late polyamine spike) or directly into basal "ALIM" + polyamines did not yield notable growth. Further, varying the levels of polyamines in both strategies was also not successful in achieving multi-passage growth.

This speaks to the complexity of yeast extract, and that likely at the residual levels from the first passage into basal "ALIM", a lack of polyamines is limiting growth, but in subsequent passages some other micronutrient is now limiting growth. Residual yeast extract amounts from the first passage would contain enough of said micronutrient(s) to allow for growth, but beyond that they also become limiting as yeast extract levels approach zero. Additionally, these polyamine spike experiments were not always repeatable, once again demonstrating the inconsistencies arising due to residual yeast extract in basal "ALIM". Similarly, utilizing this polyamine spike at the beginning of first passage basal "ALIM" culture also yielded variable growth results, ranging from typical first passage basal "ALIM" growth (i.e. 1.5 - 2 $\times 10^6$ cells/mL; no improvement over baseline), to as high as 3×10^6 cells/mL (data not shown). While it is not possible to draw conclusions regarding the influence of polyamines on cell proliferation from these preliminary experiments, it is worth exploring their impact further and including the polyamines as pGICs in the feed additive. Concentrations are based on the results of preliminary experimentation and may be seen in Table 4.1. The entire polyamine family was included due to all being variously reported as important in literature.

4.3.3.3 Nucleic Material

Nucleosides have been shown to be present at significant levels in yeast extract [6, 109]. Uridine, inosine, and adenosine were chosen as pGICs as they were observed to be the most prevalant nucleic compounds present in the yeast extract used with "ALIM" media. By adding nucleosides to the feed additive, the cells are being provided with 'pools' of these compounds, which will potentially reduce growth lag after passaging. Concentrations were

chosen to mimic 4 g/L yeast extract.

4.3.3.4 Other Compounds

Other compounds elected to be included in the feed additive as "pGICs" were as follows:

- Sodium ascorbate: Can act as an antioxidant, which is especially important in the trace metal enriched medium [56]. Ascorbic acid may play a more complex role in cell metabolism [57] and is likely to be an important compound in chemically defined insect cell media [99, 58].
- Ethanolamine: Has been shown to be essential to or important for growth in many serum-free media for mammalian cell culture [97, 114, 115]. Ethanolamine may not be necessary for insect cell culture [116] but this has not been thoroughly investigated.
- Selenium: Has been shown to be critical for serum-free cell growth in most cell lines; often acts as an antioxidant co-factor [97, 68].

These compounds were added based on levels seen in literature, and were tested to ensure they were tolerated by the Sf-9 cells (data not shown).

4.3.3.5 Summary

All compounds added to the feed additive as pGICs and their concentrations (final media concentration; i.e. 1/20th of their concentration in the feed additive) are summarized in Table 4.1.

Table 4.1: Potential growth inducing compounds and their final concentrations in "ALIM + 5% YX-free F.A. with pGICs".

Compound	**Form**	**Final Concentration (μM)**
betaine	betaine	1000
ornithine	ornithine · HCl	40
putrescine	putrescine · 2HCl	10
spermidine	spermidine · 3HCl	10
spermine	spermine · 4HCl	10
adenosine	adenosine	100
inosine	inosine	1000
uridine	uridine	1000
ascorbate	sodium ascorbate	5
ethanolamine	ethanolamine · HCl	25
selenium	sodium selenite	0.3

4.4 Consistent Low Yeast Extract Growth

4.4.1 Improved Feed Additive Testing

Next, it was of interest to incorporate these pGICs into the feed additive, and use this to enhance "ALIM" media at 5% volume. To determine the effects of this improved feed

additive, previous feed additive experiments (Figures 4.9 and 4.10) were repeated. The results of these experiments are shown in Figures 4.13, 4.14 and 4.15.

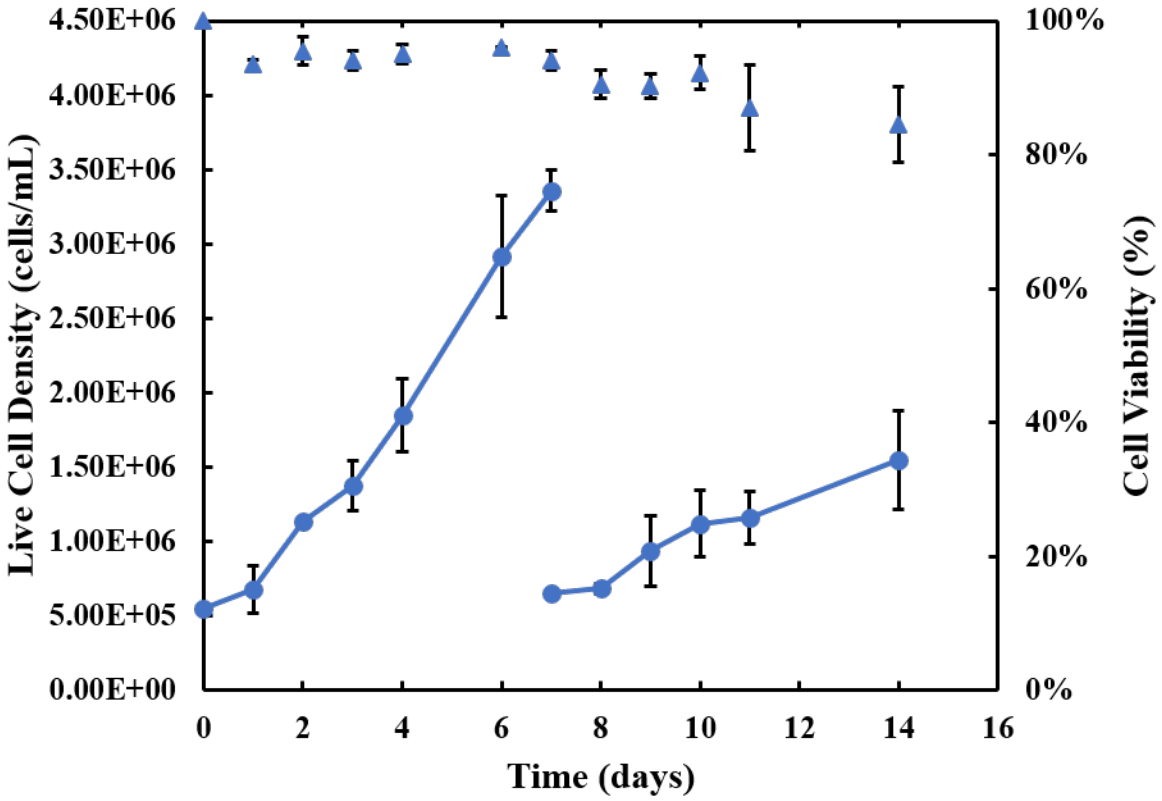

Figure 4.13: Growth profile and cell viability of first and second passage basal "ALIM" supplemented with 5% yeast extract free feed additive with pGICs by volume. Triangles represent viability. Error bars represent the range of duplicate cultures.

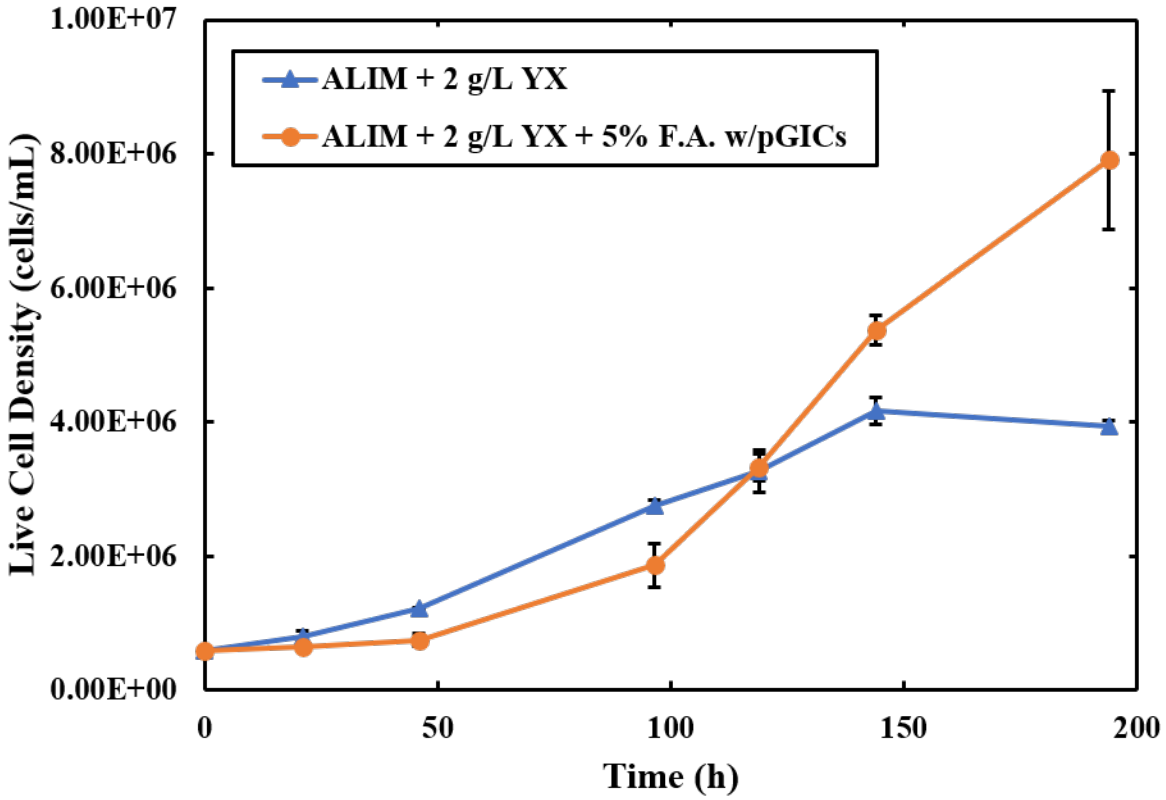

Figure 4.14: Growth profile comparison of Sf-9 cells in "ALIM + 2 g/L YX" medium and in "ALIM + 2 g/L YX" medium supplemented with 5% yeast extract free feed additive with pGICs by volume. Error bars represent the range of duplicate cultures.

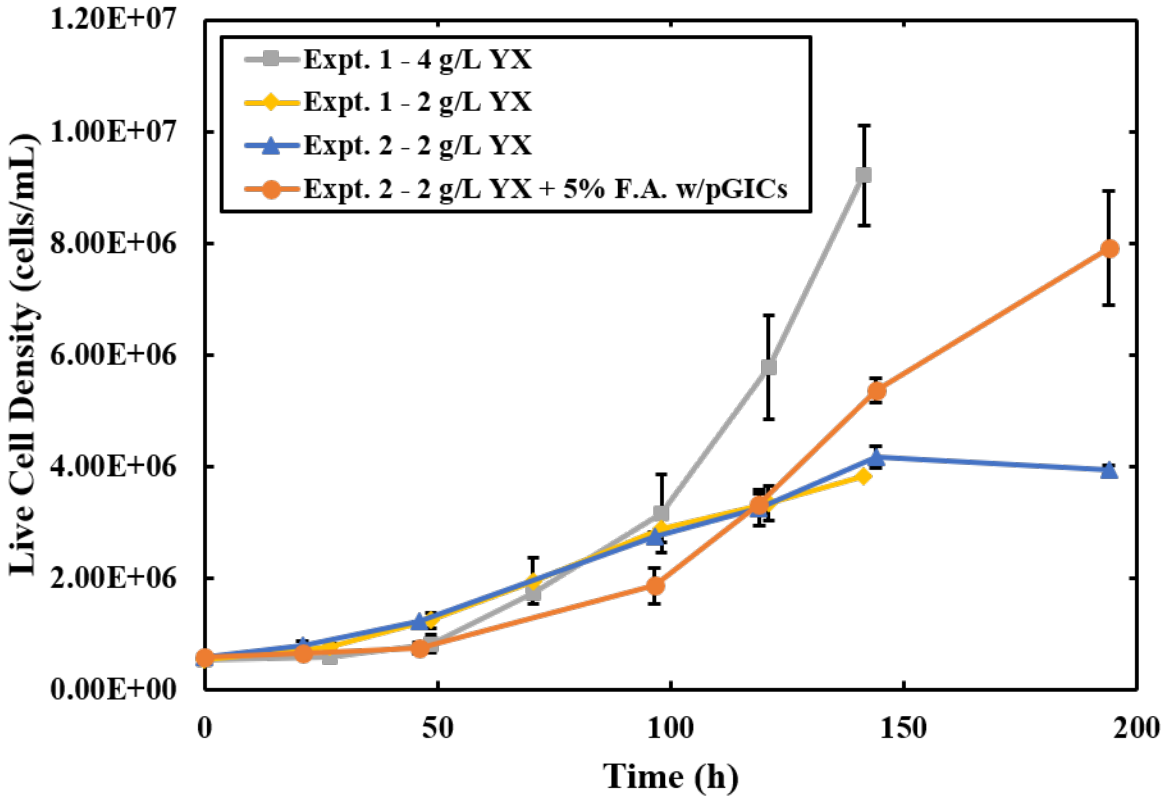

Figure 4.15: Growth profile comparison of "ALIM" media with 4 g/L yeast extract to 2 g/L yeast extract with and without 5% feed additive supplement with pGICs by volume. Error bars represent the range of duplicate cultures.

Figure 4.13 shows that supplementing basal "ALIM" with the defined feed additive with pGICs greatly improves first passage growth as compared to basal "ALIM" without feed additive, and further, as compared to basal "ALIM" with 5% defined feed additive without pGICs (see Figure 4.9). A second passage into the same conditions even achieves nearly two cell density doublings, which is more than any previous basal "ALIM" experimental

condition (none of which have achieved any second passage growth). Subsequent passages do not yield any cell growth, once again indicating that while additional hurdles in replacing yeast extract seem to have been overcome, still more compounds are likely needed. Figure 4.14 compares growth of "ALIM + 2g/L YX" to that with 5% of the improved feed additive. It is clear that this feed additive restores a significant amount of cell growth which was stunted by halving the "ALIM" yeast extract content. Comparing this to Figure 4.10, where the initial yeast extract free feed additive failed to restore growth, indicates that the defined pGICs are responsible for this growth restoration. Figure 4.15 compares these growth profiles with the original yeast extract levels experiment (Figure 4.5) in order to demonstrate the growth restoration as compared to "ALIM + YX". It is clear that while growth is not restored to that of "ALIM + YX", the improvement is significant.

These experiments clearly demonstrate that the pGICs improve the feed additive and are capable of restoring at least some of the growth lost with yeast extract reduction in "ALIM" media. The combination of a vitamin and trace metal enriching feed additive and a variety of potential growth inducing compounds is a viable strategy for achieving growth in "ALIM" with reduced yeast extract. As is the case with most media alterations, it follows that an adaptation to low yeast extract in the presence of the feed additive with pGICs should be carried out in order to ascertain the true effects and viability of this enhanced media.

4.4.2 Low Yeast Extract Adaptation

Due to the promising results seen in preliminary feed additive with pGIC experiments, it was decided to attempt to reduce the yeast extract concentration in "ALIM" over multiple passages. This was done utilizing a feed additive with pGIC supplement at 5% volume in each flask. Figure 4.16 shows the cell growth profiles over the course of this adaptation.

