Continuous production and supercritical extraction of acetone, butanol and ethanol by *Clostridium acetobutylicum* fermentation

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Abstract

A batch and continuous fermentation of *Clostridium acetobutylicum* ATCC 824 was tested for the production of acetone, butanol and ethanol. It was found that the batch process with a glucose-based medium supplemented with yeast extract grew reliably at 1-L scale at pH 5.0, 37 ^{o}C, inoculated with a seed meeting the criteria of pH 4.8 ± 0.7 and a glucose concentration of 9.6 ± 4.9 g/L, and producing 0.5 ± 0.1% (w/w) final concentration of butanol. Seeds which do not meet these criteria do not reliably result in growth in production medium.

A stable continuous fermentation was developed based on the batch process. The continuous fermentation without cell recycling ran stably at a dilution rate of 0.16 h^{-1} with a filter-sterilised feed medium, producing oscillating levels of butanol between 0% and 2%, as well as copious amounts of polysaccharide slime. As a solution to the issues faced by the continuous fermentation (incomplete conversion, large amounts of slime, and oscillating solvent production levels), a two-stage reactor is proposed, with potential for a commercial development of this fermentation with multiple stages. Multiple stages will allow the fermentation to run to completion, resolving all issues and producing the maximum yield of solvents.

Supercritical extraction has been tested for the extraction of ABE from fermentation broth. It was determined to be unsuitable for solvent extraction at the present time. The extract concentrations reached up to 7.58% with very high variance, which is not sufficiently high to warrant further testing with a view to commercialisation.

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1 Introduction

1.1 Bioprocessing and fermentation

Fermentation has been known for thousands of years. Mostly it has been used to make alcoholic drinks, but since the 20th century, other applications have been found (Stanbury, Whitaker, and Hall 1999). Fermentation is carried out by cells – usually bacteria or fungi, though algae, modified human or animal cells have also been used in recent times. The cells or organisms are referred to as whole-cell biocatalysts, as they take on the role of a catalyst in the conversion of substrates to products. On occasion, the microbial culture itself is the desired product, as in the case of protein substitutes. In addition to recreational and food purposes, fermentation is widely used for pharmaceutical and wastewater treatment purposes, and to produce biofuels (El-Mansi et al. 2012).

With the advent of genetic manipulation technology, fermentation using genetically modified organisms has also become much more common. For example, *Escherichia coli* (*E. coli*) is now used to produce human growth hormone (HGH); as the previous technique involved harvesting HGH from the pituitary glands of corpses, this is a significant improvement in economic terms. Genetic manipulation is also used to make organisms produce the desired product at a faster rate or under more adverse conditions. This is usually done via random mutagenesis and selection of better strains (Stanbury, Whitaker, and Hall 1999).

Fermentation can be done in batch culture, which is a closed culture system containing an initial, limited amount of nutrient (Stanbury, Whitaker, and Hall 1999). During batch fermentation, the microbial culture goes through four main phases: the lag, exponential, stationary and death phases (Figure 1). After the fermentation has run its course, the vat is emptied. If separation is required, usually the biomass is separated from medium first, followed by further downstream processing to purify a desired product if necessary (El-Mansi et al. 2012).

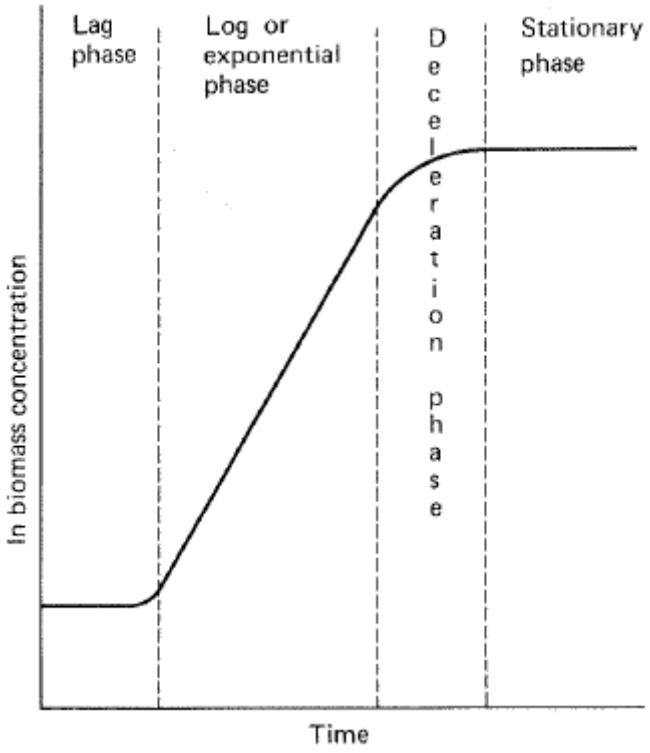


Figure 1 Microbes go through different phases of growth in batch culture. (Not shown: death phase, taking place after stationary phase, in which cells die as nutrients run out.) (Stanbury, Whitaker, and Hall 1999)

Alternatively, continuous culture may be used. There are advantages to continuous culture, most notably in that downtime for startup, shutdown, inoculation and cleaning represents a smaller proportion of total time, along with higher volumetric productivity (El-Mansi et al. 2012). There are also challenges to overcome issues such as contamination, strain drift and highly specific equipment that cannot be used in other processes (Stanbury, Whitaker, and Hall 1999). Whether batch or continuous culture is used depends on the specific requirements of the process and its microbial catalyst; when it can be done both ways, economics is the deciding factor (Stanbury, Whitaker, and Hall 1999).

1.2 Production of acetone, butanol and ethanol (ABE)

Some bacteria of the class Clostridia, of which the most studied is *Clostridium acetobutylicum*, produce solvents by fermentation of sugars (Durre 2007)(Nasib Qureshi, Hodge, and Vertès 2014). Acetone, butanol and ethanol (ABE) are produced in the ratio 3:6:1

during batch fermentation, by way of two sequential production phases: first is the acid phase, in which butyric acid and acetic acid are produced; then the solvent phase, in which the acids are converted into acetone, butanol and ethanol. The phase shift is triggered by pH and acid concentration (Hubert Bahl et al. 1982; Monot, Engasser, and Petitdemange 1984). Butanol is toxic to Clostridia; consequently, solvent build-up leads directly to the end of the fermentation batch, and limits the initial glucose concentration to about 60 g/L.

Commercial production of ABE began in World War 1 (Durre 2007). Usually, commercial ABE production by Clostridia resulted in up to 1.2% w/v butanol (approximately 13 g/L) before cell sporulation in batch fermentation starts (Köhler et al. 2015). Production continued until after the 1950s, but was subsequently phased out in most of the world as production of ABE from petrochemicals became cheaper. Fortunately, research into ABE production via Clostridium fermentation did not stop at the same time commercial production did. Research into ABE fermentation primarily focuses on two main avenues: genetic improvement and process modifications (Köhler et al. 2015). By and large, genetic engineering has focused on creating improved strains of Clostridia, usually by random mutagenesis; however, there have also been efforts to implement the metabolic pathway in *E. coli* (Figure 2) (Köhler et al. 2015).

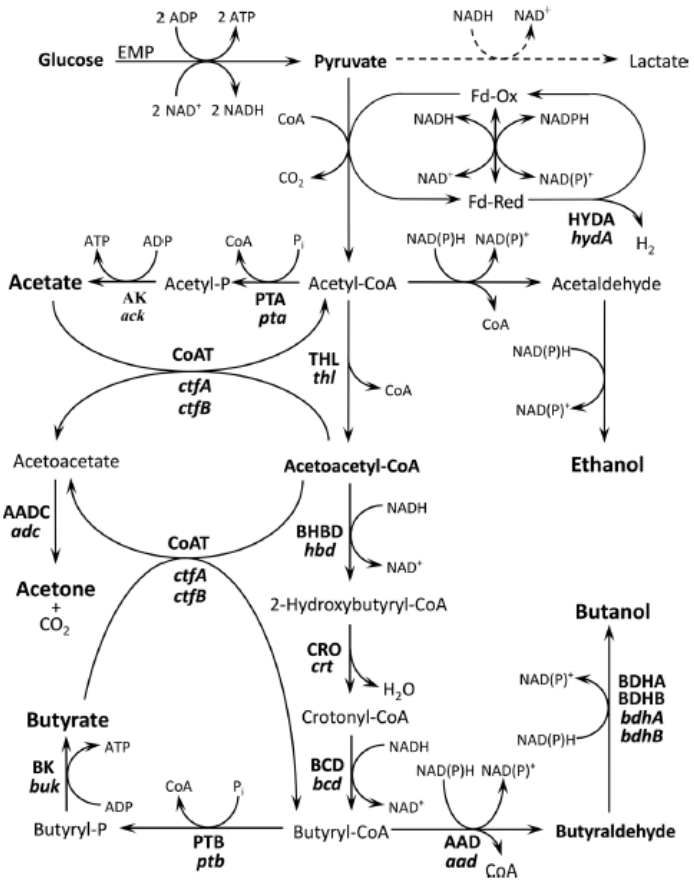


Figure 2 Metabolic pathway of solvent production: Glucose is converted to acetate and butyrate, which are re-metabolised to form acetone, butanol and ethanol. (Köhler et al. 2015)

Process modifications may be conducted on the fermentation itself or on the downstream processing. While ABE-producing continuous processes have been operated at bench scale, they have generally not yet been scaled up for industrial use. The exceptions are a few plants in China which were shut down after the economic opening of the country, though recently reopened (Chiao and Sun 2007). Continuous culture for ABE allows for higher productivity compared to batch culture, since solvents are secondary metabolites and continuous culture bypasses the long lag time before production of solvents begins in batch culture. However, contamination is a major issue in the one existing industrial-scale production system (Chiao and Sun 2007). There is also the option of cell immobilisation, in which non-growing cells are adsorbed or otherwise attached to immobilisation matrices, thus

bypassing the biomass separation step. This has not been scaled up for industrial use yet (Köhler et al. 2015).

At the time when ABE production by fermentation fell out of favour, the primary extraction processes used were distillation and fractional distillation. Since that time, advances have been made in many other methods of separation. Some of these are applicable to ABE production; most notably, much attention has been paid to gas stripping using the gaseous byproducts of the ABE fermentation itself. Other techniques under investigation include liquid-liquid extraction and pervaporation (Durre 2007).

1.3 Improving fermentation productivity

There are many ways to improve and optimise Clostridium fermentations. One way to improve production is by choosing the correct substrate. It has been well established that fermentation with different substrates results in a different ratio of solvents produced, large quantities of the intermediate acids in the final broth, or even different end products entirely (H. Bahl et al. 1986)(Raganati et al. 2015)(Choi et al. 2012). In addition, the presence of mineral salts can inhibit or even stop a fermentation in its tracks (Maddox, Qureshi, and Roberts-Thomson 1995). The choice of substrates is a contentious one. In addition to their effects on the fermentation, economic factors must be considered. Whey permeate, corn mash, processed lignins, etc. have all been suggested for various economic reasons (Nasib Qureshi, Hodge, and Vertès 2014). As for corn mash, there is backlash in the US for its use in producing bioethanol, as this use for it directly competes with food supply (Martin 2007)(Rosenthal 2011). The same argument would apply to using it for production of biobutanol.

