DEVELOPMENT OF A FED-BATCH PROCESS TO PRODUCE BETA-CAROTENE USING ENGINEERED *SACCHAROMYCES CEREVISIAE*

A Thesis

by

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ABSTRACT

Biocatalysts have been increasingly targeted to produce bio-fuels, pharmaceuticals and synthetic and natural chemicals due to a rising interest in sustainability and safety. The use of biocatalysts can eliminate the need for brute chemical synthesis and the toxic materials utilized in the process. Beta-carotene is a favorable candidate for microbial production as it is naturally produced in several different organisms and the pathway of production is well characterized. A brightly colored pigment which is used in a wide array of industries such as nutraceuticals, food and cosmetics, beta-carotene has a predicted market value of 247 million dollars by 2019. This work focuses on applying upstream and midstream approaches to maximize titer, yield and productivity of beta-carotene production under fed-batch operation using a carotenoid hyper-producer (SM14), an engineered strain of *Saccharomyces cerevisiae*. Bench-top bioreactor experiments determined ethanol feeding, when compared to glucose feeding, results in a 64% improvement in productivity. Glucose and ethanol feeding increased the maximum titer to 179 mg/L which is a 44% improvement when compared to batch experiments. In addition, yield was increased to 21 mg/g DCW, a 22% improvement compared to batch results. Response Surface Methodology (RSM) was utilized to optimize minimal YNB media for maximum biomass and titer production. Sixteen different compositions were studied and the analysis showed that optimal composition results in an improvement of 243.35% in biomass production, 198.21% in titer and 106.49% in productivity.

DEDICATION

To my family and friends.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. M. Nazmul Karim, for all his support and guidance through finishing my Master's thesis. I would also like to thank Dr. Katy Kao and Dr. Arul Jayaraman for their advice and for serving as members of my committee.

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1. INTRODUCTION

1.1 Process Development for Microbial Production

Microbial production of pharmaceuticals, synthetic and natural chemicals and bio-fuels is a growing field of research and development due to an increasing interest in producing the materials required to sustain human life in a more sustainable manner. However, a significant challenge in implementing microbial production at industrial scale is the optimization and development of the numerous stages of the process.

The three main stages of production (summarized in Figure 1) - upstream, midstream and downstream – need rigorous optimization in order to maximize the three basic metrics of a microbial process – yield, titer and productivity (Lee & Kim, 2015).

Upstream development includes selection of a host and target pathway for producing the bio-product of interest. Thorough metabolic flux analysis and metabolic balances are required to optimize the yield and titer of the process. At this stage, shake flask experiments are used to study if the selected host and pathway have sufficient productivity to advance to later stages of development.

Midstream development involves refining the parameters of production for larger scale. For example, an optimal growth medium is required to maintain substrates and nutrients at levels that are productive while minimizing the cost of raw materials for the process. In addition, scale-up is especially difficult because the factors that affect the transition from shake flask scale to bench top bioreactor scale greatly vary depending on the host, strain and target product. The mode of fermentation – batch, fed-batch or

continuous – is also investigated in this stage of process development. Fed-batch and continuous cultures require optimized feeding profiles and can also demand controllers that manipulate feeds using online variables as inputs. Greater analysis of parameters such as pH, temperature and dissolved oxygen (DO) are also required to maintain and/or increase the yields and titers achieved at smaller scales. Iterative experiments testing several different conditions and feeding profiles are required to optimize yield, titer and productivity.

Downstream development involves optimizing the purification and separation of the product of interest. Often times, recycling of media and cells can also be utilized in order to increase titer and productivity and decrease costs of raw materials. Separations and purification of the target product is most often the costliest portion of microbial production.

This work will focus on upstream and midstream approaches to developing a fed-batch process to maximize the production of beta-carotene.

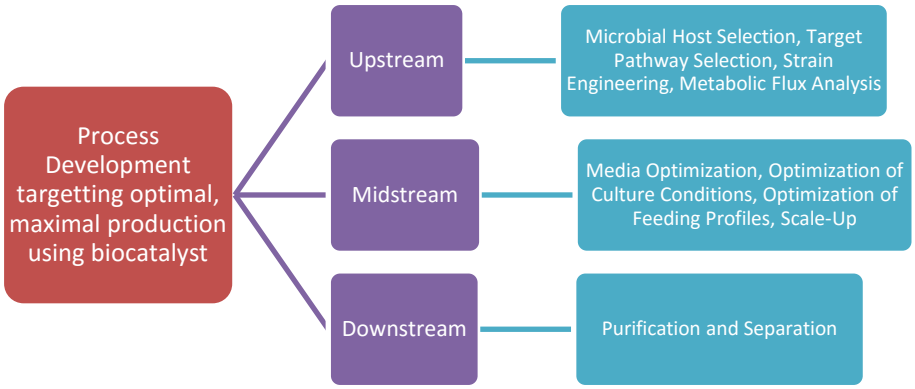

Figure 1 Summary of the stages of development for microbial production processes (Lee & Kim, 2015).

1.2 Upstream Development

1.2.1 Microbial Host Selection

Microorganisms have been used for centuries to produce fine alcohols, cheeses and breads. In recent decades, the advantages of microbial growth have been harnessed as biocatalysts to produce organic products at an industrial scale without the use of dangerous and toxic starting materials such as heavy metal catalysts, organic solvents and strong acids and bases. Moreover, biocatalysts typically produce the compounds with the desired stereochemistry naturally which is of particular importance when producing chemicals for human consumption (Du, Shao, & Zhao, 2011). Additionally, microbes have been targeted to use cheap and renewable carbon sources such as glucose to produce value added compounds. Tractable microbes, such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*), are some of the safest and well characterized industrial organisms.

Historically, biocatalyst utilization was limited by our ability to manipulate the pathways that produce target chemicals. However, due to the emergence of molecular biology techniques capable of rapid genetic engineering and whole cell profiling, the aim to manipulate biocatalysts to achieve profitable yields and productivities is an achievable task (Hong & Nielsen, 2012; I.-K. Kim, Roldão, Siewers, & Nielsen, 2012; Sagt, 2013).

While efforts to implement microbial production of chemicals have progressed, several issues still need to be addressed in order to increase productivity of the organisms. The challenges include identifying the appropriate biocatalyst and pathway for producing the product of interest, establishing the optimal growth conditions for the cultivation of the biocatalyst, identifying bottlenecks of production by parsing through metabolic flux information and developing high throughput methods to engineer the production pathways for higher yields and screen for higher producers (Lee & Kim, 2015).

Advances in metabolic engineering techniques and merging of computational and experimental approaches have been able to increase the productivity and yields of industrial strains and address the challenges described above. The recent development of the CRISPR-Cas9 (Sander & Joung, 2014) method allows for rapid genome editing with higher efficiency and accuracy than ever before. CRISPR-Cas9 also allows for several gene disruptions (knock-outs) to be performed simultaneously, which drastically decreases the time required for gene editing. With the ability to rapidly modify

organisms in parallel, a systems approach to optimization of biocatalyst production is feasible.

The baker's yeast *S. cerevisiae* has GRAS (Generally Regarded as Safe) status and has been used as a biocatalyst for centuries. Its genome was sequenced in 1996 (Goffeau et al., 1996) and has been well annotated which allows for both modeling and genetic modifications with greater ease. In addition, S. cerevisiae is highly tolerant to a vast array of challenging conditions with varying pH, temperature and toxicity which makes it a great candidate for industrial applications (Hong & Nielsen, 2012).

Under aerobic conditions, *S. cerevisiae* metabolizes glucose to produce energy for cell maintenance and growth. Under anaerobic (oxygen limited) conditions, energy is derived from metabolizing glucose to ethanol (fermentation), which may result in decreased growth rates due to the toxic effects of ethanol if concentrations reach inhibitory levels. A defining feature of *S. cerevisiae* is its ability to grow under anaerobic conditions (van Dijken, Weusthuis, & Pronk, 1993), another reason it is a preferred biocatalyst for industrial application. Ethanol fermentation can also occur when glucose concentrations are maintained above a critical threshold. This phenomenon, called overflow metabolism or the Crabtree effect, is a significant challenge in fermentations involving *S. cerevisiae* (Postma, Verduyn, Scheffers, & Van Dijken, 1989).

1.2.2 Ethanol Toxicity

The toxic effects of ethanol can affect growth rate, cell vitality and cell death (Birch & Walker, 2000). It can affect many aspects of the cell metabolism by activating

production of heat shock-like proteins. This results in changes in RNA and protein synthesis as well. In the presence of ethanol, cell membranes are altered by increased fluidity which decreases integrity (D. Stanley, Bandara, Fraser, Chambers, & Stanley, 2010). Ethanol tolerance is of interest in this work as increased ethanol concentrations in the culture allow for higher titers of beta-carotene. Therefore, managing the stress of ethanol with its positive effects on yield, titer and productivity are crucial.

One approach to combating overflow metabolism in *S. cerevisiae* is utilizing fermentation modes such as fed-batch or chemostat in which the concentrations of the substrates can be controlled. Fed-batch operation with *S. cerevisiae* is a well-developed topic with established methods for optimization and control of feeding policies as well (Aiba, Nagai, & Nishizawa, 1976; Pham, Larsson, & Enfors, 1998; van Hoek, De Hulster, Van Dijken, & Pronk, 2000). A second approach to combating overflow metabolism is to engineer the biocatalyst to better tolerate the inhibitory effects of ethanol production. Evolving the strain to grow in ethanol-stressed environments (Çakar, Seker, Tamerler, Sonderegger, & Sauer, 2005; Dragana Stanley, Fraser, Chambers, Rogers, & Stanley, 2010; Wallace-Salinas & Gorwa-Grauslund, 2013) and rational strain engineering to overexpress genes that confer ethanol tolerance (Hirasawa et al., 2007; Lewis, Elkon, McGee, Higbee, & Gasch, 2010; Watanabe, Watanabe, Akao, & Shimoi, 2009) have been used to address ethanol toxicity. However, the success of the approach is highly variable and depends on the target product and pathway.

A second approach to minimizing the effects of ethanol toxicity is to re-engineer the strain to increase its ethanol tolerance. A higher tolerance to ethanol could minimize the slowing down of growth rate and therefore result in higher yields. Furthermore, this approach could be especially important in producing β-carotene since specific yield from ethanol is significantly higher than specific yield from glucose (Reyes, Gomez, & Kao, 2014). In identifying which genes should be targeted in order to increase ethanol tolerance, genes that are involved in the pathway of mevalonate synthesis (a pathway upstream of β-carotene synthesis) and also involved in minimize the negative effects of ethanol on growth could be the most beneficial to target. IDI1 (isopentenyl diphosphate:dimethylallyl disphospate isomerase) was identified as being involved in converting Δ^3-isopentenyl-PP to dimethyllallyl-pyrophosphate and has been shown to be related to ethanol tolerance (Teixeira, Raposo, Mira, Lourenco, & Sa-Correia, 2009).

1.2.3 Target Product: Beta-Carotene

Beta-carotene is a brightly pigmented orange organic compound naturally produced in algae, plants, fungi and bacteria. The perfectly symmetrical molecule has a system of conjugated double bonds and is flanked by two beta-ionone rings. It is part of a larger class of molecules called carotenoids, marked by the 40 carbon composition and can be differentiated into carotenes (carotenoids with only carbon) and xanthophylls (carotenoids with oxygen) (Donhowe & Kong, 2014). Carotenoids are part of a larger class of molecules called terpenoids (isoprenoids) which are formed by 8 units of isoprene. The trans-beta carotene form can be cleaved into two units of vitamin A,

making it a valuable nutrition source for humans. It functions as an anti-oxidant in the human body and has been implicated in vision health (Grune et al., 2010). In photosynthetic microbes and plants, carotenoids are of high importance as they are utilized in absorption of light for photosynthesis and as protection against photo-oxidation (Armstrong & Hearst, 1996).

Beta-carotene is used in such industries as nutraceuticals, food and cosmetics. Market research showed that in 2014, the market value of beta-carotene was $197 million dollars and is projected to grow to $247 million dollars in 2019 (Marz, 2015). The largest market for beta-carotene is North America where it is most commonly used in food and nutraceutical industries.

The production of beta-carotene dates back to 1940 when it was first synthesized at Roche in an effort to synthesize vitamin A using Grignard reactions. In the 1970's, public concerns about the quality of food forced Roche to turn to microbial production of beta-carotene using micro-algae. Today, most of the beta-carotene produced is done so using chemical synthesis and in smaller quantities, algae extraction and fermentation are also used. It is typically produced into crystalline tablets for safe transport and ease of storage.

1.2.4 Strain Engineering Approaches

The foundation of microbial production is the choice of organism and the product synthesis pathway to produce the bio-product of interest. Historically, the transfer of knowledge of strain engineering techniques from academic pursuits to industry has been

sluggish which presents hinders the development of the field and in increasing yield and productivity to surpass the benefits of brute chemical synthesis (Lee & Kim, 2015). Conversely, academics possess the resources and knowledge to quickly develop biocatalysts that produce bio-products of high value but fail to further develop the process by incorporating a systems level approach to microbial production (Hong & Nielsen, 2012). Fortunately, significant gains have been achieved in the field due to the integration of high-throughput approaches with feedback between the upstream, midstream and upstream variables. A more thorough understanding of a biocatalyst is achievable due to the development of genomic computational techniques and metabolic flux prediction capabilities.