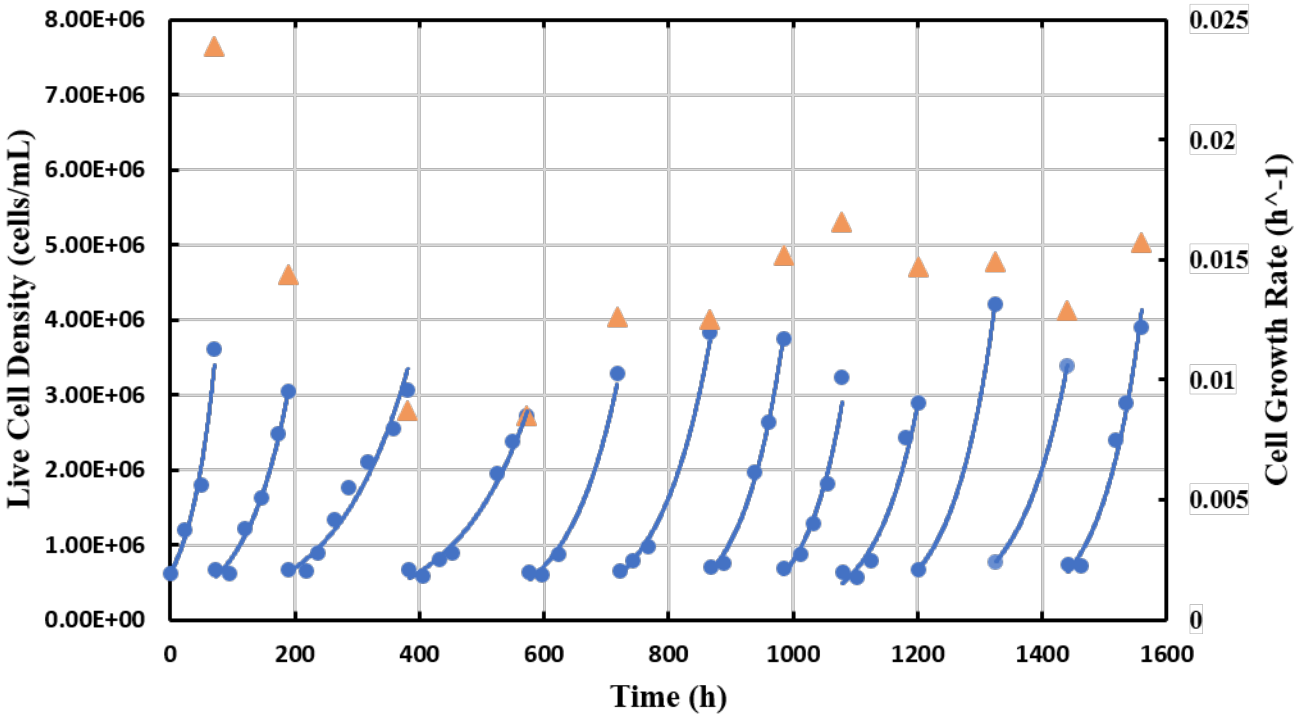

Figure 4.16: Growth profiles and rates of 12 passage adaptation from "ALIM + YX" into 1/10th YX medium with 5% F.A. and pGICs. Triangles represent cell growth rate.

As can be seen in Figure 4.16, over the course of 12 passages, the yeast extract concentration in "ALIM" was successfully reduced to 1/10th of its original concentration (0.4g/L),

and further, returned to a stable growth rate. Passage 1 was reduced to 40% yeast extract, but saw little drop in growth from that of "ALIM + YX", likely due to residual combined with fresh yeast extract. Passage 2 had 20% yeast extract, and passages 3-12 had 10% yeast extract. Each reduction in yeast extract resulted in a reduced exponential growth rate; however, this growth was recovered with subsequent passaging. Attempting to reduce the yeast extract further than this resulted in inconsistent growth and unhealthy cells. Although the stabilized growth rate was lower than that in "ALIM + YX", it was consistent. This adaptation was shown to be repeatable in two duplicate experiments and to maintain consistent growth for at least 40 passages (data not shown).

4.4.3 Discussion

The "ALIM" + 5% feed additive with pGICs and low yeast extract was combined into one medium and this was able to maintain stable growth. Other minor alterations were made to the medium: yeast extract concentration was raised from 0.4 to 0.5 g/L as this was observed to further stabilize growth rate; L-cysteine was added (previously only supplied in the form of L-cystine), and glucose concentration was reduced to "ALIM + YX" levels as it was already provided in excess. The lower growth rate than "ALIM + YX" was beneficial for the purposes of testing compound effects on growth rate and yeast extract replacement as more minute improvements were observable. This medium formulation was named "Enhanced low YX ALIM" (Table A.3).

Attempts to transition from "Enhanced low YX ALIM" to the same media without pGICs did not result in sustainable growth, confirming that at least some of the pGICs are

necessary. Individual effects of the pGICs should be investigated more thoroughly to gain a better understanding of this system and to remove unnecessary additives. Determining the individual effects of the pGICs is important for streamlining the medium.

It was further attempted to transition from "Enhanced low YX ALIM" directly to the chemically defined basal "ALIM" with pGICs. It was hypothesized that the long term adaptation to low yeast extract growth may allow for the cells to transition into fully defined growth in the presence of the pGICs. Once again, chemically defined sustainable growth was not achieved, confirming that the yeast extract is still the sole provider of some necessary compounds.

Chapter 5

Utilizing the Low Yeast Extract Medium and Compound Screening

5.1 Potential Growth Inducing Compound Analysis

Although consistent low yeast extract growth was achieved with the utilization of a feed additive and a number of pGICs ("Enhanced low YX ALIM"), the exact effects of these compounds and their necessity remained unknown. In order to streamline the medium and acquire more information as to the individual effects of the pGICs, it was decided to perform additional experimentation prior to utilizing the medium platform to test additional compounds. Since there were 11 pGICs a 12 run, 11 factor Plackett-Burman design [117] was selected in order to efficiently gain insight and allow for low yeast extract growth optimization. The experimental design and levels are summarized in Tables 5.1 and 5.2.

Table 5.1: Plackett-Burman 12 run design for testing of pGIC cell growth effects.

Run	*spermidine*	*ethanolamine*	*inosine*	*adenosine*	*betaine*	*ornithine*	*spermine*	*uridine*	*putrescine*	*ascorbate*	*selenium*
1	+	-	+	-	-	-	+	+	+	-	+
2	+	+	-	+	-	-	-	+	+	+	-
3	-	+	+	-	+	-	-	-	+	+	+
4	+	-	+	+	-	+	-	-	-	+	+
5	+	+	-	+	+	-	+	-	-	-	+
6	+	+	+	-	+	+	-	+	-	-	-
7	-	+	+	+	-	+	+	-	+	-	-
8	-	-	+	+	+	-	+	+	-	+	-
9	-	-	-	+	+	+	-	+	+	-	+
10	+	-	-	-	+	+	+	-	+	+	-
11	-	+	-	-	-	+	+	+	-	+	+
12	-	-	-	-	-	-	-	-	-	-	-

Table 5.2: Low and high pGIC levels for low yeast extract medium optimization

Compound	**Concentration (μM)**	
	Low (-1)	**High (+1)**
selenium	0	0.3
inosine	0	1000
uridine	0	1000
adenosine	0	100
betaine	0	1000
ethanolamine	0	25
ascorbate	0	10
ornithine	0	40
putrescine	0	10
spermidine	0	10
spermine	0	10

Compounds were ordered randomly in generating Table 5.1. As is seen in Table 5.2, the "low" level tested for all compounds was zero. These zero levels were selected in order to ascertain whether or not all of these compounds were required in the medium. The "high" levels were set as the level currently used in the low yeast extract formulation. Prior to beginning this experiment, cells were passaged from "enhanced low YX ALIM" into the same media without pGICs. This allowed for enough residual growth to achieve passagable densities and enabled the pGICs to be tested without convolution from residual media after passaging.

5.1.1 Individual Effects

Results of the pGIC Plackett-Burman experiment are presented in Figures 5.1 and 5.2. Live cell density is used to analyze compound effects.

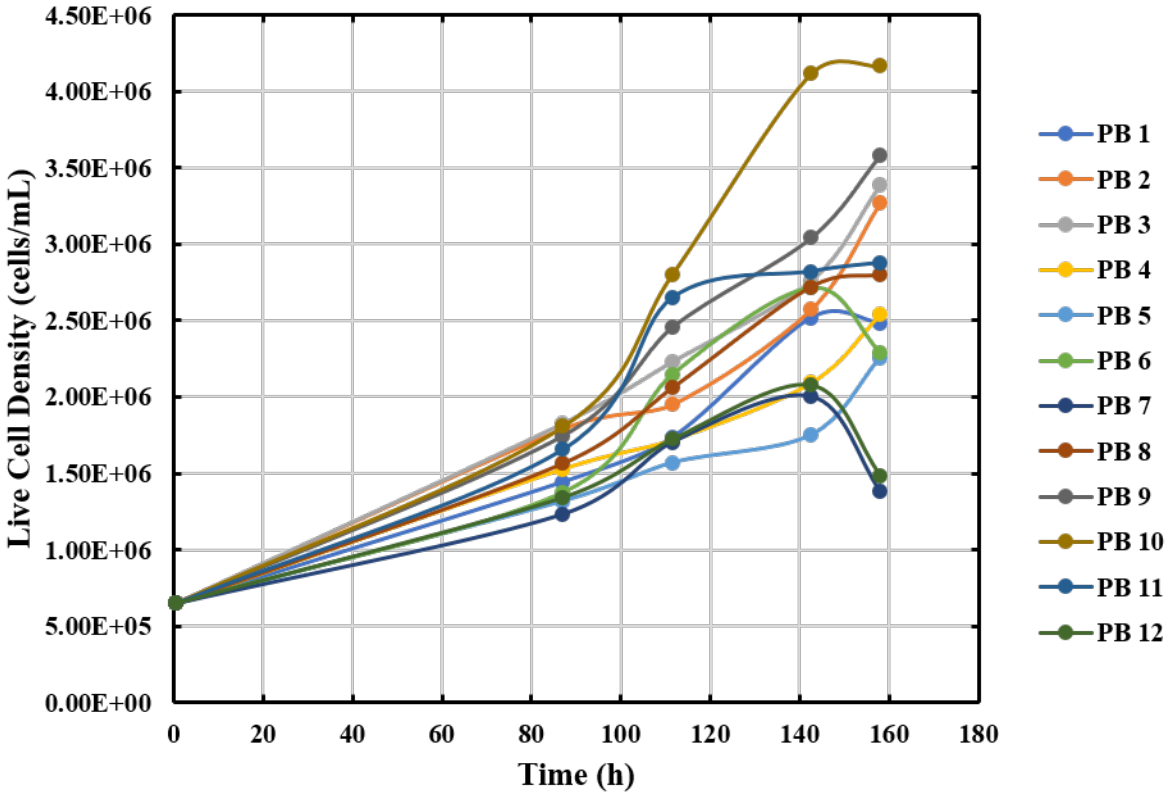

Figure 5.1: Growth profiles of all pGIC Plackett-Burman conditions.

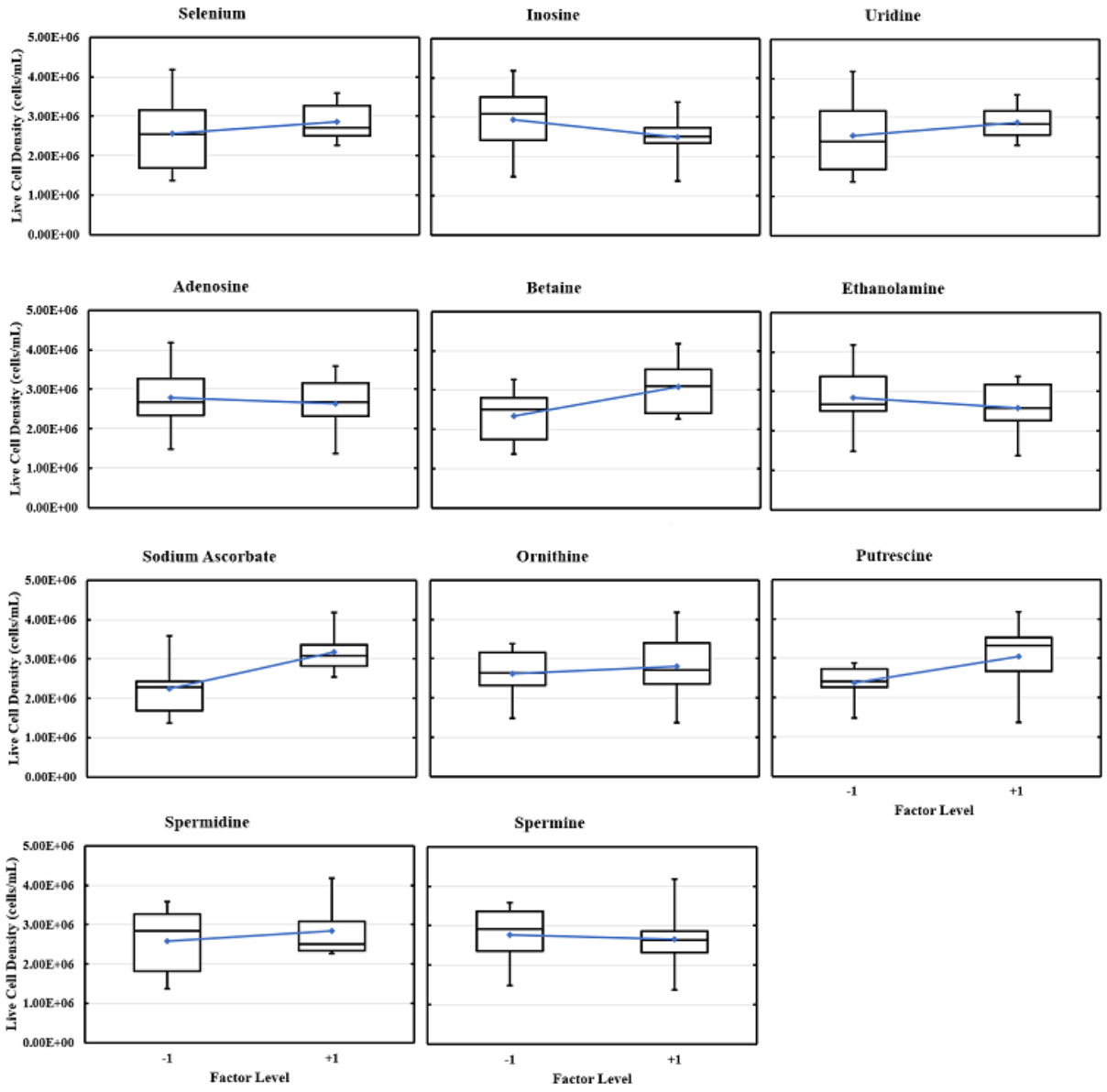

Figure 5.2: Factor level effects of all 11 pGIC compounds tested. Blue dots represent the mean cell density at each factor level.

Figure 5.1 shows the growth profiles over the course of the cultures. It is clear that the different combinations of pGICs result in significant growth differences. It is also apparent that a lack of all pGICs (flask 12) does not result in significant growth, as expected and as was demonstrated previously. Figure 5.2 utilizes box plots to demonstrate the factor level effects of each individual pGIC. The majority of compounds tested do not show significant effects. Three of the compounds (betaine, sodium ascorbate, putrescine) show significant positive effects when present (i.e. "High" factor level), improving mean cell density by 32, 41, and 28% respectively (see Table 5.3). This experiment indicated that while not all of the pGICs appeared to be strictly necessary for growth, none inhibited growth.

Flasks showing the strongest growth (conditions 2,3,9,10) all contained putrescine and at least one of sodium ascorbate or betaine, and the flask showing the best growth (condition 10) contained all three. While the flask showing the worst cell growth (condition 7) also contained putrescine, it contained neither sodium ascorbate nor betaine. It is possible that the cells required putrescine for growth and also at least one "protective" agent due to the richness of the medium and lack of hydrolysate. The results of this experiment supported the hypothesis that antioxidant compounds (e.g. sodium ascorbate) are beneficial to Sf-9 cell culture, particularly in a trace mineral rich medium such as "Enhanced low YX ALIM". The benefits of putrescine are in line with previous reports and indicate that it may be the most important polyamine for Sf-9 cell culture. This makes sense as the cells are likely able to synthesize the other polyamines in the pathway when provided with putrescine. The strong benefits of betaine imply that it may in fact act as an osmoprotectant in insect cell culture as it does in other cell lines. The medium used is enriched, and as such, high in osmolality; while yeast extract generally contains betaine, the low yeast

extract levels used in this experiment may not provide sufficient osmoprotection. This speaks to the usefulness of the low yeast extract platform, as previous attempts at testing betaine in this work proved inconclusive (Appendix B), likely due to convolution from its presence in yeast extract or from other osmoprotective compounds present.