Glucose, lactose and more exotic feedstocks have all been studied (Raganati et al. 2015). Many cellulose feedstocks require pre-processing to render them suitable for use in fermentation, and while this is advantageous as it puts to use plant matter which would otherwise be wasted, it also leaves behind trace chemicals which may be toxic. For example, hydrolysation of lignocellulose feedstocks produces acetic and formic acid, so that bacteria which are used to ferment them need to be tolerant (Cho, Shin, and Kim 2012). Impurities, not only from the processing but also from the original feedstock, always need to be accounted for. The addition of butyric acid has also been shown to stimulate butanol production in a chemostat, whereas the addition of acetic acid has no effect (Hubert Bahl et

al. 1982). On the other hand, phosphate limitation has been shown to stimulate solvent production (Hubert Bahl, Andersch, and Gottschalk 1982).

Another popular method to improve productivity is genetic engineering, which can increase the productivity of commercial fermentations and can also be used in conjunction with other methods of process improvement. Strains have been engineered for increased rate of solvent production (up to 29 g/L, approximately 3% w/v), as well as for increased solvent tolerance (Jang et al. 2012)(N. Qureshi and Blaschek 2001). In addition, resistance to bacteriophages and other disruptions, such as changing conditions, can prove invaluable. There have been efforts to engineer strains which produce cellulases, so that plant waste matter can be used directly without the hydrolysis step that introduces toxic impurities (Dürre 2008).

1.4 Design of other systems in the literature

Besides genetic engineering and changing the substrate, process improvements of all sorts have been tried. The original ABE fermentations were all done in batch mode (Stanbury, Whitaker, and Hall 1999), but fed-batch and continuous have been tested (Köhler et al. 2015). The idea of having continuous fermentations is not new, and many kinds of systems have been developed at lab scale. These systems can be sorted into two major groups: suspended and immobilised cells. Innovations are also being made in product recovery, which can be done in situ or after the fermentation process.

In systems that use non-growing, immobilised cells, no removal of biomass from fermentation broth needs to be done. The lack of a filtration requirement is one less mode of mechanical failure to worry about, besides the cost and maintenance issues. Genetic drift is not a problem, since there is no cell growth and division, so no opportunity for replication errors to be introduced. The feedstock is also not diverted to biomass production, so all of it can be converted to the final product. Cells are usually adsorbed on a matrix, which can be made of lignocellulosic material, calcium alginate or other suitable substances (Survase, van Heiningen, and Granström 2012)(Schoutens, Nieuwenhuizen, and Kossen 1985).

When cells are growing in suspension, there is substrate utilisation for biomass production, resulting in a lower process efficiency. It has been suggested that acidogenic and solventogenic bacteria coexist in a single culture, instead of all bacteria being in the same

stage of growth (Napoli et al. 2011). Two-stage continuous bioreactors have also been developed which have an acidogenic culture in the first stage and solventogenic culture in the second stage, with conditions optimised for each (Kayaalp 2013). Actively growing cells in suspension are also prone to genetic drift, and one result is the accumulation of non-solvent-producing mutants (Maddox et al. 2000). Filtration of the broth is naturally required, with all its attendant - but very well known and studied – complications (Stanbury, Whitaker, and Hall 1999).

Without cell recycling, washout limits dilution rate to approximately 0.26 h^{-1}(Tashiro et al. 2005). With cell recycling, filtration becomes paramount, but adds complexity. However, the payoff for this increased complexity is greatly increased production, as dilution rates of up to 0.85 h^{-1} have been achieved(Tashiro et al. 2005).

The traditional method of product recovery is distillation after filtration, which has the advantages of simplicity and isolation (Stanbury, Whitaker, and Hall 1999). Recently, there have been attempts to integrate product separation into the fermentation process (Qureshi, Hodge, and Vertès 2014). This may be by means of gas stripping using the gaseous by-products of fermentation (H_2, CO_2), pervaporation, or other techniques (Köhler et al. 2015; Xue et al. 2012; Wu et al. 2012; Bankar et al. 2012). This avenue of research has the natural limitation imposed by cell biology, as in situ product recovery requires a method that is benign to the cells. Instead of distillation, liquid-liquid extraction (LLE) with 2-ethyl-1-hexanol was proposed to separate the solvents from the aqueous broth after filtration (van der Merwe et al. 2013). This avoids the costly operations of ethanol/water and butanol/water separations, both of which have azeotropes. The process has been modelled, using N-hexyl acetate instead (99.9% recovered), and shown to be cost-effective in theory (Sánchez-Ramírez et al. 2015).

1.5 Commercial ABE fermentation

In Russia, a "continual" ABE fermentation was developed in the mid-20th century, with several fed-batch fermentations in parallel run at staggered times, so that the substrate production and separation operations could be constantly running (Zverlov et al. 2006). In this process, 5 tonnes of dry flour resulted in 58.8 tonnes of liquid substrate and produced 1 tonne of solvents and 1.7 tonnes of gases (CO_2 and H_2). Fermentations took place in working volumes of up to 2000 m^3, consisting of several joined fermenters each with volumes up to

275 m^3. The process could run for up to 50-60 hours before being forced to stop due to acidification (accumulation of non-solventogenic mutants) or infection. Fermentation sludge was used as substrate for a thermophilic methanogenic fermentation, providing process heat, and producing vitamin B12, biogas, and fodder yeast for animal feed. Solvents were isolated by distillation and fractionation. The last ABE fermentation plants in Russia closed in the late 1980s, thanks to the reduced economic power of the Soviet bloc and intense competition from petrochemicals.

The *C. acetobutylicum* strain used was independently isolated and showed phage resistance, but the fermentation was susceptible to lactic acid bacteria, and the process was run in batch mode until the continual mode was developed. The substrate used in the batch processes was originally starch from barley, rye and other food products, but molasses was introduced as substrate due to shortages. Later on, lignocellulosic hydrolysates were used in one plant in the continuous process. Although product composition changed due to the change in substrate, and solvent yield technically decreased, the lower cost of lignocellulosic hydrolysates and a broader substrate basis more than compensated for it.

Hydrolysates formed up to 7.5% of the substrate used in the commercial continual production, though in lab operations successful fermentations were conducted with up to 70% hydrolysates. Difficulties in the use of hydrolysates resulted largely from the impurities introduced during the hydrolysis process. Acetic and formic acids and furfural were the major inhibitory products, though arsenate (likely a contaminant of the sulphuric acid used), acrylamides and amino acids were also found (Zverlov et al. 2006).

To date, a true continuous ABE fermentation process has been operated only in China (Ni and Sun 2009). It was developed in the 1960s, and the last plant closed in the 1990s due to petrochemical competition. Production resumed in 2006, except for a hiatus during the 2008 financial crisis. In all plants, corn mash or corn starch (mash with the proteins and oil removed) is used as the substrate. The strain used was isolated and bred locally, suited to the local substrate and production conditions. Genetic engineering was used to create hyper-butanol producing strains. Products were separated by distillation and fractionation, and the gas byproducts are passed through an absorption tower to recapture solvents. The final productivity is 20-50% higher than the equivalent batch process.

The process, as used in China, consists of 6-8 tanks in series, with fresh substrate added to the first two. The periodic addition of seed culture broth, also to the first two tanks, prevents acidification as in the Russian process. Microscope inspection of samples is used to detect abnormalities and contamination, neither of which cause noticeable impact on the final production of solvents by the plants; if a tank is found contaminated, it is replaced. The fermentation runs for 170-480 hours.

The original fall of industrial ABE fermentation was due largely to the petrochemical industry, which lowered solvent prices to the point where fermentation as a production method could not compete (Durre 2007). Now, as the process continues to be developed with a view towards re-commercialisation, internal and external factors both have a great effect on its economic viability. External factors include the price of oil, and the general economic condition (Nasib Qureshi, Hodge, and Vertès 2014). Internal factors include substrate cost, product yield and recovery, and the possible use of all by-products. Commercial ABE fermentation requires both reducing the overall production cost, and increasing the revenue that may be generated, in order to be viable (Nasib Qureshi, Hodge, and Vertès 2014).

1.6 Acid crash and acidogenic kinetics

Clostridium acetobutylicum does not always produce ABE; sometimes, the fermentation produces primarily acetic and butyric acid instead. There are several ways that this may occur. Genetic drift may cause accumulation of mutant bacteria that lack the ability to produce solvents. High concentrations of mineral salts have been shown to inhibit production of ABE but not its precursor acids, leading to an acidogenic fermentation (Maddox, Qureshi, and Roberts-Thomson 1995). Alternatively a fermentation run at a high pH can produce mostly or all acids, as the concentration of undissociated butyric acid never reaches the threshold of 1.5 g/L at which solvent production begins (Monot, Engasser, and Petitdemange 1984). On occasion, a fermentation which meets none of these criteria simply fails to transition to solvent production as expected. This is known as acid crash.

It was found that acid crash fermentations reached a higher maximum concentration of acids than normal fermentations under the same conditions: 59 mM or higher, as compared to 53 mM. These fermentations also recommenced after the concentration of acids dropped below a threshold of 55 mM. Thus, the high combined concentration of undissociated acetic and butyric acids was suggested as a probable cause of acid crash (Maddox et al. 2000).

Butyric acid is known to be a microbial inhibitor, so the popular theory is that acid crash occurs when *C. acetobutylicum* is at or near its maximum growth rate and fails to make the switch to solventogenesis and acid consumption before the inhibitory effect stops growth (Maddox et al. 2000). An alternative theory proposed that formic acid is the cause of the crash (Wang et al. 2011).

To avoid acid crash, several methods are proposed. Both of the following involve reducing the concentration of undissociated acids: pH may be controlled so that the majority of acids are dissociated, which also results in relatively higher concentrations of acids in the broth after the fermentation runs its course. The fermentation may also be run at lower temperatures so that acid production – and the entire fermentation - is slower, thus preventing the inhibitory effect from taking place until solventogenesis begins (Maddox et al. 2000). Strains of *C. acetobutylicum* have also been engineered to produce formate dehydrogenase (Wang et al. 2011).

1.7 Polysaccharide production in *C. acetobutylicum* fermentations

When certain external conditions are met, bacteria will produce polysaccharides for various reasons, including but not limited to energy storage and support matrices (Sutherland 1990). The presence and production of polysaccharides affects fermentation yield and productivity, and may also have a physical effect on the fermentation.

Bacteria in the Clostridia family produce intracellular polysaccharides collectively termed granulose. Granulose accumulation normally occurs at the end of exponential growth before the onset of sporulation and is usually degraded during spore formation (Reysenbach et al. 1986). The two events (granulose degradation and spore formation) are linked. In fact, a single gene (Spo0A) has been identified in *C. beijerinckii*, which when knocked out causes the bacterium to be asporogenous and unable to produce granulose or solvents (Wilkinson et al. 1995). Regulatory pathways which granulose production, sporulation and solventogenesis have in common have also been discovered, including a quorum sensing system (Steiner et al. 2012).