In general, there are three overarching approaches to strain engineering: rational metabolic engineering, inverse metabolic engineering and evolutionary strategies (I.-K. Kim et al., 2012). Rational engineering allows for genomic changes to re-direct metabolic fluxes to produce a product of interest. This can be achieved through gene knock-outs or over-expression of genes, promoters and inducers. However, rational engineering approaches suffer from a host of difficulties such as the *a priori* knowledge of genomic functions, predicting the downstream and upstream effects of genomic edits and the difficulty of making precise and efficient genetic changes, especially in non-model organisms. However, the large wealth of information available of widely used microbial platforms such as *S. cerevisiae* and *E. coli*, the emerging technologies in gene editing tools (such as CRISPR-Cas9) intracellular flux modelling (such as constraint

based flux balance analysis) (Long, Ong, & Reed, 2015; Reed, Famili, Thiele, & Palsson, 2006) allow for more systems level approaches to rational strain engineering. Inverse metabolic engineering (adaptive evolution, genomic libraries) is the methodology in which strains with genetic modifications that confer a desired characteristic are first selected or screened; these strains can be further rationally engineered or subsequently used to gain greater understanding of the determinants of the desired trait (Portnoy, Bezdan, & Zengler, 2011; Winkler & Kao, 2014). Using selective pressures, in adaptive laboratory evolution, the biocatalyst is cultivated to grow in the preferred conditions without enforcing strict regulations on the genomic changes occurring over time. Once the strain is able to adapt, the genomic, proteomic and metabolic changes that occurred in the process of adaptive evolution are studied. With careful strain design and selection of pressures on growth, adaptive laboratory evolution can be used to select for more successful producers.

1.2.5 Development of SM14

Transforming genes from other organisms into *S. cerevisiae* to produce bio-products is of interest since the wealth of genetic information of the species allows for greater ease of genetic manipulation. Yeast species such as *Xanthophyllomyces dendrorhous (X. dendrorhous),* previously known as *Phaffia rhodozyma* (*P. rhodozyma*), produce carotenoids and the corresponding genes have been cloned into other organisms. In the following studies, an evolved strain of *S. cerevisiae* is used to over-produce beta-carotene. Both *X. dendrorhous* and *S. cerevisiae* naturally biosynthesize geranylgeranyl

diphosphate (GGPP), a precursor of the carotenoid pathways, which allows for transfer of the carotenoid cassette into *S. cerevisiae* to produce the β-carotene (Verwaal et al., 2007). The pathway was cloned into *S. cerevisiae* to generate the strain YLH2 capable of beta-carotene production. The strain used in this study is a carotenoid hyper-producer derived from YLH2 named SM14. SM14 was developed in the Kao Lab using adaptive evolution techniques. The Kao lab utilized the anti-oxidant properties of beta-carotene to select for higher producers in the presence of oxidative agent hydrogen peroxide (Reyes et al., 2014) . The pathway for carotenoid production is summarized in Figure 2.

The carotenoid synthesis pathway involves some key intermediates such as acetyl CoA (the starting material for the pathway), mevalonic acid (MVA), isopentenyl pyrophosphate (IPP), dimethyllayl pyrophosphate (DMAPP) and geranyl geranyl pyrophosphate (GGPP) (Mata-Gómez, Montañez, Méndez-Zavala, & Aguilar, 2014). GGPP can be combined to produce phytoene which forms lycopene – the basis of many other carotenoids including beta-carotene. Ethanol has been implicated in upregulating the activities of some key enzymes in this pathway, which allows for higher productivity in the ethanol uptake phase of the fermentations utilizing SM14. It has been postulated that ethanol has a positive effect on the production of carotenoids due to the increased pool of acetyl-coA (a precursor to the mevalonate pathway). This phenomena has been studied in *P. rhodozyma* (Gu, An, & Johnson, 1997) from which the beta-carotene producing pathway is transplanted into ancestral strain of SM14. Ethanol has also been shown to stimulate activity in alcohol dehydrogenase and hydroxyl-methyl-glutaryl-CoA

(HMG-Co-A) reductase which increases flux through the mevalonate pathway (G. J. Kim, Park, Kim, & Ryu, 2003). This effect is less studied in *S. cerevisiae*.

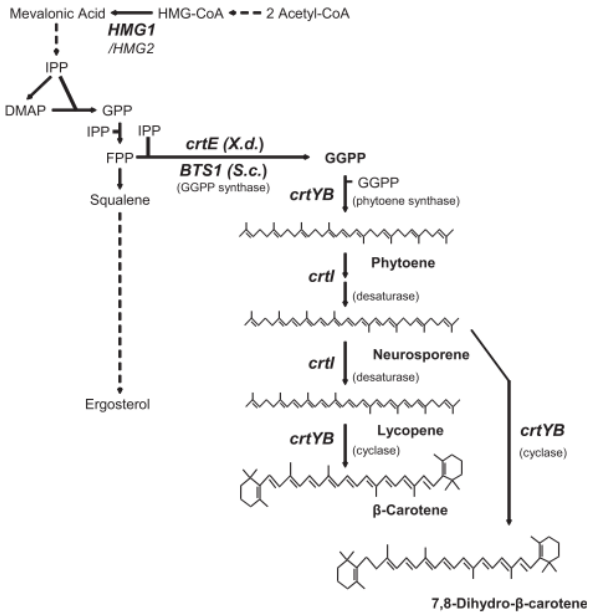

Figure 2 Summary of the pathway that was added to S. cerevisiae (Verwaal et al., 2007).

Bioreactor conditions for SM14 were optimized for a batch mode of operation in Kao lab (M. Olson, 2014). It was observed that culture conditions of pH 4, 30 °C, stirring rate of 800 rpm result in the highest titers and therefore the same conditions will be maintained in the fed-batch experiments also.

1.3 Midstream Development

1.3.1 Fed-batch Mode of Operation

Fed-batch operation has been developed since the early 1900's to increase yield and productivity. One of the earliest uses of fed-batch operation was in the production of yeast using malt wort (Lim & Shin, 2013). Using high malt concentrations would inhibit the cell growth due to anaerobic production of ethanol, which is toxic to yeast. Therefore, malt wort was fed to the culture throughout the process to minimize the production of ethanol and increase biomass production. The success of this progress in microbial production during the era of World War I, led to the application of fed-batch to increase the production of penicillin using glucose as the carbon source.

The fed-batch mode of operation is a variation of batch cultures where nutrients, carbon source or other necessary inputs for continued cell growth and product formation are fed into the reactor during the fermentation (Lim & Shin, 2013). Feed rates and the different feed compositions can be controlled and manipulated to influence the concentrations of the ingredients within the reactor. As the fermentation continues, the volume of the culture broth increases until the cells are harvested to extract the product of interest. Fed-batch allows for finer control of the concentrations of reactants required for the chemical and biochemical reactions taking place in the culture. This strategy is especially relevant when product formation results in feedback inhibition or reaction rates are highly dependent on reactant concentrations.

As suggested above, one method for combating overflow metabolism is to implement a fed-batch mode of operation for fermentation. Slow feeding of glucose allows the operator to maintain productive glucose levels while avoiding the sudden buildup of ethanol. This strategy prevents decreases in growth rate and allows for replenishing of nutrients to maintain production.

In addition to circumventing overflow metabolism, fed-batch operation is commonly also utilized in preventing catabolite repression. When glucose is rapidly metabolized by the microorganism resulting in accelerated growth rates, intracellular cAMP (cyclic AMP) concentrations decrease which results in reduced enzyme biosynthesis (Minihane & Brown, 1986). This phenomenon, called catabolite repression, can be limited by slow feeding of carbon sources, such as glucose. However, slow feed rates can regress product formation rates due to slower growth rates. To combat this issue, an optimized feeding profile can be developed using online measurements such as carbon dioxide evolution rate (CER) or oxygen uptake rate (OUR).

A significant challenge in fed-batch operation is determining the optimal feeding profile to maximize yield and productivity. Commonly studied feeding patterns include constant, linearly increasing or decreasing, exponential and intermittent feed rates. It is of interest, depending on the particular aim, to control the concentrations of components such as substrates, nitrogen, phosphate or intermediates in the culture broth (Lim & Shin, 2013). Material balance equations can be used to describe the dynamics of these components. A simplified mathematical description of fed-batch operation would

include material balances for the cell mass, substrate, intermediate metabolites and the product. In this study, we applied a fed-batch mode of operation to guide the improvement of yields and productivities in *S. cerevisiae* using a simple constant feed pattern to study the effect of substrate feeding on the production of beta-carotene.

1.3.2 Design of Experiments Using Response Surface Methodology

There exists a myriad of experimental approaches to either confirming or nullifying a hypothesis. Most often, experiments are designed intuitively that offer insight into the problem that is being probed. However, a systematic approach to gathering data is appropriate when investigating a large number of variables whose effect is largely unknown. This approach, called design of experiments (DOE), applies statistics to develop models that relate input variables (called factors) to output variables (called responses) with higher efficiency than traditional methods (Hockman & Berengut, 1995). DOE allows for systematic changes to factors in order to study the response in a given experimental space. The traditional "one-factor at a time" approach can be laborious and may lead to incomplete analysis of the way in which the factors interact with each other (Li, Bai, Cai, & Ouyang, 2002).

In this study, the response surface methodology (RSM) approach will be utilized to optimize the components of minimal Yeast Nitrogen Base (YNB) media. RSM is a set of statistical and mathematical techniques used to design experiments, deduce a statistical model and determine the response due to changes in factors in a given experimental space (Khuri & Mukhopadhyay, 2010; Li et al., 2002). Each factor is

varied over a certain range simultaneously with all the factors being tested, thereby creating a "surface" of responses. The surface can then be analyzed to find the appropriate conditions to match the desired effect (maximizing or minimizing the response). In general, the relationship between the factors and the response can be summarized by (Khuri & Mukhopadhyay, 2010):

$$y = f'(x)\beta + \epsilon$$

Equation 1 Generalized equation for modeling the relationship between factors and responses.

$where\ x_1, x_2, \dots, x_k\ are\ k\ number\ of\ factors$

$x = (x_1, x_2, \dots, x_k)'$

$f(x)$

$= vector\ function\ of\ p\ elements\ that\ consist\ of\ powers\ and\ cross\ products\ of\ powers\ of\ x_1, x_2, \dots, x_k\ up\ to\ a\ certain\ degree\ denoted\ by\ d$

$\beta = vector\ p\ unknown\ coeffecients\ (parameters)$

$\in = random\ experimental\ error\ with\ a\ mean\ of\ zero$

There are two main models that can be utilized for RSM: first-degree model (Equation 2) and second-degree model (Equation 3). This study will utilize a second-order design.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \epsilon$$

Equation 2 First-degree model for RSM

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \sum_{i<1} \sum \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \epsilon$$

Equation 3 Second-degree model for RSM

RSM utilizes factorial design since it allows for all factors to be varied simultaneously which greatly reduces the number of experiments that need to be conducted (Plackett & Burman, 1946). The most common second-order designs are 3^k factorial, central composite and Box-Behnken. In this study, a central composite design (CCD) is used to study the effects of varying media compositions on titer and biomass.

Often, DOE is useful in optimizing a mixture of factors. For example, when determining the optimal compositions of media components targeted for maximum cell density, DOE can be used to experimentally study the effect of varying compositions. Then, a statistical model relating the composition to cell growth can be determined and utilized to relate composition and cell density.

The advantages of using minimal media, such as YNB, include greater control of the composition and cheaper raw material costs when scaling up (Yee & Blanch, 1993). Furthermore, richer media often includes a high protein content, which makes downstream purification of bio-products more difficult. Therefore, this study aims to enhance the production of beta-carotene by optimizing the concentrations of the three

components of YNB media: glucose, ammonium sulfate and YNB (a mixture of nitrogen sources, trace elements, salts and vitamins).

2. MATERIALS AND METHODS

The fermentation experiments were conducted in a 3 Liter and 7 Liter autoclavable bioreactors (Applikon, Foster City). Before autoclaving, the pH probe was calibrated with pH 4 and pH 7 buffers. Dissolved oxygen (DO) probe was calibrated after stabilizing the probe with maximum air flow for 6-8 hours after autoclaving. The temperature, pH and stirring rate were maintained constant throughout the experiments using Applikon ezControl. The temperature was maintained at 30 °C, the pH was maintained at 4 using automatic addition of 2M HCl and 2M NaOH and the stirring rate was maintained at 800 rpm. Online measurements including pH, temperature, stirring rate, dissolved oxygen (DO), CO_2 off-gas and O_2 off-gas were also recorded using the Applikon ez Control and a BlueSens off-gas analyzer.

Frozen stock cultures of SM14 were stored at -80°C. For bioreactor experiments, 50 mL YNB inoculum cultures were started from a single colony of SM14 grown on a YNB+Agar plate. The inoculum culture was then grown for at least 48 hours to ensure exponential growth at 200 rpm and 30 °C. YNB media consists of 20g/L glucose, 1.7 g/L YNB and 5 g/L ammonium sulfate and always filter sterilized.

For batch experiments, a total of 3L of YNB were inoculated and duplicate samples of 3 mL were drawn periodically for further analysis. Samples were stored at -20°C.

2.1 Fed-Batch Experiments:

2.1.1 Glucose Feed Type 1

In this experiment, 3 L of 20 g/L glucose in YNB is inoculated with the 50 mL SM14 culture. Then, the experiment is conducted in batch mode for 10 hours to ensure the culture reaches the beginning of the glucose consumption phase (which coincides with the beginning of the exponential phase). At 10 hours, 1 L of 200 g/L glucose in YNB is fed at the glucose consumption rate of 0.02133 L/hr continuously (calculated from several batch experiments). When the 1 L of feed was finished, the pump was turned off and the experiment was allowed to continue in batch mode until all carbon sources (glucose, ethanol and acetic acid) had been exhausted in the culture broth. The concentrations of the carbon sources were tested using HPLC analysis.

2.1.2 Glucose Feed Type II

This experiment was repeated using an initial 2 L of YNB media inoculated by the 50 mL SM14 culture. Then, the culture was allowed to reach a state of glucose depletion (reaching a target glucose concentration of 1 g/L). The timing of this state was calculated using previous batch experiments. At this point, a 1 L of 20 g/L glucose in YNB was fed at a rate of 0.1574 L/hr. The glucose consumption rates when the glucose concentrations ranged from 5 g/L to 1 g/L in previous batch experiments were calculated and used as the feed rate during this experiment. After a 1 L of the feed had been finished, the experiment was allowed to continue in batch mode until all carbon sources had been exhausted (as described previously).