Table 5.3: Factor level effects of all pGICs tested in terms of mean live cell density at time = 158h. Highlighted compounds represent the most significant effects.

Mean Cell Density (cells/mL)			
Compound	**Factor Level**		**% Change**
	-1	**+1**	
selenium	2.57E+06	2.85E+06	11%
inosine	2.94E+06	2.48E+06	-16%
uridine	2.54E+06	2.88E+06	14%
adenosine	2.78E+06	2.64E+06	-5%
betaine	2.34E+06	3.08E+06	32%
ethanolamine	2.84E+06	2.58E+06	-9%
ascorbate	2.25E+06	3.17E+06	41%
ornithine	2.61E+06	2.81E+06	7%
putrescine	2.38E+06	3.04E+06	28%
spermidine	2.58E+06	2.84E+06	10%
spermine	2.76E+06	2.66E+06	-4%

5.1.2 Discussion

The Plackett-Burman experiment done here yielded useful information on the importance of certain compounds used in the "Enhanced low YX ALIM" medium platform. It would next be beneficial to perform a factorial experiment to elucidate the interactions and optimal levels of the three most notable compounds (sodium ascorbate, betaine, putrescine). As none of the pGICs tested demonstrate significant negative effects, their immediate elimination from the platform is not a priority, however inosine levels should be slightly reduced.

The significance of the positive results in this experiment were supported by the fact that all flasks were seeded simultaneously from the same flask, and due to the consistent growth repeatedly observed in "Enhanced low YX ALIM". Testing many compounds simultaneously without introducing variation due to seeding from different flasks, or due to splitting the experiment into multiple sections, is only possible utilizing an approach such as the Plackett-Burman design. This experiment demonstrated the usefulness of this approach, combined with a low yeast extract medium platform, to distinguish effects of micronutrients in an efficient manner. This usefulness instilled confidence in utilizing the approach to simultaneously screen a variety of new micronutrients for potential growth stimulating effects.

5.2 Towards Defined Growth - Compound Screening

Due to the promising results of the pGIC Plackett-Burman experiment, it was decided to utilize this approach for screening new micronutrients. While the previous experiment did not provide in-depth information on the effects of compounds or their optimal levels, it did demonstrate that strong effects may be observed and that yeast extract convolution could be minimized. For the sake of experimental ease and efficiency it was decided to continue with the 11 factor, 12 run Plackett-Burman approach for the screening of new compounds.

5.2.1 Compound Screening Experimental Design

Compounds chosen for the Plackett-Burman screening of new micronutrients in "Enhanced low YX ALIM" were as follows:

- Sugars (trehalose and glucose): Trehalose is a disaccharide composed of 2 glucose molecules which is able to be broken down by Sf-9 cells [6]). A significant amount of trehalose is present both in insect hemolymph and in yeast extract [35, 6]. An equivalent amount of glucose was also included in the screening to help determine if any effects which may be seen with trehalose addition are simply related to an increased sugar concentration rather than being trehalose specific.

- TCA cycle intermediates (α-ketoglutarate, fumarate, malate, succinate): Organic acids known to be present in high concentrations in insect hemolymph and which are often included in classic Sf-9 cell media (e.g. IPL-41). Could play a role in chela-

tion [65], which is important in a trace metal rich environment such as "Enhanced low YX ALIM".

- L-citrulline: Related to arginine metabolism and ornithine and therefore may relate to the polyamine synthesis pathway. Citrulline has been found to be present in soy hydrolysate [118]. It is worth investigating citrulline for the sake of thoroughly exploring the polyamine pathways.

- Guanosine: Nucleoside present in yeast extract [6], and not previously tested in this work.

- Lipoic acid: Present in a variety of serum-free media formulations (e.g. Ham's F12). Plays a number of cellular roles (e.g. enzyme co-factor, antioxidant) [119, 120].

- Reduced glutathione: Tripeptide with a variety of roles in cell culture. The antioxidant role of glutathione has been investigated in Sf-9 insect cells [56] and glutathione has been included in a chemically defined insect cell culture medium (MTCM-1520 [99]).

- Corning^{TM} Trace Elements B (Thermo Fisher Scientific): Contains a variety of trace elements not present in the "ALIM" media. Table 5.4 lists the components contained in the solution.

Table 5.4: Trace elements and their form contained in Corning^{TM} Trace Elements B solution.

Corning^{TM} Trace Elements B

Trace Element	**Form**
Mo	ammonium molybdate
V	ammonium vanadate
Mn	manganese sulfate
Ni	nickel sulfate
Si	sodium silicate
Sn	stannous chloride

The Plackett-Burman experimental design and levels are summarized in Tables 5.5 and 5.6. Compounds were ordered randomly in generating Table 5.5.

Table 5.5: Plackett-Burman 12 run design for screening of new compounds in low yeast extract medium.

Run	*glutathione*	*malate*	*glucose*	*guanosine*	*succinate*	*fumarate*	*α-ketoglutarate*	*TM B*	*trehalose*	*citrulline*	*lipoic acid*
1	+	-	+	-	-	-	+	+	+	-	+
2	+	+	-	+	-	-	-	+	+	+	-
3	-	+	+	-	+	-	-	-	+	+	+
4	+	-	+	+	-	+	-	-	-	+	+
5	+	+	-	+	+	-	+	-	-	-	+
6	+	+	+	-	+	+	-	+	-	-	-
7	-	+	+	+	-	+	+	-	+	-	-
8	-	-	+	+	+	-	+	+	-	+	-
9	-	-	-	+	+	+	-	+	+	-	+
10	+	-	-	-	+	+	+	-	+	+	-
11	-	+	-	-	-	+	+	+	-	+	+
12	-	-	-	-	-	-	-	-	-	-	-

Table 5.6: Low and high compound levels for Plackett-Burman screening experiment in "Enhanced low YX ALIM".

Compound	**Concentration (μM)**	
	Low (-1)	**High (+1)**
succinate	0	200
α-ketoglutarate	0	100
fumarate	0	50
malate	0	50
L-citrulline	0	100
lipoic acid	0	5
glucose	0	10000
trehalose	0	5000
glutathione	0	1000
guanosine	0	50
TM B	0	10 μL

As is seen in Table 5.6, the "low" level tested for all compounds other than glucose was zero. This is due to the fact that glucose is already present in basal "ALIM" as the primary energy source. The 10 mM increase from "low" to "high" glucose levels is equivalent to the 5 mM increase of trehalose (i.e. there are two glucose molecules in each molecule of trehalose). The "high" levels were based on a combination of available literature values, available media formulations and yeast extract composition. Since *Corning*^{TM} *Trace Elements B* is a 1000x concentrate solution, it was added as a volume amount. *Trace Elements B* was added at

slightly less than the recommended levels (10 μL instead of 13 μL) due to the fact that enhanced basal "ALIM" with GICs is already very rich in trace metal content.

5.2.2 New Compound Effects

Results of the new compound screening Plackett-Burman experiment are presented in Figures 5.3 and 5.4. Live cell density is used to analyze compound effects.

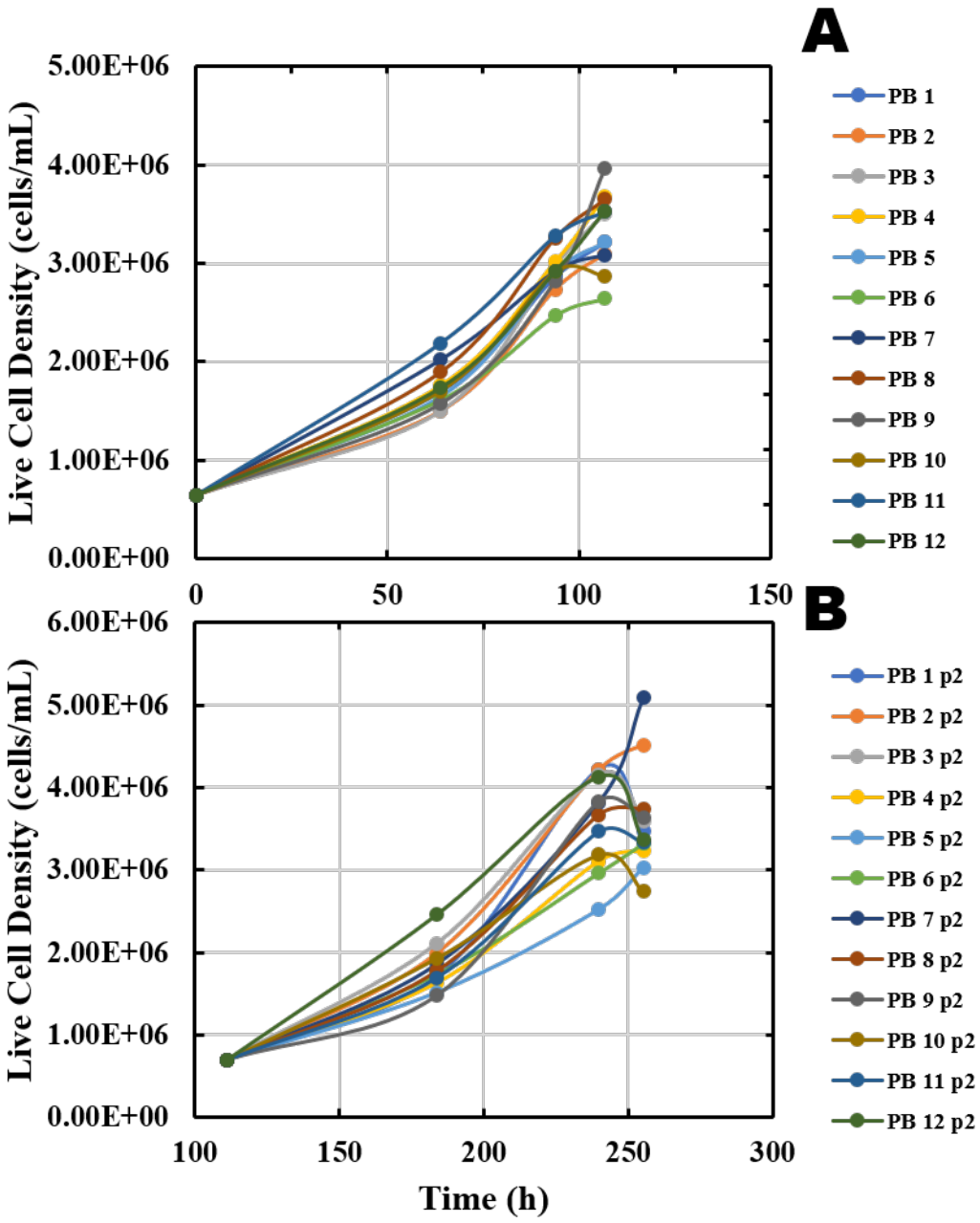

Figure 5.3: Growth profiles of all compound screening Plackett-Burman conditions. A) First passage; B) Second passage.

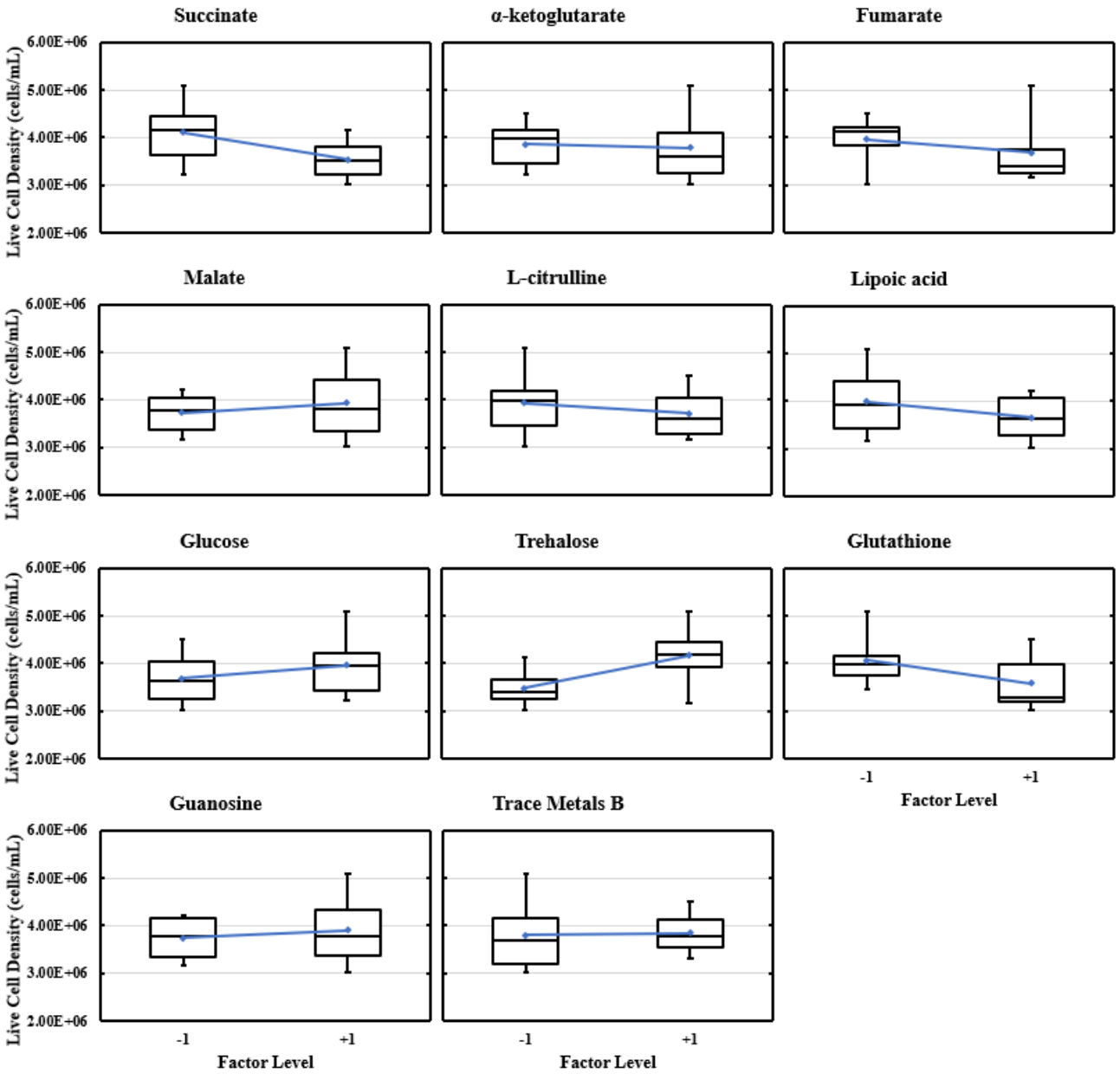

Figure 5.4: Factor level effects of all 11 compounds screened. Blue dots represent the mean cell density at each factor level.

Figure 5.3 shows the growth profiles over the course of both passages in each condition. Growth profiles showed very little variation in the first passage reaffirming the observation that growth effects often require multiple passages to observe. Figure 5.4 utilizes box plots to demonstrate the factor level effects of each individual compound screened in this experiment. This data was based on the maximum cell density achieved during passage 2 of each condition. Most of the compounds screened do not show significant effects. The largest factor effect was the addition of 5 mM trehalose, which resulted in a mean cell density increase of approximately 19%. All four conditions showing higher maximum passage 2 cell density than the control (condition 12) contained trehalose. Compound factor level effects are summarized in Table 5.7.

Table 5.7: Factor level effects of all compounds screened in terms of mean live cell density at time = 144h (of passage 2). Highlighted compounds represent the most significant effects.

