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Novel fed-batch process with in-situ product recovery for glycerol fermentation to butanol using Clostridium pasteurianum

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Abstract

Butanol, a next-generation biofuel, can be produced by fermenting glycerol using *Clostridium pasteurianum.* To address product inhibition, an integrated system that combined a fed-batch process with pervaporation was assessed against conventional batch and fed-batch fermentations. This study showed that with the novel process configuration, the productive fermentation time could be extended, translating to a 2.4-fold and 1.9-fold increase in butanol production relative to baseline fed-batch and batch operation, respectively. Further, it was demonstrated that butanol concentrations were able to be maintained below inhibitory levels throughout the fermentation. Despite this outcome, metabolic oscillations were revealed, indicating instability in the process. The introduction of secondary sugar substrates into the modified system improved the butanol selectivity and did not result in fluctuations of the product profile. Overall, these findings provide strong evidence of the advantages of *in-situ* product recovery in glycerol fermentation which can be aided by the addition of secondary carbon sources.

Keywords

Butanol, Glycerol, Fermentation, *Clostridium pasteurianum*, Pervaporation, Fed-batch, Biofuels

Summary for Lay Audience

Efforts to improve the sustainability of biofuel production practices have redirected attention to new generation biofuels, such as biobutanol. Butanol is an ideal biofuel candidate that can be easily integrated into current infrastructure and provides a higher carbon content relative to other biofuels such as ethanol. Several avenues exist to produce biobutanol, yet an attractive route is via fermentation with *Clostridium pasteurianum*. This anaerobic process is unique in that it can exclusively use crude glycerol, a 'waste' product from the biodiesel industry as a low-cost feedstock. However, current limitations of low productivity render the process economically unfeasible on an industrial level. One factor that restricts the efficiency of the fermentation in batch operation is butanol toxicity. A solution to minimize the accumulation of butanol in the reactor is to incorporate *in-situ* product removal.

The focus of this project is to develop a new process configuration for glycerol fermentation that addresses the accumulation of butanol in the reactor. To accomplish this goal, a membrane-assisted fed-batch fermentation was performed using pervaporation, a selective method of butanol recovery. In this system, fermentation broth is pumped out from the bioreactor and across a membrane, where butanol diffuses through the surface while the remaining cell-rich broth is returned to the fermenter.

When compared to a baseline fed-batch control without pervaporation beginning with the same initial glycerol concentration, the modified set-up resulted in a 2.4-fold increase in butanol production and extended the fermentation time by 19.5 hours. No evidence of butanol inhibition was observed as concentrations were maintained below inhibitory levels. Despite this improvement, the process was variable, with fluctuations observed in gas production and glycerol consumption, indicative of metabolic shifts of the culture. To overcome this oscillatory behaviour, additional simple sugar substrates were introduced into the modified system. Under co-substrate fermentation, the system performed more consistently, without observed oscillations but an imbalance between removal capacity and production resulted in toxic butanol levels, prematurely ending the fermentation. This outcome indicates the need for more refinement of process conditions but overall highlights the value of *in-situ* product removal in glycerol fermentation.

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Chapter 1

1 Introduction

1.1 Background and Motivation

As fossil fuels continue to dominate the transportation industry, rising greenhouse gas emissions and depleting resources are motivating the adoption and sustainable production of biofuels (Jeswani et al., 2020). With biodiesel currently experiencing the fastest rate of growth relative to any other biofuel, the accompanied production of crude glycerol, a by-product, contributes to a global surplus of unrefined glycerol with estimates indicating 5.87 billion pounds was generated just within 2020 (Kumar et al., 2019; Mahlia et al., 2020; Taconi et al., 2009). As such, the commercial and industrial value of crude glycerol has continued to decline, while purification costs remain high, leading to the handling of crude glycerol as more of a waste product than a by-product (Kosamia et al., 2020). This combination of trends in the glycerol market suggests an opportunity to valorize crude glycerol into a profitable compound.

The microbe, *Clostridium pasteurianum*, has been recognized for its ability to anaerobically ferment crude glycerol to produce a variety of products including 1,3-propanediol (PDO), ethanol, and butanol (Biebl, 2001). The main product in this fermentation, butanol, is a versatile compound frequently used as a solvent, or generated as an intermediate chemical (Ndaba et al., 2015). Beyond the traditional uses of butanol, it is more recently gaining traction as a new generation biofuel. Butanol is an ideal biofuel candidate due to its capability of blending at high concentrations with gasoline and diesel, as well as having low volatility and high energy content (Mariano et al., 2011; Zheng et al., 2009). Despite the benefits of applying butanol as a gasoline additive, economic feasibility is a major barrier to the commercialization of this fermentation process which is plagued by low butanol yields and low volumetric butanol productivity. As a result, glycerol fermentation using *C. pasteurianum* is not yet industrialized (Zheng et al., 2009).

One known limitation that hinders productivity is that of product inhibition which has been known to occur when the concentration of butanol in the reactor reaches 12-17 g/L (Kießlich et al., 2017). Strategies focused on overcoming this barrier have primarily been to incorporate *in-situ* product removal within batch fermentations, with the ultimate goal of maintaining butanol concentrations below toxic levels. Research efforts have utilized a variety of approaches including adsorption, gas stripping, and pervaporation as a means to strategically remove butanol during fermentation (Sarchami, Munch, et al., 2016). While each strategy has associated advantages and drawbacks, the low energy requirement, high selectivity, and ability to operate at ideal fermentation conditions were all factors considered in selecting pervaporation as the mode of product recovery in this study (Kießlich et al., 2017; Sarchami, Munch, et al., 2016).

Pervaporation is a membrane-based technique for separating volatile components from a bulk fluid. As a unit, it functions by applying a tangential flow across a selective membrane, allowing a volatile compound such as butanol to diffuse through to the other side as a gas and be collected as permeate (Kujawska et al., 2015). Using cell-free broth obtained from glycerol fermentation using the same bacterium, *C. pasteurianum,* pervaporation has already been demonstrated by Kießlich et. al. to be effective at removing butanol from the complex fermentation broth (Kießlich et al., 2017).

The proposed solution in this work introduces a novel process configuration by coupling a pervaporation unit with fed-batch fermentation. In this modified fed-batch approach, fermentation broth is continuously removed from the system and directed through the pervaporation unit where butanol is selectively removed, and the remaining fermentation broth returned into the fermenter. This semi-continuous process involves a constant stream of media supplementation, preventing the culture from experiencing substrate limitation. Unlike continuous processes, the mass flow into the reactor greatly exceeds the amount removed, resulting in volume accumulation. The proposed process design is intended to extend fermentation time and result in greater butanol production.

1.2 Research Objectives

The main motivation for this study was to determine if incorporating *in-situ* butanol removal via pervaporation with a fed-batch fermentation, would result in greater butanol productivity and yield a greater cell density in the reactor compared to a batch-mode operation. This hypothesis was evaluated by accomplishing the following three objectives.

- Aim 1: Refine predictive simulations of the proposed fed-batch system with *in-situ* removal
- Aim 2: Compare and evaluate the performance of a modified fed-batch system with conventional methods of batch and fed-batch fermentation
- Aim 3: Adjust fermentation parameters to improve productive fermentation time and volumetric butanol productivity in the modified system

Chapter 2

2 Literature Review

The following chapter provides a detailed scientific review of foundational research in glycerol fermentation directed at butanol production. First establishing background as to the industrial importance of butanol, the chapter further leads to the fermentative methods of production, the associated challenges, and the strategies in development to overcome them. The various types of fermentation configurations will also be discussed along with their impact on volumetric butanol productivity.

2.1 Butanol: Characteristics and Applications

Butanol is an industrially relevant chemical, having applications as a solvent as well as being a precursor to other marketable chemicals such as butyl acrylate and methacrylate esters (Lee et al., 2008). Estimations suggest that over 55% of butanol production goes towards the manufacture of butyl acrylate and butyl acetate, which are staple chemicals in latex paints and other surface coatings (Uyttebroek et al., 2015). Although the production and value of butanol have traditionally relied on its function as an intermediate chemical, there is emerging interest in the use of butanol as a transport fuel (Ni & Sun, 2009).

When derived through biochemical routes, this four-carbon alcohol is promising as a new generation biofuel (Ndaba et al., 2015). Inherent to its chemical properties, biobutanol offers significant advantages relative to other biofuels. Specifically, when compared to bioethanol, butanol is less volatile, has higher energy content, and has greater miscibility with both diesel and gasoline (Jin et al., 2011). From an infrastructure perspective, butanol is also less corrosive than ethanol, making it easier to transport via pipelines (Harvey & Meylemans, 2011). Butanol can also be used as a direct fuel in internal combustion engines, but more research is needed to guide the applicability of butanol as a biofuel in other engine types. Although there are some known limitations of butanol in that it has a lower heating value compared to low-carbon alcohols as well as a lower octane number, it is typically regarded as a superior alternative to current biofuels on the market (Jin et al., 2011). Despite the proven benefits of butanol as a gasoline additive, current barriers in fermentative

production routes have limited the use of biobutanol as a transport fuel (Birgen et al., 2019). Recently, research efforts focused on improving some of these limitations such as increasing product titers, are gaining new momentum (Jiang et al., 2015). With advancing global understanding of the consequences of environmental dependence on fossil fuels, there is increasing investment into developing sustainable production processes for biofuels, such as biobutanol.

2.2 Methods of Butanol Production

2.2.1 Acetone, Butanol, and Ethanol Fermentation (ABE)

After the initial discovery of fermentative routes of butanol production made by Louis Pasteur in 1862, Chaim Weizmann, using *Clostridium acetobutylicum* and a starch-rich substate, identified a new production avenue for butanol, acetone, and ethanol (Sauer, 2016). The newly coined, "Weizmann" process, now more commonly referred to as acetone, butanol, ethanol fermentation (ABE fermentation) gained industrial traction beginning in 1912 when there was a high demand for acetone (Jin et al., 2011). ABE fermentation rose to become the second-largest fermentation process in the 20th century, with prevalent interest in butanol as a solvent for car lacquers (Sauer, 2016; Uyttebroek et al., 2015). Despite the initial commercial success of ABE fermentation, more profitable routes of butanol production, derived from petrochemicals emerged in the 1950s (Green, 2011).

ABE fermentation is an anaerobic process that employs gram-positive solventogenic *Clostridia* such as *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum, and C. saccharoperbutylacetonicum* (Gu et al., 2014). These microbes are robust and capable of metabolizing different carbon-rich sources from simple sugars, complex starchy carbohydrates, fibrous sources such as inulin to other carbon sources such as glycerol (Mayank et al., 2013; Patakova et al., 2013). This versatility suggests an opportunity for the use of low-cost feedstocks for ABE fermentation, an approach that continues to be investigated in literature (Mayank et al., 2013)

These *Clostridia* species exhibit biphasic behaviour, which refers to a two-stage metabolism. In the first stage which occurs during the exponential stage of cell growth,

acetic acid, butyric acid, hydrogen, and carbon dioxide are produced. This is commonly referred to as the acidogenic phase (Jin et al., 2011). Next, in the solventogenic phase which is initiated at decreased pH levels, the acids are subsequently converted to solvents (Mayank et al., 2013).

Currently, ABE fermentation is generally considered to be economically impractical at commercial levels due to low titers and productivity, as well as high costs for downstream product recovery and substrates (N. Qureshi & Blaschek, 2001).

2.2.2 Chemical Synthesis

Currently, the majority of butanol is produced commercially through petrochemical synthesis (Jiang et al., 2015). Most prominently, butanol is formed via a hydroformylation reaction followed by hydrogenation of the aldehydes that are generated, a process known as oxo synthesis (Brito & Martins, 2017). In this process, carbon monoxide and hydrogen are fed to a propylene mixture in the presence of a catalyst such as Cobalt, Rhodium, or Ruthenium (Lee et al., 2008).

Butanol can also be produced chemically from bioethanol, in a three-stage reaction. Dehydrated bioethanol is first oxidized using a catalyst, such as aluminum isopropanolate (Kolesinska et al., 2019). Secondly the product, acetaldehyde, undergoes aldol condensation to form crotonaldehyde. Lastly, a hydrogenation reaction is facilitated to obtain butanol (Kolesinska et al., 2019; Ndaba et al., 2015). There have been increased efforts to identify promising catalysts to improve conversion efficiencies of butanol from bioethanol as an alternative to microbial production methods (Ndaba et al., 2015).

2.2.3 1,3-Propanediol, Butanol, and Ethanol Fermentation (PBE)

Another attractive route for butanol production is that of glycerol fermentation with the gram-positive bacterium, *Clostridium pasteurianum.* This microorganism in tandem with glycerol, a reductive substrate, yields a diverse solvent profile consisting of 1,3-propanediol (PDO), butanol and ethanol (PBE). This fermentation is also known to produce carbon dioxide, hydrogen, acetic acid, butyric acid, and lactic acid (E. Johnson et al., 2016). Literature has suggested a possible metabolic pathway for glycerol consumption in *C.*

pasteurianum, yet further exploration is needed to further characterize the complex breakdown of this carbon source (Figure 1).

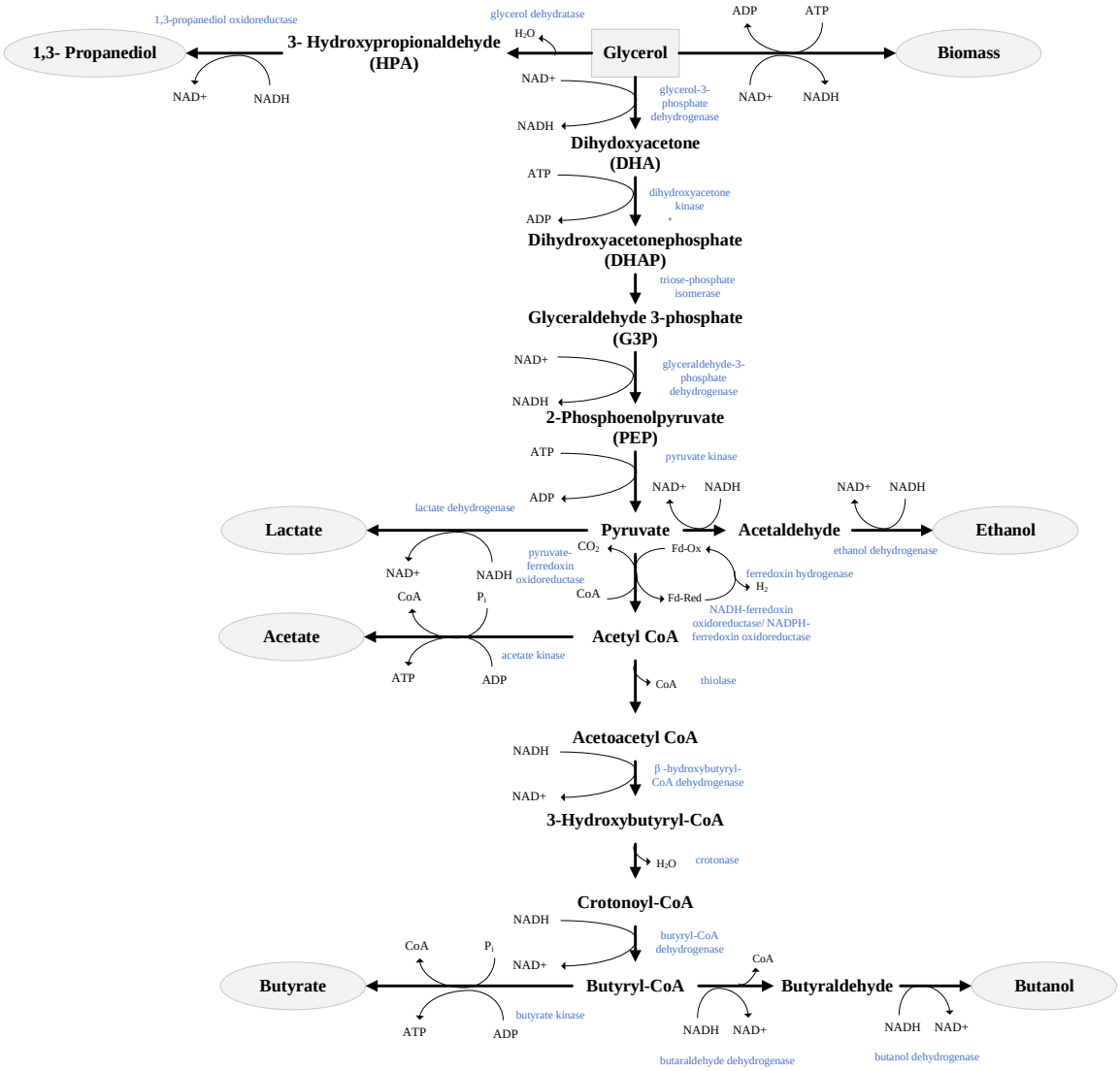

Figure 1: Glycerol utilization pathway in *C. pasteurianum*. Metabolic enzymes are highlighted in blue text. (adapted from: Gallardo et al., 2017; Johnson et al., 2016; Schwarz et al., 2017)

The oxidative pathway to pyruvate from glycerol necessitates a variety of enzymes, converting the carbon chain to several intermediate molecules, and generating NADH in the process. Hydrogen is produced in the conversion of pyruvate to acetyl CoA via pyruvate ferredoxin oxidoreductase, which operates in tandem with ferredoxin hydrogenase, facilitating the transfer of electrons to a final acceptor (E. E. Johnson & Rehmann, 2020; Schwarz et al., 2017). This step is also closely associated with CO_2 production. The reductive pathways of the cell metabolism lead to the formation of butanol, ethanol, and 1,3-PDO, and the co-factor, NAD (E. E. Johnson & Rehmann, 2020). Literature suggests that 1,3-PDO production, a glycolysis independent pathway, is used by the cells to effectively regulate the redox balance during biomass formation, where an excess of reducing equivalents (NADH) are formed (E. Johnson et al., 2016; Pyne et al., 2016).

Product selectivity or inactivation of certain pathways can be influenced by several factors such as media composition, operational parameters such as pH and directed mutations. For example, iron limitation can play a role in diverting the carbon pathway of *C. pasteurianum* toward 1,3-PDO (Biebl, 2001). This switch in preferred pathways is likely related to the critical function of the iron-dependent enzymes, such as ferredoxin oxidoreductase, in glycolysis (Dabrock et al., 1992; E. E. Johnson & Rehmann, 2020).

For glucose metabolism in *C. pasteurianum*, the pathways resulting in the production of acetic acid and butyric acid are more active. One contributing factor may be in the degree of reduction, glucose having a reductive number of 4 compared to 4.67 for glycerol. As a result, the theoretical yield of butanol can be 17% lower with a glucose substrate than glycerol (Sandoval et al., 2015).

Notably, the biphasic behavior seen with many species of *Clostridium*, has not been observed in *C. pasteurianum.* At industrial scale, the co-production of acids and solvents negates the requirement of two-stage fermenters which have typically been used to carry out ABE fermentations (E. Johnson et al., 2016).

