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## Fermentative Processes Requiring Low Solubility Feed Gases: an Investigation into Gas-Dependent Microorganisms

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

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## Abstract

Two bioprocesses were separately investigated based on their common interest of using gaseous substrates that have low solubility. The first process involved the development of a mixed culture using two organisms capable of utilizing glycerol and carbon monoxide separately to increase biobutanol production, while the second process involved an investigation of different production media used in aerobic xanthan production of *Xanthomonas campestris* with pressurization effects. It was determined that *Clostridium pasteurianum* should be used with an organism like *Clostridium carboxidivorans* or *Eubacterium limosum* in order for butyrate uptake at 3 g/L to occur with a minimum 0.1 g/L butyrate production. Likewise, a good-performing medium for xanthan production was chosen and increasing pressure saw increases to oxygen mass transfer coefficients for better yields up to 13 g/L biomass and 250 mPa·s of viscosity. Future work should be applied in connecting the results of the second process to that of the first.

## Keywords

Butanol, Mixed Culture, Glycerol, Carbon Monoxide, Biofuels Production, Bacterial Selection, Xanthan, Media Optimization, Pressurized Fermentation, Gas Solubility

## Co-Authorship Statement

**Chapter 4, 4.1.1-4.1.3:** Establishing an anaerobic mixed culture system for butanol formation from glycerol

**Authors:** A. Staaden

This set of experiments was used in the master's thesis for A. Staaden.

All experiments were planned and conducted by E.W. Doerr and A. Staaden, under supervision of supervisors Dr. L. Rehmann and co-supervisor Dr. L. Regestein. The manuscript was written by A. Staaden. Experiments were performed in full co-operation with all authors present and in equal contribution to the body of work. Data collection and analysis were performed together, while data interpretation and usage were performed independently and within the scope of each associated thesis work.

**Chapter 4, 4.1.5:** Impact of butyric acid on butanol formation by *Clostridium pasteurianum*

**Authors:** L. Regestein, E.W. Doerr, A. Staaden, L. Rehmann

The experiments of this section are published in *Bioresource Technology* (Bioresource Technology 196 (2015) 153–159).

The manuscript was prepared by Dr. L. Regestein and subsequently revised by Dr. L. Rehmann, E.W. Doerr, and A. Staaden. Experiments were performed in full co-operation with all authors present and in equal contribution to the body of work. Data collection, analysis, and interpretation were performed together, while final manuscript writing was completed by Dr. L. Regestein.

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I dedicate this work to my family – progenitors in my being, sustainers in what I've been able to become, and the continual reflection of everything that I could hope to aspire to. Thank you for all you have done.

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## Preface

The majority of this dissertation is original, unpublished, independent work by the author. Any collaborations have been noted in the Co-Authorship Statement above.

# Chapter 1

## 1 Introduction

How a fermentation is described has been defined and redefined as time has progressed with scientific discovery. Originally used to describe the ancient procedure of beer- and wine-making with ancestral peoples, the traditional definition of fermentation consisted of any anaerobic process producing some sort of biochemical through a specific metabolic pathway. However, a more accurate and modern description of the process can be extended to include aerobic processes following similar metabolic patterns; as such, it can be said that a fermentation is ‘any process for the production of product by the mass culture of a micro-organism’ (Stanbury, Whitaker, & Hall, 2003). With this definition in mind, many of the bioprocesses and biotechnologies developed today involve some sort of fermentation within their industrial process plan. The rise of research interest into diverse topics such as biorefinery design, advanced bioreactor conceptualization, and optimization of unilaterally standard bioprocesses continue to lead the way into exciting and innovative implementations (Kamm, Schönlicke, & Hille, 2016).

The intent of this work is to lead an investigation into two fermentative processes that require the input of low-solubility feed gases within their respective systems. The first process is an experimental inquiry into the decades-old anaerobic acetone-butanol-ethanol (ABE) fermentation scheme; the proposed system requires a primary organism to degrade glycerol in order to produce biochemicals such as butanol, ethanol, and 1,3-propanediol (1,3-PDO) (Dabrock, Bahl, & Gottschalk, 1992). With the primary focus in butanol synthesis due to its high octane rating, increased energy content in comparison to other alcohols, and its ability to blend with already existing fuel sources, the ABE fermentation will take a deviation from its traditional processing with the introduction of a non-competitive secondary microorganism (Maity, 2015). This secondary organism can be classified as a carboxydophilic bacteria – a bacterial strain capable of consuming carbon monoxide (CO) as both a carbon and energy source in order to produce useful intermediate compounds utilized by the primary organism in question (Henstra, Sipma, Rinzema, & Stams, 2007).

The second process under scrutiny is the well-understood aerobic fermentation of xanthan gum. Xanthan's widespread applications in the pharmaceutical, oil extraction and refining, and food and beverage industries as a mostly inert emulsifier/thickening agent has produced a multitude of studies into its production, properties, and industrial extraction (García-Ochoa, Santos, Casas, & Gómez, 2000). While a host of studies and research has been performed in the understanding of the fermentation's kinetics, oxygen transfer requirements, and fluid dynamics behaviour, fewer efforts have been made in determining pressure effects in pilot-scale bioreactors (García-Ochoa, Castro, & Santos, 2000). The addition of pressure within a fermentation would theoretically increase the amount of solubilized gas within the bulk liquid, and in turn increase the mass transfer coefficients to associatively increase product yield. In addition to the exploration into pressure and xanthan, an array of several different production media were analyzed with one ultimately chosen as the best-performing in terms of healthy cell density and increased xanthan yield.

The basis of the work performed relies on the low-solubility of these feed gases – carbon monoxide and oxygen, respectively – within their separate systems. Distinct efforts were made in each fermentation scheme to attempt gas solubilisation in the system to provide an increased yield of desired product, being either butanol or xanthan.

## 1.1 Anaerobic Butanol Fermentation

An understanding of the proposed process requires investigation into what substrates are available for consumption. Within the biodiesel industry, approximately 10 lbs of crude glycerol are generated whenever 100 lbs of biodiesel are synthesized. In the past decade, the price of this crude glycerol has declined despite the increase in production yields (Yazdani & Gonzalez, 2007). There are currently a host of potential applications to which this crude glycerol can be used, from thermochemical conversions for high-platform petrochemicals to feedstock additives for several ruminant and non-ruminant animal species (Yang, Hanna, & Sun, 2012), and it appears the applicability and versatility of such a raw material is nearly boundless. Even more impressive still, several studies have begun an investigation into the utilization of glycerol in anaerobic ABE fermentation settings to yield several important biofuels and other chemical constituents (Tracy, Jones, Fast, Indurthi, & Papoutsakis, 2012). The overarching pertinence of such an impressive substrate demands further investigation

and necessary experimentation into other biochemical systems attempting to achieve similar goals.

Another high-profile waste material of many industries, chemical production facilities, and oil refineries comes in the form of noxious carbon monoxide (Henstra et al., 2007). Carbon monoxide, much like crude glycerol, has many opportunities for further refinement and usage in thermochemical applications, such as the Fischer-Tropsch process and the Mond process for production of hydrocarbon compounds and separation of metallic compounds, respectively (Bell, 1972). However, these extensive, high-energy processes often require high temperatures and pressures as driving forces to complete the necessary reactions. A suggested alternative to these operational conditions comes in the form of carboxydrotrophic microorganisms – a range of anaerobic bacterial cultures capable of fixating carbon monoxide as a carbon source through the Wood-Ljungdahl metabolic pathway (Köpke, Mihalcea, Bromley, & Simpson, 2011). These organisms are able to oxidize CO using a biological version of the Water-Gas Shift (WGS) reaction in order to produce Acetyl CoA, the precursor to several organic acids and alcohols in their metabolism.

As it currently appears, both of these ‘waste’ substrates do in fact have some further use within their respective biochemical systems. What, if possible, would happen if an attempt was made in combining these biotechnical aspects into one unified system capable of increasing the working yields of already established processes? The root of this area of work aims to answer this vital question.

Several studies have inspected the metabolism of clostridial species in the specific shift of acidogenesis to solventogenesis in an attempt to understand how these mechanisms work (Tracy et al., 2012). In particular, heightened interest has been placed into the pursuit of butanol production from these microbial species. Several works have investigated the effects of intermediate compound supplementation – chemicals such as acetic and butyric acid – into fermentation systems in the hopes of an increased butanol yield (Sabra, Groeger, Sharma, & Zeng, 2014). Consequentially, some species of carboxydrotrophic bacteria are able to produce volatile organic acids such as the two aforementioned when supplemented with the necessary amount of carbon monoxide (Munasinghe & Khanal, 2010). As such, the idea has been proposed to operate a mixed culture system, consisting of a primary microorganism capable

of consuming glycerol to produce butanol, along with a secondary organism fixating carbon monoxide in its gaseous form to produce butyric acid as a secondary carbon source under glycerol limitation. It would be critical then that both organisms should behave non-competitively with each other's substrates and additionally be able to tolerate moderate levels of butanol toxicity within an isolated system (Yazdani & Gonzalez, 2007).

## 1.2 Aerobic Xanthan Fermentation

The solubility of gasses is, along with several other parameters, a function of pressure. By increasing the pressure within a fermentation vessel, one would therefore increase the solubility of a specific gas. During this work, a 50-L pressure fermenter was available for use at the biochemical engineering institute at the RWTH Aachen, Germany. However, this vessel was not certified for the use of CO nor for H<sub>2</sub> production, and as such a different process involving the transfer of oxygen into a rheologically complex medium was investigated. The results from this area of work could be anticipated for possible transfer of knowledge from the experimental results with aerobic fermentation to the envisioned anaerobic CO process discussed prior.

Studies related to xanthan gum and its various applications have been recorded since its first publishing in 1961. The industrial process has remained relatively standard since its days of conception – a liquid medium containing adequate amounts of carbon and nitrogen sources along with several nutrient salts are well-mixed and well-aerated with cellular cultures (Katzbauer, 1998). Once the fermentation has reached maximum xanthan yield and undergoes either oxygen or carbon limitation, the broth undergoes sterilization and xanthan gum is precipitated via isopropyl alcohol. The demand for xanthan has increasingly grown as time has progressed due to its remarkable pseudoplastic, shear-thinning properties. A multitude of industries, such as the pharmaceutical, cosmetic, food and beverage, and oil recovery sectors, use xanthan gum as an emulsion stabilizer, fluid thickener, and lubricant, among other claims (Petri, 2015).

Despite the tested-and-true nature of xanthan production, there are still several process aspects that require further inquiry and analysis. Fundamental differences observed in media composition, such as the carbon-to-nitrogen (C/N) ratio, during the growth and production phases of the microorganism require differing media compositions with each

separate phase (Casas, Santos, & García-Ochoa, 2000). Some studies have focused on whether the addition of organic or inorganic nitrogen has significant impact on the quantity and quality of xanthan produced, while other research has looked into step-by-step medium component addition and analysis (García-Ochoa, Santos, et al., 2000). Likewise, the subject of homogeneous and effective oxygen transfer within a bioreactor setting remains an open issue to successfully solve; possible solutions to increase oxygen transfer rate include dynamic agitation and gassing systems, impeller design and configuration, and several other methods (Palaniraj & Jayaraman, 2011). However, few studies to date have not included the effects of a pressurized environment as a means to increase dissolved oxygen content and in turn theoretically increase xanthan yield.

Decidedly, the following work aims to determine production medium well-suited for the xanthan fermentation process from a studied array of several different complex and minimal options. In addition, the presence of a universally applicable trace elements solution should be developed as a means to standardize the various solutions available within this domain of research. Finally, the effects of a pressurized system will provide valuable information on whether the selected production medium and new trace elements solution have contributed to a more efficient process with greater productivity.

### 1.3 Cumulative Results

Jointly, the combination of work collected on these two bioprocesses will shed new light on gas-liquid mass transfer in complex systems. The data and trends collected on the pressurized reaction system in particular will help to overcome some of these limitations. The results obtained during the xanthan production will be relevant for future design of applicable syngas fermentation processes, as current work has only tested pressurization at a much smaller scale (Najafpour & Younesi, 2006; Younesi, Najafpour, & Mohamed, 2005). In turn, the progressive experimental method of determining syngas cultures and other necessary parameters followed by a model process to stand-in for increasing gas-liquid solubility issues will provide prospective work with the fundamental research needed for eventual scale-up and operation of an industrial syngas fermentation system.

## 1.4 Experimental Objectives

To fully develop the work on anaerobic butanol fermentation, the following objectives are to:

1. Determine an ideal primary organism capable of glycerol consumption, moderate butanol yield, and general robustness/ability to adapt in several conditions;
2. Examine and choose an ideal secondary organism exhibiting carboxydrotrophic properties, ability to grow and produce butyric acid in specified media, inability to consume glycerol, and have a moderate tolerance towards butanol; and,
3. Record and analyze the secondary organism's ability to consume carbon monoxide and its resulting product profile, along with noting the primary organism's ability to uptake butyrate and consequentially produce butanol.

In the case of aerobic xanthan fermentation, the following objectives are to:

1. Determine which of an array of different production media and whether the addition of a newly developed trace elements solution proves the most effective in terms of biomass growth, viscosity and associated xanthan concentration, effective oxygen transfer metabolic profile, and other necessary criteria;
2. Develop a scale-up procedure based on constant volumetric power and mass transfer coefficients within an aerobic system from flask to pilot-plant levels; and,
3. Study and analyze the effects of pressurization in relation to the oxygen transfer rate within the pilot plant fermentation

With these objectives complete, future work could develop on the connecting aspects between these two bioprocesses and the development of a model and common techniques aimed at increasing gas solubility in systems dependent on effective mass transfer to produce greater amounts of product with healthy cellular growth.

## Chapter 2

### 2 Literature Review

The following literature review is structured into two major sections, Anaerobic Butanol Fermentation (ANBF) and Aerobic Xanthan Fermentation (AEXF). Each section will discuss the potential microorganisms that could be used along with their benefits and drawbacks, the substrates, intermediates, and products/by-products to be expected, and the governing physical phenomena from mass transfer, heat transfer, and fluid dynamics principles bounding these systems. Finally, discussion will be made into the production media studies performed with keynote options fully defined.

#### 2.1 Anaerobic Butanol Fermentation (ANBF)

The start of biological butanol production begins with the founding experiments by Louis Pasteur in 1861, leading to his fundamental discovery of the eponymous ‘Pasteurization’ process. However, the upscaling and commercialization of biobutanol was not truly pursued until 1916, where Chaim Weizmann had successfully isolated *Clostridium acetobutylicum* and patented the claims to this process (Jones & Woods, 1986). From thereon, expansion and demand in ABE fermentation rapidly grew across the globe until the utilization of petrochemical technologies in the 1950s and 1960s. Even as the interest in ABE fermentation began to decline in this period, China still produced approximately 170 000 tons of ABE-derived solvents by 1980 (Chiao & Sun, 2007). Returned interest in this bioprocess has occurred in recent years due to the demand for more environmentally sustainable practices, diversification of renewable energy sources, and the concern for general costs and availabilities of existing fossil fuel deposits (Yazdani & Gonzalez, 2007).

Current studies have concluded that typical butanol yields tend to not surpass the value of 2% w/v (Jones & Woods, 1986; Xue, Zhao, et al., 2014) during fermentation because of the toxic effects on cellular cultures. Because of this natural limitation, several innovative techniques are under examination in the hopes of surpassing this major threshold. One possibility in increasing butanol yields lies in the domain of *in situ* removal, with the integration of technical concepts such as pervaporation, gas stripping, two-phase liquid-liquid extraction, and resin adsorption technologies (Jones & Woods, 1986; Wiehn, 2013; Xue, Du,

et al., 2014). Regardless of whatever alternative is used, all *in situ* technologies are able to decrease the amount of desired butanol within a selected system through specific physical principles that separate a required compound. In addition to this caveat, further recovery and purification techniques necessary to achieve a certain percentage of compound purity for commercial standards become much simpler when this type of removal is integral to the fermentation. However, significant drawbacks to *in situ* removal such as higher operational costs, increased energy demand, greater complexity of standard bioreactor design, and dependency on more external equipment lead to further questioning and experimental discovery (Wiehn, 2013).

### 2.1.1 Cellular Cultures

Typically, any organism capable of producing biofuels must be able to produce the necessary building block compounds of nearly all cellular functions – pyruvate or Acetyl-CoA. Most microorganisms are capable of producing these essential components through the regularly defined and well-understood glycolytic or pentose phosphate pathways (Fischer, Klein-Marcuschamer, & Stephanopoulos, 2008). In terms of biofuel-related microorganisms, however, several other pathways are capable of utilizing non-standard substrates to produce desired product. Glycerol, a molecule commonly associated in fatty acid biosynthesis, can be consumed by a select range of bacterial cultures capable of producing several biochemicals as a result. Some common examples of such bioactivity occur in species of bacterial families such as *Clostridium*, *Enterobacter*, *Citrobacter*, and *Klebsiella* (Yazdani & Gonzalez, 2007). In particular, clostridial species such as *Clostridium pasteurianum* have been shown to increase butanol yields using glycerol as a substrate (Dabrock et al., 1992).

Another more recently established metabolic pathway occurs in the form of the Wood-Ljungdahl pathway; since the discovery of *Clostridium ljungdahlii* in 1987, several bacterial species have been reported to produce small-chain organic acids and alcohols – such as acetic and butyric acid along with ethanol and butanol – purely based on the consumption of carbon monoxide and/or a combination of hydrogen and carbon dioxide (Munasinghe & Khanal, 2010). Aptly dubbed carboxydophilic bacteria, these organisms are able to reduce these gaseous compounds into Acetyl-CoA under the right conditions and produce associated biofuels (Henstra et al., 2007). Much like the glycerol-consuming

organisms, several species of *Clostridia* are able to convert up to 45% of the theoretical CO available within a fermentation system (Fischer et al., 2008).

As discussed in the previous section, there are several strategies in development for increasing butanol yields from an applied biotechnological approach. Primarily, research into genetic engineering, mutagenesis, and recombinant development has proven fruitful in several attempts (Tracy et al., 2012). Characterizations of genomes such as *Clostridium acetobutylicum* and *Clostridium carboxidivorans* have provided scientists with the knowledge and ability to understand what necessary traits these organisms require for solventogenesis (Bruant, Lévesque, Peter, Guiot, & Masson, 2010). However, challenges such as the need for developing clostridial-specific recombinant technologies for metabolic pathway merging or further investigation into genetic mutations for enhancement of solventogenesis still demand more exploration (Tracy et al., 2012).

#### 2.1.1.1 Primary Organism – Glycerol-consuming Bacteria

As previously discussed, the knowledge and applications discovered regarding the glycerol-consuming metabolic pathway in select organisms has provided industry and academia alike with the opportunity to utilize an inexpensive substrate, much unlike pure glucose and other similar saccharidic compounds. Although the main discussion on glycerol and its associated organisms has been primarily done on its ability to produce butanol, several other distinct species can produce a host of niche, in-demand chemicals for use in today's economy. For example, under photo-fermentative conditions, cultures such as *Rhodospirillum rubrum* and *Enterobacter aerogenes* were able to yield ethanol and hydrogen at significant quantities, with the latter product up to 75% mol/mol of its theoretical value (Yang et al., 2012). Likewise, *Klebsiella planticola* was also able to produce ethanol and formic acid – two platform chemicals for any major manufacturer – at concentrations up to 2 g/L (Yazdani & Gonzalez, 2007). However, these cultures require a significant amount of energy and total fermenting time when compared to the case of butanol production, and in turn do not provide yields substantial enough for profitable commercialization. Of the possibilities and potential microbiological systems available, butanol production via *C. pasteurianum* or *C. acetobutylicum* appear as the most promising choices.

#### 2.1.1.1.1 *Clostridium pasteurianum*

*C. pasteurianum*, a spore-forming, obligate anaerobe, was first discovered capable of fermenting glycerol when converting a mixture of glycerol along with algal biomass of five separate *Dunaliella* species into butanol, ethanol, acetate, and 1,3-propanediol (Nakas, Schaedle, Parkinson, Coonley, & Tanenbaum, 1983). Since this cornerstone work was performed, a multitude of studies have been conducted to analyze and understand many effects prevalent in this glycerol-consuming organism. Another mixed-substrate system was performed where glycerol was supplemented with glucose that had enhanced significant cellular growth along with acid production with a maximum butanol concentration of 21 g/L achieved (Sabra et al., 2014). Additionally, an acidic fermentation system consisting of glycerol supplemented with butyric acid was found to increase butanol yields when sufficient levels of glycerol were still present (Gallardo, Alves, & Rodrigues, 2014).

Other pertinent research prospects have come in the form of operational and gassing factors; when continuously sparged with 15% v/v carbon monoxide, the major products of glucose consumption with *C. pasteurianum* was seen to consist of acetate, butyrate, ethanol, butanol, and lactate, as opposed to the CO-free scenario where butyrate and acetate were the major components. This phenomenon could be attributed to the inhibiting effects CO has on hydrogenase enzymes (Dabrock et al., 1992). In terms of nitrogenous sources, it is important to note that fermentation times increase by approximately 50% when the sole source of nitrogen within a fermenter is supplied via gaseous nitrogen (Biebl, 2001). In terms of pH and temperature regulation, cultures of *C. pasteurianum* have been seen to operate at a pH range between 4.5-7.5 and temperatures between 30-39 °C, with the most common studies operating their experiments at a pH between 5.5-6.0 and 35 °C, respectively (Regestein, Doerr, Staaden, & Rehmann, 2015; Sabra et al., 2014).

### 2.1.1.1.2 *Clostridium acetobutylicum*

*C. acetobutylicum*, initially known as the ‘Weizmann Organism’ at the advent of its discovery, proves itself as the industry standard when discussing traditional ABE fermentation. Originally using the long-chain polysaccharides present in potato starches and maize, *C. acetobutylicum* gained its original appeal for its ability to produce acetone instead of either butanol or ethanol, at a product-mass ratio of 3:6:1 for acetone:butanol:ethanol respectively (Jones & Woods, 1986). More modern research interests have looked into the ability of polysaccharidic decomposition by *C. acetobutylicum* of several low-cost feedstocks such as fibrous corn wastes as a means of utilizing the undesirable components within industry and society (Gheshlaghi, Scharer, Moo-Young, & Chou, 2009a). Likewise, it was determined that, when grown on glucose as a substrate, gassing with select levels of CO had improved glucose uptake rates by 300% while simultaneously supporting complete hydrogen product inhibition (Meyer, Roos, & Papoutsakis, 1986). As these two caveats of data prove favourable in the support of *C. acetobutylicum* as a potential candidate as an ideal primary organism, there are some concerns to note regarding its performance overall.

While a recombinant strain of this bacteria is able to consume glycerol with 1,3-PDO as the primary product synthesized, the wild *C. acetobutylicum* is unable to do so (Gheshlaghi, Scharer, Moo-Young, & Chou, 2009b). Additionally, some studies have made direct comparisons of butanol yields grown on glycerol; *C. pasteurianum* was able to produce titres at 0.3 g butanol/g glycerol, nearly doubling that of *C. acetobutylicum* at 0.15-0.20 g/g (Yang et al., 2012). Even though both clostridial strains operate at nearly identical temperature and pH ranges, *C. acetobutylicum* proves more advantageous in having a wider pH range for the culture to grow compared to *C. pasteurianum*. Despite this, the sensitivity of pH is less influential in terms of the latter organism’s productivity patterns than that of the former (Dabrock et al., 1992).

### 2.1.1.2 Secondary Organism – Carboxydrotrophic Bacteria

As discussed in Section 2.1.1, this category of microorganisms are able to convert gaseous substrates of carbons monoxide and dioxide along with hydrogen into a range of organic acids and alcohols. In the first step of substrate fixation, an organism will reduce either carbon oxide to a corresponding methyl group in a series of increasingly reductive reactions. Afterwards, the newly formed methyl group is synthesized in conjunction with an existing carbonyl and CoA group to produce the elementary unit of any fermentative product – Acetyl-CoA (Munasinghe & Khanal, 2010). The utilization of this set of microorganisms provides a unique list of advantages and drawbacks to consider.

Primarily, in comparison to thermochemical conversions of syngas via the Fischer-Tropsch process, carboxydrotrophic fermentations are able to achieve greater product specificities, operate in considerably lower pressure and temperature ranges, and achieve flexibility in feed gas composition all with a self-propagating microorganism (Perez, Richter, Loftus, & Angenent, 2013). In comparison to other bioprocesses, carboxydrotrophs are able to better utilize the entirety of a substrate in comparison to lignin-based bioconversion; additionally, the elimination of expensive pretreatment and enzyme stages are removed when dealing with a lignocellulosically-derived syngas fermentation (Munasinghe & Khanal, 2010). Existing challenges that require resolution with these microorganisms include multiple product formation with lower yields, poor mass transfer of gaseous substrates, and creating effective yet efficient product recovery operations (Yasin et al., 2015). While stratagem and experimentation have been conducted in the hopes of providing real solutions, there is still a necessity to continue research into these issues for better fermentative control and productivities.

The majority of carboxydrotrophic organisms investigated were mesophilic species such as *Acetobacterium*, *Clostridium*, and *Eubacterium*; however, thermophilic organisms such as *Moorella thermoacetica* and *Carboxydotherrmus hydrogenoformans* could prove advantageous in biochemical operations desiring to integrate both fermentation and product recovery, such as gas stripping, into singular units (Yasin et al., 2015). Despite this consideration, these thermophiles would be non-ideal in a mixed culture fermentation setting designed for the control of a primarily mesophilic bacterium. With the above discussion in mind, it was observed that *Acetobacterium woodii*, *Clostridium autoethanogenum*,

*Clostridium carboxidivorans*, *Clostridium ljungdahlii*, and *Eubacterium limosum* proved the most promising in the hopes of this fermentative proposal.

#### 2.1.1.2.1 *Acetobacterium woodii*

In the original study of *A. woodii*, it was observed that this organism was able to oxidize hydrogen while reducing carbon dioxide to produce acetic acid; in addition to its carboxydrotrophic properties, the gram-positive, fastidiously anaerobic bacterium was also able to ferment fructose, glucose, lactate, glycerate, and formic acid (Balch, Schoberth, Tanner, & Wolfe, 1977). It was not until several years later that *A. woodii* was also able to utilize carbon monoxide as a carbon and electron source. When comparing which carbon oxide proved the better carboxyl group in Acetyl-CoA synthesis, carbon monoxide was determined to be optimal and helped better understand what mechanisms occur in the Wood-Ljungdahl pathway (Kerby, Niemczura, & Zeikus, 1983). The majority of these studies operated at 30 °C with pH adjusted to 7, usually with buffering supplemented via CaCO<sub>3</sub> or other similar agents. Growth on glucose was still observable at pH values as low as 4.7 (Genthner & Bryant, 1987).

Studies regarding supplementation with phosphate had subjected *A. woodii* to produce acetate and trace amounts of alanine as its sole products with virtually no ethanol detected. In addition, it was also observed that consumption of low-carbon alcohols such as ethanol, propanol, and butanol provided conversion to their respective fatty acids with minimal growth detected (Buschhorn, Dürre, & Gottschalk, 1989). In a fermentation setting devoted to producing these high-value alcohols like butanol, the potential of counterproductive mechanisms such as this could prove hindering in achieving desirable yields. Genetic manipulation of the wild type strain has seen an increase in acetate concentration, but the full applicability of such work may go unrealized within this mixed culture (Yasin et al., 2015).

### 2.1.1.2.2 *Clostridium autoethanogenum*

With its original isolation from rabbit faeces, the discovery of *C. autoethanogenum* had reported metabolization on compounds such as CO and CO<sub>2</sub> with H<sub>2</sub>, pyruvate, xylose, arabinose, and fructose to produce significant amounts of acetate, ethanol, and 2,3-butanediol (2,3-BDO) (Abrini, Naveau, & Nyns, 1994). Being a strictly anaerobic, spore-forming clostridial culture similar in nature to other species within this body of work, this culture was observed to have ideal growth conditions at 37 °C and at a pH between 5.8 to 6.0 (Henstra et al., 2007). In terms of optimizing production conditions for ethanol, it was detected that reductions in the pH level and concentration of yeast extract to values around 4.75 and 0.60 g/L, respectively, had enhanced maximum ethanol production up to 200% from its ideal growth conditions. In the same study, an increase to system overpressure set to 1.6 bar had only promoted minor improvement to ethanol production, but significant increases to both acetate and biomass concentrations were reported (Abubackar, Veiga, & Kennes, 2012). Such observations could prove synonymous amongst other carboxydrotrophic organisms, as similar metabolic pathways and products are common with this category of bacterial species.

In terms of operational studies done with *C. autoethanogenum*, it was determined that higher gaseous flow rates resulted in greater end product formation with little to no influence on changes in ethanol:acetate ratios in a batch fermentation setting (Cotter, Chinn, & Grunden, 2009). With a continuous feed gas flow rate increasing in a batch liquid system, the solubility of the gas will only increase with the flow rate until a specific saturation value is achieved. It was also determined that genes for ferredoxin and other iron-containing enzyme complexes are not expressed while growth is experienced on CO using *C. autoethanogenum*; such a finding further confirms the deactivation of these complexes when carbon monoxide is present. Carbon monoxide remains a preferential gaseous feedstock even when hydrogen and carbon dioxide are simultaneously present (Wang et al., 2013).

### 2.1.1.2.3 *Clostridium carboxidivorans*

With its original classification as strain P7, *C. carboxidivorans* was reported to be a robust microorganism capable of metabolizing a plethora of organic matter, including CO, H<sub>2</sub>/CO<sub>2</sub>, glucose, galactose, fructose, and several other cellulosic sugars and compounds. With original isolation from a settling lagoon and primarily displaying acetogenic behaviour, this Gram-positive, spore-forming anaerobe proved exceptional in both its metabolism and product profile (Liou, Balkwill, Drake, & Tanner, 2005). Not only has *C. carboxidivorans* been reported to produce noteworthy amounts of acetate, ethanol, butyrate, and butanol from syngas fermentation, but C<sub>6</sub> compounds such as hexanoic acid and hexanol were detected at maximum concentrations of 0.36 g/L and 0.94 g/L respectively (Phillips et al., 2015). The ability to achieve this advanced level of product formation in carboxydrotrophic bacteria is especially rare for an organism solely relying on C<sub>1</sub> compounds as substrate.

While being able to optimally grow at 38 °C and at a pH of 6.2, several studies conducted at pH levels below 5.8 have typically resulted in enhanced solvents production as expected by most solventogenic organisms (Munasinghe & Khanal, 2010). With genetic characterization via genome sequencing performed on *C. carboxidivorans*, it was determined that this organism possessed the same genetic abilities of ABE fermentation bacteria such as *C. acetobutylicum* (excluding acetone production) along with its inherent use of the Wood-Ljungdahl pathway (Bruant et al., 2010). Indeed, when the partial pressure of carbon monoxide was able to be increased within an isolated system to 2.0 atm, a nearly 440% increase in cell concentration was reported along with conversion of acetic acid occurring in systems greater than 1.35 atm (Hurst & Lewis, 2010). Further proof of the versatility and adaptability of this microorganism was shown when exposed to syngas compositions mixed with tars present in the mixture; while the tars induced cell dormancy and reduced end product distribution to more acidogenic favourability, *C. carboxidivorans* was able to adapt to tars presence after prolonged exposure (Ahmed, Cateni, Huhnke, & Lewis, 2006). Such findings can denote a microorganism that exhibits less sensitivity towards differing operational conditions and the ability to adjust the metabolic and growth processes from external controls such as gas composition, pH regulation, and other methods with ease.

#### 2.1.1.2.4 *Clostridium ljungdahlii*

Like other species discussed previously, *C. ljungdahlii* was isolated from chicken yard waste, where it too was able to metabolize the typical carboxydrotrophic gas compounds along with glucose, fructose, arabinose, and xylose. With reported metabolites such as acetate, ethanol, and 2,3-butanediol and optimal growth conditions at 37 °C and a pH of 6.0, *C. ljungdahlii* appears to be nearly identical to that of *C. autoethanogenum* (Tanner, Miller, & Yang, 1993). A major difference between these two organisms, however, can be seen with molar ethanol: acetate ratios at pH 6.8 where the former and the latter are able to produce 1:8 and 1:1.13 respectively using syngas. Despite this, higher gaseous flow rates did not result in increased productivity as hoped in *C. ljungdahlii* as it had been observed with *C. autoethanogenum* (Cotter et al., 2009).

Scale-up experiments performed in several different types of bioreactor systems have been done with *C. ljungdahlii*. One study conducted a series of fermentations using a 4.5 L bubble-column reactor with an artificial blend of synthesis gas; with this setup, it was determined that the introduction of hydrogen gas into the system allowed for increased ethanol production after 60% of the CO had been utilized to form CO<sub>2</sub>. It was also suggested that the ability to increase cellular concentrations ten-fold as in commercial bioreactors should permit ethanol-based syngas fermentations as an economically viable option assuming a lack of macronutrient limitation (Rajagopalan, P. Datar, & Lewis, 2002). The confirming possibility of this hypothesis can likely be extended to the realm of butanol fermentation, with the additional consideration of the toxic effects this substance has on most cellular cultures. Finally, similar to studies done on *C. autoethanogenum*, the effects of pressure on both gaseous compounds and the cellular culture was conducted with *C. ljungdahlii*. While maximum ethanol concentration of 0.6 g/L was achieved at both 1.6 and 1.8 atm, maximum acetate production peaked at 1.4 atm with a value of 1.3 g/L (Younesi et al., 2005). Furthermore, it was determined that no growth inhibition occurred at higher pressure values and that a target molar production ratio of 5:1 for ethanol to acetate was realizable at these conditions (Najafpour & Younesi, 2006).

### 2.1.1.2.5 *Eubacterium limosum*

The existence of the *Eubacterium* genus has been studied since the initial discovery made by André-Romain Prévot in 1938; however, it was not until the 1980s that *E. limosum* was made the type species of the entire genus (Cato, Holdeman, & Moore, 1981). Being a gram-positive, non-spore forming anaerobe similar to other acetogenic bacteria like *A. woodii*, there are some notable differences to consider between these two cultures. When introduced to a fermentation system consisting of gaseous CO, CO<sub>2</sub>, and H<sub>2</sub> along with aqueous formate, it was observed that only the *E. limosum* was able to effectively use all substrates unlike *A. woodii*, which was unable to utilize formic acid likely due to its concentration and/or acidity effects. In the same study, the former bacteria had been reported to produce butyrate along with acetate and ethanol like *A. woodii*, with growth conditions still satisfied at pH values as low as 4.7 (Genthner & Bryant, 1987).

While these lower pH values are more acceptable for acidogenic and solventogenic phases, optimal growth was observed at pH values ranging from 7.0 to 7.2 and at a temperature of either 38 or 39 °C. While growth had been observed up to 75% v/v CO within the useable gas phase, growth was truly uninhibited up to 50% v/v CO. The ability to increase the fraction of carbon monoxide within the gas phase should theoretically allow greater solubility within a bulk liquid and thus translate to enhanced growth and acid production (Genthner & Bryant, 1982). In a study performed using *E. limosum* in anaerobic digestion waste systems, the product profile had extended not only to include the typical acetate and butyrate but an inclusion of extended-chain derivatives such as isobutyrate and variations of valerate (Sharak Genthner, Davis, & Bryant, 1981). In actuality, a more recent study has proposed, like in several studies discussed prior, that the influence of ferredoxin-derived activity plays a major role in end products formed. It was determined that significant amounts of butyrate are formed when CO is used versus CO<sub>2</sub> with H<sub>2</sub> as the energetic benefit of hydrogenase inhibition helps create a metabolic pathway geared towards butyrate synthesis (Jeong et al., 2015).

