SEPARATION AND RECOVERY OF INTRACELLULAR BETA-CAROTENE USING A PROCESS SYNTHESIS FRAMEWORK WITH TARGETED EXPERIMENTS

A Thesis

by

ALEXANDER MICHAEL SABOL

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Chair of Committee,	M. Nazmul Karim
Committee Members,	Ahmad Hilaly
	Arul Jayaraman
Head of Department,	Nazmul Karim

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ABSTRACT

Systematic process synthesis approaches are widely applied to traditional chemical process industries, but have seen limited use in the bioprocessing industry due to the limited or non-existent availability of thermodynamic or kinetic data. In this work, the process synthesis problem for the bio-manufacturing of high-value intracellular compounds is addressed using a systematic framework that allows for the user to input key process parameters from literature or experiments. The framework is based on a superstructure optimization approach and integrates various methods and tools, including a generic model and a database for data management. We propose the following five steps: (1) problem formulation, (2) data collection and superstructure generation, (3) solution of the optimization problem, and (4) process parameter analysis and (5) experimentation with informed design and then determination of the optimal process design. The framework is implemented in Super-O, software which guides the user through the formulation and solution of synthesis problems. This thesis demonstrates the proposed framework though an illustrative case study on the production of beta-carotene from recombinant Saccharomyces cerevisiae (SM14) via continuous cultivation using experimental, simulation and literature values.

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This work was supervised by a thesis committee consisting of advisor, Professor Dr. M. Nazmul Karim, and committee members, Professor Dr. Ahmad Hilaly, of the Department of Chemical Engineering and Professor Dr. Arul Jayaraman of the Department of Biomedical Engineering.

The software, Super-O depicted in Section III was created by Mari-Ona Bertan and Dr. Raquel Gani at the Technical University of Denmark in the Department of Chemical Engineering and published in 2017. The data collection presented in Section III was assisted by Mari-Ona Bertan during her two week collaboration period at Texas A&M University during November of 2016. All other work conducted for the thesis was completed by the student independently.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

I. INTRODUCTION

In this work, we will discuss a novel process synthesis process that follows these five steps: (1) problem formulation, (2) data collection and superstructure generation, (3) solution of the optimization problem, (4) process parameter analysis and (5) experimentation with informed design and then determination of the optimal process design. This process synthesis problem will be input into software named Super-O, which guides the user through the formulation and solution of synthesis problems.

The generic process model consists of a series of processing tasks, namely mixing, reaction, waste removal and product separation, for which the model parameters need to be provided by the user. However, the limited availability of technology data for bioprocesses is a bottleneck in the superstructure development. In this work, experimental studies are used to determine estimates for key process parameters for their integration into the synthesis problem. These experimental values are used to complement data available in the literature and from simulations.

As a case study to exemplify this framework, the production of beta-carotene from recombinant Saccharomyces cerevisiae (SM14) consuming glucose via cultivation is analyzed. Beta-carotene has important industry relevance as a colorant for food products and antioxidant and cancer prevention agent in supplements. The processing tasks for the beta-carotene production process were taken from literature and experiments. The synthesis of a beta-carotene production process has been posed as a profit maximization problem, using capital expenditures (CAPEX), where given the raw

material and product, the optimal process topology is determined. This work opens the door to the synthesis of processes for other key intracellular compounds of interest, such as chemotherapy agents and biofuels.

II. LITERATURE REVIEW*

II.I Market and Process for Bio-Products

The market for non-energetic bio-products, including chemicals and pharmaceuticals, is projected to reach $472.8 billion in 2018, with a compounded annual growth rate of 14.9%. (Gobina, 2014) The nutraceutical and herbal/botanical market, which is the main focus of this thesis, make up 30% of the 2013 market value. This number is expected to rise if downstream separation costs are reduced from the current 60-80% of the total production costs. (Kiss et al., 2015) Downstream processing costs are heavily dependent on the nature of the bio-product. Currently most industrial produced bio-products are extracellular, which means they are secreted out of the cell. However, there remain a significant number of useful bio-products that are intracellular and not secreted to the extracellular environment. (Chisti and Moo-Young, 1986) Recovery of these useful intracellular products requires more expensive processing methods, as cell homogenization and purification from the resulting debris are necessary. (Balasundaram et al., 2009) A literature analysis of 100 articles about the order of purification process stages that was conducted by Bonnerjea et. al (1986) and shown in figure 1 below, indicates that homogenization, or the destruction of the microbe's outer barrier has to be done first, but the process step variability at each stage increases after that because of the complex nature of bio-products and similarities between the bio-product and bio-waste. (Bonnerjea et al., 1986)

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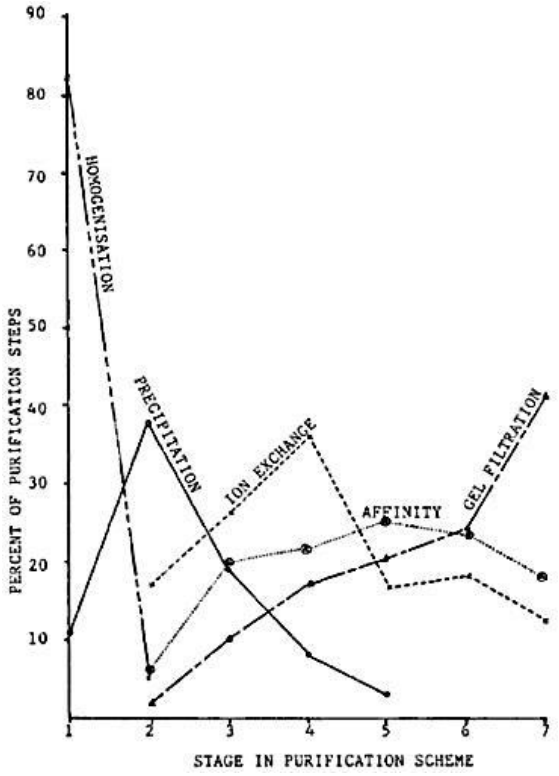

Figure 1 - Purification Steps Literature Review, reprinted from Bonnerjea et al., 1986

Unlike chemical processes, which typically produce multiple products from one process line, intracellular bio-products exhibit a high level of process variability when switching between cell lines or bioproducts and should, therefore, have different optimal process topologies. Therefore, in the bio-product industry most products are not optimally produced especially when considering that most bio-products are produced in a batch process instead of the more optimal continuous process.

Though continuous manufacturing has been implemented in almost every other industry, the biopharmaceutical industry has been reluctant to change from the archaic batch processing model. In the past, the main concern of the biopharmaceutical industry

was the regulatory authorities' definition of a batch, but the FDA and European Medical Agency (EMEA) has defined a batch as a specific quantity of a drug that is intended to have uniform character and quality within specified limits, putting more emphasis on drugs meeting specific quality standards and less emphasis on the means of production. (Jungbauer, 2013) Therefore, the bio-products industry should be pursuing integrated continuous biomanufacturing platforms as these methods have been shown to reduce costs (net present value) by 55% relative to conventional batch processing. (Zydney, 2015) However, the bio-product industry is still reluctant to pursue integrated continuous biomanufacturing platforms for intracellular products because of the supposed costs of the downstream processing and because the intracellular cell culture is difficult to maintain continuously, specifically due to the bottlenecks in the normally batch-wise separation processes, mainly chromatography. An article published by Cachumba et al. (2016), stated an optimized extraction and purification train has been estimated to save 50-80% of the total production cost. (Cachumba et al., 2016) Therefore, a simultaneous process synthesis method is needed to evaluate multiple process technologies and design alternatives in a processing network that converts raw materials into high-value intracellular products which could be paired with a superstructure optimization algorithm to determine an optimal process.

II.II Superstructure Optimization

Process synthesis deals with the selection of the topology of a process out of various options. Researchers have proposed three different types of methods to solve a process synthesis problem: 1) heuristics- or knowledge-based, 2) mathematical modeling

or programming, and 3) hybrid methods. Mathematical modeling is used to solve process synthesis problems using a systematic method in various studies by Grossman, Kravanja and Yeomans. (Grossmann, 1985; Kravanja and Grossmann, 1997; Yeomans and Grossmann, 1999) When the synthesis problem is setup mathematically as an optimization problem, shown in figure 2 below, Floudas (1995) discusses different solution methods and algorithms. (Floudas, 1995)

$$\begin{cases} \min\limits_{x,y} f(x,y) \\ \text{s.t. } h(x,y) \\ \quad\quad g(x,y) \\ \quad\quad x \in X \leq R^n \\ \quad\quad y \in Y = \{0,1\}^l \end{cases}$$

Figure 2 – Mathematically defined process synthesis problem, reprinted from Floudas, 1995

In figure 2, continuous variables are input as a vector in variable x and binary variables are input as a vector in variable y. The first line in figure 2 is the objective function which is set up as a minimization. Lines two through five are constraints which are used to constrain the feasible region in which the objective function can search for the minimum point. These constraints stated in lines two through five of figure 2 can be defined as equalities or inequalities.

Superstructure-based optimization techniques have been developed to evaluate the design space and identify the optimal processing network, but very few have been applied to the bio-product space, especially intracellular products. (Yeomans and

Grossmann, 1999) Biochemical processes require the use of a simultaneous synthesis method to evaluate all of the economic trade-offs and interactions that are involved in the process synthesis and design. A generic framework for synthesis of biomass conversion processes which incorporates generic mathematical models and a software interface called Super-O was developed by Bertran et al. (2016, 2017), based on the framework initially proposed by Quaglia et al. (2012). (Bertran et al., 2016, 2017; Quaglia et al., 2012) The idea behind the framework proposed by Quaglia et al. (2012) was to develop an integrated business and engineering framework that also accepts generic processing models based on the processing intervals. The generic processing model is shown below.