2.2.4 Glycerol as a Substrate for Butanol Production

Glycerol, also commonly referred to as glycerin, is a three-carbon compound having many traditional commercial uses as a humectant, solvent, and sweetener (Monteiro et al., 2018). The majority of the world's global supply of glycerol is currently produced as a by-product from the biodiesel sector (Kaur et al., 2020). During the transesterification of oils to produce biodiesel, the main by-product is crude glycerol, which contains several impurities such as methanol or ethanol, soap, fatty acid methyl esters, and free fatty acids (Xiao et al., 2013). The exact composition of the impurities present in crude glycerol is characteristic of the feedstock and catalyst used in the process. The relative abundance of impurities is also variable and can also range from 20-60% (Monteiro et al., 2018; Yang et al., 2012). The presence of these impurities limits the possible direct applications of crude glycerol but it is often used for in-house energy generation for biodiesel plants (Aline et al., 2016). Purified glycerol holds more value and can be used for a vast number of applications in the food, pharmaceutical and medical sectors, however, costs for refining crude glycerol are high and the purification process can be energy-intensive (Kaur et al., 2020; Quispe et al., 2013).

With the expansion of the global biodiesel industry, projections are suggesting there will be a 4.5% annual production growth, translating to 41 million m^3 of biodiesel produced worldwide in 2022 (Monteiro et al., 2018). The accompanied production of crude glycerol, of which 1 kg is generated per 10 kg of biodiesel, has led to a surplus in this potential feedstock (Kumar et al., 2019; Monteiro et al., 2018). The abundance of available crude glycerol presents an opportunity for valorization. One such application of crude glycerol is as a substrate for bioconversion.

There are many known microorganisms capable of using glycerol as a sole substrate such as *Citrobacter* and *Klebsiella* bacteria, which are pathogenic and therefore not typically desirable for industrial fermentation. Other *Clostridium* species can metabolize glycerol exclusively such as *C. butyricum*, and *C. butylicum*, although butanol is not a product of these fermentations (E. Johnson et al., 2016; Venkataramanan et al., 2012). Alternatively, other butanol-producing bacteria such as *C. acetobutylicum* have only been shown to metabolize glycerol when in the presence of glucose (Taconi et al., 2009). As such, there

has been extensive work related to improving glycerol fermentation for butanol production with *C. pasteurianum*, using both pure refined glycerol as well as crude feedstock.

In terms of substrate limitations, *C. pasteurianum* has only been reported to exhibit signs of inhibition with very high concentrations of glycerol, and as such is able to tolerate up to 17 (wt/vol %) of purified glycerol (Dabrock et al., 1992; Kalafatakis et al., 2019).

2.3 Butanol Inhibition and *in-situ* Butanol Removal Strategies

Inhibitory effects from butanol accumulation have been observed in *C. pasteurianum* starting as low as 12 g/L (Kießlich et al., 2017). This phenomenon holds true for other butanol-producing *Clostridia* spp. where butanol levels often become toxic above 2%. The mechanism of inhibition is often attributed to the interaction of butanol with the cell membrane. At high concentrations, butanol has been observed to alter the cell membrane in *C. acetobutylicum*, resulting in an increase in cell membrane fluidity thereby affecting cellular function (S. Liu & Qureshi, 2009). The effect of butanol accumulation can inhibit glucose uptake and cause internal pH to decrease in *C. acetobutylicum* (Ellefson & Bowles, 1985). There are many strategies in development aimed to overcome butanol inhibition, many of which involve *in-situ* removal processes.

2.3.1 Butanol Removal Strategy: Distillation

The conventional method of butanol separation from fermentation broth is distillation. This process requires high energy investment, and due to the azeotropic mixtures of water and butanol being present in the broth, this approach generally has low selectivity (Kujawska et al., 2015). Integrated methods of recovery not only aim to reduce product inhibition but can also be a means to minimize downstream separation costs (Outram et al., 2017; Sarchami, Munch, et al., 2016).

Vacuum distillation or vacuum stripping operates under reduced pressures, forcing volatile components into the gas phase (Outram et al., 2017; Sarchami, Munch, et al., 2016). This process has been demonstrated to be effective at removing and maintaining a low butanol concentration in fermentation broth without adversely impacting the cells in ABE

fermentation (Mariano et al., 2011). Inefficiencies in condensation methods used to recover butanol are a limitation for vacuum stripping (Outram et al., 2017).

2.3.2 Butanol Removal Strategy: Adsorption

Adsorption as an *in-situ* removal technique involves the addition of adsorbents such as activated carbon and silicates into the fermentation broth. Butanol is then recovered through desorption by heat treatment or via displacers (Outram et al., 2017; Zheng et al., 2009). Drawbacks to this process are associated with challenges with regenerating the adsorbent, and limited capacity of the absorbent (Outram et al., 2017; Sarchami, Munch, et al., 2016).

2.3.3 Butanol Removal Strategy: Gas Stripping

Due to the volatility of butanol, gas stripping is an attractive and relatively simple approach for product recovery. Gas stripping involves sparging the fermentation broth with an inert, oxygen-free gas, enabling the volatile solvents to vaporize and escape the fermenter with the effluent gas (Figure 2). The effluent gas can then be condensed and the butanol recovered (Zheng et al., 2009). Attempts to reduce costs of stripping gases in PBE fermentation have been made by capturing and recycling the effluent gases containing the microbially produced CO_2 and H_2. Groeger et. al. investigated glycerol fermentation with *C. pasteurianum* coupled with effluent gas stripping and found that at high stripping flowrates the concentration of butanol could be sustained below inhibitory levels (Groeger et al., 2016). However, gas stripping can be energy-intensive, and the presence of gas bubbles may require the addition of antifoam (Kujawska et al., 2015; Sarchami, Munch, et al., 2016). Gas stripping has also been evaluated in a hybrid model as a precursor to other *in-situ* techniques such as pervaporation or after liquid extraction with varying success (Sarchami, Munch, et al., 2016).

2.3.4 Butanol Removal Strategy: Liquid-Liquid Extraction

Liquid-liquid extraction requires the addition of another solvent to the broth, relying on a difference in solubility between compounds (Outram et al., 2017). Solvents that have previously been applied to ABE and PBE fermentations include: n-decol and oleyl alcohol which can, at high concentrations, be toxic to the bacteria (Kujawska et al., 2015). Although high selectivity has been reported in this process, butanol recovery post-fermentation due to the formation of emulsions poses additional challenges and there is limited research evaluating the ability to recycle the solvents (Kujawska et al., 2015; Outram et al., 2017). Perstraction is an alternative technique (visualized in Figure 2) associated with liquid-liquid extraction where the organic solvent and fermentation broth are separated via a membrane (Outram et al., 2017; Zheng et al., 2009). This approach aims to remove barriers such as solvent toxicity, phase dispersion, and emulsion formation (Ezeji et al., 2007).

2.3.5 Butanol Removal Strategy: Pervaporation

Pervaporation is a membrane-based approach that can be used to separate volatile compounds from complex liquid mixtures (Figure 2). The working principle relies on having a difference in chemical potential between both sides of the membrane (Vane, 2005). This can be done in a variety of ways either by manipulating temperature, applying a purge gas, or creating a pressure difference using a vacuum (Kujawska et al., 2015). In vacuum pervaporation, liquid is pumped tangentially across a membrane while a vacuum is applied to the opposite side. The difference in pressure across the membrane drives the desired component selectively to the permeate side as a vapour. The permeate vapours are then condensed back into a liquid state (Zheng et al., 2009). The effectiveness and suitability of a membrane are dictated by the butanol flux and selectiveness (N. Qureshi & Blaschek, 1999).

Owing to low operating temperatures, low energy consumption and having high selectivity enabling substrates and nutrients to be retained in the media, pervaporation is particularly promising for microbial fermentations (Kießlich et al., 2017; Sarchami, Munch, et al., 2016). The type of membrane needed for pervaporation is highly dependent on its

application. For butanol recovery, organophilic membranes are the most widely used. Silicone-based membranes such as polydimethylsiloxane (PDMS) membranes are typically employed for applications with ABE fermentation broth, due to low manufacturing costs, and superior performance in permeate selectively (G. Liu et al., 2014; Nasibuddin Qureshi & Blaschek, 1999).

Disadvantages of pervaporation include membrane fouling, which can lead to decreased separation performance. This phenomenon was seen by Qureshi et. al. who reported up to a 3 times reduction in selectivity with active ABE fermentation broth compared to spent broth, suggesting the presence of growing cells may contribute to membrane fouling (G. Liu et al., 2014; Nasibuddin Qureshi & Blaschek, 1999). In practice, the performance of membranes prone to fouling may be improved by operating at high flowrates and pressures. These membranes also require routine cleaning protocols, to remain effective. Kießlich et. al. evaluated the performance of a PDMS membrane for the removal of butanol from PBE broth, concluding that temperature and flowrate were significant variables showing improved butanol flux at 50℃ compared to 35℃ (Kießlich et al., 2017).

There have been several attempts to integrate PBE fermentation with concurrent product recovery (Table 1). Only one study, conducted by Chen et. al., has applied pervaporation to a fed-batch glycerol PBE fermentation. The group used a wild-type strain of *C. pasteurianum*, CT7, and incorporated a composite tubular membrane of PDMS and ceramic materials (ZrO_2 and Al_2O_3) to facilitate *in-situ* removal of butanol. Pulse feeding of media and nutrients was used to maintain glycerol concentrations above 20 g/L. The reported volumetric butanol productivity was 0.21 g/L·h with an accompanied yield of 0.41 g/g (Chen et al., 2019; Xin et al., 2017).

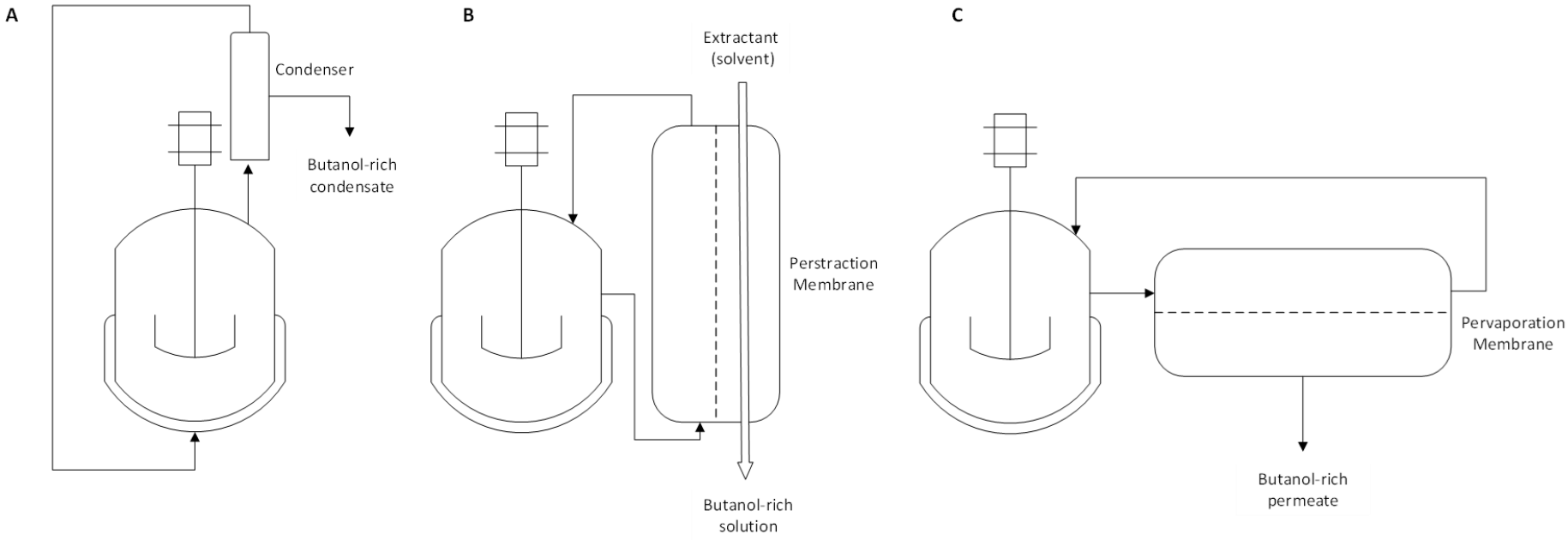

Figure 2: Simplified schematic overview of various *in-situ* removal technologies; A) Gas stripping B) Perstraction C) Pervaporation (adapted from (Lee et al., 2008))

Table 1: Summary of recent literature evaluating *in-situ* butanol removal for PBE fermentation using glycerol

Mode of Operation	Strain	Substrate and initial concentration (g/L)	Overall Butanol Productivity (g/L·h)	Fermentation Time (h)	Reference
Fed-batch with pervaporation	CT7	Glycerol (60)	0.21	240	(Chen et al., 2019)
Fed-batch with gas stripping	DMSZ 525	Crude glycerol (100)	1.3	70	(Jensen, Kvist, Mikkelsen, Christensen, et al., 2012)
Fed-batch with gas stripping	Mutant DMSZ 525 (MNO6)	Crude glycerol (122)	1.8	75	(Jensen, Kvist, Mikkelsen, & Westermann, 2012)
Fed-batch with gas stripping	DSM 525	Glycerol (≈120) Glucose (≈120)	0.19	70	(Groeger et al., 2016)
Batch with vacuum membrane distillation and butyrate addition	CH_4	Glycerol (≈100)	0.29	100	(Lin et al., 2015)
Fed-batch with liquid-liquid extraction	GL11	Glycerol (60)	0.18	160	(Xin et al., 2016)
Batch with liquid-liquid extraction	SE-5	Glycerol (80)	0.33	72	(Zhang et al., 2013)

2.4 Strategies for Improving Butanol Productivity and Yield

Bioprocesses tend to be complex in nature, with a multitude of factors playing a combined role in process efficiency. As such, there are a variety of different approaches aimed to address low butanol yields, titers, and productivity. These strategies range from manipulating genetic material, to refining process parameters, redirecting metabolic pathways through the addition of new substrates, and adjusting media components to favour butanol production.

2.4.1 Metabolic Engineering

Genetic modification of *C. pasteurianum* to obtain strains that have greater butanol and crude glycerol tolerance or that can divert more carbon towards butanol production rather than organic acids is a rapidly expanding area of research. Mutagenesis can be accomplished randomly via chemical techniques or selectively with tools such as electrotransformation (Pyne et al., 2013). Malaviya et. al. successfully employed chemical mutagenesis for *C. pasteurianum* ATCC 6103 using N-methyl-N′-nitro-N-nitrosoguanidine (NTG) and screened for enhanced butanol production, resulting in a hyper producing strain, MBEL_GLY2. In batch studies, the strain produced 17.8 g/L butanol, compared to 7.6 g/L produced with the original strain (Malaviya et al., 2012). Sandoval et. al. focused on the development of a strain (M150B) that is capable of tolerating high concentrations of crude glycerol using chemical mutagenesis via NTG. In the same study, a knockout strain of the gene responsible for spore formation (SpoA), was developed with similar tolerance to crude glycerol as M150B (Sandoval et al., 2015).

Through inactivation of the 1,3-PDO dehydrogenase gene (dhaT), Pyne et. al., were able to identify a pathway for 1,2-PDO formation. This alternative pathway was likely activated to oxidize the reducing equivalents generated during cell growth as well as glycolysis via the ferredoxin oxidoreductase enzyme. This yield improvement strategy resulted in greater butanol selectivity and did not affect cell growth (Pyne et al., 2016).

2.4.2 Bioprocess Design

Parameter and system design are some of the many strategies available to address poor volumetric butanol productivity. Conventional industrial fermentations are typically operated in batch-mode, due to the inherent simplicity and reliable process control of a closed system (S. Y. Li et al., 2011; Veza et al., 2021). Despite being relatively easy to maintain, batch systems, allow for the accumulation of metabolites that can hinder productivity. Fed-batch operations, which involve either continuous or intermittent addition of substrate can dilute the fermentation broth which may lessen the effects of butanol toxicity in ABE fermentation (S. Y. Li et al., 2011). Conversely, a study comparing the product profile of *C. pasteurianum* grown on glycerol in both batch and fed-batch modes found no significant differences in butanol productivity or product yield between both operations (Biebl, 2001).

Continuous modes of operation, which simultaneously provide an influent stream of nutrients to the reactor vessel and remove production broth as effluent, can offer a number of industrial advantages (T. Li et al., 2014). For example, the steady-state system allows for fewer disruptions to production and can improve productivity by minimizing the time spent in the lag phase of cell growth. In microbial butanol production, the dilution of the fermentation broth with media along with constant removal of products are particularly relevant design aspects for limiting product inhibition (S. Y. Li et al., 2011). However, continuous fermentations are vulnerable to contamination and subsequent operational challenges (T. Li et al., 2014). Additionally, in continuous reactors, cell-wash out leads to low cell density within the reactor, which can be counteracted by incorporating cell-recycling units or through cell immobilization (Veza et al., 2021). In one attempt to achieve high cell density, Gallazzi et. al. utilized a packed bed reactor containing corn stover with surface-immobilized cells of *C. pasteurianum* and were able to increase butanol productivity from 0.1 g/L·h in suspended culture to 4.2 g/L·h with cell immobilization (Gallazzi et al., 2015). Cell-recycling has also been shown to be a promising tool in continuous PBE fermentation. To date, the highest butanol titer obtained during glycerol fermentation using *C. pasteurianum* was 17.8 g/L which was reported by Malaviya et. al., using a mutant strain under continuous operation with cell recycle (Malaviya et al., 2012).

In both *C. acetobutylicum and C. pasteurianum*, an oscillatory metabolism has been observed during continuous fermentations under various conditions. In a suspended culture of *C. pasteurianum* operated in a continuous packed bed reactor Gallazi et. al., observed cyclic behavior of the cells in terms of butanol production, substrate utilization, and cell density at various dilution rates (Gallazzi et al., 2015). Johnston et. al. studied the influence of varying operating parameters on the duration of oscillations in the measured redox potential and gas production, a phenomenon believed to be related to the result of enzyme activation and inhibition by redox equivalents. Varying of parameters such as temperature and dilution rate were found to delay or eradicate these oscillations (E. E. Johnson & Rehmann, 2020).

Another significant factor that affects the desired product profile, is the operating conditions set during fermentation, such as temperature and the controlled pH. For example, an investigation into the role of pH in glycerol fermentation with *C. pasteurianum* found that basic conditions up to pH 5.9 favoured 1,3-PDO production over butanol (E. E. Johnson & Rehmann, 2016). In another study, temperature, inoculum age, and initial cell density were optimized for butanol production using crude glycerol in PBE fermentation, reporting that a lower operating temperature of 30 ℃ resulted in the highest butanol titers (Sarchami, Johnson, et al., 2016a).