In addition to granulose, *C. acetobutylicum* produces a currently unnamed exocellular polysaccharide, first described in 1984 by investigating material balances, and not yet characterised. It had been found that excess glucose was consumed during acidogenesis

which could not be accounted for by acid production. In the same fermentations, excess solvents production could not be accounted for by the combined consumption of acids and glucose during solventogenesis. The exopolysaccharide was proposed as an intermediate product to explain this phenomenon, and subsequent experiments proved its existence (Haggstrom and Forberg 1986).

This exopolysaccharide causes the fermentation to become viscous, an effect which is frequently observed in *C. acetobutylicum* fermentations, and which is a known effect of exocellular polysaccharides (Sutherland 1990). This viscosity dissipates as rapidly as it appears, as the exopolysaccharide is consumed for solvent production or spore formation. It is likely that exopolysaccharide consumption, like solvent production, is linked to sporulation. The exopolysaccharide shows a high degree of acetylation (Haggstrom and Forberg 1986). Its production is independent of acetic and butyric acid concentration, as demonstrated when those acids were added to fermentations (Junelles et al. 1989). It has been hypothesised that the polysaccharide is produced when there is a high demand for reducing power, but the conditions are not good for the formation of end products (Haggstrom and Forberg 1986).

It is preferable to reduce or eliminate exopolysaccharide production, as polysaccharides have adverse effects on filtration mechanisms, and highly viscous solutions cause other difficulties. A viscous fermentation requires more energy to keep agitated. The risk of stagnant zones also increases greatly, especially in pseudoplastic fluids, which most microbial polysaccharides behave as (Stanbury, Whitaker, and Hall 1999). In a continuous stirred-tank fermentation, exopolysaccharides are washed out without being converted to a final product, thus reducing yield. It is known that medium composition is frequently a factor in the production of exopolysaccharides, so altering the medium is a solution to unwanted polysaccharide production (Sutherland 1990). It has been proposed that careful control of stoichiometric ratios is the key to reducing the presentation of exopolysaccharides in a *C. acetobutylicum* fermentation, especially since its presence appears necessary to the smooth running of the fermentation (Haggstrom and Forberg 1986).

1.8 Supercritical fluid extraction

A supercritical fluid (SCF) is a substance at a temperature and pressure above its critical point (Figure 3). Above the critical point, the liquid and vapour phases become

indistinguishable. A SCF behaves as a dense gas phase. By going through the SCF "phase", it is possible to carry out a process which transforms a liquid into a gas or vice versa without any phase transitions, that is, no interface between liquid and vapour can be observed during the process (Hołyst and Poniewierski 2012). Table 1 shows a comparison of properties for gases, liquids and SCFs.

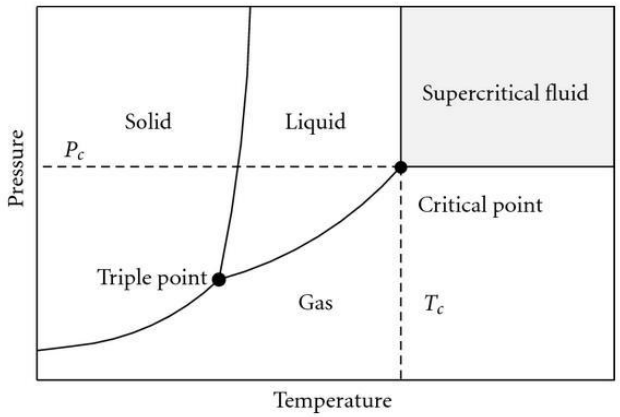


Figure 3 P-T diagram showing the critical point

Table 1 A comparison of the typical properties of gases, supercritical fluids and liquids

	Density (kg/m^3)	Viscosity (μPa·s)	Diffusivity (mm^2/s)
Gases	1	10	1-10
Supercritical fluids	100-1000	50-100	0.01-0.1
Liquids	1000	500-1000	0.001

SCFs can be used in separations, much like non-critical fluids, but with distinct advantages. Extraction with SCFs and near-critical fluids are often treated collectively, as there are many similarities. Diffusivity of solutes is much higher in SCFs than in liquids. The viscosity (μ) and density (ρ) of SCFs can be controlled by changing the pressure and temperature, especially near the critical point, where large changes in μ and ρ can result from

relatively small changes in pressure (P) and temperature (T). In addition, recovery of the product can be easily carried out by changing the conditions of the fluid (McHugh and Krukonis 1994). However, supercritical extraction can be expensive, largely due to the pressure requirement.

Carbon dioxide, which is inexpensive, environmentally friendly and safe, is the most widely used SCF for extraction purposes, sometimes with co-solvents to dissolve polar compounds. It has a supercritical temperature of 31.1 °C, just slightly above room temperature, and critical pressure of 73.8 bar. Supercritical extraction by CO_2 is used in many areas of industry, such as polymer processing and natural products (e.g. decaffeination) (McHugh and Krukonis 1994). The use of supercritical CO_2 for the extraction of ABE from aqueous solutions has been studied before (Moreno et al. 2012; Moreno, Tallon, and Catchpole 2014). CO_2 can be used to extract acetone and butanol from fermentation broth, though not ethanol, as its partition coefficient is too low (Moreno et al. 2012).

1.9 Aims of this thesis

The main aim of this project is to develop a system in which *C. acetobutylicum* can be kept viable and productive under continuous fermentation conditions. A secondary aim is to combine this system with supercritical extraction as an alternative to distillation, if possible. Continuous fermentation of ABE has been done before, as has extraction of ABE from fermentation broth using supercritical CO_2 (Hubert Bahl, Andersch, and Gottschalk 1982)(Moreno et al. 2012). But there has not been a combined effort to create a working system whereby the products of fermentation are separated from the broth by supercritical extraction. Other relevant aims of this research include the investigation of technologies to enhance solvent recovery, thus increasing the yield, and the optimisation of operating conditions and feed stock, thus improving the overall cost effectiveness of the project. Ideally, work done here will go some way towards establishing a commercially viable ABE fermentation and separation system.

There are some major design issues to overcome. Firstly, there is the question of separation: ethanol has a much lower partition coefficient than either acetone or butanol in the CO_2 extraction system. At 100 bar and 40 °C, acetone has a partition coefficient of 6.4, butanol at 2.2, and ethanol at 0.12 in supercritical CO_2 (Moreno, Tallon, and Catchpole 2014). Thus, most of the ethanol will remain in the raffinate, either to be disposed of, or to be

recycled to the bioreactor. If recycled, then toxicity is likely to become an issue. Based on the literature, the production rate of ethanol in continuous process is less than in batch process, suggesting that ethanol build-up should not reach toxic levels before removal (Li et al. 2011; Pierrot, Fick, and Engasser 1986).

In developing a process for the fermenter, there are many factors to examine. The dilution rate for this fermentation, without cell recycling, is a maximum of 0.26 h^{-1}, above which there is cell washout. With cell recycling, the dilution rate can be raised. However, this may lead to the cell concentration becoming very high, leading to foaming. Thus, cell bleeding must be used (Tashiro et al. 2005; Jang, Malaviya, and Lee 2013). For an application such as this, filtration and cell recycling can be used to maintain the cell concentration at a high level, with a bleed stream to counter the effect of cell growth.

Process control is another major hurdle in designing a continuous production system for ABE. There have been efforts made towards designing systems which work, but real-time process control is likely to remain challenging (Ewanick et al. 2013; Finch et al. 2011). This is particularly difficult as it is not simple to directly measure the current concentrations of solvents in any given stream. With simple chemical reactions such as the Haber process, a certain set of reaction conditions will return a given result, so process control can focus on maintaining those reaction conditions. With whole-cell biocatalysts, keeping the reaction conditions within certain ranges is necessary but not sufficient. The system can be perturbed by contamination with bacteriophages, or genetic drift may occur in longer-running fermentations (Zverlov et al. 2006; Maddox et al. 2000).

In this thesis, specific conditions of fermentation will be investigated, to determine a reliable set of conditions under which batch fermentations can be run. This will be used as the basis for developing a continuous system for *C. acetobutylicum* fermentation. At the same time, supercritical extraction of ABE will be investigated, with the aim of integrating it into the system if possible. Finally, the question of future systems and next steps will be considered, with a view towards eventual commercial production of ABE by *C. acetobutylicum* fermentation.

2 Materials and Methods

2.1 Fermentation

Table 2 gives a full accounting of the fermentation experiments done.

Table 2 List of all experiments done; see Table 3 for details of fermenters

Designation	Purpose	Fermenters used	Comment
1	Replication of previous results	FER100	
2 (1)	Scale-down	FER_A1	
2 (2)		FER_A3	pH control attempted
3 (1)	pH control testing	FER_A1	No pH control
3 (2)		FER_B1	
3 (3)		FER_A2	pH control with base
3 (4)		FER_B2	
3 (5)		FER_A3	pH control with base and acid
3 (6)		FER_B3	
4 (1-3)	Solvent evaporation	FER_A1 through A3	See section 2.1
5.1 (1-8)	Media study	Small bottles	20mL working volume
5.2 (1-8)			15mL working volume
6.1 (1-6)	Seed study	100-mL Duran bottles	
6.2 (1-5)			
6.3 (1-3)			
6.4 (1-2)			
6.5 (1-4)		FER_A1, A2, B1, B2	
7	Filtered media testing	FER_A2	
8	Continuous fermentation (cell recycling)	FER_A1	500 mL working volume intended
9		FER_A1	
10.1-10.5	Continuous fermentation (no cell recycling)	FER_A1	Runs done consecutively in the same fermenter

2.1.1 Cultures

The strain used in all experiments was *Clostridium acetobutylicum* ATCC 824, strain IRL 542, a 15% glycerol stock which had been stored at -80 °C. Seed medium was inoculated with 0.5-1 mL of the vial which had been thawed at room temperature. After the seed culture had grown, it was inoculated into production at a 10% inoculation rate (Bankar et al. 2012). Appendix C details failed attempts at using other strains.

2.1.2 Medium

Clostridial reactor medium (CRM) (Monot et al. 1982), was used for the seed and production experiments. The culture medium contained, per litre, 0.5 g of KH_2PO_4, 0.5 g of $K_2HPO_4.3H_2O$, 0.2 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g NaCl, 2.2g of ammonium acetate, 1 mg para-aminobenzoic acid, and 10 μg biotin. The seed medium contained 20 g/L glucose, and the production medium contained 60 g/L glucose. In the media study, a medium containing an additional 5 g/L of yeast extract, 5 g/L of tryptone and a combination of the two at a 1:3 ratio respectively were tested (Al-Shorgani et al. 2016). The medium used in subsequent experiments contained an additional 5 g/L of yeast extract. Antifoam 204 (Sigma-Aldrich) was used to control foaming.