2.1.3 Ethanol Feed with 25 g/L Ethanol in YNB

In this experiment, 3 L of 20 g/L glucose in YNB is inoculated with the 50 mL SM14 culture. The experiment was conducted in batch mode for 24 hours to ensure that the glucose had been depleted and that the culture had reached the ethanol consumption phase. The glucose depletion time was predicted using data from several batch experiments. At 24 hours, the ethanol YNB media was fed at a rate of 0.0268 L/hr continuously at the ethanol consumption rate (also calculated from previous batch experiments). The ethanol YNB media was prepared using 25 g/L ethanol, 5 g/L ammonium sulfate and 1.7 g/L of YNB. When 1 L of the feed was finished, the experiment was allowed to continue in batch mode until all carbon sources had been exhausted as in previous experiments.

2.1.4 Beta-carotene Analysis

An assay was developed by the Kao group for the analysis of beta-carotene, which is described here. A 500 µL sample of the culture is added to an O-ring tube. The sample is then centrifuged for 2 minutes at 13,000 rpm. The supernatant is removed using vacuum aspiration and the cell pellet is left undisturbed. To the pellet, 1 mL of dodecane and an approximate aliquot of 200 µL of acid-washed glass beads are added. The O-ring tube is then placed on a cell disruptor and the cell pellet is disrupted for 6 minutes twice. The sample is again centrifuged for 2 minutes at 13,000 rpm. If a noticeable cell pellet still remains, the disruption steps and subsequent centrifugation step is repeated until the pellet is broken and the β-carotene has been dissolved into the

dodecane phase. Then 200 µL of the sample can be added to a black, clear-bottomed 96 cell culture plate and analyzed using a TECAN. This method is compatible with high throughput analysis which allows for many bioreactor samples to be analyzed in very little time. The raw data from the TECAN analysis was copied into an excel worksheet that included equations to convert the spectrophotometric analysis to concentrations of beta-carotene in the samples. The equations were found through creating a calibration curve using pure beta-carotene.

2.1.5 HPLC Analysis

The glucose, ethanol and acetic acid content of the culture broth were analyzed using the HPLC (Agilent 1260 Infinity, Santa Clara CA) with an Aminex HPX-87H ion exchange column. Samples drawn from the bioreactor experiments (an approximate volume of 3 mL) were centrifuged at 8000 rpm for 8 minutes. The supernatant was then filtered through a syringe filter (0.22µm filter) into a HPLC vial. The conditions used for the column include: a flow rate of solvent at 0.6 mL/hour and a column temperature of 50 °C. The RID analyzer was used.

2.2 Media Optimization Experiments

Design of experiments (DOE) was utilized to optimize the YNB defined and minimal media for the production of beta-carotene. Using the statistical software JMP, shake flask experiments were planned and analyzed through RSM. The three components of YNB are glucose (20 g/L), YNB (1.7 g/L) and ammonium sulfate (5 g/L). Each of the components were assigned a minimum and maximum value (Listed in

Table 1) and listed as a factor. The responses were targeted to be maximized and were defined as biomass and titer.

The resulting DOE included 16 shake flask experiments with two biological replicates. Each flask containing a different composition of glucose, YNB and ammonium sulfate was started from a culture grown in non-supplemented YNB media. The non-supplemented YNB media culture was started from a single colony from a YNB plate. The culture was grown at 30C and 200 rpm for 48 hours. Then, 500 µL starter culture was added to each of the flasks in the DOE. The new cultures were grown at 30C and 200 rpm for 72 hours. At the end of the 72 hours, biomass was measured and beta-carotene analysis was conducted.

Component	Minimum (g/L)	Maximum (g/L)
Glucose	20	100
YNB	1.7	40
Ammonium Sulfate	5	15

Table 1 A summary of the minimum and maximum set for each component for the media optimization experiments.

3. RESULTS

3.1 Batch Experiments

In batch experiments using SM14 and 20g/L YNB media, the results showed an average maximum titer of 123.15 mg/L, average yield of 17.25 mg/g DCW and an average productivity of 1.95 mg/L*hr (Table 4). These results served as targets that we sought to surpass by utilizing fed-batch. Many trends are very similar in batch and fed-batch experiments. For example, the maximum titer coincides with the conclusion of the ethanol consumption phase. In addition, acetic acid trends were similar in that there is a build-up, depletion, build-up and depletion pattern in both batch and fed-batch. The batch experiments were extended to fed-batch using the same controlled parameters – pH 4, temperature of 30°C and agitation of 800 rpm (M. Olson, 2014; M. L. Olson et al., 2016)

Using batch experiments as a guide, glucose uptake rate for type I and type II and ethanol uptake rate were calculated. A simple mass balance was used to target steady state (Equation 4 and Equation 5). The equations were extended to ethanol uptake by replacing the glucose terms with ethanol terms.

$$G_2 = \frac{[G_1V_1 + G_{con}(t_2 - t_1) + F_{in}G_{in}(t_2 - t_1)]}{V_2}$$

Equation 4 Mass balance equation used to calculate the feed rates for fed-batch experiments.

$$V_2 = V_1 + F_{in}(t_2 - t_1)$$

Equation 5 Volume balance equation used to calculate feed rates for fed-batch equation.

$$where\ G1 = concentration\ of\ glucose\ at\ t1$$

$$G2 = concentration\ of\ glucose\ at\ t2$$

$$Fin = flow\ rate\ of\ the\ inlet\ feed$$

$$Gin = glucose\ concentration\ of\ the\ inlet\ feed$$

$$V1 = volume\ of\ culture\ at\ t1$$

$$V2 = volume\ of\ culture\ at\ t2$$

These equations were used in Microsoft Excel's solver in order to target a specific steady state concentration. For glucose feed type I, 20 g/L of glucose was targeted. For glucose feed type II, 1g/L of glucose was targeted. And lastly for ethanol feed, 10g/L of ethanol was targeted. Using glucose and ethanol concentration data from batch experiment profiles (Figures 3 and 4), we calculated the uptake rates listed in Table 2. In glucose feed type II, glucose uptake rate of only the last 3-4 hours was considered to account for any differences in the uptake rate when lower concentrations of glucose are present in the culture.

	Data from batch experiments used to calculate uptake rate	Uptake Rate (L/hr)	Uptake Rate (g/hr)
Glucose Feed Type I	Average of entire glucose consumption phase	1.286	3.827
Glucose Feed Type II	Average of the last 3-4 hours of glucose consumption phase	0.997	2.991
Ethanol Feed	Average of the entire ethanol consumption phase	0.134	0.402

Table 2 A summary of the uptake rates calculated from batch fermentation data.

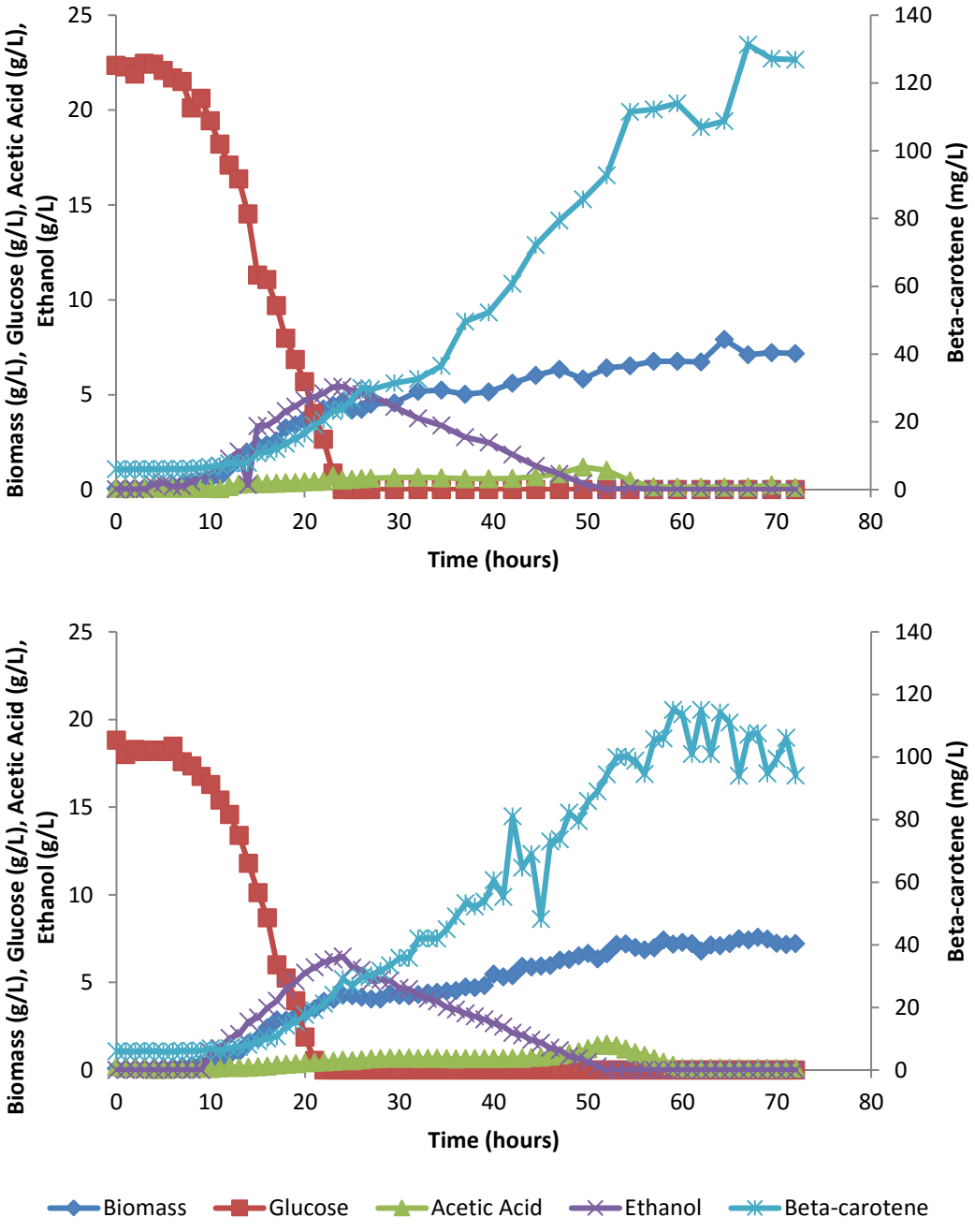

Figure 3 Profiles of duplicate batch fermentations of SM14.

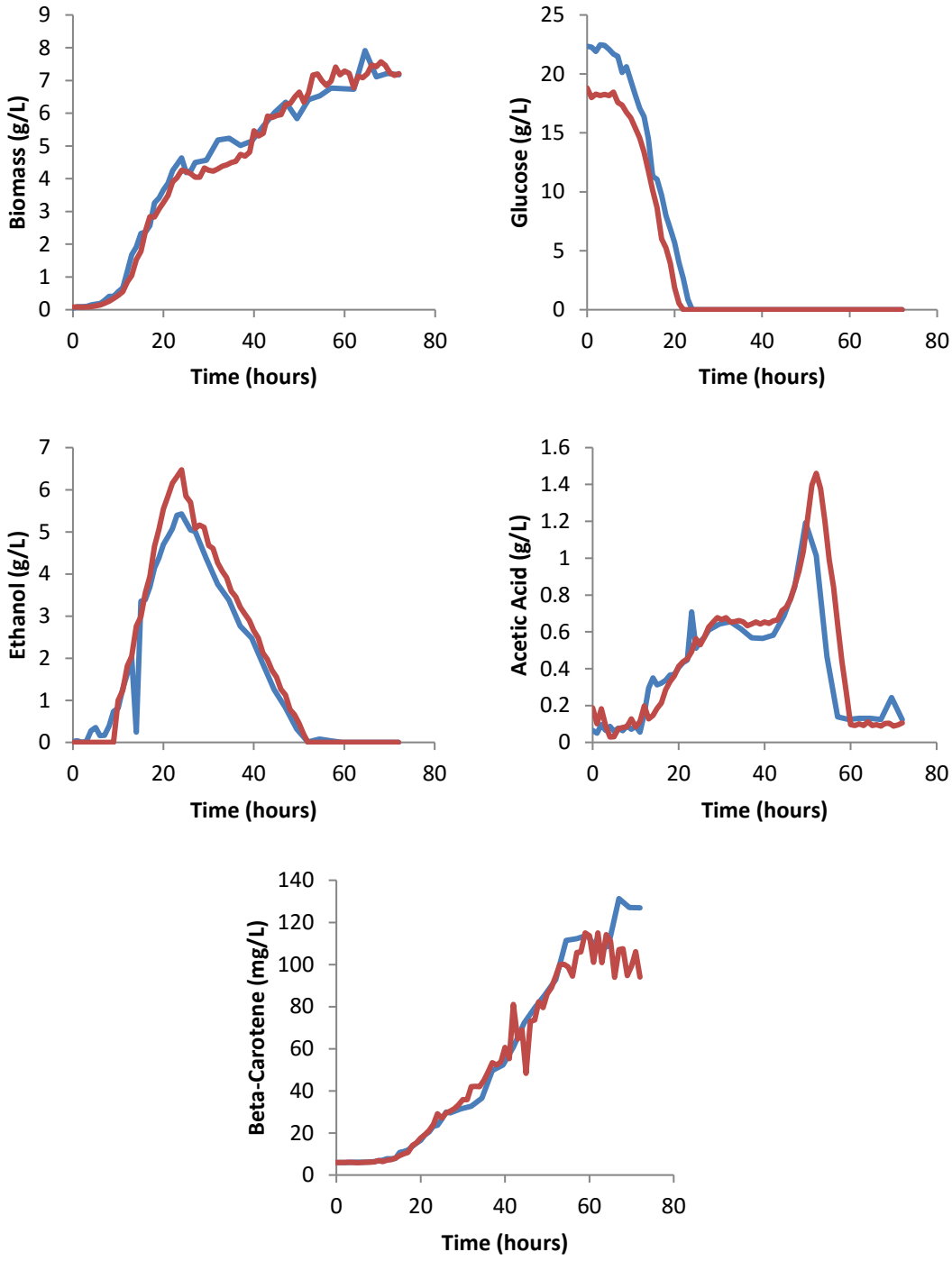

Figure 4 Comparison of each metabolite in duplicate batch fermentations of SM14.