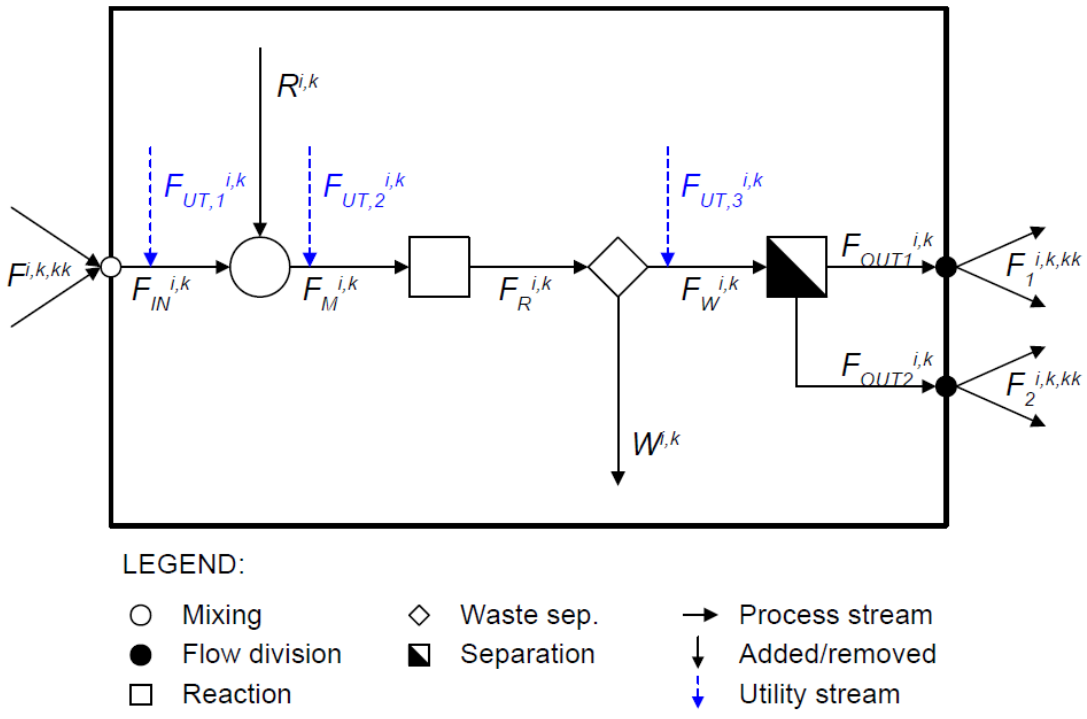

Figure 3 – Generic Process Intervals Schematic with internal variables, reprinted from Bertran et al., 2016

The generic process interval structure shown in figure 3 has five major processing tasks: 1) chemical mixing, 2) reaction, 3) waste separation, 4) product separation, and 5) utility consumption. The chemical mixing is shown in figure 3 as the larger circle and has two inputs and one output. The amount of chemicals that are added to the system, $R^{i,k}$, is based on a user defined chemical addition fraction, $\mu^{i,ii,kk}$, which is multiplied by the total amount of chemicals coming into the chemical mixing processing task,$F_{in}^{i,k}$ which is shown in equation 1 below. (Bertran et al., 2016)

$$R^{i,kk} = \sum_{ii} \mu^{i,ii,kk} F_{in}^{ii,kk} \tag{1}$$

The chemical addition flow rate, $R^{i,kk}$, calculated in equation 1 is added to the amount of the chemicals coming into the chemical mixing processing task, $F_{in}^{i,k}$, to give a mass balance around the chemical mixing processing step and input to the next processing task, $F_{M}^{i,kk}$. The next processing task is a reaction task which is shown in figure 3 as a blank square with one input and one output. The mathematical model for the reaction processing task is shown below:

$$F_{R}^{i,kk} = F_{M}^{i,kk} + \sum_{rr,react} F_{M}^{react,kk} \theta^{react,kk,rr} \frac{\gamma^{i,kk,rr}}{\gamma^{i,kk,react}} \frac{MW^{i}}{MW^{react}} \tag{2}$$

Equation 2 comes from Bertran et. al (2016) and is based on a stoichiometric reaction equation from Biegler, Grossman and Westerberg (1997). (Bertran et al., 2016; Biegler et al., 1997) For this model, $\theta^{react,kk,rr}$is the fraction of conversion based on the limiting reactant, and $\gamma^{i,kk,rr}$is the stoichiometry of the chemical reaction. Both of these terms are user defined. Following the reaction processing task is the waste separation

processing task which is shown in figure 3 as a diamond. The mathematical model for this processing task has two equations: 1) mass balance and 2) waste flow rate. The equation that determines the waste flow rate from Bertran et. al (2016) is shown below in equation 3: (Bertran et al., 2016)

$$F_W^{i,kk} = F_R^{i,kk}\left(1 - \delta^{i,kk}\right) \tag{3}$$

In equation 3, the user defines a split fraction,$\delta^{i,kk}$, which has to be less than 1 and multiplied by the reactor effluent to equal the waste flow rate. The mass balance around the waste separation processing task has one input, reactor effluent, and two outputs: 1) waste flow rate and 2) waste separation effluent. The product separation processing task is setup the same way as the waste separation processing task in which the user defines a split fraction which will determine how much goes to the primary outlet and how much goes to the secondary outlet. This equation is shown below:

$$F_{OUT1}^{i,kk} = F_W^{i,kk}\sigma^{i,kk} \tag{4}$$

Equation 4 is used to determine how much flow rate goes to the primary outlet. The user defined split fraction defined in equation 4, $\sigma^{i,kk}$, has to be less than or equal to 1. The flow rate of the secondary outlet is calculated from the product separation processing task mass balance, which has one inlet, waste separation effluent, and two outlets, 1) primary flow rate, $F_{OUT1}^{i,kk}$, and 2) secondary flow rate, $F_{OUT2}^{i,kk}$. The final processing task is the consumption of utilities which is calculated in three locations: 1) influent of the chemical mixing processing task, $F_{UT,1}^{ut,kk}$, 2) effluent of the chemical mixing processing task, $F_{UT,2}^{ut,kk}$, and 3) effluent of the waste separation processing task,

$F_{UT,3}^{ut,kk}$, as shown in figure 3. The mathematical model of the consumption of utilities for the effluent of the chemical mixing processing task is shown below:

$$F_{UT,2}^{ut,kk} = \beta_2^{ut,kk} \sum_{ii} F_{in}^{ii,kk} \tag{5}$$

As seen in equation 5, the utility consumption is based on the total flowrate of the selected stream, effluent or influent. The utility consumption factor, $\beta_2^{ut,kk}$, can have multiple utilities for any one given stream and is similar to the split fraction, because the user defines the amount of utility that is added in relation to the total flow rate of the selected streams processing task.

These generic processing tasks inside the integrated business and engineering framework has been adapted in this work for the synthesis of process flowsheets in the production of intracellular compounds, integrating literature, experimental results, and simulation data. By integrating a feedback loop that uses targeted data collection, this method helps to overcome the limitations of data collection for different processing steps. The generic mathematical framework allows for adaptation of scientific literature or lab-scale experiments to design a preliminary flowsheet which can be further analyzed in the software interface. The applicability of this framework is demonstrated using the production of the high-value intracellular product beta-carotene as a case study.

II.III Beta-Carotene Relevance

Beta-carotene is a naturally occurring orange pigment that can be found in many plants like carrots and peppers, as well as a select few bacteria or fungal species. There

are two major applications of beta-carotene, 1) colorant for food products and 2) antioxidant and cancer prevention agent in supplements. Other minor applications include 1) fertility increasing agents in the farming industry and 2) natural bronzing agent and pro-vitamin A source in cosmetics. (Marz, 2015) For these applications, there are three methods to produce beta-carotene, 1) synthetically, 2) natural product extraction and 3) fermentation. Because of the complex structure of beta-carotene, the synthetic pathway is complex and with a yield of only 60% from Roche and 85% from BASF with an extensive recovery process. (Ribeiro et al., 2011) Natural product extraction of beta-carotene is usually performed on vegetables, like palm oil, but uses harsh chemicals, like acetone, which then need to be subsequently removed before human consumption. (Rodriguez-Amaya, 2001) The natural form of beta-carotene has been shown to have a greater antioxidant activity versus the synthetic counterpart in studies published by Britton and Stahl and Sies due to an increased fat solubility of natural beta-carotene. (Britton, 1995; Stahl and Sies, 2005) However, natural beta-carotene is only found in micrograms per gram in natural products versus milligrams per gram in yeast and algae which will decrease the processing costs due to higher concentrations. For these reasons, *Saccharomyces cerevisiae* is an excellent example organism. (Ribeiro et al., 2011) The market price for natural beta-carotene is significantly higher than the synthetic beta-carotene which makes it a model compound for analyzing the profitability of downstream separation in bioprocessing. (Marz, 2015)

A case study of the bio-manufacturing of the intracellular molecule beta-carotene using a recombinant strain of *Saccharomyces cerevisiae*, or baker's yeast, is used to

exemplify this process synthesis framework. This strain, named SM14, has been optimized to increase the yield of beta-carotene per gram dry cell weight by three times compared to wild type S. cerevisiae through chromosomal integration and adaptive evolution. (Olson et al., 2016; Reyes et al., 2014) The natural form of beta-carotene has been shown to have a greater antioxidant properties when ingesting beta-carotene, therefore the synthetically produced molecule is significantly cheaper when compared to its naturally produced counterpart. (Raftery et al., 2017)

II.IV Bio-Manufacturing of Beta-Carotene Processing Intervals

The five steps proposed in this work for novel process synthesis are: (1) problem formulation, (2) data collection and superstructure generation, (3) solution of the optimization problem, and (4) process parameter analysis and (5) experimentation with informed design and then determination of the optimal process design. The second step, data collection and superstructure generation, is essential for trusting the results given by process synthesis optimization. In section II.II, Superstructure Optimization, there are five main processing tasks: 1) chemical mixing, 2) reaction, 3) waste separation, 4) product separation, and 5) utility consumption which describe all processing intervals in the case study of bio-manufacturing beta-carotene. This section will break down each process interval into the five main processing tasks describing the where the data was collected and the assumptions made to fit into each process interval. Table 1below breaks down the case study of bio-manufacturing beta-carotene into individual processing steps, which are composed of processing intervals. Each processing interval contains the five processing tasks that are described in section II.II.