2.4.3 Butyrate Addition and Co-substrate Fermentation

The role of butyric acid as an intermediate to butanol formation has been explored as an approach to refining product selectivity. The motivation for this strategy is that through supplementing the amount of butyrate available to the cells by means of direct addition or via co-substrate production, the energetically favourable metabolic pathway from butyrate to butanol can be further simulated, thereby increasing butanol yield (Regestein et al., 2015). Munch et. al. investigated the addition of both pure and microbially produced butyric acid in PBE fermentation, concluding that the highest uptake of butyric acid occurred during delayed addition as opposed to initial supplementation. Further, it was reported that butanol selectivity was increased resulting in a carbon yield of 0.519 mols $_{\text{carbon in product}}$/ mols $_{\text{carbon in substrate}}$ with late-stage addition (Munch et al., 2020). In contrast, Regestein et. al., reported that with moderate levels (3 g/L) of butyric acid addition at the

start of batch fermentation, butanol yield could be improved, however with higher levels of butyric acid (4 g/L), cell metabolism was adversely affected and the lag phase prolonged. Specifically, pH was found to be an important factor in butyric acid uptake, with greater conversion occurring at pH 5.3 compared to pH 6.3 (Regestein et al., 2015). Sabra et. al. also investigated butyric acid addition to PBE fermentation in pH-controlled conditions (pH 6), finding that butanol selectivity was increased, however butanol productivity and cell growth suffered as a result (Sabra et al., 2014).

External acetate addition to production media has also been shown to direct *C. pasteurianum* metabolism. In a study evaluating acetate addition at concentrations up to 5 g/L found that at 3 g/L the addition of acetate improved butanol yield by 10.7% and did not impact productivity (Sarchami & Rehmann, 2019). Similar results were reported by Moon et. al., who found a positive correlation between acetate addition and butanol production (Moon et al., 2015).

Mixed substrate fermentations can offer an alterative route for increased selectivity for butanol production. During glucose utilization in *C. pasteurianum*, the product profile is typically skewed towards the production of acids (E. Johnson et al., 2016). Due to this observation, sugar-based substrates have been incorporated with glycerol fermentations to enhance the reductive butanol pathway. Sabra et. al. achieved a record high butanol titer of 21.5 g/L and volumetric productivity of 0.96 g/L·h with a 1:1 glucose to glycerol ratio during batch fermentation under optimized pH conditions. Interestingly, glycerol consumption was found to occur only after glucose was fully depleted (Sabra et al., 2014). Sarchami et. al., studied co-substrate fermentation using crude glycerol and Jerusalem artichoke tuber hydrolysate, comprised primarily of fructose and glucose sugars. The study achieved a productivity of 0.55 g/L·h and a yield of 0.28 $g_{butanol}/ g_{substrate}$ in a bench-scale batch fermentation (Sarchami & Rehmann, 2019).

2.4.4 Media Selection

Augmenting media composition is another means to direct the product profile for *C. pasteurianum.* Two medium compositions were evaluated by Sarchami et. al., Biebl media and MP2 media, which contain differing amounts of nitrogen sources as well as trace

elements. This investigation demonstrated that Biebl media was more suitable for butanol production under the same optimized conditions (Sarchami, Johnson, et al., 2016a).

Iron-dependent enzymes are crucial for maintaining redox within *Clostridia*, and as such the amount of iron available in the media can influence product selectivity (Groeger et al., 2017). As reported by Dabrock et. al., iron limitation during glycerol fermentation with *C. pasteurianum* has been shown to divert carbon toward 1,3-PDO production (Dabrock et al., 1992). Similarly, in a study conducted by Groeger et. al., a 5-fold increase in 1,3-PDO production was seen in iron-limited conditions. Along with an upregulation of key enzymes such as pyruvate: ferredoxin oxidoreductase and an increase in hydrogen production during iron-rich conditions suggested that iron supply is critical in establishing redox balance and product selectivity (Groeger et al., 2017).

Trace elements present in the production media can also influence the activity level of enzymes in the metabolic pathway from glycerol to butanol. Nimbalkar et. al. studied the role of trace elements in the metabolism of C. *acetobutylicum* and found that nickel chloride and sodium selenite act as enzyme cofactors for butanol dehydrogenase. By optimizing the concentrations of these two trace elements in the media, a 2-fold increase in solvent production relative to control conditions was reported (Nimbalkar et al., 2018).

2.4.5 Review of Current Research Gaps

In summary, as the role of butanol is gradually expanding from primarily being an industrial chemical to becoming a new generation biofuel, a means to develop a sustainable and economically competitive route for butanol production from bio-based sources is needed (Xin et al., 2016). A promising direction for the future of butanol production is through PBE fermentation with *C. pasteurianum.* This species in particular has a unique metabolism that allows it to produce butanol from crude glycerol, a waste stream from the biodiesel sector (E. Johnson et al., 2016). The ability to use this low-cost feedstock provides economic incentives to establish this fermentation pathway for butanol production. However challenges with low productivity and high separation costs render translation of the process to industrial scale unprofitable with current methods of bioprocessing (Sarchami, Munch, et al., 2016).

One of the greatest hindrances to glycerol fermentation is that of product toxicity, a common limitation in batch and fed-batch fermentations (S. Liu & Qureshi, 2009). Some attempts to overcome this barrier to commercialization incorporate *in-situ* butanol removal, allowing the concentrations of butanol in the bioreactor to be maintained below inhibitory levels (Outram et al., 2017). Several strategies were reviewed in this chapter such as adsorption, liquid-liquid extraction, gas stripping and pervaporation. Other improvement strategies aimed to further streamline the fermentation and achieve greater butanol yields have implemented genetic engineering, manipulation of process parameters, multiple substrate systems, as well as optimization of media components.

Some key observations made in literature discussed in this chapter are summarized below:

- Butanol is a known inhibitor to *C. pasteurianum*, with recognized and adverse influences to the fermentation occurring from 12 to 20 g/L (Dabrock et al., 1992; Kießlich et al., 2017).
- The addition of continuous *in-situ* butanol removal is presently being evaluated using multiple techniques for PBE fermentations. For example, stripping butanol from the fermentation broth using the off-gas generated by the fermentation itself, a concept known as gas stripping has seen moderate success, at the limitation of being an energy intensive process, requiring high flowrates of stripping gas (Groeger et al., 2016).
- PDMS membranes, used in the context of pervaporation, have been demonstrated to be effective at selective butanol removal from PBE fermentation broth, with temperature, and flowrates being key parameters in the unit's efficiency (Kießlich et al., 2017).

A review of literature in the area of glycerol fermentation to butanol, has resulted in the identification of the following research gaps:

- For ABE fermentations, membrane fouling has been reported to be greater with living cultures compared to cell- free broth (G. Liu et al., 2014; Nasibuddin Qureshi & Blaschek, 1999). However, for *C. pasteurianum*, there are limited studies demonstrating how organophilic membranes perform with complex fermentation broths containing live and growing cultures.

- There is currently only one study evaluating the addition of a pervaporation module to a fed-batch PBE fermentation, yet the influence on cell density within the bioreactor was not reported and a low yielding strain was used for the study resulting in low productivities (Chen et al., 2019). As such, there has been no attempt to study how volumetric butanol productivity is influenced in a semi-continuous process with pervaporation using the DSM 525 strain of *C. pasteurianum*.

Chapter 3

3 Fed-batch Glycerol Fermentation with *in-situ* Pervaporation

This chapter focuses on the integration of a pervaporation unit with a fed-batch glycerol fermentation in attempt to overcome the core challenge of butanol inhibition, intrinsic to traditional batch and fed-batch processes. With the aim of prolonging fermentation time and improving volumetric butanol productivity, this novel process configuration was evaluated at lab scale through a series of iterative experiments.

Starting with a comprehensive explanation of the materials and equipment employed during the execution of the experiments, this chapter moves on to describe the experimental procedures, and the analytical methods used. Following the description of experimental methodology, simulations that were used to model the process and provide insight into the expected advantages of the new process configuration are described, providing background into the equations, parameters and assumptions used. Next, preliminary experiments are described as performed. These initial experiments were done to isolate the influence of the external pump that connects the pervaporation unit to the bioreactor. As a next step, an experiment is summarized that employed a batch fermentation with *in-situ* butanol removal, used to evaluate the suitability of the membrane. Following the overall positive outcome of this experiment, the results from the first iteration of the proposed process configuration are presented along with succeeding experiments. The results from these experiments are compared to predicted outcomes from the simulations as well as compared against batch and fed-batch controls.

3.1 Materials and Methods

This section provides an overview of the methodology used to execute the modified fed-batch studies. Materials used for media formulation are discussed, followed by the procedures used to incrementally scale-up the bacterial cultures and prepare the inoculum. Operating conditions are stated along with a detailed summary of the experimental set-up and the equipment that was employed. Pre-experimental procedures and aseptic techniques

used are reviewed leading to a summary of the operating procedures. Lastly, an outline of the analytical methods used to evaluate the performance of the fermentations is provided.

3.1.1 Chemicals

For the seed train, an established growth medium, reinforced clostridium media (RCM) was prepared as described in literature which has been shown to be sufficient for culturing *C. pasteurianum* (Munch et al., 2020; Sarchami, Johnson, et al., 2016a; Venkataramanan et al., 2012). The chemicals used in the preparation of RCM are listed in Table 2.

Table 2: Summary of components used in growth media

Media Component	Manufacturer (Location)
Peptone, Yeast, Extract, Dextrose/Glucose, Resazurin	Thermo Fisher Scientific (Ottawa, ON)
Beef Extract	BD-Becton, Dickinson and Company (New Jersey, USA)
Sodium acetate	Ward Chemical (Edmonton, AB, Canada)
Sodium chloride	BDH Chemicals (Radnor, PA, USA)
L-cysteine hydrochloride	Sigma Aldrich (St. Louis, MO, USA)
Soluble starch, Potassium hydroxide	Alfa Aesar (Ward Hills, MA, USA)

To skew the product profile towards butanol production, an established medium, Biebl medium, was prepared with the components in Table 3, as stated in literature with modified amounts of substrate (Biebl, 2001). Compared to other standard medium compositions, such as MP2 media, Biebl media is considered to have superior performance, resulting in greater butanol yields under the same operating conditions (Sarchami, Johnson, et al., 2016b).

Table 3: Summary of components used in Biebl Media

Media Component	Manufacturer (Location)
Glycerol, Dextrose/Glucose Potassium phosphate monobasic, Ammonium sulphate, Magnesium sulphate, Yeast extract	Thermo Fisher Scientific (Ottawa, ON)
Potassium phosphate dibasic	Anachemia (Lachine, QC, Canada)
Calcium chloride	EMD Millipore (Gibbstown, NJ, USA)
Ferrous sulphate, Fructose	Sigma Aldrich (St. Louis, MO, USA)

In addition to the standard components of Biebl media, a solution containing various trace elements essential for basic cellular functions was added to the media, with the components used listed in Table 4. This trace element solution, known as SL7 was prepared as described in literature (E. E. Johnson & Rehmann, 2020; Venkataramanan et al., 2012).

Table 4: Summary of components used in trace element solution, SL7

Media Component	Manufacturer (Location)
Ferrous chloride tetrahydrate, Sodium molybdate dihydrate, Cobalt (II) chloride hexahydrate	EMD Millipore (Gibbstown, NJ, USA)
Nickel (II) chloride, Zinc chloride	Alfa Aesar (Ward Hills, MA, USA)
Hydrochloric acid	BDH Chemicals (Radnor, PA, USA)
Manganous choride-4-hydrate	JT Baker (Phillipsburg, NJ, USA)
Boric acid	Amersco (Solon, OH, USA)

3.1.2 Microorganisms and Media

In this work, frozen cultures of *Clostridium pasteurianum* DSM 525 were obtained from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The 10 % (v/v) glycerol stock cultures were stored at -80 ℃ in 1 mL aliquots until use. All seed culture work was performed within an anaerobic chamber (Plas-Labs, MI, USA, model 855-ACB-EXP) filled with mixed gas (5% CO_2, 10 % H_2 and 85% N_2). Catalyst heaters containing aluminized palladium were used to both control the temperature in the chamber at 35 ℃ and to react with any present oxygen (Plas-Labs, MI, USA, model 800-HEATER). Anaerobic indicators containing resazurin were used to verify if oxygen was present in the chamber environment prior to use (Thermo Fisher Scientific).

Pre-cultures were scaled up in three stages at 10% (v/v) during the log phase of cell growth. The initial seed cultures were prepared from 500 µL of glycerol stock and 4.5 mL of RCM. After 16-17 hours of incubation on a stir plate set at 320 rpm, the 5 mL culture was added to 45 mL of RCM and allowed to incubate in the anaerobic chamber for another 7-8 hours or until the optical density reading was above 1.0 AU. Once log phase growth was confirmed via optical density measurement, the 50 mL culture was added to 450 mL of RCM and again incubated for 7-8 hours or until OD_{600} reading was above 1.0 AU.

The RCM was prepared with the following ingredients (per liter of deionized water): 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g dextrose, 5 g sodium chloride, 1 g soluble starch, 3 g sodium acetate, 4 ml of resazurin solution diluted to 0.025% with deionized water, and 0.5 g L-cysteine hydrochloride. The pH of the media was adjusted to 6.8 using 3M KOH. The media was purged with nitrogen, subsequently sterilized and stored within the anaerobic chamber.

The production or modified Biebl media contained (per liter of deionized water): 50 g glycerol (batch fermentation) or 20 g glycerol (modified fed-batch), 0.5 g potassium phosphate dibasic, 0.5 g potassium phosphate monobasic, 5g ammonium sulphate, 0.02 g calcium chloride, 0.1g ferrous sulphate, 1g yeast extract, 0.2 mL antifoam 204, 2mL trace element solution. The prepared media was added to the bioreactor and the entire bioreactor was autoclaved at 121 ℃ for 50 min.

For co-substrate fermentation, the glycerol concentration in the reactor was modified to 14 g/L, glucose was added at 54 g/L and fructose at 18 g/L, all other components were added in the same amounts as described above. A concentrated solution containing the glucose and fructose was autoclaved separately from the other components of the media and aseptically pumped into the reactor before inoculation to avoid caramelization of the media and sugar degradation (Wang & Hsiao, 1995).

The production media used for feeding was prepared with the same chemicals and concentrations as previously mentioned however the glycerol concentration was increased to 250 g/L. The 4 L feed bottle and tubing were autoclaved together at 121 ℃ for 20 min. However, for co-substrate fermentation, the media was prepared with 180 g/L glycerol, 4.5 g/L glucose, and 1.5 g/L fructose. A concentrated solution of the sugars was prepared and sterile filtered, while the other portion of the media was autoclaved as previously described.

Trace element solution (SL7) was prepared with the following (per liter of deionized water): 1.5 g ferrous chloride tetrahydrate, 10 mL of hydrochloric acid diluted to 25% with deionized water, 0.19 g cobalt (II) chloride hexahydrate, 0.10 g manganous choride-4-hydrate, 0.07 g zinc chloride, 0.062 boric acid, 0.03 g sodium molybdate dihydrate, 0.024g nickel (II) chloride. The SL7 was sterile vacuum filtered through a 0.2 µm filter prior to use (Pall Corporation, Portsmouth, United Kingdom).

3.1.3 Inoculum Preparation

Pre-culture preparation was conducted as described in section 3.1.2. Once an OD_{600} of greater than 1.0 AU was achieved for the 500 mL culture, a volume equivalent to 10 % of the initial working volume of the fermenter was added to the inoculum bottle. The bottle was then removed from the chamber, with the exit standpipe clamped to prevent the introduction of air to the bottle. Aseptic technique was used through seed preparation and during inoculation. Sterile nitrogen was used to drive the inoculum through the sterilized tubing and into the bioreactor.

3.1.4 Fermentation Conditions

All fermentations were conducted using a 7 L bench top bioreactor (Infors HT, Bottmingen/Basel, Switzerland, model: LabFors 4). The working volume for batch fermentations was 5 L. For fed-batch fermentation, the initial working volume was 3 L and 4 L for the modified fed-batch fermentations. The reactor was sparged with nitrogen gas at a flowrate of 0.5 L/h and the impeller was set to rotate at 200 rpm. All nitrogen gas used in this work was purchased from Praxair (London, ON, Canada). pH was controlled at 5.0 using 3 M KOH and 1.5 M H_2SO_4. The temperature was controlled at 35 ℃ for all configurations.

3.1.5 Experimental Configuration of Modified Fed-batch Operation

In this design, fermentation broth was pumped from the bioreactor and directed through the tangential flow membrane filter where butanol was selectively absorbed and diffused across the membrane. Cells, unused substate, and most by-products were returned into the fermenter and the product was recovered in the permeate (Figure 3).

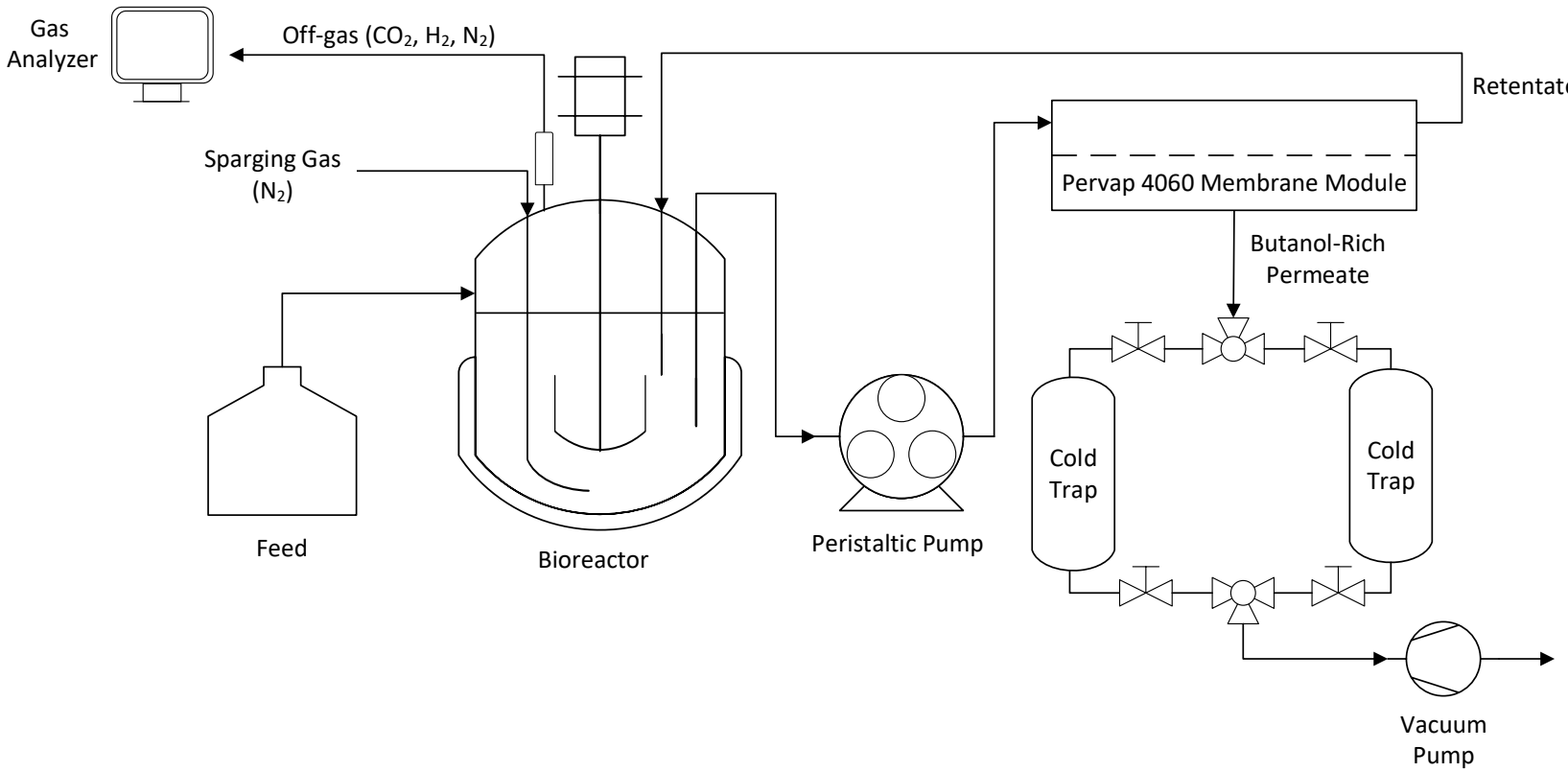

Figure 3: Schematic overview of the modified-fed batch process

The experimental configuration (Appendix 1), consisted of three main components: the bioreactor, the peristaltic pump and the pervaporation unit. The bioreactor (Infors HT) was fitted with a stainless-steel standpipe (2.5 cm inner diameter) allowing fermentation broth to be pumped out of the fermenter and into the plate and frame pervaporation module (Sulzer Chemtech, Model Pervap 4060, Winterthur, Switzerland). The test cell was equipped with a flat sheet polydimethylsiloxane (PDMS) membrane, with an area of 170 cm^2 and an approximate thickness of 6 µm (Sulzer Chemtech). This thin commercial membrane was selected due to its suitability to remove butanol in complex fermentation broth, with extensive literature applying PDMS-based membranes in ABE fermentations (Outram et al., 2017; N. Qureshi & Blaschek, 2001). Further, using cell-free spent fermentation broth, it has already been determined that butanol can be removed from PBE fermentation broth at relatively high fluxes using the Pervap 4060 module, compared to other commercial PDMS membranes (Kießlich et al., 2017). Although other polymeric membrane materials are capable of removing butanol from binary solutions with water such as poly(ether block amide) (PEBA) and poly[1-(trimethylsilyl)-1-pro-pyne] (PTMSP), they either fail to achieve comparable selectively to PDMS or have greater fabrication costs. Although, PDMS-membranes continue to be the standard for butanol separation applications, research efforts are investigating composite PDMS-based membranes as well as zeolite membranes to improve separation efficiencies (G. Liu et al., 2014). A pressure gauge was incorporated prior to the membrane inlet to ensure the pressure through the module did not exceed the operating limits (16 bar) indicated by the manufacturer.