In the bioreactor scale experiments, four different modes of operation are compared and summarized in

Table 3. The resulting titer, productivity and yield of each type of experiment are summarized in

Table 4 and Figure 11.

	Feed Composition	**Feed Rate (L/hr)**	**Time of Feeding (hours post inoculation)**	**Target steady state concentration (g/L)**	**Initial Volume (L)**	**Final Volume (L)**
Batch	N/A	N/A	N/A	N/A	3	3
Glucose Feed Type I	1 L of 200 g/L Glucose in YNB Media	0.02133	10-56	20	3	4
Glucose Feed Type II	1L of 20g/L Glucose in YNB Media	0.1574	21-27.5	1	2	3
Ethanol Feed	1 L of 25 g/L Ethanol in YNB Media (without glucose)	0.0268	25.5-62.8	10	3	4

Table 3 A summary of the experimental conditions of bioreactor scale experiments.

	Average Max Titer (mg/L)	Std. Dev Max Titer (mg/L)	Average Productivity (mg/L*hr)	Std. Dev Productivity (mg/L*hr)	Average Yield (mg/g DCW)	Std. Dev Yield (mg/g DCW)
Batch	123.15	8.15	1.95	0.01	17.25	1.22
Glucose Feed Type I	177.83	1.01	0.94	0.09	20.80	1.55
Glucose Feed Type II	123.13	1.75	1.69	0.20	15.16	0.33
Ethanol Feed	179.09	1.48	1.55	0.35	21.18	0.68

Table 4 A summary of the maximum titer, productivity and yield of each of the four types of bioreactor scale experiments.

3.2 Glucose Feed Type I

In the glucose feed type I experiments, the goal was to maintain a steady state glucose concentration of 20 g/L to allow for increased biomass production by maintaining availability of the primary carbon source, glucose. Feeding at the glucose uptake rate would prevent overwhelming the system with glucose which can lead to build up of ethanol at toxic levels (overflow metabolism). This required feeding glucose at the glucose uptake rate beginning at the onset of the glucose consumption phase (10 hours post inoculation).

This experiment was repeated twice. As seen in Figures 5 and 6, the glucose uptake rate increases as a result of feeding. In the first experiment (top panel of Figure 5), the glucose uptake rate increases till 32 hours and then holds steady until 56 hours, at which

point the feeding finished. Then, the glucose is consumed till completely depleted. In the second experiment (bottom panel of Figure 5), the glucose uptake rate increased steadily and steady state was not observed. These discrepancies between experiments are attributed to differences between the inoculum cultures and inconsistency in the pump flow rates. Although the pumps were calibrated before each experiment, it was difficult to prevent stretching in the tubing.

The glucose constant feed experiments increased the maximum titer by 44.4% when compared to batch experiments. This is attributed to the higher concentration of ethanol that resulted from an increased availability of glucose in the culture broth (Figure 5 and Figure 6). However, there was no significant increase in biomass which resulted in increased yields by 20.6%. Productivity is lower in glucose feed type I due to the elongated glucose consumption phase.

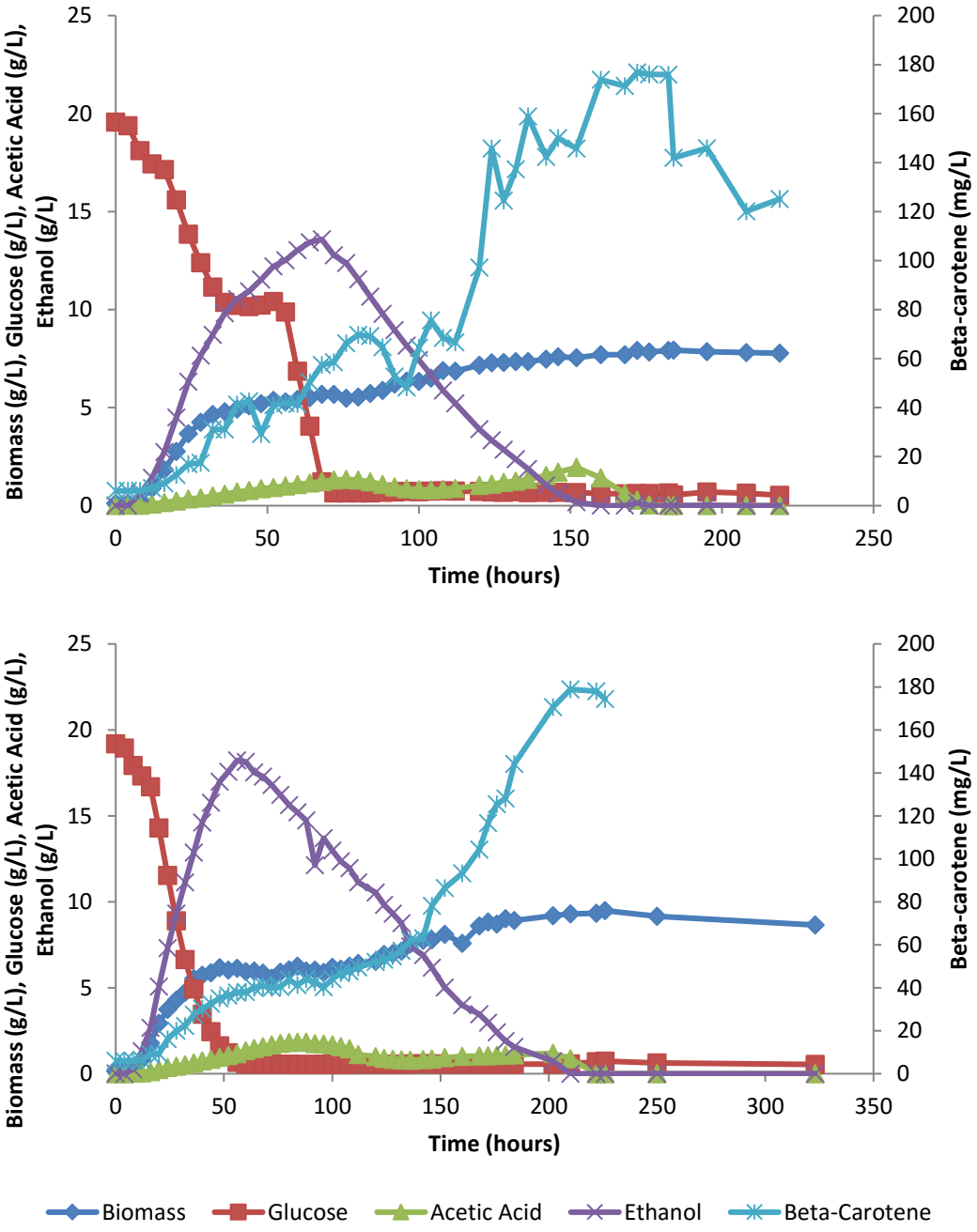

Figure 5 Profiles of duplicate glucose feed type I fermentations of SM14.

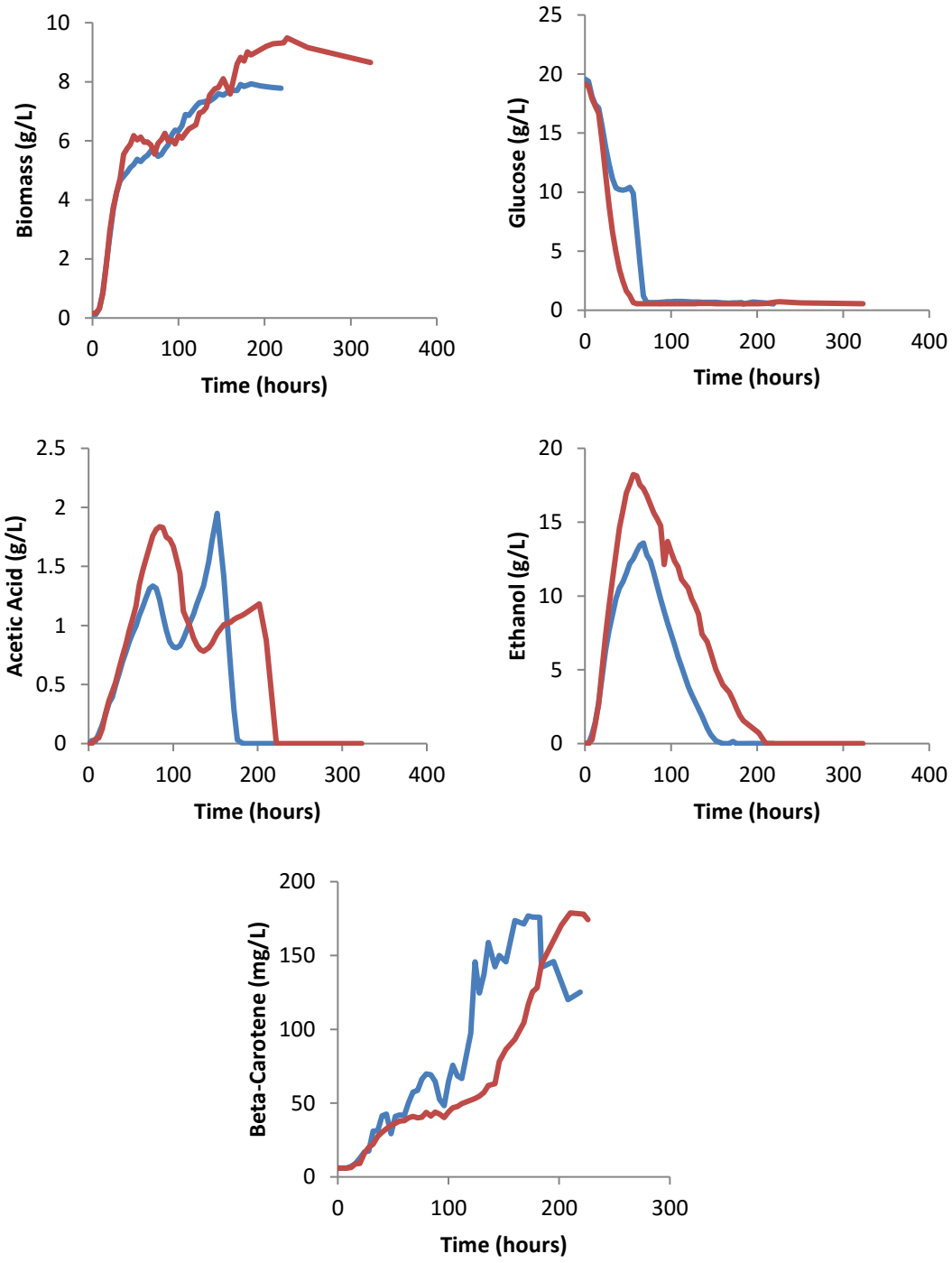

Figure 6 Comparison of each metabolite in duplicate glucose feed type I fermentations of SM14.

3.3 Glucose Feed Type II

Glucose feed type II experiments (Figure 7 and Figure 8) served to observe the effects of maintaining low levels of glucose compared to maintaining higher levels as in glucose feed type I experiments. In addition, they also served in isolating the effects of fed-batch operation while maintaining the same amount of carbon source as compared to batch experiments (60g of glucose).

The fermentation began with 2 liters of 20 g/L of glucose in YNB media and an additional 1 Liter of 20 g/L of glucose in YNB media was fed at the end of the glucose consumption phase (21 hours post inoculation) to maintain 1 g/L of glucose in the culture broth. The feed finished at 27.5 hours and continued in batch mode until acetic acid was depleted. By the end of the fed-batch phase, the glucose feed type II experiments were identical to batch experiments in terms of total glucose, nutrients and volume. As summarized in

Table 4, fed-batch operation in the glucose feed type II experiments did not significantly increase the maximum titer and decreased the productivity and yield by 13% and 12% respectively when compared to batch experiments. This finding signified that culture required a larger carbon source in addition to fed-batch operation in order to benefit in terms of increase in yield and titer. In addition, maintaining low levels of glucose did not allow for increased production of ethanol and therefore did not result in higher titers.

The result of glucose feed type II experiment demonstrates the challenge in optimizing this fed-batch profile. While feeding high concentrations of glucose lead to increased production of ethanol, the time required to consume the glucose extends run time and thereby decreases productivity. Moreover, increased ethanol concentrations lower growth rate of *S. cerevisiae.* However, ethanol is the carbon source that results in larger titers, yields and productivity when compared to glucose.