### 2.1.1.3 Pure vs. Mixed Cultures

Typically, the majority of research conducted in both glycerol- and syngas-based biofuel fermentations use one microbiological culture for experimentation. A pure culture system allows the opportunity to understand the general growth and metabolic behaviour of a single organism, left unaffected by any other constraints from foreign species and able to be studied under various effects and conditions for comprehension. However, the reality of maintaining a pure culture under full-scale industrialization provides both process-related and economic challenges that could be seen as unnecessarily redundant. For example, previous work done on a syngas fermentation system using the carboxydophilic *Alkalibaculum bacchi* with propionic acid-producing *Clostridium propionicum* was compared to a fermentation using *A. bacchi* as a monoculture. As a result of these organisms feeding into each other's metabolisms, the mixed culture was able to produce ethanol, propanol, and butanol from a syngas feed and had been able to convert 50% more of any propionic, butyric, and hexanoic acid to their respective alcohol counterparts in comparison to the pure culture case (Liu et al., 2014). The use of completely open mixed culture systems had also been performed in a mixed substrate system using glucose and glycerol. With a combination of anaerobic sludge from distillery wastewater and potato starch processing tank residue combined in one consortium of microflora, the resulting fermentation had provided a stable and predictable product profile consisting of acetate, ethanol, 1,3-PDO, formate, and other desirable organics without any sterilization required (Temudo, Poldermans, Kleerebezem, & Van Loosdrecht, 2008). Such applicative, innovative uses of stable mixed cultures had been observed to not only reduce operational costs with sterilization and pure culture preparations (Fava et al., 2015), but the ability to fixate and produce longer-chain alcohols such as butanol and hexanol had proved as successful if not more so in these mixed culture conditions (Tracy et al., 2012). The necessity to regulate, control, and quantify the amount of each microorganism present in a mixed culture fermentation remains a critical step in advancing this set of bioprocesses.

## 2.1.2 Substrates

### 2.1.2.1 Solid Aqueous & Liquid Sources

#### 2.1.2.1.1 Glucose

Glucose can be consumed by a host of microorganisms in its monosaccharidic or complex carbohydrate forms – feedstocks such as sugar cane and beet molasses, corn, cassava, and many other residual waste streams can provide a means of lower-cost fermentative conditions. The majority of cellulolytic and solventogenic clostridial species can use glucose along with other six-carbon ring sugars and their derivatives simultaneously as an effective means to produce biofuels (Tracy et al., 2012). Separate studies conducted on *C. acetobutylicum* and *C. pasteurianum* had both concluded that consumption of glucose in the presence of CO had provided a more reduced-favoured product profile (i.e., ethanol and butanol) versus the standard anaerobic glucose fermentation (Dabrock et al., 1992). Furthermore, in a mixed substrate fermentation of glucose and glycerol by *C. pasteurianum*, it was observed that glucose limitation had caused either reduction and/or inhibition of glycerol uptake; as such, an avoidance in either substrate's limitation was recommended to meet target butanol quantities (Sabra et al., 2014). The presence of glucose in a mixed culture system along with a mix of both gaseous and aqueous substrates could lead to biological competitive interaction, decreased product yields, and other adverse effects.

#### 2.1.2.1.2 Glycerol

The applicability of glycerol as a substrate for biofuels production stems from the surplus presented from existing biodiesel industries intent on maximizing useful product from every output stream generated. As such, research interest with use of both pure and crude sources of glycerol has begun to extend itself with further studies done. The use of glycerol by *C. pasteurianum* as the sole substrate in contrast to glucose had provided greater molar quantities of ethanol, butanol, and 1,3-propanediol with glycerol tolerances up to 17% w/v without inhibition recorded. With iron limitation occurring during glycerol consumption, the product profile was in favour of 1,3-PDO synthesis (Dabrock et al., 1992). In a study utilizing glycerol from thin stillage, a greater productivity yielding 0.32-0.44 g butanol/g glycerol was found to be marginally better or on par with the definitive work conducted by Biebl (0.14-0.31 g butanol/g glycerol). The presence of lactic acid in the thin stillage had the

dual occupation of both an effective buffering agent along with acting as a secondary substrate to enhance butanol yield to about 8.7 g/L from 6.5 g/L (Ahn, Sang, & Um, 2011). It was also observed that increasing the glycerol concentration up to a value of 35 g/L had resulted in favourability of the butanol pathway being utilized versus the 1,-3-PDO pathway in a fermentation of crude glycerol by *C. pasteurianum* (Gallardo et al., 2014). As discussed previously, the mixed substrate fermentation of glucose and glycerol provided challenges with substrate limitation and potential competitive interaction between microbes, but the presence of both substrates had been able to enhance growth and acid production (Sabra et al., 2014). Although the cost of ethanol produced from glycerol is approximately \$ 0.39 less per gallon in comparison to corn-derived ethanol, a combination of these processes together could prove lucrative in utilizing a wide range of waste materials for biofuel production (Yazdani & Gonzalez, 2007).

## 2.1.2.2 Gaseous Sources

### 2.1.2.2.1 Carbon Monoxide

There is no doubt that carbon monoxide is capable of providing itself as a theoretically abundant feedstock for a host of carboxydophilic and acetogenic microorganisms from industrially-produced syngas. The composition of impurities in syngas greatly vary depending on the industry from which it originates, but the major concerns of elevated concentrations in ammonia, nitric oxide, or tar particulates surpassing a size of 0.025  $\mu\text{m}$  have a greater effect on hindering fermentations. Through potential shifts in the redox potential of the system or damaging cellular growth due to acute toxicity, a recommendation in an initial gas cleanup prior to fermentation should be performed to ensure ideal conditions are met (Xu, Tree, & Lewis, 2011). Despite this pretreatment concern, a host of interesting results have been discovered regarding carbon monoxide utilization. The onset of butanol production had been seen with two non-carboxydophilic organisms in the presence of CO. *C. pasteurianum* was observed to produce significantly more solventogenic products such as ethanol and butanol with CO concentrations as low as 1% v/v within the fermentation atmosphere (Dabrock et al., 1992). Likewise, similar titres in solvent production were seen with *C. acetobutylicum* grown on glucose in the presence of CO, along with a noted absence of acetone production in the bulk liquid (Meyer et al., 1986).

Initial studies with CO and acetogenic cultures had determined that CO was selectively incorporated into the carboxyl group of acetate when used in the Wood-Ljungdahl metabolic pathway (Diekert & Ritter, 1983). Further research had helped identify which species had actually used CO as an agent of both growth and production, as exemplified in a comparative study of *E. limosum* exhibiting this desired behaviour while *Methanobacterium thermoautotrophicum* could not (Genthner & Bryant, 1982). To assist in increasing the uptake of gaseous CO within a bulk liquid, additions of reducing agents to alter the electron flow in microorganisms to more solventogenic means along with the onset of sporulation in applicable cultures saw an increase in solvent titre and CO uptake (Klasson, Ackerson, Clausen, & Gaddy, 1992). In initial work done as an attempt to establish a continuous syngas fermentation system, artificial blends of CO and CO<sub>2</sub> using either *C. carboxidivorans* or *C. ljungdahlii* had seen steady-state molar yields of ethanol, butanol, and acetic acid at 0.15, 0.075, and 0.025 mol/mol CO, respectively, along with a cellular yield of 0.25 g/mol CO observed (Rajagopalan et al., 2002). More recent work with *C. carboxidivorans* showed promising consumption of CO in rates ranging between 50-80%. Additionally, operating at a pH-controlled system at a value of 5.75 had produced concentrations of ethanol and butanol at 5.55 and 2.66 g/L, respectively, due to initial CO consumption along with residual consumption of organic acids produced in the starting period of the fermentation (Fernández-Naveira, Abubackar, Veiga, & Kennes, 2016). The promising results from the realm of CO-consuming organisms can lead to the development of bioindustrial processes capable of converting an existing demand of residual syngas formation as a means to develop realizable biofuel production.

#### 2.1.2.2.2 Hydrogen & Carbon Dioxide

The dual-needed nature of hydrogen and carbon dioxide as gaseous substrates to both acetogenic and carboxydrotrophic organisms generally appear to assist in the utilization of carbon monoxide in artificially- and industrially-made syngas systems. While acetogens such as *A. woodii* have been reported to not be able to produce products greater than C<sub>2</sub> compounds from H<sub>2</sub>/CO<sub>2</sub> mixtures, cultures containing the necessary genetic traits such as *E. limosum* had been seen to produce compounds as long as butyric and hexanoic acids from this simple C<sub>1</sub> gas (Sharak Genthner et al., 1981). In the presence of CO, CO<sub>2</sub>, and H<sub>2</sub>, preferential utilization of CO had occurred before any significant levels of H<sub>2</sub> or CO<sub>2</sub> had

been recorded with *E. limosum* (Genthner & Bryant, 1982). Furthermore, other carboxydrotrophs exhibited similar behaviour such as *C. ljungdahlii*; in fact, with general stoichiometric relations developed in terms of syngas fermentation, the theoretical increase in CO and H<sub>2</sub> components in a gas mixture would initiate production and then consumption of biologically-synthesized CO<sub>2</sub> in the pursuit of organic acids and solvents (Klasson et al., 1992). To combat the use of nearly 60% of available CO<sub>2</sub> during syngas fermentation with carboxydrotrophic cultures, recommendations to double the amount of hydrogen available in comparison to CO<sub>2</sub> in a syngas feedstock would assist in diverting conversion from gaseous products to more desirable liquid fuels instead (Rajagopalan et al., 2002). Recent studies comparing the use of synthetic producer gas with actual producer gas had observed the immediate cessation of hydrogen use with the actual producer gas cases – this phenomenon was caused due to high levels of nitric oxide and acetylene being present in the mixture. As such, any attempt for hydrogenase enzymes to be activated had been nullified by these gaseous impurities and limited the overall consumption rate and end product concentration of *C. carboxidivorans* (Ahmed et al., 2006). As such, before any work on true syngas fermentations is to be conducted, an investigation into effective cleanup technologies should be employed to avoid any sort of inhibition from a substrate already difficult to utilize.

### 2.1.3 Intermediates

#### 2.1.3.1 Acetic Acid

Common to all organisms presented in this review, the formation of acetic acid acts as the initial indicator that product formation begins in biofuels production. The acquisition of acetic acid by either chemical or biological means is well-practiced and understood, with examples such as the Reppe process used by BASF being synonymous with anaerobic fermentation of carbon monoxide, as the same base of utilizing methanol and CO chemically also exhibits the same carboxyl-forming behaviour that anaerobic species use CO for in producing acetate (Diekert & Ritter, 1983). However, the formation and/or utilization of acetic acid is not only prevalent with syngas fermentation. While undergoing glucose fermentation with phosphate limitation, *C. pasteurianum* produced acetate and butyrate as major products independent of growth rate or the pH of the fermentation system. However, under glycerol fermentation, trace amounts of these acids were present while their solvent counterparts, along with 1,3-PDO, proved more favourable (Dabrock et al., 1992). Further

work with glycerol fermentation of *C. pasteurianum* had also concluded that external acetic acid additions up to 5 g/L had only slightly increased the final concentration of butanol during fermentation compared to butyric acid addition within the same added range (Sabra et al., 2014).

To return to syngas fermentations, a comparative study between *C. autoethanogenum* and *C. ljungdahlii* had observed the greatest acetate formation at near-neutral conditions at a syngas flow rate of 10 mL/min, in addition to a smaller ratio of ethanol to acetate as pH decreased and/or gas flow rates increased (Cotter et al., 2009). Finally, a concurrent syngas fermentation was done with carboxylic acid addition ranging from propionic to hexanoic acid in terms of molecular carbon size. Both acetic acid and ethanol had been produced during all fermentations in a near 1:1 ratio between these two products, with the amount of these products decreasing as carboxylic acid size increased (Perez et al., 2013).

### 2.1.3.2 Butyric Acid

As previously mentioned, the formation of butyric acid from both glycerol-consuming and carboxydophilic bacteria is present to some degree depending on the conditions of cultivation. For glycerol fermentation undergoing phosphate limitation and in the presence of CO, butyrate formation was observed in trace amounts compared to the bulk of butanol and ethanol produced with *C. pasteurianum* (Dabrock et al., 1992). Instead of hypothesizing that this substance was not produced during fermentation, later research had concluded that the majority of this C4 acid had been utilized for increased butanol titres. It had been determined that supplementation of butyric acid to a fermentation medium had not only increased butanol yield by 45%, but decreased overall fermentation time along with a reduction in 1,3-propanediol formation using this same microorganism (Gallardo et al., 2014). Further evidence in this butyric acid uptake by *C. pasteurianum* had been seen in a mixed substrate fermentation of glucose and glycerol. In this system under pH control, a second claim to higher butanol-to-1,3-PDO ratios had been recorded with butyric acid addition, yet with a diminished growth rate noted after an initial addition of the acid versus a later addition when pH was 6.0 or 5.5 (Sabra et al., 2014). The inclusion of a perstractive membrane as a means of effectively separating tolerable organics like butyrate from toxic products such as butanol during fermentation had been noted as beneficial in maintaining the

vitality of a microorganism capable of converting these acids with *in situ* product removal (Xue, Zhao, et al., 2014).

In terms of enhancing the available quantity of butyric acid during fermentation, a secondary carboxydrotrophic organism can naturally supply a primary organism like *C. pasteurianum* with an alternative substrate for butanol synthesis. A multitude of solvent-producing species, including several carboxydrotrophs, have been genetically sequenced and recognized to produce butyrate without any sort of genetic modification. It was detected that *C. carboxidivorans*, under an environment of 100% v/v CO, had produced equimolar amounts of ethanol and butyrate combined (Bruant et al., 2010). Should other acetogens and carboxydrotrophs likely contain the same genomes as *C. carboxidivorans* or *E. limosum*, the onset of butyric acid formation should be expected during mid-exponential growth once enzymes like thiolase, crotonase, and butyryl-CoA dehydrogenase have been synthesized during fermentation (Jeong et al., 2015). Strategies to enhance effective butyric acid formation leading to further butanol production follows a similar stream of thought. Concepts such as butyric acid feeding and manipulation of the redox potential with carboxydrotrophic organisms would allow a greater titre of butyrate for reutilization (Perez et al., 2013).

## 2.1.4 Products & Byproducts

### 2.1.4.1 Ethanol

The recognition of ethanol worldwide is almost always contributed to the presence of microorganisms degrading some sort of carbohydrate compound. Excluding yeast fermentations to produce alcoholic beverages, it can be widely understood that the foundational ABE fermentation is one of the best known bioprocesses to date. However, despite its widespread use and popularity, current research interests in biofuel acquisition have underplayed this chemical in favour of its more energy-intensive relative, butanol (Jang et al., 2012). Even more interesting, the pursuit of both these biofuels has been underway even in the domain of syngas fermentation. Initial studies in the pursuit of a continuous syngas fermentation using *C. carboxidivorans* had found that when growth on a mixture of CO and N<sub>2</sub> without the presence of H<sub>2</sub> or CO<sub>2</sub>, fermentation still resulted in ethanol production with little to no cellular growth observed, thus demonstrating the microbiological affinity this compound possesses (Rajagopalan et al., 2002). Likewise, pressurized

fermentations of another clostridial relative, *C. ljungdahlii*, had seen its highest ethanol concentration of 0.6 g/L at pressures between 1.6-1.8 atm using a 55:20:10 % v/v blend of CO:H<sub>2</sub>:CO<sub>2</sub> with argon as the residual makeup gas. Several indications had been made in this study that the ability of hydrogen to act as an electron acceptor in this system could possibly promote the production of ethanol (Younesi et al., 2005). Within these studies, ethanol production had achieved steady-state following 60 hrs post-inoculation, and the desired ethanol:acetate product ratio of 5:1 was achievable at a pressure of 1.8 atm, providing promising data for future scale-up with this model (Najafpour & Younesi, 2006). A review recently conducted in pursuit of bioethanol from carbon monoxide had stated that the requirement of available sulfur, iron, nickel, and zinc along with the limitation of cobalt, phosphate, and particular B-vitamins were found to increase ethanol production while limiting co-production of acetate as well (Köpke, Mihalcea, Bromley, et al., 2011). Further ethanol studies had also concluded that a reduction in initial pH and yeast extract with an increase to initial system pressure and reducing agent concentration contributed to a 200% increase in ethanol production using *C. autoethanogenum* (Abubackar et al., 2012). While the pursuit of butanol generally attempts to limit the amount of ethanol produced, the ability to pursue both alcohols at reasonable titres is not necessarily out of the question. Recent work using *C. carboxidivorans* confirms this statement with achieving 2.66 g/L and 5.55 g/L of butanol and ethanol, respectively, using 100% v/v CO fed in a 2 L bioreactor setting at a rate of 10 mL/min (Fernández-Naveira et al., 2016).

#### 2.1.4.2 Butanol

The entire focus of this work hinges on the ability for these microorganisms to effectively produce butanol while retaining viable cellular growth within a closed fermentation system. The necessity of butanol as the biofuel of the future arrived in the advent of the new millennium – originally used as a chemical precursor for several polymer, solvent, and additive applications, the initiation of interest with this four-carbon alcohol as a superior biofuel has spurred a relentless period of research and experimentation into achieving biologically-feasible quantities for industrialization (Jang et al., 2012). The story of butanol, like bacterially-derived ethanol, derives from ABE fermentation during the spur of biochemical applications in the 20<sup>th</sup> century. While largely abandoned in later years, a resurgence into this area of research brought about several significant findings. With the use

of the Weizmann organism – *C. acetobutylicum* – a two-stage fermentation consisting of an initial growth period followed by a production period under continuous gassing by CO had concluded that this gas inhibited further growth and acetate/butyrate formation, but had allowed solventogenic products such as ethanol and butanol to begin formation. After CO sparging, a drop in butanol production was also noted (Meyer et al., 1986). Much like its clostridial cousin, *C. pasteurianum* exhibited the same behaviour with fermentation on glycerol, where further experiments also concluded increasing available iron and inducing phosphate limitation would allow greater butanol yields (Dabrock et al., 1992). The formation of butanol from either glucose or glycerol is seen to be redox balanced and exhibits the formation of other byproducts, such as hydrogen or 1,3-propanediol, as a means to regulate electron flow throughout its metabolism (Fischer et al., 2008). The ability to produce butanol from raw and/or unrefined substrate sources also remains viable as well; experimentation done with crude glycerol-rich thin stillage on *C. pasteurianum* had produced approximately 6.2-7.2 g/L butanol, with this concentration reaching 8.7 g/L when lactic acid was included in the feedstock. Additionally, the lactic acid also acted as an adjunct buffering agent to maintain a pH within the bounds of exhibiting solventogenic behaviour during fermentation (Ahn et al., 2011). In the attempts to use a co-substrate system of glucose and glycerol, a butanol concentration of 21 g/L at a productivity of 0.9 g/L·hr was made possible when neither substrate was limited. This co-substrate fermentation was able to retain high levels of butanol and organic acids while reducing the amount of 1,3-PDO produced, proving much more favourable in enriching butanol titres (Sabra et al., 2014).

In terms of butanol from syngas fermentations, several studies make a noteworthy case regarding the possibility carboxydrotrophic organisms have in either directly producing this solvent or contributing intermediate compounds that could aid in further butanol yields. A series of fermentations coupled with genomic analysis of *C. carboxidivorans* had seen molar quantities of butanol at about 891 mmol/mol CO under a 100% v/v CO atmosphere. In this fermentation, it was also made prominent that fermentation had stopped once cultures achieved stationary phase, as CO uptake and CO<sub>2</sub> production completely ceased without any further solvent generation (Bruant et al., 2010). While undergoing syngas fermentation blended with an array of carboxylic acids prior to fermentation, *C. ljungdahlii* was observed to convert organic acids such as acetate, butyrate, and hexanoate to their associated alcohols

of ethanol, butanol, and hexanol. The study had also remarked that the replacement of H<sub>2</sub> for CO in this organic acid reduction system would likely avoid any byproduct formation and strictly focus on near-full conversion to butanol (Perez et al., 2013). Finally, as discussed above while covering ethanol, a fermentation using pure CO in a bench bioreactor setting produced significant titres of ethanol and butanol using *C. carboxidivorans*. After a period of time, part of the medium was removed and flushed with freshly prepared medium as to remove the solvents, and it was seen that any acids produced following this flush had undergone near-complete conversion to their counterpart ethanol or butanol constituents as pH decreased likewise (Fernández-Naveira et al., 2016). All in all, the above discussion, along with a combination of techniques, cultures, and understanding of microbiological functionality, could lead the way into realizing butanol production at a true industrial level.

#### 2.1.4.3 2,3-Butanediol

The presence of the less commonly pursued 2,3-butanediol establishes this substance more as a byproduct than a desired product like butanol due to a smaller amount of applications and industrial uses in comparison. Nevertheless, 2,3-BDO has several applications in biopolymer synthesis and precursor chemical usage in certain epoxides. For example, American-based waste gas conversion company LanzaTech recently developed a process converting steel mill off-gas to ethanol and 2,3-BDO using company-developed microbes (Köpke, Mihalcea, Liew, et al., 2011). In terms of native clostridial species capable of producing this diol, both *C. ljungdahlii* and *C. autoethanogenum* were the first of what could be potentially many able to synthesize this compound (Tracy et al., 2012). While the ability to produce 2,3-BDO exists, actual production rates are comparatively slow for *C. autoethanogenum* at a specific rate of about 0.06 mol/L·day (Wang et al., 2013). This caveat, along with the inability for an organism such as *C. pasteurianum* to consume 2,3-BDO, leads very little interest in understanding the production parameters of this biochemical (Ahn et al., 2011).

#### 2.1.4.4 1,3-Propanediol

Much like 2,3-BDO, 1,3-propanediol is a popular chemical building block for several polymers, adhesives, and coatings such as polyethylene terephthalate (PETE). Unlike 2,3-BDO, however, this byproduct derives from glycerol-based fermentations from organisms such as *C. pasteurianum* and *C. acetobutylicum*; with these organisms, the production of 1,3-PDO acts as a general regulator for controlling internal redox balance within a system (Dabrock et al., 1992). To expand on this, it was determined that available  $\text{NADH}_2$  was able to predict the yield of 1,3-PDO per molecule of glycerol used. It was also noted that an increase in butyrate formation was found to decrease 1,3-PDO yield in clostridial cultures (Biebl, Menzel, Zeng, & Deckwer, 1999). Research done on both pure and industrially obtained glycerol on mixed cultures had been able to produce significant amounts of 1,3-PDO and hydrogen, but the same fermentation on glucose had only been able to yield hydrogen (Selemba, Perez, Lloyd, & Logan, 2009). As previously mentioned, the addition of butyrate along with a decrease in pH had been seen to decrease the butanol:1,3-PDO ratio by stopping 1,3-PDO production altogether, and in turn increase the yield of butanol (Sabra et al., 2014). Once again, another study that had increased crude glycerol concentration and/or decreased nitrogen source with *C. pasteurianum* had seen a decrease in this byproduct's formation with further increase to butanol yield, and as such the necessity to purify this substrate at an industrial scale appears unlikely when optimizing for butanol (Gallardo et al., 2014). All efforts to limit the production of 1,3-PDO until desirable levels of butanol can be achieved with uninhibited cellular growth should be pursued as necessary.

### 2.1.5 Physical Phenomenon

#### 2.1.5.1 Mass Transfer

The major considerations in mass transfer limitations come in the form of effective gassing distribution and solubility constraints within a major bulk liquid as opposed to the interaction of already aqueous substrates with cellular cultures. Despite this, improvements for mass transfer with glycerol-consuming microorganisms could be suggested with the use of immobilized cell systems in a fermenter. A review discussing the entirety of ABE fermentation research had concluded that an application of thin-layer adsorbed cells on a low-cost inert support versus traditional gel encapsulation would provide an economical,

low-maintenance method to retain a high-throughput of fermented broth without major toxic or substrate-limited effects occurring (Jones & Woods, 1986). With this still in mind, it is important to note that the greatest challenge in a gas-substrate fermentation system is almost always related to the effective solubility of the gas in a liquid and finding opportunities to overcome any limitations. While the choice of a bioreactor defines what sort of mass transfer occurs, the most common choice in industry remains the continuous stirred-tank reactor (CSTR). A comparison in two agitation rates had noted that a 50% increase in the agitation rate of a CSTR system provided a 360% increase in the value of the mass transfer coefficient  $k_{LA}$  due to vortex mixing within the fermenter (Klasson et al., 1992). A comprehensive study into the impeller design and schematic setup in an artificial CO-water system compared power demand to  $k_{LA}$  values achieved; it was determined that the best mass transfer per unit power drawn had been made possible with the use of a dual impeller setup with axial flow impellers at the top of the impeller shaft (Ungerma & Heindel, 2007).

Operation of a 100-L pilot fermenter had run for nearly 60 days during continuous syngas fermentation using *Clostridium ragsdalei*. Further use of a dual impeller system combined with a microsparger had allowed an improvement in mass transfer performance without any changes needed in impeller configuration or increasing gas flow rate (Kundiya, Huhnke, & Wilkins, 2010). These improvements, united with advancements in understanding fluid flow patterns, gassing power efficiency, and baffle design would significantly improve the volumetric mass transfer coefficient during syngas fermentation. Substantial work done on the application of hollow fiber membranes (HFM) as an effective means of gaseous diffusion had seen vast improvements in CO mass transfer (Munasinghe & Khanal, 2010). These considerations in mass transfer principles must be included in additional process economics such as minimal gas and medium reprocessing, lowering product separation cost, and achieving homogenous mixing as a twofold method to achieve optimal performance (Köpke, Mihalcea, Bromley, et al., 2011). Further work should be executed in the pursuit of maintaining either a high driving force or volumetric mass transfer to achieve the most efficient conversion on syngas. Applications of high-cell density cultures, possibly from pressurization, could prove ultimately successful in achieving truly obtainable levels of CO for substrate utilization (Yasin et al., 2015).

### 2.1.5.2 Heat Transfer

The heat control and transfer operations mainly derive from biological and mechanical heat generation during fermentation, and as the majority of these fermentations operate at conditions slightly above standard temperature and pressure (STP), little concern is placed in this area of reactor design with the use of mesophilic bacteria. Future plans to upgrade and industrialize these processes must consider the necessity to cool influent syngas entering a fermenter to tolerable levels (Henstra et al., 2007). The integration of a heat recovery system used in unison between an exit gasifier stream and the temperature jacket of a fermenter could provide a very efficient and economical manner in distributing heat optimally (Munasinghe & Khanal, 2010). As well, the application of photosynthetic fuel cells were proposed for *C. ljungdahlii* – photosensitive particles capture the right amount of energy to convert CO<sub>2</sub> to CO and in turn have these cells consume this CO as an effective driving force from heat transfer principles (Köpke, Mihalcea, Bromley, et al., 2011).

### 2.1.6 Production Media Composition

The majority of work performed in both areas of glycerol and syngas fermentation have their own definitive production medium used. The majority of syngas fermentation-patented material references PETC medium used in the defining study of *C. ljungdahlii*, likely due to its simplistic design and role involved in works such as this (Tanner et al., 1993). For glycerol fermentation, Biebl medium utilizes a significant amount of dissolved iron to better activate ferredoxin and other iron-related enzymes necessary to propel solvent production (Biebl, 2001). An additional nutrition study regarding the growth and production of *C. carboxidivorans* on artificial syngas had revealed the necessity of nickel, tungsten, molybdenum, iron, and zinc in trace amounts to better regulate the metabolism of the Wood-Ljungdahl pathway (Phillips et al., 2015).

## 2.2 Aerobic Xanthan Fermentation (AEXF)

Equipment limitations regarding the use of CO in larger scale fermenters such as the pressure fermentation used made syngas fermentation unavailable for these later experiments. As such, an analogous series of experiments using the xanthan fermentation as a model process for solubility-limited fermentations was investigated to provide heuristic solutions with future work. The available knowledge regarding the nature, kinetics, and operating parameters surrounding xanthan fermentation by *Xanthomonas campestris* can be seen as extensive and encompassing as time has progressed. The foundational work to industrialize this process can be understood by the middle of the previous century. As such, the standard of these fermentations were found to operate at 28 °C with a neutral pH, and that maintenance of dissolved oxygen content above 20% provided ideal growth and production conditions. The major limitation in this aerobic fermentation is the notable decline in specific product formation due to increasing viscosity caused by the product therein (Moraine & Rogovin, 1973). With clearance provided by the United States Food and Drug Administration (USFDA) by 1969 as a thickener and stabilizer with the same clearance offered by the European Commission (EC) in 1980, it can be more than acceptable to state that this biopolymer is a safe and major commodity derived from the bioprocess industry. The general process involves supplementation with a carbohydrate and nitrogenous source in a defined salt solution over a set period of time, where once completed, the broth is heat-sterilized, precipitated, dried, milled, and finally distributed as xanthan gum of a certain degree or quality (Katzbauer, 1998).

Due to the complexity of the xanthan molecule, a host of factors and operational considerations can change some fundamental properties of the polymer produced. Conditions such as temperature, nitrogenous source type and concentration, and oxygen mass transfer rates can dramatically affect the molecular weight of xanthan post-fermentation. Additionally, changes in quality due to the effects of acetylation and/or pyruvilation can alter the rheological behaviour of xanthan when suspended in solutions or mixed with other biopolymer matrices (Casas et al., 2000). The versatility of xanthan remains remarkable in terms of applicability in various temperature, pH and salts content ranges that would usually hinder the ability other polymers possess. Significant consideration, however, must be made in the cultivation conditions of *Xanthomonas* when it produces this biopolymer, as fluxes in

temperature, pH, medium composition, available oxygen content, and the effect of pseudoplasticity greatly influence the quality/quantity of xanthan made (García-Ochoa, Santos, et al., 2000). This shear-thinning property can prove the most challenging parameter to consider during operation, as areas of anoxic ‘dead zones’ can form, creating non-uniform growth and production profiles in a fermenter. A study done in increasing the agitation speed of a fermenter as means to avoid such behaviour had denoted higher pyruvate contents with increased speed, but no effect made on the molecular weight of the final product (Papagianni et al., 2001). A hefty focus on the mass transfer principles undergoing xanthan fermentation provide a better titre and quality of xanthan produced; attempts to maintain constant  $k_{La}$  coefficients through a host of process control monitoring and/or adjusting agitation, superficial gas flow, volumetric aeration, and other parameters have been pursued extensively (Palaniraj & Jayaraman, 2011).

More recent work attempts to upgrade current batch operations to continuous systems capable of cell retention while efficiently removing this pseudoplastic product. A study modelling the use of internal and external cell recycling had determined that these systems could provide xanthan production rates approximately 12-17 times higher than the standard batch fermentation used (Zabot et al., 2012). Future demand and consideration of further applications with xanthan gum appear to be increasing as research progresses in this process. Approximately 61% of 5,929 publications on xanthan from 2005 to 2014 had been related to patented technologies, with progressive focuses into the fields of aerogels, nanocrystals, and biomedical luminescent materials as potential forays into new and exciting industrial opportunities (Petri, 2015).

### 2.2.1 Cellular Cultures

Nearly all microorganisms used for the production of xanthan are derived from the *Xanthomonas* genus of the *Pseudomonaceae* family. These organisms are plant pathogens, and commonly known as ‘black rot’ on many popular vegetables consumed in day-to-day life, such as cabbage, lettuce, and beans (García-Ochoa, Santos, et al., 2000). Of the majority of strains known, *X. campestris* remains the most popular option in bioprocessing systems, given the full sequencing of its three most popular type strains and general ease of use for growth and production (Enshasy & Homosany, 2011). While several studies have been performed regarding genetic modification and plasmid insertion with *Xanthomonas* with

success, the capability to surpass existing xanthan gum yields with these new strains appears unlikely at an industrial scale (Rosalam & England, 2006).

## 2.2.1.1 Xanthan-producing Bacteria

### 2.2.1.1.1 *Xanthomonas Campestris*

As mentioned prior, the bulk of research conducted to date has used *X. campestris* or one of its three fully-sequenced type strains. These cells are Gram-negative, motile, and exist as single straight rods sized between 0.7-1.8  $\mu\text{m}$  long with a single polar flagellum between 1.7-3  $\mu\text{m}$  long. As an obligate aerobe that requires oxygen as a terminal electron acceptor for respiration, these cells exist as large (L), small (S), or very small (VS) strains, where older strains originally existing as L strains can degrade to non-xanthan producing VS strains over extended periods of time (García-Ochoa, Santos, et al., 2000). The metabolic pathway *X. campestris* utilizes is a conjugation between the Entner-Doudoroff pathway and the tricarboxylic acid cycle to effectively consume substrate, primarily glucose. This carbohydrate consumption results in xanthan biosynthesis through sequential polymerization of monosaccharides and a small routing of residual glucose into the pentose phosphate pathway for other cellular functions (Rosalam & England, 2006). Cellular functionality requires neutral conditions at a temperature between 28-30 °C for optimal growth and production kinetics to occur, as pH levels below 5.0 drastically reduces biomass and xanthan formation during fermentation. As the pH tends to decrease during product formation due to the synthesis of organic acids added to side chains of the xanthan polymer, a buffering agent or pH control system is usually recommended to prevent severe acidification (Palaniraj & Jayaraman, 2011).

## 2.2.2 Substrates

### 2.2.2.1 Solid Aqueous & Liquid Sources

#### 2.2.2.1.1 Glucose

The role of an effective carbon source in the generation of xanthan gum greatly influences not only the fermentative parameters but the quality of the polymer produced. Initial work into the effects of glucose on kinetic rates attempted to determine when glucose limitation occurs. It was established that substantial declines in consumption rates were seen at levels below 0.2% w/v, but a declining specific rate was observed at levels much higher than this. Overall, any amount of glucose above 0.5% w/v was found to not be rate-limiting to *X. campestris* (Moraine & Rogovin, 1973). Later research investigated a host of different carbon sources on the typical aerobic xanthan fermentation; of all the sugars tested, glucose at 4.0% w/v proved optimal for enhanced xanthan formation. When compared to an identical concentration of sucrose yielding similar endpoint titres, the glucose-derived xanthan had superior polymeric properties synthesized throughout the entirety of the fermentation (De Vuyst & Vermeire, 1994). Another review confirmed the efficacy glucose has over other common carbon sources with having the highest xanthan yield approaching 15 g/L. A combination of results collected had inferred that high glucose concentration should be induced once the culture achieves stationary phase, as several glucose-introducing techniques such as pulsing or C/N ratio manipulation with time had seen significant yields in xanthan produced (Rosalam & England, 2006). A co-substrate fermentation of glucose and xylose using *X. campestris* had yielded results once more in favour of glucose use. While increasing the xylose concentration had provided a higher quality xanthan compared to pure glucose synthesis, the xylose proved inhibiting to the bacterial culture and proved counterintuitive towards efficient substrate utilization (Zhang & Chen, 2010). A second investigation into the kinetic patterns had concluded that the consumption of glucose was heavily dependent on the cellular growth with production rates seen at higher levels during exponential growth compared to the stationary phase (Gilani, Najafpour, Heydarzadeh, & Zare, 2011). With all the evidence collected, it is more than apparent that glucose remains the ideal substrate in xanthan fermentation, but future pursuits in finding economically-feasible feedstocks should be inspected.