A peristaltic metering pump (Blue-White Ind., Model: Flex-Pro Norprene A4F24-MNHH, Huntington Beach, USA) was used to direct flow to the membrane at the desired flowrate. Calibration of the pump was done manually using deionized water, correlating the speed percentage of the pump with a flowrate.

A vacuum was applied to the permeate side of the membrane, allowing the butanol-rich permeate to be collected in the adjoining cold traps. To protect the vacuum pump (Edwards, Model: RV5, Crawley, UK) from potentially damaging solvent vapours, a charcoal trap

was used ahead of the pump (Labconco, Visi Trap® Model 77725, Kansas City, USA). The retentate was returned via a smaller standpipe (0.635cm inner diameter) installed in the fermenter. The cold traps (Best Value Vacs, Illinois, USA), used to collect the permeate were composed of two cylinders. The larger outer cylinder was insulated and closed to the environment during operation, whereas the inner cylinder was not subjected to vacuum and could be accessed throughout the fermentation to maintain the level of dry ice.

3.1.6 Experimental Preparation

For both fed-batch and modified fed-batch operations, the fermentation was initially operated in batch mode for the first 14 hours of fermentation, unless otherwise stated. Due to the amount of remaining substrate and upward trend in gas production at this timepoint, 14 hours was determined to be an ideal time to initiate substrate feeding. Calibration of the feed pump was done manually with deionized water (Infors HT, Feed2). The feed bottle was sparged with nitrogen throughout the duration of the fermentation and stirred at 300 rpm.

Standpipes were autoclaved separately from the bioreactor then placed aseptically into the reactor and connected to the corresponding tubing pre-inoculation. However, sterility procedures were not used to disinfect the tubing connecting the pervaporation module to the bioreactor. Although initial configurations of the system incorporated a separate sterilization loop using 70% ethanol, there were challenges identified with successfully removing the ethanol from the system, post disinfection. With the potential to introduce residual ethanol this extra sterilization step was avoided, due to risks to cell viability.

3.1.7 Experimental Procedures

To prepare for the incorporation of pervaporation, the cold traps were connected and the inner cylinder of the first trap filled hallway with reagent alcohol (Thermo Fisher Scientific, Ontario, Canada). Dry ice obtained from the BioChem Stores at Western University was then slowly added into the cold trap to maintain a temperature of approximately -70 ℃. The temperature was measured using a thermocouple (Fisher Traceable, Model 4015). With all valves to the traps closed, the vacuum pump was turned on for one hour for preparation.

Before attaching the inlet tubing to the pervaporation module, the valves corresponding to cold trap A were opened, while cold trap B valves remained closed. The valve connecting the cold trap to the membrane was then switched to the open position. This sequence in the procedure ensures that the membrane is always under vacuum prior to the flow of fermentation broth. This step is necessary to prevent creasing and folding of the membrane which can create inefficiencies for separation.

Approximately 30 minutes after substrate feeding was initiated, the peristatic pump was turned on, marking the start of *in-situ* butanol removal. The peristaltic pump was pre-set to 2 L/min (47 rpm) such that there was an immediate flowrate change once turned on.

The cold traps were switched every 5 hours to prevent frozen permeate from clogging the tubing, as well as to obtain permeate samples at intervals throughout the process. To ensure the membrane remained under vacuum, the following protocol was followed: the vacuum side of the new trap was opened, the valve of the old trap closed, the permeate valve of the new trap opened, and subsequently, the permeate valve of the old trap closed.

The permeate sample was collected by removing the dry ice and alcohol from the inner cylinder and filling it with warm deionized water. This step was done to ensure frozen permeate that collected on the outer walls of the inner cylinder thoroughly melted and collected into the outer portion of the trap. The previously recorded weight of the outer trap was subtracted from the combined weight of the permeate and the outer cylinder trap to determine the mass of permeate collected (Sartorius, Model TE3102S, Göttingen, Germany). The permeate was then diluted with deionized water, transferred to a glass beaker and stirred until a homogenous solution was achieved. Samples were stored at -20 °C until analysis.

Cleaning protocols for the membrane were adapted from Kießlich et al., and used post-fermentation (Kießlich et al., 2017). The tubing joining the membrane inlet and outlet was disconnected from the bioreactor. The peristaltic metering pump was turned on at 4 L/min and any remaining fermentation broth in the tubing was collected and disposed of according to biological waste protocols. The tubing was then connected to a rubber flask stopper containing two standpipes and used to cycle 1 L of deionized water through the

membrane. This rinsing step was repeated 5 times. After which, a 1% (w/w) solution of NaOH, pre heated to 50 ℃, was cycled for 30 min. This step was followed by another 5 cycles of rinsing via deionized water. Next, a 1% (w/w) solution of citric acid, also pre heated to 50 ℃, was cycled for 30 min. Deionized water was then cycled another 5 times.

3.1.8 Analytical Methods

High-performance liquid chromatography (HPLC) was used to determine substrate and metabolite concentrations throughout this work. The majority of samples were analyzed using the Waters - Breeze™ 2 HPLC system (Waters Corp. Milford, USA). The system is comprised of an isocratic pump (Waters model 1515), autosampler (Waters model 2707), and refractive index detector (RID) (Waters model 2414). The Hi-Plex H column and respective guard column, designed for carbohydrates, alcohols, and acids were used for analysis (Agilent, Santa Cara, USA). The analytical method operated at a flowrate of 0.8 mL/min using mobile phase consisting of 5 mM sulphuric acid. Additional method parameters included a 20 µL injection volume per sample, column temperature of 35 ℃, and RID temperature of 40 ℃. For each analysis, a standard sample containing known quantities of each analyte was used to confirm the precision of the results within 3%. All samples were centrifuged for 5 min at 13000 rpm (Spectrafuge™, Model 24D, New Jersey, USA) subsequently filtered with 0.22 µm cellulose filters (Maple Lab Systems, Mississauga, ON).

Samples obtained from co-substrate fermentation were analyzed using the Agilent 1260 infinity system (Agilent USA, Santa Clara) with a Hi-Plex H column and RID detector. The parameters for analysis were modified to allow for distinct separation of fructose and glucose peaks. The flow rate through the column was set to 0.45 mL/min and the column temperature was held at 50℃ while the detector was set to 35℃. Each sample was run for 80 minutes, with a 20µL injection volume. Sample preparation was performed as described above.

During bench-scale fermentations, pH was measured using the Hamilton EasyFerm Plus K8 325 pH probe (Reno, NV, USA). Online redox measurements were done using the

Mettler Toledo Ingold probe (Wilmington, DE, USA). For off-gas composition measurement, the BlueSens Bluevary sensor (Sensor ID: 3078 and 31068, Herten, Germany) was used. The off-gas flowrate was measured using (Aalborg, Model TIO, New York, USA). Iris Software V5 Pro was used to record all online measurements (Infors HT, Bottmingen/Basel, Switzerland). Offline dry cell weight measurements were done in duplicate using 5 mL of fermentation broth and glass microfiber filters with a particle retention of 1.5 µm (VWR, Radnor, PA, USA).

3.2 Results and Discussion

In the following section, the findings of this work are presented. MATLAB generated simulations were performed as an initial step to assessing the impact of a coupled fed-batch and pervaporation process on butanol production. Parameters related to the selectivity of the membrane were identified through preliminary experiments with the pervaporation module. The simulations supported the translation of the modelled process to an experimental scale. As a transitionary step, a batch process coupled with product removal was performed indicating that the PDMS membrane was capable of sustaining butanol selectivity with live culture. Next, the results of the first lab-scale run through of the modified fed-batch process are presented along with subsequent iterations in which parameters such as feeding rate were altered.

3.2.1 Fed-batch and Integrated Fed-batch Modelling

3.2.1.1 Determination of Flux through Membrane using PBE Fermentation Broth

In order to accurately model an integrated fermentation system with product recovery, the water, and product flux through the Pervap 4060 membrane was first determined by evaluating the pervaporation system separately from an active fermentation. This evaluation was accomplished using spent fermentation broth from *C. pasteurianum* grown on glycerol, collected from a completed batch fermentation. In this preliminary experiment, a 2-L vessel containing cell-free supernatant from settled fermentation broth was connected to the Pervap 4060 frame and plate module (Appendix 2, Appendix 3). Notably, a similar experiment was performed by Kießlich et al., where the effectiveness of the same

membrane was tested with PBE fermentation broth under different operating conditions (Kießlich et al., 2017).

In the pre-experiment, the broth was maintained at 35 °C and pumped through the membrane and back into the vessel at a volumetric flowrate of 4 L/min. Water flux was found to be independent of butanol concentration in the vessel and the average water flux over a 7-hour period was determined to be 498 $g/m^2 \cdot h$. Similar to observations made by Kießlich et al., a linear relationship was found to exist between butanol flux and the butanol concentration in the vessel with a fitted slope of 14.78 $m^{-2} \cdot h^{-1}$. It is also important to note that the permeate in this experiment contained primarily butanol and water, but some ethanol was also recovered. No other identified by-products were detected via HPLC analysis. The average separation factor (Eqn. 3-1) for butanol was estimated to be 26.6, confirming the suitability of the membrane for the intended application.

$$\beta_{butanol} = \frac{y_{butanol}/y_{water}}{x_{butanol}/x_{water}} \qquad \text{Eqn. 3-1}$$

In the calculation of the average separation factor ($\beta_{butanol}$), $y_{butanol}$ refers to the molar fraction of butanol in the permeate, y_{water} refers to the molar fraction of water in the permeate, $x_{butanol}$ refers to the molar fraction of butanol in the vessel, and x_{water} refers to the molar fraction of water in the vessel.

3.2.1.2 Monod Kinetics: Fed-Batch

The average water flux, identified from the previous experiment, was then used to model product inhibited growth with and without pervaporation under the same operating conditions.

For the simulation, Monod kinetics were used to estimate the biological growth rate and product profile. For the fed-batch simulation, a system of four differential equations were used to describe the rate of change of glycerol (Eqn. 3-2), butanol (Eqn. 3-5), biomass (Eqn. 3-7), and volume in the reactor (Eqn. 3-8).

$$\frac{dS}{dt} = \frac{Q}{V(t)}\left(S_f - S(t)\right) + r_s(t) \qquad \text{Eqn. 3-2}$$

The rate of change of glycerol, dS/dt, is dependent of on the volumetric flowrate of media into the reactor, Q, the volume of the reactor, V, the concentration of substrate in the feed, S_f, the concentration of substrate in the reactor, S, and the substrate utilization rate, r_s (Eqn. 3-3).

$$r_s = \left(\frac{-1}{Y_{xs}}\right) r_x(t) \qquad \text{Eqn. 3-3}$$

The substrate utilization rate, r_s, is linked to the biomass yield, Y_{xs}, and the rate of biomass production, r_x (Eqn. 3-4).

$$r_x = \mu_{max}\left(1 - \frac{p(t)}{p_i}\right)^n \left(\frac{S(t)}{K_s \cdot \left(1 - \frac{p(t)}{p_i}\right)^n + S(t)}\right) X(t) \qquad \text{Eqn. 3-4}$$

The biomass production rate, r_x, is linked to the maximum specific growth rate, μmax, the concentration of butanol in the reactor, p, the inhibitory concentration, p_i, the inhibition term, n, the concentration of substrate in the reactor, S, the Monod Constant, K_s, and the cell concentration in the reactor, X.

$$\frac{dP}{dt} = r_p(t) - \left(\frac{Q}{V(t)}\right) p(t) \qquad \text{Eqn. 3-5}$$

The rate of change of butanol, dP/dt, is dependent on the rate of butanol production, r_p, the volumetric flowrate of media into the reactor, Q, the volume of the reactor, V, and the concentration of butanol in the reactor, p.

$$r_p = \left(-Y_{ps}\right) r_s(t) \qquad \text{Eqn. 3-6}$$

The rate of butanol product, r_p, is linked to the butanol yield constant and the rate of substrate utilization, r_s.

$$\frac{dx}{dt} = r_x(t) - \left(\frac{Q}{V(t)}\right)X(t) \qquad \text{Eqn. 3-7}$$

The rate of change of biomass in the reactor, dx/dt, is dependent on the rate of biomass production, r_x, the volumetric flowrate of media into the reactor, Q, the volume of the reactor, V, and the cell concentration in the reactor, X.

$$\frac{dV}{dt} = Q \qquad \text{Eqn. 3-8}$$

The rate of change of volume, dV/dt, is dependent only on the volumetric flowrate of media into the reactor, Q.

3.2.1.3 Monod Kinetics: Fed-Batch with Pervaporation

For the fed-batch simulation coupled with pervaporation, the same differential equations for the rate of change of substrate and biomass apply as in the fed-batch model, but the rate of change of both volume (Eqn. 3-9) and butanol in the reactor (Eqn. 3-10) are affected.

$$\frac{dP}{dt} = r_p(t) - \left(\frac{Q}{V(t)}\right)p(t) - \frac{p(t)(M_{butanol})A}{V(t)} \qquad \text{Eqn. 3-9}$$

The rate of change of butanol, dP/dt, is dependent on the rate of butanol production, r_p, the volumetric flowrate of media into the reactor, Q, the volume of the reactor, V, the concentration of butanol in the reactor, p, the slope constant relating butanol in reactor to butanol flux, $M_{butanol}$, and area of the membrane, A.

$$\frac{dV}{dt} = Q - \frac{J_{water}}{\rho} \qquad \text{Eqn. 3-10}$$

The rate of change of volume, dV/dt, is dependent on the volumetric flowrate of media into the reactor, Q, the average water flux, J_{water}, and the density of water, ρ.

3.2.1.4 Simulations

The system of differential equations for both fed-batch and modified fed-batch with pervaporation processes was solved numerically using an adaptive $2^{nd}/3^{rd}$ order Runge Kutta algorithm (ode23, Mathworks, Matlab 2021b - Figure 4, Figure 5).

For this work, the kinetic constants, maximum specific growth rate, μ_{max} ($0.171h^{-1}$), and the Monod constant, K_s (0.243 g/L), were obtained from unpublished continuous fermentation studies with *C. pasteurianum* completed by Erin Johnston (2017). It should be noted that a mutant strain of DSM 525 was used to evaluate these kinetic constants, which highlights a limitation of the simulation. Yield constants, Y_{xs} (0.07 g/g), and Y_{ps} (0.26 g/g) were calculated as an average from a batch fermentation. A moderate estimate of the butanol inhibition concentration was selected to be above 14 g/L, as predicted from preliminary batch fermentations with DSM 525 which showed a maximum butanol concentration of 13 g/ L could be achieved before growth and product formation slowed. The inhibition term, n, for biomass production was estimated to be 1, for simplicity.

Both simulations in Figure 4 and Figure 5 were made with an initial glycerol concentration of 20 g/L and a feeding rate of 7 g/h of glycerol. Under fed-batch operation, the effects of butanol inhibition are predicted to begin after 30 hours, beyond which the rate of substrate utilization decreases and cell growth slows. However, in the simulation incorporating product removal, the fermentation can be operated for the full 100 hours without reaching inhibitory butanol concentrations. This predicted ability to overcome inhibition translates to an overall volumetric productivity of 0.31 g/L·h with pervaporation compared to 0.13 g/L·h in unmodified fed-batch over 100 hours. Further, 172 g of butanol in total is expected to be produced with product removal versus 81 g without. These differences provide motivation to extend the simulations to bench-top fermentation studies to evaluate the validity of the assumptions, and how external influences may change the expected outcome. For example, both models fail to account for differences in the kinetic constants between both bacterial strains, operational limitations such as the maximum allowable volume in the reactor, the delayed start of the pervaporation unit, the accumulation of intermediates and their potential effects on the cell metabolism, as well as potential fouling of the membrane and decline of membrane efficiency over time. As such, the predictions

may carry a wide degree of error and it is necessary to perform the experiment to scale to better understand whether *in-situ* product removal truly provides a benefit to the fermentation.