The medium was steam-sterilised for times appropriate for the volume at 121 °C. To test the effect of autoclaving, sterile filtration was tested in one experiment. To get the correct concentration of production medium in a clean bioreactor, the bioreactor was steam-sterilised containing water up to 50% of the required initial volume (450 mL). The remaining 50% of the required volume (450 mL) of production medium was made at 2x concentration, filtered into a sterile bottle, and added aseptically to the bioreactor. Subsequently, seed medium (in bottles) and production medium (in bioreactors) were steam-sterilised before inoculating, while the feed medium for continuous runs was filter-sterilised before use.

2.1.3 Seed

Seed medium was prepared in Duran bottles (working volumes 1-L for 20-L fermenter, 100/250mL for 1L fermenters). The seed culture was inoculated with 0.15% glycerol stock of IRL 542 and N_2 bubbled through it before inoculating to ensure an

anaerobic environment. It was grown at 37 °C in an anaerobic chamber (BD GasPak™ System), agitated at 50 RPM in a shaking-incubator (Infors Multitron II). Seeds were allowed to grow for either a set period of time (24-72 hours) (Moreno et al. 2012; Tashiro et al. 2005)or until the seed reached pH = 4.8 ± 0.7 and [glucose] = 9.6 ± 4.9 g/L.

2.1.4 Production fermenters

The initial fermentation was conducted in a 30-L fermenter (FER100, working volume 20-L) with a 7.5% inoculation rate (1.5 L of seed culture) (Appendix A). As it was impractical to conduct a continuous fermentation in the large fermenters, all subsequent fermentations were conducted in 1-L fermenters. The 1-L fermenters were batched with 900 mL production CRM and inoculated with 100 mL seed culture (10% inoculation rate) unless otherwise stated. Details of fermenters are shown in Table 3.

Table 3 Details of fermenters used

Fermenter	Manufacturer	Model	Working volume	Comments	Used in experiments
FER100	B. Braun Biotech Int'l	Biostat C-DCU13	20-L (30-L nominal)	Steam-in-place	Appendix A
FER_A1 FER_A2 FER_A3 FER_B1 FER_B2 FER_B3	Sartorius Stedim	Biostat Q plus	1-L (1.3-L nominal)	Autoclave sterilisation required	All except media and seed studies

Temperature was controlled at 37 °C, with stirring at 50 RPM, and flow of N_2 (0.1 LPM except in the 20-L fermenter where 0.8 LPM was used) to maintain dissolved oxygen (DO) at 0%. Where pH control was used, it was with 3% aqueous ammonia or 1 M NaOH. Samples were taken through a sampling port twice a day in the mornings and evenings on

weekdays, and once a day on weekends, then stored at -20 °C until processed for solvent analysis.

For continuous operation, the fermentation was run in batch mode until the pH reached the first peak in pH level. This normally took place between 21 and 30 hours from inoculation, and corresponded with the period when butanol was being produced at the highest rate. At this point, or as close as reasonably possible, the fermentation was switched to continuous operation.

The working volume in the 1-L fermenters during continuous operation was 600 mL, unless otherwise noted. For fermentations with cell recycling, an extraction pump was used in addition to the feed pump. For fermentations without cell recycling, level control was accomplished with constant overflow by pumping liquid out at a slightly higher rate than the inlet flow rate. A Y-connector on the base of the sampling port (Figure 4) minimised the impact of foaming on the level control. An additional sampling port was placed to allow sampling before chemostat operation.

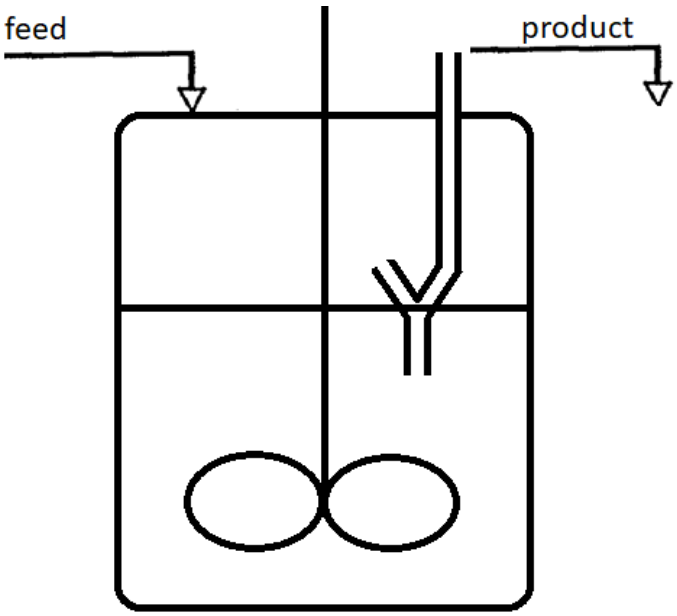


Figure 4 Level control using a Y-connector

2.1.5 Production bottles

The media study fermentations (Experiment 5) were conducted in a series of 24 glass bottles with volume 25 mL (working volume 20 mL in the first, 15 mL in the replicate). Seed medium was prepared as described above. Production medium, prepared in a 100-mL Duran bottle, was inoculated from seed to OD_{600} = 0.3 ± 0.03, and dispensed into the 25-mL bottles which had been autoclaved prior to filling. Nitrogen was bubbled through the samples for 1 minute, to reach 0% DO. Temperature was controlled at 37 °C, with agitation at 50 RPM. The pH was not controlled in this experiment. In the first run, samples were taken once a day, then stored at -20 °C until processed for solvent analysis. In the second run, samples were taken at different intervals, more often in the earlier part of the fermentation, to gain a finer-grained view of the exponential phase. For the second run, IPA stock was used instead of IPB.

The seed characterisation study fermentation (Experiment 6.1) was conducted in six Duran bottles, each with working volume 100 mL. The bottles were each inoculated with 10% inoculum (v/v) from the same seed culture, 12 hours apart for each successive bottle starting from 20 hours. Nitrogen was bubbled through the 100-mL bottles for 1 minute each, to reach 0% dissolved oxygen (DO). Temperature was controlled at 37 °C, with agitation at 50 RPM. pH control was not used in this experiment. Samples were taken once every 12 hours, analysis conducted, then stored at -20 °C until processed for solvent analysis. The study was repeated, with a series of five Duran bottles instead of six. (Experiment 6.2)

Experiment 6.3 was conducted in three Duran bottles with working volume 100 mL, with a separate seed culture for each. All bottles were inoculated with 10% inoculum (v/v) after the seeds had grown for 36 hours. Nitrogen was bubbled through the 100-mL bottles for 1 minute each, to reach 0% dissolved oxygen (DO). Temperature was controlled at 37°C, with agitation at 50 RPM. pH control was not used in this experiment. Samples were taken twice a day, analysis conducted, then stored at -20°C until processed for solvent analysis.

2.1.6 Pumps and filters

Different sizes of peroxide-cured silicon tubing from Cole-Parmer and Elastomer Products Ltd were used where required. Table 4 describes the pumps used. For peristaltic

pumps, the location of the pump on the tubing was changed every 24 hours at minimum to prevent tubing fatigue.

Table 4 Standalone pumps used in this thesis

Type	Manufacturer/model	Used in experiments
Peristaltic	LongerPump YZ1515x (x2)	8-10
	Watson-Marlow 101U/R	10
	Watson-Marlow 323 (x2)	8
Digital gear pump	Cole-Parmer 75211-35	Appendix B

For cell separation and recycling during continuous fermentation, several filters were tested. Two hollow fibre ultrafiltration modules were used: Scepter Microfiltration ceramic membranes, 0.1 µm and 0.02 µm pore diameters, single tube single pass modules with 316" stainless construction and Tri Clover fitting (Custom orders from Graver Technologies). A bench-scale tangential flow filtration (TFF) system, the Sartocon Slice module, was also tested in Experiment 9 with a 0.2 µm PES membrane and a SciLog tandem model 1082 pump feeding it. The TFF unit could be run under constant flow rate or constant transmembrane pressure settings.

For filtration of production medium, Corning bottle top filter was used with 0.2 µm PES membrane. For filtration of feed medium, Sartorius Sartobran capsule filter was used, which is a 2-stage filter with 0.45 µm + 0.2 µm.

2.1.7 Testing for solvent evaporation

A test solution was made up using 1% butanol, 0.5% acetone and 0.1% ethanol (w/v). It was piped to the reactors as shown in Table 5.

Table 5 Gas flow to bioreactors for evaporation study

Sub-experiment	Bioreactor	Gas flow	To location
6.1	FER_B1	ON	Head space
6.2	FER_B2	ON	Sparger
6.3	FER_B3	OFF	N/A

2.2 Supercritical extraction

2.2.1 Feed

In the experiments testing extraction conditions, a stock solution consisting of 1% butanol and 0.5% acetone (w/v) was used as feed. In the others, the broth from fermentation experiments was centrifuged at 7333 RCF for 45 minutes, and the supernatant used as the feed in this experiment.

2.2.2 Contactor

The contactor used for supercritical extraction is made of microporous hydrophic isotactic (semicrystalline) polypropylene with 70% porosity (Mentec). Contactor specifications are listed in Table 6.

Table 6 Specifications of the contactor used for SCE

ID	16.6 mm
Fibre ID	0.25 mm
Fibre OD	0.54 mm
Number of fibres	566
Effective length of fibres	270 mm
Effective contact area	0.26 m^2
Packing density (phi)	0.6

2.2.3 Gas

CO_2 was obtained from BOC New Zealand. CO_2 flowing counter currently through the inside of the fibres was more efficient than CO_2 flowing through the shell side, and this configuration was used in the present work.

2.2.4 Procedure

The Hollow Fibre Membrane (HFM) contactor was placed horizontally inside a temperature-controlled water bath. The system was initially filled with CO_2 using ISCO HL-Series syringe pumps. The CO_2 was preheated to the operating temperature before entering the fibres. Once the system was stabilised at the desired operating pressure, feed material was introduced using ISCO D-Series syringe pumps. The syringe pumps were used to increase control over the flow rate and pressure in the system (flow accuracy 0.5% of set point and standard pressure accuracy 0.5%). This setup is shown in Figure 5.