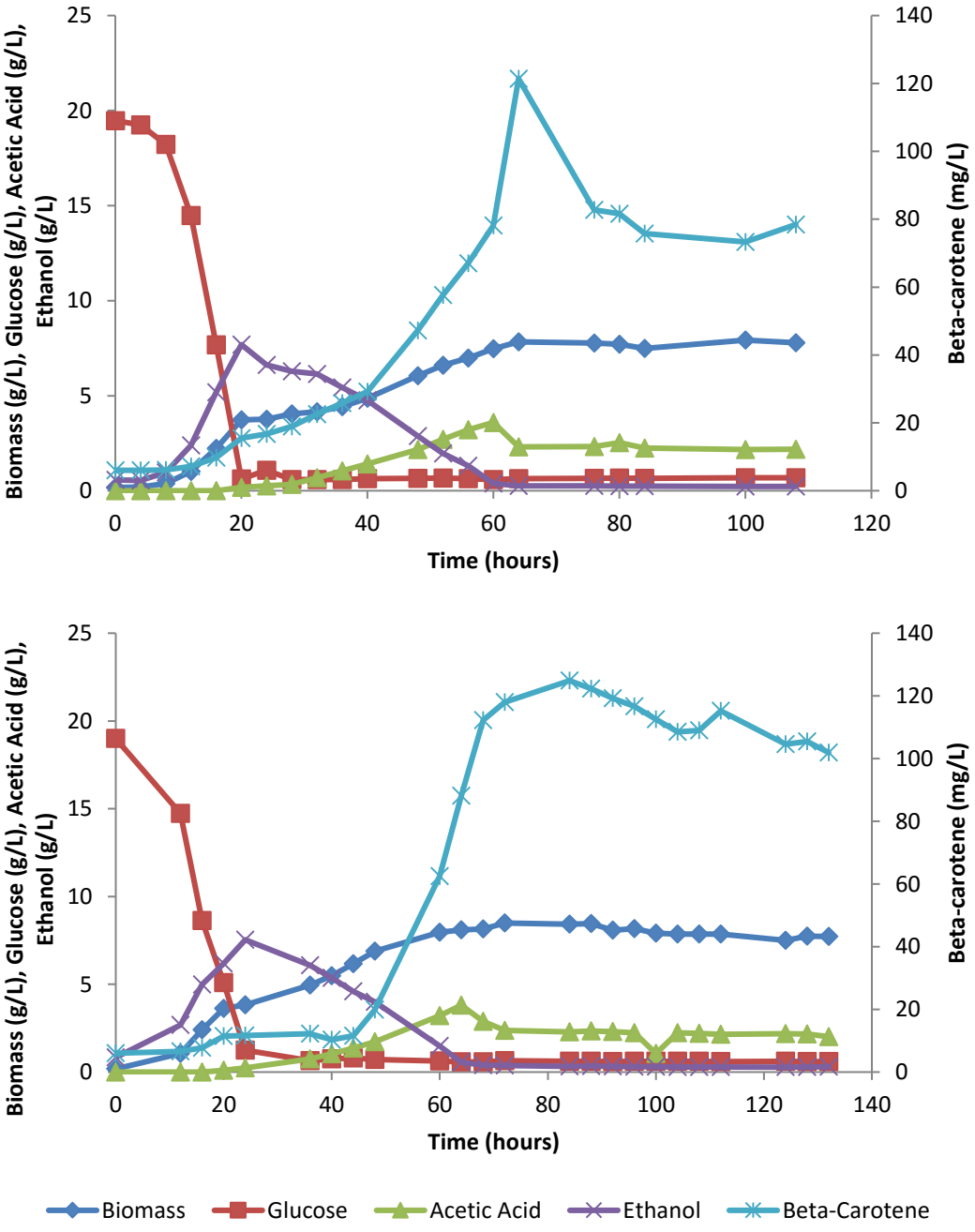

Figure 7 Profiles of duplicate glucose feed type II fermentations of SM14.

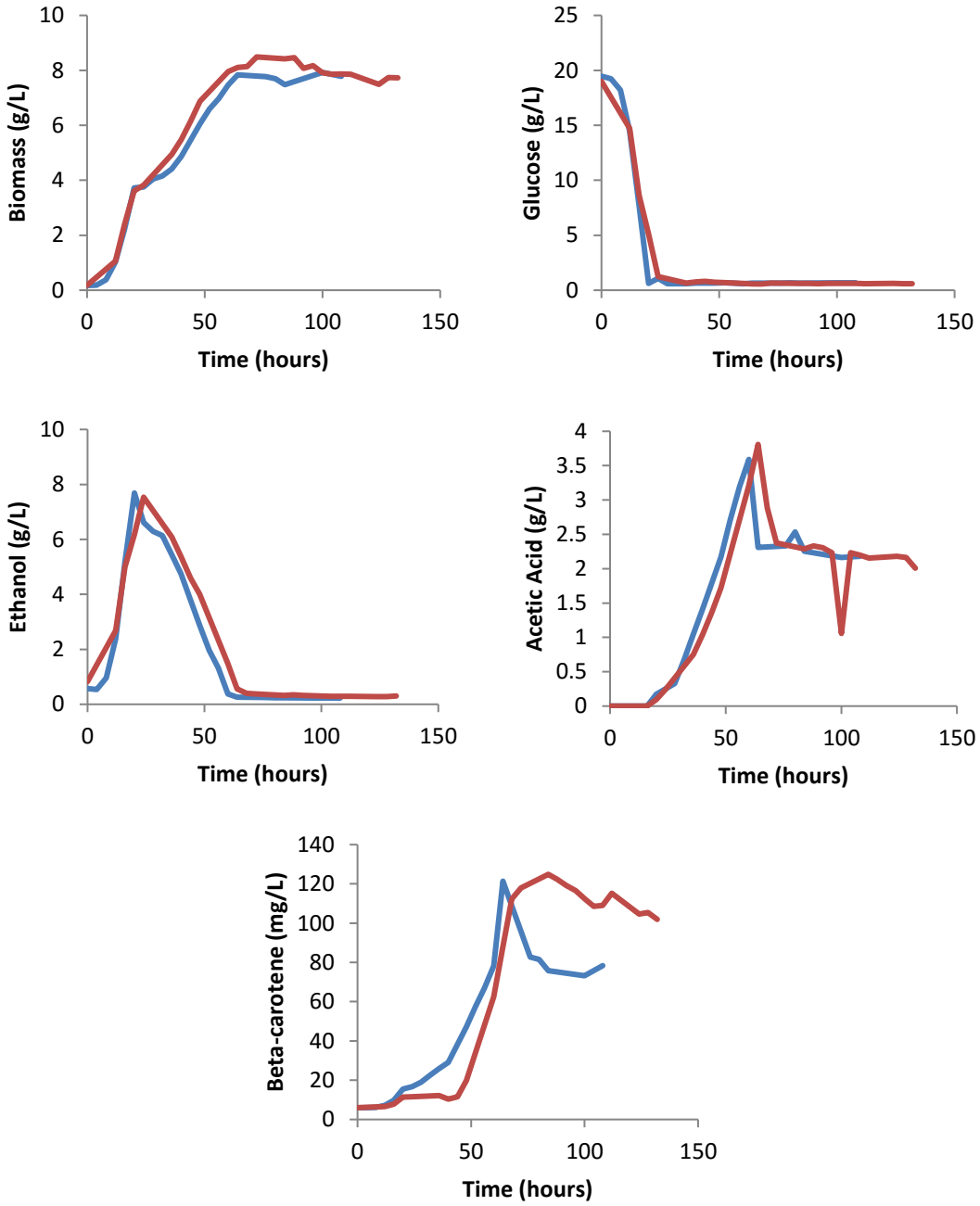

Figure 8 A comparison of each metabolite in duplicate glucose feed type II fermentations of SM14.

3.4 Ethanol Feed

In ethanol feeding experiments, the objective was to maintain a constant ethanol concentration in the culture broth by feeding 25g/L ethanol in YNB at the rate of ethanol uptake since batch experiments show highest productivity during the ethanol consumption phase. In the duplicate experiments (Figure 9 and Figure 10), the ethanol uptake rate increased along with the feeding of ethanol, so a clear steady state was not observed. Moreover, the second experiment of this type was able to consume the ethanol completely within 88 hours while the first experiment required 136 hours. This discrepancy was attributed to differences in inoculum cultures or pump control issues as described above.

The ethanol feeding experiments resulted in a maximum titer of 179.09 mg/L which increased the yield by 22.4 % and titer by 45% when compared to batch experiments. On average, the ethanol feed also resulted in higher productivity when compared to glucose feed type I experiments. This was due to a shortened glucose consumption phase, which allows for ethanol consumption to begin earlier and achieve the maximum titer at earlier stages in the experiment. Biomass growth was not affected by the ethanol feeding, largely due to the fact that ethanol concentrations remained below 10 g/L. Earlier inhibition studies in Karim lab have shown that growth inhibition begins around 30g/L of ethanol (Ordoñez et al., 2016). Other studies have shown that ethanol feeding can increase production of carotenoids in yeast. Gu et al. showed that an additional 0.2% ethanol concentration resulted in a yield of 2170 µg/g yeast compared to

1680 µg/g yeast in *P. rhodozyma* cultures without any additional ethanol (Gu et al., 1997). They also concluded that carotenoid production using ethanol effect the activity of HMG-CoA reductase, which is under feed-back regulation by mevalonate in *S. cerevisiae* (Dimster-Denk, Thorsness, & Rine, 1994). This led them to believe that deregulation of HMG-CoA reductase increases flux through the isoprenoid pathway as a result of ethanol.

Ethanol feeding in microbial production is an uncommon practice due to higher average cost and variability in cost. However, large margins of profit due to the product value of beta-carotene would allow ethanol feeding a viable means of production.

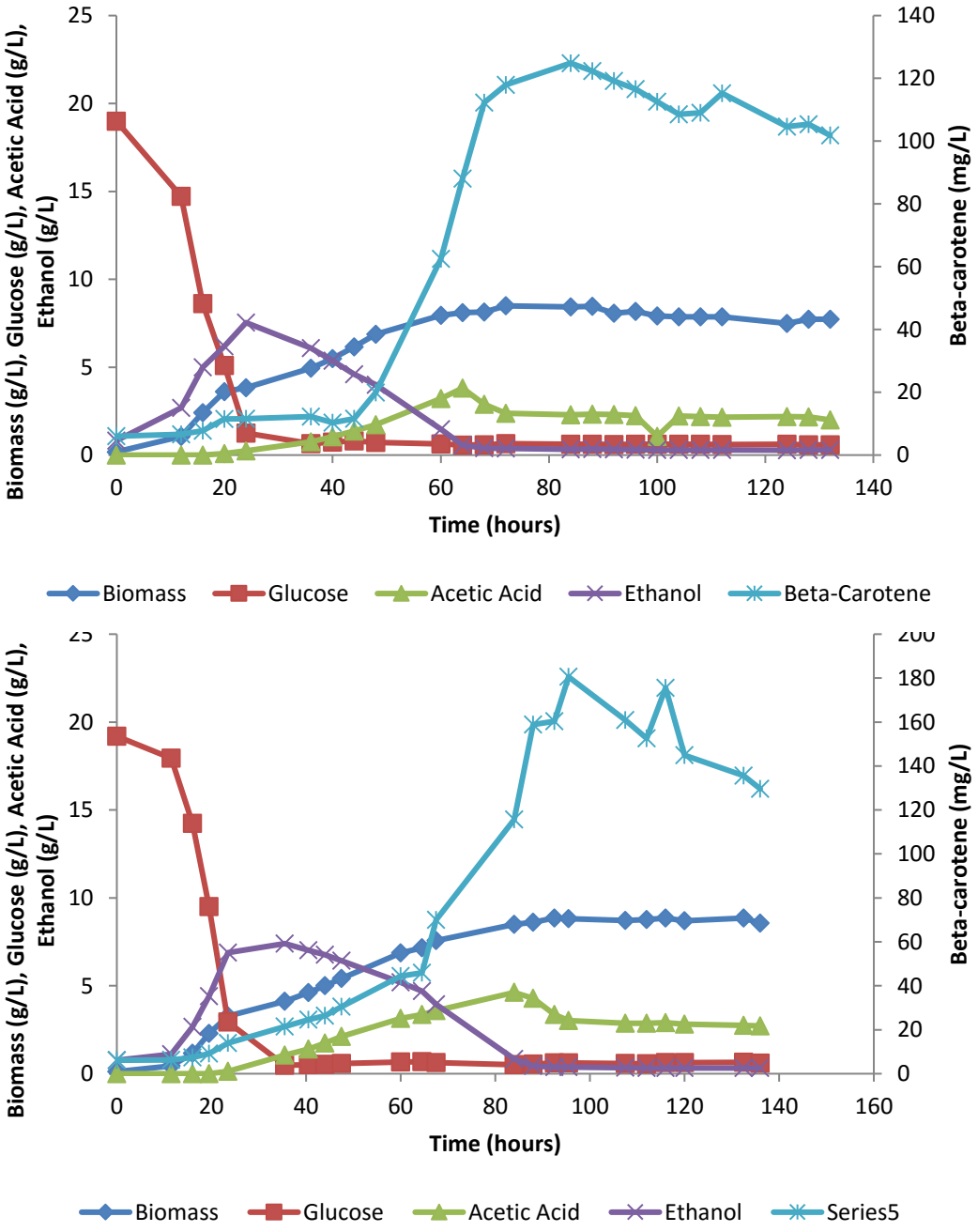

Figure 9 Profiles of duplicate ethanol feed fermentations of SM14.