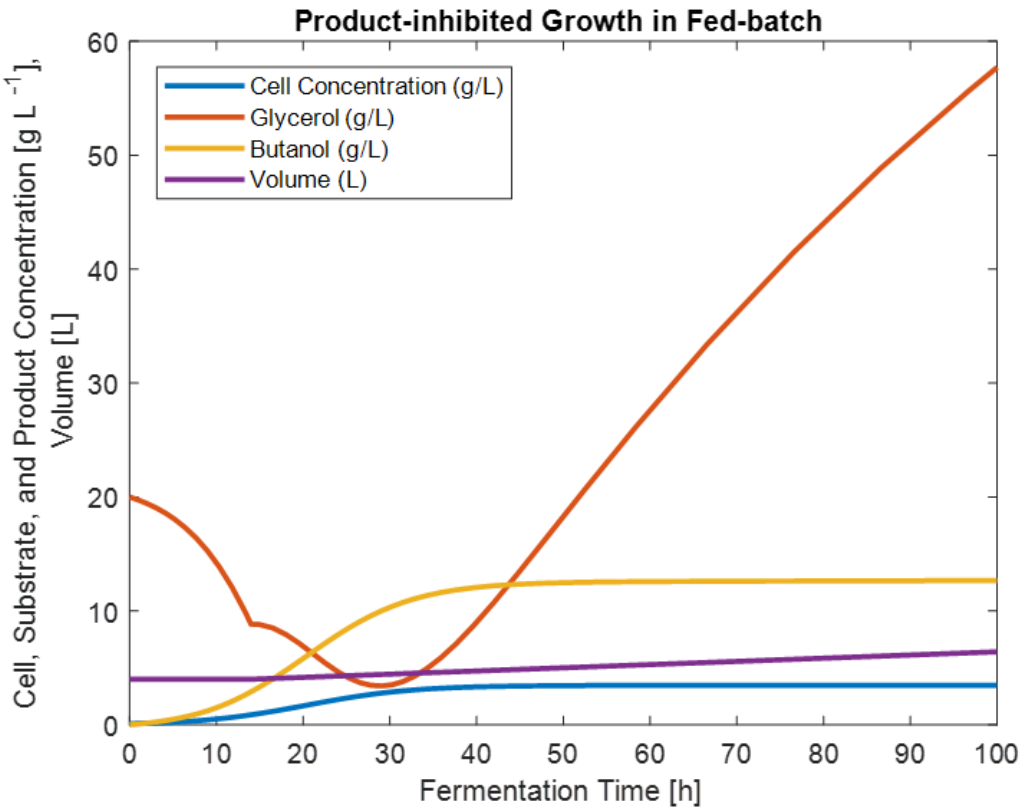

Figure 4: MATLAB generated simulation of fed-batch fermentation without pervaporation; Initial glycerol concentration (S_0) = 20 g/L; Initial volume in reactor (V) = 4 L; Initial glycerol concentration in feed (S_f) = 250 g/L; Time to fed-batch (t_f)= 14 h; Volumetric flowrate into reactor (Q) = 0.028 L/h; Kinetic Constants: μ_{max}= 0.171 h^{-1}, K_s= 0.243 g/L; Initial Cell concentration (X_o) = 0.1 g/L; Estimated Yield Constants: Y_{xs}= 0.07 g/g, Y_{ps}= 0.26 g/g; Inhibition Concentration (p_i)= 14 g/L; Inhibition Term (n)= 1

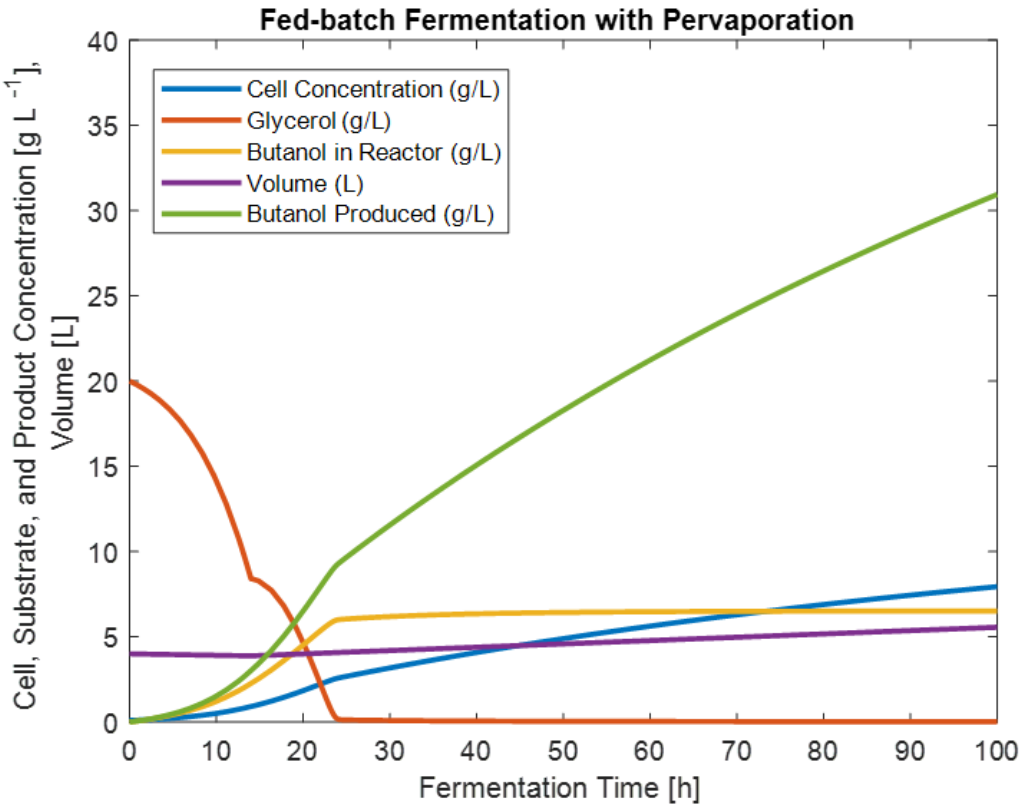

Figure 5: MATLAB generated simulation of fed-batch fermentation with pervaporation; Initial glycerol concentration (S_0) = 20 g/L; Initial volume in reactor (V) = 4 L; Initial glycerol concentration in feed (S_f) = 250 g/L; Time to fed-batch (t_f)= 14 h; Volumetric flowrate into reactor (Q) = 0.028 L/h; Kinetic Constants: μ_{max}= 0.171 h^{-1}, K_s= 0.243 g/L; Initial Cell concentration (X_o) = 0.1 g/L; Estimated Yield Constants: Y_{xs}= 0.07 g/g, Y_{ps}= 0.26 g/g; Inhibition Concentration (p_i)= 14 g/L; Inhibition Term (n)= 1; Water Flux= 498 $g/m^2 \cdot h$; Slope of Butanol Flux/ Butanol Concentration in Reactor=14.78 $m^{-2} \cdot h^{-1}$

3.2.2 Batch Fermentation Coupled with *in-situ* Pervaporation

3.2.2.1 Investigation of Flowrate Setting and Batch Performance

In a pre-experiment isolating the effects of shear or other disturbance by the peristatic pump on a *C. pasteurianum* culture, two consecutive batch fermentations were performed at 4 L/min, and 2 L/min without incorporation of the pervaporation unit (Appendix 4, Appendix 5). At the higher flowrate, an immediate and adverse effect to gas production, substrate utilization and butanol production was evident with the start of the pump. Once the flowrate was reduced to zero, the culture appeared to recover with gas production increasing, redox potential stabilizing and a higher rate of product formation. However, at 2 L/min, despite a brief decline in the same parameters, the culture recovered without any reduction in flowrate.

It should be noted that at 2 L/min, a two-phase flow was observed, referring to large nitrogen gas bubbles that were present throughout the tubing. These gas bubbles were smaller and distributed throughout the broth at 4 L/min. The higher flowrate also created greater turbulence at the liquid return standpipe. This change in flow pattern could have had a significant impact on the sheer stress and pressure imposed on the cells in the system and may help to explain why the lower flowrate had a better outcome. However, gas distribution and turbidity were not factors evaluated separately in this study, so their true impact cannot be concluded.

As a result of this preliminary test, a flowrate of 2 L/min was selected for subsequent experiments in attempt to select the best operating parameters for cell survival.

3.2.2.2 Batch Fermentation with *in-situ* Product Removal

As a succeeding step to assess the removal capacity of the membrane itself using a live culture, a batch fermentation was performed with simultaneous butanol removal (Figure 6). The reactor was supplied with 20 g/L glycerol with no additional feeding. The fermentation was run for a total of 24 hours, with product removal beginning at 14.5 hours.

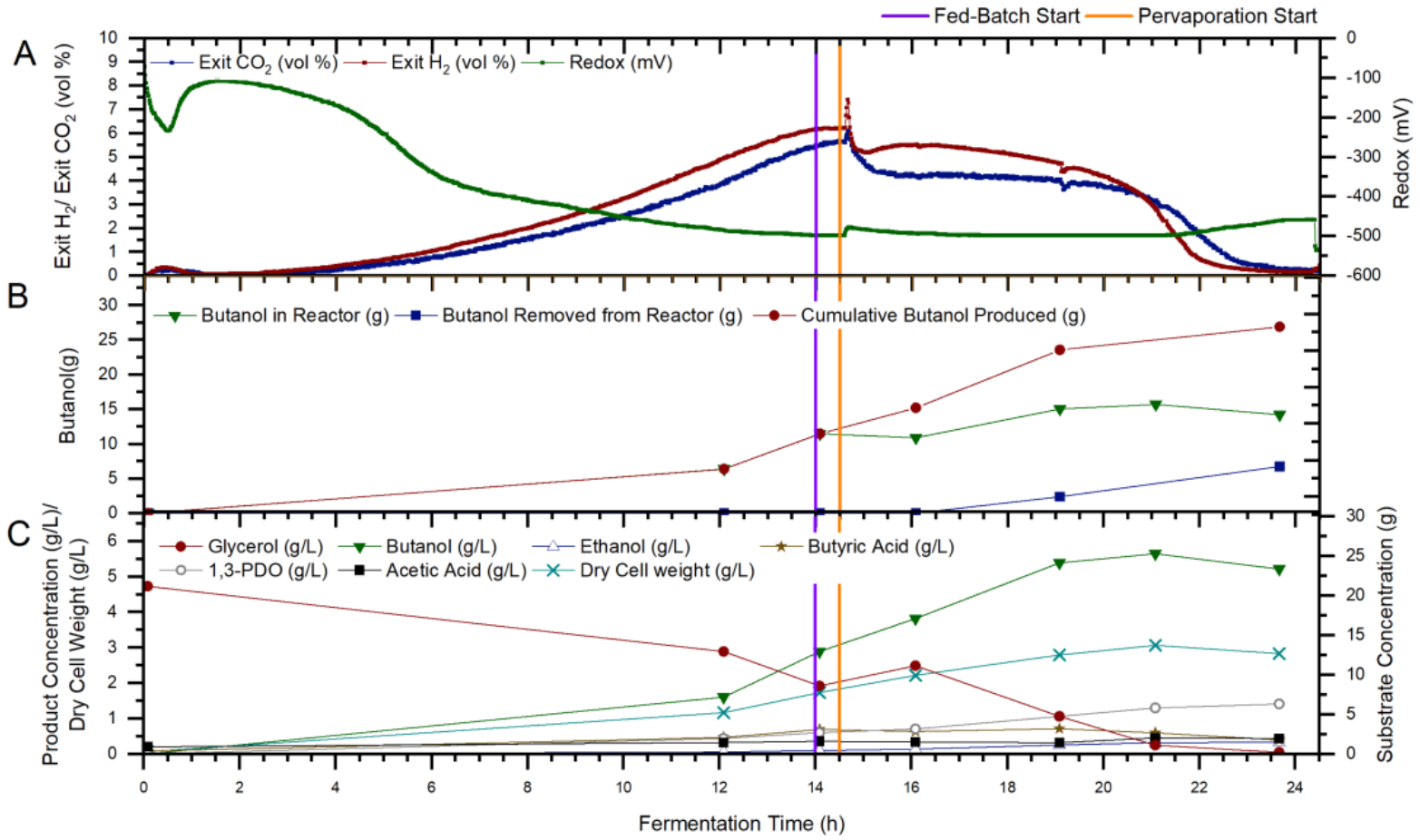

Figure 6: Overview of a batch glycerol fermentation integrated with pervaporation; 35 ℃, pH=5.0, pump flowrate = 2 L/min; **A:** Online data-gas production and redox potential, **B:** Butanol profile in total grams produced, removed and in reactor, **C:** Substrate and product concentrations in reactor

Similar to observations made without the pervaporation module, the initiation of the peristaltic pump had an immediate effect on online gas production and redox signal. Redox potential spiked from a stable reading of- 498 mV to a maximum of -478 mV in under 5 minutes. The signal preceded to return to -498 mV after 3.5 hours. This fluctuation in redox value can indicate a variety of events occurring in the reactor. One possible explanation could be the introduction of small amounts of oxygen into the system due to the residual air remaining in the tubing or the suction pressure formed at the reactor outlet allowing atmospheric air to enter the system. The latter is a less likely scenario as it would not be expected that the redox returns to the baseline value in the case that air was continuously being entrained. This theory could not be supported using the off-gas flow meter due to the alternating flow pattern of exit gas through the detector, characteristic of the peristatic pump, as well as the sensitivity of the flow meter. Alternatively, a small decline of the temperature in the fermentation broth as well turbidity created by the return standpipe may influence the signal from the redox probe.

In terms of gas production, off-gas CO_2 and H_2 readings spiked in accordance with the onset of the pump and did not stabilize to previous levels but remained steady between 4 to 5 vol % until substrate became limited. The sudden increase and subsequent decline in gas production could be a result of a sudden influx of gas through the analyzer as opposed to a reflection of true gas production from the culture. The tubing connecting the pervaporation unit to the reactor was previously subjected to atmospheric air which was pulled through the analyzer, creating a misleading trend. Despite having slowed gas production after the initial spike, dry cell weight continued to increase along with butanol production indicating that the modifications to the batch fermentation were not creating an undesirable or intolerable environment for the cells. The steady trend of gas production from 15 to 21 hours and correspondingly relatively stable levels of butanol in the reactor are also indicative of some extent of metabolic regulation in the reactor.

In this case, product inhibition was not encountered, as the butanol titer in the reactor reached a maximum of 5.6 g/L, well below the known inhibitory concentration. The total amount of butanol produced was restricted to 26.8 g due to substrate limitation, with

complete glycerol depletion after approximately 24 hours. Overall butanol productivity in this experiment was 0.29 g/L·h comparable to another batch fermentation without pervaporation (0.29 g/L·h) with higher substrate concentrations of 50 g/L.

Throughout the experiment, there was no indication of membrane fouling or reduced performance in butanol separation. In fact, from 14.5 hours to 19 hours through the fermentation the butanol flux was measured to be 28.3 g/h·m^2 and increased to 55.9 g/h·m^2 over the next 5 hours. The water flux was stable at an average of 448 g/h·m^2 suggesting the impact of live culture on the membrane is minimal.

3.2.3 Fed-batch Fermentation Coupled with *in-situ* Pervaporation

To sustain a longer, more productive fermentation, an experiment with continuous substrate and nutrient supplementation was performed (Figure 7). The reactor was supplied with 15 g/h of glycerol, beginning at 14 hours. The initial glycerol concentration was kept at 20 g/L to remain comparable to previous experiments. Pervaporation was initiated at 14.5 hours.

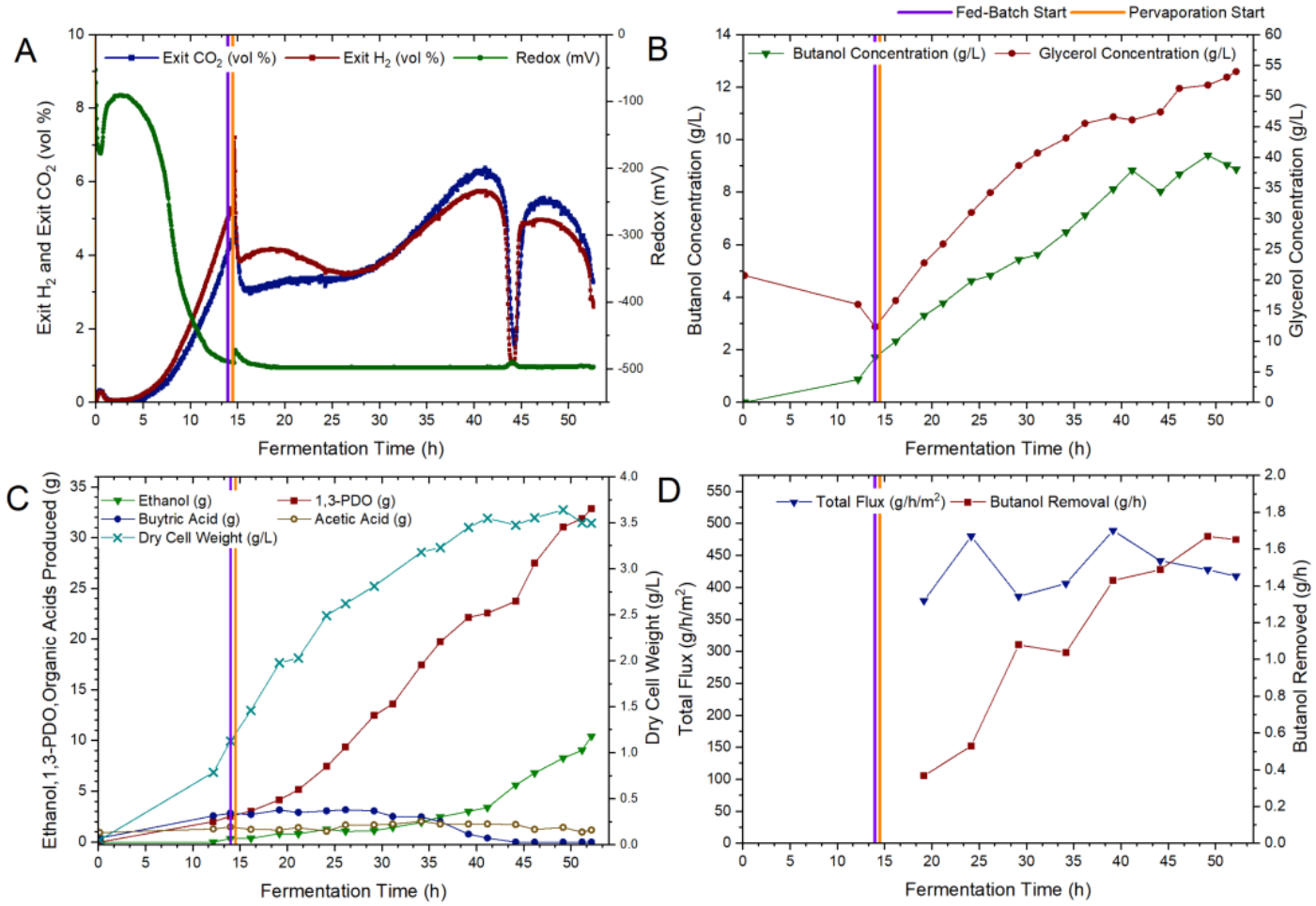

Figure 7: Overview of fed-batch glycerol fermentation integrated with pervaporation; 35 ℃, pH=5.0, glycerol feeding rate = 15 g/h, pump flowrate = 2 L/min; **A:** Online data-gas production and redox potential, **B:** Concentrations of substrate and butanol in the reactor (g/L), **C:** Total mass of by-products and dry cell weight (g/L) in reactor **D:** Flux through Pervap 4060 membrane and rate of butanol removal

An expected shift in redox potential and gas production occurred with the onset of the pump. There was an increase in the oxidative capacity in the bioreactor in the magnitude of 15 mV, however metabolic processes recovered quickly rebalancing the redox level within 1.5 hours. In terms of off-gas data, H_2 production dominated over the formation of CO_2 for the first 30 hours of fermentation, after which the trend was reversed. This result is contrary to the proposed metabolic breakdown of glycerol in *C. pasteurianum* reported in literature, which suggests that CO_2 and H_2 are generated in the same step converting pyruvate to acetyl-CoA. This suggests that the current pathway in literature is incomplete and/or that the specific enzymes that facilitate the production of gases are regulated differently under conditions of stress.