Mean Cell Density (cells/mL)			
Compound	**Factor Level**		**% Change**
	-1	**+1**	
succinate	4.11E+06	3.55E+06	-14%
α-ketoglutarate	3.87E+06	3.79E+06	-2%
fumarate	3.97E+06	3.69E+06	-7%
malate	3.72E+06	3.93E+06	6%
L-citrulline	3.94E+06	3.72E+06	-6%
lipoic acid	4.00E+06	3.66E+06	-8%
glucose	3.69E+06	3.96E+06	7%
trehalose	3.49E+06	4.17E+06	19%
glutathione	4.07E+06	3.59E+06	-12%
TM B	3.81E+06	3.85E+06	1%
guanosine	3.75E+06	3.91E+06	4%

5.2.3 Discussion

Since all of the compounds except trehalose showed negative or non-significant positive effects they were ruled out at those levels in future screening experiments. The positive

effect seen with trehalose is of interest due to the fact that the medium already has an excess of glucose, and due to the fact that an analogous glucose spike did not result in significant improvement. This indicates that perhaps trehalose is operating in a manner beyond simply as a carbohydrate source. Further investigation is required to fully elucidate the effects of trehalose addition in a low yeast extract growth medium.

This screening experiment illustrated that growth effects or lack thereof may be observed simultaneously and efficiently for many compounds by utilizing the "Enhanced low YX ALIM" medium and designed experiments. The low growth of the medium (relative to commercial media and "ALIM + YX") allowed for the observation of small effects. This strategy should be continued for multiple experiments, eliminating conditions which showed negative or no effects. Conditions which display potential positive effects (e.g. trehalose) should be added to the medium formulation and carried forward for subsequent experimentation. Designed experiments with more degrees of freedom should be run in order to better quantify significance and move forward with confidence.

Chapter 6

Media Cost Analysis and Optimization

6.1 In-house Medium Cost Analysis

6.1.1 Component Cost Analysis

A secondary benefit to utilizing an in-house medium with a known formulation is the potential for significant cost reductions compared to purchasing commercial media. A detailed cost analysis was performed on the "ALIM + YX" formulation, the results of which are shown in Table 6.1 (Component pricing current to 2019).

Table 6.1: "ALIM + YX" in-house medium cost analysis summarized by class of components.

Component Class	**Cost ($/L)**	
	Normal	**Bulk**
Amino Acids	$ 18.84	$ 11.46
Trace Metals[1]	$ 0.00	$ 0.00
Vitamins	$ 0.10	$ 0.05
Lipids	$ 24.24	$ 24.24
Other[2]	$ 4.41	$ 3.25
Total:	$ 47.59	$ 39.01

[1]trace metal cost per litre is less than $0.01.
[2]"other" includes sugars, salts, hydrolysate.

The analysis was performed using both small scale and bulk pricing. All compounds were of cell culture/ bioreagent grade and the majority of prices were taken from Sigma-Aldrich (the only exception being yeast extract, which was priced through Fisher Bioreagents).

Compared to commercially available Sf-9 insect cell culture media (Table 6.2), "ALIM + YX" was significantly less expensive.

Table 6.2: Commercial media versus in-house "ALIM + YX" cost comparison.

Medium	Manufacturer	Cost ($/L) Normal	Bulk
Sf-900^{TM}III SFM	ThermoFisher	$ 126.00	$ 106.75
Sf-900^{TM}II SFM	ThermoFisher	$ 162.00	$ 80.40
ALIM + YX	In-house	$ 47.59	$ 39.01

This potential savings clearly demonstrates that in-house medium provides economic benefits, in addition to the previously described benefits of control and customizability.

6.1.2 Feed Additive and Micronutrients

When considering the feed additives, the cost of "ALIM + YX" is not significantly increased as the vitamins and trace metals are at such low concentrations that their cost per litre is essentially negligible (even when increased nearly 3-fold in "Enhanced low YX ALIM"). Potential growth inducing compounds (pGICs) mentioned previously also do not have a significant impact on costs, as shown in Table 6.3.

Table 6.3: Cost analysis of potential growth inducing compounds at their desired concentration provided via feed additive.

Compound	Conc. (g/L)	Cost ($/L) Normal	Bulk
inosine	0.2682	$ 1.25	$ 1.25
uridine	0.2442	$ 1.28	$ 0.99
adenosine	0.0267	$ 0.27	$ 0.16
ornithine · HCl	0.0067	$ 0.01	$ 0.01
putrescine · 2HCl	0.0016	$ 0.01	$ 0.01
spermidine · 3HCl	0.0025	$ 0.15	$ 0.07
spermine · 4HCl	0.0035	$ 0.27	$ 0.19
ethanolamine · HCl	0.0024	$ 0.00	$ 0.00
Na_2SeO_3	0.0001	$ 0.00	$ 0.00
betaine	0.1172	$ 0.17	$ 0.09
sodium ascorbate	0.0020	$ 0.00	$ 0.00
	Total:	$ 3.41	$ 2.78

Compounds showing a cost per litre of $0.00 cost less than $0.01 per litre. Nucleosides are priced through ThermoFisher at the highest available purity (99%+). All other compounds included in Table 6.3 are priced from Sigma-Aldrich at Bioreagent or higher grade.

6.1.3 Potential for Cost Reduction

Looking more closely at the "ALIM + YX" cost breakdown, it is apparent that the Sigma *Chemically Defined Lipid Mixture 1* is contributing a significant percentage of the cost (50.9%). As such, the lipids are an obvious starting point for potentially further reducing the cost of the medium. Table 6.4 shows the cost breakdown if the lipid mixture was

produced in-house.

Table 6.4: Individual component cost analysis of lipid mixture at in-house media concentrations.

Compound	**Conc. (g/L)**	**Cost ($/L) Normal**	**Bulk**
arachidonic acid	0.0001	$ 0.09	$ 0.06
linoleic acid	0.0003	$ 1.45	$ 1.45
linolenic acid	0.0003	$ 0.06	$ 0.03
myristic acid	0.0003	$ 0.00	$ 0.00
oleic acid	0.0003	$ 0.01	$ 0.01
palmitic acid	0.0003	$ 0.00	$ 0.00
stearic acid	0.0003	$ 0.01	$ 0.00
cholesterol	0.0066	$ 0.42	$ 0.13
Tween-80	0.0660	$ 0.03	$ 0.01
tocopherol acetate	0.0021	$ 0.01	$ 0.01
Pluronic F-68	3.0000	$ 9.81	$ 9.81
	Total:	$ 11.89	$ 11.52

As shown in Table 6.4, good cost reduction is possible by making the lipid mixture in-house. However, due to the labour involved, and the difficulty of consistently producing the solution (as compared to e.g. amino acid solution, which is trivial), it is likely not worthwhile for the minor reduction in "ALIM + YX" cost per litre. A thorough investigation of the necessity of such high lipid levels is therefore warranted. The potential savings due to lipid mixture concentration reduction are outlined in Table 6.5, highlighting the importance of this avenue of investigation.

Table 6.5: Cost saving potential of utilizing less defined lipid mixture in in-house medium.

Medium	Lipid Mixture Conc. (mL/L)	Cost ($/L)		Savings (%)	
		Normal	Bulk	Normal	Bulk
ALIM + YX	30	$ 47.59	$ 39.01	-	-
ALIM + YX	20	$ 39.51	$ 30.93	17%	21%
ALIM + YX	10	$ 31.43	$ 22.85	34%	41%

As outlined in Table 6.5, reducing the level of lipid mixture concentration in the medium would provide significant cost savings while also maintaining consistency in a portion of the medium which is typically difficult to produce. It is reasonable to hypothesize that such a reduction is possible due to the higher than typical amounts used in the medium.

6.2 Lipid Optimization and Cost Reduction

6.2.1 Premise

The originally used in-house "ALIM + YX" formulation called for 30 mL lipid mixture per 1 L media. This amount had been chosen prior to this work as preliminary experiments had shown a growth improvement when increasing the lipid mixture concentration from the typical 10 mL/L (*Lipid Mixture 1* is sold as a 100x concentrate). *Lipid Mixture 1* and its component contributions to "ALIM + YX" in-house media may be seen in Table 6.6.

Table 6.6: Sigma-Aldrich *Lipid Mixture 1* component concentrations and final concentrations in original in-house "ALIM + YX" formulation.

Compound	**Soln Conc. (μg/mL)**	**ALIM Conc. (g/L)**
arachidonic acid	2	0.00006
linoleic acid	10	0.00030
linolenic acid	10	0.00030
myristic acid	10	0.00030
oleic acid	10	0.00030
palmitic acid	10	0.00030
stearic acid	10	0.00030
cholesterol	220	0.00660
Tween-80	2200	0.06600
tocopherol acetate	70	0.00210
Pluronic F-68	100000	3

6.2.2 Preliminary Experimentation: Lipid Reduction

First, the lipid levels were reduced in fully adapted "ALIM + YX" cells to confirm that the growth was, in fact, impacted. Figures 6.1 and 6.2 show the exponential growth as well as maximum growth of duplicate flasks of each: 30mL lipid mixture per 1L"ALIM + YX" (control), 20mL/L, and 10mL/L.

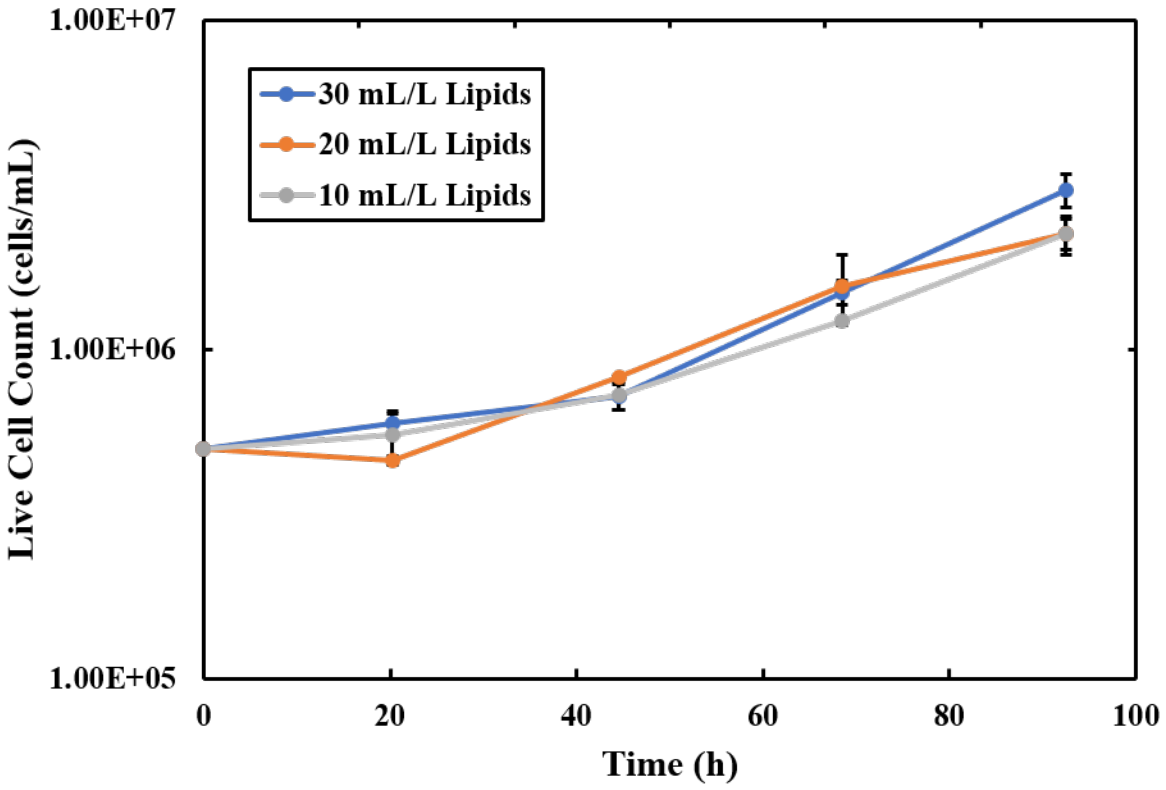

Figure 6.1: Semi-log plot showing exponential growth effects of lipid mixture reduction in media. Error bars represent the range of duplicate cultures.

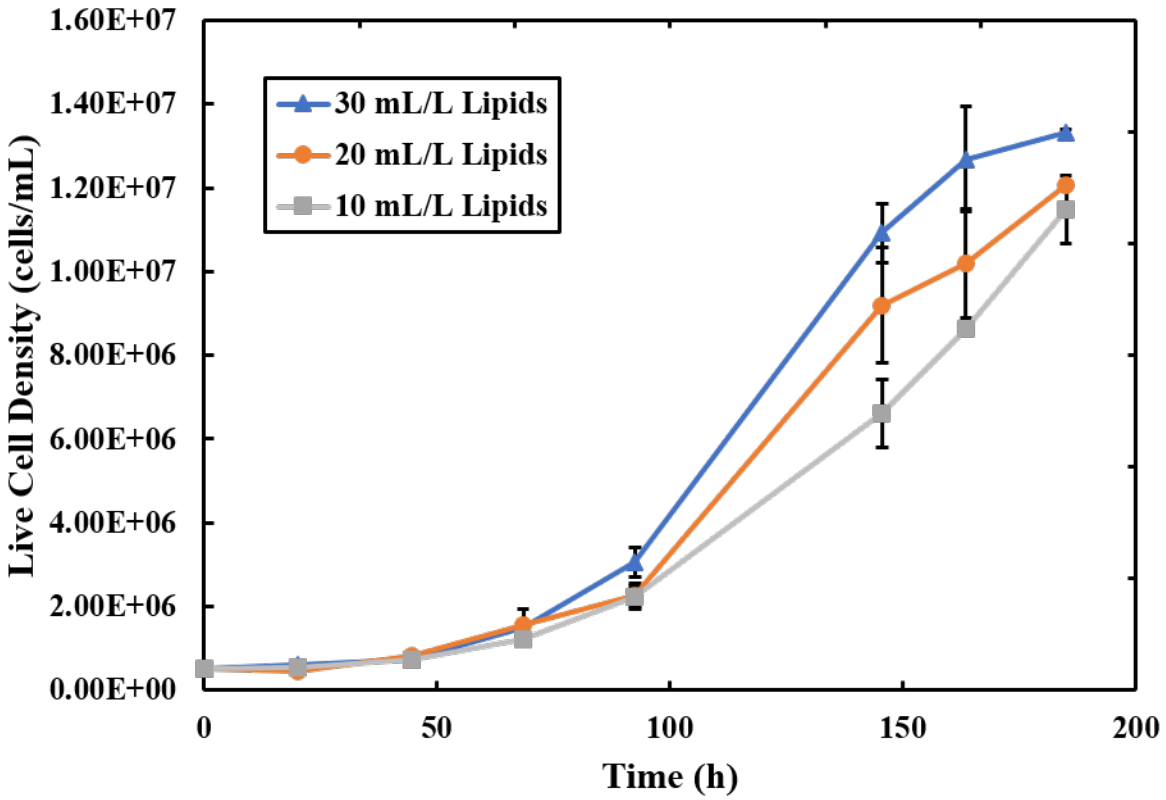

Figure 6.2: Complete growth profile of lipid mixture reduction in media. Error bars represent the range of duplicate cultures.

As can be seen in Figures 6.1 and 6.2, the reduction of lipid concentration did have a detrimental effect on cell proliferation as well as maximum density. Calculated maximum exponential growth rates were 0.030 h^{-1}, 0.025 h^{-1}, and 0.023 h^{-1} for 30mL/L, 20mL/L, and 10mL/L lipid mixture respectively. The greater the lipid reduction, the stronger the detrimental effect (i.e. 10mL/L lipid mixture performs worse than 20mL/L). The effects on exponential growth are of particular concern.

6.2.2.1 Adaptation

Since the growth was not completely inhibited by reducing the lipid mixture concentration, it was thought that the cells may be fine at lower levels but simply needed to adapt. The same experiment was repeated, with the cells being passaged into their same lipid level when densities of 2 - 5 $\times$ 10^6 cells/mL were reached. This was continued for 3 passages for each condition and the results are presented in Figure 6.3.