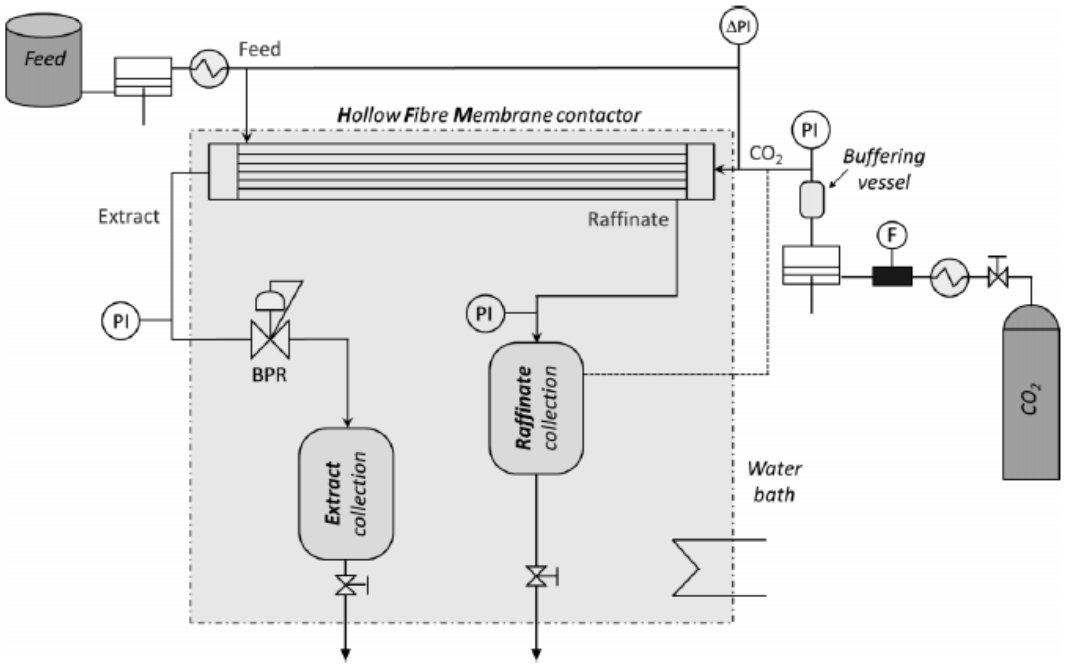


Figure 5: Experimental setup described by Moreno et al. and also used in this thesis (Moreno, Tallon, and Catchpole 2014)

Solvent flow rate varied between 6 and 15 g/min; feed flow rate varied between 4 and 12 g/min. Operating pressure was 100 bar and temperature ranged from 18 to 40 °C. Solvent to feed (S/F) ratio ranged from 1.0 to 2.0. The aqueous outlet was connected to the CO_2 inlet in order to equalise the shell and tube side pressures, thereby preventing the occurrence of a high trans-membrane pressure drop. The butanol-lean raffinate was collected through a manually operated valve from the bottom of the equalising vessel, while the butanol-enriched extract was collected from the bottom of the separator. In some experiments, a second water bath was added to raise the temperature of the exit streams for collection (Figure 6).

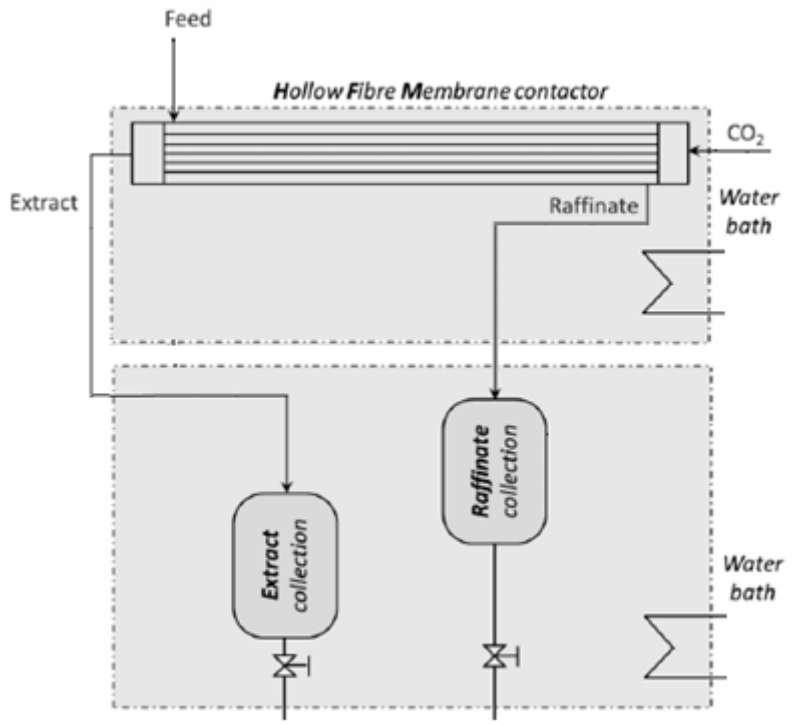


Figure 6 Setup with different collection temperature

Steady state was assumed when at least five volumes of the aqueous phase had passed through the contactor (10-20 minutes) (Moreno et al. 2012). At that point, samples of raffinate and extract were collected for analysis. The fibres were periodically regenerated between runs by flowing ethanol (using ISCO D-Series syringe pumps) through them

followed by flushing with compressed CO_2 in order to remove any water that might have entered the membrane. Each set of conditions was tested in duplicate.

2.3 Analysis

2.3.1 Optical density

Optical density (OD_{600}) measurements were performed on a Spectronic 2000 (Thermo Scientific) spectrophotometer or a model 83059-15 spectrophotometer from Cole-Parmer. Samples were diluted with PBS where necessary, so that the final absorbance reading was between 0.1 and 1.0. OD_{600} was calculated by multiplying the absorbance reading by the dilution factor.

2.3.2 Sample analysis

Automatic sample analysis was done by gas chromatography on a Trace GC Ultra (Thermo Fisher Scientific) gas chromatograph equipped with flame ionization detector (FID), TriPlus HeadSpace autosampler and BP1 (60 m x 0.32 mm x 0.25 µm) capillary column (SGE), using 1-propanol as internal standard. Helium was used as carrier gas, and a split ratio of 1:50 was used. Injector and detector temperatures were both 200 °C. Oven temperature was raised from 90 °C to 110 °C at a rate of 3 °C/min, followed by a rise to 200 °C at a rate of 20 °C/min. Each sample was analysed in triplicate.

Some samples were manually analysed with the following method: Samples were heated in a water bath at 50 C for at least 15 mins and syringe was heated in the oven at approximately 60 °C to 70 °C. In between samples, the syringe was flushed with air three times before it was placed in the oven. Once the syringe was heated, it was flushed with the new sample once. Approximately 100 µL of sample was extracted with the syringe and injected to the gas chromatograph. In between trials of the same sample, the syringe was not flushed. A Shimadzu GC-2010 gas chromatograph was used for these samples, with settings as described in Tables 7-9. The column used was a Restek Stabilwax with Integra-Guard (30 m x 0.25 mm x 0.25 µm).

Table 7 Injector details for GC manual sampling

Temperature	180 °C
Injection Mode	Split
Sampling Time	0.50
Carrier Gas	He
Flow Control Mode	Linear Velocity
Pressure	121.0 kPa
Total Flow	28.7 mL/min
Column Flow	1.23 mL/min
Linear Velocity	27.1 cm/sec
Purge Flow	3.0 mL/min
Split Ratio	20.0

Table 8 Column details for GC manual sampling

Temperature	50.0 °C
Equilibration Time	1.0 min
Column Max. Temp	250 C
Length	40.0 m (Column length + guard length)
Inner Diameter	0.25 mm ID
Film Thickness	0.25 µm

Rate	Temperature	Hold Time
-	50.0	1.00
5.00	83.0	0.00
40.00	190.0	3.00

Table 9 FID details for GC manual sampling

Temperature	200.0 °C	
Sampling Rate	40 ms	
Stop Time	13.27 min	
Delay Time	0.00 min	
Subtract Detector	None	
Row Program	Make up	
Rate	Row	Hold Time
-	30.0	0.00

3 Batch fermentation

3.1 Reproduction of previous results

An initial experiment was done at 20-L scale to reproduce previous results (Moreno et al. 2012). It produced a final butanol concentration of 0.8% in a fermentation time of 161 hours. The maximum OD_{600} reached was 10. Details of Experiment 1 may be found in Appendix A.

3.2 Scale down

Subsequently in this project, experiments were conducted to establish a baseline growth curve, fermentation profile, and solvent production at 1-L scale. This scale down was necessary as continuous fermentations of *C. acetobutylicum* can reach a dilution rate of 0.85 h^{-1} with cell recycling, not suitable for testing at 20-L scale with available equipment and resources. Figure 7 shows the OD_{600} and pH of the two duplicate runs in this experiment, while Figure 8 shows the solvent concentrations over the course of Experiment 2.

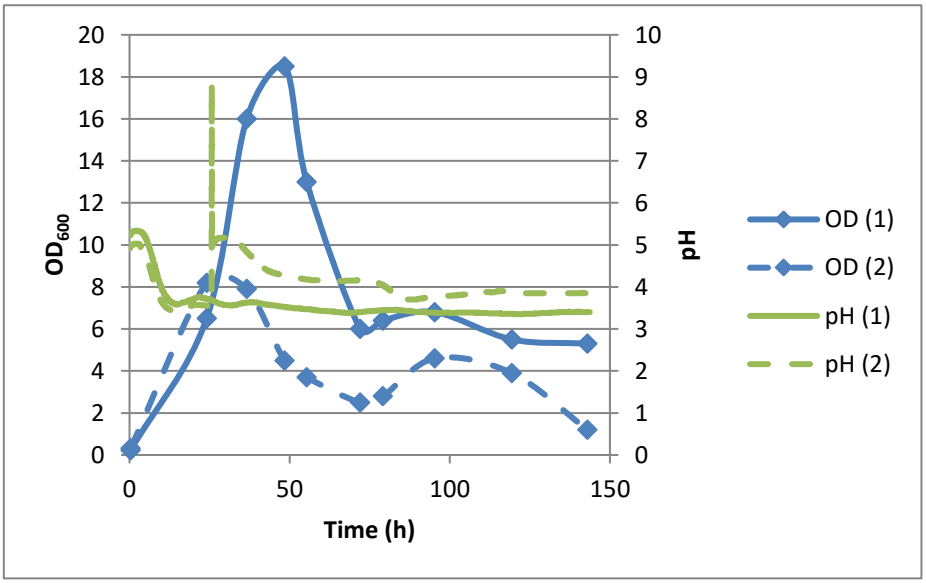


Figure 7 Growth of *C. acetobutylicum* in batch mode in a 1-L fermenter with production medium containing 54 g/L glucose at 37 °C, runs 2(1) and 2(2)