Beginning at approximately 43 hours, there is a noticeable decline and resurgence of gas production occurring over a period of 3 hours. Concurrently, there is also a decline in overall exit gas flow from an average of 0.6 L/min to 0.55 L/min (data not shown). This trend aligns with an increase in redox potential, however, there is no measured trend change in the production of solvents during this time period. With manual sampling accompanied by a change of the cold traps at 44 hours, initial reasoning for the shift was attributed to operational errors. This initial theory was made as the switching of cold traps coincided with gas production reaching it's minima. The rationale being that in the cold trap that was operated from 39 to 44 hours, frozen permeate could have accumulated in the tubing and created a blockage, preventing the pervaporation unit from maintaining a full vacuum. However, taking a closer look at the data, the butanol flux continued to trend upwards during this time, suggesting that the initial reasoning was incorrect as membrane performance was not impacted as originally suspected. It is, therefore, more likely that this observation is due to a shift in metabolic activity. It is unclear what events triggered this change.

In this experiment, there was a total of 94.3 g butanol produced with an overall volumetric butanol productivity of 0.31 g/L·h. Compared to a batch fermentation in which 53 g of butanol was produced over 36 hours, the addition of pervaporation was able to extend the productive time of the culture to yield nearly double the amount of butanol.

From the glycerol concentration over time, it is evident that the system was supplied with more carbon than required. Substrate accumulated in the reactor in a mainly linear fashion with approximately 7.4 g/h of glycerol remaining unconsumed. As such, the rate of butanol production was greater than that of removal. This imbalance created a circumstance in which butanol concentration increased over time in the reactor. This trend is undesirable as near inhibitory concentrations of 9 g/L were eventually reached after approximately 48 hours. This factor may contribute to the subsequent decline in gas production and biomass formation, ending the fermentation.

In order to address the imbalance without compromising the experimental set-up or altering the pervaporation unit, butanol production was limited by slowing cell growth through substrate limitation. In a follow-up experiment, the feeding rate of glycerol was adjusted to 7 g/h by lowering the total feed flowrate to 0.028 L/h and maintaining the same glycerol concentration in the feed bottle of 250 g/L (Figure 8).

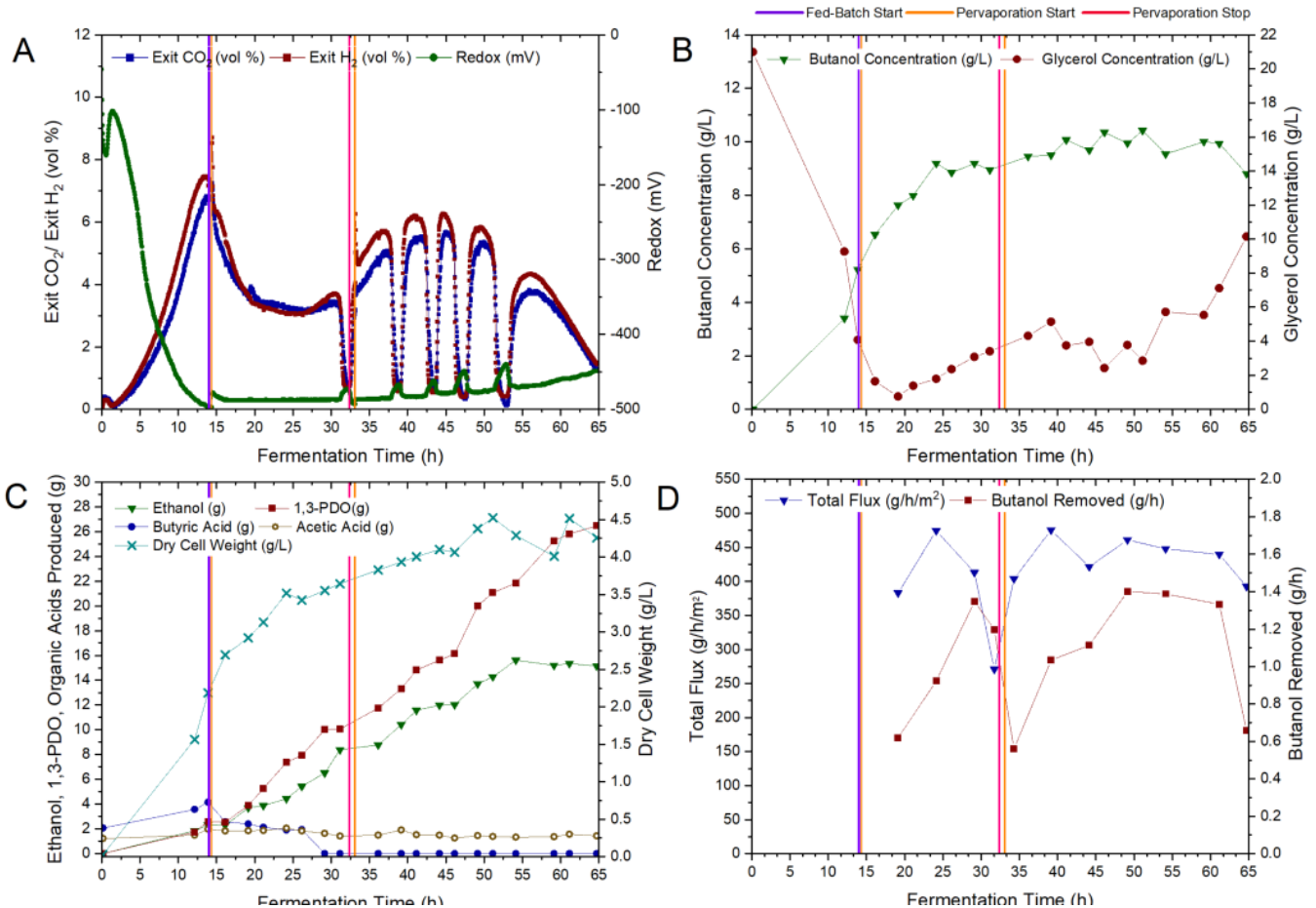

Figure 8: Overview of fed-batch glycerol fermentation integrated with pervaporation; 35 ℃, pH=5.0, glycerol feeding rate = 7 g/h, pump flowrate = 2 L/min; **A:** Online data-gas production and redox potential, **B:** Concentrations of substrate and butanol in the reactor (g/L), **C:** Total mass of by-products and dry cell weight (g/L) in reactor **D:** Flux through Pervap 4060 membrane and rate of butanol removal

With a lower supplementation rate, the culture began to decline prior to the start of fed-batch operation at 14 hours, at which point glycerol concentration in the reactor was measured to be 4 g/L. Evidence of this decline is seen in the slowing of gas production, which continued beyond the initiation of the peristaltic pump at 14.25 hours. From 20 to 30 hours of fermentation, gas production began to plateau as butanol and biomass production continued to increase.

At 32 hours, a sudden decline in gas production and surge in redox potential was observed. To assess whether the cause of the initial downward trend in gas production was influenced by the pervaporation unit, the flowrate through the peristatic pump was reduced to zero at 32.4 hours and turned back on again at 33.1 hours. Apart from an initial spike in gas production with the restart of the pump, there was no other measured impact of the pump.

Further, between 30 to 54 hours, the cells exhibited an oscillatory behavior, alternating between periods of low and high gas production and substrate utilization. Data obtained from the off-gas flowmeter showed fluctuations between 0.53 L/min to 0.61 L/min (data not shown). The period between oscillations varied between approximately 4 to 7 hours. Oscillations, in the reverse direction, were seen in redox potential with periods of low gas production coinciding with periods of higher redox potential. The peaks in redox balance increased with each cycle, reaching a maximum of -440 mV and returning to -478 mV.

In continuous studies, oscillatory metabolic regulation in *C. pasteurianum* has been previously reported. Gallazzi et. al was one of the first groups to report periodic oscillations with varying dilution rates of suspended cells. These oscillations were described in terms of cell density, butanol, glycerol, and butyric acid concentrations. The cycles lasted approximately 75 h and no correlation was found between cell sporulation and the observed phenomenon. It was hypothesized that butanol toxicity played a role in the unsteady state conditions (Gallazzi et al., 2015). Oscillatory patterns in cell growth, solvent yield and organic acid production in other *Clostridium* species such as *C. acetobutylicum* have also been observed, however, it was postulated that sporulation of the cells was the underlying mechanism for the shift in metabolism in addition to fluctuating proportions of acid and solvent producing *Clostridia* (Clarke et al., 1988). More recently, Johnson et. al.,

investigated the oscillatory metabolism in *C. pasteurianum*, evaluating the effects of varying fermentation parameters. In this study, feed glycerol concentration, pH, dilution rate and temperature were found to be significant factors affecting frequency of the oscillations. In this case, the regulation of cofactors, NAD and NADH, was suspected to be a key contributor to the resultant oscillatory behavior, an indicator of the redirection of the metabolic pathways (E. E. Johnson & Rehmann, 2020).

In the modified fed-batch reactor with pervaporation, there were many similarities to continuous culture such as consistent removal of liquid and the addition of fresh media. Despite these similarities, the tested experiment did not operate at a constant volume and consequently allowed for the accumulation of by-products, particularly 1,3-PDO. In a continuous operation, the concentration of by-products is less likely to accumulate as there is a constant volume of fermentation broth leaving the system, unlike the pervaporation-focused bioprocess. At the three dilution rates ($0.066h^{-1}$, $0.080h^{-1}$, $0.092h^{-1}$,) tested by Johnston et. al., oscillations persisted in all scenarios (E. E. Johnson & Rehmann, 2020). In this experiment however, the dilution rate was not constant, changing with volume. Initially, the calculated dilution rate was approximately $0.0070h^{-1}$, ending at $0.0058h^{-1}$.

To the authour's knowledge, this is the first report highlighting the oscillatory mechanism in *C. pasteurianum* in a non-continuous fermentation.

Butanol concentration in the reactor was maintained between 9 to 10 g/L from 40 to 60 hours. The fermentation then showed a slow decline beginning at 54 hours, when the butanol concentration was 9.5 g/L.

In this experiment, 101 g of butanol was produced in a period of 64 hours while butanol productivity was determined to be 0.32 g/L·h. The maximum dry cell weight reached 4.5 g/L, compared to 3.3 g/L achieved in the batch fermentation control.

As seen in Figure 9, the experimental concentrations of butanol align well with those simulated. Notably, butanol removal was overestimated, requiring adjustments to the model. Estimates of substrate utilization, however, did not align as expected with the trends seen in the experimental results. In practice, glycerol was consumed at a faster rate initially

in batch mode, and slower during the modified set-up. Overall, the simulations proved to be a valuable tool in predicting the outcome of the process, within a margin of error. Further iterations and a thorough model validation would need to be implemented to obtain a more precise predictor of the process.

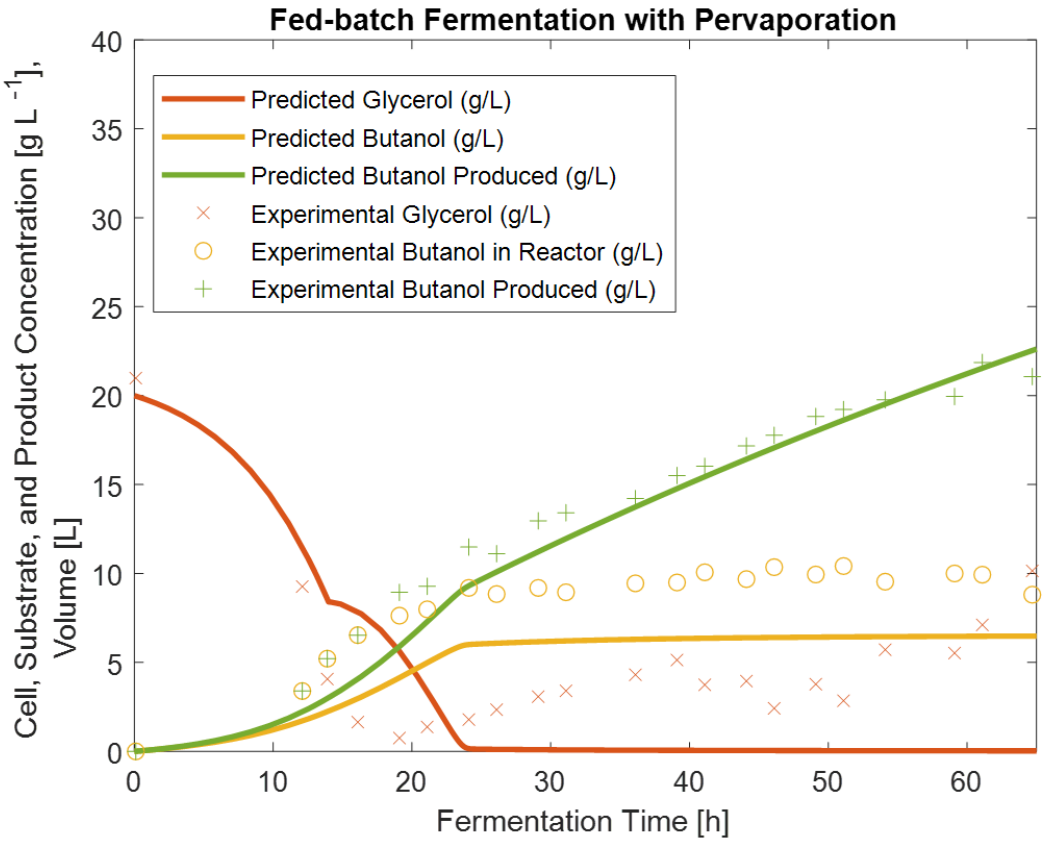

Figure 9: Comparison of experimental results and simulated results in a fed-batch fermentation with incorporated pervaporation

To evaluate the performance of the new configuration, the total amount of butanol produced was compared to controls in batch and fed-batch operation (Figure 10). The outcomes of these experiments are further summarized in Table 5. Although volumetric butanol productivity allows for consistent comparison between process configurations, both overall butanol produced and fermentation time must be considered to gain a comprehensive understanding of the impact of the new operation.

Relative to the controls, the fermentation with the modified process was able to successfully run for 19.5 hours longer than fed-batch and 28.5 hours compared to batch.

This extended fermentation time allowed for greater butanol production, 59 g more than fed-batch, a 2.4-fold increase, and 48 g more than batch, 1.9-fold increase. Although there was a slight improvement in volumetric butanol productivity, there was only a difference of 0.03 g/L·h, likely influenced by periods of reduced product formation during the oscillation period.

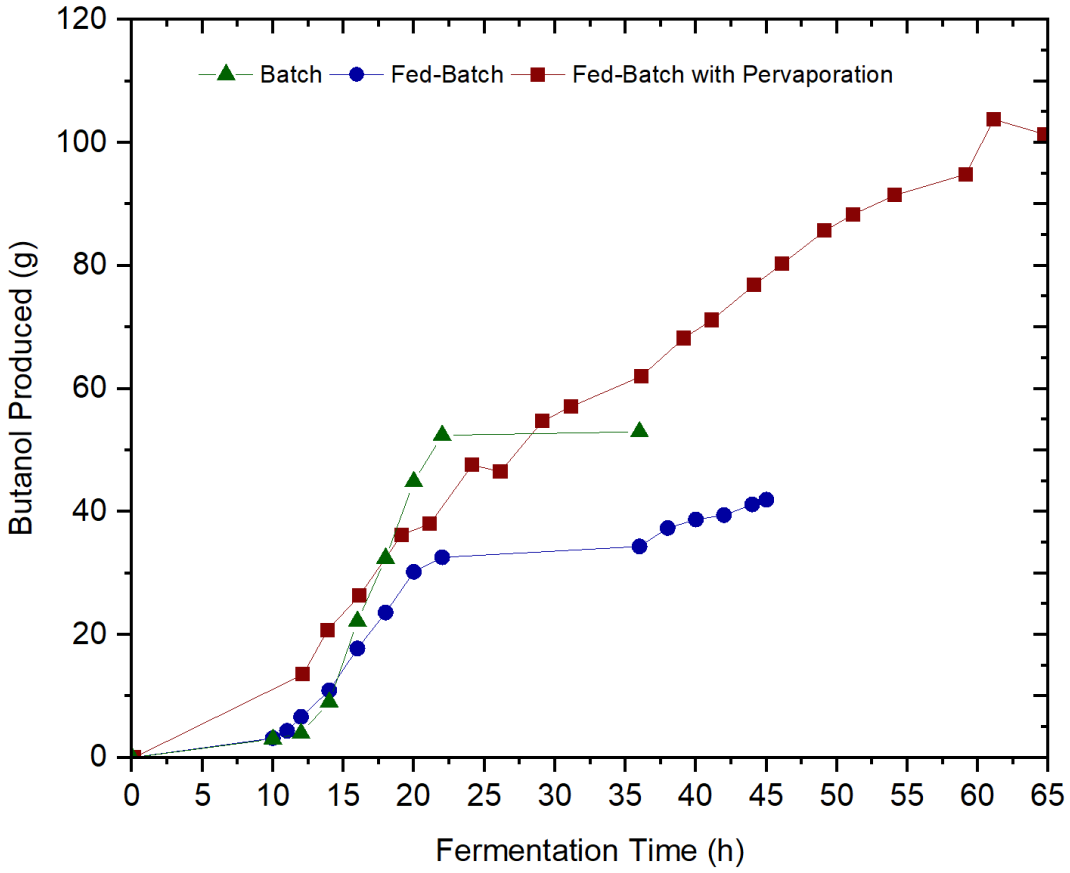

Figure 10: Comparison of butanol production in various operational configurations: batch, fed-batch and fed-batch with pervaporation

Table 5: Summary of outcomes from batch, fed-batch, and modified fed-batch glycerol fermentation configurations

Mode of Operation	**Total Fermentation Time (h)**	**Initial Glycerol Concentration (g/L)**	**Maximum Dry Cell Weight** (g/L)	**Glycerol Feeding Rate (g/h)**	**Total Butanol Produced (g)**	**Overall Volumetric Butanol Productivity (g/L·h)**	**Overall Butanol Yield ($g_{butanol}$/ $g_{substrate}$)**
Batch	36	50	3.3	N/Ap	53	0.29	0.26
Fed-Batch	45	20	3.5	15	42	0.20	0.21
Fed-Batch with Pervaporation	64.5	20	4.5	7	101	0.32	0.25

3.2.4 Co-substrate Fed-Batch Fermentation with *in-situ* Pervaporation

Further exploring how the modified fed-batch system can be used to enhance butanol productivity, which based on the previous experiment, was in-part limited by instability in cell metabolism, led to the introduction of additional substrates (Figure 11). With the goal to overcome the oscillatory cellular response, the system was supplied with other carbon sources. Further, co-substrate fermentation with sugar has repeatedly been shown to increase butanol selectivity through the conversion of saccharides to organic acids and subsequently to butanol (Munch et al., 2020; Sarchami & Rehmann, 2019).