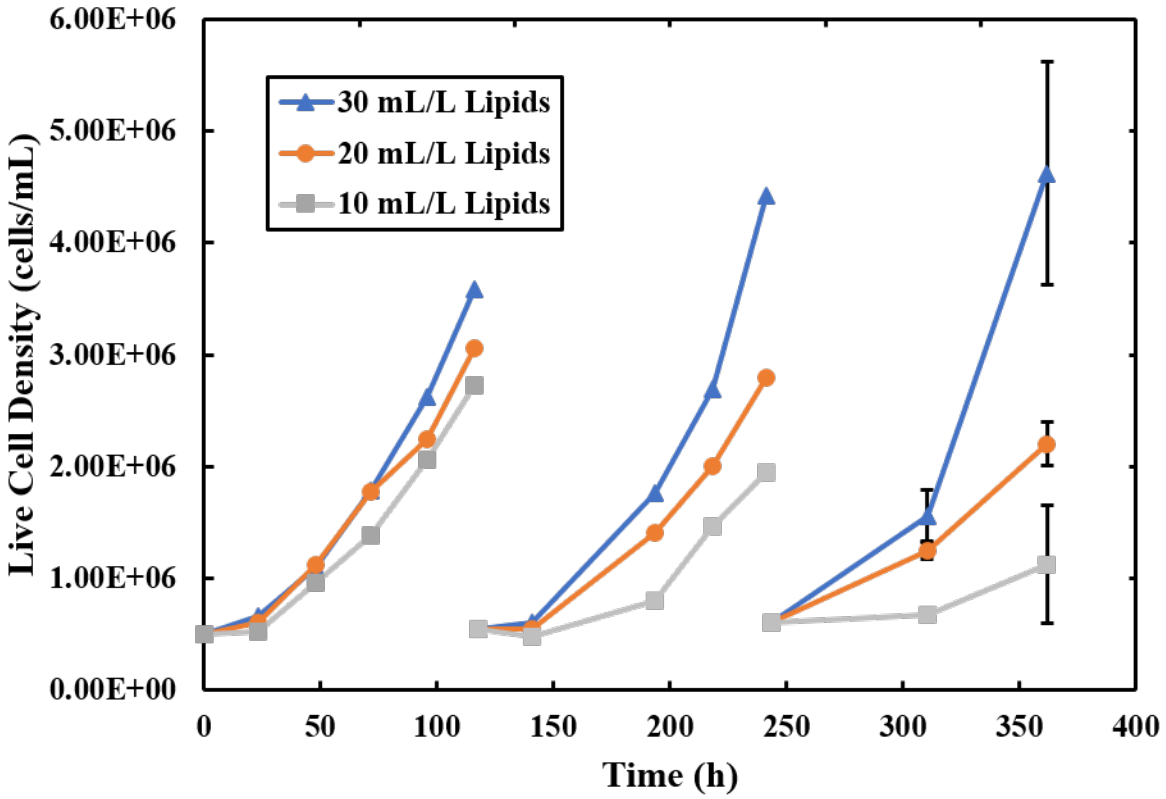

Figure 6.3: Three passage reduced lipid adaptation growth profiles. Connected data points comprise a single passage. Error bars represent the range of duplicate cultures.

Figure 6.3 clearly demonstrates that reducing the lipid mixture concentration is not a viable cost saving solution. Adaptation to lower lipid levels was not successful; in fact, at reduced lipid concentrations, cell growth slows down with subsequent passages. This effect is particularly noticeable at 10mL/L lipid mixture (which did not even reach passageable cell densities during passage 3). The control (30mL/L) lipid amount maintains consistent growth throughout the three passages; the appearance of growth rate improvement is simply related to experimental variation and increasing seed densities (i.e. passage 1 flasks were seeded at 5.0×10^5 cells/mL, while passage 2 and 3 flasks were seeded at 5.5×10^5 and 6.0×10^5 cells/mL respectively). The fact that the cell growth is less affected by lipid reduction in the initial passage is likely due to carry over volume and perhaps the cells holding on to components of the lipid mixture. For example, the effects of Pluronic F-68 on insect cell culture have been demonstrated to persist even after elimination of the polyol from the medium [121].

6.2.3 Pluronic F-68 Replacement

The previous results indicated that it was not a viable strategy to simply reduce the concentration of lipid mixture used. It followed that an investigation of specific components of the mixture might yield a cost reduction strategy that maintains cell proliferation rates. Previous work by Gilbert et al focused on the importance of lipids during cell growth, specifically in insect cells (Sf-9 and High-Five^{TM}) [64]. This study demonstrated that a key component to growth present in typical lipid mixtures is Pluronic^{TM} F-68 polyol (Poloxamer 188), while other lipid mixture components investigated were not strictly required

for cell growth. Pluronic F-68 is a polyol often included in cell culture media largely as a shear protectant and as a stabilizing agent in lipid emulsions. As can be seen in Table 6.6, *Lipid Mixture 1* provides a significant amount of Pluronic F-68 to "ALIM + YX" in-house media (3g/L).

It was hypothesized that the only component requiring the medium concentration provided by 30mL/L *Lipid Mixture 1* was Pluronic F-68, while the other lipid mixture components were being provided in excess. Based on this, it was decided to pursue *Lipid Mixture 1* reduction with simultaneous Pluronic F-68 replacement. Duplicate flasks of the 5 experimental conditions were seeded simultaneously from the same "seeding flask". The experimental conditions were: 30mL/L lipids (+ve control), 20mL/L lipids + Pluronic F-68, 10mL/L lipids + Pluronic F-68, 0mL/L lipids + Pluronic F-68, 0mL/L lipids (-ve control). Pluronic F-68 was added such that the total Pluronic F-68 concentration was equivalent to that which would be provided by 30mL lipid mixture per 1L in-house media (i.e. all flasks in the experiment had the same concentration of Pluronic F-68 except for the -ve control which did not contain any). The results of this experiment are presented in Figures 6.4 and 6.5.

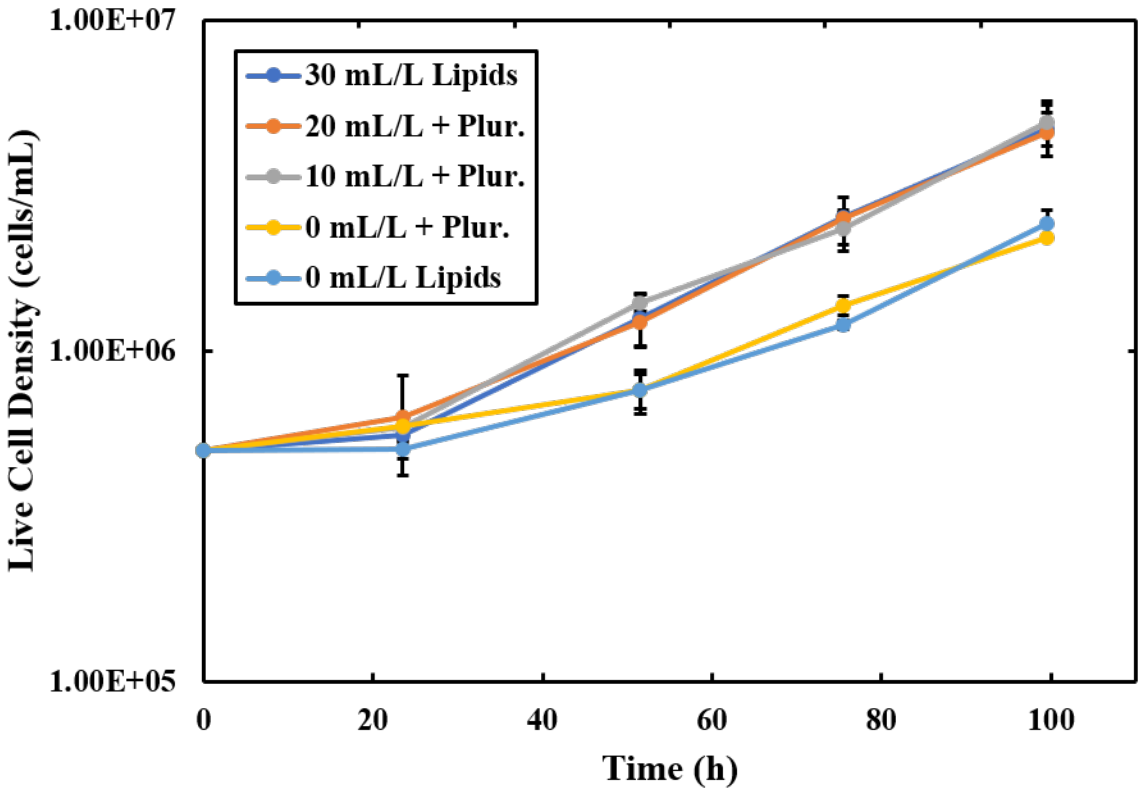

Figure 6.4: Semi-log plot showing exponential growth effects of lipid level reduction plus Pluronic F-68 replacement in media. Error bars represent the range of duplicate cultures.

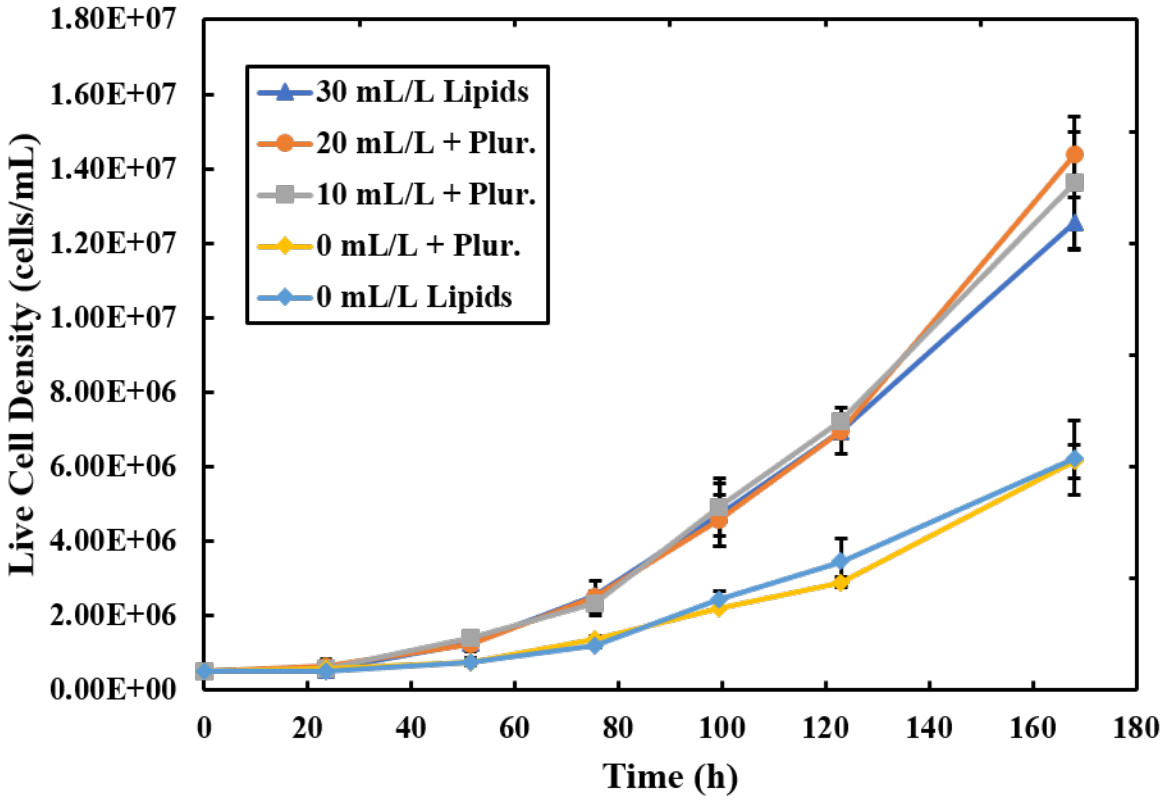

Figure 6.5: Complete growth profiles of lipid level reduction plus Pluronic F-68 replacement in media. Error bars represent the range of duplicate cultures.

As can be seen in Figures 6.4 and 6.5, the replacement of Pluronic F-68 in media with reduced lipid mixture concentrations restored growth to that of media with full (30mL/L) lipid mixture concentration. Figure 6.4 highlights the exponential growth recovery via Pluronic F-68 replacement, in contrast to Figure 6.1. This supports the hypothesis that the only component of the lipid mixture necessary at such high concentration initially provided was, in fact, the Pluronic F-68. Flasks without lipid mixture showed very slow growth,

likely due to carry-over lipids and Pluronic F-68 from passaging. Pluronic F-68 addition did not appear to help in the absence of lipid mixture, indicating that at least some level of the lipids themselves (e.g. cholesterol) or another component (i.e. Tween-80 or Vitamin E) are necessary for exponential growth. Figure 6.5 presents the complete growth profile, demonstrating that maximum cell density is also recovered. Figure 6.5 shows the possibility that maximum cell density is actually increased in the flasks with reduced lipid mixture and Pluronic F-68 replacement, however further investigation would be required in order to determine whether or not this is the case in a manner that is statistically significant. If that were the case, one explanation might be that the reduction in lipid mixture means that there is less ethanol in the medium, which can have a negative effect at high concentrations. It is possible that the importance of Pluronic F-68 in Sf-9 insect cells is simply related to shear protection, although it has been demonstrated to be incorporated into the cellular membrane and perhaps plays a larger role in cell proliferation [121].

It was then necessary to determine if Pluronic F-68 replacement was a viable strategy to restore cell growth from lipid mixture reduction over multiple passages. Figure 6.6 repeats the experiment shown in Figure 6.3 but with Pluronic F-68 replacement for the 20mL/L and 10mL/L *Lipid Mixture 1* cultures. Pluronic F-68 was replaced as before, i.e. to mimic total Pluronic F-68 present in media with 30 mL/L *Lipid Mixture 1*.

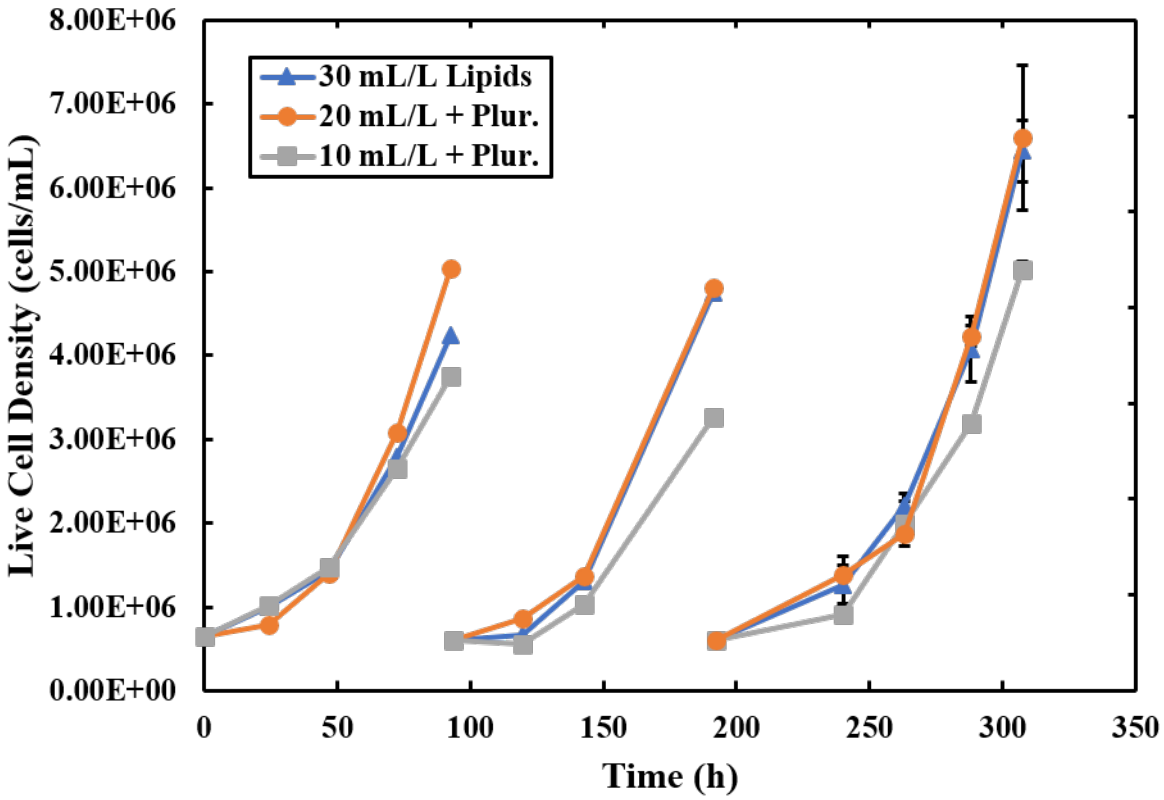

Figure 6.6: Three passage reduced lipid adaptation growth profiles with Pluronic F-68 replacement. Error bars represent the range of duplicate cultures.