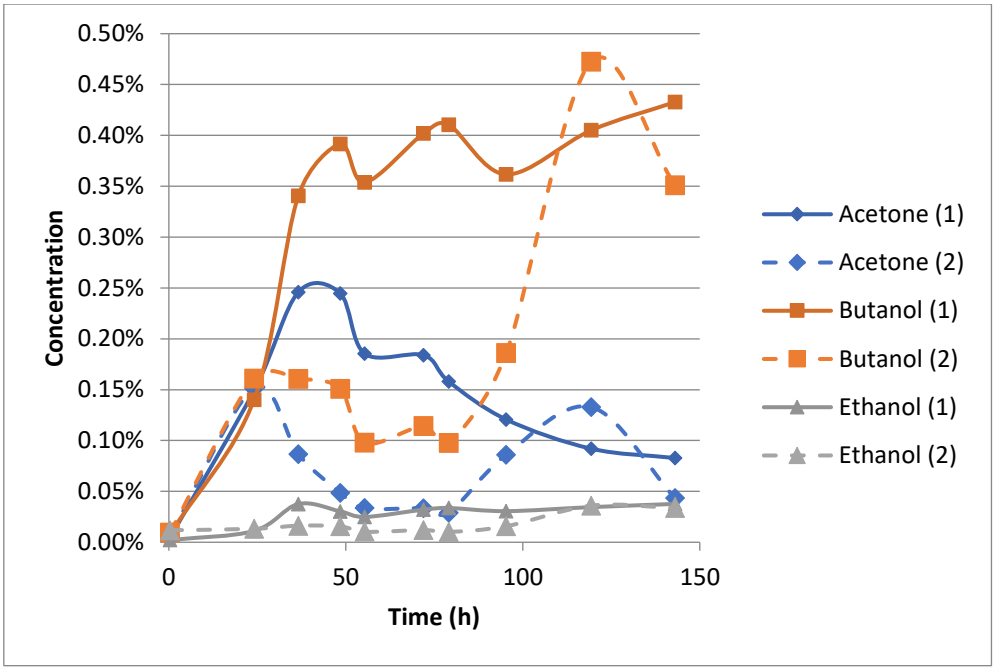


Figure 8 Solvent production of *C. acetobutylicum* in batch mode in a 1-L fermenter with production medium containing 54 g/L glucose at 37°C, runs 2(1) and 2(2)

The experiment ran smoothly, except for one deviation. The pH in 2(2) dropped to below 3.5, which was at risk of an "acid crash" (Maddox et al. 2000). Therefore, pH control using 3% aqueous ammonia was implemented in run 2, with a pH set point of 4.5 (refer section 1.6 and (Maddox et al. 2000)). The settings on the pH-control system (proportional-integral-derivative, PID) were set for a previous aerobic bacterial fermentation at pH 7 that responded differently than the present anaerobic *C. acetobutylicum* process. These tuning parameters were too aggressive for this system and the pH set-point of 4.5 was overshot; the pH of the culture went from 3.42 to 8.84 over the course of ca. 10 minutes. (The parameters were adjusted for future experiments and this event was not repeated.) In an effort to salvage the culture, a 3% solution of H_3PO_4 was filter-sterilized and manually added to the bioreactor, bringing the pH down to 4.96. Due to this difference in experimental conditions, the final results obtained from the two experiments will be discussed separately.

The contents of the bioreactor were a buffered solution: ammonium acetate was part of the medium formula, and acetic acid (pKa 4.76 in aqueous solution) is produced by *C. acetobutylicum*. Therefore, the behaviour of the fermentation batch, with a non-linear

response to base addition, was entirely in character. The pH control parameters used in later runs took this behaviour into account.

Biomass peaked at OD_{600} = 18 in 2(1) at 48 hours (Figure 7) and then declined, but with a slight bump around 100 hours. This is almost double the maximum OD from the previous experiment (Appendix A). Solvent production (Figure 8) was much lower than expected, with less than 0.5% final butanol concentration, as opposed to 0.8% which was obtained in the prior attempt at reproduction (Appendix A), or 1.2% which was reported in the previous work (Moreno et al. 2012). The level of acetone dropped over the course of the experiment, which had not been previously observed. A gas stripping effect may have been responsible, which will be discussed later in the section.

These differences between this fermentation and the 20-L run may be due to change in experimental conditions, or they may be due to inherent process variability. The primary differences between the 20-L run and this one were the size of the bioreactor (20-L vs. 1-L) and sterilisation method (steam-in-place vs autoclaved). These differences may have resulted in subtle process changes that affected culture performance, but it is also likely that there were differences in sample handling and preparation.

The butanol production in 2(2) was unexpected, given the pH deviation and subsequent delayed culture growth. The likely reason is that the pH shock did not kill all the cells, and so the fermentation could proceed as normal once the remaining cells recovered, but took longer to reach the point of maximum solvent production. The solvent production in this experiment reached the same level as in 2(1), but the OD did not. Along with the result in the 20-L fermentation, which reached a lower maximum OD of 10 but had a higher final butanol level at 0.8%, this seems to suggest that there may not be a correlation between OD and solvent production in a batch fermentation, or that other environmental factors contribute more to final solvent concentration.

3.3 pH control

Acid crash, the phenomenon where acids are over-produced resulting in complete cessation of fermentation, is known to happen sometimes in fermentations that do not use pH control (Maddox et al. 2000). In view of the potential for acid crash, it was determined that pH control should be used. The fermentations to test pH control ran for 4 days. Runs 3(1) and

3(2) were used as the control, with no pH control. Runs 3(3) and 3(4) were done with base pH control using 3% aqueous ammonia, and 3(5) and 3(6) with both base control and acid control using 3% phosphoric acid. Table 10 shows the graph designations corresponding to each fermentation run. Figure 9 shows the pH and biomass as a function of time, while Figure 10 shows the concentration of each solvent produced over the course of the experiment. The fermentations with both acid and base control had a setpoint of 4.5 ± 0.1, and the fermentations with only base control had a setpoint of 5.0.

Table 10 Run designations in pH control experiment (Experiment 3)

Run	Control used	Label in graphs
3(1)	None	None (1)
3(2)		None (2)
3(3)	3% NH_3(aq) to pH 5.0 ± 0.1	Base (1)
3(4)		Base (2)
3(5)	3% H_3PO_4(aq) and 3% NH_3(aq) to pH 4.5 ± 0.1	Both (1)
3(6)		Both (2)

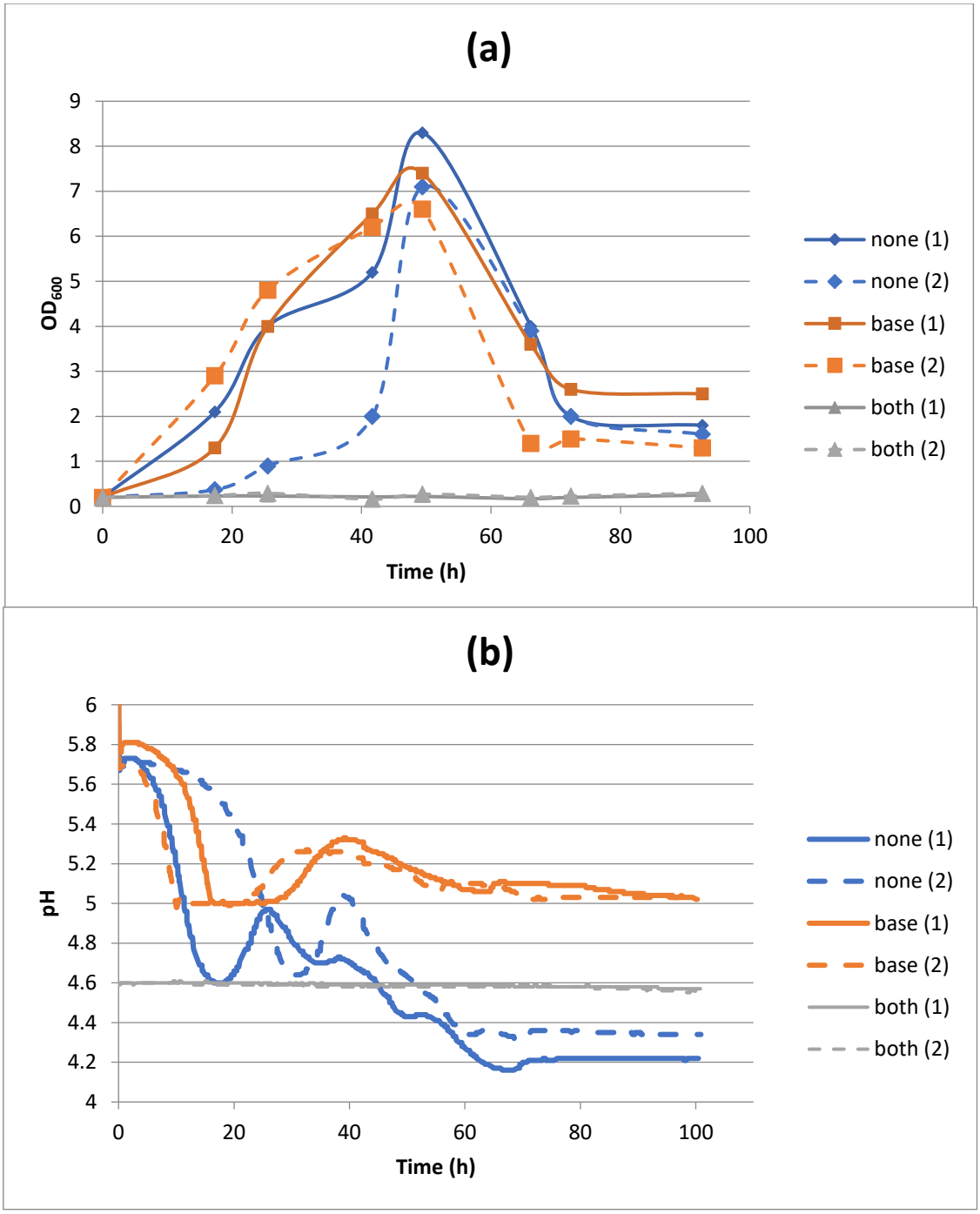


Figure 9 (a) OD_{600} of *C. acetobutylicum* fermentation in batch mode in a 1-L fermenter with production medium containing 54 g/L glucose at 37°C, with and without pH control (b) pH of the same fermentations