Table 1 – Summary of Process Intervals with Processing Tasks

Section #	Step	Interval	Processing Tasks			
			Reaction	Waste	ProdSep	Chem Add
II.IV.I	RM	RM-GLU				
II.IV.II	FERM	FERM-1	X	X		X
II.IV.III	CHARV	CHARV-CENT		X		
II.IV.III	CHARV	CHARV-MF		X		
II.IV.III	CHARV2	CHARV2-CENT		X		
II.IV.III	CHARV2	CHARV2-MF		X		
II.IV.IV	DISR	DISR-BMILL	X			
II.IV.IV	DISR	DISR-HOMO	X			
II.IV.V	SOLV	SOLV-DOD		X		X
II.IV.V	SOLV	SOLV-HEX		X		X
II.IV.V	SOLV	SOLV-DEE		X		X
II.IV.V	SOLV	SOLV-ETAC		X		X
II.IV.V	SOLV	SOLV-CYHX		X		X
II.IV.V	SOLV	SOLV-TOL		X		X
II.IV.VI	BMFL	BMFL-DCT-SN		X		X
II.IV.VI	BMFL	BMFL-DCT-NI		X		X
II.IV.VII	CRY	CRY-1		X		
II.IV.VIII	ETH	ETH-WSH		X		X
II.IV.IX	PROD	PROD-BC				

II.IV.I Chemical Added and Raw Materials

Materials that are initialized in the first processing step are called ‘raw materials.’ In the case study of bio-manufacturing beta-carotene, the only raw material is glucose. Chemicals that are added during the chemical addition processing task have a special distinction. In table 2 shown below, all of the chemicals added and raw materials as part of this case study are shown.

Table 2 – List of Chemicals Added and Raw Materials

Compound	Chemical Added	Cost 2017 ($/kg)	Reference
Cyclohexane	X	$ 0.82	(Chang, 2006)
Diethyl Ether	X	$ 1.75	(Chang, 2006)
Dodecane	X	$ 14.08	(Chen et al., 2001)
Ethanol	X	$ 0.67	(Chang, 2006)
Ethyl Acetate	X	$ 1.50	(Chang, 2006)
Glucose		$ 0.23	(Korovessi and Linninger, 2005)
Hexane	X	$ 0.51	(Chang, 2006)
Nickel	X	$ 25.43	(Chang, 2006)
$SnCl_4$	X	$ 10.65	(Chang, 2006)
Toluene	X	$ 1.13	(Chang, 2006)
WFI	X	$ 0.02	(Harrison et al., 2015)
		$ 0.24	(Harrison et al., 2015)

Water for injection (WFI) is an ultra-purified water that is used in this bioprocess for the media in the bioreactor for the *Saccharomyces cerevisiae* to grow. In Harrison et. al (2015) the price for water for injection (WFI) is an order of magnitude difference because of the different compositions of the potable water and process technologies. This huge price variance in prices difference heavily contributes to the chemical cost of the bio-manufacturing case study which is why we did a process parameter analysis around the water cost using a high and low cost for WFI.

II.IV.II Fermenter

The first processing step is the fermenter that uses *Saccharomyces cerevisiae* strain SM14 to convert glucose to beta-carotene, biomass, acetic acid, ethanol, and gaseous carbon dioxide, in which the conversion is a reaction processing task. In this processing interval, there is also a chemical mixing processing task of adding 79 times the amount of WFI to the glucose flow rate. There is also a reaction processing task,

which uses a stoichiometric reaction for the processing interval which was adopted from Raftery et al. (2017) for a continuous bioreactor. (Raftery et al., 2017) It is assumed that the carbon dioxide product is gaseous and vented from the reactor during the fermentation process and is not considered in the downstream processing.

II.IV.III Cell Harvesting

Cell harvesting is the process of reducing the amount of water in cell broth, therefore increasing the cell concentration for the following process steps. The waste separation processing step is used in both of the cell harvesting steps and intervals for the separation of the liquid waste from the cell broth. As seen in table 1, cell harvesting is broken up into two sections for the purposes of the mathematical model because of the influent constraints of the cell disruption processing step, which will be discussed in the next section. In this bio-manufacturing case study, the two process technologies that are evaluated are centrifugation and cross-flow microfiltration. Centrifugation uses centripetal force to separate products based on density. In this work, we made the assumption that the *Saccharomyces cerevisiae* would not deteriorate during centrifugation because of the strong cell walls of the *Saccharomyces cerevisiae.* (Mohn, 1988) Cross-flow microfiltration increases the concentration of *Saccharomyces cerevisiae* by removing water from the mother liquor via the permeate of the microfiltration membrane. We made the assumption of no concentration polarization, which states the cells won't stick to the membrane if the flux of the membrane is kept below a critical flux. (Gerardo et al., 2015; Kwon et al., 2000) Therefore, both process technologies don't result in any loss of biomass.

II.IV.IV Cell Disruption

For intracellular products, like beta-carotene in this bio-manufacturing case study, the cell needs to be broken to access the product. Bypassing the cell disruption step results in very low yields, especially when dealing with cells that have a cell wall. The two process technologies that are identified for cell disruption are homogenization and bead milling. Homogenization is the process of pressurizing a fluid through a small orifice. Bead milling uses many beads inside of a drum that is rotated perpendicularly to the flow of fluid to break or grind the solid-liquid slurry. The solids concentration is limited to 5% percent for the homogenizer, but results in a 95% disruption of the yeast cells. (Lovitt and Coss, n.d.) On the other hand, the bead mill requires a solids concentration of at least 40% with a resulting 98% disruption of yeast cells. (Kula and Schütte, 1987) The solids concentration differences in the influent flow to the cell disruption lead to the two step cell harvesting, where the first cell harvesting stage went to 5% solids concentration and the second cell harvesting stage went to 40% solids concentration.

II.IV.V Solvent Addition

After the cell disruption there are two processing pathways considered: (1) extraction from disrupted cells or (2) direct extraction from undisrupted cells. Both pathways have six different solvent addition processing steps which are comprised of two processing tasks, chemical mixing and waste separation. The chemical processing task has a user defined input which is the chemical addition fraction for each solvent and the waste separation processing task has a user defined input which is the waste

separation fractions for each solvent. These user defined inputs for the solvent processing step are specific to the cell and product type. After conducting a literature review there is no reliable data available for the extraction of beta-carotene from *Saccharomyces cerevisiae*. Therefore, in this case study, we determined these user defined inputs by experimentation using disrupted and undisrupted *Saccharomyces cerevisiae* (SM 14) cell lines, which will be discussed in section IV.

Table 3 – Beta-Carotene Solvent Solubility and Hydrophobicity

Solvents	Solubility Beta-Carotene (mg/L)	Is solvent soluble in water?	Reference
Dodecane	n/a	No	(National Center for Biotechnology Information., n.d.)
Hexane	600	No	(National Center for Biotechnology Information., n.d.)
Diethyl Ether	1000	Slightly	(National Center for Biotechnology Information, n.d.)
Ethyl Acetate	500	Slightly	(National Center for Biotechnology Information., n.d.)
Cyclohexane	2000	No	(National Center for Biotechnology Information, n.d.)
Toluene	4000	No	(National Center for Biotechnology Information., n.d.)

The following solvents were selected due to their high solubility of crystalline beta-carotene and hydrophobic nature: (1) dodecane, (2) hexane, (3) diethyl ether, (4) ethyl acetate, (5) cyclohexane, and (6) toluene. As seen in table 3, the solubility of beta-carotene varies which leads to beta-carotene being disposed of in the waste stream. The hydrophobic nature will help with the solids removal and two phase separation of the later processing steps.

II.IV.VI Solids Removal

In the solids removal step, the flocculating agent is added to the system to initiate settling of the disrupted and undisrupted biomass to the bottom of the decanter. Therefore in this processing step, there are two processing tasks: 1) chemical addition and 2) waste separation. The first option for solids removal was using tin (IV) chloride ($SnCl_4$) as a flocculent which would be added in the chemical addition processing task. A study by Nishihara et. al (1982) used tin (IV) chloride to flocculate complete yeast cells and disrupted yeast cell walls in the presence of salt which is a common ingredient of cell media solution. (Nishihara et al., 1982) The second option for solids removal was using nickel (Ni) powder as a flocculating agent in the chemical addition processing task. Weeks et.al (1983) published a study which flocculated undisrupted *Saccharomyces cerevisiae* where the temperature and pH had little effect. (Weeks et al., 1983) In this case study, we assume that disrupted and undisrupted *Saccharomyces cerevisiae* will act the same way in the presence of nickel powder. The overarching assumptions for this processing step is that the tin (IV) chloride and nickel powder aren't effected by the presence of organic solvents and that all hydrophilic and flocculated cellular components settle into the heavier aqueous phase, which is sent to waste, while the organic, extracted, hydrophobic beta-carotene goes into the organic phase. The organic phase with the beta-carotene product is then sent to the next processing step.

II.IV.VII Crystallization

Since the aqueous phase has been removed, the beta-carotene has to be separated from the organic solvents. The crystallization processing step uses vacuum evaporation

to perform the separation. In this step, the waste separation processing task is used to dispose or recycle the spent solvent. At first, the economics of this process were not viable with disposal of the solvent because the extraction yield of beta-carotene is extremely low. Recycling was implemented during the process parameter analysis, which will be discussed in section V. In this case study, we assumed that none of the beta-carotene will travel with the solvent in the waste stream and that there are no interactions between the solvent and beta-carotene. Based on vendor calculation, we assume that vacuum evaporation processes use about 170 KWh per cubic meter. ("The basis of vacuum evaporation - Environmental engineering," 2015)

II.IV.VIII Ethanol Wash

The ethanol wash processing step was implemented to remove all residual solvents for the preparation for human consumption. Based on the recommendation of Atkinson and Mavituna (1991), we added 4 grams of ethanol per gram of beta-carotene to remove residual solvents. (Atkinson and Mavituna, 1991) We assume all residual solvents are removed in this step.