As can be seen in Figure 6.6, Pluronic F-68 replacement does maintain restored cell growth for media with 20mL/L lipid mixture. However, while Pluronic F-68 does restore some of the growth lost at 10mL/L lipid solution, it does not maintain pace with the 30mL/L control, unlike in the single passage experiment (Figure 6.5).

6.3 Discussion

A cost analysis of in-house "ALIM + YX" revealed that while the medium is already cheaper than commercially available products, there was potential for further cost savings. It was successfully demonstrated that reducing the amount of the expensive *Lipid Mixture 1* was possible only alongside Pluronic F-68 replacement. These effects should be investigated with respect to virus amplification and protein production in IC-BEVs, where it is known that lipid levels (particularly cholesterol) play a role [64].

Based on these results, it is economically beneficial to reduce the concentration of defined lipid mixture used, while replacing the polyol Pluronic F-68 that was removed while doing so. This should not have a detrimental effect on exponential cell proliferation or maximum cell density. It appears viable to reduce the lipid mixture content of in-house "ALIM + YX" media by 1/3, or in some cases 2/3, to 20 or 10 mL/L (in line with manufacturer recommended levels). This would result in an in-house media production cost savings of approximately 17-41% (see Table 6.5). It is likely that Pluronic F-68 addition would prove even more necessary in large-scale cultures due to the greater shear forces in such systems. Further investigation is required before applying these changes to IC-BEVS applications.

Chapter 7

Conclusions

The objective of this work was to develop an Sf-9 cell culture medium platform based on yeast extract composition and media designed for other cell lines, with which micronutrient compound effects could be properly tested in order to progress towards a fully chemically defined growth medium for Sf-9 insect cells.

- A trace metal and B vitamin fortified medium in combination with a selection of "growth inducing" compounds was shown to be necessary to, and effective in, the development of a low yeast extract growth medium for Sf-9 insect cells. The medium was capable of consistent growth over at least 40 passages with a yeast extract concentration of only 0.5 g/L. Initial yeast extract concentration was 4 g/L.
- At low yeast extract concentration (0.5 g/L), multi-passage growth was only achievable with the inclusion of the determined "growth inducing" compounds.

- The low yeast extract medium was instrumental in elucidating the positive effects of compounds which were otherwise convoluted by their presence, or the presence of similar compounds in full yeast extract concentration medium. Betaine in particular was shown to be of significance in the low yeast extract medium while preliminary experiments in the full yeast extract medium proved inconclusive.

- Glycine betaine (1 mM), ascorbic acid (10 μM), and putrescine (10 μM) were shown to be necessary supplements when reducing yeast extract concentration, improving maximum cell density in the low yeast extract medium by 32%, 41%, and 28% respectively (in comparison to their exclusion from the medium).

- There still remains other components which are necessary for fully chemically defined growth which are yet to be identified.

- The low yeast extract medium is cost-effective compared to commercially available Sf-9 insect media.

- Pluronic F-68 is an important component in Sf-9 cell media and its supplementation allows for the reduction of chemically defined lipid solution concentration in the medium. This allows for further cost-saving benefits of the in-house medium.

Chapter 8

Recommendations

- The low yeast extract medium platform should be used for further designed screening experiments (e.g. Plackett-Burman) in order to identify the remaining compounds necessary for a fully defined growth medium. Hormones, growth factors and synthetic oligopeptides in particular should be tested.

- Further refine the low yeast extract medium by testing the elimination of pGIC compounds deemed to have insignificant effects in the Plackett-Burman experiment in this study. Further experimentation is necessary to ensure that compounds which are required are currently at optimal levels (e.g. full factorial experiment to optimize betaine, ascorbate, and putrescine levels).

- Enhancement of the medium to improve growth rate and maximum cell density would be beneficial regardless of whether, but especially if, chemically defined growth is achieved.

- The enhanced low yeast extract medium should be studied with respect to virus replication and protein production utilizing IC-BEVS, as well as with stably transfected cell lines, in order to determine the feasibility of utilizing it as a more-defined, lower-cost alternative to commercially available media over a wider range of applications.

References

[1] Rachael S Felberbaum. The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. *Biotechnology Journal*, 10:702–714, 2015.

[2] TDC Grace. Establishment of Four Strains of Cells from Insect Tissues Grown In Vitro. *Nature*, 195:788–789, 1962.

[3] S A Weiss, G C Smith, S S Kalter, and J L Vaughn. Improved Method for the Production of Insect Cell Cultures in Large Volume. *In Vitro*, 17(6):495–502, 1981.

[4] Brian Maiorella, Duane Inlow, Andrea Shauger, and David Harano. Large-Scale Insect Cell-Culture for Recombinant Protein Production. *Nature Biotechnology*, 6:1406–1410, 1988.

[5] Duane Inlow, Andrea Shauger, and Brian Maiorella. Insect Cell Culture and Baculovirus Propagation in Protein-Free Medium. *Journal of Tissue Culture Methods*, 12(1):13–16, 1989.

[6] Marco Quattrociocchi. Partial Characterization of Yeast Hydrolysates for Insights on Chemically Defined Media for Sf-9 Insect Cells. *University of Waterloo MASc Thesis*, 2017.

[7] Michael L Shuler and Fikret Kargi. *Bioprocess Engineering: Basic Concepts*. Prentice Hall, Upper Saddle River, NJ, 2^{nd} edition, 2002.

[8] WHO. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. Technical report, World Health Organization, 2010.

[9] WHO. Acceptability of cell substrates for production of biologicals: Report of a WHO study group. Technical report, World Health Organization, 1987.

[10] Peter Castle and James S. Robertson. Animal Sera, Animal Sera Derivatives and Substitutes Used in the Manufacture of Pharmaceuticals. *Biologicals*, 26:365–368, 1998.

[11] Max D Summers and Gale E Smith. *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. Texas Agricultural Experiment Station, College Station TX, 1987.

[12] L Sondergaard. Drosophila Cells can be Grown to High Cell Densities. *Biotechnology Techniques*, 10(3):161–166, 1996.

[13] Hans-Dieter Klenk. Post-translational modifications in insect cells. *Cytotechnology*, 20:139–144, 1996.

[14] Marija Vrljic, Pavel Strop, Ryan C Hill, Kirk C Hansen, Steven Chu, and Axel T Brunger. Post-Translational Modifications and Lipid Binding Profile of Insect Cell-Expressed Full-Length Mammalian Synaptotagmin 1. *Biochemistry*, 50:9998–10012, 2011.

[15] Hilary A Overton, Yoichi Fujii, Ian R Price, and Ian M Jones. The Protease and *gag* Gene Products of the Human lmmunodeficiency Virus: Authentic Cleavage and Post-translational Modification in an Insect Cell Expression System. *Virology*, 170:107–116, 1989.

[16] Dwight E Lynn. Novel Techniques to Establish New Insect Cell Lines. *In Vitro Cellular & Developmental Biology - Animal*, 37:319–321, 2001.

[17] Dwight E Lynn and Robert L Harrison. Available Lepidopteran Insect Cell Lines. In David W Murhammer, editor, *Baculovirus and Insect Cell Expression Protocols*, chapter 6, pages 119–137. Humana Press, New York, 3^{rd} edition, 2016.

[18] J L Vaughn, R H Goodwin, G J Tompkins, and P Mccawley. The Establishment of Two Cell Lines from the Insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In Vitro*, 13(4):213–217, 1977.

[19] G R Gardiner and H Stockdale. Two Tissue Culture Media for Production of Lepidopteran Cells and Nuclear Polyhedrosis Viruses. *Journal of Invertebrate Pathology*, 25:363–370, 1975.

[20] Robert R Granados, Guoxun Li, and GW Blissard. Insect Cell Culture and Biotechnology. *Virologica Sinica*, 22(2):83–93, 2007.

[21] PV Vail, G Sutter, DL Jay, and D Gough. Reciprocal Infectivity of Nuclear Polyhedrosis Viruses of the Cabbage Looper and Alfalka Looper. *Journal of Invertebrate Pathology*, 17:383–388, 1971.

[22] Gale E Smith, M J Fraser, and Max D Summers. Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations Within the Polyhedrin Gene. *Journal of Virology*, 46(2):584–593, 1983.

[23] Gale E Smith, Max D Summers, and M J Fraser. Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector. *Molecular and Cellular Biology*, 3(12):2156–2165, 1983.

[24] Monique M van Oers, Gorben P Pijlman, and Just M Vlak. Thirty years of baculovirus insect cell protein expression: from dark horse to mainstream technology. *Journal of General Virology*, 96:6–23, 2015.

[25] Thomas A Kost, J Patrick Condreay, and Donald L Jarvis. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature Biotechnology*, 23(5):567–575, 2005.

[26] S S Wyatt. Culture In Vitro of Tissue from the Silkworm, *Bombyx Mori* L. *The Journal of General Physiology*, 39(6):841–852, 1956.

[27] WF Hink. Established Insect Cell Line from the Cabbage Looper, *Trichoplusia ni*. *Nature*, 226:466–467, 1970.

[28] Ronald H Goodwin. Insect Cell Culture: Improved Media and Methods for Initiating Attached Cell Lines from the Lepidoptera. *In Vitro*, 11(6):369–378, 1975.

[29] Charles Bédard, Rosanne Tom, and Amine Kamen. Growth, Nutrient Consumption, and End-Product Accumulation in Sf-9 and BTI-EAA Insect Cell Cultures: Insights into Growth Limitation and Metabolism. *Biotechnology Progress*, 9:615–624, 1993.

[30] Ronaldo Z Mendonca, Laura A Palomares, and Octavio T Ramirez. An insight into insect cell metabolism through selective nutrient manipulation. *Journal of Biotechnology*, 72:61–75, 1999.

[31] Jerome P Ferrance, Akshay Goel, and Mohammad M Ataai. Utilization of Glucose and Amino Acids in Insect Cell Cultures: Quantifying the Metabolic Flows within the Primary Pathways and Medium Development. *Biotechnology and Bioengineering*, 42:697–707, 1993.

[32] L Ohman, J Ljunggren, and L Haggstrom. Induction of a metabolic switch in insect cells by substrate-limited fed batch cultures. *Applied Microbiology and Biotechnology*, 43:1006–1013, 1995.

[33] Monika Drews, Magnus Doverskog, Lars Ohman, Bogdan E Chapman, Ulla Jacobsson, Philip W Kuchel, and Lena Haggstrom. Pathways of glutamine metabolism in *Spodoptera frugiperda* (Sf9) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by $^{1}H/^{15}N$ NMR. *Journal of Biotechnology*, 78:23–37, 2000.

[34] Monika Drews, Toomas Paalme, and Raivo Vilu. The growth and nutrient utilization of the insect cell line *Spodoptera frugiperda* Sf9 in batch and continuous culture. *Journal of Biotechnology*, 40:187–198, 1995.

[35] G R Wyatt. The Biochemistry of Insect Hemolymph. *Annual Review of Entomology*, 6:75–102, 1961.

[36] Gilles B Tremblays, Narciso R Mejia, and Robert E MacKenzie. The NADP-dependent Methylenetetrahydrofolate Dehydrogenase- Methenyltetrahydrofolate Cyclohydrolase-Formyltetrahydrofolate Synthetase Is Not Expressed in *Spodoptera frugiperda* Cells. *The Journal of Biological Chemistry*, 267(12):8281–8285, 1992.

[37] Chouki Benslimane, Cynthia B Elias, Jalal Hawari, and Amine Kamen. Insights into the Central Metabolism of *Spodoptera frugiperda* (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5) Insect Cells by Radiolabeling Studies. *Biotechnology Progress*, 21:78–86, 2005.

[38] Lars Ohman, Maria Alarcon, Jan Ljunggren, Anna-Karin Ramqvist, and Lena Haggstrom. Glutamine is not an Essential Amino Acid for Sf-9 Insect Cells. *Biotechnology Letters*, 3(7):765–770, 1996.

[39] Jun Mitsuhashi. Amino acid requirements of some continuous cell lines of insects. *Applied Entomology and Zoology*, 13:170–175, 1978.

[40] Michael Brüggert, Till Rehm, Sreejesh Shanker, Julia Georgescu, and Tad A Holak. A novel medium for expression of proteins selectively labeled with ^{15}N-amino acids in *Spodoptera frugiperda* (Sf9) insect cells. *Journal of Biomolecular NMR*, 25:335–348, 2003.

[41] T W Chiou, Y C Hsieh, and C S Ho. High density culture of insect cells using rational medium design and feeding strategy. *Bioprocess Engineering*, 22:483–491, 2000.

[42] R C L Marteijn, O Jurrius, J Dhont, C D de Gooijer, J Tramper, and D E Martens. Optimization of a Feed Medium for Fed-Batch Culture of Insect Cells Using a Genetic Algorithm. *Biotechnology and Bioengineering*, 81(3):269–278, 2003.

[43] Mohd Iqbal Yatoo, Archana Saxena, Padinjare Melepad Deepa, Biju Peer Habeab, Sarita Devi, Ranbir Singh Jatav, and Umesh Dimri. Role of trace elements in animals: a review. *Veterinary World*, 6:963–967, 2013.

[44] Joel G Anderson and Keith M Erikson. The Importance of Trace Elements for Neurological Function. In V Preedy, R Watson, and C Martin, editors, *Handbook of Behavior, Food and Nutrition*, pages 423–439. Springer, New York, 2011.

[45] Tatsuma Yao and Yuta Asayama. Animal-cell culture media: History, characteristics, and current issues. *Reproductive Medicine and Biology*, 16:99–117, 2017.

[46] Armand B Glassman, Raymond S Rydzewski, and Carol E Bennett. Trace Metal Levels in Commercially Prepared Tissue Culture Media. *Tissue and Cell*, 12(4):613–617, 1980.

[47] Joanne Keenan, Karina Horgan, Martin Clynes, Indre Sinkunaite, Patrick Ward, Richard Murphy, and Finbarr O'Sullivan. Unexpected fluctuations of trace element levels in cell culture medium in vitro: caveat emptor. *In Vitro Cellular & Developmental Biology - Animal*, 54:555–558, 2018.

[48] R Forrer, K Gautschi, A Stroh, and H Lutz. Direct Determination of Selenium and other Trace Elements in Serum Samples by ICP-MS. *Journal of Trace Elements in Medicine and Biology*, 12:240–247, 1998.

[49] BD Bionutrients. BD Bionutrients Technical Manual - Advanced Bioprocessing Third Edition, 2006.

[50] W Gregory Hamilton and Richard G Ham. Clonal Growth of Chinese Hamster Cell Lines in Protein-Free Media. *In Vitro*, 13(9):537–547, 1977.

[51] Y M Qi, P F Greenfield, and S Reid. Evaluation of a simple protein free medium that supports high levels of monoclonal antibody production. *Cytotechnology*, 21:95–109, 1996.

[52] Robert W Kenerson. Cell Culture Medium Comprising Transition Metals or Trace Elements;US 0287666 A1, 2005.

[53] William L Cleveland and Bernard F Erlanger. Protein-Free Culture Medium;4767704, 1988.