### 2.2.2.1.2 Other Sugar-based Compounds

As suggested above, several alternatives to expensive and purified glucose feedstocks have been explored in the attempts to meet, and if possible exceed, the bulk of optimal fermentation parameters glucose sets. An investigation into the use of sucrose, molasses, and corn syrup supplied by Sirodex was performed to find if any of these substrates proved worthwhile for fermentation by *X. campestris*. When compared to glucose, the xanthan produced by sucrose had lower viscosity achieved in solution and was indicative of a poorer-grade xanthan. While molasses was comparable to the ideal glucose fermentation in terms of endpoint concentration and quality of xanthan, a greater concentration of molasses was needed along with significant adjustments to precultures to account for unaccounted lag time. The corn syrup had yielded xanthan concentrations lower than sucrose and had been comparable in lag and reduced kinetics with that of the molasses scenario (De Vuyst & Vermeire, 1994). Further studies on beet root molasses had indicated that a neutral pH with 4 g/L  $K_2HPO_4$  and 175 g/L molasses proved optimal and comparable to the standard glucose-run xanthan fermentation (Kalogiannis, Iakovidou, Liakopoulou-Kyriakides, Kyriakidis, & Skaracis, 2003). Review into several unmodified and partially hydrolyzed starch feedstocks – such as rice, barley, corn flour, among others – had hypothesized that the effects of certain functional groups, differing composition per substrate used, byproduct formation, possible influence of inhibitors, and high concentrations of non-reacted compounds directly influenced the low quantity and quality obtained during fermentation (Rosalam & England, 2006). Another study into pretreated, hydrolyzed molasses had determined that the yield from pretreated molasses had provided xanthan concentrations approximately 1-2 g/L greater than the raw study done prior (Gilani et al., 2011). All in all, several steps in either pretreating these feedstocks and/or conditioning precultures prior to fermentation must be done to increase yields to realistic values.

## 2.2.2.2 Gaseous Sources

### 2.2.2.2.1 Oxygen

As with any aerobic bioprocess, oxygen is the key element for not only cellular propagation but enhanced product formation and enzymatic activity for a given system. In some of the definitive work aimed at resolving the kinetics involving oxygen and *X. campestris*, dissolved oxygen tension was observed to remain at about 50% during regular operation. When sparging air was switched off, the oxygen tension decreased at an almost constant rate where any value below 20% dissolved oxygen significantly decreased the oxygen consumption rate of the microorganism (Moraine & Rogovin, 1973). One study investigated the relationship linking kinetics, mixing, and drawn power as a means to predict xanthan formation; it was observed that during stationary phase or in oxygen-limited regimes, the mean molecular weight (MMW) of xanthan was linearly dependent on the specific oxygen uptake rate, thus directly affecting the quality of the polymer. When oxygen was not limited, the level of DOT within the fermenter directly affected the xanthan concentration and rheology, and as such, the model developed proved to instead increase the consistency index of the broth and therefore increase the xanthan quality in comparison to a DOT-controlled system (Serrano-Carreón, Corona, Sánchez, & Galindo, 1998). In the determination to create a model capable of influencing the oxygen mass transfer and uptake rates for *X. campestris*, results had shown a slow decrease in oxygen concentration during lag but near depletion of oxygen as the exponential phase was reached. With increasing biomass and therefore increasing biological oxygen demand, the stirrer speed of the system had to increase in order to retain constant oxygen mass transfer properties (García-Ochoa, Castro, et al., 2000). Recent work had resolved another oxygen-controlling model with the use of heat transfer coefficients and on-line viscosity prediction, as the use of biomass stoichiometry combined with calorimetric data could derive specific oxygen uptake rates via cellular respiration (Schelden, 2015).

## 2.2.3 Products & Byproducts

### 2.2.3.1 Xanthan

To understand the driving force and challenges behind this fermentation, a fundamental understanding into the xanthan molecule, how it is created, and its intrinsic properties must be done. The main chain of xanthan is comparable to that of cellulose as it consists of 1,4-linked  $\beta$ -D-glucose residues, while the protruding side chains are composed of  $\beta$ -D-mannose-1,4- $\beta$ -D-glucuronic acid-1,2- $\alpha$ -D-mannose – these side chains can also experience acetylation and/or pyruvilation at the internal and terminal mannose residues, respectively (Katzbauer, 1998). When in aqueous solution, these branches change their interactional behaviour from disordered to ordered conformations when factors such as shear rate, temperature, and ionic strength comes into play. This property, in unison with a wide range of stability regarding pH, temperature, and pK values, has provided xanthan with use as an emulsifier, thickener, and lubricant in a host of industries (Casas et al., 2000). Shifts in fermentation conditions, such as medium composition, concentration type of carbon/nitrogen source, and temperature can drastically change the expected molecular weight and structure of the polymer. Additionally, the increase in xanthan or salts concentration in solution was found to significantly increase the viscosity of the fermentation broth, which poses several challenges in operation and product recovery (García-Ochoa, Santos, et al., 2000).

The standard recovery of xanthan after fermentation involves sterilization, precipitation, and milling for further use. Attempts to retain a cell-free broth for ease of extraction were pursued in the use of heat treatment and ultrafiltration. While a cell-free broth was maintained to some success, the excessive fouling of the membrane during operation would prove too frequent and impractical for full-scale industrialization (Rosalam & England, 2006). Precipitation techniques aimed at increasing recovery of xanthan from clarified broth include organic solvent extraction using ethanol, isopropyl alcohol, and solvent mixtures with trivalent and tetravalent salts like aluminum. These solvents/mixtures were found to be addable to fermentation broth without major inhibition occurring while allowing a washout of impurities prior to downstream precipitation (Palaniraj & Jayaraman, 2011). Once this xanthan is purified, the boundless applications that this biopolymer presents grows with time. Recent studies have used combinations of xanthan with glycerides to greatly improve drilling fluids in typical oil recovery operations. At a micromolecular level,

low-concentration injections of xanthan into articular joints were found to act as an elastic shock absorber and able lubricant at low and high impact movements, respectively (Petri, 2015). With this discussion in mind, the necessity to pursue and perfect the xanthan fermentation remain relevant in the hopes to meet growing demands and expand the inventive nature of this biopolymer.

## 2.2.4 Physical Phenomenon

### 2.2.4.1 Mass Transfer

The ability to solubilize necessary amounts of oxygen into a fermentation system where notable fluxes in viscosity and consequentially fluid dynamics are apparent presents a host of challenges and opportunities to correct this problem. Fundamental work performed on a fermentation of *X. campestris* in a stirred tank bioreactor had used operational variables such as stirrer speed, superficial gas velocity, and broth viscosity as a means to enhance mass transfer in accordance with power law principles pertaining mostly to viscosity. As such, a dimensionless correlation utilizing the Sherwood number as the function of the Reynolds number along with provisions described by the Aeration and Weber's numbers had allowed an accurate prediction of  $k_{La}$  using two different rheological models (García-Ochoa, Castro, et al., 2000). Another study utilized a pH-uncontrolled bench fermenter with enriched oxygen-air to independently record the effects of aeration without hydrodynamic influence on xanthan production rates. This work, along with a host of others, had determined a linear relationship between agitation and specific production rates by noting that lower agitation provides lower production due to a lessened amount of cells able to utilize the dissolved oxygen within a system (Papagianni et al., 2001).

Further work expounded on the influence of agitation in terms of effective mass transfer achieved, by remarking that an increasing viscosity enlarges the film resistance to effectively transport oxygen to cell cultures. Furthermore, strict adherence to maintaining an either constant or increasing OTR remains essential in bioreactor design and implementation to successfully yield xanthan in a manageable time period (Garcia-Ochoa & Gomez, 2009). The ability to maintain a constant volumetric aeration rate is critical in providing effective and efficient bioreactor operation, but the ability to develop a dynamic agitation control system could prove considerably useful for growth and production kinetics. Studies that have

employed a varied agitation rate, beginning with low stirrer speeds to allow stronger growth before late-exponential and stationary phases while slowly progressing to a higher maximum agitation to better distribute oxygen for productive means once a substantial biomass concentration had been reached, have seen success in maintaining higher production rates and end yields for xanthan (Palaniraj & Jayaraman, 2011). More recent research done on a 50-L fermenter testing six different impeller combinations to compare power consumption with local and average volumetric mass transfer coefficients was performed using xanthan gum solutions. A correlation combining specific gassed power input, effective viscosity, and gas velocity, which had shown strong dependence on the first two criteria, was calculated for each impeller combination. In turn, it was seen that ‘large-diameter’ impeller configurations displayed more homogeneous  $k_{La}$  distribution than ‘small-diameter’ configurations, but poorer gas dispersion became apparent as viscosity increased (Xie et al., 2014). Little to no work has been observed regarding the effects of pressurization for enhanced mass transfer capabilities with *X. campestris* fermentations.

#### 2.2.4.2 Heat Transfer

The ability to maintain constant and reliable heat control in a system that has changing fluid properties much like this aerobic xanthan fermentation becomes a challenge and opportunity to utilize the issue of viscosity. To begin, it should be noted that as limitations on mass transfer increase with viscosity, the ability to properly transfer heat grows more challenging as higher volumetric power inputs are needed to maintain constant conditions. It can be rather difficult to estimate the true aerated power used during such a fermentation as calculation of the aerated power number is a complex function involving turbulence, aeration, and rheological conditions within a fermenter (Serrano-Carreón et al., 1998). Overall, while the heating conditions around xanthan production are rather mild in comparison to many petro- and thermochemical operations currently in use, a greater interest in the external influence of heat transfer processes such as cooling jacket and product recovery components should be considered. For example, over-application of heat post-fermentation as a means to sterilize and recover xanthan can prove ineffective at high viscosities or cause polymeric degradation (Palaniraj & Jayaraman, 2011).

To combat this issue and to provide further online analysis of a fermentation, the recording and utilization of various heat generation coefficients can be performed to better understand and protect the biopolymer. Past research had concluded that the measurement of biological heat generation could prove universal at any scale in calculating the amount of product synthesized at any point during fermentation (Regestein, Giese, Zavrel, & Büchs, 2013). Further work done on this subject had developed two techniques, with one based on cooling jacket mass flows and jacket temperature differences and the other based on varying the universal heat transfer coefficients with the temperature difference between the fermenter and the jacket, that can accurately quantify product yield on-line (Regestein, Maskow, et al., 2013). Explicit application of the aforementioned second method had recently yielded successful results and understanding with a xanthan fermentation run using different conditions and media (Schelden, 2015).

## 2.2.5 Production Media Composition

### 2.2.5.1 Complex Media

The use of complex and minimal media recipes requires understanding and insight into how these different mixtures work. Complex media will contain one or more compounds that cannot be accurately defined by its chemical composition due to the complexes of potential carbohydrate, protein, and elemental sources they may contain. More often than not, an industrial-scale batch cultivation of xanthan will use a complex media over a minimal recipe due to a general ease of use and provision of supplementary chemicals that can prove valuable for a growing culture (Palaniraj & Jayaraman, 2011). Earlier work done on complex medium composition had seen maximal xanthan production when a C/N ratio of 23 was achieved, along with the addition of  $\text{CaCO}_3$  and  $\text{KH}_2\text{PO}_4$  as light buffering agents and ionic supports for polymer propagation (Roseiro, Esgalhado, Amaral Collaço, & Emery, 1992). Another study, aimed at determining the interaction between pH and temperature on the growth and production cycle of *X. campestris*, had used an adjusted complex medium of the previous work discussed. It was determined that optimal conditions for growth and production were realizable at a pH of 6.0-7.5 and 7.0-8.0 along with temperatures between 25-27 °C and 25-30 °C, respectively (Esgalhado, Roseiro, & Collago, 1995). Later work provided the joint use of a complex carbohydrate source such as molasses with a complex medium containing yeast extract in the attempts to observe a kinetic model for xanthan

production; a maximum concentration of 17.1 g/L was achieved when the temperature, agitation, and initial glucose concentration were set at 32 °C, 500 RPM, and 30 g/L (Gilani et al., 2011). Finally, a series of differing complex media recipes were examined with the intent of replacing the long-used YM medium in the first stage of building growth in the xanthan bioprocess. Maximum cellular yield with a notable achieved viscosity had involved the use of 0.5% w/v yeast extract with 0.25% w/v  $(\text{NH}_4)_2\text{HPO}_4$ , where initial inoculum quality positively influenced the xanthan production and quality in the second stage of fermentation (Carignatto, Mustafé Oliveira, Gomes de Lima, & de Oliva Neto, 2011).

#### 2.2.5.2 Minimal Media

Minimal media, meanwhile, can be classified as a growth or production medium lacking any complex components and thus containing a set of compounds that can fully define the carbon and nitrogen content it possesses. Several key pieces of literature utilize minimal media recipes at all different scales of fermentation due to their reliability and reproducibility in experimental design combined with ease in determining exact yields for elemental and component mass balances. For example, a nutritional study aimed at determining which components prove most effective in growth and production kinetics was done in a newly developed minimal medium produced from factorial design analysis. Based on the results, magnesium and sulfur were able to benefit the growth and production phases separately, but the presence of nitrogen and phosphorous proved beneficial during both phases of cellular activity (García-Ochoa, Santos, & Fritsch, 1992). Furthermore, a study designed for increasing the reproducibility of pilot-scale fermentations aimed to reduce the variance of *X. campestris* cultures using a strict inoculation strategy between different fermenter runs. Over two years, there was no evidence of culture degradation and results collected remained in good agreement at the 14, 20, and 150 L fermenters tested (Amanullah, Serrano, Galindo, & Nienow, 1996). More recent work done on the several genetic modification strategies employed with the wild type of *X. campestris* attempted to resolve higher yields and an understanding of carbohydrate consumption using a minimal medium. A testing of 95 different carbon sources in the same minimal medium had provided a genetic model capable of determining which genomes were required for effective degradation and utilization of the carbon source during fermentation (Montforts, 2009).

### 2.2.5.3 Trace Elements Solution

The provision of a trace elements solution for xanthan fermentation assists to meet the demands *X. campestris* has for effective production purposes. A depletion in available phosphate can lower biomass accumulation with an overall decrease in pyruvyl substitution because of metabolic constraint (Rosalam & England, 2006). The influence of polyvalent cations in smaller quantities can prove beneficial in polymeric support in solution along with an easier ability to precipitate product following fermentation. The influence of compounds like calcium, aluminum, and quaternary ammonium salts proved most effective for precipitation, as monovalent salts such as sodium and potassium are unable to cause any sort of precipitation (Palaniraj & Jayaraman, 2011). Further investigation and a survey into the role of the trace elements solution and the components that make up all of it should be pursued for enhanced nutrient supplementation.

## Chapter 3

### 3 Materials & Methods

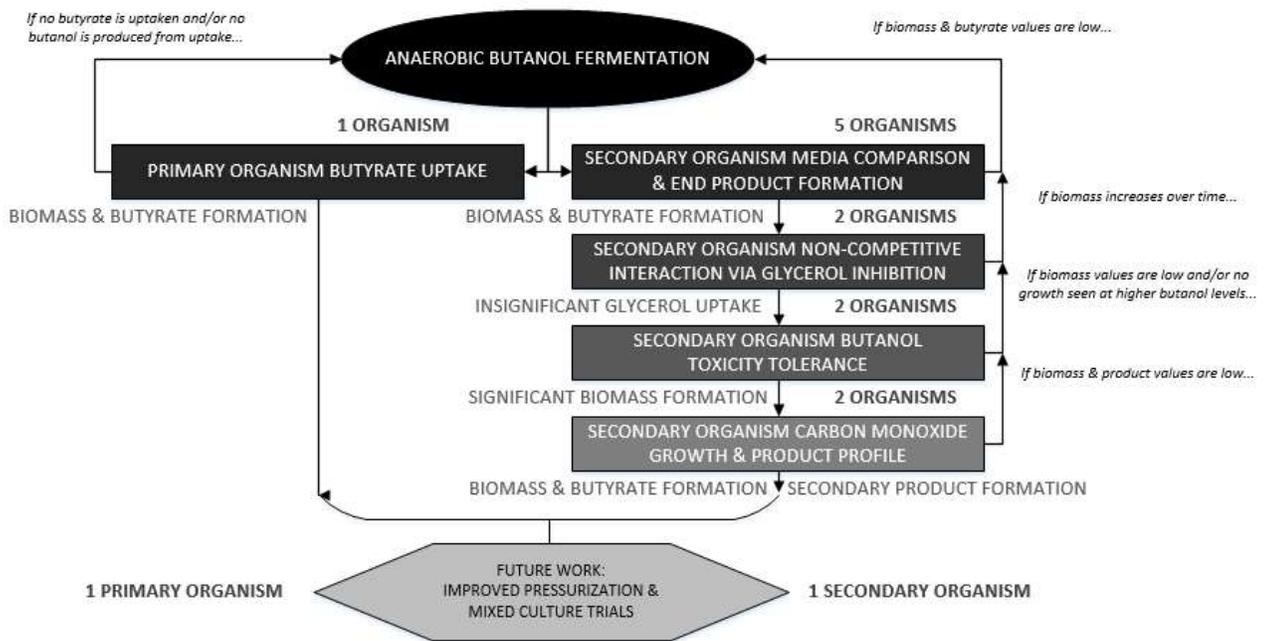
#### 3.1 Anaerobic Butanol Fermentation

##### 3.1.1 Research Design

The experimental methodology behind this section of research depends on the development of a mixed culture that is capable of producing butanol from glycerol and biologically synthesized butyrate with the primary organism along with promoting healthy, continuous growth between all organisms involved. Additionally, the produced butyrate must come from a significant amount of carbon monoxide converted by the secondary organism to justify the use of this adjunct species in a mixed culture setting. Efforts to achieve these demands have been highlighted in the experimental guidelines as mapped out in Figure 3.1.1. The primary aspects of this research must first independently investigate the ability for the primary organism, *C. pasteurianum*, to properly consume butyrate within the system and in turn increase the butanol titre in comparison to the typical fermentation scheme. Should a lack of butyrate uptake or associated butanol formation not be found, further research and inquiry into a suitable primary organism would have to be conducted.

The second part of this experimentation involves the screening of five potential carboxydrotrophic organisms with a series of experiments aimed at complementing the growth conditions of the primary organism. First, an investigation into prominent media comparisons and the growth/production profiles developed would assist in understanding which organisms are able to exhibit both healthy, strong growth along with the necessary formation of butyrate. Assuming at least two organisms exhibit this behaviour, further research could be done to determine whether these carboxydrotrophic bacteria are not able to consume glycerol in a glycerol-rich system. This aspect is important to determine in order for these organisms to exist in a non-competitive, substrate-independent environment. A lack of this behaviour would result in the exhaustion of the weaker species and render the mixed culture useless. Once two species have been found to be non-competitive with the primary organism, a test observing the tolerance towards lengthy exposure to butanol within a butanol-producing system. As the threshold value is well reported with a primary organism like *C. pasteurianum*, the ability to effectively grow and produce in the presence of

carboxydrotrophs must be present for the mixed culture to work. A lack of growth would therefore require another survey into other organisms that can meet the demands necessary for this system. Once found, the final round of experimentation for the secondary organisms would test the ability to effectively uptake carbon monoxide to produce more biomass and, more importantly, utilizable amounts of butyrate in order for enhanced butanol production to occur. When both aspects of this work have been established, future work involving actual mixed culture trials and the testing of higher-performing pressurization techniques could be performed to better utilize and understand the system proposed.



**Figure 3.1.1 – Experimental Decision Matrix Highlighting Work to be Done for the Anaerobic Butanol Fermentation.**

### 3.1.1.1 Chemicals & Reagents

#### 3.1.1.1.1 Production Media

For the following media used, all components, save for the trace elements and vitamins solutions along with  $\text{CaCl}_2$  for select media, were added prior to sparging with nitrogen and autoclaving at 121 °C for 25 minutes. Once sterile, these solutions were added to the bulk medium in an anaerobic chamber using aseptic techniques. Likewise, a solution of concentrated glucose or glycerol was added in a similar matter prior to inoculation with a preculture. Biebl medium was prepared as defined in its original work, save for the substitution of  $\text{CaCO}_3$  for 10 g/L MES as a buffering agent for shake flask experiments, as seen in Appendix 1. Adjustments to reach a pH of 6.5 was achieved using 3 M KOH and/or  $\text{H}_2\text{SO}_4$  respectively. The same acid/base combination was used for pH control at the bench-scale fermentation. Typical glucose concentrations for the shaker flask experiments include either 60, 30, or 20 g/L while glycerol concentrations at this level were adjusted to 30 g/L. At the bench-scale fermentation, 45 g/L of glycerol was added.

PETC medium was prepared as defined in its original work as seen in Appendix 2. Adjustments to reach a pH of 6.5 was achieved using 3 M KOH and/or  $\text{H}_2\text{SO}_4$  respectively. Glucose concentration for the shaker flask experiments was adjusted to 60 g/L. Phillips medium was prepared as defined in its original work as seen in Appendix 3. Adjustments to reach a pH of 6.5 was achieved using 3 M KOH and/or  $\text{H}_2\text{SO}_4$  respectively. Once fully prepared and inoculated, the media was sparged with a gas mixture consisting of 35 % v/v  $\text{CO}$ , 10% v/v  $\text{H}_2$ , 5% v/v  $\text{CO}_2$ , and 50% v/v  $\text{N}_2$  as to add the sole gaseous substrate within the bulk liquid. The media was sparged for 90 s with either 10 s or 30 s of overpressure allowed within the vessel.

#### 3.1.1.1.2 Growth Media

All precultures were grown using reinforced clostridial medium (RCM) as originally defined in its founding work as seen in Appendix 4 (Staadén, 2015). All components except for cysteine-HCl were added prior to solution boiling under continuous nitrogen sparging. If necessary, a 1% v/v solution of resazurin indicator was also added prior to boiling if an anaerobic indicator was deemed essential. Once the solution had boiled and cooled, the cysteine-HCl was added to scavenge any remaining oxygen and the solution was autoclaved

for 25 minutes at 121 °C. Once ready, typical precultures were inoculated 10% v/v from cryogenic vials with a typical glucose concentration of 20 g/L added unless otherwise specified. These precultures were grown until exponential phase was achieved and then used in the experimentation under analysis.

#### 3.1.1.1.3 Vitamins & Trace Elements Solutions

All components were added as seen in Appendix 5-10, with the exceptions of the addition of 1 mL of 25% v/v HCl for effective dissolution of all minerals for Appendix 5 and the addition of nitrilotriacetic acid first for effective dissolution of all minerals for Appendix 6-7. The pH was adjusted to 6.5 using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub>. Once fully mixed and soluble, the solution was sterile filtered into clean glassware and stored either in a 4 °C refrigerator or -80 °C cold storage refrigerator until required.

#### 3.1.1.2 Microbial Strains

The following microorganisms were obtained and prepared from the protocol/instructions provided by the microbial library collection provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) located in Braunschweig, Germany: *Acetobacterium woodii* (DSMZ-1030, or also classified as ATCC-29683 and JCM-2381), *Clostridium acetobutylicum* (DSMZ-792, or also classified as ATCC-824 and VKM B-1787), *Clostridium autoethanogenum* (DSMZ-10061), *Clostridium carboxidivorans* (DSMZ-15243, or also classified as ATCC BAA-624 and KCTCC-15178), *Clostridium ljungdahlii* (DSMZ-13528, or also classified as ATCC-55383 and KCTCC-15179), *Clostridium pasteurianum* (DSMZ-525, or also classified as ATCC-6013, McClung-2300, NCIB-9486, and VKM B-1774), and *E. limosum* (DSMZ-20543, or also classified as ATCC-8486 and NCIB-9763). All freeze-dried cultures were regenerated in their respective liquid media under anaerobic conditions and, once at exponential growth, was mixed with 20% w/v glycerol solution. Once mixed, the cultures were pipetted into a specific amount of cryo-vials and stored in a -80 °C refrigerator until use as required.

## 3.1.2 Research Instruments & Validation

### 3.1.2.1 Erlenmeyer Flasks, Serum Bottles, & Shakers

For the flask level of experiments, experimentation was performed in either 125 mL Erlenmeyer flasks or in 125 mL sealed serum bottles if the effect of pressurization was required. The working volumes in these flasks were done at 25 and 50 mL, respectively, with the typical experimental case containing 10% v/v inoculum from a preculture at exponential phase and 30 g/L of aqueous substrate. Any deviations in this standard have been noted as necessary. Sampling at specific intervals was done to determine the growth of a microorganism via absorbance readings along with component analysis using high-performance liquid chromatography (HPLC) techniques.

### 3.1.2.2 Anaerobic Chamber

The majority of preculture growth along with experimental flask work was performed in an anaerobic chamber purchased from PlasLabs (Lansing, MI, USA). Dry and anaerobic conditions were provided by a gas mixture consisting of 10% v/v H<sub>2</sub>, 5% v/v CO<sub>2</sub>, and 85% v/v N<sub>2</sub> with the use of catalytic space heaters to maintain a constant temperature of 35 °C. Unless otherwise specified, shakers contained within the anaerobic chamber kept liquid within the flasks agitated at a rate of 200 RPM. Any deviations in this chamber setup have been noted as necessary.

### 3.1.2.3 Fermenter

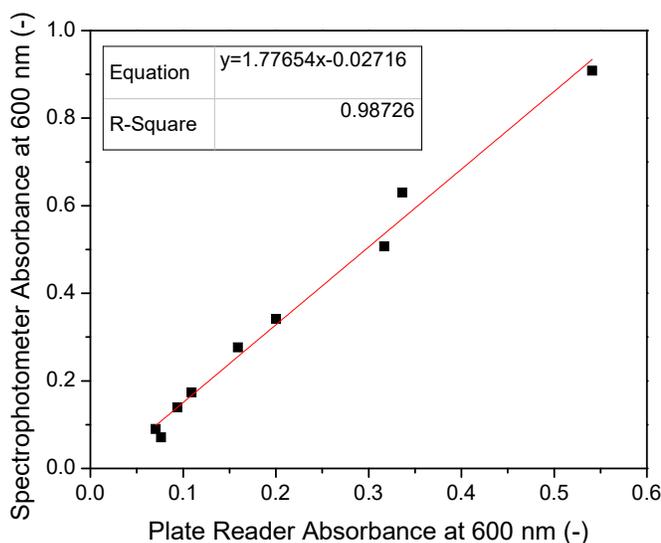
A 7-L stirred-tank fermenter was used for any bench-scale fermentations done within this body of work. Provided by Infors located in Bottmingen, Switzerland, the Labfors fermenter and operating system provides online control and analysis of many control parameters used in this area of research. Prior to experimentation, the vessel was prepared with 5 L of any specified production medium, with any necessary probes calibrated, added, and sealed into the system along with the screw-top plate. Once fully sealed, the fermenter was autoclaved for 45 minutes at 121 °C. Once cooled, the fermenter was re-attached to the control base with the cooling jacket, probe sensors, and other required systems reconnected. Prior to inoculation, the system was fully sparged with nitrogen gas to retain anaerobicity. Agitation was set at 400 RPM along with a constant gas flow rate of 0.6 L/min at a

temperature of 35 °C. Any pH control was either done using a buffering agent such as 10 g/L MES or through a pH control system using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub>. Any unnecessary foaming was controlled through the manual addition of Antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA). Online sampling such as temperature, pH, redox potential, and exit CO<sub>2</sub> mass percentage were calculated from their respective probes. Periodic offline sampling determined the component production profile using HPLC analysis.

### 3.1.3 Data Gathering & Processing

#### 3.1.3.1 Spectrophotometer & Plate Reader

Two pieces of equipment were used for absorbance readings based on the nature of experimentation. For the initial flask work performed, an Evolution 60S UV-Visible Spectrophotometer supplied by Thermo Scientific (Waltham, MA, USA) was used. When a sample was taken, the optical density of that sample was measured at 600 nm in semi-micro cuvettes. A blank was first prepared and analyzed using distilled water, then the sample was diluted, if necessary, to remain within the spectrophotometer's linear range (within 0.2 to 0.8). At least two measurements were made for every sample taken. In terms of the plate reader, a Tecan Infinite 200 PRO Series microplate reader also supplied by Thermo Scientific was used for the serum bottle pressurization experiments that consumed CO as the carbon source. Once 1 mL samples had been extracted with a sterile syringe from each serum bottle, 0.2 mL were dispensed in duplicates in their respective microwells. Once the plate was prepared, it was loaded onto the plate reader and a series of absorbance measurements were made at 600 nm. Figure 3.1.2 depicts the correlation between the spectrophotometric and plate reader measurements using three different cellular cultures as a basis to interpret these results between these pieces of equipment.



**Figure 3.1.2 - Correlation between Plate Reader and Spectrophotometer Absorbance Measurements.**

### 3.1.3.2 High-Performance Liquid Chromatography (HPLC)

Determination of concentrations (i.e., glucose, glycerol, acetic acid, ethanol, butyric acid, butanol, and 1,3-propanediol) within an experimental medium were obtained by HPLC analysis using an Agilent 1260 Infinity Quaternary HPLC System supplied by Agilent located in Santa Clara, CA, USA. An Agilent Hi-Plex H (7.7x300 mm) column at 35 °C using 5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase was used for all experiments. For fermentation broth containing glucose or any other sort of saccharidic carbohydrate, a refractive index detector (RID) at 40 °C was used at a flow rate of 0.4 mL/min to fully elute all compounds. For sugar-free compounds, the RID was operated at 70 °C and a flow rate of 0.7 mL/min for clear elution of all organic acids and alcohols. Prior to sample injection at 20 µL, samples were filtered and appropriately diluted using a 0.2 µm sterile filter.

### 3.1.3.3 pH Meter

The pH measurement used during both sequences of flasks and pressurized serum bottle experiments was done by a SympHony Benchtop pH meter supplied by VWR (Radnor, PA, USA). Prior to the measurement of the fermentation broth, a two-point calibration was done using buffer agents at a constant pH of 4 and 7.

### 3.1.3.4 On-Line Fermentation Analyses

#### 3.1.3.4.1 pH Probe

Online detection of pH was done through the use of a pH probe supplied by Hamilton (Reno, NV, USA). Before installation into the bench-scale fermenter and sterilization, a two-point calibration was done using buffer agents at a constant pH of 4 and 7.

#### 3.1.3.4.2 Optical Density Probe

Optical density was continuously monitored using a TruCell2 OD probe supplied by Finesse Solutions (Santa Clara, CA, USA). Adjustment was done prior to the sterilization and fermentation with a one-point calibration by zeroing the probe in the pre-sterilized fermentation broth.

#### 3.1.3.4.3 Redox Probe

Redox potential during a fermentation was detected using a Mettler Toledo ORP probe (Columbus, OH, USA). The probe was calibrated at -250 mV in a one-point calibration prior to sterilization using a solution provided by the same supplier.

#### 3.1.3.4.4 Gas Mass Flow Rate Meter

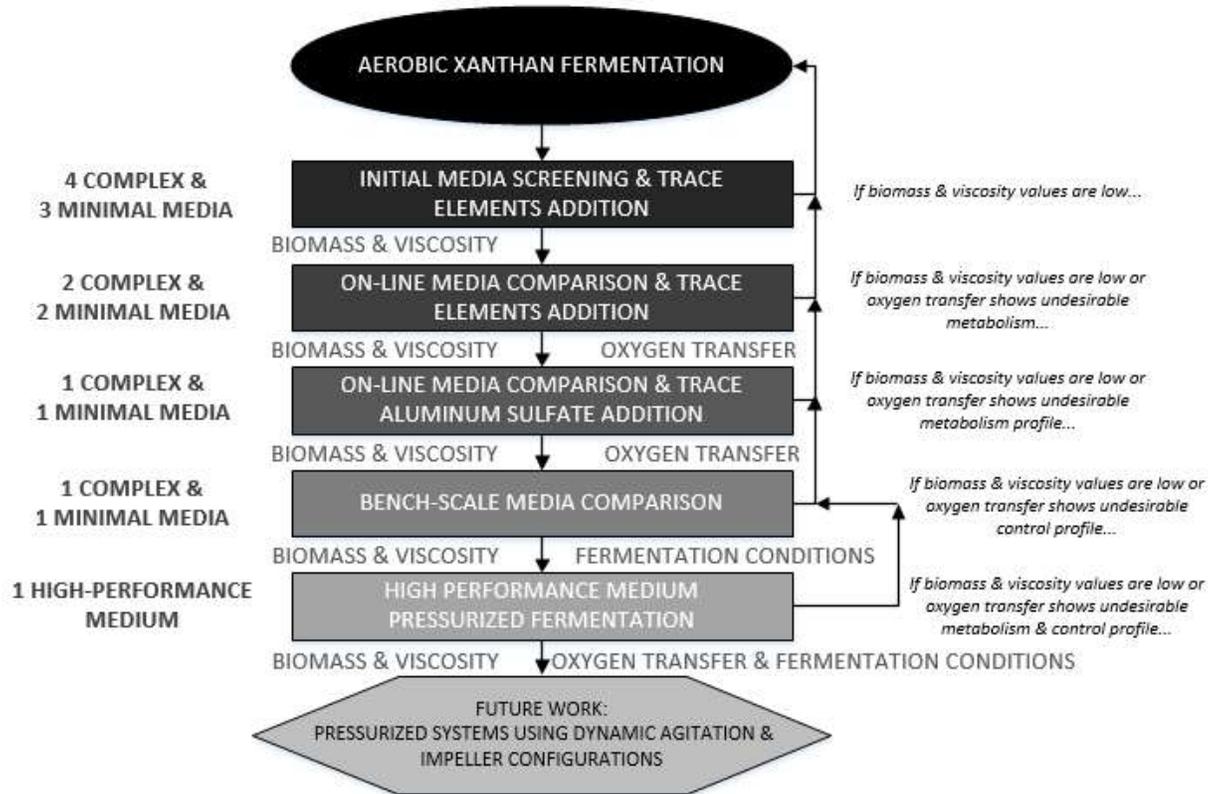
An exhaust gas analyzer, designed to measure the mass percentages of oxygen and carbon dioxide from the fermenter, was measured using an INFORS HT Gas Analyzer (Bottmingen, Switzerland). Calibration was done using nitrogen gas to act as the zero-point with a second calibration gas consisting of 8.04% v/v CO<sub>2</sub>, 3% v/v H<sub>2</sub>, and 88.96% v/v N<sub>2</sub> to determine the potential maximum value of CO<sub>2</sub> produced from this fermentation.

## 3.2 Aerobic Xanthan Fermentation

### 3.2.1 Research Design

Much like the work conducted on butanol production above, a visualization of the experimental research to be performed was developed for better comprehension on why and how the aerobic xanthan research was performed as seen in Figure 3.2.1. The bulk of this experimentation required the eventual decision of a high-performing production medium from an array of seven different options, but several other factors and variables could be investigated in simultaneous discovery of this best medium. For example, the first two sets of experiments – a simple shaker flask experiment followed by a more detailed on-line flask monitoring system – investigated the effects of the newly developed trace elements solution in conjunction with the determination of which production media provided better growth and viscosity values. From this aspect of work, the seven different media could be drawn down to a direct comparison between the best performing complex and minimal media, along with adjustments made to the trace elements solution based on more controversial compounds added. Should all media show little or insignificant development of growth or viscosity during these trials, a review of other existing media could be tested until suitable values were obtained.

Following this, the next two experiments would provide a direct comparison with these two media in the on-line measuring flasks along with a scale-up bench fermentation aimed at exploring the process limitations in conjunction with the biological constraints in place. Prior to the latter part occurring, the determination of continued use regarding aluminum sulfate within the trace elements solution would be required in order for future experiments to guarantee that the trace elements solution was fully aiding in cellular growth and/or production of xanthan. Once concluded with both metabolic profiles understood with their oxygen demand and process requirements recorded, the final pilot-plant pressurized fermentation could take place once the best of the potential production medium had been chosen. From here onwards, all process, biological, and other conditions could be directly applied to how pressurization either helps or hinders the xanthan production process, leading to potential future work investigating more pressurized systems with dynamic variables such as agitation rate or impeller configuration to aid in better growth and/or production.