II.IV.IX Product

Since this case study only has one product, beta-carotene, there is only one product processing interval. The price of bio-manufactured beta-carotene is $2,065.97 per kilogram which comes from Caswell and Zilberman. (Caswell and Zilberman, 2001)

III. SUPER-O METHODS*

The framework developed in this thesis uses an iterative approach between experimentation and superstructure optimization to solve the synthesis problem under the constraint of limited availability of reliable data. The iterative framework consists of five steps: (1) problem formulation, (2) data collection and superstructure generation, (3) determination of the optimal process topology, and (4) experimentation with informed design and (5) process parameter analysis and determination of the optimal process design. The flowsheet for this iterative framework is shown in Figure 4.

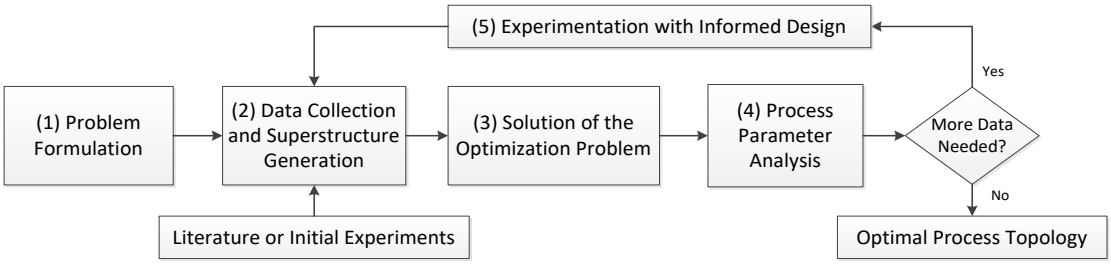

Figure 4 – Flow diagram for the iterative methodology for solving the process synthesis problem under the constraint of limited availability of reliable data.

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III.I Step 1: Problem Formulation

The objective of this step is to define the process synthesis problem that will be solved by specifying the following characteristics: the set of raw materials, the set of products, the set of locations, the set of processing steps, and the set of technologies. Based on the characteristics of the problem, each problem can be put into five different categories shown below.

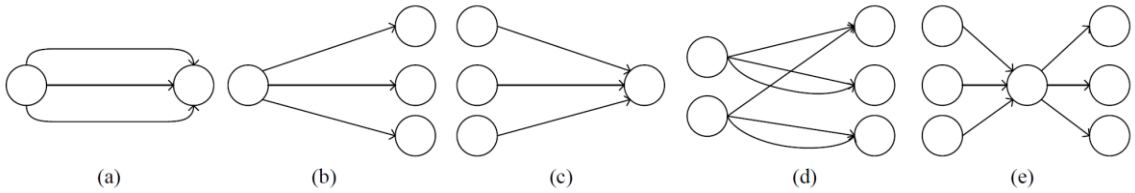


Figure 5 – Different problem types in network optimization problems: (a) route selection, (b) product selection, (c) raw material selection, (d) simultaneous raw material, route and product selection, and (e) raw material and product selection via intermediate, reprinted from Bertran et al., 2016

Figure 5 displays the five different types of network optimization problems: (a) route selection, (b) product selection, (c) raw material selection, (d) simultaneous raw material, route and product selection, and (e) raw material and product selection via intermediate. In problem type (a), route selection, there are a number of alternative processing routes, but the raw material(s) and product(s) are specified. In problem type (b), raw material selection, the raw material and route to product are fixed, but there are multiple different products. On the other hand, raw material selection or problem type (c) has multiple raw materials, but the route to a single product is fixed. Problem type (d) is the most complex because it is solving a simultaneous raw material, route and product selection based on the optimization algorithm. In problem type (e), raw material and

product selection via intermediate, the route is specified, but the raw materials and product are not. The case study for the bio-manufacturing of beta-carotene is a problem type (a) because we know the raw material, glucose, and the product, beta-carotene.

III.II Step 2: Data Collection and Superstructure Generation

The purpose of this step is to analyze the problem defined in Step 1 to determine and collect all necessary data, and then generate a superstructure of possible alternatives. Data can be collected from literature (online databases, academic literature or industrial partners) or generated through simulation software, i.e. Aspen Plus, SuperPro Designer, and SolventPro. When estimation is not possible, data can be initially generated experimentally through designing experiments that will specifically fit the requirements for that alternative material, route or technology within the confines of the synthesis framework. Once the data is collected it can be stored in a database for future use and a process superstructure can be generated.

III.III Step 3: Determination of the Optimal Process Topology

The generated superstructure can now be utilized with the user interface Super-O to solve the synthesis problem. The processing alternatives that are represented in this superstructure encompass what is being considered for this process synthesis problem. The synthesis problem is solved by entering the necessary superstructure and process data into the Super-O user-interface which exports the data into a generic process model which is saved by Super-O automatically as a .csv file. This file then can be read by GAMS and solved using MIP/MINLP optimization solvers. Then GAMS saves the output as a .csv file that Super-O can read to give the user the optimal process topology.

III.IV Step 4: Experimentation with Informed dDesign

Data generated from the optimal synthesis problem can be analyzed to determined areas where more accurate data may be needed. This is then used to develop and conduct informed experiments to generate more accurate and reliable data. This data is incorporated into Step 2 of the framework and the process synthesis problem is optimized again with a higher level of reliability.

III.V Step 5: Process Parameter Analysis and Determination of the Optimal Process Design

Once a sufficient level of data reliability has been reached, a process parameter analysis can be performed directly in the Super-O user interface to (i) understand the effect of external variation of parameters and (ii) identify the key process parameters, parameters that greatly affect the output of the system. Experimental data can be used to determine the sensitivity limits on key process parameters. Once the process parameter analysis is performed, the optimal process design is determined.

IV. EXPERIMENTAL MATERIALS AND METHODS

As discussed in section II.IV.V, there is no reliable data for the extraction of beta-carotene from *Saccharomyces cerevisiae*. Therefore for this case study, we conducted two experiments to get the necessary extraction data: 1) bioreactor harvesting of *Saccharomyces cerevisiae* (SM 14), and 2) solvent extraction with disrupted and undisrupted *Saccharomyces cerevisiae* (SM 14) using different amounts of solvents. The beta-carotene extracted from *Saccharomyces cerevisiae* (SM 14) was quantified using a colorimetric spectrophotometry analysis with a previously calibrated assay using pure beta-carotene.

IV.I Experiment Materials

IV.I.I Bioreactor Harvesting of Saccharomyces cerevisiae (SM 14)

The *Saccharomyces cerevisiae* (SM 14) cell with the intracellular beta-carotene was harvested from a 7 Liter autoclavable bioreactor (Applikon, Foster City). From the 7L bioreactor, there is approximately three liters of working volume which was transferred to approximately sixty 50 mL centrifuge tubes to be used for all of the extraction experiments. The sixty centrifuge tubes were frozen to prevent deterioration of the SM14 cells. The procedure for running the bioreactor was discussed in Jaladi's thesis (2016). (Jaladi, 2016)

IV.I.II Solvent extraction with Disrupted and Undisrupted SM 14 Cells

The solvents extraction experiments using disrupted and undisrupted SM 14 cell were used to create find the user defined inputs for the chemical mixing processing task

and the waste separation processing task in the solvent addition processing step. The user defined inputs are the chemical addition fraction and waste separation fractions for each solvent. The solvents that were tested are dodecane, diethyl ether, hexane, cyclohexane, toluene, and ethyl acetate because of the solubility and hydrophobicity of beta-carotene. This experiment analyzed the amount the carotenoids extracted before and after disruption of the cell using different amounts of solvents. The final concentration of beta-carotene was determined through spectrophotometry through a previously calibrated assay using pure beta-carotene and will be discussed in section IV.II.

IV.I.II.I Solvent Extraction Procedure with Undisrupted SM 14 Cells

The extraction of beta-carotene using six different solvents from undisrupted *Saccharomyces cerevisiae* (SM 14) will be discussed first. One 50 mL centrifuge tube was taken out of the freezer and left at room temperature for one hour to defrost. The samples of culture broth were not defrosted more than once because the cyclical process of defrosting and re-freezing weakened the cell membrane and wall. Once defrosted, the samples were vortexed until well mixed. Then 500µL of the well-mixed culture broth was collected from the centrifuge tube and transferred to a 2 mL o-ring tube with a cap. The o-ring tube was centrifuged for 1 min at 15,000 rpm to form a pellet at the bottom of the o-ring tube. Next, the supernatant was removed from each o-ring tube by aspiration using a vacuum pump without disturbing the small pellet at the bottom of the o-ring tube which contains the SM 14 cells. The next step is the solvent addition step. For this experiment we tested these five amounts of solvents for each of the six different solvents

without disrupting the cell wall: 1) 500 uL, 2) 750 uL, 3) 1 mL, 4) 1.25 mL, and 5) 1.5 mL. The o-ring tubes were placed in the Disruptor Genie ® Cell Disruptor Homogenizer from Scientific Industries for two 6 minute intervals. Disruptor Genie ® is a device that simultaneously agitates and vortex's at high speeds, but without beads the disruption occurs from the cells hitting the wall and each other, therefore it doesn't act as bead mill or homogenizer. After 12 minutes of disruption, the samples were centrifuged for 1 min. If an orange colored cell pellet still remained, the o-ring tubes were placed back on the Disruptor Genie ® again for 12 minutes and centrifuged again for 1 min. After disruption, 200 µL of the beta-carotene cell extract, which is the supernatant in the o-ring tube, was placed into a well on a clear bottom 96 well polypropylene plate. A blank of the pure corresponding solvent was also added to a well on the clear bottom 96 well polypropylene plate.