Based on their high prevalence in low-cost biomass feedstocks such as molasses, Jerusalem artichokes, and other lignocellulosic sources, glucose and fructose were selected to demonstrate the effect of the simultaneous consumption of simple sugars and glycerol. Ratios were chosen based on two studies that investigated and optimized ratios of molasses and Jerusalem artichoke hydrolysate in batch processes (Munch et al., 2020; Sarchami & Rehmann, 2019). As such, the experiment was performed with a substrate composition of approximately 7% fructose, 21% glucose and 71% glycerol. The fructose to glucose ratio was modified from optimal conditions suggested in the work by Munch et. al. to accommodate for limited fructose availability. The same carbon molar equivalent as used in previous experiments was applied for this multi-substrate system.

The reactor was supplied with concentrated media starting from 12.4 hours post-inoculation at a rate of 0.028 L/h. Pervaporation was initiated at 14 hours and operated continuously for the duration of the fermentation, which was ended prematurely at 79 hours. Constraints imposed by the volume of the reactor influenced the decision to end the fermentation before the culture showed clear signs of decline in solvent production, and substrate utilization.

Although, there were no samples collected from 0 to 10 hours when the fermentation was operated in batch mode, a clear drop in gas production and increase in redox potential is evident between 4.7 and 5 hours followed by a larger drop starting at 7.2 hours. These sudden dips in the online data are likely a result of complete consumption of fructose

followed by depletion of glucose. This theory is supported by the fact that at 12 hours, neither sugar could be detected in the reactor. Further there is a clear trend change in H_2 production compared to CO_2. Sabra et. al. reported that ferredoxin independent hydrogenase enzymes were downregulated upon glucose limitation in *C. pasteurianum* resulting in a greater ratio of CO_2 to H_2 production. A similar trend seen by Munch et. al in a dual substrate experiment, showed an initial decline in off-gas aligning with depletion of fructose and a second in glucose. There was also a clear deviation of H_2 production seen in this study following the consumption of the sugar substrates (Munch et al., 2020).

A lag phase, in which biomass production plateaued, gas generation slowed, and glycerol was accumulated, occurred after the start of pervaporation and continued until the 42-hour mark. Beyond this time point, the culture appeared to recover, with both sugars fully consumed by 59 hours, accompanied by a sharp decrease in gas production. During this stage both CO_2 and H_2 production were synchronized, however, once the environment became sugar limited, H_2 production remained steady while CO_2 continued to increase. After 73 hours, a maxima was reached, after which a steady decline in both CO_2 and H_2 was observed.

The substrate profile in the reactor suggested that although sugar was the preferred substrate over glycerol, glucose consumption slowed from 39 to 54 hours while no fructose was accumulated in the reactor, indicating a preference for fructose over glucose. Further, glycerol consumption during this period was reduced to an average of 1 g/L which continued to increase reaching a consumption rate of 7.5 g/h towards the end of the fermentation (from 76 to 79 hours).

There was a noticeable increase in butanol production in the mixed substrate experiment relative to when glycerol was the sole substrate. Over 79 hours, the total butanol produced reached 128 g, having an overall productivity of 0.31 g/L·h. Comparatively, 27 g more of butanol was achieved over an extended 14.5 hours. There was ultimately greater conversion to butanol with additional substrate compared in terms of overall butanol yield which was calculated to be 0.29 ($g_{butanol}/ g_{glucose + fructose +glycerol}$). The production of 1,3-PDO however was limited under the parameters, with an overall yield of 0.015 ($g_{butanol}/ g$

$_{glycerol}$) versus 0.066 (g $_{butanol}$/ g $_{glucose + fructose + glycerol}$) when there were no sugars present. The rate of 1,3-PDO formation decreased beyond 30 hours of fermentation.

Redox potential fluctuated throughout the experiment, increasing during times of slow substrate utilization but stabilizing briefly from 52 to 76 hours at -439 mV. This balance in redox is different from a system running only with glycerol, however, it is expected that having multiple substrates with varying degrees of reduction would influence this parameter.

Another notable observation is that of diffusion of butyric acid across the membrane. Butyric acid was not recovered in the permeate when glycerol was used as the sole substrate. The increased concentration of butyric acid directed by the metabolism of the sugars, allowed a total of 0.5 g of butyric acid to be collected as permeate. This is not desirable as this fraction of butyric acid cannot be converted to its desired form, butanol.

Otherwise, the total flux through the membrane aligned with glycerol utilization, with a declining trend observed when glycerol was not being efficiently consumed and an increase during periods of high butanol production and concentration in the reactor. Interestingly, the final butanol concentration was above expected inhibitory values, 12.9 g/L. A contributing factor could be the reduced flux between 44 and 49 hours as well as 69 to 74 hours. Qualitative observations made during this period suggest that frozen permeate may have created a blockage at the cold trap inlet, preventing the accumulation of water, ethanol and butanol.

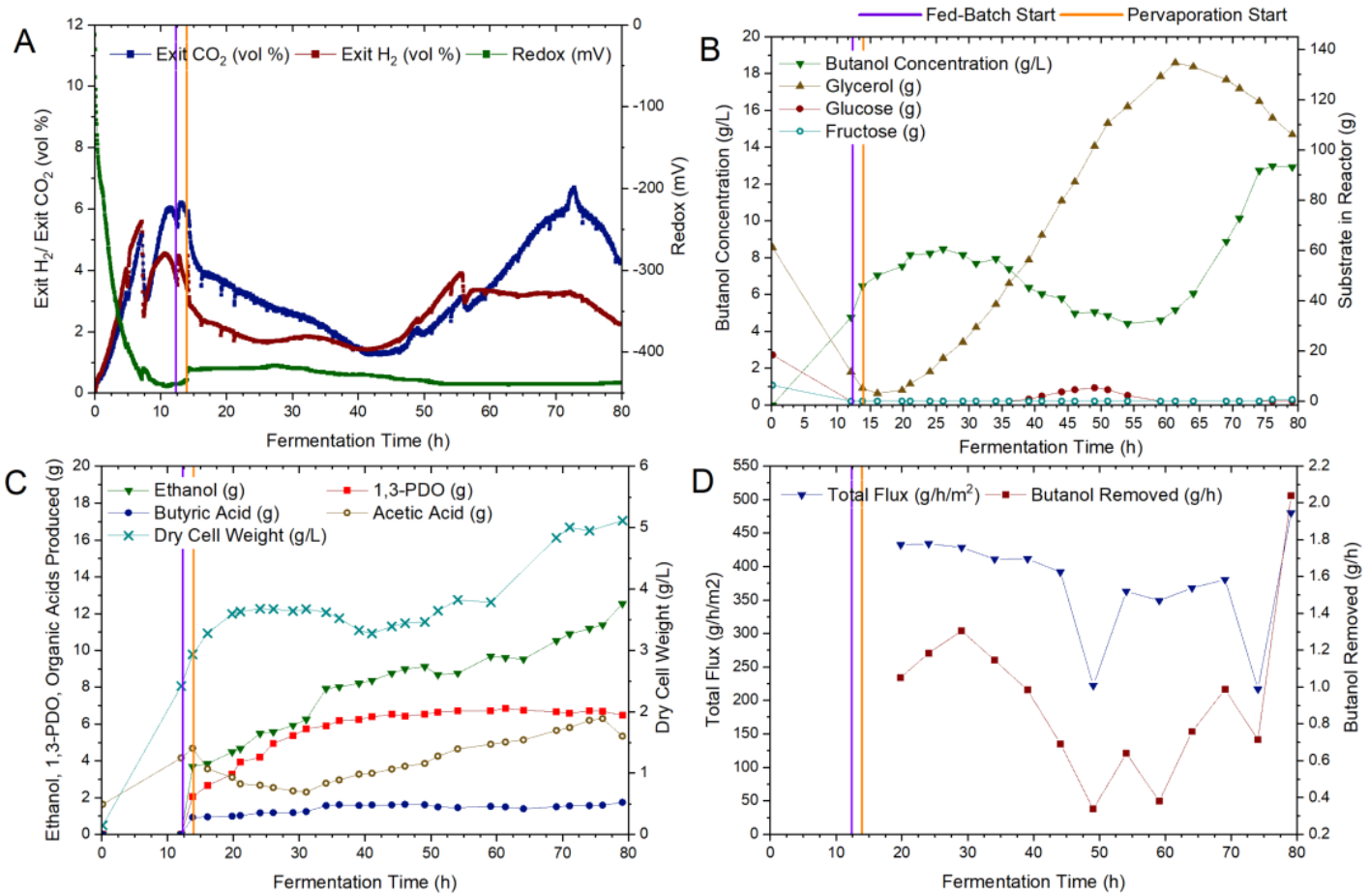

Figure 11: Overview of fed-batch co-substrate fermentation integrated with pervaporation; 35 ℃, pH=5.0, glycerol feeding rate = 5 g/h, glucose feeding rate = 1.5 g/h, fructose feeding rate = 0.5 g/h, pump flowrate = 2 L/min; **A:** Online data-gas production and redox potential, **B:** Concentrations of butanol and total mass of substrate in the reactor, **C:** Total mass of by-products and dry cell weight (g/L) in reactor **D:** Flux through Pervap 4060 membrane and rate of butanol removal

Chapter 4

4 Conclusions and Recommendations

4.1 Conclusions

With the fundamental goal of advancing research in the field of biobutanol production, this study was designed to provide insight into glycerol fermentation with *C. pasteurianum* operated in a semi-continuous bioprocess with *in-situ* product removal.

During a preliminary investigation of a batch fermentation with a simple external loop, the flowrate of the broth removed from and returning to the vessel was determined to be a significant factor in cell survival. In fact, at a flowrate of 4 L/min, the culture showed immediate signs of decline while at a lower flowrate of 2 L/min the cells were able to adjust to the turbulent conditions and ultimately thrive. The reason for this discrepancy is not fully understood but its is theorized to be attributed a difference in gas distribution, turbulence, and/or shear stresses imposed on the cells at greater speeds. This explanation has yet to be confirmed.

In the following study, batch fermentation was coupled with the pervaporation unit to evaluate the removal capacity of the membrane with a live culture. The outcome of this experiment showed reliable membrane performance over the duration of the fermentation. As expected, trends in butanol flux correlated with the concentration of butanol in the reactor and there was no evidence suggesting membrane fouling or cell cake formation.

The main body of this work evaluated the fully integrated fed-batch system with butanol removal. Relative to batch operation, productive fermentation time was enhanced, but there was no significant improvement to overall volumetric butanol productivity as postulated. Under conditions of excess substrate supplementation, butanol production exceeded the capacity of removal resulting in accumulation of product thereby creating a possibly toxic environment within the reactor. To compensate, in the following experiment the feeding rate was adjusted to meet the limitations of the membrane, restricting the rate of cell growth as well as product formation. In this scenario, butanol concentrations did not reach inhibitory concentrations however the culture experienced metabolic fluctuations. It is

unclear what may have triggered this cellular response to environmental conditions, but the presence of these oscillations is undesirable for efficient process control.

As predicted, the modified reactor achieved a higher cell density compared to batch operation. This result suggests a future opportunity to utilize the modified system as a tool to study cell dense systems, which can be difficult to achieve at a lab-scale, typically requiring ultrafiltration membranes for cell-recycling or centrifugation.

Simulations that were generated to model the integrated system using Monod kinetics were a useful predictor of experimental outcomes in terms of cell density and overall butanol yield. However, the models were poor at estimating trends in substrate utilization and over-represented the butanol flux exiting in the permeate. To make the models more robust, modifications of kinetic constants, and a refined parameter for butanol flux are required.

To better direct the product profile to butanol, co-substrate fermentation with glucose, fructose, and glycerol was performed using the fed-batch system with pervaporation. Under the tested conditions, butanol selectivity did improve, and no oscillations were observed. The operation was able to run almost twice as long compared with batch fermentation. Despite this success, an imbalance in butanol removal and production justifies further parameter adjustment.

The significance of this work highlights the advantages of *in-situ* butanol removal, within a semi-continuous system, of which findings can be further used in the development of an economical viable commercial processes for glycerol fermentation.

4.2 Recommendations

To further expand on the scope of this work and obtain a comprehensive understanding of how *in-situ* product removal can play a role in the future commercialization of biobutanol production, small-scale research is still needed.

In the current work, the methodology followed an iterative approach to process design, though statistical analysis of the results was not employed. To have a more representative data set, fermentation runs in both controls and experimental groups could be done in duplicate or triplicate. This strategy would create tolerance for variations caused by operational issues, or possible differences in inoculum cell density. Ensuring that the data is reproducible and consistent strengthens the conclusions that can be drawn from the results.

Another addition would be to include more direct controls for contamination detection within the design of the experiments. One way to ensure the culture is not supporting the growth of other organisms would be to plate samples of the culture, incubate and evaluate colony growth visually under a simple compound microscope. This extension of work could include differential staining methods such as gram staining as an inexpensive tool to uncover the presence of certain types of bacteria. Alternatively, more advanced methods have emerged to verify potential contamination including fluorescent microscopy detection, PCR, and gel electrophoresis methods. However, these strategies may be costly and add unnecessary complexity to the process.

Initial work suggested that the flowrate of fermentation broth through the peristaltic pump influenced cell viability, yet the reason for this correlation was not explored. A separate investigation into the fluid dynamics of the system may yield information as to what pressures and shear stresses the cells are subjected to and how gas is distributed within the system, factors that may influence the ability of the cells to survive. This knowledge could then be used to improve the overall design of the process, locating areas of poor mixing, or high turbidity and thereby potentially improving cell growth within this modified operation. Further, as membrane performance is known to be dependent on flowrate, being able to limit undesired environmental stresses on the culture through further process design

while also operating the system at a higher flowrate, would also likely enhance butanol removal.

In tandem with better defining fluid flow within the system, a worth-while step and simple experiment to explore may be to adjust the height of the sparger to minimize product stripping as well as to better control the formation of nitrogen gas pockets within the tubing.

To complement this work, a population viability assay should be performed to determine the ratio of living to dead cells throughout the fermentation and identify how this ratio changes with the incorporation of the pervaporation unit. The current approach to determining cell density, dry cell weight, does not account for cell viability over the course of the fermentation. Gallazzi et al. successfully applied flow cytometry after staining with a dual fluorescent probe strategy of propidium iodide (PI) and carboxy fluorescein diacetate (CFDA) to measure cell viability in a culture of *C. pasteurianum* (Gallazzi et al., 2015). The addition of this monitoring step would provide more insight into the accumulation of nonfunctioning, dead cells and how it may contribute to growth inhibition.

In all experiments conducted with the peristaltic pump, an immediate change in gas production was detected, along with an influx in gas flowrate detected from the off-gas flow meter. Further investigation is required to determine for certain if this insurgence of H_2 and CO_2 is truly representative of the metabolic occurrences or if it can be attributed to the sensitivity of the gas analyzer.

For future work, efforts should also be applied to investigate the metabolic activity occurring during oscillations. This can be done in a variety of ways. One example would be to apply proteomic techniques to identify the expression of proteins that are upregulated and down-regulated at different stages of fermentation. A similar approach was used by Sabra et al as well as Groeger et al., to assess changes in metabolic regulation in glucose-limited conditions and iron-limited conditions, respectively (Groeger et al., 2017; Sabra et al., 2016). Proteins associated with stress signaling would be of particular interest in the modified fed-batch system. In addition to this approach, intercellular quantification of NAD, and NADH levels through enzymatic assays, previously applied to *C. pasteurianum* by Arbter et al., could be used to better understand redox homeostasis occurring during

periods of oscillation. Additionally, mass spectrometry could be used as another analytical tool, which has previously been shown to be effective at determining relative concentrations of metabolic intermediates such as pyruvate (Arbter et al., 2021). In combination with previously discussed proteomics, this analysis could be used to generate a cohesive overview of metabolic regulation of the culture under the experimental conditions.

Under the proposed approach, the accumulation of untargeted products which are impermeable to the membrane such as 1,3-PDO is unavoidable. Therefore, it may be necessary to perform small-scale experiments evaluating the tolerance of this by-product in a smaller scale design of experiments (DOE) approach. Accompanied by higher cell concentrations, accumulation of intermediates such as butyric acid, or associated cell debris from bacteria in the death phase can also influence the fermentation more prominently in the modified fed-batch set-up compared to continuous modes of operation suggesting an opportunity for further investigation.

Supplementary attempts to refine fermentation parameters and overall system design are needed to improve process feasibility. For example, in one iteration of the modified fed-batch process, an imbalance in butanol production to removal was observed. To account for this, substrate supplementation was limited to slow butanol production. While practical for avoiding a design overhaul, it is not the ideal scenario. Alternatively, butanol removal could be improved through the implementation of a larger membrane with greater surface area or through changing operating conditions such as temperature to increase selectivity. An attempt to operate at a pseudo-steady state could also be made, matching inlet flowrates to the permeate flowrate. This would allow the volume to remain constant throughout the fermentation and not create limitations in terms of the capacity of the vessel.

A relevant observation made during the trial reported in this work was the operational issue of the blockage of permeate valve from the membrane to the cold trap. This largely impacted the butanol flux, with inhibitory concentrations of product in the fermenter being detected after periods of reduced flux. A simple solution to this issue may be to alternate

between cold traps more frequently or to install a valve with a greater diameter to accommodate for the collection of frozen permeate.

During HPLC analysis, an unidentified yet prominent peak was observed in the chromatographs, eluting from the column before any other constituents. This observation was continually seen at time zero in all experiments, taken at the time of inoculation and increasing in area over the duration of the fermentation. However, when the pervaporation unit was used, this peak appeared to decrease over time. As the unidentified compound was also detected in the permeate, it suggests a possible intermediate or by-product formed in the fermentation that was able to diffuse across the membrane. A systemic approach should be used to identify this peak, comparing possible compounds such as lactic acid to the retention time of this compound.

The objective of this work focused on comparing the performance of the modified fed-batch system to that of conventional fed-batch and batch models. This assessment could further be extended to continuous reactors. With the ability to have a steady-state operation, and reduce turnover time, continuous fermentation is becoming an industrially advantageous practice. As such, it would be relevant to identify parameters such as volumetric butanol productivity under both conditions. As cell concentration in continuous modes is inherently lower, the addition of a cell-recycle unit may provide a better baseline for comparison.