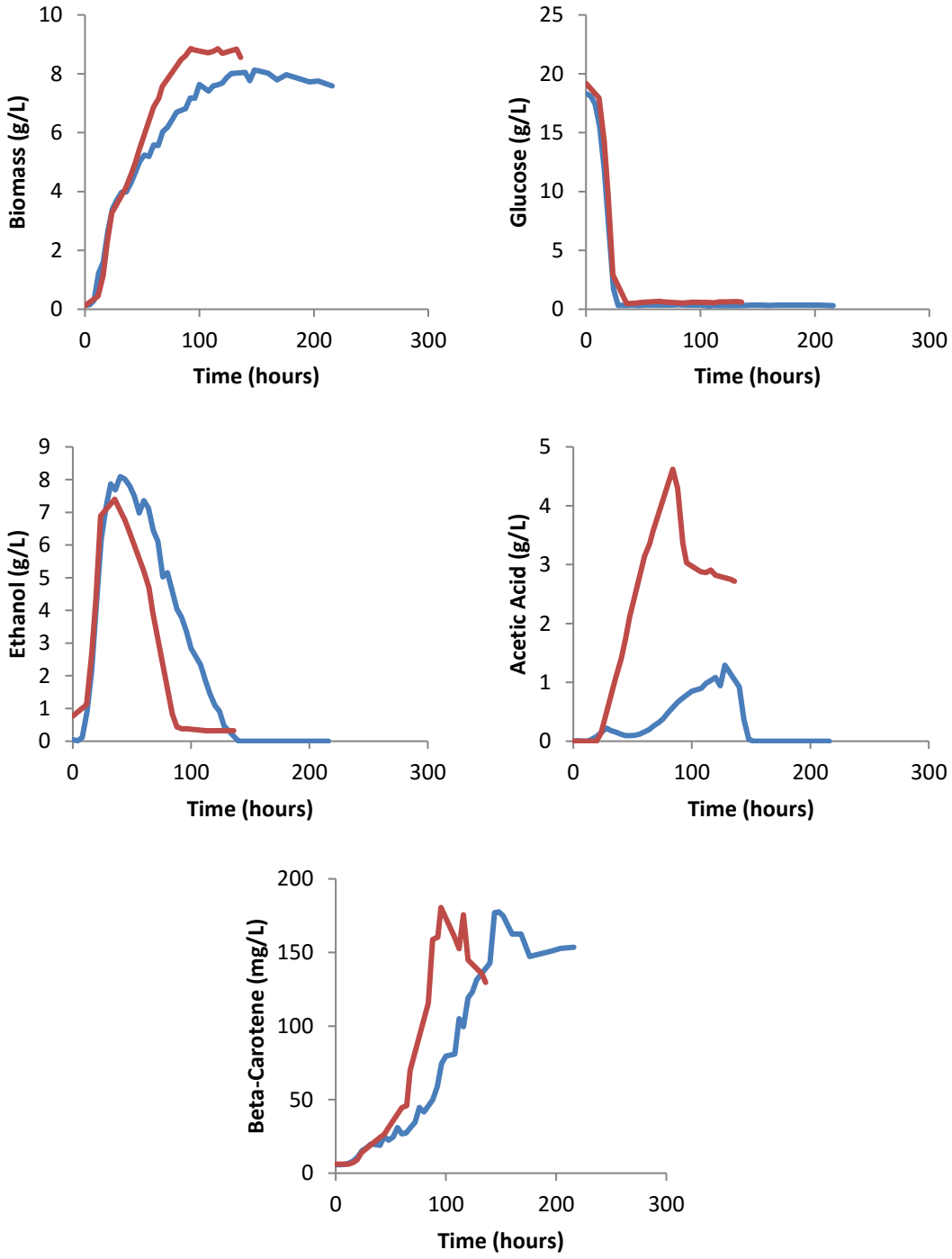

Figure 10 Comparison of each metabolite in duplicate ethanol feed fermentations of SM14.

3.5 Summary of Bioreactor Scale Experiments

Fed-batch experiments of glucose feed type I and ethanol feed increased the tier and yield compared to batch experiments (as summarized in Figure 11). Average cellular yield for glucose feed type I was the highest among the four types of experiments compared. However, glucose yield was highest for ethanol yield by a very large margin. Surprisingly, ethanol yield was lower for fed-batch experiments than batch experiments. This indicates that after reaching maximum titer, ethanol cannot be used for biomass growth or increased beta-carotene production. This is also evidence that a physiological limitation is being reached in the fed-batch experiments since an approximate 180mg/L titer is achieved and further feeding of carbon sources and nutrients do not contribute to any further production of beta-carotene. Further optimization of the growth conditions, feed profiles and metabolic fluxes can increase the maximum titer of SM14 by increasing biomass growth. This can be achieved through high cell density fed-batch fermentations (Riesenberg & Guthke, 1999).

For comparison with other beta-carotene producing biocatalysts, Yu Lab engineered a strain of S. cerevisiae using an inducer/inhibiter free control system whereby they were able to control expression of MVA and squalene pathways by controlling the glucose concentration in the culture (Xie, Ye, Lv, Xu, & Yu, 2015). Using fed-batch operation and high-cell density fermentations (rich media composition), they were able to produce 1156 mg/L of carotenoids (39.06% beta-carotene). The yields

for their fermentation are comparable to fed-batch fermentations presented in this work (20.79 mg/g DCW).

A significant challenge in analyzing the performance of each bioreactor experiment was deciphering the variability from run to run. Variability is minimized by maintaining the same reactor conditions such as pH, temperature, stirring rate, inlet flow rate and inoculum size. However, disturbances such as failure of pumps and differences in inoculum contribute to variability between the runs. Moreover, the difficulty in extracting beta-carotene from the cell creates a low signal to noise ratio. Each cell pellet was visually inspected to assure that the maximum number of cells was disrupted. Still, we noticed high variability between duplicate samples.

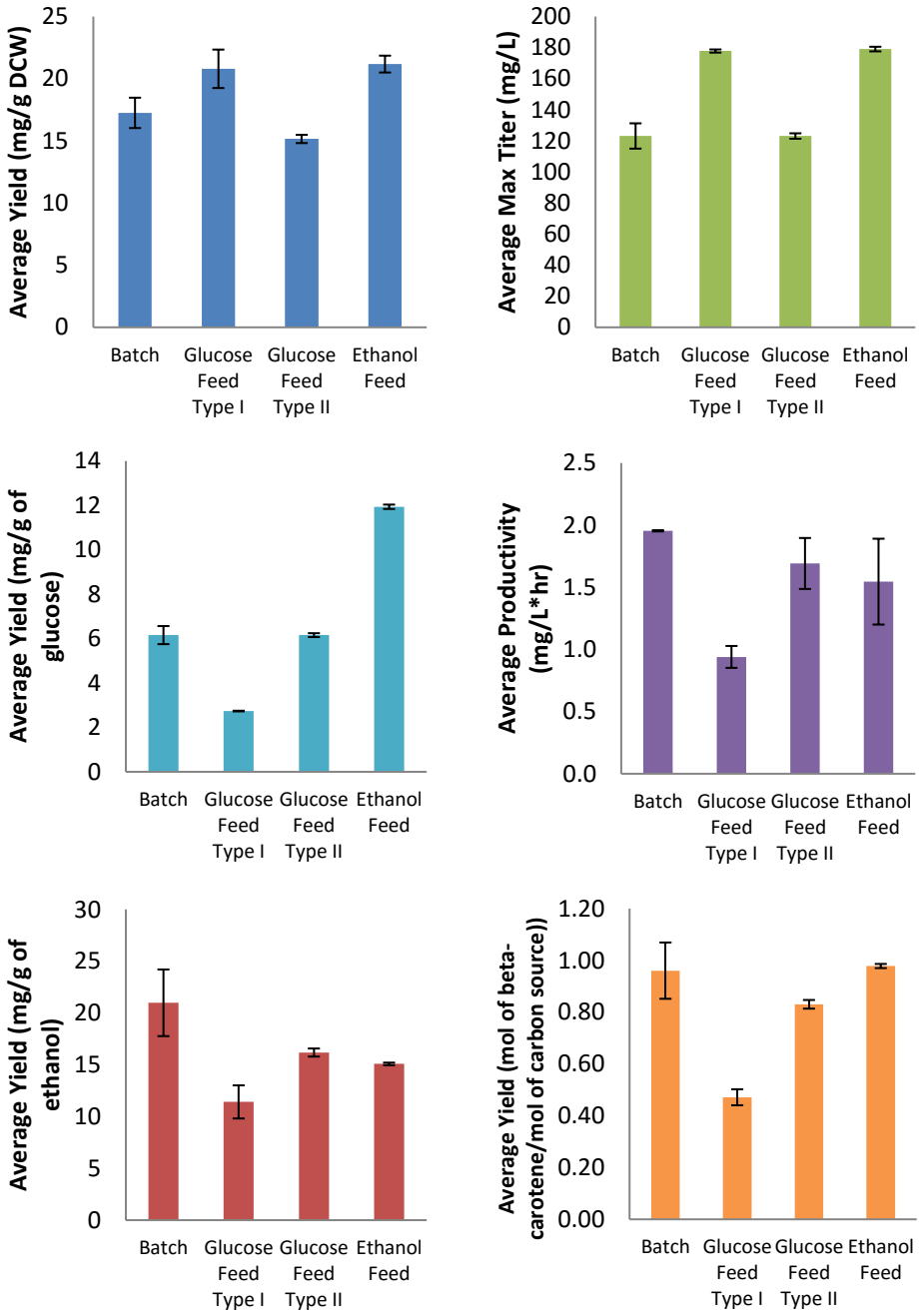

Figure 11 Summary of average yield, average maximum titer and average productivity from the four different types of fermentation experiments.

3.6 Media Optimization using Response Surface Methodology

Although fed-batch operation using SM14 increased yields and titers as shown above, productivity needed improvement in order to substantiate the use of fed-batch operation. In addition, fed-batch operation did not result in significant gains in biomass when compared to batch experiments even while feeding larger amounts of carbon source. To address these issues, we explored media optimization in order to increase biomass which would in turn increase titer and productivity.

The statistical software JMP, design of experiments (DOE) and RSM were utilized to investigate varying concentrations of glucose, YNB (vitamins, trace elements and salts) and ammonium sulfate – the three components of defined YNB media. Glucose serves as the main carbon source, YNB provides nutrients, trace elements and nitrogen and ammonium sulfate is the main nitrogen source. The 16 different compositions of the media and the resulting biomass and final titer that were investigated are summarized in Table 5, Table 6 and Figure 13. In general, the trends showed that higher glucose and YNB concentrations resulted in higher titer and biomass. However, increasing ammonium sulfate had little effect on increasing biomass or titer.

Flask	Glucose (g/L)	Ammonium Sulfate (g/L)	YNB (g/L)	Titer (mg/L)	Biomass (g/L)	Predicted Titer (mg/L)	Predicted Biomass (g/L)
1	77.6	12.5	21.85	46.46633	8.817702	49.29454	8.939677
2	20	12.5	21.85	52.73711	7.960849	47.392	7.346367
3	60	12.5	42	49.0284	8.021808	41.9862	7.416293
4	20	5	42	42.06246	6.353058	43.84649	6.472223
5	100	5	1.7	21.13924	4.061385	17.57987	3.747782
6	100	20	42	36.42785	8.934286	35.50487	8.734981
7	20	20	1.7	14.98239	2.905072	14.67992	2.698487
8	100	20	1.7	19.09211	4.230927	18.46772	4.194497
9	60	5	21.85	52.7977	9.160977	51.02828	8.784058
10	100	5	42	37.80315	8.886662	39.26527	9.17598
11	20	20	42	27.19571	4.307126	31.91474	4.703463
12	20	12.5	21.85	48.91276	7.221722	47.392	7.346367
13	77.6	12.5	21.85	40.61828	8.240879	49.29454	8.939677
14	20	5	1.7	19.88077	3.297494	21.96341	3.579534
15	60	20	21.85	48.37551	8.077052	45.50633	8.123035
16	60	12.5	1.7	20.1225	3.425127	22.52609	3.699706
Control	20	5	1.7	19.45991	3.230821	-	-

Table 5 A summary of the compositions of each of the flask with varying concentrations of glucose, ammonium sulfate and YNB.

Flask	Average Biomass (g/L)	Std. Deviation of Biomass (g/L)	Average Titer (mg/L)	Std. Deviation of Titer (mg/L)	Yield (mg/g)
1	8.82	0.01	46.47	0.76	5.27
2	7.96	0.66	52.74	2.70	6.62
3	8.02	0.46	49.03	2.33	6.11
4	6.35	0.49	42.06	1.53	6.62
5	4.06	0.21	21.14	0.16	5.20
6	8.93	0.28	36.43	1.70	4.08
7	2.91	0.09	14.98	0.91	5.16
8	4.23	0.07	19.09	0.81	4.51
9	9.16	0.12	52.80	0.30	5.76
10	8.89	0.00	37.80	0.29	4.25
11	4.31	1.29	27.20	11.52	6.31
12	7.22	0.48	48.91	2.01	6.77
13	8.24	0.21	40.62	1.60	4.93
14	3.30	0.18	19.88	0.64	6.03
15	8.08	0.08	48.38	0.63	5.99
16	3.43	0.06	20.12	0.09	5.87
Control	3.23	0.13	19.46	0.15	6.02

Table 6 Summary of results from RSM shake flask experiments.

The general process used to optimize the media composition using JMP is as follows. First, the user defines the factors and responses that are being investigated in the experiment. In the case of optimizing media composition for SM14 media, the factors are glucose, YNB and ammonium sulfate concentrations while the responses are titer and biomass. Then, each of the responses requires an objective which can be in the form of maximizing, minimizing or targeting a specific value. Here, we chose to maximize the titer and biomass production. Next, JMP uses face-centered central composite design to determine the compositions that should be tested experimentally. The data from the

experiments was then used to fit a generalized second order model. In RSM, there are two main generalized models – first order and second order. Second order (Equation 6) was used in these studies since it provides a greater understanding of the relationship between separate factors.

$$y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_{AB} AB + \beta_{AC} AC + \beta_{BC} BC + \beta_{AA} A^2 + \beta_{BB} B^2 + \beta_{CC} C^2$$

Equation 6 Generalized second order model used in RSM to fit experimental data.

Using least squared error methods, JMP fits the experimental data to the generalized second order model and determines the values of the parameters (all β terms in Equation 6). Each of the factors is represented by A, B or C (Table 7). The estimated parameters are summarized in Table 8. There were two models created – one describing titer (beta-carotene) production and the other describing biomass production. The significance of each of the parameters is calculated using log-worth (Equation 7). If a p-value of 0.01 is considered as significant, the equivalent log-worth would be 2. Analyzing the log-worth of each of the parameters for both models (listed in Table 8), it can be seen that the most significant parameters are the ones pertaining to glucose and YNB. Due to their low significance, parameters C, AC, BC A^2 and C^2 were removed. This resulted in a low R-squared value (below 0.9) when comparing the experimental data to the predicted data. Therefore, all parameters were left remaining in the model to maximize the fit. The comparison of experimental data to predicted model data is shown in Figure 12.

Factor	
Glucose (g/L)	A
YNB (g/L)	B
Ammonium Sulfate (g/L)	C

Table 7 The corresponding variable used for each factor in the generalized second order model.

Source	Parameter Estimate (Beta-Carotene)	Parameter Estimate (Biomass)	Log-Worth
B	9.73	1.86	4.282
B^2	-17.65	-2.94	3.769
A	-2.76	1.05	2.832
AB	2.04	0.63	1.679
C	-2.71	-0.33	0.920
AC	-1.16	0.33	0.810
BC	-1.64	-0.22	0.497
A^2	-0.19	-0.10	0.276
C^2	-0.05	-0.04	0.167

Table 8 A summary of all parameters for beta-carotene and biomass production and the corresponding significance as indicated by log-worth.

$$\boldsymbol{LogWorth = -log_{10}(p\ value)}$$

Equation 7 Conversion from p-value to LogWorth.