**Figure 3.2.1 – Experimental Decision Matrix Highlighting Work to be Done for the Aerobic Xanthan Fermentation.**

### 3.2.1.1 Chemicals & Reagents

#### 3.2.1.1.1 Complex Production Media

The media recipes used for this section of work can be found in Appendix 11-14. All media investigated had made adjustments to reach a pH of 7.0 was achieved using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub> respectively. The same acid/base combination was used for pH control at the bench-scale fermentation. All components, save for the FeCl<sub>3</sub>, CaCO<sub>3</sub>, ZnO, and trace elements solutions, were added prior to autoclaving at 121 °C for 25 minutes. Once sterile, the solutions were added to the bulk medium. Unless otherwise specified, the glucose concentration was adjusted to 10 g/L from a concentrated sterile solution. If flask work was to be performed, 30 g/L MOPS was added from another concentrated sterile solution as well.

### 3.2.1.1.2 Minimal Production Media

The media recipes used for this section of work can be found in Appendix 15-17. All media investigated had made adjustments to reach a pH of 7.0 was achieved using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub> respectively. The same acid/base combination was used for pH control at the bench-scale fermentation. All components, save for the FeCl<sub>3</sub>, CaCl<sub>2</sub>, CaCO<sub>3</sub>, ZnO, ZnCl<sub>2</sub>, and trace elements solutions, were added prior to autoclaving at 121 °C for 25 minutes. Once sterile, the solutions were added to the bulk medium. Unless otherwise specified, the glucose concentration was adjusted to 10 g/L from a concentrated sterile solution. If flask work was to be performed, 30 g/L MOPS was added from another concentrated sterile solution as well.

### 3.2.1.1.3 Growth Media

All precultures were grown using yeast-malt (YM) growth medium as defined throughout *Xanthomonas*-related literature as seen in Appendix 18. All components were added and the solution was autoclaved for 25 minutes at 121 °C. Once ready, typical precultures were inoculated 5% v/v from cryogenic vials with a typical glucose concentration of 10 g/L added unless otherwise specified. These precultures were grown until exponential phase was achieved and then used in the experimentation under analysis.

### 3.2.1.1.4 Vitamins & Trace Elements Solutions

The solution recipe as seen in Appendix 19 highlights all essential minerals covered and averaged on a survey of literature regarding these solutions, excluding Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·16H<sub>2</sub>O as this was added for experimental purposes. All components were added, except for FeSO<sub>4</sub> and CaCl<sub>2</sub> which were both prepared separately, with the addition of Na<sub>2</sub>(EDTA) first for effective dissolution of all minerals. Once fully mixed and soluble, the solutions was sterile filtered into clean glassware and stored in a 4 °C refrigerator to prevent any sort of contamination.

### 3.2.1.2 Microbial Strains

The following microorganism was obtained and prepared from the protocol/instructions provided by the microbial library collection provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) located in Braunschweig, Germany: *Xanthomonas campestris* (DSM-19000, or also classified as ATCC-13951 and NRRL B-1459). All freeze-dried cultures were regenerated in their respective liquid media under anaerobic conditions and, once at exponential growth, was mixed with 20% w/v glycerol solution in the medium defined in Appendix 20. Once mixed, the cultures were pipetted into a specific amount of cryo-vials and stored in a -80 °C refrigerator until use as required.

## 3.2.2 Research Instruments & Validation

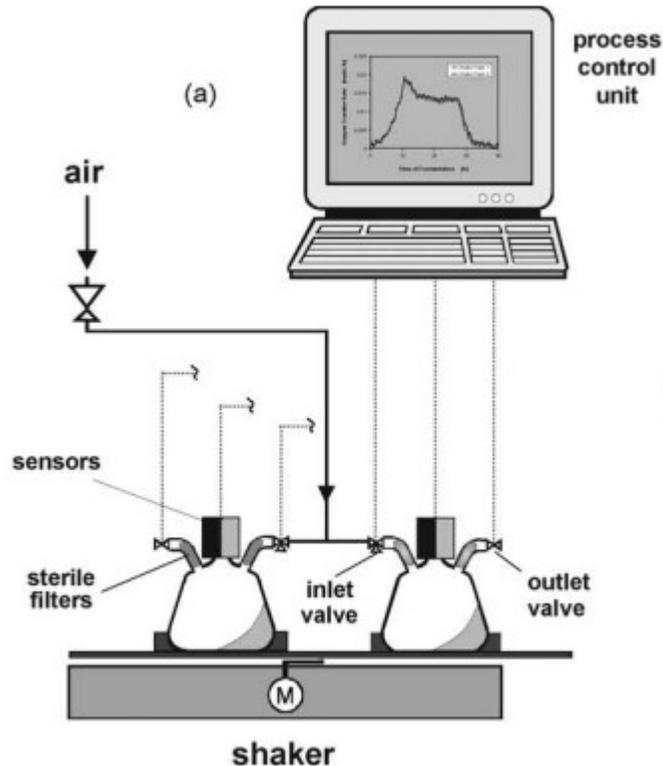
### 3.2.2.1 Erlenmeyer Flasks & Shakers

For the flask level of experiments, experimentation was performed in 125 mL Erlenmeyer flasks. The working volume in these flasks was done at 20mL, with the typical experimental case containing 5% v/v inoculum from a preculture at exponential phase and 10 g/L of glucose. MOPS buffer was used at 30 g/L to maintain a constant pH of 7. Any deviations in this standard have been noted as necessary. Sampling at the end of the fermentation was done to determine the growth of *X. campestris* via dry cell weight (DCW) and viscosity to roughly determine the amount of xanthan present in a specific medium.

### 3.2.2.2 Respiration Activity Monitoring System (RAMOS)

For the online-measuring flask level of experiments, experimentation was performed in custom-made 125 mL RAMOS flasks as observed in Figure 3.2.2. The working volume in these flasks was done at 20mL, with the typical experimental case containing 5% v/v inoculum from a preculture at exponential phase and 10 g/L of glucose. MOPS buffer was used at 30 g/L to maintain a constant pH of 7 along with continuous aeration at 1 vvm. Any deviations in this standard have been noted as necessary. Online detection of oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) was continuously measured based on the available partial pressures in the gaseous headspace. Sampling at the end of the fermentation

was done to determine the growth of *X. campestris* via dry cell weight (DCW) and viscosity to roughly determine the amount of xanthan present in a specific medium.



**Figure 3.2.2 - Schematic of RAMOS Device and Operating System as used in Anderlei et al., 2004.**

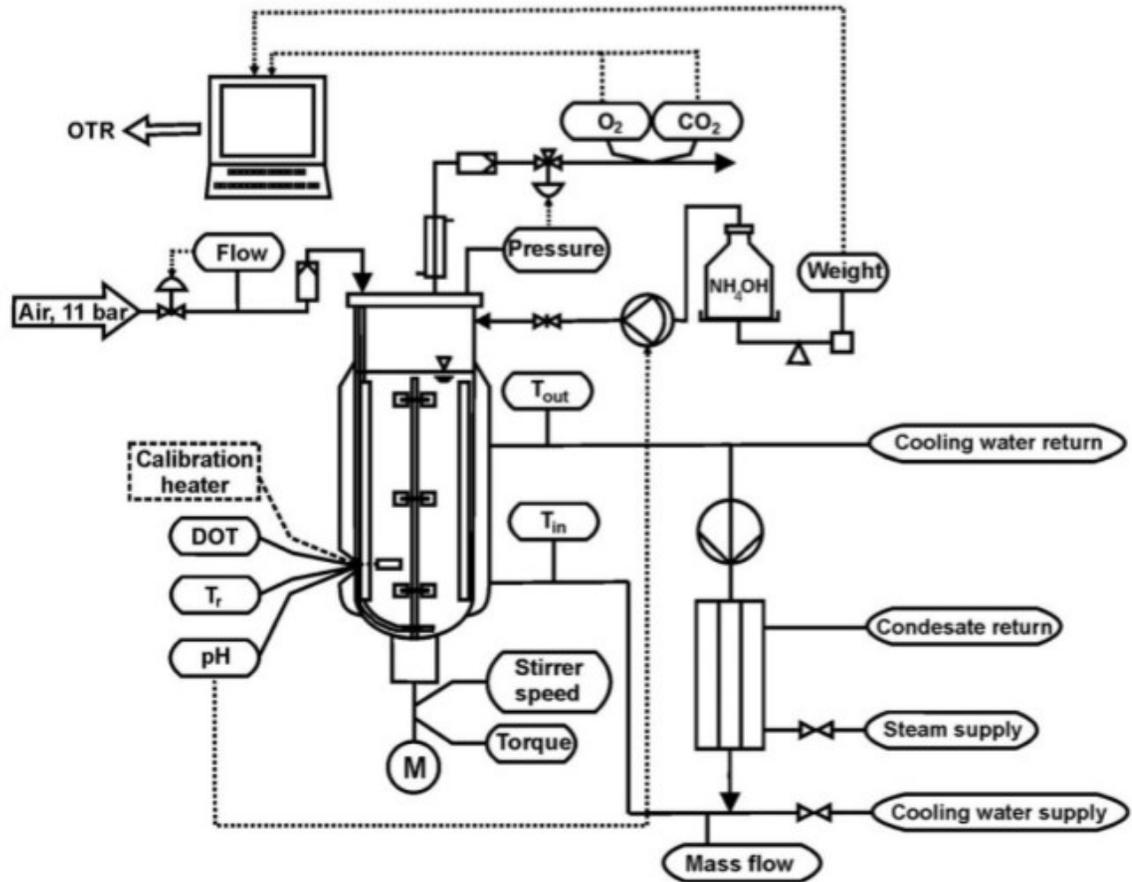
### 3.2.2.3 Bench-scale Fermenter

A 2-L stirred-tank fermenter was used for any bench-scale fermentations done within this body of work. Provided by Eppendorf located in Hamburg, Germany, the New Brunswick BioFlo 115 fermenter and associated operating system provides online control and analysis of many parameters used in this area of research. Prior to experimentation, the vessel was prepared with 1.5 L of any specified production medium, with any necessary probes calibrated, added, and sealed into the system along with the screw-top plate fixed. Once fully sealed, the fermenter was autoclaved for 45 minutes at 121 °C. Once cooled, the fermenter was re-attached to the control base with the cooling jacket, probe sensors, and other required systems reconnected. Prior to inoculation, the system was fully sparged with nitrogen gas followed by air to calibrate the dissolved oxygen tension (DOT) probe.

Agitation was set as an output controlled determined by DOT % with a range between 250 to 450 RPM along with a constant gas flow rate of 1 vvm at a temperature of 30 °C. Any pH control was either done using a buffering agent such as 30 g/L MOPS or through a pH control system using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub>. Any unnecessary foaming was controlled through the manual addition of antifoaming agent Plurafac (BASF, Ludwigshafen, Germany). Online sampling such as temperature, pH, DOT, and agitation were calculated from their respective probes. Periodic offline sampling determined the biomass concentration from the DCW method discussed and glucose consumption profile using HPLC analysis.

#### 3.2.2.4 50-L Pressure Fermenter

A Bioengineering AG LP351 50-L stirred-tank fermenter was used for the final pressurized fermentation with the high-performing production medium as seen in Figure 3.2.3 (Wald, Switzerland). Prior to experimentation, the vessel was prepared with 27 L of any specified production medium, with any necessary probes calibrated, added, and sealed into the system along with the screw-top plate fixed. Once fully sealed, the fermenter was autoclaved for 45 minutes at 121 °C. Prior to inoculation, the system was fully sparged with nitrogen gas followed by air to calibrate the dissolved oxygen tension (DOT) probe. Agitation was set as at 500 RPM along with a constant gas flow rate of 1 vvm at a temperature of 30 °C. When pressure was added, the gas flow rate was also increased to ensure that a constant 1 vvm occurred through the whole fermentation. Any pH control was either done using 3 M KOH and/or H<sub>2</sub>SO<sub>4</sub> with manual additions. Any unnecessary foaming was controlled through the manual addition of antifoaming agent Plurafac (BASF, Ludwigshafen, Germany). Online sampling such as temperature, pH, DOT, off-gas O<sub>2</sub> and CO<sub>2</sub> mass percentages, and agitation were calculated from their respective probes. Periodic offline sampling determined the biomass concentration from the DCW method discussed and glucose consumption profile using HPLC analysis.



**Figure 3.2.3 - Schematic of 50 L Pressurized Fermenter and Operating System as used in Regestein et al., 2013.**

### 3.2.3 Data Gathering & Processing

#### 3.2.3.1 High-Performance Liquid Chromatography (HPLC)

Determination of concentrations (i.e., glucose) within an experimental medium were obtained by HPLC analysis using an HPLC Ultimate 3000 System supplied by Dionex located in Sunnyvale, CA, USA. An AMINEX HPX 87H (7.7x300 mm) column along with a pre-column (4.6x30 mm) at 35 °C using 5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase was used for all experiments. For fermentation broth, a refractive index detector (RID) at 80 °C was used at a flow rate of 0.4 mL/min to fully elute all compounds. Prior to sample injection at 20 µL, samples were filtered and appropriately diluted using a 0.2 µm sterile filter.

### 3.2.3.2 Rheometer

An Anton Paar Physica MCR 301 rheometer was used to measure the viscosity of a fermentation broth at a shear rate of  $300 \text{ s}^{-1}$  (Graz, Austria). Using a cone-plate type spindle (CP50-0.5/TG) at a range from 30 to  $30\,000 \text{ s}^{-1}$ , approximately 450 to 800  $\mu\text{L}$  of pure fermentation broth was measured at least in duplicate.

### 3.2.3.3 pH Meter

The pH measurement used during both flask and RAMOS experiments was done by a benchtop pH meter supplied by Hanna Instruments (Woonsocket, RI, USA). Prior to the measurement of the fermentation broth, a two-point calibration was done using buffer agents at a constant pH of 4 and 7.

### 3.2.3.4 On-Line Fermentation Analyses

#### 3.2.3.4.1 pH Probe

Online detection of pH was done through the use of a pH probe supplied by Bioengineering AG (Wald, Switzerland). Before installation into the fermenter and sterilization, a two-point calibration was done using buffer agents at a constant pH of 4 and 7.

#### 3.2.3.4.2 Calibration Heater

An external cooling jacket calibration heater was used to determine the biological heat formation of the organism, and was supplied by Bioengineering AG (Wald, Switzerland). The calibration heater was operated at 250 W and switched on and off in cycles of 0.5 and 1.0 hrs, respectively.

#### 3.2.3.4.3 Dissolved Oxygen Tension (DOT) Probe

Dissolved oxygen tension was continuously monitored using a probe supplied by Bioengineering AG (Wald, Switzerland). Calibration involved a two-point calibration using nitrogen gas as the zero point and enriched air under full liquid saturation. As the DOT approached 30%, increases to aeration/pressure was made for the majority of the fermentation, excluding any time past 100 hrs.

## Chapter 4

### 4 Results & Discussion

#### 4.1 Anaerobic Butanol Fermentation

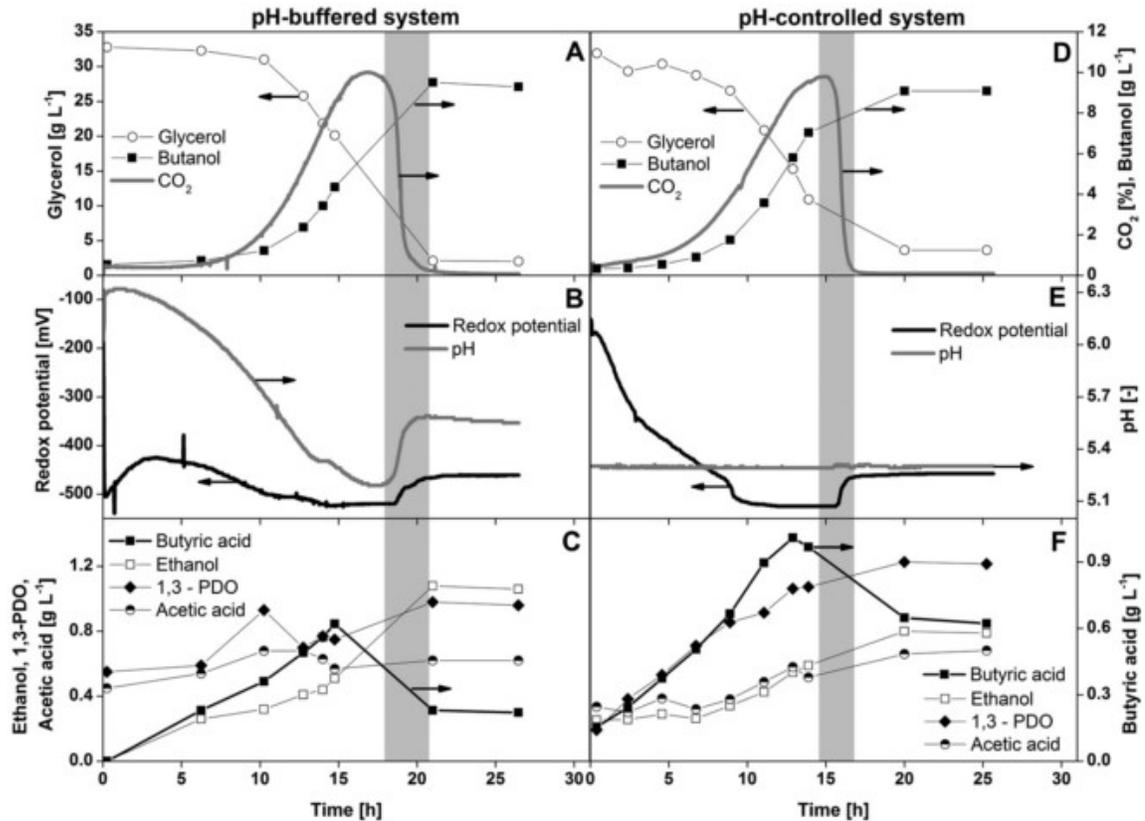
##### 4.1.1 Butyrate Uptake with Glycerol-Consuming Organism

A series of developmental techniques have been established over time to enhance the butanol yield and productivity with this grouping of fermentations. An avenue generally left uninvestigated in the hopes of process improvement is the use of a mixed culture system capable of utilizing a multi-substrate fermentation system to produce biobutanol. The proposition of a primary organism capable of producing butanol as well as consuming the metabolites from a secondary organism would theoretically increase the amount of butanol. Before such a system can be explored, an examination into the ability for a primary organism such as *C. pasteurianum* to consume a secondary metabolite like butyric acid from a secondary organism should be proven for feasibility. Given the nature of butyric acid, the secondary effects of system pH were also studied to determine how effective the uptake of butyric acid was in a buffered and pH-controlled environment. The change in pH within a buffered system could also act as additional evidence of butyrate uptake by the primary organism *C. pasteurianum*.

A first preculture consisting of 15 g/L glycerol was prepared for *C. pasteurianum* operating at 35 °C and 200 RPM at 10 mL for 17 hrs. The second and third stages were maintained at the same conditions with increasing liquid volumes of 50 and 250 mL for 8 and 12 hrs, respectively. While the precultures were reaching an exponential phase of growth, a bioreactor was prepared with 5 L of Biebl medium and sterilized as necessary. Adjustments to the pH system were either made into a buffer system consisting of 2 g/L CaCO<sub>3</sub> at a pH value of 6.3 or into a pH-controlled system at pH 5.3 using 3M KOH and H<sub>2</sub>SO<sub>4</sub>. The fermenter was prepared with an initial glycerol concentration of 45 g/L. Probes to detect the pH, redox, temperature, and other necessary parameters were calibrated and utilized during the fermentation. Additionally, an offgas mass flow detector was used to find the mass percentage of CO<sub>2</sub> leaving the fermenter. Once prepared, the final preculture was inoculated into the fermenter and run at 35 °C and 400 RPM with a constant gas flow of nitrogen at 0.6

L/min. Offline samples determining several important compound concentrations, such as glycerol, butanol, and butyric acid among others, were gathered using HPLC analysis.

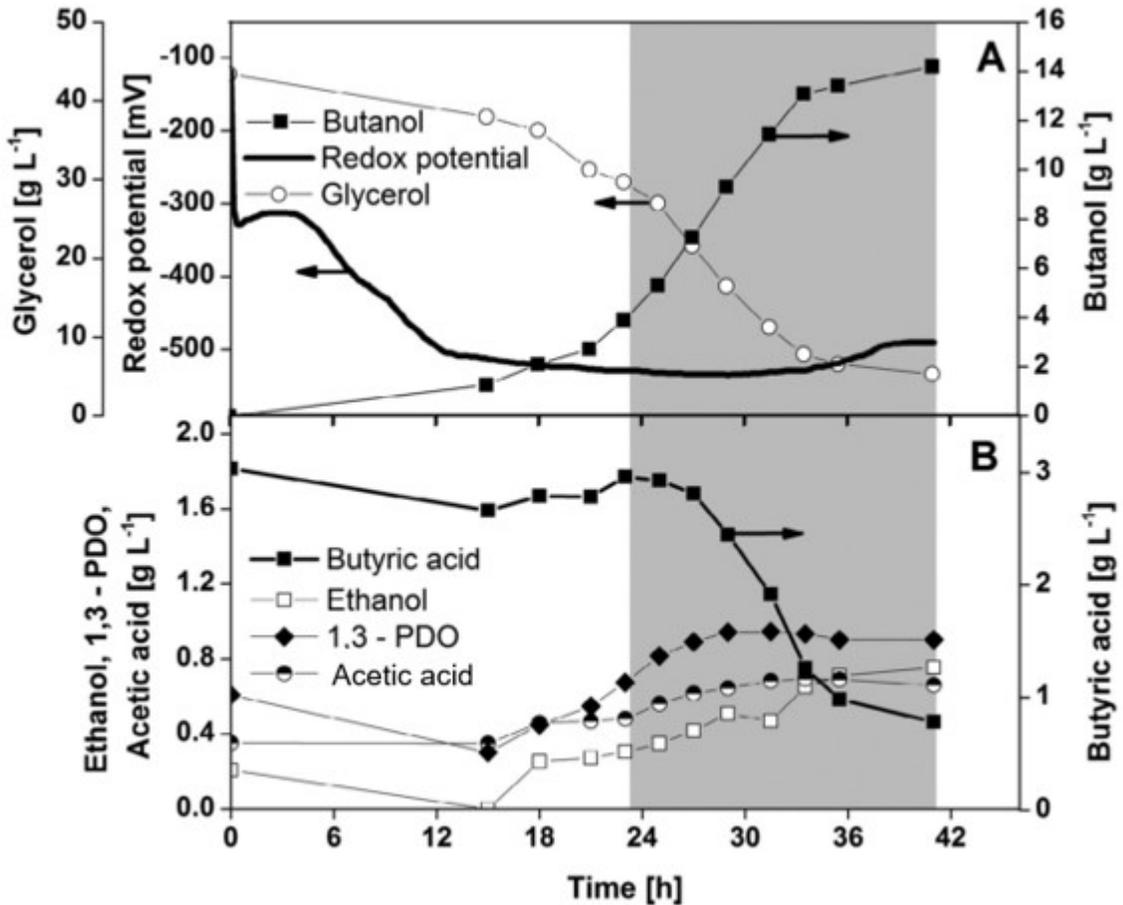
Figure 4.1.1 displays the substrate consumption, product formation, and pH and redox flux throughout the pH-buffered and pH-controlled fermentation systems. The glycerol/butanol data collected in both cases appear nearly identical in both rate of changes recorded along with similar end points reached. The major differences between Figure 4.1.1A,D is the peak position of the CO<sub>2</sub> curve; the pH-controlled system climaxed approximately 5 hrs earlier than the pH-buffered system. A justification for this increase in gas production can come into fruition with the results gathered in Figure 4.1.1B,E. The comparison between the redox and pH curves appear to switch positions for each case, as redox is relatively constant like the pH is in the pH-controlled system. Likewise, familiar curve shapes can be compared from the pH-buffered curve related to the second system's redox curve. As such, a pH-controlled system proves to offer a quicker and thus more efficient fermentation in comparison to the buffered system. The last two portions of Figure 4.1.1C,F display some significant differences between the fermentations under review. The amount of butyric acid and 1,3-propanediol is greater in the pH-controlled case versus the pH-buffered one, where a similar amount of acetic acid but a greater amount of ethanol was detected. While these differences in metabolites remain important in the understanding of this fermentative process, the major article of significance from this data is in the apparent uptake of butyric acid within the same time range as the CO<sub>2</sub> peak recorded above. While this decrease in butyric acid does attest to a potential ability in uptake by *C. pasteurianum*, an external source of butyric acid should be supplied in this fermentation system to truly prove that uptake of this compound is possible for justification of a future mixed culture fermentation.



**Figure 4.1.1 - pH Control Conditions as an effect on Butyric Acid Uptake with *C. pasteurianum*. A-D display the glycerol consumption and butanol/CO<sub>2</sub> formation. B-E display the redox potential and pH flux. C-F display the secondary metabolite patterns throughout the fermentation.**

Prior to the data collection as seen in Figure 4.1.2, a similar fermentation was prepared using 4 g/L butyric acid as displayed in Appendix 21. It was observed that such a high level of butyric acid proved inhibiting towards *C. pasteurianum*, and to counteract this limitation, the initial butyric acid concentration was decreased to 3 g/L. Using pH control at a value of 5.3, similar profiles in glycerol consumption and product formation were observed like in Figure 4.1.1D-F excluding a significant change in the butyric acid profile. With the initial increase in butyric acid, no formation from *C. pasteurianum* can be detected, yet the concurrent consumption of both glycerol and butyric acid is recorded after 24 hrs. With this substrate and intermediate consumption made, a final butanol yield approximately at 14 g/L was recorded, being about 5 g/L greater than in Figure 4.1.1. This evidence offers solid proof

to authorize butyrate addition as a means to increase butanol titres from either biologically- or chemically-adjusted means.



**Figure 4.1.2 - On-line & Off-line Data Analysis of *C. pasteurianum* fermentation with 3 g/L butyrate addition.**

With the above experimentation complete, it can be justified that a mixed culture system using *C. pasteurianum* as a primary organism capable of fixating both glycerol and butyric acid is feasible for this biobutanol production. Likewise, the provision of butyric acid from a biological process such as syngas fermentation using carboxydrotrophic organisms could deliver this secondary metabolism capable of converting more butanol in a streamlined and methodical system. When reviewing the objectives made necessary from Figure 3.1.1, the achievement of production and consumption of butyric acid at 3 g/L with uninhibited growth justifies the use of *C. pasteurianum* as the primary organism in this mixed culture proposal. Future work should consider the interactions between these organisms in a

fermentative setting and observe the effects of when cultures were added, monitoring of individual cultures using independent metabolite detection or other methods, and the efficacy of fermentative kinetics in comparison to the pure culture case to validate this process' usage. Attentions into any other intermediate compound interactions and other defensive mechanisms that these cultures may provide in a fermentative setting should be considered when designing the proposed mixed culture fermentation.

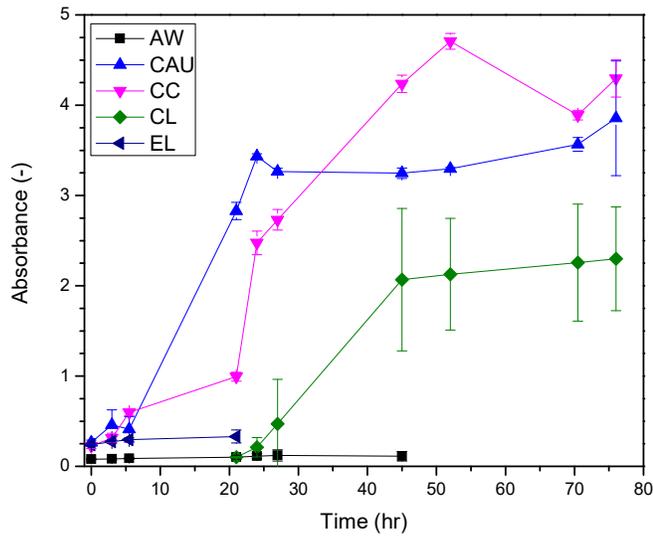
#### 4.1.2 Production Media Comparison & Product Formation

To begin experimentation into the proposed fermentation setup, it is necessary to understand which cultures reviewed will provide the greatest yields for butanol production. As discussed in Section 2.1.1, the primary organism of this process must be able to consume glycerol. The previous work done on *C. pasteurianum* exhibits an organism capable of both glycerol and butyric acid uptake to produce higher butanol yields. Next, a secondary organism must be able to fixate carbon monoxide along with the potentially helpful ability to utilize carbon dioxide and hydrogen gas as substrates in order to produce essential intermediate compounds such as acetic and butyric acid to feed into the primary organism's metabolic pathway. Furthermore, if the secondary carboxydrotroph is able to produce butanol from carbon monoxide alone, greater consideration for the use of such an effective bacteria will be strongly reflected in actual culture design.

The determination of culture selection therefore relies on a well-thought screening process. In order for both organisms to thrive within this mixed culture, three criteria must be met for theoretical success in increased biofuel yields; healthy growth with both organisms in a designated production medium, non-competitive growth conditions with two independent substrates, and butanol tolerances able to withstand prolonged exposure to this substance without major inhibition. The following section will look into the effects that two different media compositions have on the growth and endpoint production profiles with the five organisms currently under investigation. It should be noted that the three criteria experiments were performed on glucose instead of CO as the use of this gas was not possible in an anaerobic chamber setting due to safety concerns. While the growth and product conditions may prove different with use of differing substrates, glucose use should provide some estimated similarities with CO fermentation.

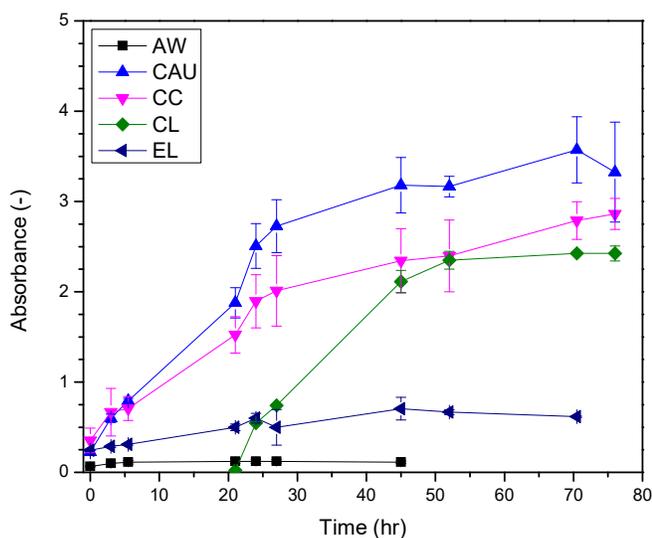
As stated in Section 2.1.6, the two media under scrutiny have been individually planned to fulfill the necessary nutrient requirements particular to that type of microorganism. The Biebl medium, originally designed for glycerol fermentation via *C. pasteurianum*, and PETC medium, prepared for several carboxydrotrophic organisms, are the options available for this fermentation. Prior to experimentation, all cultures were grown in RCM medium until absorbance data was at least above a value of 1.0 before inoculation to ensure adequate growth. Once at this point, flasks containing both media were inoculated at 30 g/L glucose. To ensure effective growth of these organisms, all flasks were inoculated at 10 % v/v of the total liquid volume. In addition, these flasks were agitated at 200 RPM and kept at a constant temperature of 35 °C during experimentation, with a starting pH value of 6.5 in order to balance healthy growth with acid formation. When there was no change in absorbance observed, the applicable flasks were removed and product concentration analysis was performed.

Figure 4.1.3 displays the absorbance curves for the five organisms analyzed; it can be observed that the growth patterns yielding the shortest times until stationary phase and highest recorded absorbance values come from *C. autoethanogenum* and *C. carboxidivorans*, despite other cultures showing similar behaviour. While the former organism appears to peak at an absorbance around 3.5 with stationary achieved around 22 hrs, the latter does not peak until 52 hrs yet reaches an absorbance of nearly 5.0. In this scenario, both acetogenic cultures of *E. limosum* and *A. woodii* show little to no growth. *A. woodii* and its lack of activity can be explained due to elevated temperatures within the anaerobic chamber, as this culture's optimal temperature is at 30 °C. While *E. limosum* does not show similar growth curves like the other organisms studied, an interesting profile of products are still achieved and will be discussed later. The final organism, *C. ljungdahlii*, provides a similarly moderate absorbance profile with one major difference between the others. The absorbance data collected from *C. ljungdahlii* displays significant standard deviation in its latter points, which can likely be explained from spectral interference from both dissolved gas bubbles and substantial biofilm formation together with flocculated and resettled biofilm within the extracted sample.



**Figure 4.1.3 – Growth Comparison of Five Carboxydophilic Bacteria using Biebl Media.** The cultures investigated are *A. woodii* (AW), *C. autoethanogenum* (CAU), *C. carboxidivorans* (CC), *C. ljungdahlii* (CL), and *E. limosum* (EL). Experiments were run in duplicate and the standard deviation was recorded for each data point.

Figure 4.1.4 shows the same strains growing on a different medium. Overall, it would appear that Biebl medium is able to support more growth given the higher absorbance values achieved. Additionally, PETC medium curves generally do not achieve a stationary phase with most cultures until after five hours, thus creating a greater exponential growth phase to consider. Another interesting difference between these two media is the marginal increase in absorbance level with *E. limosum*. Changes in the media composition such as decreased concentration of available nitrogen and iron compounds could be a contributing factor to the elongated and reduced absorbance curve observed. It is also worthwhile to note that the data for *C. ljungdahlii* has a much smaller standard deviation in comparison to the Biebl case; the issue of excessive biofilm formation and flocculation along with reduced microbubbles appeared to provide more accurate results in this scenario. Despite the discussion on these growth curves, further decision-making and the closing-in on the choice of microbes for this process requires an examination into the product profiles expected from these microorganisms.



**Figure 4.1.4 – Growth Comparison of Five Carboxydrotrophic Bacteria using PETC Media.** The cultures investigated are *A. woodii* (AW), *C. autoethanogenum* (CAU), *C. carboxidivorans* (CC), *C. ljungdahlii* (CL), and *E. limosum* (EL). Experiments were run in duplicate and the standard deviation was recorded for each data point.

The results displayed in Table 4.1.1 give further insight into the patterns recorded from Figure 4.1.3 and Figure 4.1.4. It is important to note that certain cultures like *A. woodii* and *E. limosum* indicate endpoint glucose concentrations above 30 g/L, which was the concentration provided in the flask experiment. This higher than expected concentration comes from residual glucose remaining from the preculture when inoculated into the flask experiment. The residual glycerol detected can be explained from the remainder left over from cryogenic culture storage. At any rate, consumption of glucose was observed in all culture cases and the product profile generated leads to further questioning in the pursuit of ideal organisms.

All cultures in both media had produced acetic acid and ethanol from values between 0.07 to 0.56 g/L and 0.70 to 2.87 g/L, respectively. While the production of C2 compounds is non-essential and generally seen as undesired in the pursuit of C4 compounds like butyric acid and butanol, an ability to produce higher titres of both levels of organic acids and

solvents could prove opportunistic to exploit in a multi-product, integrated biorefinery system as discussed in prior sections.