IV.I.II.II Solvent Extraction Procedure with Disrupted SM 14 Cells

The extraction of beta-carotene from disrupted *Saccharomyces cerevisiae* (SM 14) using six different solvents is discussed. One of the 50 mL centrifuge tubes was taken out of the freezer and left at room temperature for one hour to defrost. The samples of culture broth were not defrosted more than once because the cyclical process of defrosting and re-freezing weakened the cell wall. Once defrosted, the samples were vortexed until well mixed. Then 500µL of the well mixed culture broth was collected from the centrifuge tube and transferred to a 2 mL o-ring tube with a cap. The o-ring tube was centrifuged for 1 min at 15,000 rpm to form a pellet at the bottom of the o-ring tube. Next, the supernatant was removed from each o-ring tube by aspiration using a

vacuum pump without disturbing the small pellet at the bottom of the o-ring tube which contains the SM 14 cells. Approximately 250µL of glass beads were added to the o-ring tube to aid in disrupting the cell membrane and wall. The solvent was then added to the o-ring tube. For this experiment we tested these five amounts of solvents for each of the six different solvents: 1) 500 uL, 2) 750 uL, 3) 1 mL, 4) 1.25 mL, and 5) 1.5 mL. The o-ring tubes were placed in the Disruptor Genie ® Cell Disruptor Homogenizer from Scientific Industries for two 6 minute intervals. After 12 minutes of disruption, the samples were centrifuged for 1 min. If an orange colored cell pellet still remained, the o-ring tubes were placed back on the Disruptor Genie ® for 12 minutes and centrifuged again for 1 min. After disruption, 200 µL of the beta-carotene cell extract, which is the supernatant in the o-ring tube, was placed into a well on a clear bottom 96 well polypropylene plate. A blank of the pure corresponding solvent was added to a well on the clear bottom 96 well polypropylene plate as well.

IV.II Methods for Analysis of Solvents

Once all of the aliquots of 200 µL of beta-carotene cell extract were placed in their respective wells on the clear bottom 96 well polypropylene plate, the plate was ready for the spectrophotometry assay. Spectrophotometry is a quantification method that measures how much light a chemical absorbs by determining the intensity of the light beam that passes through solution. For each beta-carotene and solvent combination there is a corresponding wavelength at which to check the absorbance.

Table 4 – Beta-Carotene and Solvent Corresponding Wavelengths

Solvent	Wavelength (nm)	Reference
Dodecane	454	(Craft and Soares, 1992)
Hexane	454	(Craft and Soares, 1992)
Diethyl ether	448	(Craft and Soares, 1992)
Cyclohexane	454	(Craft and Soares, 1992)
Toluene	462	(Craft and Soares, 1992)
Ethyl acetate	452	(Craft and Soares, 1992)

Therefore, as seen in table 4, the device which measures the wavelength needs to have the capability to measure wavelength between 448 nm and 462 nm. The Karim Group has access to two devices which were used for getting the absorbance reading: 1) TECAN Infinite M200 and 2) Molecular Devices Spectra Max Gemini XPS. Both of these devices can measure the absorbance of the beta-carotene cell extract from 0, the lowest absorbance or all of the light passed through, to 4, which means none of the light passed through and it was all absorbed. After the following procedures were run, the raw data generated is contained in an excel file. The raw data was then transferred to an excel worksheet created by Reyes et. al (2014) that converts the absorbance from the spectrophotometric analysis to concentration of beta-carotene in sample. (Reyes et al., 2014) These correlations came from a calibration curve which Reyes et. al (2014) created using pure crystalline beta-carotene at different concentration that was dissolved in dodecane. (Reyes et al., 2014)

IV.II.I TECAN Absorbance Procedure

After the samples were plated in the clear bottom 96 well plate, the absorbance was measured immediately on the TECAN Infinite M200. The wavelength range was adjusted to measure absorbance between 425 nm and 475 nm.

IV.II.II Molecular Devices Spectra Max Gemini XPS Absorbance Procedure

After the samples were plated in the clear bottom 96 well plate, if the absorbance was not measured on the TECAN Infinite M200, it was measured on the Spectra Max Gemini XPS plate reader. The wavelength preference was selected by pressing the setup button, then wavelengths, and then each wavelength was entered.

V. RESULTS AND DISCUSSION*

In this section, we will discuss the results from 1) the solvent extraction with disrupted and undisrupted *Saccharomyces cerevisiae* (SM 14) cells and 2) the superstructure optimization using Super-O.

V.I Solvent Extraction with Disrupted and Undisrupted *Saccharomyces cerevisiae* (SM 14) Cells

As discussed in section IV.II.II, two experiments were run using the *Saccharomyces cerevisiae* (SM 14) bioreactor broth shown in figure 6.

Figure 6 - *Saccharomyces cerevisiae* (SM 14) bioreactor broth

*Part of this section is reprinted with permission from "Separation and recovery of intracellular beta-carotene using a process synthesis framework" by Alexander M. Sabol, Maria-Ona Bertran, Jonathan P. Raftery, John M. Woodley, Rafiqul Gani, M. Nazmul Karim, 2017. Computer Aided Chemical Engineering, Vol. 40, 2851-2856, Copyright 2017 by Elsevier.

The orange color from the broth comes from the *Saccharomyces cerevisiae* (SM 14) cells producing beta-carotene which has an orange color as well. Therefore, an eye test can be done to tell if any beta-carotene was extracted by looking at the color of the extraction solvent. The first experiment tested direct extraction of beta-carotene from *Saccharomyces cerevisiae*.

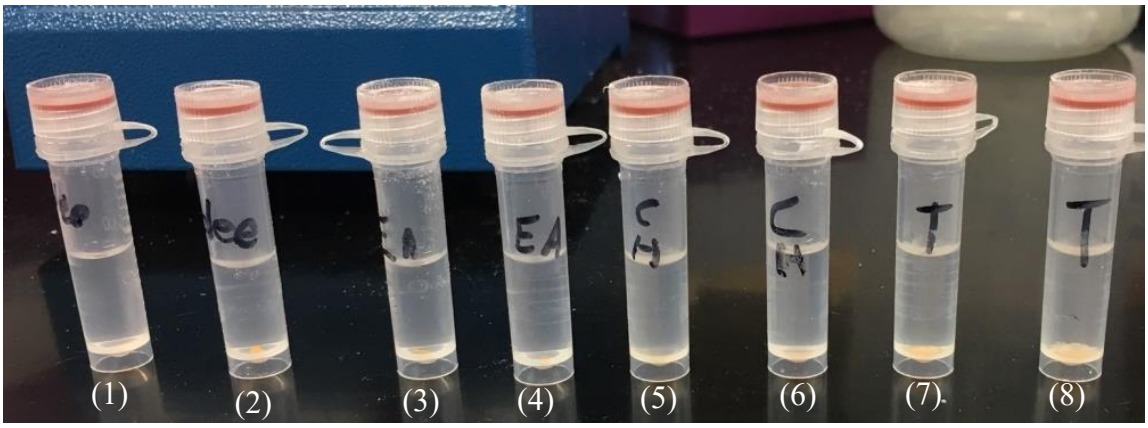


Figure 7 – Results from the Undisrupted Extraction test; (1) and (2) is diethyl ether; (3) and (4) is ethyl acetate; (5) and (6) is cyclohexane; (7) and (8) is toluene

The tubes shown in figure 7 were shaken vigorously using the Cell Genie ® for 24 minutes and after spinning down in the centrifuge, there was still an orange pellet on the bottom. Therefore, all of the solvent shown in figure 7 didn't extract any beta-carotene from undisrupted *Saccharomyces cerevisiae* (SM 14) cells. It should be noted that hexane and dodecane had similar results to the four solvents shown in figure 7 but no picture was taken. Therefore, it was determined that a negligible amount of beta-carotene could be recovered due to the durable cell wall of *Saccharomyces cerevisiae*.

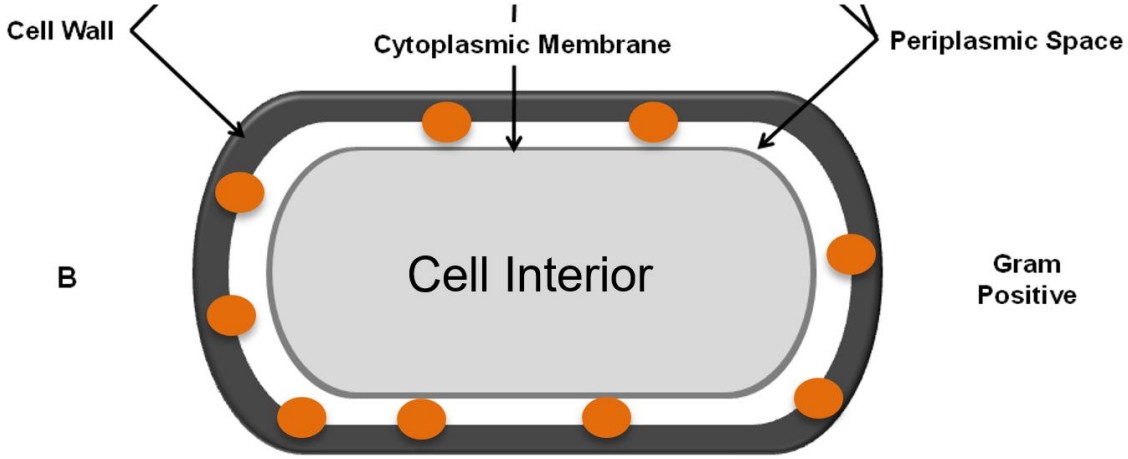


Figure 8 – Animated representation of where beta-carotene is located in *Saccharomyces cerevisiae (SM14)*

The orange dots shown in figure 8 represent beta-carotene and how it collects in between the cell membrane and cell wall. The location of the beta-carotene in between the cell membrane and cell wall is due to the hydrophobic structure of the molecule beta-carotene. The cell interior is comprised primarily of water, whereas the cell wall and membrane is composed of lipids which have the hydrophobic heads to the outside and hydrophilic tails to the inside. The strength of the cell wall coupled with the location of the beta-carotene lead to no extraction of beta-carotene from the undisrupted *Saccharomyces cerevisiae.* Therefore, these results removed the direct extraction approach from consideration in the process superstructure discussed in section V.II.