[54] Stephen F Gorfien, Richard M Fike, Glenn P Godwin, Joyce L Dzimian, David A Epstein, Dale Gruber, Don McClure, and Paul J Price. Serum-Free Mammalian Cell Culture Medium, and Uses Thereof;US 8198084 B2, 2012.

[55] Gerald F Combs Jr. and James P McClung. *The Vitamins: Fundamental Aspects in Nutrition and Health*. Academic Press, 5^{th} edition, 2016.

[56] Ying Wang, Larry W Oberley, and David W Murhammer. Antioxidant Defense Systems of Two Lipidopteran Insect Cell Lines. *Free Radical Biology & Medicine*, 30(11):1254–1262, 2001.

[57] Oreste Arrigoni and Mario C De Tullio. Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta*, 1569:1–9, 2002.

[58] Jeremiah Zartman, Miranda Burnette, Jun Li, and Teresa Brito-Robinson. Cell Culture Medium and Bioprocess Optimization; US0251614 A1, 2016.

[59] Alisa Schnellbaecher, Dennis Binder, Stephanie Bellmaine, and Aline Zimmer. Vitamins in cell culture media: Stability and stabilization strategies. *Biotechnology and Bioengineering*, 116(6):1537–1555, 2019.

[60] A A Spector, S N Mathur, T L Kaduce, and B T Hyman. Lipid Nutrition and Metabolism of Cultured Mammalian Cells. *Progress in Lipid Research*, 19:155–186, 1981.

[61] Kathrin Marheineke, Sylvia Grunewald, William Christie, and Helmut Reilander. Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. *FEBS Letters*, 441:49–52, 1998.

[62] T E Mittler and R H Dadd. *Metabolic Aspects of Lipid Nutrition in Insects*. Westview Press, 1983.

[63] James L Vaughn. Insect Cell Nutrition: Emphasis on Sterols and Fatty Acids. *In Vitro*, 9(2):122–128, 1973.

[64] Rebecca S Gilbert, Yuichi Nagano, Tadafumi Yokota, Sun Fon Hwan, Tom Fletcher, and Ken Lydersen. Effect of lipids on insect cell growth and expression of recombinant proteins in serum-free medium. *Cytotechnology*, 22:211–216, 1996.

[65] Ernst Jürgen Schlaeger. Medium design for insect cell culture. *Cytotechnology*, 20:57–70, 1996.

[66] Ravinder Bhatia, Gary Jesionowski, Jerome Ferrance, and Mohammad M Ataai. Insect cell physiology. *Cytotechnology*, 24:1–9, 1997.

[67] Barry Halliwell. Cell Culture, Oxidative Stress, and Antioxidants: Avoiding Pitfalls. *Biomedical Journal*, 37(3):99–105, 2014.

[68] Yoshiro Saito, Yasukazu Yoshida, Takashi Akazawa, Kazuhiko Takahashi, and Etsuo Niki. Cell Death Caused by Selenium Deficiency and Protective Effect of Antioxidants. *The Journal of Biological Chemistry*, 278(41):39428–39434, 2003.

[69] Ying Wang, Larry W Oberley, and David W Murhammer. Evidence of Oxidative Stress Following the Viral Infection of Two Lepidopteran Insect Cell Lines. *Free Radical Biology & Medicine*, 31(11):1448–1455, 2001.

[70] Valentina C Ciccarone, Dale Gruber, and Shelly Bennett. Culture Medium for Cell Growth and Transfection; US0096414 A1, 2003.

[71] Jorg Neermann and Roland Wagner. Comparative Analysis of Glucose and Glutamine Metabolism in Transformed Mammalian Cell Lines, Insect and Primary Liver Cells. *Journal of Cellular Physiology*, 166:152–169, 1996.

[72] Suh-Chin Wu, Bruce E Dale, and James C Liao. Kinetic Characterization of Baculovirus-Induced Cell Death in Insect Cell Cultures. *Biotechnology and Bioengineering*, 41:104–110, 1993.

[73] Chad Brocker, David C Thompson, and Vasilis Vasiliou. The role of hyperosmotic stress in inflammation and disease. *BioMolecular Concepts*, 3:345–364, 2012.

[74] Yu V Natochin and R G Parnova. Osmolality and Electrolyte Concentration of Hemolymph and the Problem of Ion and Volume Regulation of Cells in Higher Insects. *Comparative Biochemistry and Physiology*, 88(3):563–570, 1987.

[75] Junli Zhang, Nicolas Kalogerakis, and Leo A Behie. Optimization of the physiochemical parameters for the culture of *Bombyx mori* insect cells used in recombinant protein production. *Journal of Biotechnology*, 33:249–258, 1994.

[76] S N Agathos, Y-H Jeong, and K Venkat. Growth Kinetics of Free and Immobilized Insect Cell Cultures. *Annals of the New York Academy of Sciences*, 589:372–398, 1990.

[77] Karin Oyaas, Trond E Ellingsen, Nils Dyrset, and David W. Levine. Utilization of Osmoprotective Compounds by Hybridoma Cells Exposed to Hyperosmotic Stress. *Biotechnology and Bioengineering*, 43:77–89, 1994.

[78] Vivian M DeZengotita, Lisa R Abston, Albert E Schmelzer, Shinie Shaw, and William M Miller. Selected Amino Acids Protect Hybridoma and CHO Cells from Elevated Carbon Dioxide and Osmolality. *Biotechnology and Bioengineering*, 78(7):741–752, 2002.

[79] J Tramper, J B Williamst, D Joustra, and J M Vlak. Shear sensitivity of insect cells in suspension. *Enzyme and Microbial Technology*, 8:33–36, 1986.

[80] James D Michaels, Jonathan F Petersen, Larry V Mclntire, and Eleftherios T Papoutsakis. Protection Mechanisms of Freely Suspended Animal Cells (CRL 8018) from Fluid-Mechanical Injury. Viscometric and Bioreactor Studies Using Serum, Pluronic F68 and Polyethylene Glycol. *Biotechnology and Bioengineering*, 38:169–180, 1991.

[81] David W Murhammer and Charles F Goochee. Scaleup of Insect Cell Cultures: Protective Effects of Pluronic F-68. *Nature Biotechnology*, 6:1411–1418, 1988.

[82] Leonard J Schiff. Review: Production, Characterization, and Testing of Banked Mammalian Cell Substrates Used to Produce Biological Products. *In Vitro Cellular & Developmental Biology - Animal*, 41:65–70, 2005.

[83] S J Wessman and R L Levings. Benefits and risks due to animal serum used in cell culture production. *Developments in Biological Standardization*, 99:3–8, 1999.

[84] Otto-Wilhelm Merten. Development of serum-free media for cell growth and production of viruses/viral vaccines – safety issues of animal products used in serum-free media. *Developments in Biologicals*, 111:233–257, 2002.

[85] Ronaldo Zucatelli Mendonça, Elizabeth Cristina de Oliveira, Carlos Augusto Pereira, and Ivo Lebrun. Effect of bioactive peptides isolated from yeastolate, lactalbumin and NZCase in the insect cell growth. *Bioprocess and Biosystems Engineering*, 30(3):157–164, 2007.

[86] Ernst-Jürgen Schlaeger. The protein hydrolysate, Primatone RL, is a cost-effective multiple growth promoter of mammalian cell culture in serum-containing and serum-

free media and displays anti-apoptosis properties. *Journal of Immunological Methods*, 194:191–199, 1996.

[87] Caroline C Burteau, Fran I S R Verhoeye, Johann F Mols, Jean-si Bastien Ballez, Spiros N Agathos, and Yves-jacques Schneider. Fortification of a protein-free cell culture medium with plant peptones improves cultivation and productivity of an interferon-γ-producing CHO cell line. *In Vitro Cellular & Developmental Biology - Animal*, 39(7):291–296, 2003.

[88] Michael S Donaldson and Michael L Shuler. Low-Cost Serum-Free Medium for the BTI-Tn5B1-4 Insect Cell Line. *Biotechnology Progress*, 14:573–579, 1998.

[89] L Ikonomou, G Bastin, YJ Schneider, and SN Agathose. Design of an Efficient Medium for Insect Cell Growth and Recombinant Protein Production. *In Vitro Cellular & Developmental Biology - Animal*, 37:549–559, 2001.

[90] Rolf Sommer. Yeast Extracts: Production, Properties and Components. In *9th International Symposium on Yeasts*, 1996.

[91] I.M.P.L.V.O. Ferreira, O Pinho, E Vieira, and J. G. Tavarela. Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. *Trends in Food Science and Technology*, 21:77–84, 2010.

[92] Juliet Lobo-Alfonso, Paul Price, and David Jayme. Benefits and Limitations of Protein Hydrolysates as Components of Serum-Free Media for Animal Cell Culture Applications: Protein Hydrolysates in Serum Free Media. In Vijai K Pasupuleti and

Arnold L Demain, editors, *Protein Hydrolysates in Biotechnology*, chapter 4, pages 55–78. Springer, 2010.

[93] Hasan Tanguler and Huseyin Erten. Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproducts Processing*, 86(4):317–321, 2008.

[94] Hee Jeong Chae, Hyun Joo, and Man-Jin In. Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, 76:253–258, 2001.

[95] John Conway, Hélène Gaudreau, and Claude P Champagne. The effect of the addition of proteases and glucanases during yeast autolysis on the production and properties of yeast extracts. *Canadian Journal of Microbiology*, 47:18–24, 2001.

[96] Man-Jin In, Dong Chung Kim, and Hee Jeong Chae. Downstream process for the production of yeast extract using brewer's yeast cells. *Biotechnology and Bioprocess Engineering*, 10(1):85–90, 2005.

[97] J van der Valk, D Brunner, K De Smet, Å Fex Svenningsen, P Honegger, L E Knudsen, T Lindl, J Noraberg, A Price, M L Scarino, and G Gstraunthaler. Optimization of chemically defined cell culture media Replacing fetal bovine serum in mammalian *in vitro* methods. *Toxicology in Vitro*, 24(4):1053–1063, 2010.

[98] GE Wilkie, H Stockdale, and SV Pirt. Chemically-defined media for production of insect cells and viruses in vitro. *Developments in Biological Standardization*, 46:29–37, 1980.

[99] Jun Mitsuhashi. Preliminary formulation of a chemically defined medium for insect cell cultures. *Methods in Cell Science*, 18:293–298, 1996.

[100] Jun Mitsuhashi. Polyamine as a Growth Promoter for Cultured Insect Cells. *In Vitro Cellular & Developmental Biology - Animal*, 34(8):619–621, 1998.

[101] M Burnette, T Brito-Robinson, J Li, and J Zartman. An inverse small molecule screen to design a chemically defined medium supporting long-term growth of *Drosophila* cell lines. *Molecular BioSystems*, 10:2713–2723, 2014.

[102] V Deparis, C Durrieu, M Schweizer, I Marc, J L Goergen, I Chevalot, and A Marc. Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth. *Cytotechnology*, 42:75–85, 2003.

[103] Cheng-chung Chou. Identification of bioactive yeastolate fractions responsible for insect cell growth and baculovirus production. *Journal of Biochemical Technology*, 4:611–615, 2013.

[104] Maureen Spearman, Carly Lodewyks, Meika Richmond, and Michael Butler. The Bioactivity and Fractionation of Peptide Hydrolysates in Cultures of CHO Cells. *Biotechnology Progress*, 30(3):584–593, 2014.

[105] Bok-Hwan Chun, Jong-Hwang Kim, Ho-Joung Lee, and Namhyun Chung. Usability of size-excluded fractions of soy protein hydrolysates for growth and viability of Chi-

nese hamster ovary cells in protein-free suspension culture. *Bioresource Technology*, 98:1000–1005, 2007.

[106] Maureen Spearman, Sarah Chan, Vince Jung, Vanessa Kowbel, Meg Mendoza, Vivian Miranda, and Michael Butler. Components of yeast (*Sacchromyces cervisiae*) extract as defined media additives that support the growth and productivity of CHO cells. *Journal of Biotechnology*, 233:129–142, 2016.

[107] Parlog Raluca Maria, Alina Nicula, A T Nicula, and Carmen Socaciu. The Optimization of Extraction and HPLC Analysis of Vitamins B from Yeast Products. In *Bulletin of University of Agricultural Sciences and Veterinary Medicine*, pages 323–328, 2008.

[108] Lawrence W Dick Jr., Jeffrey A Kakaley, David Mahon, Difei Qiu, and Kuangchuan Cheng. Investigation of Proteins and Peptides from Yeastolate and Subsequent Impurity Testing of Drug Product. *Biotechnology Progress*, 25(2):570–577, 2009.

[109] Jian Zhao and Graham H. Fleet. Degradation of RNA during the autolysis of *Saccharomyces cerevisiae* produces predominantly ribonucleotides. *Journal of Industrial Microbiology and Biotechnology*, 32:415–423, 2005.

[110] Richard G Ham. Clonal Growth of Mammalian Cells in a Chemically Defined, Synthetic Medium. *Microbiology*, 53:288–293, 1965.

[111] František Franek. Oligopeptides as External Molecular Signals Affecting Growth and Death in Animal Cell Cultures. In Vijai K Pasupuleti and Arnold L Demain, editors, *Protein Hydrolysates in Biotechnology*, chapter 5, pages 79–89. Springer, 2010.

[112] C Bédard, S Perret, and AA Kamen. Fed-batch culture of Sf-9 cells supports 3×10^7 cells per ml and improves baculovirus-expressed recombinant protein yields. *Biotechnology Letters*, 19:629–632, 1997.

[113] Marc G Aucoin. Characterization and optimization of the production of adeno-associated viral vectors using a baculovirus expression vector/insect cell system, 2007.

[114] Hiroki Murakami, Hideo Masui, Gordon H Sato, Noboru Sueoka, Theresa P Chow, and Tamiko Kano-Sueoka. Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component. *Proceedings of the National Academy of Sciences*, 79:1158–1162, 1982.

[115] Huansheng Yang, Xia Xiong, Tiejun Li, and Yulong Yin. Ethanolamine enhances the proliferation of intestinal epithelial cells via the mTOR signaling pathway and mitochondrial function. *In Vitro Cellular & Developmental Biology - Animal*, 52:562–567, 2016.

[116] Hiroko Nishino and Jun Mitsuhashi. Effects of some mammalian growth-promoting substances on insect cell cultures. *In Vitro Cellular & Developmental Biology - Animal*, 31:822–823, 1995.

[117] R L Plackett and J P Burman. The Design of Optimum Multifactorial Experiments. *Biometrika*, 33:305–325, 1946.

[118] Jason Richardson, Bhavana Shah, Pavel V Bondarenko, Prince Bhebe, Zhongqi Zhang, Michele Nicklaus, and Maua C Kombe. Metabolomics Analysis of Soy Hy-

drolysates for the Identification of Productivity Markers of Mammalian Cells for Manufacturing Therapeutic Proteins. *Biotechnology Progress*, 31:522–531, 1995.

[119] Wright Jones, Xia Li, Zhi-Chao Qu, Laureta Perriott, Richard R Whitesell, and James M May. Uptake, Recycling, and Antioxidant Actions of α-lipoic acid in Endothelial Cells. *Free Radical Biology and Medicine*, 33:83–93, 2002.

[120] Lester Packer. α-Lipoic Acid: A Metabolic Antioxidant Which Regulates NF-κB Signal Transduction and Protects Against Oxidative Injury. *Drug Metabolism Reviews*, 30:245–275, 1998.

[121] Laura A Palomares, Miranda Gonzalez, and Octavio T Ramirez. Evidence of Pluronic F-68 direct interaction with insect cells: impact on shear protection, recombinant protein, and baculovirus production. *Enzyme and Microbial Technology*, 26:324–331, 2000.

APPENDICES

Appendix A

Media Formulations

Table A.1: "ALIM + YX" medium formulation. See Materials and Methods for production protocol.