However, the main intent of this experiment was to observe the ability to produce butyric acid and butanol, if possible, and to what extent these components are produced. Of the six microbes observed, only *A. woodii* was observed to produce no butyric acid and butanol while *E. limosum* did not produce any butanol. Butanol production is non-essential yet more favourable with these carboxydrotrophic organisms, as the main intent of the mixed culture is to create intermediate butyric acid for the primary organism to consume and theoretically produce more butanol. If the secondary organism is simultaneously able to produce butanol in conjunction with butyric acid, then this arrangement of dual target production proves ideal. Of the four cultures quantified, *C. carboxidivorans* and *E. limosum* had produced amounts capable to increase desired butanol levels. Large deviations in the single cases of glucose concentration for *A. woodii* and *E. limosum* can be attributed to the low growth experienced and unanticipated amount of glucose left over from preculture.

**Table 4.1.1 - Fermentation Endpoint Component Analysis.**

Bacterial Culture	Media	Concentration (g/L)					
		Glucose	Glycerol	Acetic Acid	Ethanol	Butyric Acid	Butanol
<i>Acetobacterium woodii</i>	Biebl	38.87 ± 8.31	3.29 ± 1.03	0.15 ± 0.00	0.72 ± 0.20	0.00 ± 0.00	0.00 ± 0.00
	PETC	32.80 ± 0.18	2.74 ± 1.19	0.13 ± 0.01	1.03 ± 0.53	0.00 ± 0.00	0.00 ± 0.00
<i>Clostridium autoethanogenum</i>	Biebl	23.25 ± 0.32	5.23 ± 0.89	0.24 ± 0.03	0.89 ± 0.08	0.00 ± 0.00	1.59 ± 0.02
	PETC	18.52 ± 2.53	4.53 ± 0.55	0.00 ± 0.00	0.83 ± 0.05	0.00 ± 0.00	2.65 ± 0.70
<i>Clostridium carboxidivorans</i>	Biebl	18.51 ± 0.43	4.31 ± 0.07	0.70 ± 0.01	1.06 ± 0.02	1.58 ± 0.62	1.44 ± 0.51
	PETC	20.81 ± 0.44	5.41 ± 0.61	0.08 ± 0.01	1.66 ± 0.84	0.00 ± 0.00	2.08 ± 0.06
<i>Clostridium ljungdahlii</i>	Biebl	26.50 ± 1.11	2.38 ± 0.49	0.25 ± 0.04	1.19 ± 0.00	0.15 ± 0.04	1.05 ± 0.11
	PETC	20.85 ± 0.09	5.04 ± 0.69	0.07 ± 0.00	2.87 ± 2.44	0.00 ± 0.00	1.90 ± 0.07
<i>Eubacterium limosum</i>	Biebl	27.12 ± 10.37	5.22 ± 1.97	0.21 ± 0.03	0.70 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
	PETC	31.49 ± 0.86	2.45 ± 0.19	0.33 ± 0.01	0.96 ± 0.02	0.46 ± 0.06	0.00 ± 0.00

From the data above, several conclusions can be made regarding the desired medium and organisms to continue experimentation with. Primarily, the two carboxydophilic organisms that are likely to provide fruitful effort in this kind of mixed culture fermentation would be *C. carboxidivorans* and *E. limosum*. An inability to produce the desired compounds on a universal substrate such as glucose does not bode well with a harder to solubilize substrate such as CO; if an organism is unable to synthesize four-carbon compounds given a six-carbon sugar, major metabolic challenges will be present in the use of a one-carbon substrate. While the metabolic pathways utilized are different in their initial phases and the presence of uninhibited hydrogenase enzymes provide further conversion of substrate towards biomass and simpler C2 compounds like acetate and ethanol, at least some significant titre of butyric acid should be expected given the acidogenic-friendly fermentation conditions. Alternatively, if the inclusion of glucose and the creation of a multi-substrate, mixed system was pursued, this could complicate the metabolism of all organisms through competitive interaction and potential dominance of a single species, but the presence of residual glucose may help continued growth of the carboxydophilic organism in its initial phases.

Finally, the decision to choose Biebl medium over PETC medium proves a simpler concept to justify. Growth levels and stationary phase maintenance proved both quicker and more prevalent in the Biebl cases. A greater concentration of nitrogenous sources in the Biebl medium could account for better growth and maintenance conditions. Future work investigating the exact nutritional requirements could be done for species like *C. carboxidivorans* or *E. limosum* when directly using CO. Likewise, greater concentrations of butyric acid had been detected in the Biebl medium than in the PETC, as fermentation cultures experienced a quicker time to mature and possess the requisites needed for C4-compound synthesis. With reference to Figure 3.1.1, the ability of these organisms to produce butyric acid between 0.5-1.5 g/L and the noted growth profile experienced in Biebl medium justified the continued investigation into these two microorganisms. The utilization of Biebl medium with *C. carboxidivorans* and *E. limosum* will provide much needed insight into what the final conditions of this mixed culture system should be like.

### 4.1.3 Non-competitive Interaction via Glycerol Consumption

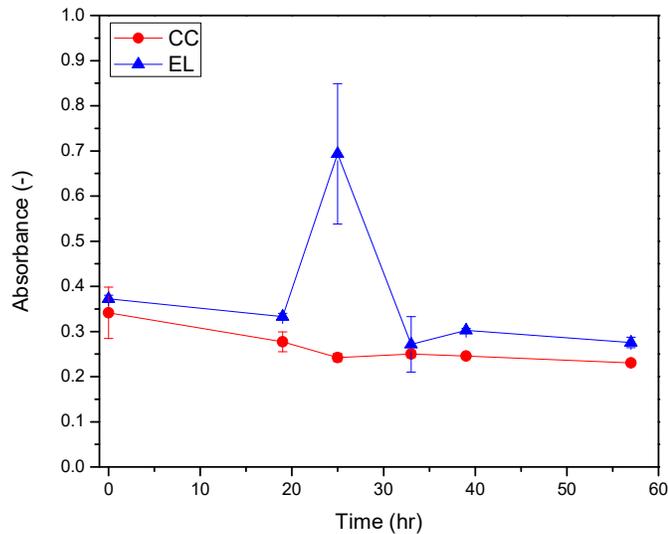
For a mixed culture system, certain metrics and design objectives help to determine which cultures are operating as desired and to what extent do these cultures interact with each other. While certain traits such as quorum sensing, symbiosis, and sustainable co-dependency between two or more organisms can prove beneficial to a biological system overall, the necessity to have independent and uninhibited growth regimes proves to be the most important characteristic in mixed cultures. As such, for a butanol fermentation system of glycerol and CO fixation by two bacterial strains to effectively occur, non-competitive growth should be sustained between such organisms.

To determine if non-competitive growth interaction is possible within this system, an experiment involving the use of glycerol as a sole substrate in Biebl medium was proposed. Undergoing the same operating conditions of 35 °C, an initial pH of 6.5, and agitation at 200 RPM, the major design change compared to previous experimentation was the concentration of substrate. As 30 g/L of substrate proved excessive in the prior case, 20 g/L of glycerol was used henceforth as the sole substrate in experimental setup. As before, precultures were grown on RCM using glucose as substrate until absorbance values from these tubes were greater than 1.0.

The results in Figure 4.1.5 mostly shows a lack of glycerol consumption with the cultures investigated. As *C. carboxidivorans* is expected to grow solely on carbon monoxide and completely ignore any available glycerol in the system as seen below, hopes that this organism could consume even minimal amounts of glycerol without the simultaneous presence of glucose as discussed previously appears possible.

Finally, the results of *E. limosum* require some additional conversation. In this scenario, *E. limosum* follows the general trend present in the other two cultures, except for a sudden 0.4 increase in absorbance at the third data point of 25 hrs. As the following points go back towards the decreasing absorbance, it is very likely that the rogue data point had proven to be an artefact within this data set. Despite *E. limosum* being reported to have very limited ability to consume glycerol, it does not appear that any growth did occur if one considers the presence of an artefact in the absorbance readings. It would be highly unlikely that this artefact would appear in reproductions of this experiment. Future work determining whether

this artefact does exist or whether a very small window of actual cellular growth followed by colony collapse should be performed to fully ensure no glycerol consumption occurs.



**Figure 4.1.5 - Non-competitive Growth Analysis via Glycerol Consumption. The cultures investigated are *C. carboxidivorans* (CC) and *E. limosum* (EL). Experiments were run in duplicate and the standard deviation was recorded for each data point.**

It can be concluded from this experiment that non-competitive growth can occur with the use of either *C. carboxidivorans* or *E. limosum*. Concerns regarding any sort of consumption from the proposed carboxydophilic organisms can be dismissed for the fermentation at hand. Further research into the difference between pure and crude versions of glycerol and any detection of uptake over a larger time period could be done to ensure that these findings are robust. Another round of experiments could determine any inhibiting effects, if any, that glycerol may have on CO uptake. With glycerol fermentation usually lasting under 50 hours during butyrate-added conditions and with no glycerol consumption attributed under 60 hours, further testing with these two organisms as outlined in Figure 3.1.1 should be concluded. To truly determine the final bacteria used in the proposed mixed culture, further decision-making in other key factors in fermentation design must be answered.

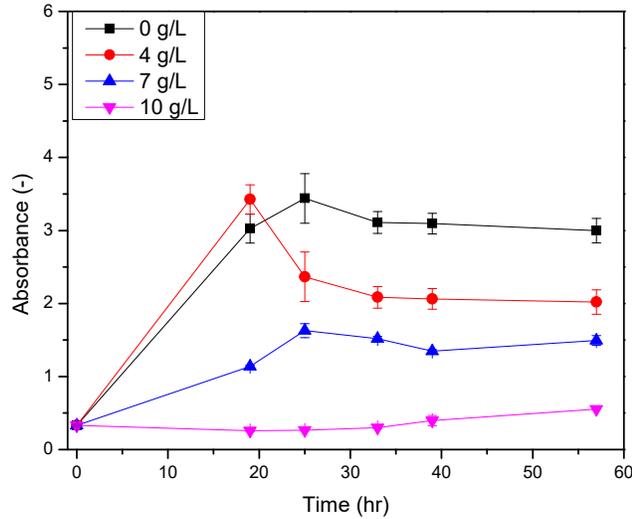
#### 4.1.4 Fermentative Feasibility via Butanol Toxicity

One of the biggest challenges that biobutanol production proposes is in the intrinsic nature of this biofuel itself; as discussed in several sections prior, the toxic effects of butanol on bacterial species remains a limiting factor for increasing desirable titres. One of the more popular proposals in current research is to include *in situ* technologies designed to extract butanol from a running bioreactor, with such principles like pervaporation, gas stripping, and two-phase extraction, among others. In addition to equipment innovation, the avenue of genetic engineering and recombinant DNA expertise could prove favourable for increasing tolerances to this compounds. The ability to increase toxicity limits along with the potential to modify and streamline butanol-producing pathways remains an optimistically cautious option. However, before any such implementation and planning can occur with either of these prospective enhancements, an understanding and determination of an organism's current limits must be understood.

The following experiment examines the effect butanol has on independent cultures of *C. carboxidivorans* and *E. limosum*. For a more precise understanding of the effects of butanol, the concentration was varied between four different levels ranging from 0 to 10 g/L. As in previous experiments, glucose was used as the sole substrate at 20 g/L with temperature and agitation occurring at 35 °C and 200 RPM respectively. With all cultures starting at a pH of 6.5, absorbance samples were periodically taken to observe what growth, if any, was possible. The experiment was concluded when the difference between two absorbance readings for all cases either remained constant or decreased in value.

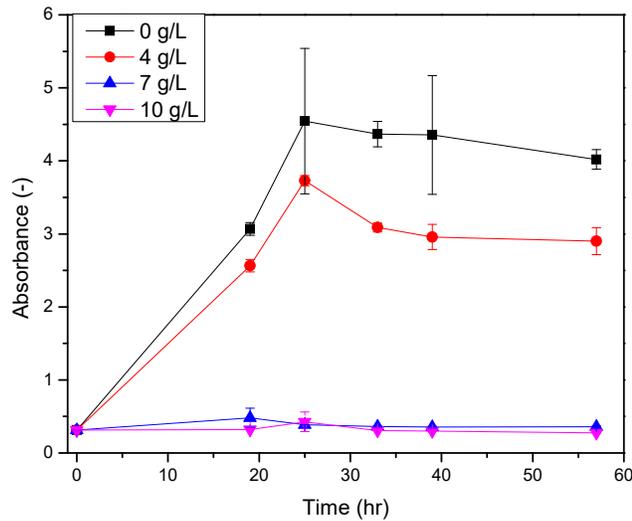
Figure 4.1.6 shows the results from the *C. carboxidivorans* case. Sampling was not done until 19 hrs as experimental focus was aimed towards whether any determinable growth was possible and the potential long-term effects this butanol could have on the analyzed cultures. It can be confirmed that growth, while increasingly inhibited as butanol concentration increased, was determined possible up to 7 g/L butanol. With the maximum level of absorbance achieved at about 3.4 in the butanol-free case, stationary phase conditions were able to be seen in the 4 and 7 g/L cases. In fact, the final few points in the 10 g/L case tend to show an increase in absorbance, which could hint at several possibilities within this scenario. Either the inhibitory effects of such a butanol concentration create a lag phase spanning close to 60 hrs, or an unexpected adaptation mechanism to butanol tolerance

could have occurred with such prolonged exposure and allow better growth as time progressed.



**Figure 4.1.6 - Butanol Tolerance for *C. carboxidivorans*.** Experiments were run in duplicate and the standard deviation was recorded for each data point.

The *E. limosum* data provides some difference in results when compared to *C. carboxidivorans*. In Figure 4.1.7, while growth had only been observed up to 4 g/L butanol, the degree of absorbance recorded was greater than in the case of *C. carboxidivorans*. A certain degree of variance can be observed in the butanol free case of growth for *E. limosum*; as discussed previously, a greater amount of biofilm flocculation had provided spectral interference with certain samples taken. Despite this concern aside from the remaining experimental analysis, it would appear that *E. limosum* could make a potential secondary organism in a biobutanol process capable of maintaining lower yields of this substance within the bulk liquid of the bioreactor.



**Figure 4.1.7 - Butanol Tolerance for *E. limosum*. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

Overall, a necessity for the robustness and wider applicability of a secondary carboxydrotrophic organism is required for a mixed culture fermentation with toxicity concerns present. Without such a consideration in mind, it would become highly improbable that effective growth and maintenance of this culture could continue as the butanol yield increases with time. After examination of this metric, it would appear that *C. carboxidivorans* is the more ideal candidate; with growth shown at nearly every butanol concentration tested and the ability to achieve a stationary phase despite inhibitory effects helps to justify why this microorganism should be the best option for this mixed culture fermentation. However, as some growth had been seen with both organisms and in accordance with the experimental plan described in Figure 3.1.1, further testing of the two bacteria should be concluded with the gassing trials. Before an absolute decision and experimental preparations can be made for future testing, the final parameter of CO behaviour in terms of consumption and production formation must be performed to effectively model the system proposed.

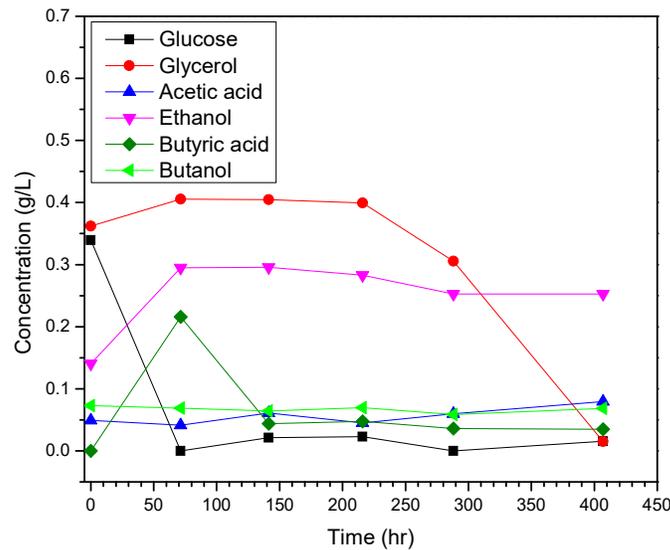
#### 4.1.5 CO Consumption & Product Formation

Several challenges exist in the attempt to determine whether a microbe can consume a gaseous substrate like CO and to what degree of success this consumption presents in terms of kinetics, production rates, and other applicable factors. Like many strains of aerobic bacteria dependent on a constantly available dissolved oxygen content within a bulk liquid medium, carboxydrotrophs must have available amounts of CO within a liquid in order for metabolic processing to occur. Like oxygen, dissolvable carbon monoxide in liquid sources proves even more challenging given its lower solubility rates at ambient temperature and pressures. Even for biological systems operating at 10 °C higher than ambient conditions, the obtainable volume of dissolved CO decreases once more; in order to conduct an idealistic experiment capable of truly showing growth and product formation on CO, some deviations must be made to prior experimental procedures.

As in previous cases, cultures of *C. carboxidivorans* and *E. limosum* were grown on RCM containing 20 g/L glucose. Once an absorbance value was recorded greater than 1.0, experimental serum bottles were inoculated at 2% v/v of the total liquid volume; a lower inoculation volume was used to limit the amount of residual glucose contributing to the serum bottle system and to effectively determine if any growth and/or production, no matter how miniscule, was recorded during experimentation. A modified medium from literature (Phillips et al., 2015) was used where 0.39 g/L NH<sub>4</sub>OH was added to the bulk liquid. Finally, a reducing agent consisting of 2% Na<sub>2</sub>S and cysteine-HCl, respectively, was used prior to inoculation in order to maintain a lower reductive potential in the hopes of a more ideal CO-consuming environment. After inoculation and distribution of 50 mL of media into the serum bottles, these containers were stopped and clamped prior to gassing with a 35/10/5/50% v/v gas mixture of CO/CO<sub>2</sub>/H<sub>2</sub>/N<sub>2</sub> for 90s. To assist in potential solubility of these gases, the 50 mL bottles were pressurized for 10 and 30s respectively. Once completely sparged and pressurized, the bottles were kept at 35 °C and at 200 RPM to ensure ideal growth conditions and increased interaction between the gas/liquid boundary layer.

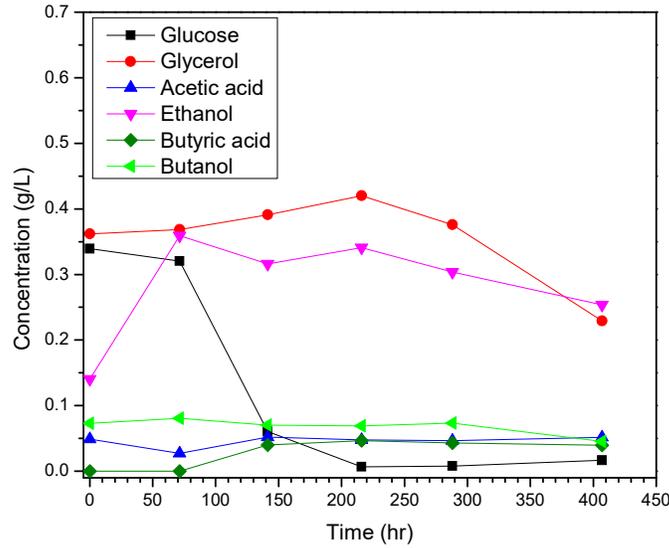
The figures below describe the consumption and production profiles for each case tested. Figure 4.1.8 and Figure 4.1.9 display the *C. carboxidivorans* profile when supplied with 10 and 30 s of gaseous overpressure. Any residual glucose left over from the preculture was fully consumed after 72 hrs; what remains a point of interest is the eventual consumption

of residual glycerol within the system after 215 hrs. As the available CO is much harder to solubilize and thus utilize, *C. carboxidivorans* resorted to the use of aqueous glycerol as a carbon source to avoid further starvation. Despite ethanol, all end products formed were under a titre of 0.1 g/L, save for the lone butyric acid peak at 72 hrs. This peak is likely caused from the residual glucose consumption, as this acid appears to be consumed after this time point to non-useable levels.



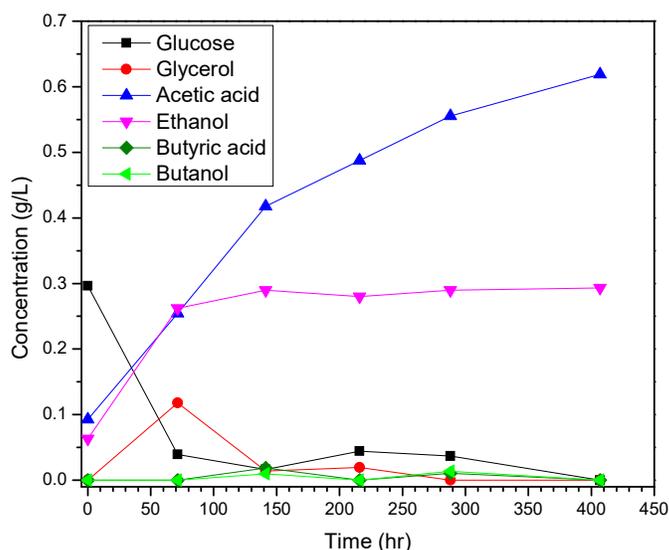
**Figure 4.1.8 - Substrate & Product Concentration Profile of *C. Carboxidivorans* Gassed with 10s Overpressure.**

The pattern observed in Figure 4.1.9 proved fairly comparable to that of Figure 4.1.8, save for some minor differences. While glycerol consumption does appear to happen in this scenario as it also happened in the 10 s overpressure case, the onset and slope of consumption takes longer to complete and is reduced in severity. Furthermore, any amount of butyric acid is not detected until 144 hrs, where no peak in production was detected as above. As the sample times are significantly spread, the point in which this peak could have occurred may have been missed. Along with the apparent glycerol inhibition, it appears that the glucose uptake is elongated as well. As such, it can be concluded that 30 s of gas overpressure caused substrate inhibition onset by either the increased CO content or excessive system pressure on the cellular cultures.



**Figure 4.1.9 - Substrate & Product Concentration Profile of *C. Carboxidivorans* Gassed with 30s Overpressure.**

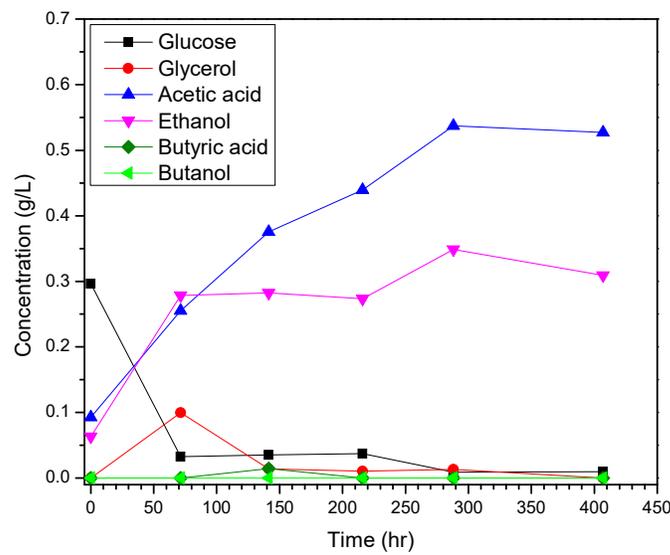
Figure 4.1.10 and Figure 4.1.11 display the consumption/production kinetics experienced by *E. limosum* with the same overpressure values as above. In comparison to *C. carboxidivorans*, *E. limosum* exhibited much faster consumption of both glucose and glycerol, with these substrates apparently being exhausted after 72 hrs. Furthermore, while ethanol titres remained at approximately 0.3 g/l for both species, *E. limosum* was able to produce six times more acetic acid with the use of CO than *C. carboxidivorans*. However, no significant amounts of butyric acid or butanol were detected from this species unlike the averaged amount of 0.05 g/L with *C. carboxidivorans*. Changes to the fermentation system setup or increasing the available content of dissolved CO would likely onset butyric acid production when greater quantities of this gas and acetic acid are able to be readily converted.



**Figure 4.1.10 - Substrate & Product Concentration Profile of *E. limosum* Gassed with 10s Overpressure.**

Very few differences can be surmised from the data collected in Figure 4.1.10 and Figure 4.1.11, respectively. This sort of close agreement between the two tested overpressure values likely notes that more dissolved CO and in turn either more gas and/or pressure would provide an increased production of the metabolites currently detected along with the potential onset of butyric acid formation that the mixed culture fermentation requires. With butyrate formation seen at concentrations below 0.1 g/L with *C. carboxidivorans* only and a greater presence of sustainable biomass growth required from both cultures, further testing and reconfiguration of experimental design should be pursued as described in Figure 3.1.1. Efforts to test greater pressures with higher concentrations of CO could be performed to determine whether any inhibitive behaviour is observed when either parameter is increased using *E. limosum*.

Future work should include the suggestions provided above, along with the use of an isolation method to separate preculture cells from the remaining broth. Certain techniques such as centrifugation and cell washing can assist in removing undesired compounds and substrates. Further research into the effects of redox potential could be implemented once a desired, established culture capable of producing butyric acid has been founded. Once completed, this work would finally determine which carboxydophilic organism would be best suited for a mixed culture fermentation.



**Figure 4.1.11 - Substrate & Product Concentration Profile of *E. limosum* Gassed with 30s Overpressure.**

## 4.2 Aerobic Xanthan Fermentation

### 4.2.1 Initial Media Screening

The natural progression of this work should follow a continued interest with CO solubility and providing a system where solubility could be increased to therefore increase possible growth/product profiles. However, as the scale-up equipment such as the 50-L pressure fermenter used was not certified for the use of CO, a model process investigating solubility challenges with the aerobic xanthan fermentation would provide some analogous results to what is experienced with syngas fermentation. As the major limitation with xanthan fermentation is the increasing liquid viscosity thereby reducing oxygen solubility, similar solutions found could be applied to syngas fermentation with future work performed.

The definition of what an optimal production medium is depends on several factors related to the fermentation at hand. A production medium could reduce lag times in cellular growth to allow a greater period for production to occur, or the exact opposite scenario could be pursued if growth-associated production is a necessity. Likewise, the ideal growth conditions of an organism may not be entirely reflective for its ideal production conditions, and the composition of what makes a production medium different from a growth medium plays a significant role in these mixtures. For the case of *X. campestris*, it is essential that a medium is able to supply an appropriate C/N ratio in the latter stages of fermentation to better maximize xanthan productivity. Moreover, the presence of key elements such as calcium, magnesium, and iron are essential not only to the metabolic pathway necessary to produce xanthan, but contribute to the stability and desired structure of the polymer while suspended in a liquid medium as discussed in Section 2.2.4.1. In turn, the discovery of a high-performing production medium for xanthan requires healthy cellular growth, effectively higher viscosities denoting a higher xanthan yield, and base characteristic compounds needed for high-quality xanthan to be synthesized.

However, the contributions towards a successful fermentation cannot be solely credited to a remarkable medium alone; the study and use of well-researched trace elements solutions aid in the particular sensitivities of molecular biology. The use of expensive but necessary biocatalytic elements such as cobalt, copper, and manganese prove their worth not in an overwhelming presence of a macro-additive sense like the elements above, but through

their use in very dilute concentrations in the initial growth phases of a bacterial colony. In the case of *X. campestris*, the presence of a trace elements solution usually provides the building blocks required to grow at an accelerated rate towards the latter phases of xanthan production once at stationary phase. While this sort of growth may prove simpler in minimal media recipes where exact quantities of compounds are definitively measured, the addition of trace elements in variable complex media proves to achieve greater growth overall. Thus, the effects of a newly developed trace elements solution were simultaneously looked into along with other factors stated above.

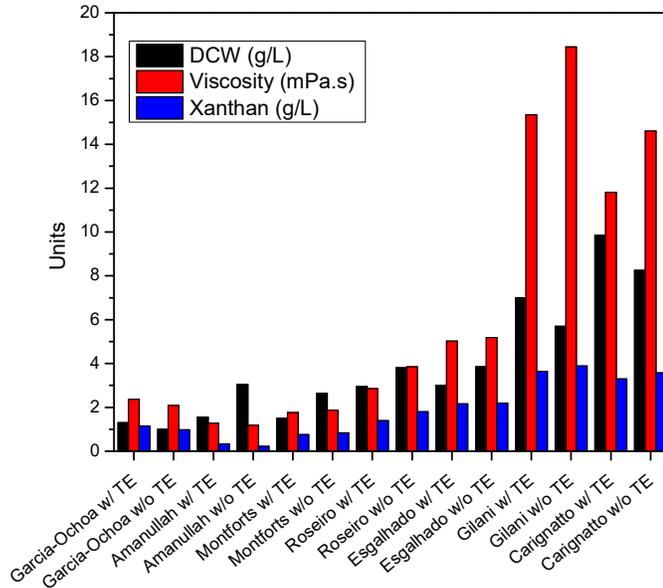
To conduct the following media screening, seven media were chosen from a review of related literature pertaining to successful xanthan fermentations and media performance studies. Cryocultures of *X. campestris* were grown on YM medium at 10 g/L glucose for a period of 20 hrs prior to experimental start. The seven different media – four complex and three minimal media recipes – were all prepared with 40 g/L glucose to guarantee growth and xanthan production. In addition, two sets of flasks were prepared with the same medium but also with or without the presence of the developed trace elements solution. Once the preculture was ready, all flasks were inoculated at 5% v/v of the 20 mL of total media; these flasks were then agitated at 300 RPM and kept at a constant temperature of 28 °C and a pH of approximately 7 with buffering from 30 g/L of MOPS. After 28 hrs, the experiment was stopped, and endpoint samples for the dry cell weight and viscosity measurements were taken. The prediction of the amount of xanthan within each flask was determined from the following correlation (Xuewu et al., 1996), Equation (1), relating reaction temperature  $T$  and medium viscosity  $\eta$  to xanthan concentration  $C_x$ :

$$C_x = \frac{\ln(\eta)}{(0.00075 \times T + 1.8531) \ln(36.7951)} \quad (1)$$

Figure 4.2.1 denotes the biomass concentration and viscosity values along with the predicted xanthan concentration obtained from each media scenario. There are several analyses one must conduct when viewing both the biomass and viscosity data from an overall perspective, a media-based perspective, and a trace elements perspective. In terms of overall results, the four cases with the highest achieving values in both biomass and viscosity result from the Carignatto and Gilani media with and without trace elements solution. Between

these four scenarios, the Gilani cases had higher viscosity values while the Carignatto cases had higher biomass concentrations; likewise, each media set had produced higher values overall when in the presence of trace elements. Several explanations can be deduced from these observances. The Carignatto medium has a lower C/N ratio than that of Gilani due to the use of peptone instead of yeast extract. The lowering of the C/N ratio has been seen to increase yields in biomass whereas the inverse of this increases achievable xanthan yields and thus viscosity too. As both of these media recipes are complex and use undefined nitrogen sources like peptone and yeast extract, the initial lag phase in colony growth is greatly reduced due to the abundance of minerals proteins, and other nutritious compounds present in these mixtures. The other two complex media, Roseiro and Esgalhado, contain nearly five times less of these complex sources and therefore have more comparable results as seen in the minimal media cases.

The minimal media cases display a much different story when compared to the complex, with nearly all recorded values being anywhere between two to nine times lower in amount. The limitation of these minimal media in this timeframe can be attributed to the necessity to buildup cellular proteins and other macromolecules before production can occur at a steadier rate. Of the minimal media cases, García-Ochoa with and without trace elements provided the highest viscosity values while the Amanullah case without trace elements had the highest biomass concentration. Like in the complex scenario described above, the greater C/N ratio of García-Ochoa had guaranteed better xanthan production instead of better biomass growth like in the Amanullah case. Among all the minimal cases, the addition or lack of trace elements slightly varied biomass growth depending on the medium and appeared to not have any influence on the achieved viscosity. A graph depicting the productivity values of viscosity along with biomass and xanthan concentrations obtained can be seen in Appendix 22.



**Figure 4.2.1 - Endpoint Analysis of Biomass, Viscosity, & Predicted Xanthan Levels after 28 hrs (TE stands for Trace Elements Solution).**

Overall, the experiment had concluded with results more favourable in the short-term production of complex media recipes over minimal media. With this in mind, it is necessary to still look into what other factors apply when allowing both media types to achieve stationary growth and production rates. On the basis of achieved viscosity and biomass concentration, the data above determines that the best complex media are the Gilani and Carignatto recipes while the best minimal media are the Garcia-Ochoa and Amanullah recipes. It was also recorded that the developed trace elements solution either had a notable difference in both studied parameters and/or provided little to no negative effect on the recipes chosen for continued experimentation. As such, further investigation into the role of this trace elements solution would be required to have a better understanding into its effects on the fermentation. Likewise, a secondary comparison of on-line metabolic behaviour via oxygen transfer rate as outlined in Figure 3.2.1 would assist in ensuring the proper media recipes were chosen for work following the flask trials.

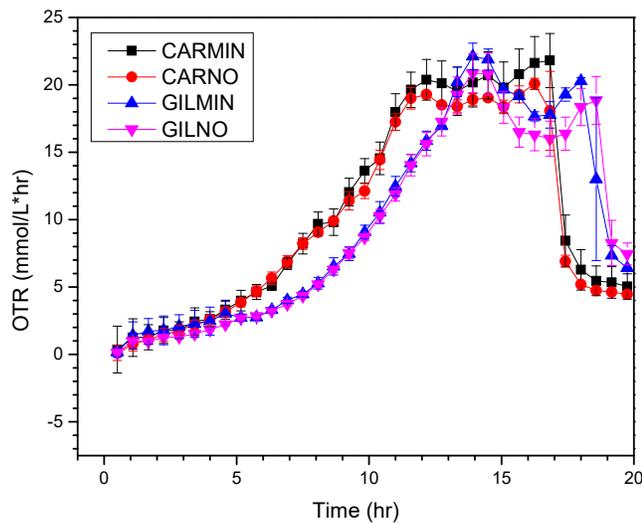
#### 4.2.2 On-line Media Comparison with Trace Elements Effect

With the best performing complex and minimal media recipes chosen, a more extensive study into the kinetic patterns through oxygen demand could be done to better understand which media provide better results. The ability to observe the oxygen and carbon dioxide transfer rates (OTR and CTR, respectively) would provide a clearer idea as to how this aerobic fermentation works and to what nutritive requirements do to these systems in place. Additionally, a more experienced analysis into what the trace elements solution does for *X. campestris* can possibly be made clear if changes in respiratory behaviour along with biomass and product formation are observed collectively.

Four media recipes – Gilani, Carignatto, Garcia-Ochoa, and Amanullah – were prepared with and without the developed trace elements solution. With an initial glucose concentration at 10 g/L and 30 g/L MOPS buffer, the initial medium was inoculated with approximately 5% v/v of *X. campestris* grown on YM medium at 28 °C and 300 RPM for 20 hrs as described previously. Once the experimental RAMOS flasks were inoculated and 20 mL of measured medium were evenly distributed into the system as needed, baseline tests ensuring the temperature, agitation, and pressure sensors detecting O<sub>2</sub> and CO<sub>2</sub> partial pressures were conducted and confirmed functional. The system was then operated at a pH of 7, 30 °C, 300 RPM, and at 20 mL liquid volume to best ensure that oxygen transfer conditions were kept uniform for all experimental trials. The experiment was stopped once it was believed that changes in either the OTR or CTR values had become negligible. Once stopped, endpoint samples of biomass concentration and viscosity values were taken. As described previously, the xanthan concentration was estimated from Equation (1).