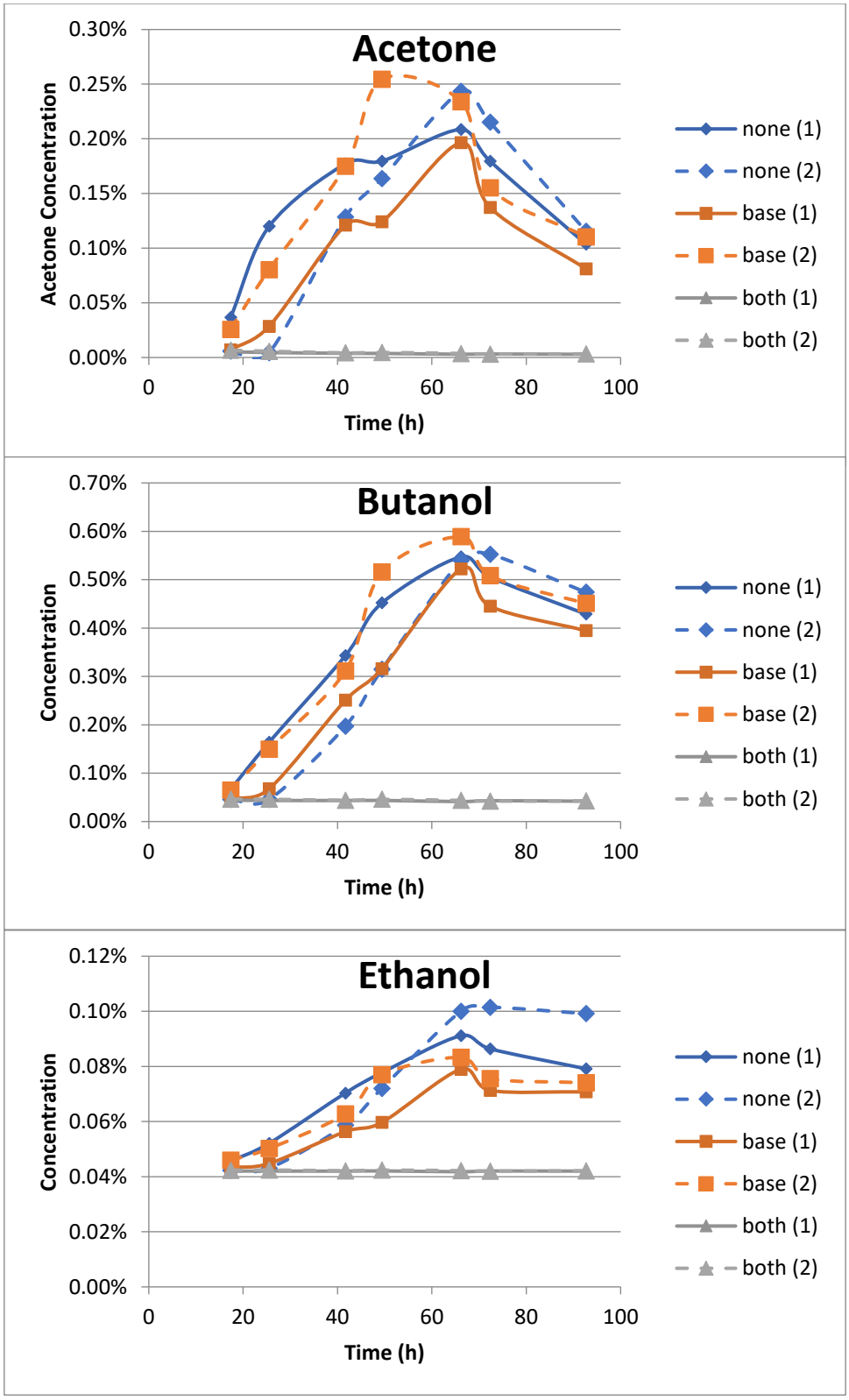


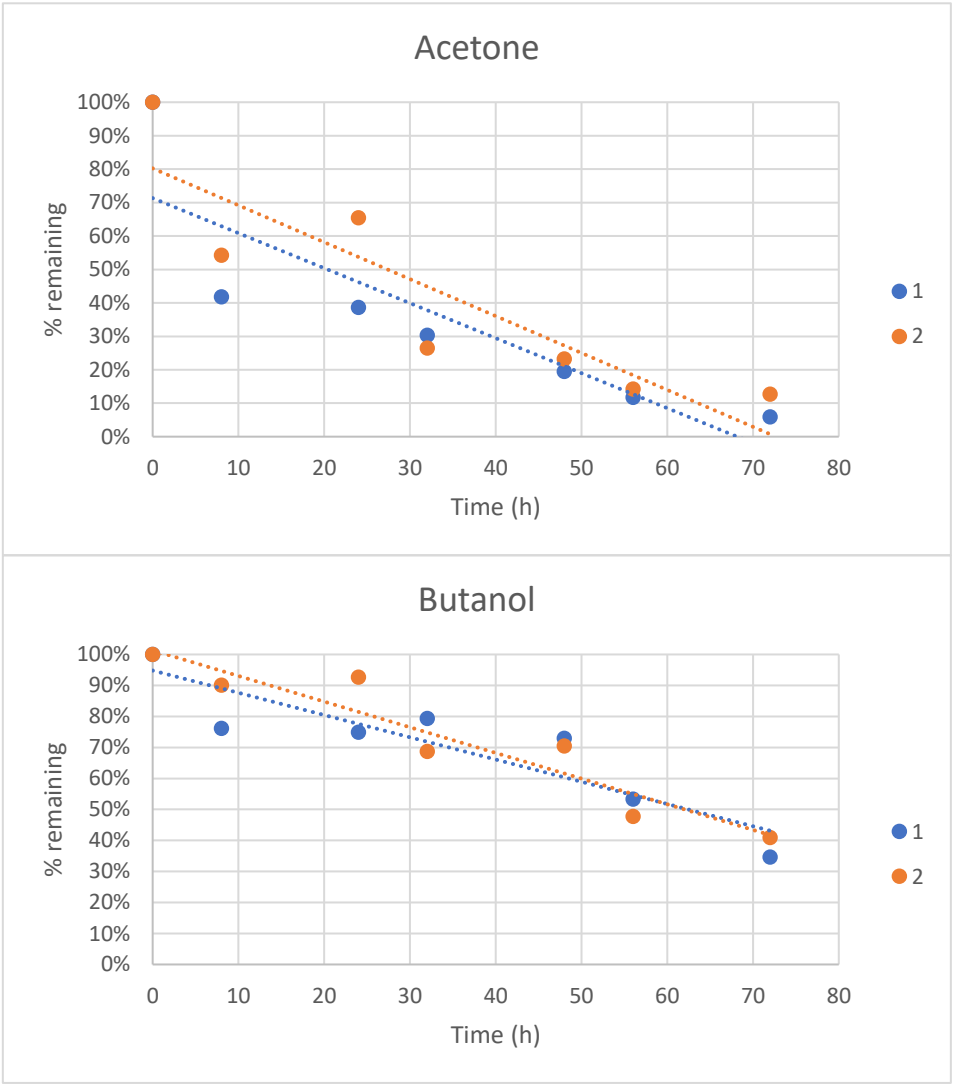
Figure 10 Solvents produced by *C. acetobutylicum* in batch mode in a 1-L fermenter with production medium containing 54 g/L glucose at 37°C, with and without pH control

The results of this experiment were mixed. The fermentations with the capacity of both acid and base pH addition did not grow at all, which may be a result of the pH adjustment made immediately before the inoculation. A pH of 4.5 is well within the standard pH range for *C. acetobutylicum* solvent production (Hubert Bahl et al. 1982), but it is possible that the acidification of the broth should have been carried out by the organisms themselves rather than externally to facilitate culture growth. While modification of production broth to a starting pH has been reported in the literature, the pH change there much smaller, with a similar medium and a final pH value of 6.0 rather than 4.5 (Jang, Malaviya, and Lee 2013). The fermentation labelled none (2), one of the control fermentations, was quite slow to start, which may be due to DO > 0% at the beginning of the experiment. This may have been caused by air diffusing into the line between the N_2 cylinder and the bioreactor, as silicon tubing was in use. Alternatively, the DO probe may have been miscalibrated; however, a poorly-calibrated probe would not explain the delay in growth as all vessels received the same N_2 sparing treatment irrespective of DO readings. The fermentation labelled base (1), a base control fermentation, also had slightly delayed growth (Figure 9a), but no cause was identified.

Other than differing growth profiles, all four runs that grew achieved similar OD values and solvent concentrations. The biomass peaked at between 6.6 and 8.3 at approximately 48 hours (Figure 9) and then declined. This represents a return to the lower maximum OD of ~10 that was seen in the 20-L fermentation, but the peak was reached at the same culture age as in the scale-down experiment. Although the peak biomass was lower, similar to Experiment 1, solvent production (Figure 10) was similar to that observed in Experiment 2 in the 1-L bioreactors (Figure 8), with approximately 0.5% final butanol concentration and about 0.25% acetone. Again, there was a drop in solvent levels at the end; however, this drop did not take place in the first experiment in the 20-L fermenter. It is possible that solvent evaporation was responsible for this drop, which will be discussed further in the next section. A pH setpoint of 5, with base control only, was chosen for all future experiments.

3.4 Solvent evaporation

The solvent concentrations obtained in the 1-L bioreactors were lower than expected from both literature and the 20-L reactors, at 0.5% instead of 1.2% (Moreno et al. 2012) or even 0.8% (Appendix A). It was hypothesised that the low solvent levels, and the solvent level drop at the end of some fermentations, were due to a gas stripping effect, as the reactors were sparged with nitrogen, but the 20-L reactor less so on a volume per volume per minute (vvm, litres sparging per litre fermentation per minute) basis. The 20-L fermentation had a rate of 0.05 vvm where the 1-L fermenters had 0.1 vvm. Thus, experiments were set up with a test solution (solvents in water, per section 2.1) as follows: 4(1) with N_2 sparging as in the previous experiments, 4(2) with gas flow into the headspace over the top of the "broth", and 4(3) with no gas flow at all, serving as the control. Gas flow was set to 0.1 vvm to mimic the previous experiments in the 1-L bioreactors. Results are shown in Figure 11.



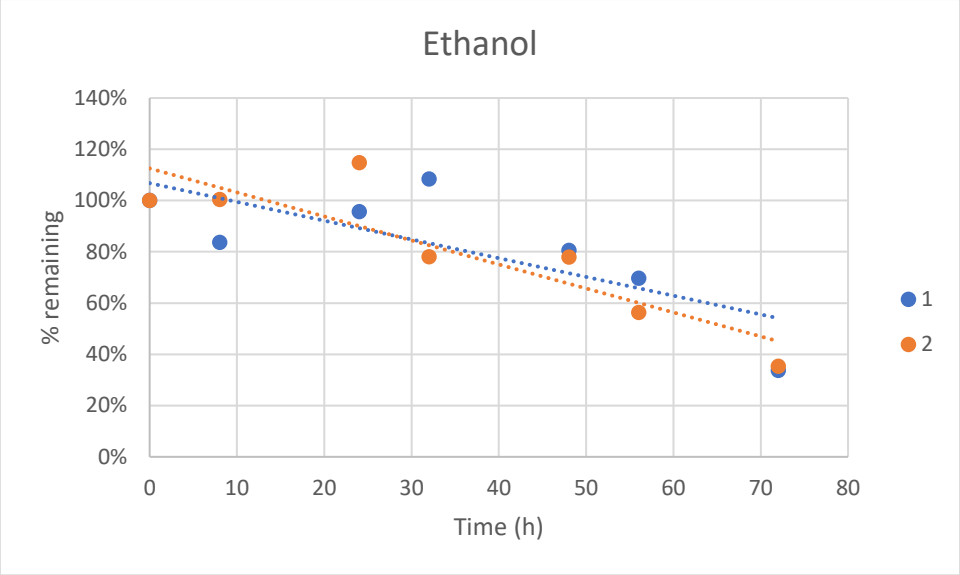


Figure 11 Solvent concentrations in bioreactors with gas flow (1) sparged (2) into the headspace, normalised against the reactor with no N_2 flow

Due to GC problems, accurate readings of solvent concentrations could not be obtained, but the trend is clear. As seen in Figure 11, the concentration of solvents in the bioreactors where gas was flowing through or over the fermentation broth decreased over time, compared to the one with no gas flow. The decrease in butanol concentration was less than the decrease in acetone concentration, which is expected since acetone is more volatile. (Henry's constant for acetone is ~2.5 x 10^{-1} mol m^{-3} Pa^{-1} under standard conditions, compared to ~1.3 mol m^{-3} Pa^{-1} for butanol and ~1.9 mol m^{-3} Pa^{-1} for ethanol (Sander 2015).)