Amino Acid Concentration (g/L)	
Arginine	1.219
Asparagine	1.300
Aspartate	1.300
Cystine	0.200
Glutamate	1.500
Glutamine	2.192
Glycine	0.200
Histidine	0.200
Isoleucine	0.750
Leucine	0.656
Lysine · HCl	0.700
Methionine	1.000
Phenylalanine	1.000
Proline	0.500
Serine	0.736
Threonine	0.596
Tryptophan	0.408
Tyrosine	0.500
Valine	0.500

Trace Metal Concentration (mg/L)	
$CoCl_2 \cdot (H_2O)_6$	0.050
$CuCl_2 \cdot (H_2O)_2$	0.200
$FeSO_4 \cdot (H_2O)_7$	0.550
$MnCl_2 \cdot (H_2O)_4$	0.020
$(NH_4)_6Mo_7O_{24} \cdot (H_2O)_4$	0.040
$ZnCl_2$	0.040

Vitamin Concentration (mg/L)	
4-Aminobenzoic acid	0.320
Biotin	0.160
Calcium pantothenate	0.008
Cyanocobalamin	0.240
Folic acid	0.080
Inositol	0.400
Niacin	0.160
Pyridoxine · HCl	0.400
Riboflavin	0.080
Thiamine · HCl	0.080

Salt Concentration (g/L)	
$CaCl_2$	0.500
Choline chloride	0.070
KCl	1.200
$MgSO_4 \cdot (H_2O)_7$	1.880
NaCl	2.505
$NaHCO_3$	0.350
NaH_2PO_4	1.000

Sugar Concentration (g/L)	
Glucose	3.895

Additive Concentration (g/L)	
Yeast Extract	4.000

Lipid Solution Concentration (mL/L)	
Lipid Mixture 1	30

Table A.2: Feed additive formulation. In experiments utilizing a yeast extract containing feed additive, concentration of yeast extract was 46 g/L.

Amino Acid Concentration (g/L)	
Arginine	1.219
Asparagine	1.300
Aspartate	1.300
Cystine	0.200
Glutamate	1.500
Glutamine	2.192
Glycine	0.200
Histidine	0.200
Isoleucine	0.750
Leucine	0.656
Lysine · HCl	0.700
Methionine	1.000
Phenylalanine	1.000
Proline	0.500
Serine	0.736
Threonine	0.596
Tryptophan	0.408
Tyrosine	0.500
Valine	0.500

Trace Metal Concentration (mg/L)	
$CoCl_2 \cdot (H_2O)_6$	1.834
$CuCl_2 \cdot (H_2O)_2$	7.336
$FeSO_4 \cdot (H_2O)_7$	20.175
$MnCl_2 \cdot (H_2O)_4$	0.734
$(NH_4)_6Mo_7O_{24} \cdot (H_2O)_4$	1.467
$ZnCl_2$	1.467

Vitamin Concentration (mg/L)	
4-Aminobenzoic acid	11.738
Biotin	5.869
Calcium pantothenate	0.293
Cyanocobalamin	8.804
Folic acid	2.935
Inositol	14.673
Niacin	5.869
Pyridoxine · HCl	14.673
Riboflavin	2.935
Thiamine · HCl	2.935

Salt Concentration (g/L)	
$CaCl_2$	0.500
Choline chloride	0.070
KCl	1.200
$MgSO_4 \cdot (H_2O)_7$	1.880
NaCl	2.505
$NaHCO_3$	0.350
NaH_2PO_4	1.000

Sugar Concentration (g/L)	
Glucose	30.675

Table A.3: "Enhanced low YX ALIM" medium formulation. Low concentration pGIC compounds added via individual freshly prepared concentrates.

Amino Acid Concentration (g/L)	
Arginine	1.219
Asparagine	1.300
Aspartate	1.300
Cysteine	0.160
Cystine	0.200
Glutamate	1.500
Glutamine	2.192
Glycine	0.200
Histidine	0.200
Isoleucine	0.750
Leucine	0.656
Lysine · HCl	0.700
Methionine	1.000
Phenylalanine	1.000
Proline	0.500
Serine	0.736
Threonine	0.596
Tryptophan	0.408
Tyrosine	0.500
Valine	0.500

Trace Metal Concentration (mg/L)	
$CoCl_2 \cdot (H_2O)_6$	0.150
$CuCl_2 \cdot (H_2O)_2$	0.600
$FeSO_4 \cdot (H_2O)_7$	1.650
$MnCl_2 \cdot (H_2O)_4$	0.060
$(NH_4)_6Mo_7O_{24} \cdot (H_2O)_4$	0.120
$ZnCl_2$	0.120

Vitamin Concentration (mg/L)	
4-Aminobenzoic acid	0.960
Biotin	0.480
Calcium pantothenate	0.024
Cyanocobalamin	0.720
Folic acid	0.240
Inositol	1.200
Niacin	0.480
Pyridoxine · HCl	1.200
Riboflavin	0.240
Thiamine · HCl	0.240

Table continues on next page

Table A.3: (Continued)

Salt Concentration (g/L)	
$CaCl_2$	0.500
Choline chloride	0.070
KCl	1.200
$MgSO_4 \cdot (H_2O)_7$	1.880
NaCl	2.505
$NaHCO_3$	0.350
NaH_2PO_4	1.000

Sugar Concentration (g/L)	
Glucose	3.895

Additive Concentration (g/L)	
Yeast Extract	0.500

Lipid Solution Concentration (mL/L)	
Lipid Mixture 1	30

pGIC Concentration (mg/L)	
Adenosine	26.724
Betaine	117.150
Ethanolamine · HCl	2.439
Inosine	268.230
Ornithine · HCl	6.745
Putrescine · 2HCl	1.610
Sodium ascorbate	1.981
Sodium selenite	0.052
Spermidine · 3HCl	2.546
Spermine · 4HCl	3.482
Uridine	244.200

Appendix B

Extended Data

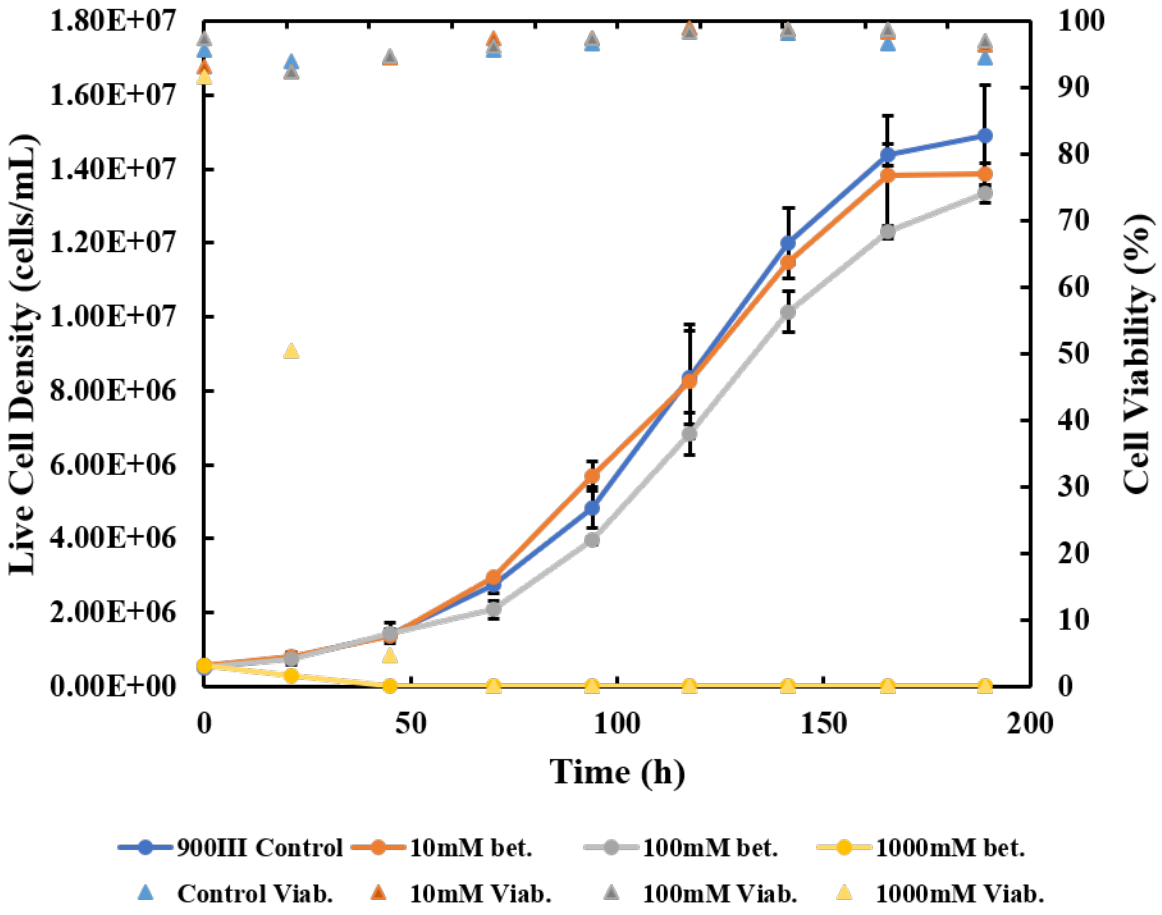

Figure B.1: Betaine addition tolerance of Sf-9 cells in 900III medium. Error bars represent the range of duplicate cultures.

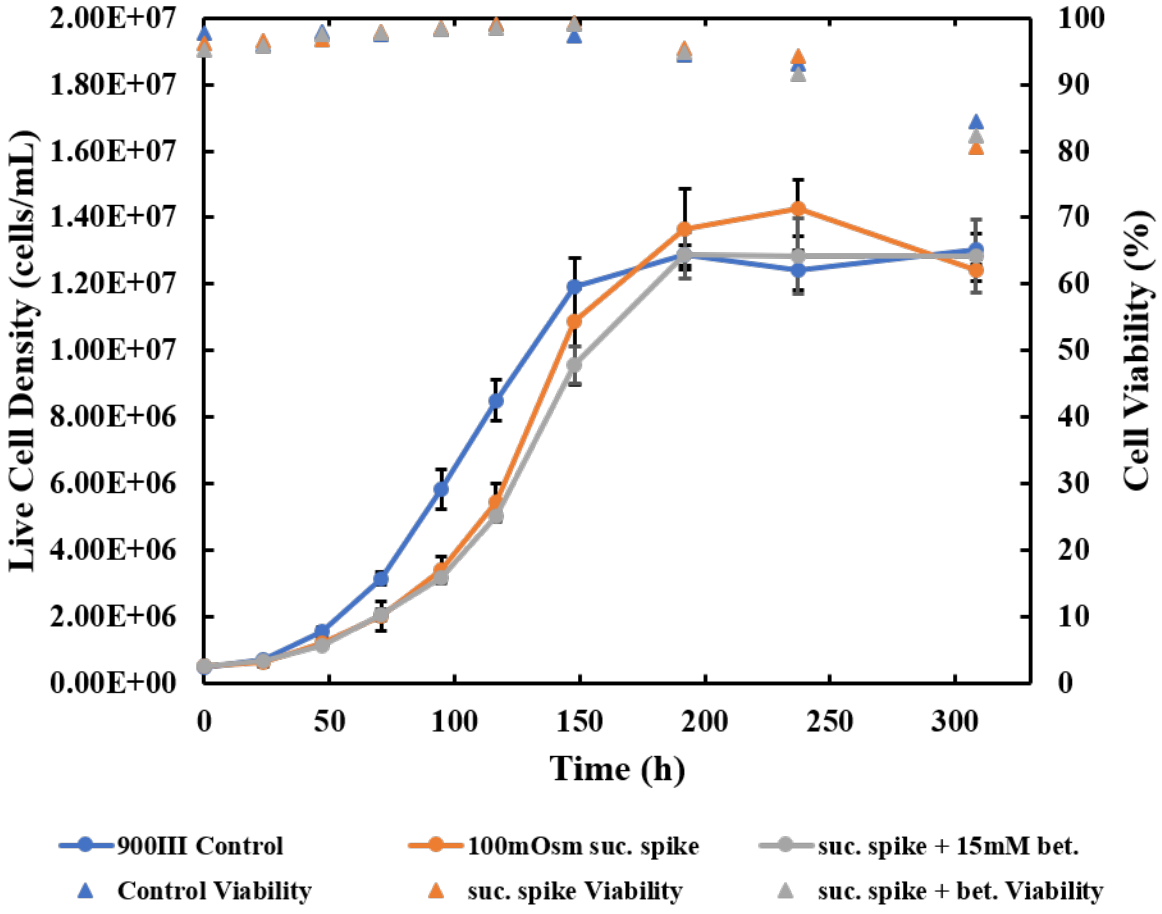

Figure B.2: Effects of betaine addition on growth in artificially osmolality-increased Sf-9 culture in 900III medium. Osmolality increased by means of approximately 100 mM spike of sucrose. Error bars represent the range of duplicate cultures.

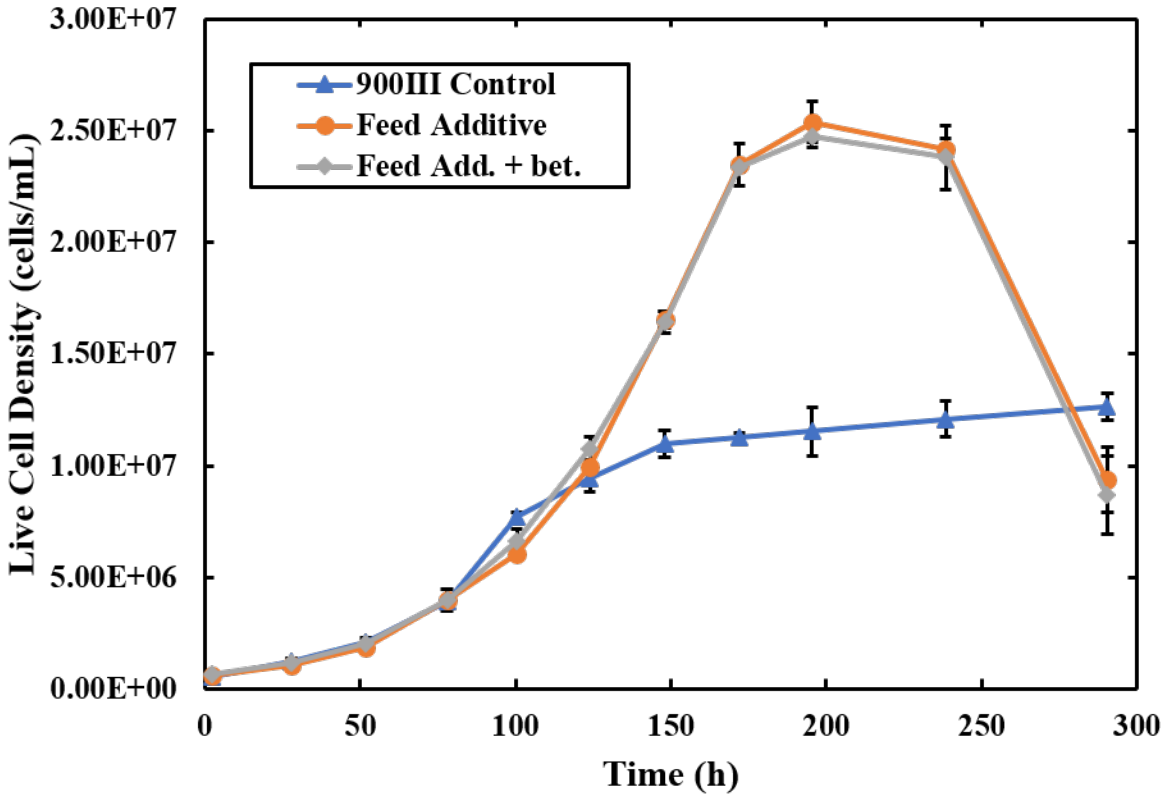

Figure B.3: Effect of betaine addition during feed additive spike regimen in Sf-9 culture in 900III medium. Betaine added simultaneously with feed additive spikes; 5mM betaine at 2% spike, 10mM betaine at 4% spike, 15 mM betaine at 6% spike. Feed additive spike regimen is as described previously. Error bars represent the range of duplicate cultures.