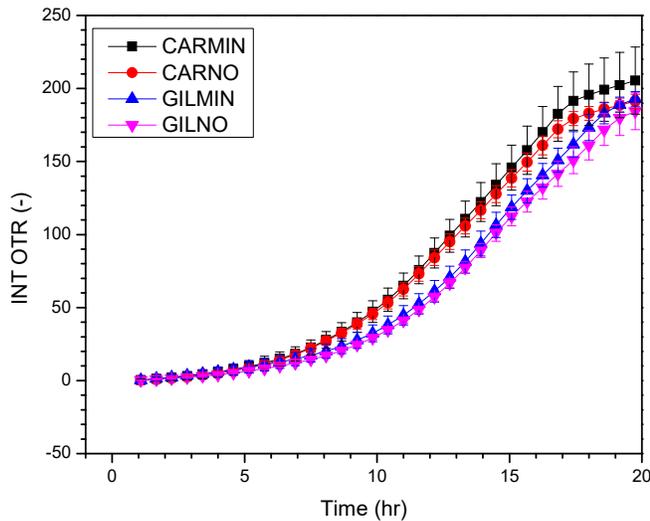
Figure 4.2.2 and Figure 4.2.3 describe the oxygen transfer rates and their associated integral curves from these values. As there is greater focus in the behaviour of oxygen in this system and the kinetic patterns produced are quite similar between OTR and CTR data, CTR-related figures can be found in Appendix 23-24. These two figures depict what is occurring for the two complex media picked for this study; as it can be seen, both variations of Carignatto medium are utilizing oxygen at a greater rate than the cultures in both Gilani variations. As understood in Section 3.2.1.2, the Gilani medium is much richer in its inclusion of macronutrients with semi-defined quantities of iron, sodium, calcium, and zinc in addition to yeast extract. However, the only components Gilani lacks other than differing

complex sources of nitrogen that the Carignatto medium possesses are glutamic and citric acids, which are two compounds well studied in terms of interaction with xanthan production. Glutamate, a secondary nitrogenous source along with being one of many amino acids necessary for protein production, lowers the C/N ratio in comparison to the Gilani medium to better favour cellular growth. The purpose of citric acid, meanwhile, helps enhance heavy metal solubilisation for metabolic pathways essential to both growth and production along with assisting in pyruvate regulation into the xanthan molecule matrix. These small additions to a simple medium such as Carignatto may just be the necessary compounds lacking in most other media recipes targeted for *X. campestris*.



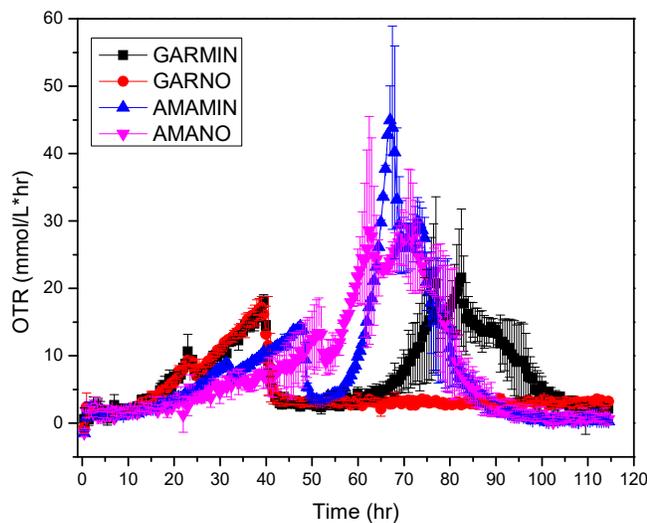
**Figure 4.2.2 - Oxygen Transfer Rates for Complex Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

The data recovered in Figure 4.2.3 further corroborates what was observed in Figure 4.2.2; while the differences in the amount of oxygen used between both complex media is rather insignificant at best, the advantage in terms of greater usage, which in turn should likely effect the greater xanthan yield, is seen in the Carignatto medium. In terms of the use of the trace elements solution, both figures show a slight benefit to the presence of these trace elements within the medium versus without. As Figure 4.2.3 shows Gompertz function-like behaviour at the 20 hr mark along with the decrease in oxygen demand in Figure 4.2.2, it was assumed that the reaction had reached its end and required offline data was collected accordingly.



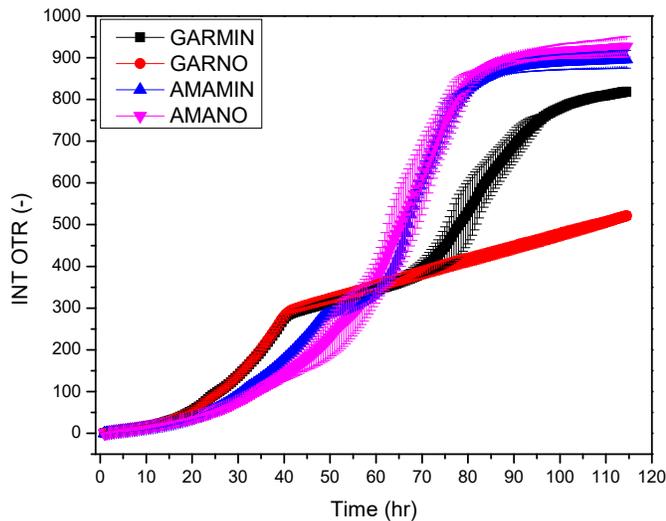
**Figure 4.2.3 - Oxygen Transfer Rate Integrated Curves for Complex Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

Despite what had been seen in terms of results in the complex media, the information gathered from the minimal media cases proved both different and exciting in metabolic nature. Figure 4.2.4 and Figure 4.2.5 show a total fermentation time of nearly 120 hrs, which is almost six times that of the complex media cases. Like in the above account, the corresponding CTR data can be found in Appendix 25-26. It is essential to note here, however, that the OTR curves present in both Garcia-Ochoa and Amanullah media demonstrate two-peak behaviour usually indicative of diauxic growth. That being said, there are several areas of interest to observe within this data. First, while the shape of the first peak is remarkably similar between both cases of Garcia-Ochoa, the OTR curve denoting Amanullah medium lacking trace elements does not have as sharp a spike downwards as Amanullah with trace elements, and instead almost appears linear in its approach to the second peak. In some form, the trace elements solution appears to inhibit some sort of metabolic switch that occurs between the two peaks. Conversely, the opposite behaviour can be observed in the Garcia-Ochoa medium, as the set lacking trace elements is unable to produce the second peak observed.



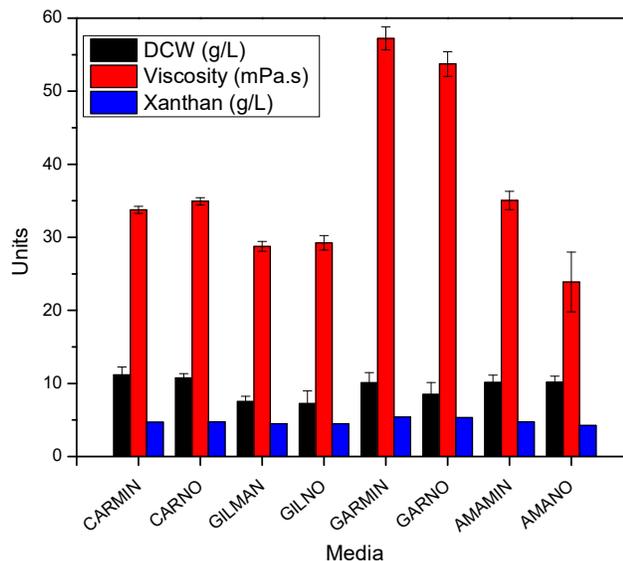
**Figure 4.2.4 - Oxygen Transfer Rates for Minimal Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

As in Figure 4.2.4, the account told in Figure 4.2.5 helps substantiate the data previously witnessed. The oxygen demand for both Amanullah cases proved slightly and significantly lower than Garcia-Ochoa with and without trace elements, respectively. Taking all of these analytical points in mind, some potential causes for these deviations should be addressed. The most significant differences between these minimal media derives from the use of either  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{NO}_3$  along with the significant increase of sodium concentration in Amanullah and Garcia-Ochoa, respectively. Interactions of heavy metals with liberated chlorine ions following nitrogen consumption could describe the difference in peak behaviour between the two cases of Amanullah medium. Likewise, the increase in sodium ions could be the contributing factor as to why oxygen demand is greater in Amanullah than Garcia-Ochoa, as respiratory conditions would be more favourable in liquids with higher salts concentrations. At any rate, these figures alone do not confirm what minimal media has a greater impact.



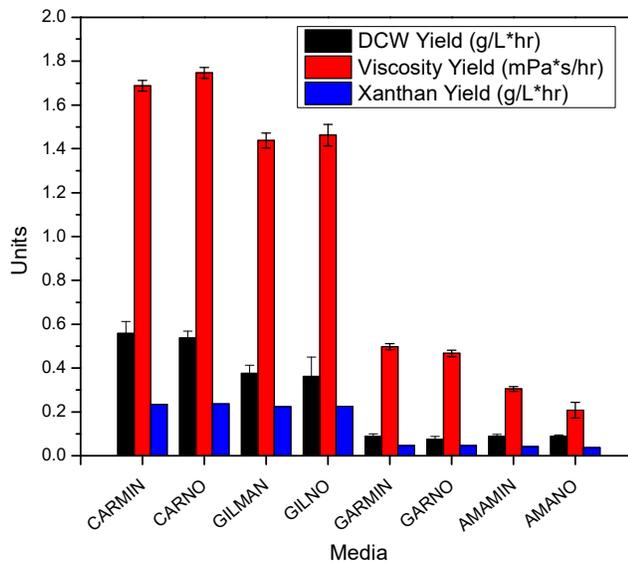
**Figure 4.2.5 - Oxygen Transfer Rate Integrated Curves for Minimal Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

Figure 4.2.6 denotes the endpoint biomass and viscosity values achieved along with the predicted xanthan model based on operating temperature and final viscosity values. It can be accurately confirmed that the majority of minimal media sets had higher viscosity systems than that of the complex media, with the Garcia-Ochoa cases proving to lead in this regard with values just below 60 mPa·s. Alternatively, the majority of biomass concentrations remain somewhat constant between all cases with values ranging either at or just below 10 g/L, which would denote that healthy cellular growth has occurred without any sort of indication that inhibition was present between media recipes. This data, when examined in conjunction with the two-peak behaviour seen above, provides the reader with the fact that the bulk of xanthan formation must occur during the first OTR peak, as there is little difference achieved between the Garcia-Ochoa media with and without trace elements. However, as the time for the complex cases was significantly shorter than that of the minimal, a study into both media types' kinetic behaviours should be done on a similar time scale. As such, if this study were to be falsified, some form of unknown secondary metabolite formation, polymer reconstruction or reconfiguration, or other unknown biological process could be occurring during the second OTR peak to cause such speculation.



**Figure 4.2.6 - Endpoint Analysis of Biomass, Viscosity, & Predicted Xanthan Levels after 20 & 115 hrs for Complex & Minimal Media, Respectively. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

Finally, the data collected in Figure 4.2.7 helps one to understand exactly how efficient each different media recipe actually was. As expected, the productivity values for the complex media are anywhere between three to nearly nine times greater than the minimal media cases. Although achieving a high and realistically feasible amount of xanthan is required to have this process happen economically and efficiently, the amount of time taken to complete the reaction is also central to proper process design and control. In this sense, once again it is observed that the complex media proves more favourable in establishing a cellular culture quicker with moderate amounts of xanthan present in the bulk liquid.



**Figure 4.2.7 - Analysis of Biomass, Viscosity, & Predicted Xanthan Productivities after 20 & 115 hrs for Complex & Minimal Media, Respectively. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

Overall, several interesting findings were concluded after careful consideration and analysis into the online measurement of oxygen transfer with *X. campestris*. Primarily, the two media of interest that proved most favourable in terms of product yield, growth behaviour, and successful interaction with the trace elements solution were the complex Carignatto and the minimal García-Ochoa. These two media both provided higher viscosity values in comparison to its competitors and had initially quicker fermentation progress as well. Although the behaviour seen in García-Ochoa proved unexpected with the dual-peak OTR system, further investigation with both media should help reduce any outstanding

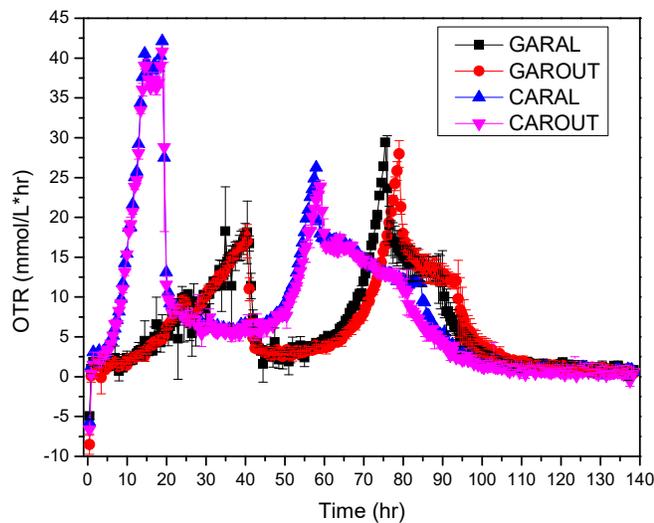
questions regarding the nature of such performances. A secondary comparison of these two phenomenon and an inquiry into the actual components of the trace elements solution should solidify that Carignatto and García-Ochoa media are the two best options for future work performed in the manner outlined in Figure 3.2.1.

#### 4.2.3 On-line Media Comparison with Aluminum Sulfate Effect

The presence of aluminum ions in downstream purification processes for xanthan recovery have been seen to increase the recovery of xanthan from fermentation broth in rather significant ways (Tempio & Zatz, 1981). Due to the higher positive charges associated with aluminum in suspensions, negatively charged xanthan polymers are attracted and form an easily recoverable metal-polymer bridged adsorption material. However, the generally low bioavailability of most microorganisms to use aluminum along with its noted toxic effects create some issues as to when and where to include this ion effectively. The dilute inclusion of aluminum in a trace elements solution could allow the desired bridging mechanism to occur prior to recovery processes along with a potential for this heavy metal to become tolerated with longer exposure to *X. campestris* while in fermentation. As such, a study into the effects of aluminum sulfate within the developed trace elements solution was performed in accordance with a time-equal study between a complex and minimal media.

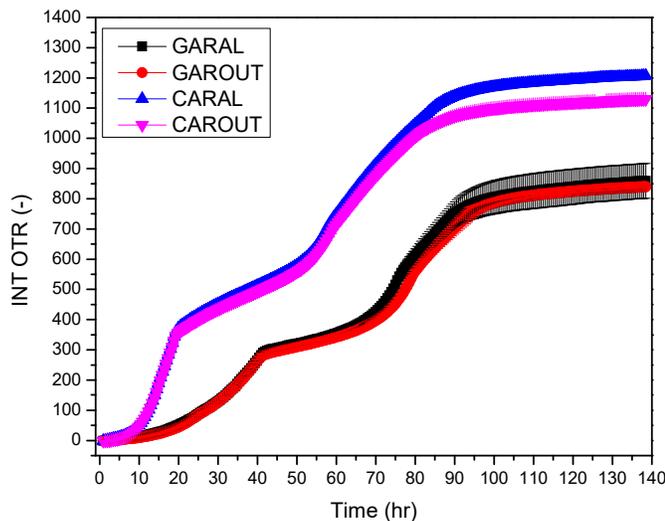
As in previous studies, *X. campestris* was grown on YM medium at 28 °C and 300 RPM. Once 20 hrs have passed, the preculture was able to inoculate the media under investigation at 5% v/v. The media preparation involved the synthesis of Carignatto and García-Ochoa recipes with 10 g/L and 30 g/L glucose and MOPS buffer, respectively. Additionally, two versions of the trace elements solution were prepared containing and lacking 0.50 mg/L of aluminum sulfate respectively, and then added to each media as required. Once prepared and inoculated, flasks containing 20 mL of the media were input into a RAMOS device operating at 30 °C, 300 RPM, and in a system of pH approximately 7.0. After configuration was done as described above, the RAMOS device was operated until the expected OTR and CTR patterns were observed. Once complete, endpoint samples of biomass concentration and xanthan were taken and xanthan concentration was estimated using Equation (1).

Figure 4.2.8 and Figure 4.2.9 remark on the OTR patterns and their integrated curves of all cases observed in this study; due to similarity and lesser amount of interest in CTR patterns, similar figures can be observed in Appendix 27-28. In Figure 4.2.8 one can observe the similarity in structure but overlying differences in slope and shape of the OTR curves generated. The Carignatto media cases prove faster overall, once again thanks to the presence of complex medium compounds within its recipe. Its first peak is narrow and achieves an OTR approximately twice of the Garcia-Ochoa media, but its second peak remains relatively similar to its competitor as well. The addition of aluminum sulfate appears to have little effect on either media's OTR, but a slight decrease in overall production time of the Garcia-Ochoa with trace aluminum case was observed. After about 110 hrs, it appears that the fermentation had reached completion.



**Figure 4.2.8 - Oxygen Transfer Rates for Two Media with & without Aluminum Sulfate Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

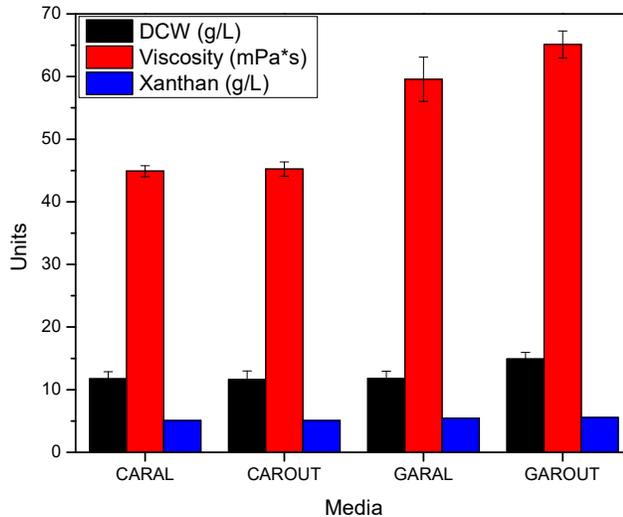
The significant difference in oxygen demand is definitively apparent from the integral curves in Figure 4.2.9. The final values for the Carignatto medium rests anywhere between 1100 to 1200 points, making this medium approximately 300 to 400 points higher than García-Ochoa. In the case of aluminum addition, it would appear that the demand for oxygen increases once the reaction nears completion, as dissolved aluminum in the bulk liquid would contribute to retaining any dissolvable amounts of oxygen made available to the system. The data from these two figures would likely indicate that the Carignatto medium would prove the greater producer in both biomass and xanthan formation, assuming that a higher oxygen transfer rate denotes increased cellular activity.



**Figure 4.2.9 - Oxygen Transfer Rate Integrated Curves for Two Media with & without Aluminum Sulfate Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

However, the results obtained from the endpoint analysis as seen in Figure 4.2.10 seem to refer to contrary beliefs. With a similar figure displaying each productivity in Appendix 29, both cases of García-Ochoa had achieved viscosities about 20 mPa·s higher than that of Carignatto. Furthermore, a biomass yield of about 15 g/L was the highest obtained from the García-Ochoa medium lacking trace aluminum, with all other scenarios achieving about 12 g/L. This revelation seems to indicate that xanthan production is more prevalent during the initial growth phases of the fermentation than previously thought. Such

efficient production could be the result of a lack of complex components such as peptone taking away from the primary focus of xanthan production for protein production instead. This caveat, along with a richer provision of macronutrients with notable increases in the amount of citrate and magnesium present, could be the contributing factors towards the Garcia-Ochoa medium.



**Figure 4.2.10 - Endpoint Analysis of Biomass, Viscosity, & Predicted Xanthan Levels after 138 hrs for Complex & Minimal Media. Experiments were run in duplicate and the standard deviation was recorded for each data point.**

In this research focus, it was determined that the increased activity of OTR in one media does not necessarily translate to a general increase in xanthan yield. Other metabolic pathways and cellular functions available with the presence of proteins and other complex molecules could be interfering with the main fermentative production of xanthan. Due to better viscosity values measured in the flasks lacking aluminum sulfate, proceeding work done on these two media excluded the compound for all future experiments as made clear in the work outline from Figure 3.2.1. For better understanding of how these media will perform in a bioreactor setting, the scaling up and direct comparison of both cases in fermentation should be made clear in operational and growth considerations.

#### 4.2.4 Media Comparison at Bench-scale Fermentation

It is through careful planning and the comprehension of fundamental bioreactor design that experimental scale-up is made feasible. To know which parameters to keep proportional from each different stage requires both knowledge and expertise in the field of study, and the aerobic fermentation of *X. campestris* is no different. To better connect previous experiments using flask agitation and constant headspace aeration to a bench-scale fermentation using impeller agitation and constant bubbling aeration, an effort should be made to keep the volumetric power requirements constant between these scenarios. With this constant maintained, the effects of volumetric mass transfer between the liquid and gas phases can be assumed the same and a greater recognition of a continuous piece of work can be formulated as such. To maintain similar conditions, the following correlations and calculation procedure was conducted from research summarizing the effects of aeration and power consumption in several agitated systems (Büchs, Maier, Milbradt, & Zoels, 2000).

To maintain constant volumetric power requirements between this and previous flask experiments, a cascade control system using dissolved oxygen content as an input controlled the impeller speed within the fermenter. At any time the dissolved oxygen tension signal dropped below a value of 30%, an increase to impeller speed was made within a certain range. To calculate this range, the following constants as displayed in Table 4.2.1 were either measured or assumed prior to experimentation and used in the calculation procedure.

**Table 4.2.1 - Constant Values Used in Agitation Range Determination**

<b>Constant</b>	<b>Symbol</b>	<b>Value</b>	<b>Units</b>
Liquid Density	$\rho$	1000	kg/m <sup>3</sup>
Flask Rotational Speed	$N_F$	5	1/s
Flask Diameter	$d_F$	0.08	m
Bench Fermenter Diameter	$d_R$	0.125	m
Volumetric Gas Flow Rate	$Q_g$	$25 \times 10^{-6}$	m <sup>3</sup> /s
Gravitational Acceleration	$G$	9.81	m/s <sup>2</sup>
Flask Liquid Volume	$V_{LF}$	$20 \times 10^{-6}$	m <sup>3</sup>
Bench Fermenter Volume	$V_{LR}$	$1.5 \times 10^{-3}$	m <sup>3</sup>
Superficial Gas Velocity	$u_g$	$2.037 \times 10^{-3}$	m/s

Once these values were obtained, a realistic viscosity range was required to fully create the desired control system. Previous maximum viscosity values were taken from Section 4.2.3 for the Carignatto and García-Ochoa media, and a lower and upper limit of 0.001 and 0.100 Pa·s was proposed as likely extreme values, as seen in Table 4.2.2. Once obtained, the Reynolds number of the previous flask experiments was calculated using Equation (2):

$$Re_F = \frac{N_F d_F^2 \rho}{\eta} \quad (2)$$

From here, the modified power number  $Ne'$  along with the flask power requirement  $P_F$  were able to be calculated using the Reynolds number, density, agitation speed, flask diameter, and total flask liquid diameter using Equations (3) and (4):

$$Ne' = \frac{70}{Re_F} + \frac{25}{Re_F^{0.6}} + \frac{1.5}{Re_F^{0.2}} \quad (3)$$

$$P_F = Ne' \rho N_F^3 d_F^4 V_L^{\frac{1}{3}} \quad (4)$$

With the flask power requirement found, it was possible to determine the unaerated volumetric power requirement used in both flask and fermenter systems. Prior to the determination of this constant, the calculation for the superficial gas flow rate had to be calculated for use in determining the gassed power requirement in the fermenter:

$$u_g = \frac{Q_g}{A_R} = \frac{4Q_g}{\pi d_R^2} \quad (5)$$

$$P = \left( \frac{P_0}{V_L} V_{L_R} \right) \left( 1 + 490 \left( \frac{u_g}{(g d_R)^{\frac{1}{2}}} \right) \right)^{-\frac{1}{2}} \quad (6)$$

Finally, as the system was to be continuously aerated while undergoing agitation simultaneously, the rotational speed for each scenario was calculated using Equation (7):

$$N_R = \left(\frac{P}{V_L}\right)^{\frac{1}{3}} \left(\frac{1}{d_R^{\frac{2}{3}}}\right) \quad (7)$$

With these calculations complete, it was resolved that the lowest possible limit of agitation would occur at approximately 250 RPM; for the case of the upper limit, it was perceived at this time that reaching a value nearing 100 mPa·s was not feasible in this scheme. As such, the average value between the Garcia-Ochoa and the originally proposed upper limit scenarios was found to be approximately 450 RPM and was used accordingly in this set of experimentation, as displayed in Table 4.2.2.

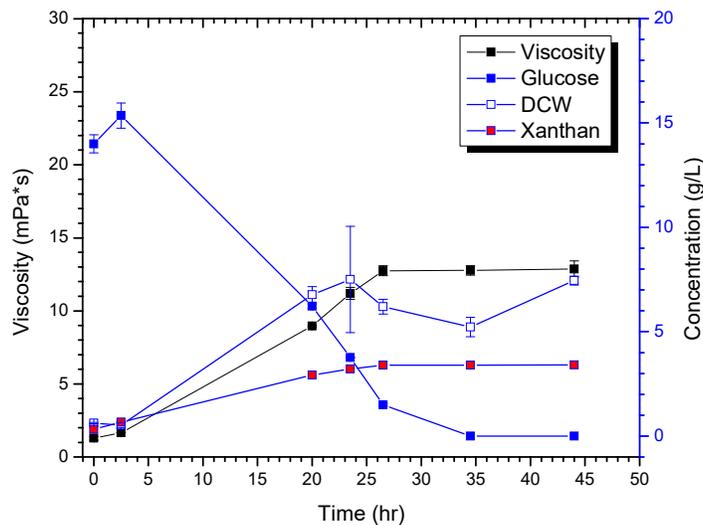
**Table 4.2.2 - Calculated Results in Determination of Impeller Agitation Limits**

<u>Parameter</u>	<u>Symbol</u>	<u>Scenarios (in order of increasing viscosity)</u>				<u>Units</u>
		<u>Lower Limit</u>	<u>Carignatto</u>	<u>Garcia-Ochoa</u>	<u>Upper Limit</u>	
<b>Viscosity (at 30 °C)</b>	$\eta$	0.001	0.045	0.062	0.100	Pa·s
<b>Reynolds Number</b>	Re	40100.251	710.054	513.298	320.000	-
<b>Modified Power Number</b>	Ne'	0.225	0.989	1.158	1.477	-
<b>Flask Power Requirement</b>	$P_F$	0.031	0.137	0.161	0.205	W
<b>Unaerated Volumetric Power Requirement</b>	$P_0/V_L$	1.564	6.870	8.047	10.263	kW/m <sup>3</sup>
<b>Aerated Power Requirement</b>	P	0.002	0.007	0.009	0.011	kW
<b>Aerated Volumetric Power Requirement</b>	$P/V_L$	1.134	4.982	5.836	7.443	kW/m <sup>3</sup>
<b>Aerated Bench Fermenter Rotational Speed</b>	$N_R$	4.172 250.293	6.832 409.908	7.202 432.100	7.810 468.587	1/s RPM

Prior to preculture setup, 1.0 L of medium under analysis were prepared for sterilization in an autoclave unit with probes capable of detecting the pH and dissolved oxygen tension (DOT) levels within the fermentation system. Once sterile, the effective amounts of trace elements and other non-autoclavable solutions, including a solution introducing 10 g/L glucose into the system, were added via sterile injection into the fermenter. The system was sparged with nitrogen and then fully aerated to determine the upper and lower limit values for the DOT probe. No pH control other than 30 g/L MOPS buffer was used in this set of experiments in order to better replicate the conditions present in

previous sections. Prior to the start of experimentation, *X. campestris* was grown in preculture at 10 g/L on either Carignatto or García-Ochoa medium depending on the contents of the bench-scale fermenter. This was done as to truly recognize the effects of the medium without any residual interference from a different growth medium. After 24 hrs, the fermenter was inoculated with 5% v/v of the preculture and an initial off-line sample was taken to later determine glucose and biomass concentration along with the viscosity measurement at this time point. The fermentation was considered complete when little to no increase was recorded in between two viscosity measurements after a certain period of time.

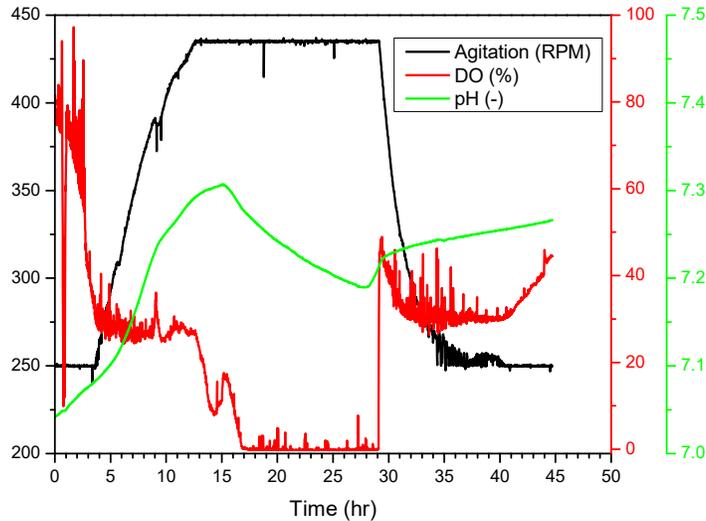
Figure 4.2.11 and Figure 4.2.12 describe the flux in several parameters when the complex Carignatto medium was tested at bench-scale. In Figure 4.2.11, the change in concentrations regarding glucose and biomass was recorded alongside viscosity flux; the amount of xanthan within this system was predicted using the model described in Equation (1). The amount of glucose initially being above the expected 10 g/L concentration can be attributed to residual glucose prior to inoculation. Furthermore, it can be observed that no significant change is recorded after 34 hrs save for the final increase in biomass concentration once more. This data on its own does not tell the complete story of what occurs within this fermenter setting, with particular questions such as the lower-than-expected viscosity values achieved in comparison to Sections 4.2.2 and 4.2.3. At some point between 26 to 34 hrs, the entirety of glucose is consumed from the system, thereby limiting any further xanthan production from the existing culture.



**Figure 4.2.11 - Glucose Consumption, Viscosity Flux, & Biomass & Xanthan Product Profiles for Complex Media Bench-scale Fermentation. The standard deviation was recorded for each data point from two, three, and four measurements for glucose concentration, viscosity measurement, and dry cell weight, respectively.**

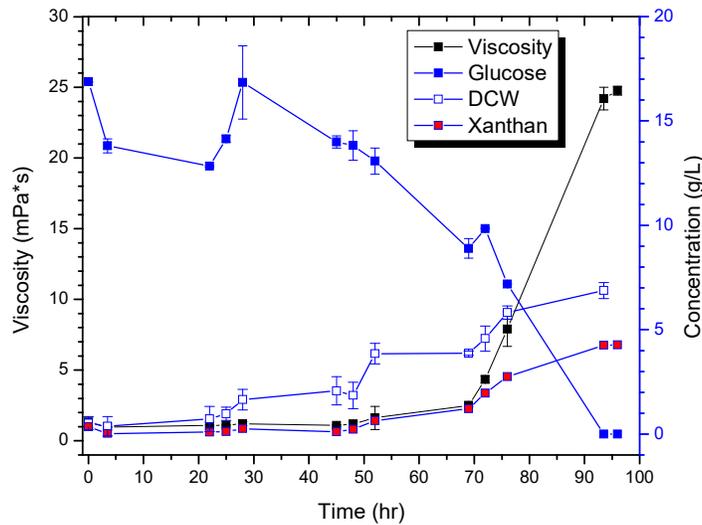
The performance data recorded in Figure 4.2.12 assists in answering some of these questions left unanswered from Figure 4.2.11. Much like the behaviour seen previously with the complex media, the *Xanthomonas* in Carignatto medium undergo an initially high demand in oxygen, with the increase in agitation speed seen as early as 4 hrs post-inoculation. By about 13 hrs, the upper limit of the agitation control has been reached, leading to complete oxygen deprivation by 17 hrs. This anaerobic system continues for another 12 hrs, which the endpoint can likely be considered the exhaustion of available glucose as noted in Figure 4.2.11. It is also at this 29 hr mark where the oxygen demand decreases dramatically with the sudden increase in dissolved oxygen tension; it can be safely assumed that the cells have fully exhausted all carbon source and begin to enter a death phase. The pH curve also appears to follow an inverse connection to the DOT remaining in the system. The shifting of behaviour from alkaline-increasing to acid-increasing once more back to alkaline-increasing could be indicative of different phases of growth and production with *Xanthomonas*. Interference of CO<sub>2</sub> production causing a decrease in the pH when DOT is depleted could be a reason as to this observed trend. This would also corroborate with the

glucose exhaustion, denoting the discontinued production of CO<sub>2</sub> and excess oxygen being once again available in the bulk liquid.



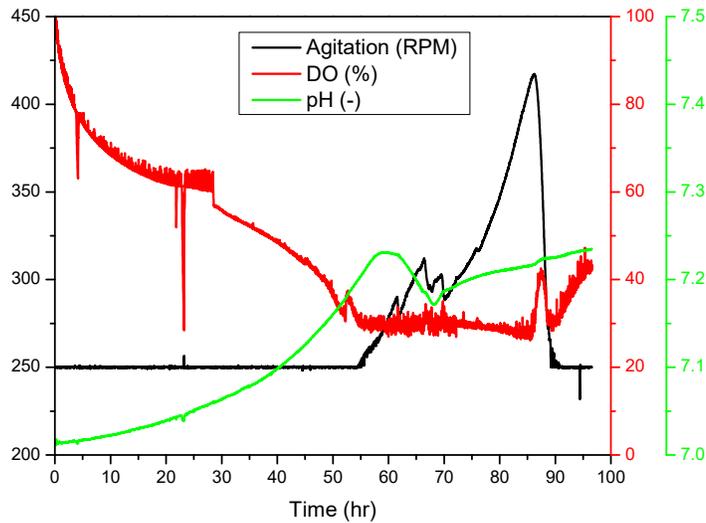
**Figure 4.2.12 - Dynamic Agitation, Dissolved Oxygen Content, & pH Profiles for Complex Media Bench-scale Fermentation.**

Figure 4.2.13 and Figure 4.2.14 represent the growth and production kinetics coupled with the fermentation parameters under investigation for the minimal Garcia-Ochoa medium. Much like the Carignatto case, the glucose concentration is seen to be higher than anticipated due to residual glucose from preculture. The kinetics and endpoint concentration of approximately 7 g/L of biomass remain similar in both cases, but significant differences are present in the total fermentation time and achieved final viscosity. With completion seen around 94 hrs, the Garcia-Ochoa scenario is nearly three times longer than that of Carignatto. However, the final viscosity value of approximately 25 mPa·s is nearly double that of the complex medium value as well. At the 72 hr mark, a sharp increase and decrease is found in viscosity and glucose concentration, respectively, as the biomass concentration surpasses 5 g/L. Similar behaviour was also observed in the Carignatto case, but to a lesser degree of slope severity.