In the second set of experiments, the *Saccharomyces cerevisiae* was disrupted and then the beta-carotene was extracted using six different solvents. In this experiment, we also tested the maximum amount of beta-carotene that can be extracted from 500 µL of fermentation broth, which is shown in figure 6. The procedure to test the maximum

amount of beta-carotene that could be extracted using the five different solvent amounts and six different solvents listed in section IV.II.II.

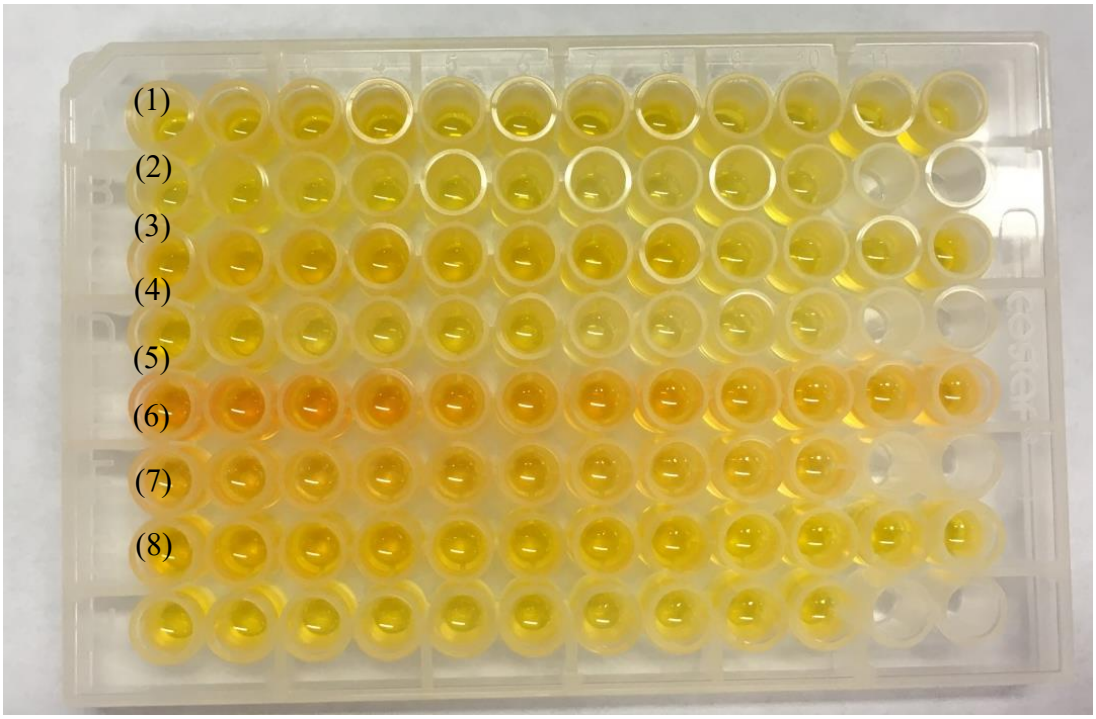


Figure 9 – Results from the Disrupted Extraction test; (1) and (2) is ethyl acetate; (3) and (4) is dodecane; (5) and (6) is toluene; (7) and (8) is cyclohexane

Figure 9 shows four of the six solvents that were tested for the maximum beta-carotene extraction using a colorimetric assay at these solvents amounts: 1) 0.50 mL, 2) 0.75 mL, 3) 1.00 mL, 4) 1.25 mL, and 5) 1.50 mL. It can be assumed that hexane and diethyl ether had a similar yellowish hue. As seen in figure 9, toluene, in rows number five and six, have more of an orange hue then the rest of the results from the disruption extraction test, therefore it can be assumed it extracted the most beta-carotene before doing the colorimetric assay. The maximum amount of beta-carotene extracted was

found to be approximately 47 mg/L-solvent using 1.50 mL of toluene or a total of 0.071 mg of beta-carotene extracted. The maximum amount of beta-carotene extracted using toluene was assumed to be the maximum amount of beta-carotene that could be extracted from the *Saccharomyces cerevisiae*, therefore it is the denominator in determining the relative extraction between solvents.

$$Relative\ Extraction = \frac{mg\ of\ beta - carotene\ in\ solvent\ x\ at\ 1.5\ mL}{mg\ of\ beta - carotene\ in\ toluene\ at\ 1.5\ mL} \quad (6)$$

As seen in equation 6, the numerator varies by both type of solvent and amount of solvent. This equation was used as the y axis for figure 10 shown below.

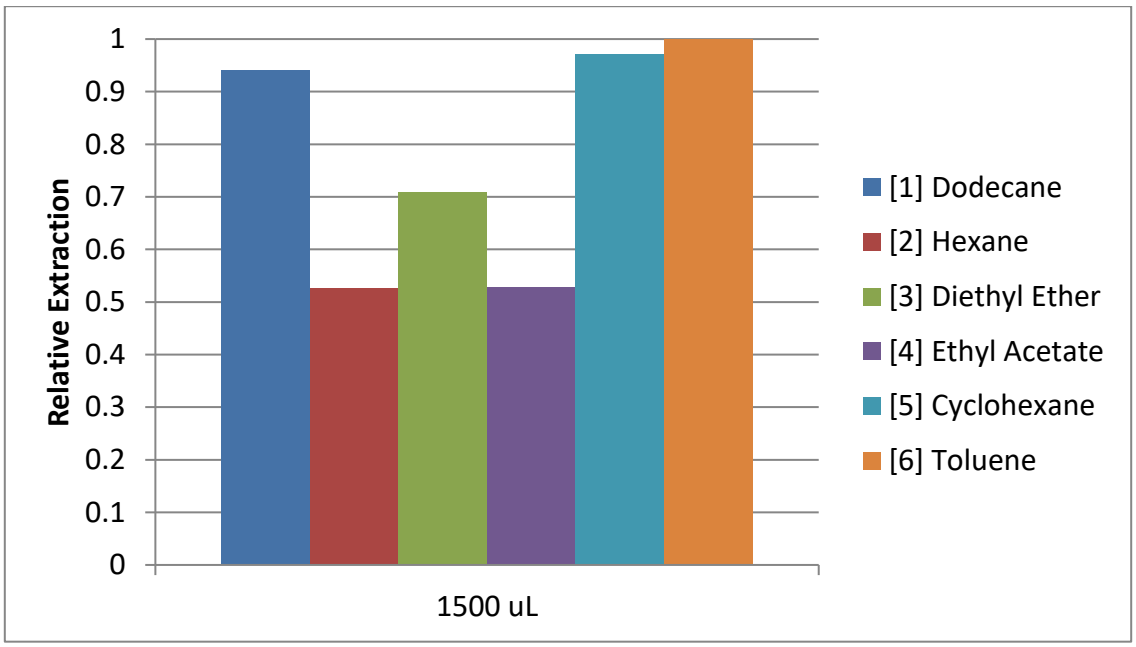


Figure 10 – Summary of the Relative Extraction for Different Solvents at 1.5 mL of solvent and 0.5 mL of broth

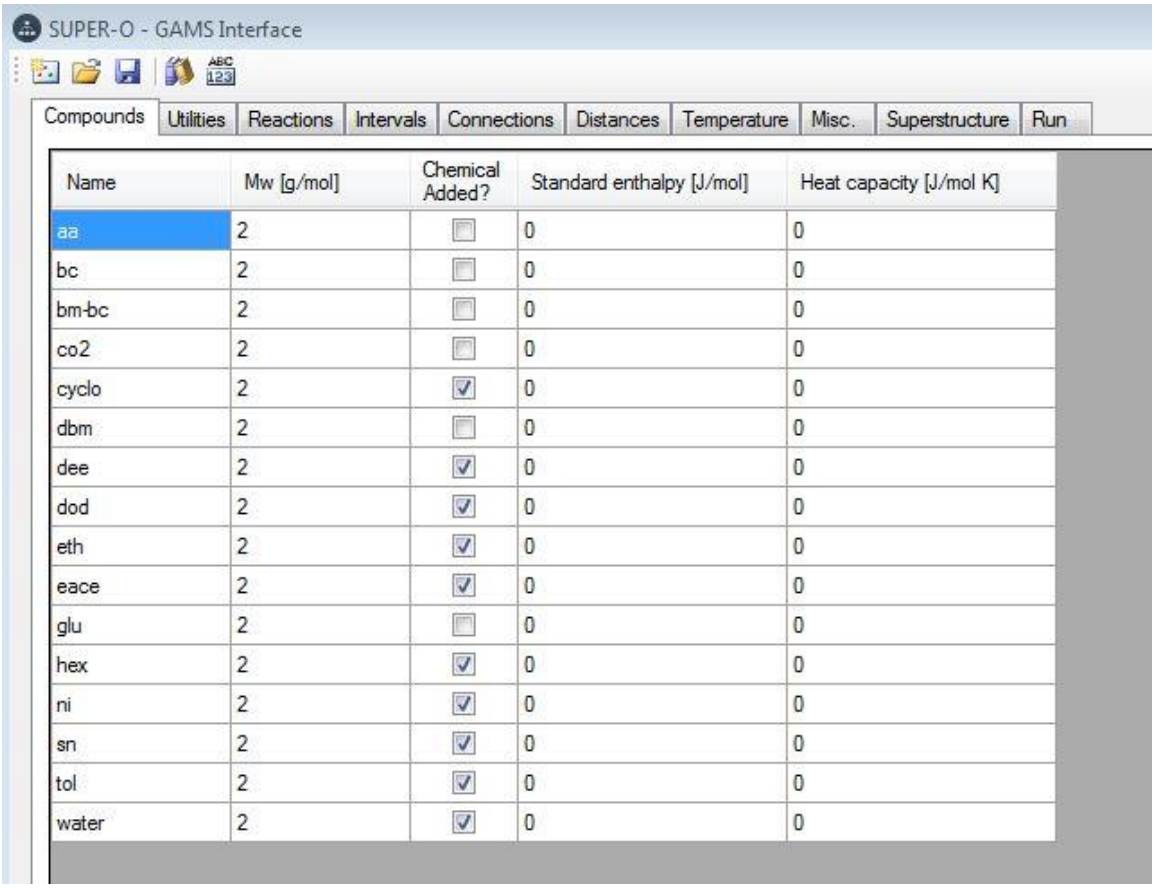
The experiment that was explained in section IV.II.II.II used five different amounts on solvents: 1) 0.50 mL, 2) 0.75 mL, 3) 1.00 mL, 4) 1.25 mL, and 5) 1.50 mL.