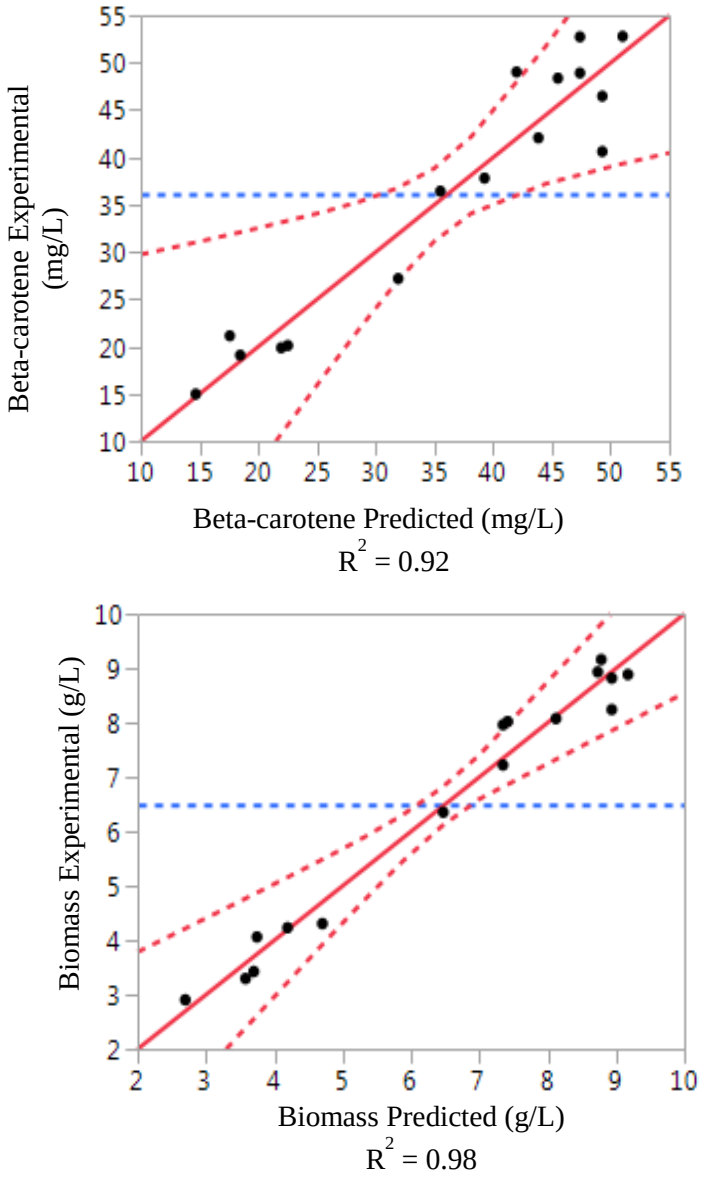

Figure 12 A comparison of the predicted beta-carotene production to experimental data (Top). A comparison of predicted biomass production to experimental data (Bottom).

Looking at biomass optimization alone, the highest biomass was achieved with the highest glucose concentration (100g/L). However, increased carbon source alone did not increase the biomass significantly. In flask 5, the glucose content was increased to 100 g/L and all other components remained the same when compared to the control (non-supplemented YNB media). The biomass increased from 3.23 g/L (control) to 4.06 g/L (flask 5) and titer increased from 19.46 mg/L (control) to 21.14 mg/L (flask 5). So, it is evident that in addition to increased carbon source, increased concentrations of vitamins and trace elements are also important in increasing the production of beta-carotene. Because beta-carotene production is a growth associated process, it is important to also maintain high levels of biomass growth. Measurements from other flasks which had higher concentrations of YNB showed a much larger increase in biomass and titer. In general, increased biomass resulted in higher titer production which resulted in decreased variation in the cellular yield across varying compositions.

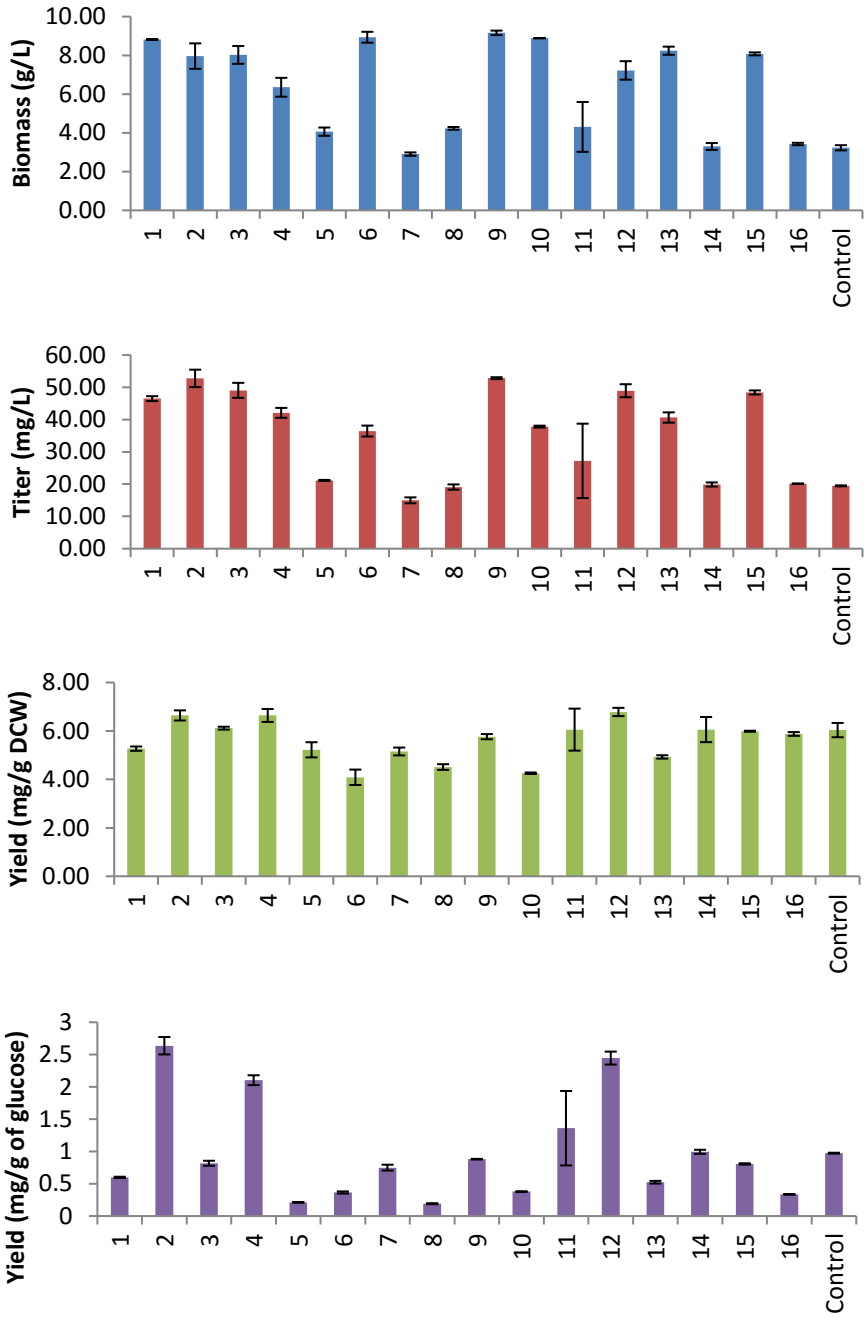

Figure 13 A summary of biomass, titer and yield from RSM experiments.

Using the model, surface and contour plots were graphed to analyze the optimal compositions (Figure 14 and Figure 15). When searching the surface for maximum biomass growth, the maximum occurs when the media includes 100 g/L of glucose. However, for optimal beta-carotene and biomass production, the maximum occurs at 79.10 g/L glucose, 5.17 g/L ammonium sulfate, 29.16 g/L YNB. At this composition, the model predicts a titer of 50.98 mg/L and a biomass of 9.58 g/L. This finding shows nutrient deficiency at the shake flask scale.

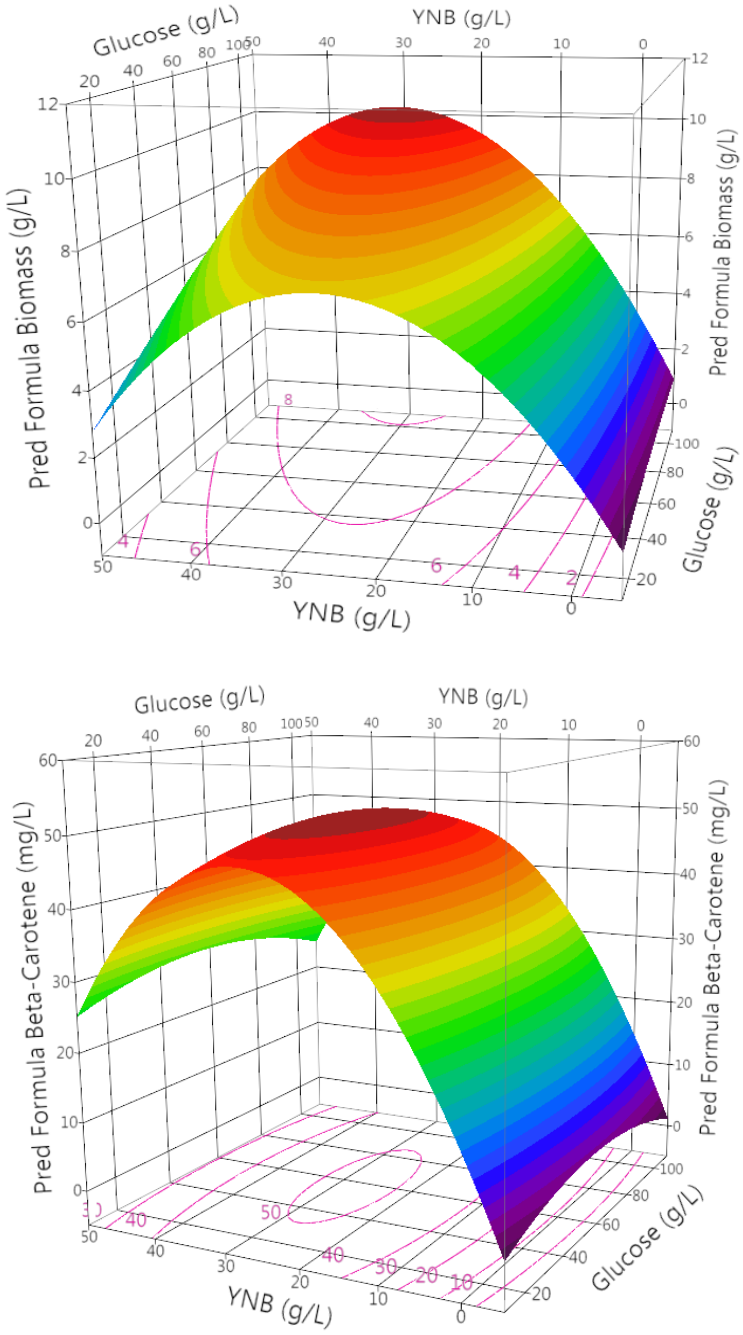

Figure 14 The response surface for predicted biomass (top) and predicted titer (bottom) by varying YNB and glucose.

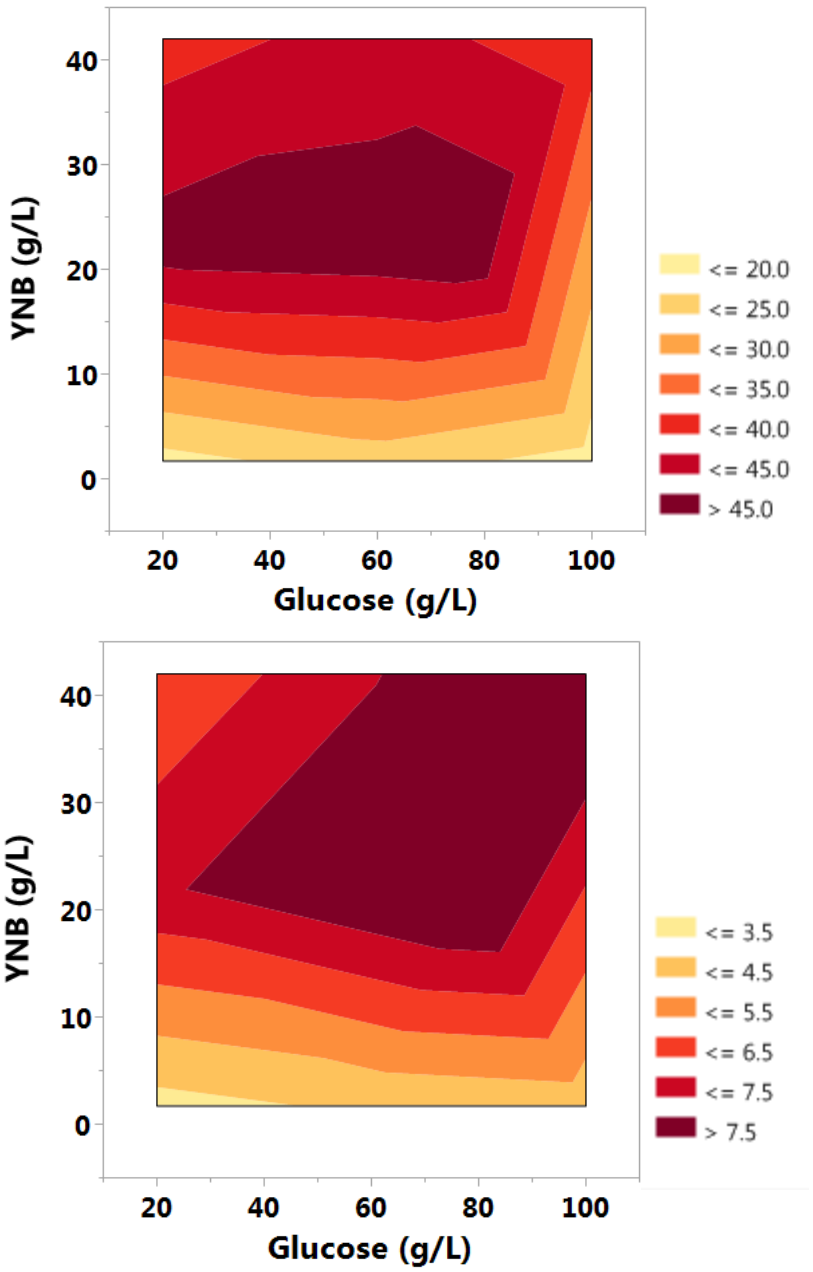

Figure 15 Contour plot of biomass production model (Top). Contour plot of beta-carotene production model (Bottom).