**Figure 4.2.13 - Glucose Consumption, Viscosity Flux, & Biomass & Xanthan Product Profiles for Minimal Media Bench-scale Fermentation. The standard deviation was recorded for each data point from two, three, and four measurements for glucose concentration, viscosity measurement, and dry cell weight, respectively.**

With Figure 4.2.14, the same patterns with pH, DOT, and agitation can be seen with the Carignatto scenario, but elongated along a greater time range. Unlike the complex medium, there is no true exhaustion of dissolved oxygen within the bulk liquid; complete deprivation of oxygen in an aerobic culture can permanently damage or kill an entire culture. With the Garcia-Ochoa medium, there is no possibility that *X. campestris* will suffer starved growth or product formation like the Carignatto case. The pH peak is about 0.5 points lower and the agitation only peaks at one short time period compared to the prior case, further suggesting a more stable and manageable process design. As noted in Figure 4.2.13 with the sudden increase in viscosity, a very similar increase in agitation behaviour is observed, denoting that some sort of connection could be made in increased oxygen demand with further xanthan formation.



**Figure 4.2.14 - Dynamic Agitation, Dissolved Oxygen Content, & pH Profiles for Minimal Media Bench-scale Fermentation.**

The challenge of scale up in bioprocesses is always presented through the deviation from theoretical calculations to practical applications. In an attempt to keep the volumetric mass transfer systems the same from flask to bench-scale fermenter, the boundaries set with the agitation speeds proved non-optimal for the Carignatto medium compared to the Garcia-Ochoa. Despite this limitation in experimental design, the Garcia-Ochoa medium has proved more successful at several different scales with a greater viscosity achieved. The combined success of better viscosity and predicted xanthan yields along with a more controllable fermentation profile made this medium the high-performing production medium to be chosen as described in Figure 3.2.1. Given this important factor and the ease of fermentative control, a final scale up to a pilot plant setting would finally determine the success in finding the highest-performing production medium.

#### 4.2.5 High-Performance Media Pressure Fermentation

The effects of oxygen solubility have often plagued designs of bioprocessing equipment in the pursuit of obtaining near theoretical yields in aerobic fermentations. When a biological system is able to operate within the bounds of typical Newtonian fluids, remedies in bioreactor design through the use of a bubble column or different sparging plates/impeller configurations assist in providing a solubility above the required biological demand for any microorganism. When the fluid dynamics of a system begins to deviate into the non-Newtonian domain, the strong effects of these design changes become either reduced or even nullified in their efficacy. As such, different performance tactics must be employed to combat increasing levels in viscosity to meet the needed biological oxygen demand. To combat this, the effect of a pressurized fermentation on an empirically and experimentally determined high-performance production medium could be investigated as a means to supply oxygen as required.

Prior to the pressurized fermentation, two precultures were prepared in a series of increasing liquid volumes. The first preculture consisted of 80 mL of YM medium inoculated at 5% v/v with cryogenic *X. campestris*. Four flasks containing 20 mL each were maintained at 28 °C and 300 RPM for a period of 20 hrs before inoculation into the second preculture. The second preculture was maintained at 30 °C in a 1.5 L bench-scale fermenter prepared with García-Ochoa medium; this medium was chosen for use at this stage in order to minimize the residual effects of any other medium during the pressurized fermentation stage. The fermenter was prepared with pH control maintained at a value of 7.0 along with a cascade control system for maintaining dissolved oxygen tension levels above a value of 30% using impeller agitation as described in Section 4.2.4. A figure depicting the fluxes in pH, DOT, and agitation can be seen for reference in Appendix 30.

While these precultures were being prepared, the 50-L pressurized fermenter was prepared with García-Ochoa medium for steam sterilization. Separate solutions of glucose, trace elements, and other reactive and/or heat-sensitive compounds were made. Once sterilized, these solutions were added to make up a total liquid volume of 27 L within the fermenter with 60 g/L glucose as an initial substrate concentration. Several probes and in-house software programs were used to record and control several fermentation parameters such as several important temperatures, headspace pressure, agitation, DOT, pH, OTR, and

gas flow rate, among others. The system was sparged with nitrogen followed by purified air to determine all necessary sensors were performing as desired. Once the second preculture had matured to the initial phases of oxygen limitations at 30% DOT, 1.35 L (5% v/v) of the inoculum was drained into the remaining 25.65 L within the pressure fermenter. The reactor was initially maintained at 30 °C with a pH value of approximately 7.0 and with a headspace pressure approximately equal to zero. Aeration rate was constantly maintained at 0.5 vvm throughout the entirety of the fermentation; when DOT values approached 30%, pressure and gas flow increases were applied in a manner to maintain this constant.

A secondary method to predict an endpoint xanthan concentration was developed from previous calorimetric studies designed to provide online measurement of products using specific types of heat generated (Regestein, Giese, et al., 2013; Regestein, Maskow, et al., 2013), given in units of W/L respectively. A table summarizing the values of constants used in the following series of calculations can be found in Appendix 31. The first set of seven equations (Equations (8)-(14)) in this set revolve around the overall energy balance surrounding the cooling jacket along with special focus on the cooling jacket heat  $q_J$ . The determination of the global heat transfer coefficient and surface area  $(UA)_{global}$  is calculated from the contributions along the reactor-side and jacket-side of the cooling jacket along with the heat transfer through the jacket wall itself. Once known, these heat transfer variables can be multiplied by the log mean temperature difference  $\Delta T_{R,J}$  in order to determine the effect is current driving force has on the fermentation at hand.

$$q_J = \dot{m}c_p^{H_2O} (T_J^{out} - T_J^{in}) = (UA)_{global}\Delta T_{R,J} = q_{mech} + q_{bio} - q_{evap} - q_{loss} \quad (8)$$

$$(UA)_{global} = \frac{A}{\frac{1}{\alpha_R} + \frac{\delta}{\lambda_{steel}} + \frac{1}{\alpha_J}} \quad (9)$$

$$\alpha_R = Nu_R \left( \frac{\lambda_R}{d_b} \right) = 0.66 Re_R^{0.66} Pr_R^{0.33} \left( \frac{\lambda_R}{d_b} \right) = 0.66 \left( \frac{\eta_R c_P^R}{\lambda_R} \right)^{0.66} \left( \frac{n d_R \rho_R}{\eta_R} \right)^{0.33} \left( \frac{\lambda_R}{d_b} \right) \quad (10)$$

$$\alpha_J = Nu_J \left( \frac{\lambda_R}{\sqrt{\left(\frac{8}{3}\right) d_s}} \right) = \left( 0.03 Re_J^{0.75} Pr_J \left[ 1 + \frac{1.74 (Pr_J - 1)}{Re_J^{0.75}} \right] \right) \left( \frac{\lambda_R}{\sqrt{\left(\frac{8}{3}\right) d_s}} \right) \quad (11)$$

$$Pr_J = \frac{\eta_J c_P^J}{\lambda_J} \quad \& \quad Re_J = 2.67 \left( \frac{u_n d_g \rho_J}{\eta_J} \right) \quad (12)$$

$$u_n = \sqrt{\frac{\dot{m}}{\pi \rho_J d_g^2} \left( \frac{\pi}{4} \right) d_b d_s \rho_R} + 0.5 \sqrt{2 g h e (T_J^{out} - T_J^{in})} \quad (13)$$

$$\Delta T_{RJ} = \frac{T_{max} - T_{min}}{\ln \left( \frac{T_{max}}{T_{min}} \right)} = \frac{\left( (T_R - T_J^{in})(T_R - T_J^{out}) \right)}{\ln \left( \frac{(T_R - T_J^{in})}{(T_R - T_J^{out})} \right)} \quad (14)$$

With the jacket heat determined, the evaporative heat loss  $q_{evap}$  can be generated from the enthalpy of water evaporation multiplied by the volumetric gas flow rate and the ratio between the medium's partial pressure with that of the total system pressure in an assumed ideal gas manner. Equation (15) assists to highlight this relations as it follows:

$$q_{evap} = \Delta H_{vap}^{H_2O} \dot{V} \left( \frac{P_{medium}}{P_T} \right) \left( \frac{M_{H_2O}}{V_{norm}} \right) \quad (15)$$

The contribution of mechanical heat via agitation and other mechanical means is considered from relations tying in the ungassed power demand on a volumetric basis. Given that the current system uses three Rushton turbines, the following equations were generated:

$$q_{mech} = \frac{P}{V} = \frac{2\pi MN}{V_L} = \frac{P_0 \rho N^3 D_i^5}{V_L} \quad (16)$$

$$P_0 = 5.9 \left( n_{bi}^{0.8} \left( \frac{h}{d} \right) f(\alpha) \right)^{0.9} \quad (17)$$

The secondary heat loss from this system was assumed to be a constant value of 1 W/L as time progressed. By determining the temperature and heat transfer difference between the cooling jacket and the surrounding environment, Equation (18) was able to accurately show this relationship.

$$q_{loss} = (UA)_{loss}(T_J - T_e) \cong 1 \frac{W}{L} \quad (18)$$

With all other heats determined, the total biological heat generation  $q_{bio}$  can be discovered from the remaining difference between these heat values. This biological heat is divided into two major components consisting of oxygenic-driven cellular growth heat  $q_{bio}^{oxy}$  and secondary product metabolic heat  $q_{bio}^{2nd}$ . As  $q_{bio}^{oxy}$  can be found from the product between the oxycaloric glucose-equivalent enthalpy and the oxygen transfer rate, a final difference between these two known values can reveal the required heat generation reflective of xanthan formation.

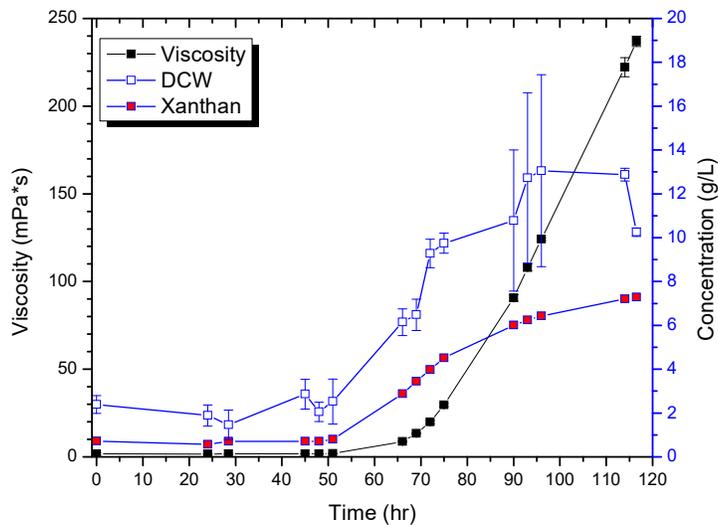
$$q_{bio} = q_{bio}^{oxy} + q_{bio}^{2nd} = \Delta H_{ox}(OTR) + q_{bio}^{2nd} \quad (19)$$

Once the range of secondary metabolic heat has been calculated, a prediction in the endpoint xanthan concentration can be found from the absolute value of this heat between two time points over the enthalpy of combustion for xanthan. These values can be summed up mathematically in Equation (20):

$$C_X = \sum_{i=1}^n \left| \frac{q_{Bio}^{2nd}(t_i - t_{i-1})}{\Delta H_{com}^X} \right| \quad (20)$$

Figure 4.2.15 displays the offline measurements made regarding the biomass concentration and viscosity values generated along with a predicted xanthan formation profile modeled using the Xuewu method. It can be observed that the first 50 hours of the fermentation provided a significant lag phase for culture development in a minimal medium. After this point, the biomass nearly doubles in quantity at the next sample point until it peaks

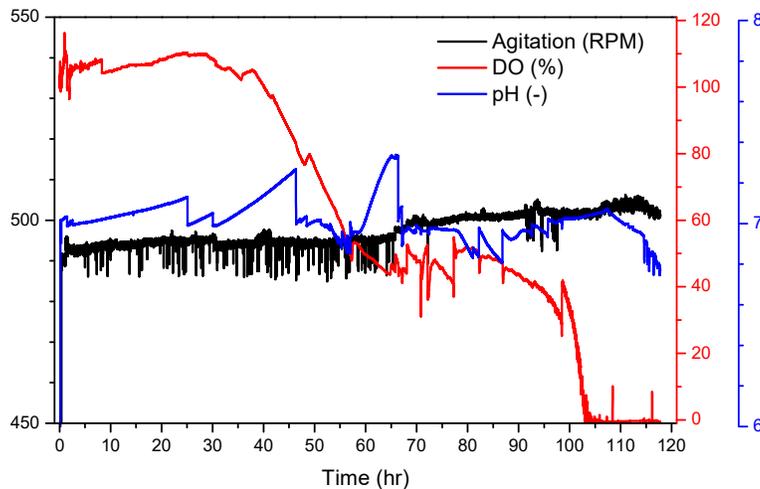
at a value of approximately 13.06 g/L at 96 hrs. Greater deviations in biomass samples can be seen at the 90, 93, and 96 hr points due to the continued increased in viscosity at this point making effective dilution and sample collection methods more difficult to conduct accurately. For the viscosity recorded, levels prior to 66 hrs remain between 1.5 to 1.9 mPa·s; after this point, the viscosity starts at 8.64 mPa·s until it peaks at the final point of approximately 116 hrs with 236.93 mPa·s. It is somewhere between 50 to 66 hrs that the onset of both cellular and xanthan production truly occur, as the Xuewu model shows a similar trend in production increase at these intervals as well.



**Figure 4.2.15 - Viscosity Flux & Biomass & Xanthan Product Profiles for High Performance Media Pressurized Fermentation. The standard deviation was recorded for each data point from three and four measurements for viscosity measurement and dry cell weight, respectively.**

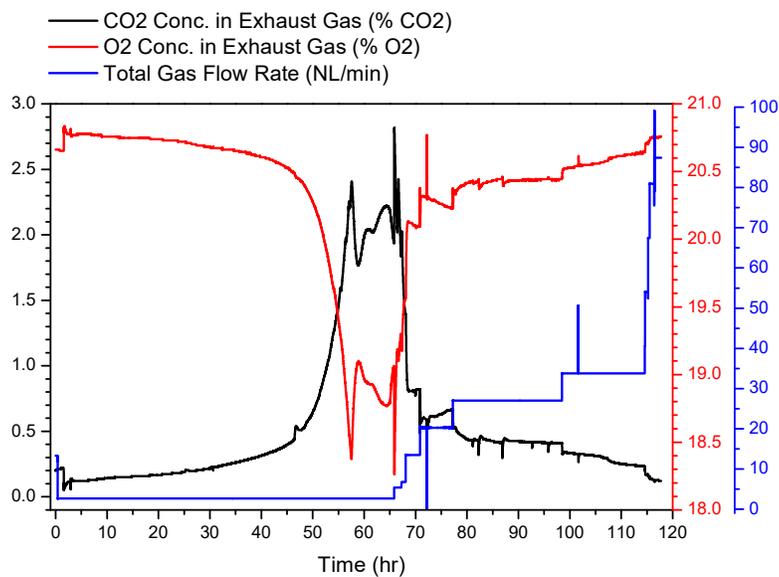
The data collected in Figure 4.2.16 helps to complement the results obtained from Figure 4.2.15 through the use of the dissolved oxygen tension in the system. As it can be observed, agitation remained mostly constant, but slight increases in value were observed when aeration and pressure buildup were increased as time progressed. The pH flux was controlled primarily with manual additions with existing pumping infrastructure in the bioreactor design; these manual additions can be clearly denoted by the sudden vertical increase or decrease in pH depending on the deviation from neutral conditions. It is

worthwhile to observe that the initial lag and early exponential phases experience tendencies to increase in alkalinity within the system. When the system begins to produce more cellular mass and xanthan past the 66 hr point, pH conditions now gravitate towards acidic preferences for almost the entire remainder of the fermentation. Effective pH control at neutral conditions is critical for *Xanthomonas* to remain at optimal efficiency in terms of both cellular growth and xanthan production, as more acidic conditions tend to degrade and thus produce a lower-grade polymer overall. Finally, the most critical set of information comes from the dissolved oxygen curve throughout the fermentation. The first area of interest in DOT appears at 40 hrs with the first major consumption of oxygen within the system, stimulating the onset of advanced cellular growth. This downward trend continues until the aforementioned 66 hr point, where the first effects of headspace pressure are made and the increase in viscosity begins its climb. With the continuing increase in pressure, the DOT is maintained above 40% for roughly 40 hrs until the 100 hr mark. At this point, pressure was kept constant until the end of the fermentation to observe what effect complete oxygen exhaustion had on this system. As reflected in this data along with Figure 4.2.15, little difference in viscosity increases were observed with the drop in biomass noted in the last offline data point collected.



**Figure 4.2.16 - Static Agitation, Dissolved Oxygen Content, & pH Profiles for High Performance Medium Pressurized Fermentation.**

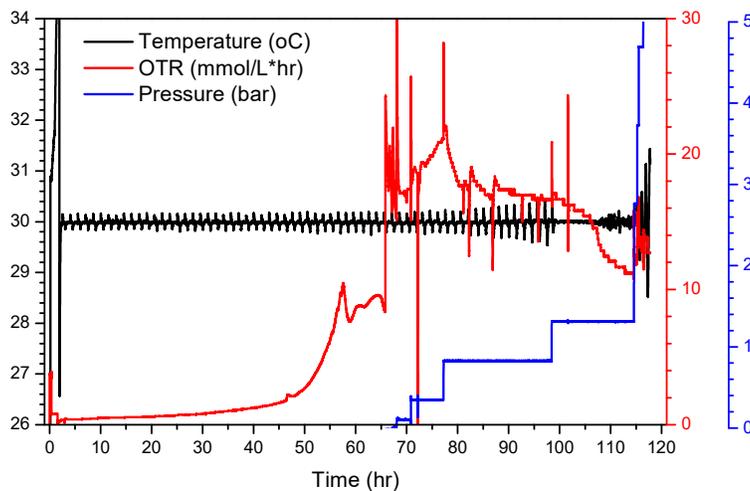
The gaseous-based data in Figure 4.2.17 assists in understanding what metabolic functions were occurring as time progressed. Total gas flow rate remained constant until pressure applications were added, thus requiring an increase in gas flow in order to keep the volumetric aeration rate constant throughout the entire fermentation. What proves to be more interesting in this data set is the directly inverse relationship shared between the mass percentages of exhaust oxygen and carbon dioxide from the fermenter. The main time frame of inversion occurs within 55 to 69 hrs, which in previous discussion has been the time frame as the onset of significant cellular and xanthan-focused production for this fermentation. Past this period, both mass percentages slowly teeter towards the original values noted in the early lag phase of the fermentation, and suggest, along with the production data from Figure 4.2.15, that a greater focus in metabolic production occurs within this time.



**Figure 4.2.17 - Total Gas Flow Rate and Exhaust Gas Compositions for High Performance Medium Pressurized Fermentation.**

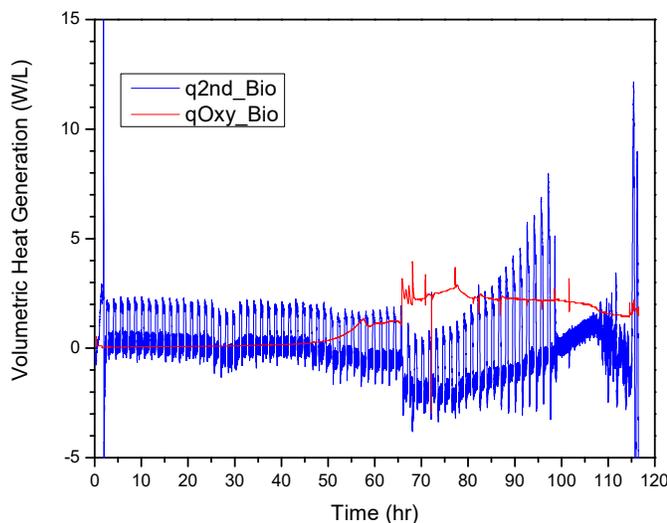
Figure 4.2.18 denotes the changes in temperature, oxygen transfer rates, and pressure additions over the entirety of the fermentation. Excluding the initial spike in temperature due to probe onset, the majority of the reactor temperature recorded appears to remain in a predictable cyclic range provided from the influence of the calibration heater probe. It is worthwhile to note, however, that visibly small increases in peak length extenuate from 70 to 100 hrs, signifying a greater demand in heat demand reflected in two specific changes in the

bioprocess. Primarily, this timeframe exhibits the temperature effect of increases in both mechanical and metabolic heat fluxes due to a greater demand in secondary product formation along with the combined process effects of increasing flow rates and headspace pressure to assist in OTR maintenance. As the calibration heater was turned off along with volumetric aeration held at its final value for nearly the remainder of the experiment after 100 hrs, it is interesting to note the eventual increase in temperature flux as the fermentation nears completion, once more highlighting the progression and conversion of remaining substrate into biomass and xanthan even in the severe lack of available oxygen highlighted from Figure 4.2.16. The dual relation between the OTR and headspace pressure comes into play at 69 hrs; even with the slightest addition of headspace pressure, the amount of oxygen available to be transferred within the bulk medium increases by at least 10 mmol/L·hr. The OTR is able to be maintained around this general range right until the increases are ceased at 100 hrs. As such, the application of pressure was able to allow the extension of aerobic product formation to occur in comparison to systems without such uses.



**Figure 4.2.18 - Temperature, Oxygen Transfer Rate, & Pressure Profiles for High Performance Medium Pressurized Fermentation.**

Finally, Figure 4.2.19 addresses both the oxygenic growth heat and secondary product formation heat produced in the fermenter. The entirety of heat balance is represented in Appendix 32, as the full presentation of heats generated is not required to understand the important aspects of this area of study. Excluding the extreme noise displayed at the start and end of this data set, the first 50 hrs of fermentation reflect the lag phase recorded and clearly understood in all data gathered. With the slow climb in oxygenic growth heat, the initial drop in formation heat at 66 hrs reflects a major devotion of energy in cellular growth versus xanthan production. However, past this point, the systematic climb in peak length of the formation heat combined with the slow decrease in growth heat help to reflect the increase in viscosity, thereby insinuating an increase in xanthan that has been found representative in some form with each figure discussed. Even with the calibration heater turned off for several hours, the next peak produced once turned on for the final sample collection appears to empirically fit the peak pattern produced prior to this segment. As such, the calculation procedure used to predict the final xanthan concentration based on the absolute value of formation heat gave a value of approximately 9.12 g/L of xanthan, a value more likely to be considered realistic in comparison to the Xuewu model's prediction of 7.23 g/L. The high viscosity achieved helps to justify this claim.



**Figure 4.2.19 - Biological Heat Generation via Oxygenic Growth & Secondary Metabolism for High Performance Medium Pressurized Fermentation.**

To truly understand what impact the choice of production medium had in a pressurized fermentation setting, a comparison of past methods and values generated from all media recipes surveyed from literature is displayed in Table 4.2.3. The current work has the highest initial substrate concentration along with the longest time taken for the fermentation to reach completion. While these factors prove somewhat disheartening upon looking at the other medium scenarios, several combined factors help to promote the success with this fermentation. While the predicted xanthan concentration is on the lower end of given samples and the biomass concentration is the second highest achieved, the viscosity level achieved proves the second-highest value recorded as well. Even with this consideration, the Carignatto viscosity has a variance of about 109 mPa·s, making the results from this work more reliable in accuracy. The high viscosity recorded denotes either xanthan concentrations higher than expected from both model estimates or that a higher quality of xanthan with greater acetylation was produced to achieve this pseudoplastic behaviour.

**Table 4.2.3 - Comparison of Results to Related Media & In-Lab Studies**

<u>Study</u>	<u>Scale</u>	<u>Medium Volume (L)</u>	<u>Initial Substrate (g/L)</u>	<u>Time (hr)</u>	<u>Biomass (g/L)</u>	<u>Xanthan (g/L)</u>	<u>Viscosity (mPa·s)</u>	<u>Flow Consistency Index (mPa·s<sup>n</sup>)</u>
Roseiro (1992)	Flask	0.1	30	24	-	11.15	-	10.35
Esgalhado (1995)	Flask	0.1	30	72	3.17	17.79	-	100.33
Amanullah (1996)	Pilot Plant	150	40	60	3.16	34.4	-	61.9
García-Ochoa (2001)	Bench Fermenter	1.5	40	30	1.421	7.06	-	-
Montforts (2009)	Pilot Plant	13	20	59	6.57 <sup>a</sup>	8.28	-	-
Carignatto (2011)	Flask	0.3	25	72	0.675	9.9	245	-
Gilani (2011)	Flask	0.3	30	72	2.5	17.1	160	-
Schelden (2015)	Pilot Plant	27	25	20	35 <sup>b</sup>	9 <sup>b</sup>	80 <sup>b</sup>	-
<b>This Work</b>	Pilot Plant	27	60	116	13.06	7.23 <sup>c</sup> 9.12 <sup>d</sup>	236	-

a. This value is actually the absorbance taken at 580 nm as no biomass concentration was given. b. These values were taken as estimations from the peaks produced in the most successful run reported. c. This value is predicted from the Xuewu model. d. This value is predicted from the calorimetry model originally developed by Regestein et al, 2012 and slightly modified in this work.

The pressurized fermentation using the Garcia-Ochoa medium as a high-performance production medium provided a host of useful information regarding the nature of this bioprocess. A high-cell density fermentation was achieved using pressurization to retain a constant OTR for *X. campestris* to utilize as needed. The inverse change in volumetric concentration of oxygen and carbon dioxide designated the onset of exponential growth along with substantial production in xanthan. Likewise, the recording and calculation of all heats produced and lost during the fermentation provided a secondary model capable of noting the endpoint xanthan concentration. Further scientific investigation of this study should include the determination of pressure limits before growth/production inhibition is observed along with the effects of changing impeller configuration and/or types used, as labelled in the latter stages of Figure 3.2.1. Additionally, control systems could be developed to dynamically regulate the agitation and pH to better disperse oxygen and allow stable growth with high-quality xanthan, respectively.

## Chapter 5

### 5 Conclusions & Recommendations

#### 5.1 Anaerobic Butanol Fermentation

The main concept covered in the first part of this thesis was the study into the development of a mixed culture fermentation system capable of producing butanol using glycerol as the primary substrate. Additionally, the fermenter would be gassed with a certain amount of carbon monoxide in the hopes of a secondary carboxydophilic organism capable of producing butyric acid as a means of introducing an intermediate compound to feed into the primary organism's metabolic pathway. The objective was to determine which organisms would prove ideal in this mixed culture based on butanol tolerance, robustness and viability in one specified media with non-competitive interaction between available substrates. The next target was to determine the effective uptake rates of glycerol and butyric acid by the primary organism and carbon monoxide by the secondary organism to prove the necessary biomechanics at play.

The first requirement prior to running the proposed mixed culture fermentation was to determine the ability of *C. pasteurianum* to uptake butyric acid and successfully produce an increased amount of butanol overall. With uptake observed from both a biological and synthetic source of butyric acid, *C. pasteurianum* was seen to increase the butanol titre to over 14 g/L butanol using 45 g/L glycerol and butyric acid concurrently. Ensuring that pH control around a level of 5.3 along with limiting the maximum amount of butyric acid to no more than 3 g/L assisted in enhancing the current fermentation scheme.

An initial screening, using an initial glucose of 30 g/L as a means to see significant growth in comparison to CO, of five carboxydophilic bacteria was conducted to directly compare which strain would be best in producing an adequate amount of butyric acid along with being tolerant enough to withstand the restrictive conditions set about by the primary organism. The first trial was an assessment of growth between Biebl and PETC medium combined with an endpoint product profile generated by each organism. Of the options available, *C. carboxidivorans* and *E. limosum* were able to produce significant growth and butyric acid concentrations proficient enough to act as the secondary organism. Finally, Biebl

medium was chosen as the production medium for this fermentation given the greater ability for these cultures to grow in at a greater and faster rate than the PETC medium.

The second experiment in this series was the determination of non-competitive interaction for the carboxydophilic organisms in terms of potential glycerol uptake. Using 20 g/L glycerol as the sole substrate, all organisms in this study provided no increase in absorbance and thus no detected growth throughout the experiment. Excluding the artefact detected at one point in the *E. limosum* case, all organisms proved unable to uptake any significant amount of glycerol. As a result of these findings, *C. carboxidivorans* and *E. limosum* were continued to be further investigated for their role as a secondary organism.

The final screening test was an analysis into the butanol toxicity and its inhibitive effects on the two organisms at hand. While *E. limosum* was able to achieve higher levels of absorbance in comparison to *C. carboxidivorans*, the latter organism was able to show growth up to 7 g/L butanol within a closed system. With such results present, the final decision in the ideal secondary organism requires an examination of their ability to use CO along with the product profile this substrate presents. A comparison of a pressurized serum bottle experiment using 35 % (v/v) CO in a batch system concluded that minimal growth was achieved during fermentation. However, the major product profile was in the form of acetic acid and eventual consumption of glycerol was observed after long periods without any other liquid substrate source. Future studies regarding this work should consider increasing pressure and/or CO content to observe whether more significant butyric acid production is possible with these cultures.

Final considerations in enhancing the practicality of this mixed culture glycerol-syngas fermentation would be the development of a culture-specific identification system that would allow an end user to establish the activity and viability of each bacteria. Outputs that can assist in such an identification process could include the analysis of key genomic markers identifying the codes and amount of codes specific to each organism. Alternatively, secondary metabolite analysis of compounds unique to a species can assist in revealing the growth expected from each culture. The potential production of hexanoic acid or hexanol from *C. carboxidivorans* remains a viable example of this compound analysis. Furthermore, a greater understanding between the relationship of pH and redox potential should be pursued

and attempted to develop some sort of mechanistic control loop proficient in controlling the separate acidogenic and solventogenic phases of the mixed culture. A pH system capable of maintaining a steady increase in acidity from neutral conditions would allow these microorganisms the chance to form a formidable amount of organic acids later to be converted into solvents like butanol at greater titres. Finally, further studies should focus on the optimal ratio between gaseous compounds such as carbons monoxide and dioxide along with hydrogen to better increase cellular growth and desired productivity of the carboxydrotrophs used in process. This understanding of the gaseous ratio should also assist the glycerol-consuming organism to better focus on butanol production thanks to the inhibition of dehydrogenase enzymes in the presence of carbon monoxide. Future work should begin the dual use of crude glycerol and syngas feeds to better realize any negative or inhibitory effects these raw sources pose for actual industrialization of this process once a mixed culture system is established and functioning properly.

## 5.2 Aerobic Xanthan Fermentation

To investigate the mass transfer principles unable to be observed during syngas fermentation due to a lack of proper equipment, the aerobic xanthan fermentation process was used as a model in understanding gas-liquid solubility constraints. A series of experiments were performed to determine which of the seven production media chosen would provide the greatest results in terms of necessary cellular growth, enhanced viscosity denoting high-quality and quantity of xanthan, and effective yields within a certain time period. Additionally, a newly developed trace elements solution was used from the weighted average of several others in an attempt to standardize what previous research has concluded. To reveal these answers, a multitude of comparisons were performed at the flask, online-measured flask, bench fermenter, and pilot plant scales as a means of choosing which medium provided the best results. Using data collected from biomass concentration, viscosity values, respiratory functions and kinetics, and other essential criteria assisted in finding what key factors enhanced the aerobic xanthan fermentation of *X. campestris*. Once the bulkhead of experiments were performed, the effects of a pressurized fermentation would be studied in the final pilot plant stage in this array of experimentation as a means to improve existing process conditions.

An initial screening of four complex and three minimal media had concluded that the Carignatto and Gilani complex media along with the García-Ochoa and Amanullah minimal media had produced the best biomass and/or viscosity values recorded. With these four media chosen, the next series of experiments used the RAMOS flask system to measure the online carbon dioxide and oxygen transfer rates of *X. campestris* to better understand the functionality these media possess. Additionally, the newly developed trace elements solution was either added or omitted from each tested medium as a means of comprehending its effective use. From this data set, it was concluded that the Carignatto and García-Ochoa media were better performing with multiple criteria achieved higher than their competing media. Likewise, the addition of the trace elements mostly assisted in accelerating and increasing the OTR and CTR values recorded, and was therefore included for future experimentation as it had supported growth/production as desired.

The next RAMOS experiment investigated the effect on growth and xanthan production of aluminum sulfate within the trace elements solution along with acting as the first direct comparison between the Carignatto and García-Ochoa media. It was observed that while the aluminum sulfate had little effect on the OTR curves experienced in both media, a slight increase in viscosity values were present in media lacking this compound. All future experimentation had used a trace elements solution without aluminum sulfate as necessary. Likewise, the primary comparison between these two media had resulted in higher biomass and viscosity values achieved in the García-Ochoa medium. A second evaluation was conducted in the form of a scale-up to a 1.5 L bench fermenter. To better replicate the mass transfer conditions occurring in the RAMOS trials, a dynamic control system adjusting agitation based on the amount of dissolved oxygen within the bulk medium was developed using a series of scale-up equations. It was determined that the limits of agitation would be between 250 to 450 RPM. As such, while the Carignatto run took half the time to produce the same amount of biomass as the García-Ochoa run, the latter fermentation provided a higher level of viscosity thus denoted a greater predicted xanthan concentration as well. Furthermore, the lack of oxygen limitation and more stable fermentative conditions of García-Ochoa helped justify the final scale-up to the pilot plant level using this medium.

The final study was the aerobic xanthan fermentation of *X. campestris* with 27 L of García-Ochoa medium. In addition to the xanthan prediction model developed by Xuewu, a

secondary xanthan production model based on calorimetry and biological heat generation was used as additional verification of this quantity. After 116 hrs, the biomass and predicted xanthan concentrations were at a value of 13.06 and between 7.23-9.12 g/L. The end viscosity achieved was approximately 236 mPa·s and, in comparison to previous studies done with the other media studied in this thesis, proved the highest achieved of all cases in terms of accurate and reliable data. The pressurization effects allowed the OTR and therefore the dissolved oxygen content to remain relatively constant for an extended period of time with increasing pressure and aeration flow rate. The temperature and heat flux data helped to determine the onset of major cellular growth and xanthan production with the increased peak height as time passed.

Several recommendations can be made to enhance the growth, production, and recovery aspects of this aerobic xanthan fermentation. Primarily, the preculture stages prior to the main stage fermentation should use a medium capable of quick and successful cellular growth. As such, a medium such as Carignatto can be used to decrease total preculture time along with decreasing the lengthy lag phase observed in the pressurized fermentation. Likewise, the pattern of shifting alkalinity to acidity back to alkalinity had been observed in each case at the bench scale or higher. It would be in the best interest for both cellular growth and high-quality xanthan yield that a dynamic pH control system is established to keep neutral conditions throughout the entirety of the fermentation. Secondary input data regarding the metabolic heat transfer can assist in determining the addition of base or acid to the system. Finally, an investigation into further research with cell immobilization could prove fruitful in a fermentation where the effects of viscosity change the mass transfer and fluid dynamics throughout the system. With cell immobilization in use, only certain areas of the fermenter will require optimal operating conditions and removal of xanthan can be made easier versus traditional fermentative processes. Considerations with the use of cheaper substrates from industrial waste streams should also be verified feasible in attempts to make the entire bioprocess more economically sound.

### 5.3 Cumulative Recommendations

The two bioprocesses studied in this thesis relate on one fundamental idea that bridges this work collectively; the use of low-solubility gases as an essential element to the proposed fermentation systems require techniques and technologies capable of either increasing these solubilities to utilizable quantities or a curtailing of the lack of these gases within a bulk liquid. The data collected from the xanthan model process will translate well to future work intended with the syngas fermentation; the application of pressure at a pilot-plant scale will increase the total solubilized gas within a bulk liquid. In turn, the significant increase in solubility will act as an additional driving force for cells to readily consume these gases and produce the products that limit these bioreactor schemes overall.