Continuation of the project would likely involve delving deeper into the impact of sugar addition in fed-batch fermentation with product recovery. Specifically, evaluating an optimal ratio of sugar to glycerol for butanol production, as well as determining the ideal time to initiate substrate feeding during fermentation. To minimize the number of experiments required, Box–Behnken design could be applied, and response surface methodology used to fit the data into a predictive model. This approach may also be useful to conclude whether oscillatory behavior is only relevant when glycerol is the sole substrate or if it emerges in a co-substrate fermentation as well.

Along this line of thinking, other sugar sources should be considered. From an economic perspective, using a feedstock that, like crude glycerol, can be obtained at a low-cost could improve the overall feasibility of the process.

As a proof-of-concept model, the system was operated using pure, refined glycerol, yet, in effort to assess the applicability of the operation in an industrial context, further experiments should consider the implementation of crude glycerol over pure glycerol.

Further expansion of this project may include the use of different strains. Research efforts that have resulted in hyper-butanol producing strains have shown great promise and would benefit from being cultured under the modified fed-batch system.

Although there are many possible future directions in continuation of this study, the contribution of this work signifies the applicability of pervaporation as an *in-situ* removal technique for mitigating the effects of butanol toxicity in glycerol fermentation. This work also further establishes that an oscillatory metabolic response can be triggered in *C. pasteurianum*, under the unique conditions of this process configuration. Additionally in this study, it was shown that secondary carbon sources were a successful strategy to prevent the occurrence of these fluctuations.

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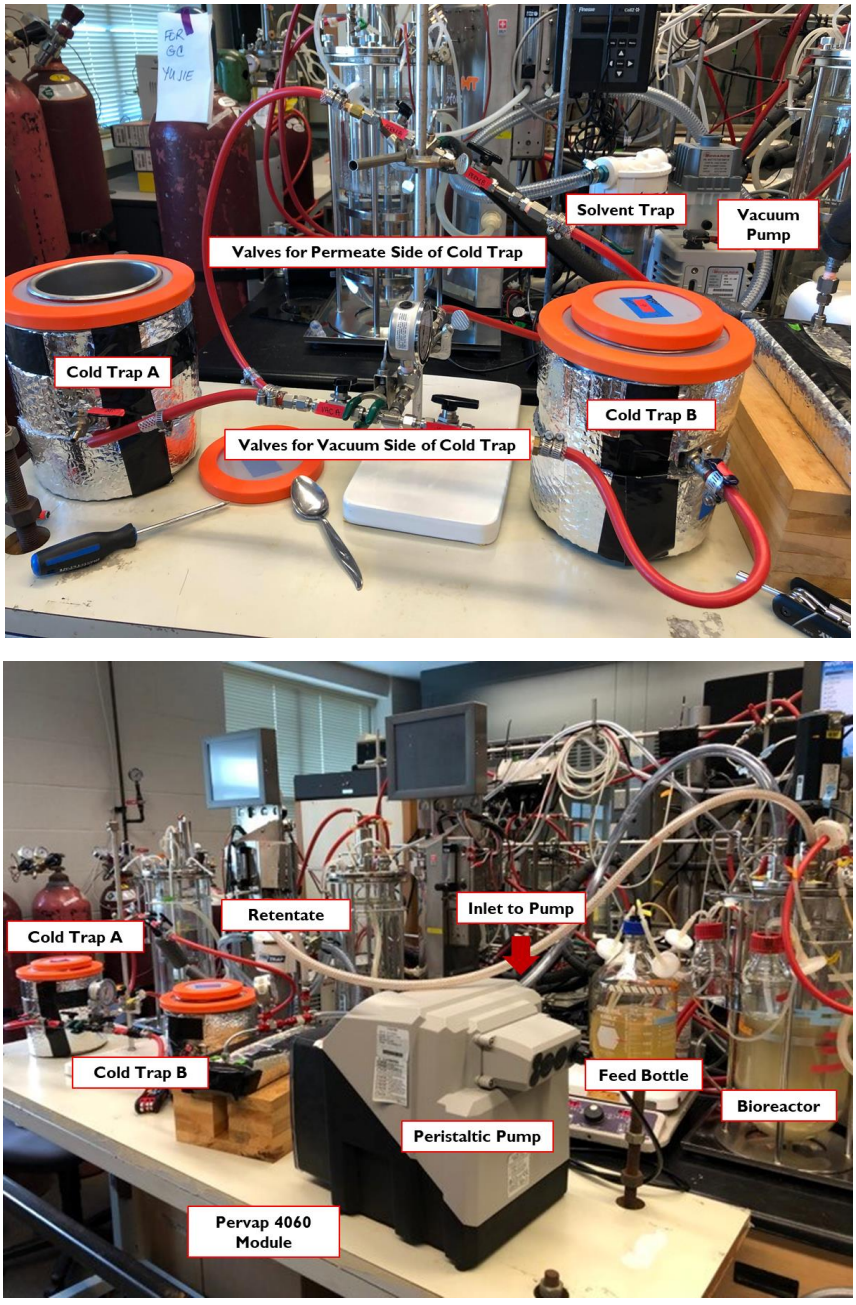
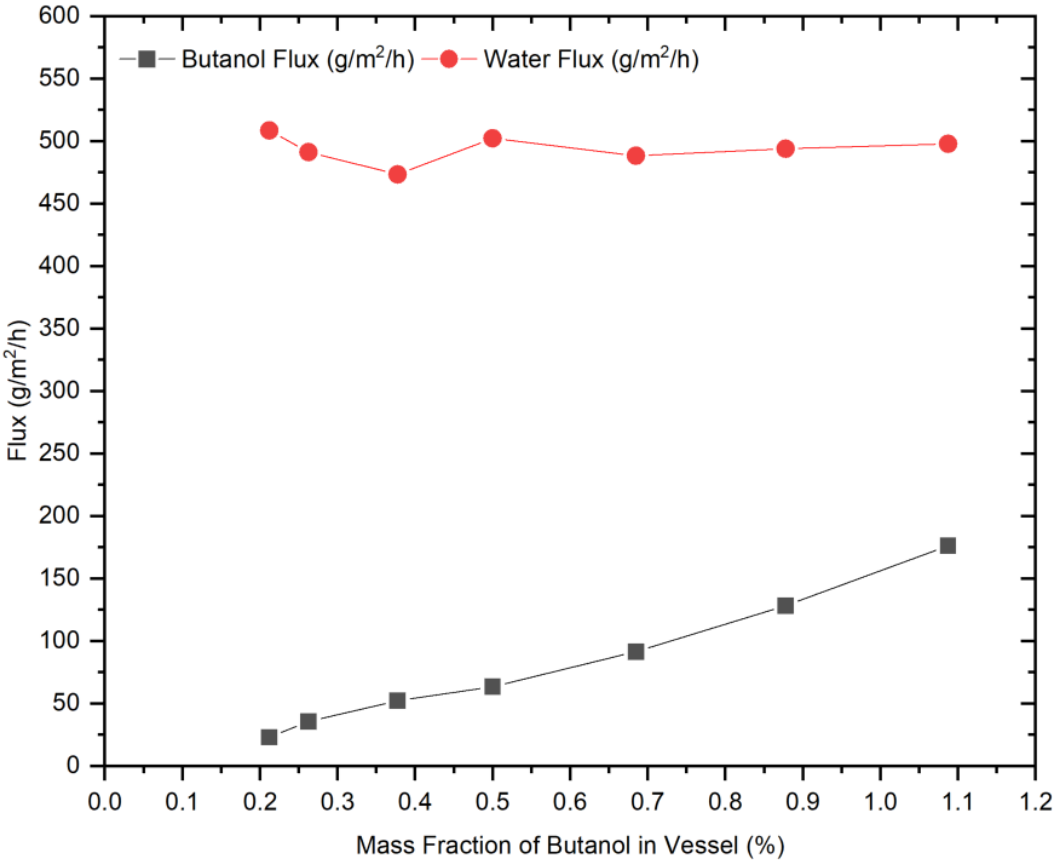
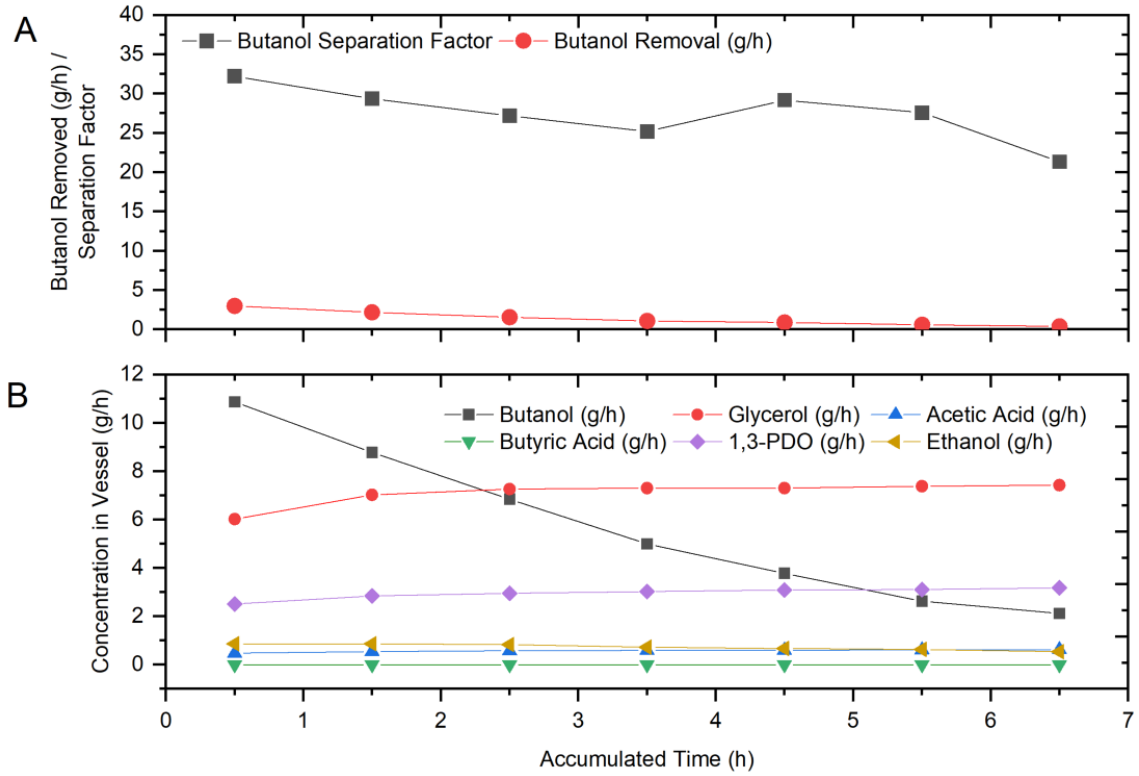
Appendices



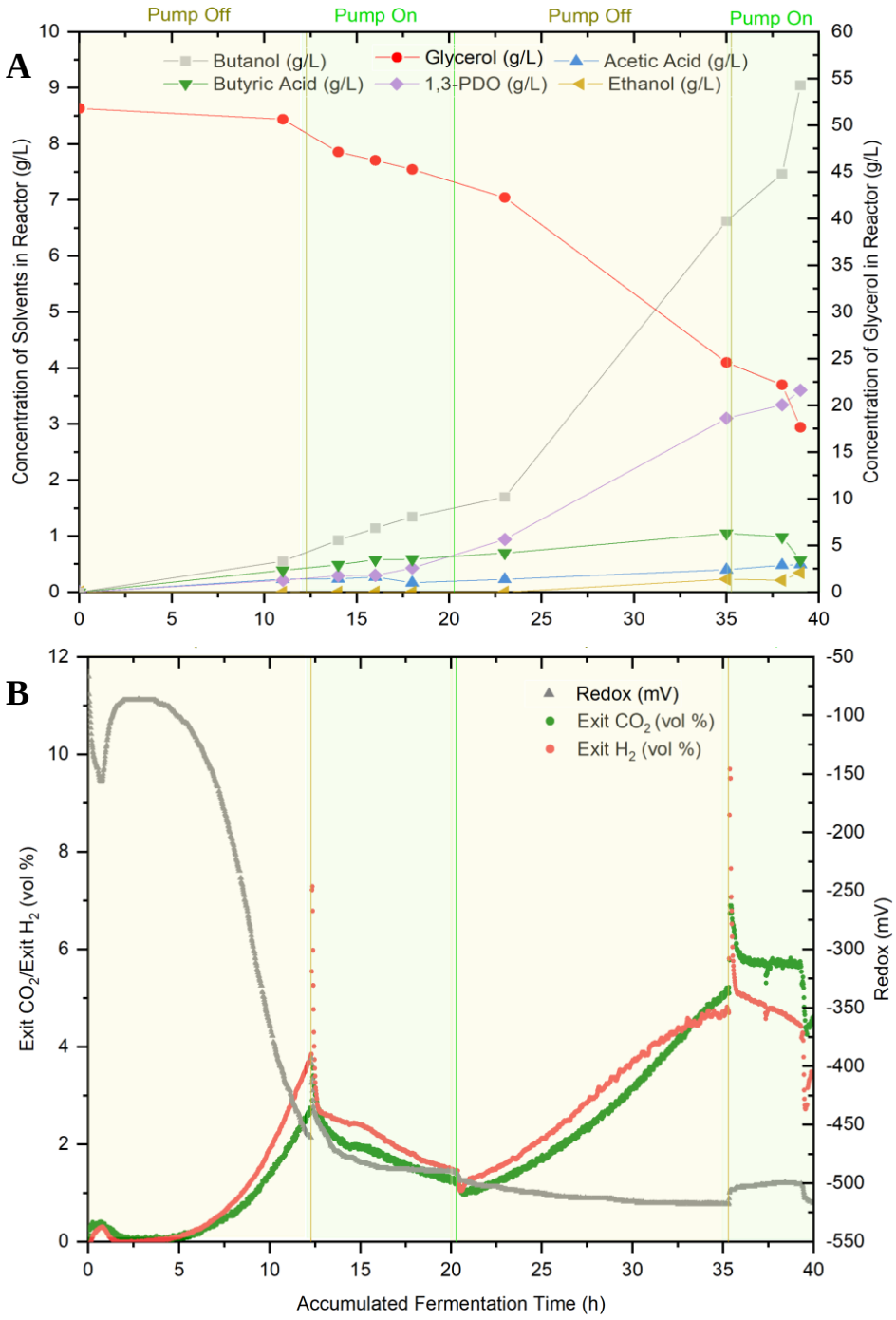
Appendix 1: Experimental set-up of modified-fed batch process



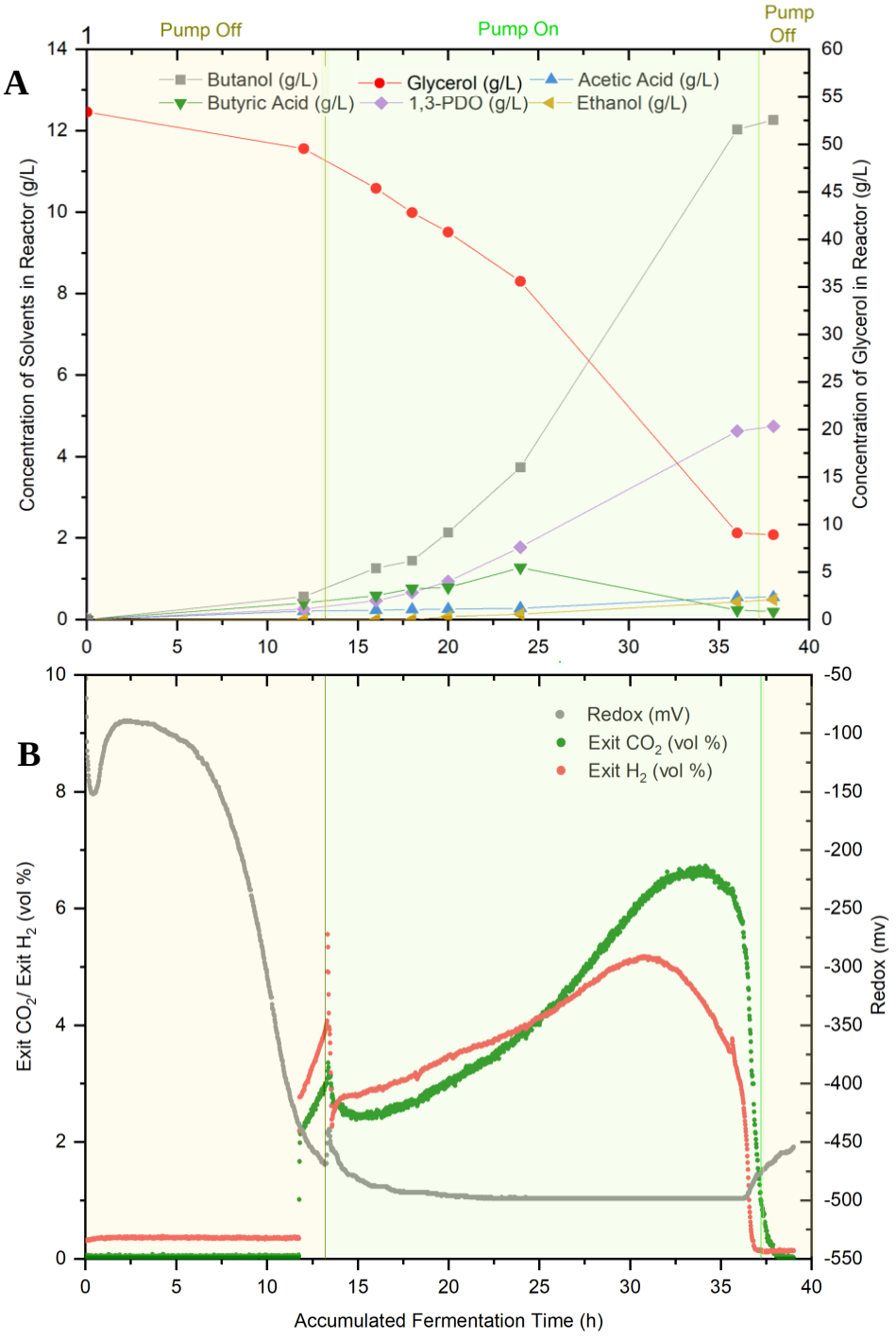
Appendix 2: Results from preliminary pervaporation experiment using fermentation broth; flux of butanol and water through Pervap 4060 membrane; starting concentration of butanol = 10.9 g/L, total pervaporation run time= 7 h, final concentration of butanol =2.1 g/L, Temperature= 35°C, flowrate = 4 L /min



Appendix 3: Results from preliminary pervaporation experiment using fermentation broth; **A:** Butanol separation factor and butanol removal in g/h over time **B:** Product profile in the vessel over time



Appendix 4: Batch fermentation with external loop through peristatic pump at 4 L/min; Temperature= 35°C; pH= 5.0; N_2 flowrate= 0.5 L/min; **A**: Concentration of glycerol and solvents in reactor; **B**: Online data-gas production and redox potential



Appendix 5: Batch fermentation with external loop through peristatic pump at 2 L/min; Temperature= 35°C; pH= 5.0; N_2 flowrate= 0.5 L/min; gas analyzer disconnected for first 12 hours (data missing); **A**: Concentration of glycerol and solvents in reactor; **B**: Online data-gas production and redox potential

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