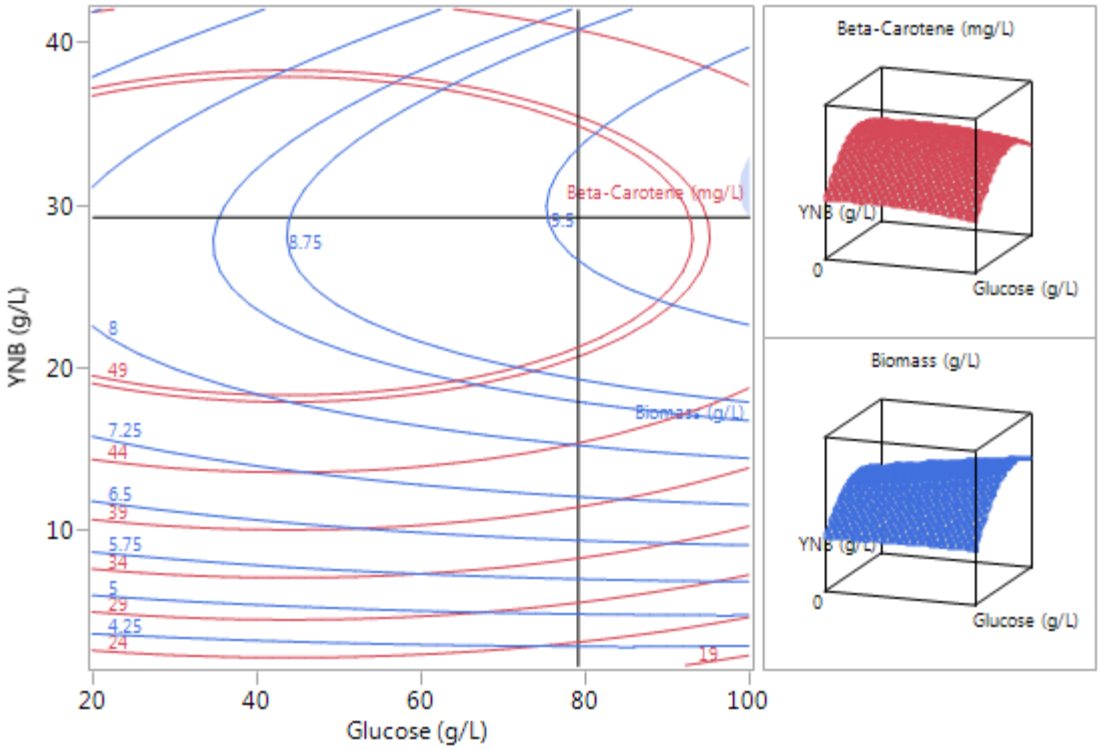

Figure 16 Overlapping contour plots of the RSM model for beta-carotene (red) and biomass (blue).

Once the model was constructed and all the parameters were assigned, the optimal composition of the media was determined by optimizing biomass and titer. In JMP, each response is assigned a desired outcome – maximize, minimize or target a value (objective function) (Rushing, Ballard, Wisnowski, & Levin, 2012). The desirability function is an optimization algorithm, built into JMP, which profiles the "desirability" as determined by the assigned desired outcome of a given response. For example, when optimizing media for cell culture, the titer could be a possible response that should be maximized through manipulation of media components (factors). After

experimentation and statistical analysis, the desirability function can be used to find the values of the factors that result in the maximum value of titer. The desirability function varies over the range of each factor and itself ranges from zero to one. When multiple responses are considered, the desirability function can optimize all responses and determine the optimal values of all the factors with respect to the objective function. Although SAS does not reveal the exact code, they do describe the minimize and maximize desirability functions as a group of three smooth piecewise functions that have exponential tails and cubic middle.

When optimizing the titer and biomass production in the media optimization described in this work, the objective function was set to maximize the factors. The two models are superimposed to find an optimal in both responses (Figure 16). In order to maximize the factors, we search the response surface for the points at which desirability is as close to one as possible (Figure 17). The resulting optimal composition was 79.10 g/L of glucose, 5.17 g/L of ammonium sulfate and 29.16 g/L of YNB. This would result in a biomass of 9.58 g/L and titer of 50.99 mg/L. Compared with the control shake flask, the new composition of YNB media results in a 196.59% increase in biomass and a 162.03% increase in titer.

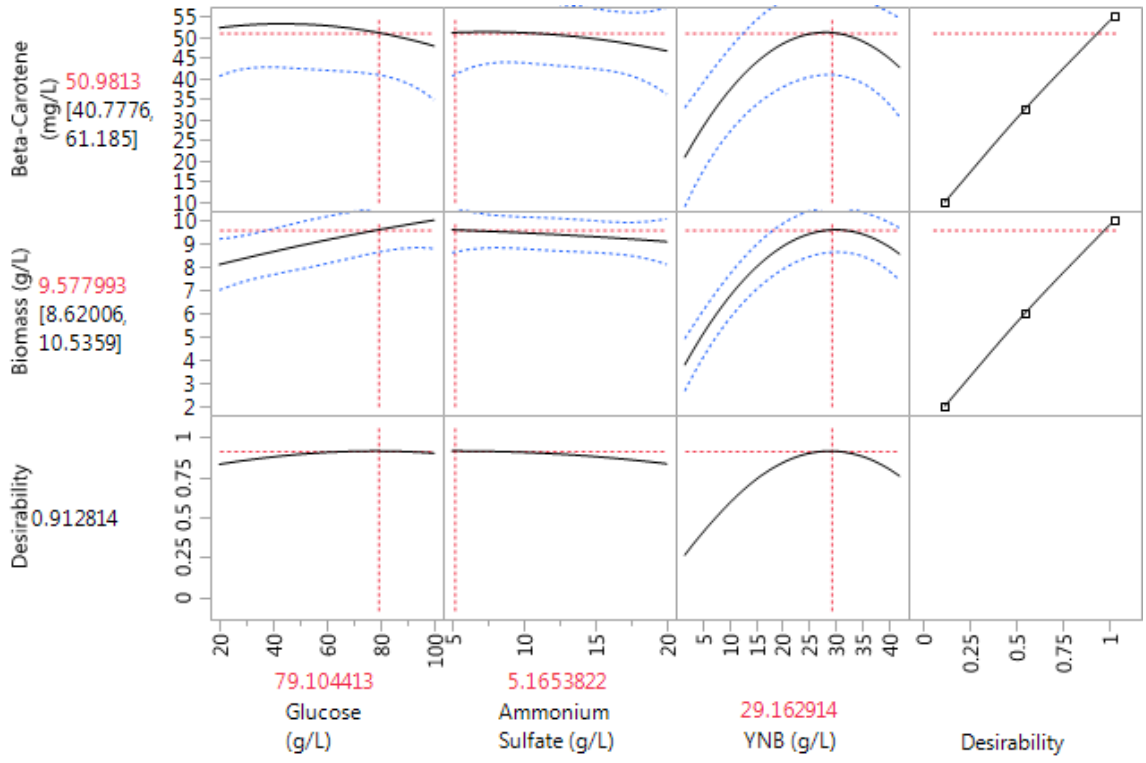

Figure 17 The predicted model for glucose, ammonium sulfate and YNB.

Using the optimal media composition found using shake flask experiments, a batch experiment at bioreactor scale was conducted (results summarized in Figure 18). The results showed a significant increase in biomass and beta-carotene production. There was a 243.35% improvement in biomass and 198.21% improvement in titer. However, cellular yield was 13% lower than typical batch fermentations. This could have resulted from the lower DO levels observed in the bioreactor due to large gains in biomass. Maintaining higher DO levels and implementing fed-batch fermentation using the optimized media could increase the cellular yield. The other metric of performance that we sought to improve was productivity. Using the optimized, productivity increased

to 4.03 mg/L*hr which is a 106.49% improvement when compared to batch experiments using SM14.

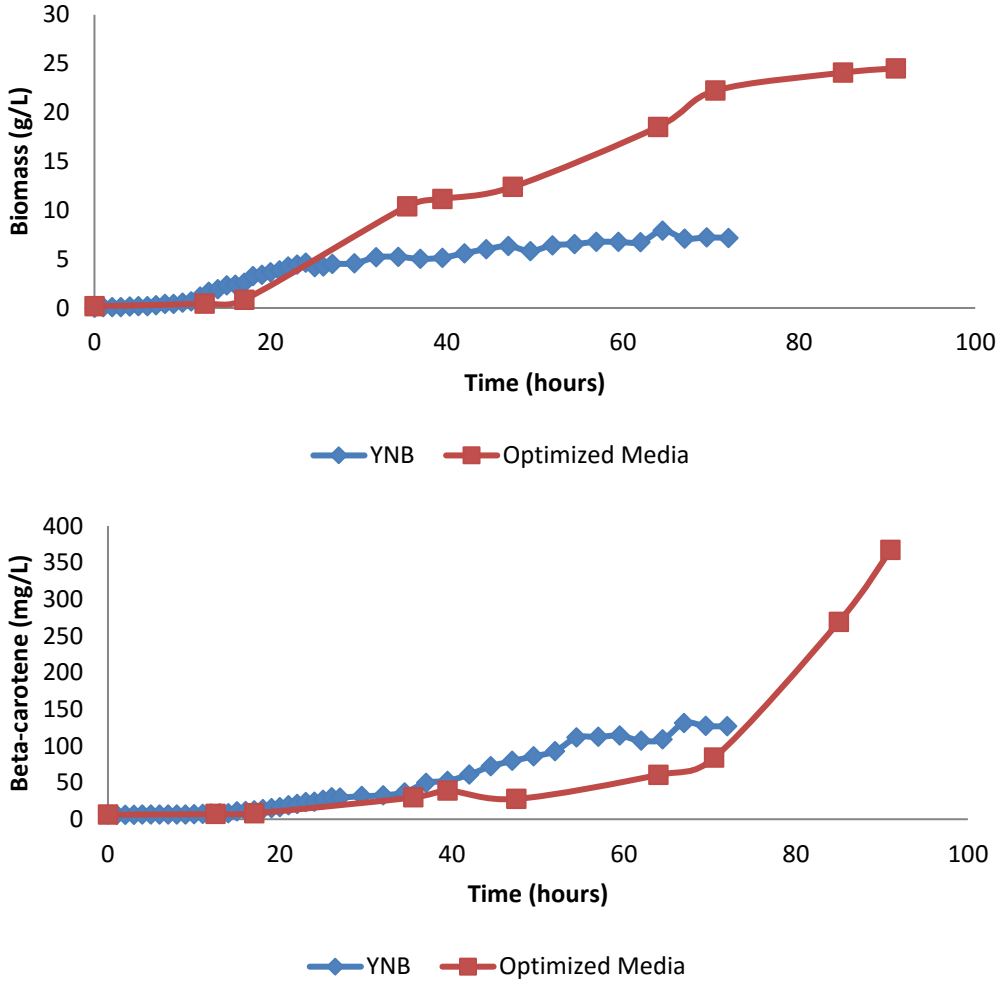

Figure 18 A comparison of biomass (Top) and beta-carotene (Bottom) production using typical YNB media and optimized media in batch operation.

4. CONCLUSIONS AND FUTURE WORK

Using fed-batch experiments, the yield and titer were increased (Table 4) when compared to batch experiments. However, productivity is slightly decreased in both glucose and ethanol feeding experiments when compared to batch due to extended glucose and ethanol consumption phases. The productivity of the ethanol feeding experiments is higher due to the shortened glucose consumption phase which allows for the ethanol consumption phase to begin and finish earlier than in the glucose feeding experiments.

The profile of each bioreactor experiment shows that maximum titer is achieved when the available ethanol is completely consumed. This presents a design challenge when determining feeding profiles as increased ethanol results in higher titers and yields; however, productivity decreases as a consequence of an extended ethanol consumption phase. This issue is highlighted in the comparison between glucose feeding experiments and ethanol feeding experiments. This issue can be remedied by increasing cell density such that higher titers can be achieved in earlier phases of the experiment.

Using DOE and RSM, we showed that fermentations with SM14 are nutrient limited and increasing the YNB and glucose concentrations of the media could significantly improve the biomass and titer production. The results from the batch experiment using the optimized media can be extended to fed-batch operation to further increase yield, titer and productivity.

In order to develop this fed-batch process for industrial implementation, computational modeling and controls would be required to optimizing feeding profiles. Currently, fed-batch modeling and an optimal controller for fed-batch are being developed in Karim group.

In addition, more midstream development is required also to better characterize the fed-batch fermentations. A significant factor of fermentation, DO, would need to be characterized for SM14 fermentations. Preliminary results of DO controlled fermentations showed that optimal DO lies in the range of 70% to 95% saturation.

Solid understanding of the fed-batch fermentations of SM14 would allow for implementation of continuous cultures. Preliminary continuous production models of SM14 (extended from batch models) show a significant increase in productivity when continuous fermentations are utilized.

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