The results displayed in figure 10 were only for 1.50 mL of each of the six different solvents because the largest volume of solvent extracts the highest amount of beta-carotene from 0.50 mL of cell culture broth. This result was expected because the amount of beta-carotene that can be put into a solvent will increase until a max solubility is reached, which we didn't hit. For the relative extraction calculated in figure 10 using equation 6, we assumed that toluene extracted all of the available beta-carotene. This was a good assumption because toluene had the highest beta-carotene solubility. (National Center for Biotechnology Information., n.d.) The relative extraction numbers shown in figure 10 were used in Super-O as the waste separation fraction because all of the available beta-carotene couldn't be extracted from dodecane, hexane, diethyl ether, ethyl acetate and cyclohexane.

V.II Superstructure Optimization using Super-O

The final step in the process of solving the process synthesis problem for the beta-carotene case study was to enter all of the relevant data into the Super-O. The Super-O interface is broken into 10 different tabs. When starting a new project one must know the number of steps (processing intervals), number of compounds (raw materials, products and chemical added), number of utilities and number of reactions. For this project, we had 10 steps, 16 compounds, 1 utility and 2 reactions. The 10 steps, which have been discussed in detail in section II.IV as processing intervals, are composed of raw materials (RM), fermentation (FERM), cell harvesting (CHARV), cell harvesting part 2 (CHARV2), disruption (DISR), solvent addition (SOLV), biomass filtering

(BMFIL), crystallization (CRY), ethanol wash (ETH), and products (PROD). The next step was to enter all of the chemicals into the Super-O interface.



Name	Mw [g/mol]	Chemical Added?	Standard enthalpy [J/mol]	Heat capacity [J/mol K]
aa	2	☐	0	0
bc	2	☐	0	0
bm-bc	2	☐	0	0
co2	2	☐	0	0
cyclo	2	☑	0	0
dbm	2	☐	0	0
dee	2	☑	0	0
dod	2	☑	0	0
eth	2	☑	0	0
eace	2	☑	0	0
glu	2	☐	0	0
hex	2	☑	0	0
ni	2	☑	0	0
sn	2	☑	0	0
tol	2	☑	0	0
water	2	☑	0	0

Figure 11 – Compound Tab in Super-O

As shown in figure 11, 16 compounds were entered into the Super-O interface with 10 of them being chemicals added. For this work, we didn't use the standard enthalpy or heat capacity feature because this work didn't feature any heat exchangers. The molecular weight (MW) was kept the same for the whole project because we had entered in all of the data as weight, therefore we didn't need to convert it to moles. The next tab is the utilities tab, but since we don't have any heat exchangers there was no

data entered in this tab besides the name of the utility which was kwh. The next tab is the reaction tab which has the stoichiometry and conversion.

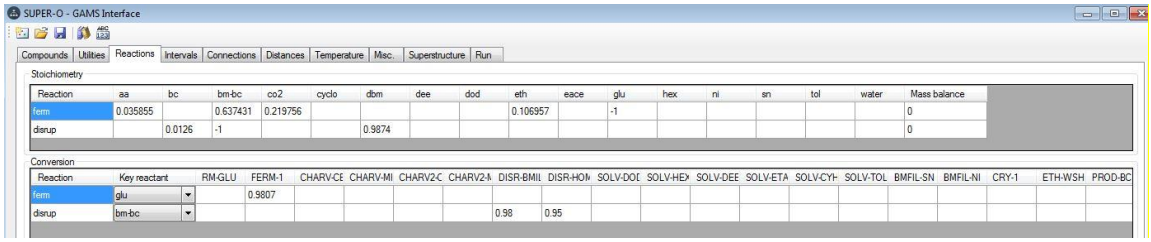


Figure 12 – Reaction Tab in Super-O

The reaction tab is made of the conversion and stoichiometry section. Super-O checks if the user has inputted a correct stoichiometry that doesn't violate the first law of thermodynamics in the mass balance column. The conversion of the reaction, which is shown on the bottom of figure 12, corresponds to the overall conversion of the key reactant which is user defined. The fermentation derived reactions that has been used in Super-O is shown below:

$$glucose \rightarrow 0.637\ biomass + 0.036\ acetic\ acid + 0.107\ ethanol + 0.220\ CO_2 \qquad (7)$$

The first reaction, shown in figure 12, was for fermentation processing interval, which has been derived from Raftery et. al which used experiments and simulations to get a continuous fermentation with a conversion of 0.981. (Raftery et al., 2017) The conversion was inputted into the bottom of the Super-O tab, which corresponds to the processing task. In equations 7 and 8, the biomass is made up of cell debris and beta-carotene.

$$biomass \rightarrow 0.987\ cell\ debris + 0.013\ beta - carotene \qquad (8)$$

The second reaction shown in figure 12 corresponds to equation 8 which is applicable to both the bead mill and homogenizer processing task in the cell disruption processing step. The conversion for the bead mill processing task is 0.98, and the homogenizer processing task, 0.95, therefore cell disruption is more efficient in the bead mill. (Kula and Schütte, 1987; Lovitt and Coss, n.d.)

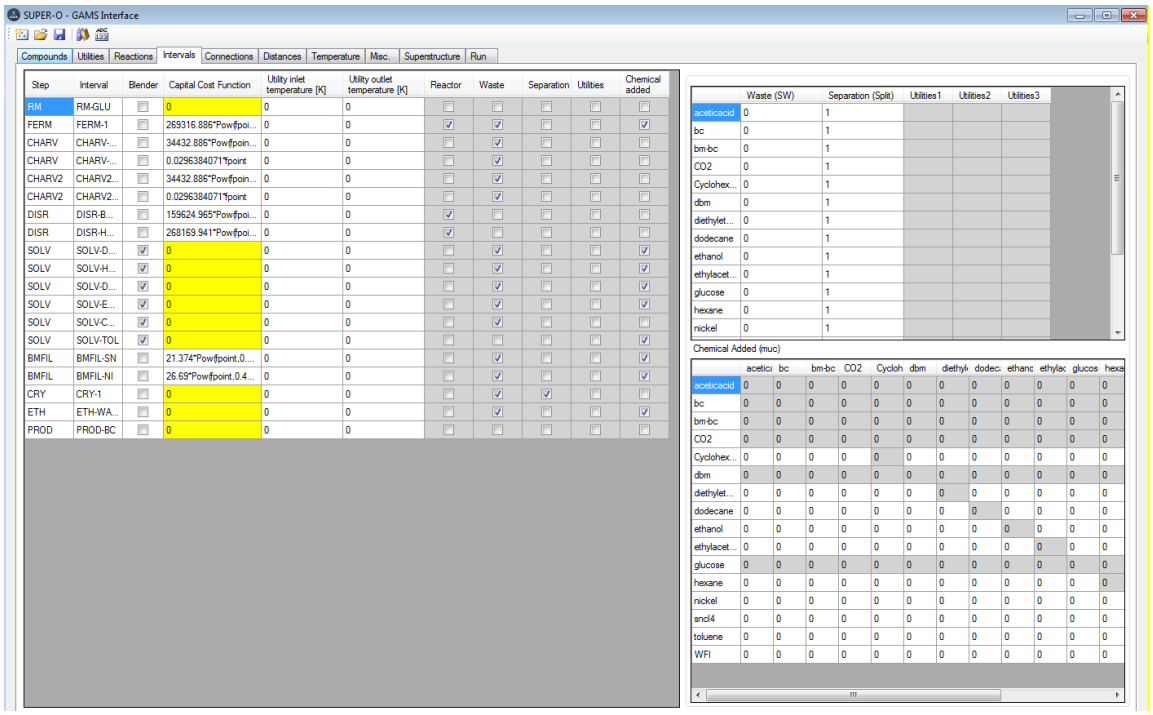


Figure 13 – Intervals Tab in Super-O

The interval tab is where all of the processing intervals are defined with the specific processing tasks. The left hand side of figure 13 breaks down each processing step into the corresponding processing intervals; within each interval there are the processing tasks and capital costs for the interval. These processing tasks are shown in table 5, which is located on top of the next page. The capital cost functions are

linearized in Python and discussed in the Misc. tab. In figure 13, the table on the top right corresponds to the split fraction for waste and product separation. The table on the bottom right of figure 13 is used for adding a specified amount of chemical A, which is chosen by the row, with respect to chemical B, which is chosen by the column. This process was done for each processing interval. The waste separation fraction and the chemical addition table played a key role in the process parameter analysis discussed later.