Both processes had also exhibited the synthesis of a desired product that proved inhibiting in greater quantities as time progressed. For the anaerobic mixed culture, increasing levels of butanol proved toxic to the microorganisms in use and would ultimately limit this end product to an estimable threshold. Similarly, increasing xanthan concentration within a liquid would thus increase the viscosity and experienced pseudoplasticity of a fluid, limiting the available mass transport of oxygen, nutrients, and other critical elements to *X. campestris*. To combat these hindrances, *in situ* removal technologies should be pursued to better the proposed bioprocesses at hand. Using techniques such as gas stripping, pervaporation, or two-phase liquid extraction can assist in removing butanol from a fermenter without greatly disturbing the existing mixed culture. The use of cell immobilization, product flocculation and collection, and viscosity-reducing salts addition would allow the *Xanthomonas* to continually produce xanthan without oxygen depletion occurring in the bulk liquid.

Equally, the monitoring and control of a dynamic pressure system for both bioprocesses would allow a greater gaseous holdup and increased solubility within a liquid medium as observed in both areas of study within this thesis. The challenges associated with such a proposal include the determination of an upper limit where system conditions deviate from optimal productivity and begin to induce cell death via lysing. Another challenge involves the deciphering of which parameter(s) should act as an effective input for pressure

control, whether this be dissolved gaseous content, off-gas composition analysis, oxygen or carbon dioxide transfer rates, or a combination therein.

Ultimately, a final consideration in the hopes of improving both mixing regimes along with gaseous solubility can potentially be solved in the use and configuration of different impeller/agitation systems during the fermentation. While the Rushton turbine impeller proves the standard in many experiments and studies performed, the changing of the impeller shape greatly influences how these fluids interact with each other and the cellular cultures at use. Furthermore, the configuration of these impellers within a fermenter greatly influence the fluid profile as well, and several additions or amendments to typical fermenter design could be investigated if deemed physically and economically feasible.

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## Appendices

### Appendix 1 - Production Medium as described by Biebl, 2001.

<b>Biebl</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5	Amresco, Solon, OH, USA
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2	EMD, Darmstadt, Germany
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.02	EMD, Darmstadt, Germany
FeSO <sub>4</sub>	0.05	Amresco, Solon, OH, USA
K <sub>2</sub> HPO <sub>4</sub>	0.5	Caledon Labs, Caledon, Canada
KH <sub>2</sub> PO <sub>4</sub>	0.5	Caledon Labs, Caledon, Canada
MES <sup>a</sup>	10	Amresco, Solon, OH, USA
Yeast Extract	1	BD, Franklin Lanes, NJ, USA
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
SL-7 Trace Elements	2	500x
Moon et al. Vitamins	2	500x

### Appendix 2 - Production Medium as described by Tanner et al., 1996.

<b>PETC</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
NH <sub>4</sub> Cl	1	Amresco, Solon, OH, USA
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2	EMD, Darmstadt, Germany
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.02	EMD, Darmstadt, Germany
KCl	0.1	EMD, Darmstadt, Germany
NaCl	0.8	Sigma-Aldrich, St. Louis, MO, USA
KH <sub>2</sub> PO <sub>4</sub>	0.1	Caledon Labs, Caledon, Canada
NaHCO <sub>3</sub>	2	Sigma-Aldrich, St. Louis, MO, USA
Yeast Extract	1	BD, Franklin Lanes, NJ, USA
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
PETC Trace Elements	10	100x
Wolfe's Vitamins	10	100x

**Appendix 3 - Production Media as described by Phillips et al., 2015.**

<b>Phillips</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Yeast Extract	0.5	BD, Franklin Lanes, NJ, USA
MES	10	Amresco, Solon, OH, USA
KOH	1.165	Alfa Aesar, Haverhill, MA, USA
NH <sub>3</sub>	0.39	Amresco, Solon, OH, USA
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.08	EMD, Darmstadt, Germany
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.4	EMD, Darmstadt, Germany
KCl	0.2	EMD, Darmstadt, Germany
KH <sub>2</sub> PO <sub>4</sub>	0.2	Caledon Labs, Caledon, Canada
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
Phillips Trace Elements	5	500x
Phillips Vitamins	0.5	5000x

**Appendix 4 - Reinforced Clostridial Growth Medium.**

<b>Reinforced Clostridial Medium (RCM)</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Beef Extract	10	BD, Franklin Lanes, NJ, USA
Peptone	10	BD, Franklin Lanes, NJ, USA
NaCl	5	Sigma-Aldrich, St. Louis, MO, USA
Yeast Extract	3	BD, Franklin Lanes, NJ, USA
Sodium Acetate	3	Sigma-Aldrich, St. Louis, MO, USA
Soluble Starch	1	Alfa Aesar, Haverhill, MA, USA
Cysteine-HCl	0.5	Caledon Labs, Caledon, Canada

**Appendix 5 - SL-7 Trace Elements Solution.**

<b>SL-7</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
FeCl <sub>2</sub> ·2H <sub>2</sub> O	1500	Amresco, Solon, OH, USA
CoCl <sub>2</sub> ·6H <sub>2</sub> O	900	EMD, Darmstadt, Germany
MnCl <sub>2</sub> ·4H <sub>2</sub> O	100	J.T. Baker, Center Valley, PA, USA
ZnCl <sub>2</sub>	70	EMD, Darmstadt, Germany
H <sub>3</sub> BO <sub>3</sub>	62	Amresco, Solon, OH, USA
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	36	Sigma-Aldrich, St. Louis, MO, USA
NiCl <sub>2</sub> ·6H <sub>2</sub> O	24	Alfa Aesar, Haverhill, MA, USA
CuCl <sub>2</sub> ·2H <sub>2</sub> O	17	Alfa Aesar, Haverhill, MA, USA

**Appendix 6 - PETC Trace Elements Solution.**

<b>PETC</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
CoCl <sub>2</sub> ·6H <sub>2</sub> O	200	EMD, Darmstadt, Germany
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	20	Sigma-Aldrich, St. Louis, MO, USA
NiCl <sub>2</sub> ·6H <sub>2</sub> O	20	Alfa Aesar, Haverhill, MA, USA
CuCl <sub>2</sub> ·2H <sub>2</sub> O	20	Alfa Aesar, Haverhill, MA, USA
Nitrilotriacetic Acid	2000	Alfa Aesar, Haverhill, MA, USA
MnSO <sub>4</sub> ·H <sub>2</sub> O	1000	J.T. Baker, Center Valley, PA, USA
Fe(SO <sub>4</sub> ) <sub>2</sub> (NH <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	800	EMD, Darmstadt, Germany
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2	EMD, Darmstadt, Germany
NaSeO <sub>4</sub>	20	Sigma-Aldrich, St. Louis, MO, USA
Na <sub>2</sub> WO <sub>4</sub>	20	Sigma-Aldrich, St. Louis, MO, USA

**Appendix 7 - Phillips Trace Elements Solution.**

<b>Phillips</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
CoCl <sub>2</sub> ·6H <sub>2</sub> O	2	EMD, Darmstadt, Germany
CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.2	Alfa Aesar, Haverhill, MA, USA
Fe(SO <sub>4</sub> ) <sub>2</sub> (NH <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	8	EMD, Darmstadt, Germany
MnSO <sub>4</sub> ·H <sub>2</sub> O	10	J.T. Baker, Center Valley, PA, USA
NiCl <sub>2</sub> ·6H <sub>2</sub> O	0.2	Alfa Aesar, Haverhill, MA, USA
Nitrilotriacetic Acid	20	Alfa Aesar, Haverhill, MA, USA
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.2	Sigma-Aldrich, St. Louis, MO, USA
Na <sub>2</sub> SeO <sub>4</sub>	0.2	Sigma-Aldrich, St. Louis, MO, USA
Na <sub>2</sub> WO <sub>4</sub> ·2H <sub>2</sub> O	0.2	Sigma-Aldrich, St. Louis, MO, USA
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2	EMD, Darmstadt, Germany

**Appendix 8 - Moon Vitamins Solution as described in Moon et al., 2010.**

<b>Moon et al.</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
Biotin	0.01	Enzo Sciences, Ann Arbor, MI, USA
Thiamin	1	Enzo Sciences, Ann Arbor, MI, USA
p-Aminobenzoic Acid	1	Alfa Aesar, Haverhill, MA, USA

**Appendix 9 - Wolfe's Vitamins Solution.**

<b>Wolfe's</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
Biotin	2	Enzo Sciences, Ann Arbor, MI, USA
Thiamin	5	Enzo Sciences, Ann Arbor, MI, USA
p-Aminobenzoic Acid	5	Alfa Aesar, Haverhill, MA, USA
Folic Acid	2	Sigma-Aldrich, St. Louis, MO, USA
Pyridoxine HCl	10	Sigma-Aldrich, St. Louis, MO, USA
Riboflavin	5	Alfa Aesar, Haverhill, MA, USA
Nicotinic Acid	5	Sigma-Aldrich, St. Louis, MO, USA
Ca-D--Pantothenate	5	Sigma-Aldrich, St. Louis, MO, USA
Vitamin B <sub>12</sub>	0.1	Sigma-Aldrich, St. Louis, MO, USA
Lipoic Acid	5	Alfa Aesar, Haverhill, MA, USA

**Appendix 10 - Phillips Vitamin Solution.**

<b>Phillips</b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
Biotin	0.02	Enzo Sciences, Ann Arbor, MI, USA
Ca-D--Pantothenate	0.05	Sigma-Aldrich, St. Louis, MO, USA
p-Aminobenzoic Acid	0.05	Alfa Aesar, Haverhill, MA, USA

**Appendix 11 - Carignatto Medium as described by Carignatto et al., 2011.**

<b>Carignatto</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Peptone	5	Roth, Karlsruhe, Germany
Glutamate	1.5	Roth, Karlsruhe, Germany
K <sub>2</sub> HPO <sub>4</sub>	1.5	Merck, Darmstadt, Germany
Citric Acid	0.5	Roth, Karlsruhe, Germany
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1	Roth, Karlsruhe, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 12 - Gilani Medium as described by Gilani et al., 2011.**

<b>Gilani</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Yeast Extract	5	Roth, Karlsruhe, Germany
KH <sub>2</sub> PO <sub>4</sub>	0.09	Merck, Darmstadt, Germany
H <sub>3</sub> BO <sub>3</sub>	0.00018	Roth, Karlsruhe, Germany
MgCl <sub>2</sub>	0.018	Roth, Karlsruhe, Germany
Na <sub>2</sub> SO <sub>4</sub>	0.003	Merck, Darmstadt, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.0006	Roth, Karlsruhe, Germany
CaCO <sub>3</sub>	0.0006	Roth, Karlsruhe, Germany
ZnO	0.00018	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 13 - Roseiro Medium as described by Roseiro et al., 1992.**

<b>Roseiro</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Yeast Extract	0.75	Roth, Karlsruhe, Germany
Peptone	0.34	Roth, Karlsruhe, Germany
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3.33	Roth, Karlsruhe, Germany
KH <sub>2</sub> PO <sub>4</sub>	7.2	Merck, Darmstadt, Germany
Citric Acid	2	Roth, Karlsruhe, Germany
H <sub>3</sub> BO <sub>3</sub>	0.0072	Roth, Karlsruhe, Germany
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.24	Roth, Karlsruhe, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.0042	Roth, Karlsruhe, Germany
CaCO <sub>3</sub>	0.029	Roth, Karlsruhe, Germany
ZnO	0.06	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 14 - Esgalhado Medium as described by Esgalhado et al., 1995.**

<b>Esgalhado</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Yeast Extract	0.75	Roth, Karlsruhe, Germany
Peptone	0.34	Roth, Karlsruhe, Germany
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3.33	Roth, Karlsruhe, Germany
KH <sub>2</sub> PO <sub>4</sub>	0.0042	Merck, Darmstadt, Germany
Citric Acid	2	Roth, Karlsruhe, Germany
H <sub>3</sub> BO <sub>3</sub>	0.0072	Roth, Karlsruhe, Germany
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.49	Roth, Karlsruhe, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.0042	Roth, Karlsruhe, Germany
CaCO <sub>3</sub>	0.029	Roth, Karlsruhe, Germany
ZnO	0.006	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 15 - Garcia-Ochoa Medium as described by Garcia-Ochoa et al., 2000B.**

<b>Garcia-Ochoa</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
NH <sub>4</sub> NO <sub>3</sub>	1.144	Merck, Darmstadt, Germany
KH <sub>2</sub> PO <sub>4</sub>	2.866	Merck, Darmstadt, Germany
Citric Acid	2.1	Roth, Karlsruhe, Germany
H <sub>3</sub> BO <sub>3</sub>	0.006	Roth, Karlsruhe, Germany
MgCl <sub>2</sub>	0.507	Roth, Karlsruhe, Germany
Na <sub>2</sub> SO <sub>4</sub>	0.089	Roth, Karlsruhe, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.02	Roth, Karlsruhe, Germany
CaCO <sub>3</sub>	0.02	Roth, Karlsruhe, Germany
ZnO	0.006	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 16 - Amanullah Medium as described by Amanullah et al., 1996.**

<b>Amanullah</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
NH <sub>4</sub> Cl	2	Roth, Karlsruhe, Germany
KH <sub>2</sub> PO <sub>4</sub>	5	Merck, Darmstadt, Germany
Citric Acid	2.3	Roth, Karlsruhe, Germany
H <sub>3</sub> BO <sub>3</sub>	0.006	Roth, Karlsruhe, Germany
MgCl <sub>2</sub>	0.065	Roth, Karlsruhe, Germany
Na <sub>2</sub> CO <sub>3</sub>	0.5	Roth, Karlsruhe, Germany
Na <sub>2</sub> SO <sub>4</sub>	0.114	Roth, Karlsruhe, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.0012	Roth, Karlsruhe, Germany
CaCl <sub>2</sub>	0.0091	Roth, Karlsruhe, Germany
ZnCl <sub>2</sub>	0.0067	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 17 - Montforts Medium as described by Montforts, 2009.**

<b>Montforts</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
NH <sub>4</sub> Cl	1.6	Roth, Karlsruhe, Germany
K <sub>2</sub> HPO <sub>4</sub>	1	Merck, Darmstadt, Germany
H <sub>3</sub> BO <sub>3</sub>	0.003	Roth, Karlsruhe, Germany
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.266	Roth, Karlsruhe, Germany
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.0017	Roth, Karlsruhe, Germany
CaCl <sub>2</sub>	0.01	Roth, Karlsruhe, Germany
ZnCl <sub>2</sub>	0.0033	Merck, Darmstadt, Germany
<b>Solution Name</b>	<b>Volume (mL)</b>	<b>Concentration of Solution</b>
This Work's Trace Elements	0.2	5000x

**Appendix 18 - Yeast-Malt Extract Growth Medium as used in Garcia-Ochoa et al., 1995.**

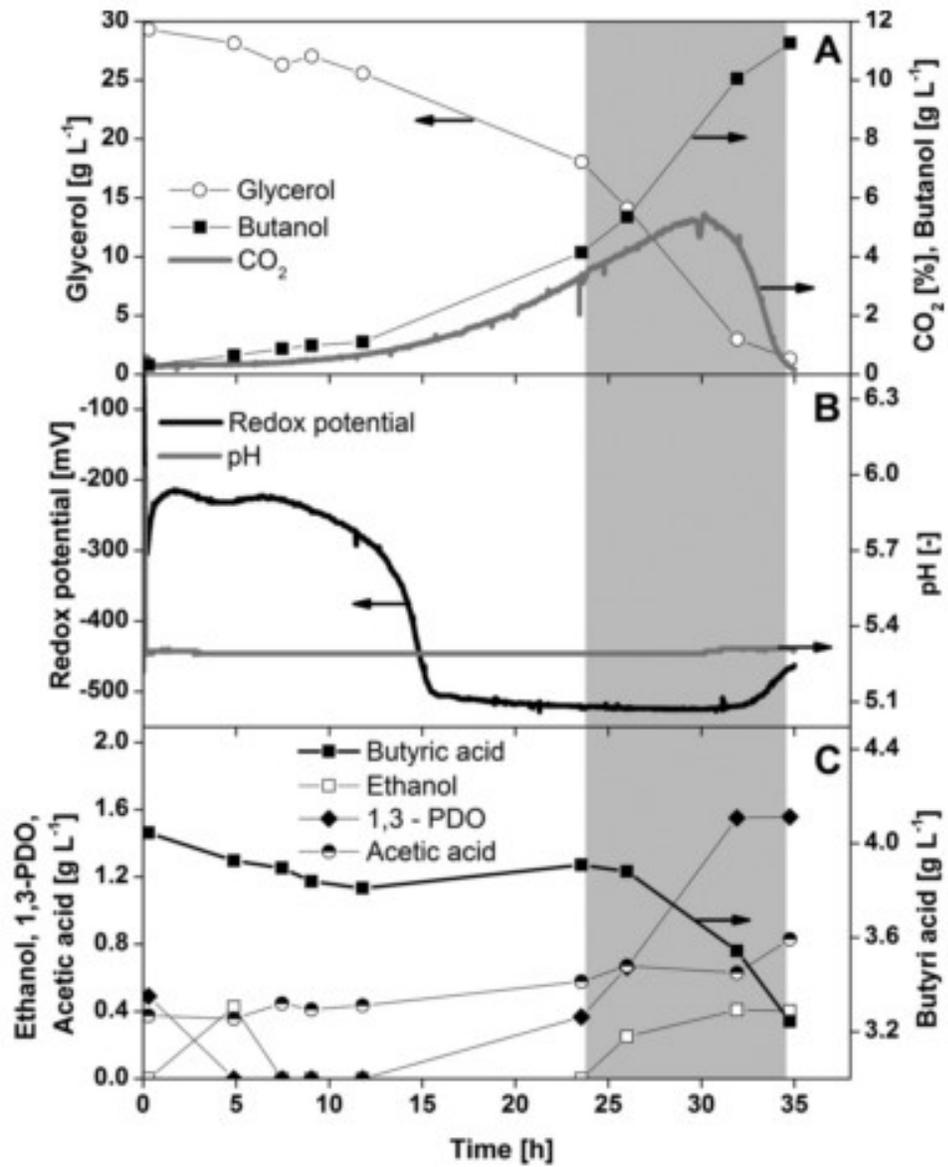
<b>Yeast-Malt (YM) Medium</b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Yeast Extract	3	Roth, Karlsruhe, Germany
Malt Extract	3	Merck, Darmstadt, Germany
Peptone	5	Roth, Karlsruhe, Germany
MOPS	30	Roth, Karlsruhe, Germany

**Appendix 19 - Trace Elements Solution as generated in This Work.**

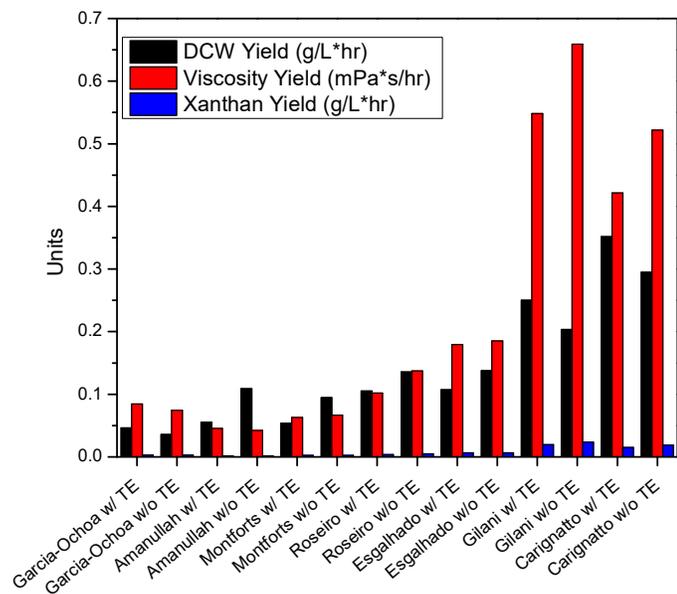
<b><u>This Work</u></b>		
<b>Component</b>	<b>Concentration (mg/L)</b>	<b>Manufacturer</b>
H <sub>3</sub> BO <sub>3</sub>	5	Roth, Karlsruhe, Germany
CoSO <sub>4</sub> ·7H <sub>2</sub> O	0.5	Merck, Darmstadt, Germany
CuSO <sub>4</sub> ·7H <sub>2</sub> O	0.5	Merck, Darmstadt, Germany
MnSO <sub>4</sub> ·H <sub>2</sub> O	0.5	Roth, Karlsruhe, Germany
Na <sub>2</sub> SO <sub>4</sub> ·2H <sub>2</sub> O	2	Roth, Karlsruhe, Germany
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	2	Merck, Darmstadt, Germany
Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·16H <sub>2</sub> O <sup>a</sup>	0.5	Merck, Darmstadt, Germany
Na <sub>2</sub> (EDTA)	1	Merck, Darmstadt, Germany
FeSO <sub>4</sub> ·7H <sub>2</sub> O	5	Roth, Karlsruhe, Germany
CaCl <sub>2</sub> ·2H <sub>2</sub> O	11	Roth, Karlsruhe, Germany

**Appendix 20 - Cell Bank (CB) Medium.**

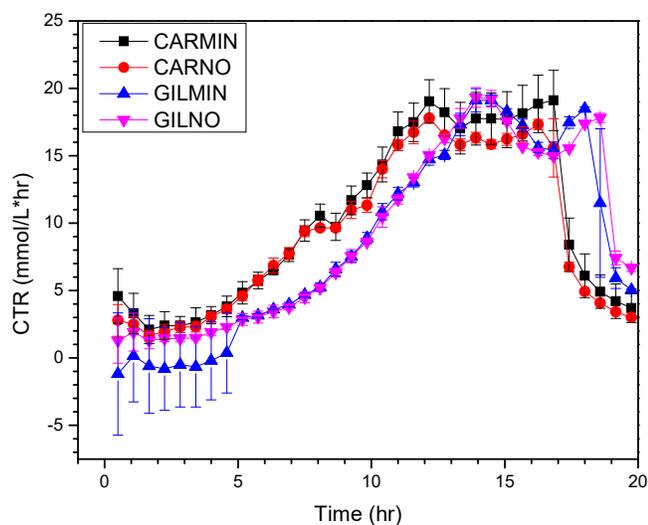
<b><u>Cell Bank (CB) Medium</u></b>		
<b>Component</b>	<b>Concentration (g/L)</b>	<b>Manufacturer</b>
Glucose	10	Roth, Karlsruhe, Germany
Yeast Extract	3	Roth, Karlsruhe, Germany
Peptone	5	Roth, Karlsruhe, Germany
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.7	Merck, Darmstadt, Germany
Streptomycin	0.8	Roth, Karlsruhe, Germany



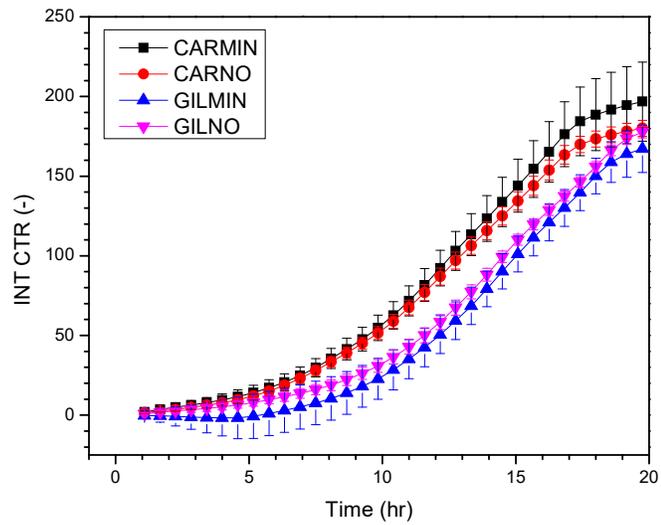
Appendix 21 - On-line & Off-line Data Analysis of *C. pasteurianum* fermentation with 4 g/L butyrate addition.



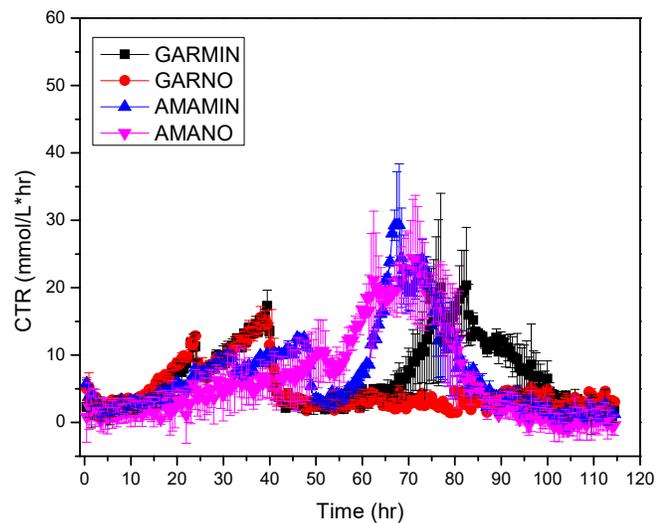
**Appendix 22 - Analysis of Biomass, Viscosity, & Predicted Xanthan Productivities after 28 hrs.**



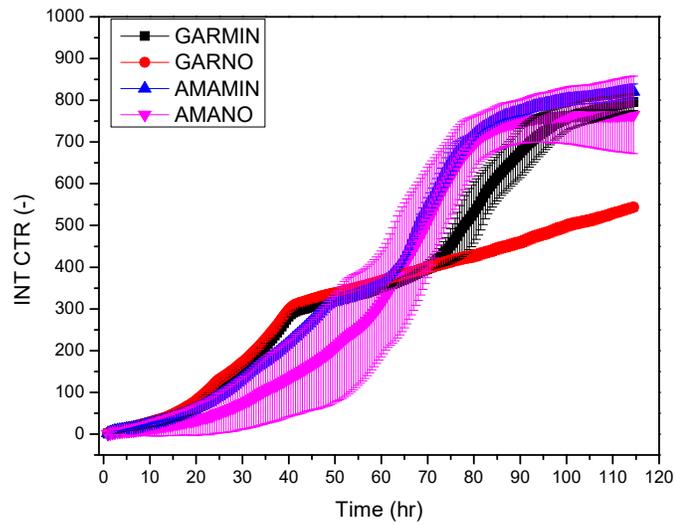
**Appendix 23 - Carbon Dioxide Transfer Rates for Complex Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



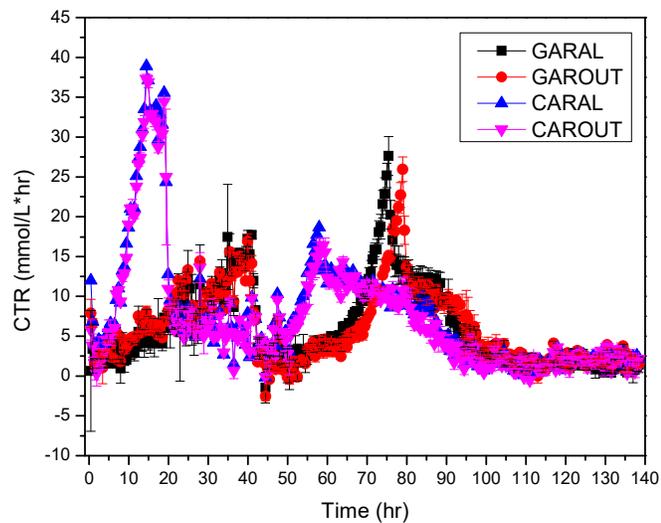
**Appendix 24 - Carbon Dioxide Transfer Rate Integrated Curves for Complex Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



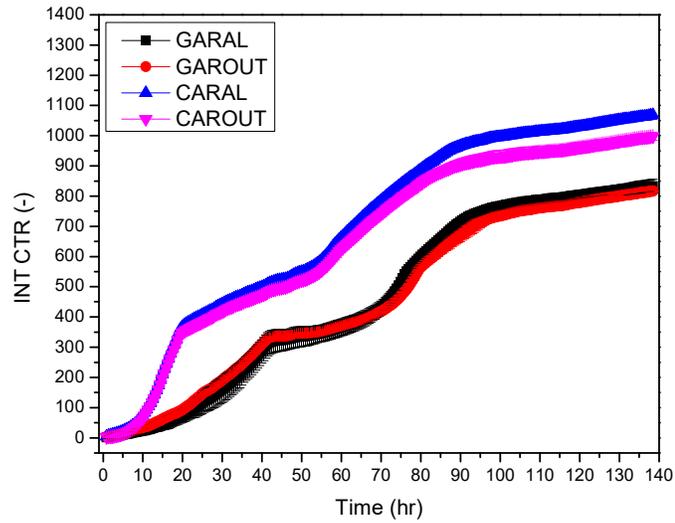
**Appendix 25 - Carbon Dioxide Transfer Rates for Minimal Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



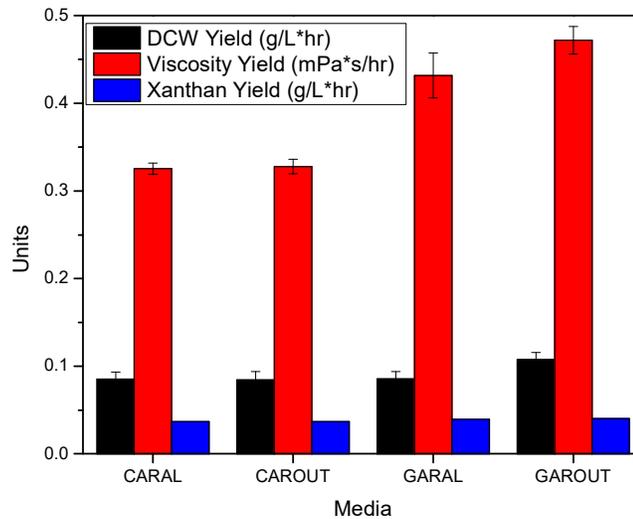
**Appendix 26 - Carbon Dioxide Transfer Rate Integrated Curves for Minimal Media with & without Trace Elements Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



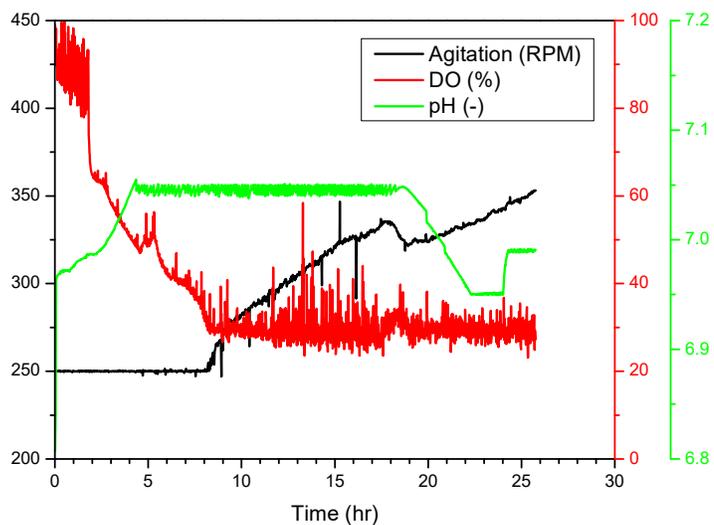
**Appendix 27 - Carbon Dioxide Transfer Rates for Two Media with & without Aluminum Sulfate Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



**Appendix 28 - Carbon Dioxide Transfer Rate Integrated Curves for Two Media with & without Aluminum Sulfate Addition. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



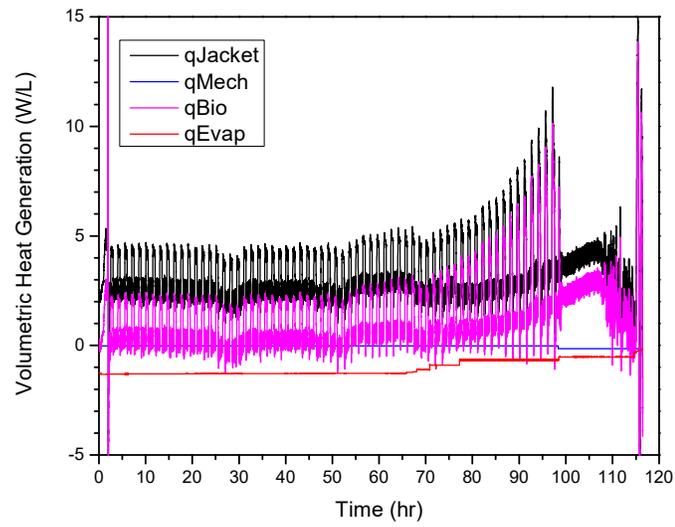
**Appendix 29 - Analysis of Biomass, Viscosity, & Predicted Xanthan Productivities after 138 hrs for Complex & Minimal Media. Experiments were run in duplicate and the standard deviation was recorded for each data point.**



**Appendix 30 - Dynamic Agitation, Dissolved Oxygen Content, & pH Profiles for Second Pre-culture for High Performance Medium Pressurized Fermentation.**

**Appendix 31 - Constants Used in Heat Transfer Calculations for High Performance Medium Pressurized Fermentation.**

<u>Constant</u>	<u>Symbol</u>	<u>Value</u>	<u>Units</u>
Cooling Jacket Area	$A_{global}$	0.45	$m^2$
Wall Thickness	$\delta$	0.005	m
Heat Conductivity of Stainless Steel 316L	$\lambda_{st}$	15	W/m·K
Heat Conductivity of Reactor Medium	$\lambda_R$	0.632	W/m·K
Inner Reactor Diameter	$d_b$	0.314	m
Stirrer Diameter	$d_R$	0.12	m
Reactor Medium Viscosity	$\eta_R$	0.0000181	Pa·s
Reactor Medium Density	$\rho_R$	1000	$kg/m^3$
Reactor Medium Heat Capacity	$cp_R$	4179	J/kg·K
Heat Conductivity of Jacket Water	$\lambda_J$	0.632	W/m·K
Inlet/Outlet Stub Diameter	$d_g$	0.12	m
Jacket Gap Width	$d_s$	0.004	m
Cooling Water Viscosity	$\eta_J$	0.00065	Pa·s
Cooling Water Density	$\rho_J$	1	$kg/m^3$
Cooling Water Heat Capacity	$cp_J$	4179	J/kg·K
Cooling Water Speed	$u_n$	Calc.	m/s
Gravitational Acceleration	$g$	9.81	$m/s^2$
Jacket Height	$h$	0.5	m
Cooling Water Expansion Coefficient	$e$	0.000304	1/K
Vaporization Enthalpy of Water	$\Delta H_{vap}^{H_2O}$	-43540	J/mol
Combustion Enthalpy of Xanthan	$\Delta H_{com}^{Xan}$	-14.477	J/g
Oxygenic Enthalpy of Cellular Growth	$\Delta H_{ox}$	-0.469	J/mol
Reactor Medium Partial Pressure	$p_{medium}$	0.0694	Bar
Molecular Mass of Water	$M_{H_2O}$	18.016	g/mol
Molar Volume of an Ideal Gas	$V_{norm}$	22.4	L/mol
Liquid Volume inside Reactor	$V_L$	27	L
Characteristic Value for Rushton Turbine	$f(\alpha)$	1	-



**Appendix 32 - Total Energy Balance between Jacket Heat and Process-Generated Heat for High Performance Medium Pressurized Fermentation.**

## Curriculum Vitae

**Name:** Eric William Doerr

**Post-secondary Education and Degrees:** The University of Western Ontario  
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The University of Western Ontario  
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