Table 5 – Summary of Literature (LIT), Experiments (EXP) or Combination (CMB) for the Process Intervals with Processing Tasks

			Processing Tasks			
Section #	**Step**	**Interval**	**Reaction**	**Waste**	**ProdSep**	**Chem Add**
II.IV.I	RM	RM-GLU				
II.IV.II	FERM	FERM-1	CMB	CMB		CMB
II.IV.III	CHARV	CHARV-CENT		LIT		
II.IV.III	CHARV	CHARV-MF		LIT		
II.IV.III	CHARV2	CHARV2-CENT		LIT		
II.IV.III	CHARV2	CHARV2-MF		LIT		
II.IV.IV	DISR	DISR-BMILL	LIT			
II.IV.IV	DISR	DISR-HOMO	LIT			
II.IV.V	SOLV	SOLV-DOD		EXP		EXP
II.IV.V	SOLV	SOLV-HEX		EXP		EXP
II.IV.V	SOLV	SOLV-DEE		EXP		EXP
II.IV.V	SOLV	SOLV-ETAC		EXP		EXP
II.IV.V	SOLV	SOLV-CYHX		EXP		EXP
II.IV.V	SOLV	SOLV-TOL		EXP		EXP
II.IV.VI	BMFL	BMFL-DCT-SN		LIT		LIT
II.IV.VI	BMFL	BMFL-DCT-NI		LIT		LIT
II.IV.VII	CRY	CRY-1		LIT		
II.IV.VIII	ETH	ETH-WSH		LIT		LIT
II.IV.IX	PROD	PROD-BC				

As seen in table 5, there are 28 different processing tasks that have to be defined in Super-O in the intervals tab shown in figure 13. Table 5 illustrate there are many processing intervals that can be improved with experimentation and with informed design to get a more accurate representation of downstream processing of beta-carotene. The input to the homogenizer can only come from the first cell harvesting processing step and the input to the bead mill can only come from the second cell harvesting step because of cell concentration constraints discussed in section II.IV.IV and inputted into Super-O in the connection tab.

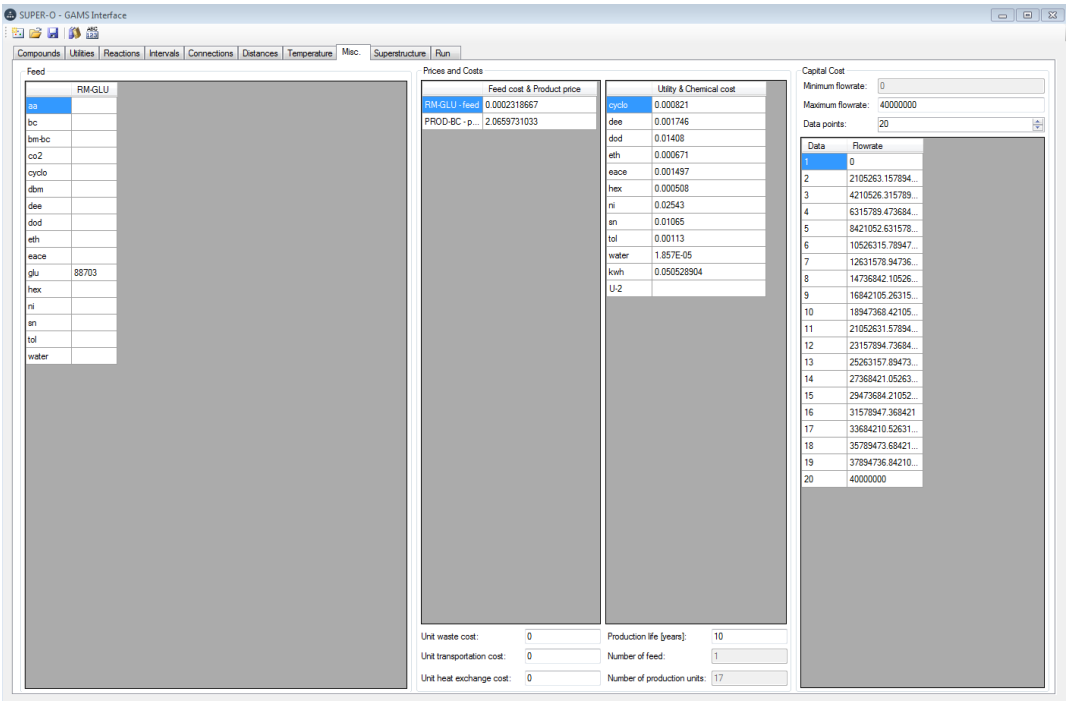


Figure 14 – Misc. Tab in Super-O

As seen in figure 14, the feed has been defined as 88,703 g/hr of glucose to give us a 4.82 tons per year of beta-carotene output, which accounts for approximately 10%

of the total beta-carotene consumption market in 2014. (Marz, 2015) The cost of glucose is $0.23 per kilogram and the price of beta-carotene which is $2,065 per kilogram is realistic based on our literature review. (Caswell and Zilberman, 2001; Korovessi and Linninger, 2005) The capital cost shown on the far right of figure 14 is the linearization done using Python. The production life of 10 years, shown on the bottom of figure 14, is a low estimate on how long this plant would run.

After all of the data is inputted into Super-O, the software exports the data into an excel file with rows and columns that correspond to the generic process interval representation shown in figure 3. This excel file was then be read by General Algebraic Modeling System (GAMS), which used the excel file as an input for the MILP solvers built into GAMS. The superstructure optimization that was used for this work was BARON. (Sahinidis, 1996)

The initial case for the beta-carotene process synthesis problem was solved using Super-O, but there was no net profit because the cost of the solvent used to extract the beta-carotene was too high. Therefore, we did a process parameter analysis around the solvent addition step to identify the amount of solvent recovery needed for the process to be profitable. The net profit per kilogram of beta-carotene is shown below while varying both the water for injection (WFI) and the yield of beta-carotene from glucose in the fermentation process step.

Table 6 – Process Parameter Analysis on the Solvent Recovery, Beta-Carotene Yield and WFI price

Solvent Recovery (%)	Gross Profit ($/year)	Operating Cost ($/year)	Net Profit ($/kg prod.)	Profit-ability
Beta-carotene Yield from Glucose in Bioreactor: 0.804% and WFI at $0.02/kg				
99	$10,994,000	$4,901,000	$1,063	124%
95	$10,994,000	$10,550,000	$1	4%
Beta-carotene Yield from Glucose in Bioreactor: 1.609% and WFI at $0.02/kg				
99	$22,025,000	$4,919,000	$1,564	348%
95	$22,025,000	$10,565,000	$1,034	108%
Beta-carotene Yield from Glucose in Bioreactor: 1.609% and WFI at $0.24/kg				
99	$22,025,000	$17,411,000	$392	27%
95	$22,025,000	$23,057,000	($138)	-4%

As seen in table 6, the optimal solution after conducting the process parameter analysis is $1,564 per kilogram, which is the case where the beta-carotene yield from glucose in bioreactor is 1.61%, the price for WFI is $0.02/kg and the solvent recovery of toluene is 99%. This result is expected because the highest beta-carotene yield from glucose, lowest WFI price, and highest solvent recovery gave the most profitable system. The optimal case produces 9.64 tons of beta-carotene per year, which is 20% of the 2014 market value. (Marz, 2015)Therefore, this process is viable if the cost of natural beta-carotene is $2,065/kg.

It should be noted that table 6 shows three of the four sensitivity analysis cases. The final sensitivity analysis case was a beta-carotene yield from glucose in bioreactor is 0.80%, the price for WFI is $0.24/kg, but even with the solvent recovery of 99 the price for the WFI is too high for any net profit. The solvent of choice for all of the cases was toluene because it had the best recovery of beta-carotene. It should be noted that the

gross profit per year is only based on the maximum amount of beta-carotene that is produced which is based on the yield of beta-carotene from glucose. The operating cost varied based on two different process parameters, 1) solvent recovery, which was directly tied to the purchase cost of toluene, and 2) chemical addition of WFI, which is located in the bioreactor processing step. The WFI price of $0.02 per kilogram was on the low end of the price range found in Harrison et. al (2015) and the WFI price of $0.24 per kilogram is the high price found in Harrison et. al (2015). (Harrison et al., 2015) The capital cost in Super-O is based on the flow rate which is the reason for the increase in capital cost as the amount of solvent recovery decreases, but it plays little effect on the net profit of the system.

The process parameter analysis, which is shown in the far right column of table 6 shows the profitability or the net profit per year over the raw material cost per year. These numbers show that for case 1 we would need at least 99% solvent recovery for a profitable solution. For case 3, we would need at least a solvent recovery of 95% for profitability with anything above that would be an economically viable process. Therefore, we will need to conduct experiments to see if we can get a 95% or better solvent recovery, which is on the low end of the spectrum for harsh solvents. For case 4, there is no solvent recovery number that is profitable; therefore showing that the WFI price plays the biggest role in the profitability of the system.

The bioreactor run that was shown in figure 6 and used for all of the solvent extraction used a cell media that had a beta-carotene yield of 0.80% from glucose. When the cell media composition was optimized in the work by Jaladi et. al (2016), the yield of

beta-carotene from glucose increased to 1.61%. (Jaladi, 2016) In this work, we assumed the processing parameters for the bioreactor would not change when twice the amount of beta-carotene was produced besides that the amount of carbon dioxide would drop by 0.80%. We also assumed that the extraction of beta-carotene using all of the solvents for a beta-carotene yield of 0.80% from glucose would directly translate to twice the beta-carotene extracted for a beta-carotene yield of 1.61% from glucose.

VI. CONCLUSION AND FUTURE WORK*

In this work, we discuss a framework for process synthesis that was able to solve via superstructure optimization under the constraint of limited availability of reliable data. The process synthesis framework lets the user do a process parameter analysis on the process parameters to determine if the data plays a key role and therefore should increase the accuracy of the data through the defined iterative process synthesis framework. The initial problem was defined to solve an optimal intracellular downstream processing train which was tested and confirmed on our downstream processing train for beta-carotene.

For the case study, the optimal process topology gave a net profit of $1,564/kg for a 200,000 L bioreactor based on optimal control algorithm of Raftery et al. (2017), with a beta-carotene yield of 1.609% from glucose, and a theoretical solvent recovery of 99%. (Raftery et al., 2017) To increase the accuracy of the case study there needs to be more experimental gathering of more accurate data for the feasibility of the solvent recovery process, optimized culture media to improve process productivity during fermentation and subsequent extraction, and the use of nickel and tin (IV) chloride for the flocculation of the disrupted beta-carotene. In the Karim group there is work going on to investigate the use of an SMB Chromatography for separation of beta-carotene. We are also looking into using SolventPro from the Dr. Gani group to find 'greener' solvents. (Brignole et al., 1986)

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