Even with GC problems, it was clear from these results that any gas flow over or through the fermentation broth would have partially evaporated solvents as they were produced. This is not unexpected, as vapour extraction is a known way to overcome the obstacle of solvent toxicity (Maddox, Qureshi, and Roberts-Thomson 1995). This is likely the cause of the lower-than-expected solvent concentrations in Experiments 2 and 3, which used the same bioreactors. Experiment 1 in the 20-L bioreactor would have been affected by solvent evaporation too, though possibly to a lesser degree as the relative gas flow (vvm) was lower.

3.5 Media study

Different fermentation media were tested in small bottles to determine their impact on bacterial growth and solvent production consistency. The original fermentation medium (defined medium) was tested, as well as variants which added yeast extract, tryptone, or both in a 1:3 ratio respectively. Table 11 shows the graph designations corresponding to each fermentation run. Figure 12 shows the results from the first media study. The experiment was replicated (Table 12, Figure 13).

Table 11 Designations of labels in media study (Experiment 5.1)

Run	Contains	Designation
5.1(1)	Defined medium	Defined (1)
5.1(2)		Defined (2)
5.1(3)	Defined medium + 5 g/L yeast extract	YA (1)
5.1(4)		YA (2)
5.1(5)	Defined medium + 5 g/L tryptone	Tryptone (1)
5.1(6)		Tryptone (2)
5.1(7)	Defined medium + 1.25 g/L yeast extract + 3.75 g/L tryptone	3:1 (1)
5.1(8)		3:1 (2)

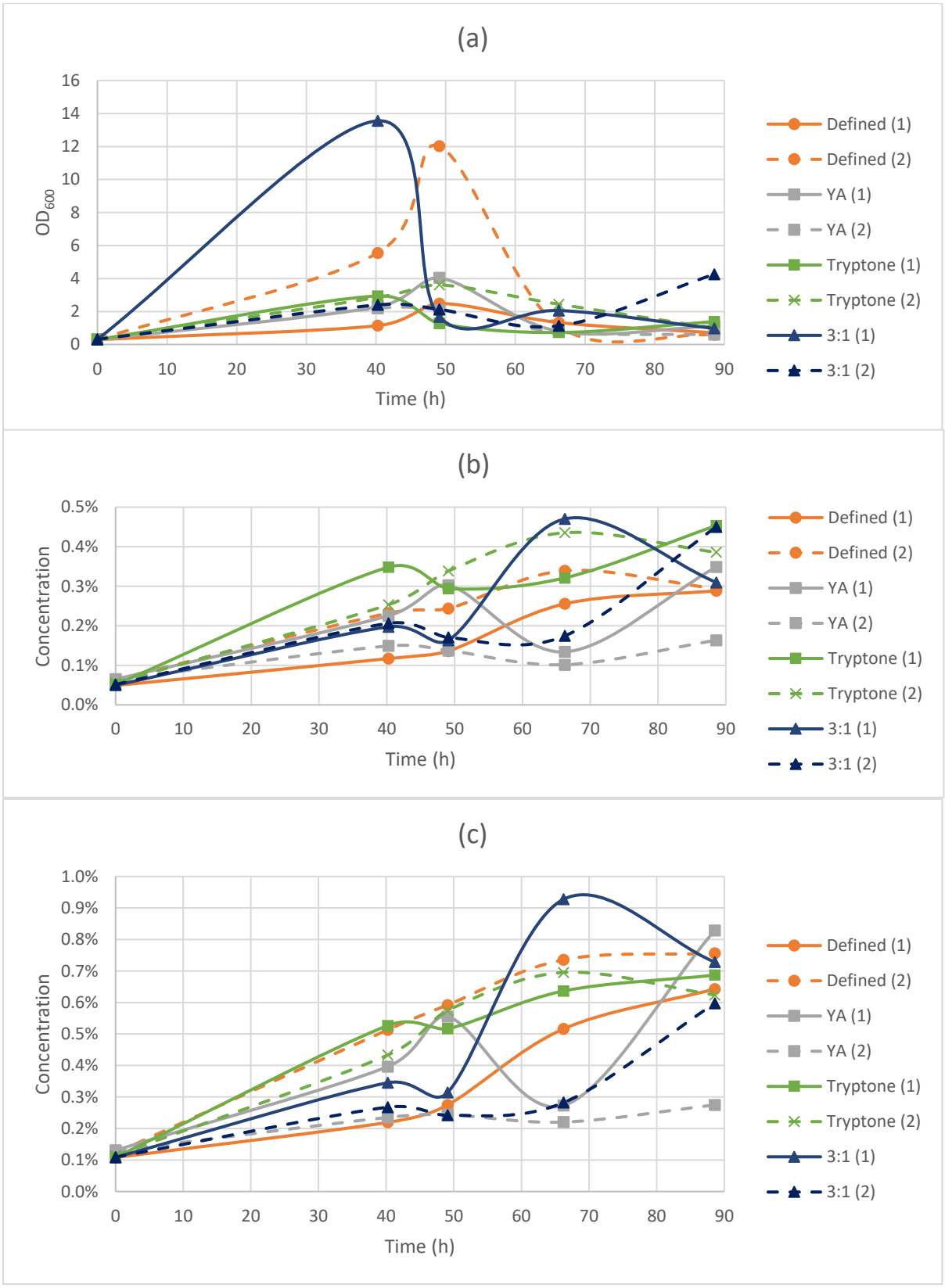


Figure 12 (a) Growth, (b) acetone and (c) butanol of Experiments 5.1(1-8), conducted in 20-mL bottles with varying media as described in Table 11

In both runs, the defined seed medium had a lower OD in the final state, so more of it was added to production so that the initial OD of production runs were the same (OD_{600} = 0.3 ± 0.03, exact amounts added not measured). Results in Experiment 5.1 were variable. OD increased and then decreased, a growth pattern that was seen previously also. Additionally, with only one sample per day, identifying the peak values was difficult. The OD had little or no effect on the production of solvents.

In Experiment 5.2, some of the bottles failed to grow at all, despite being inoculated from seeds that were confluent. In addition, of the bottles that grew, those containing defined media (inoculated from defined seed medium) grew earlier than those containing complex media, unlike the previous run. It was found that the choice of medium did not significantly affect the occurrence of growth.

Table 12 Designations of labels in media study (Experiment 5.2)

Run	Contains	Designation
5.2(1)	Defined medium	Defined (1)
5.2(2)		Defined (2)
5.2(3)	Defined medium + 5 g/L yeast extract	YA (1)
5.2(4)		YA (2)
5.2(5)	Defined medium + 5 g/L tryptone	Tryptone (1)
5.2(6)		Tryptone (2)
5.2(7)	Defined medium + 1.25 g/L yeast extract + 3.75 g/L tryptone	3:1 (1)
5.2(8)		3:1 (2)

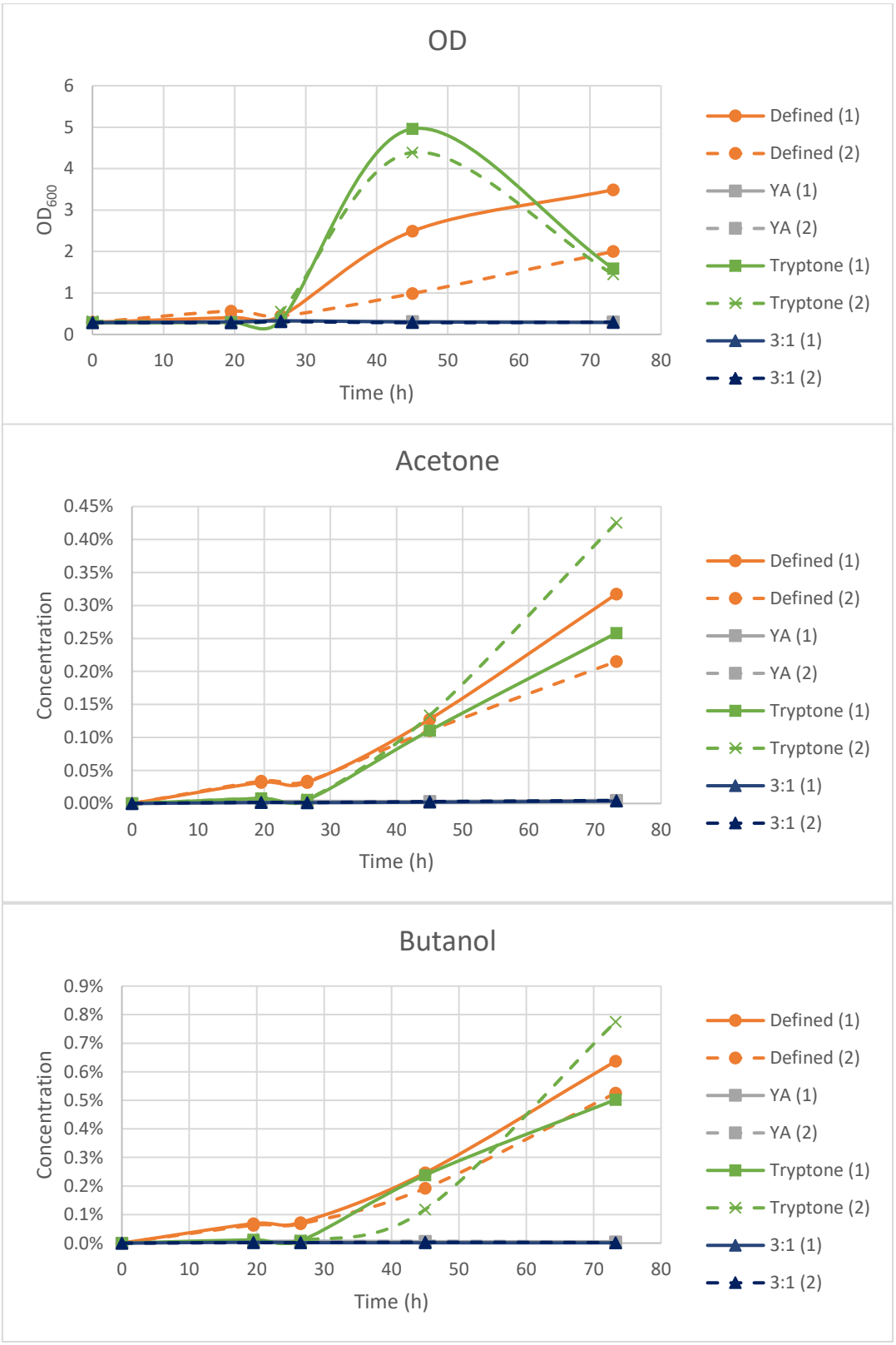
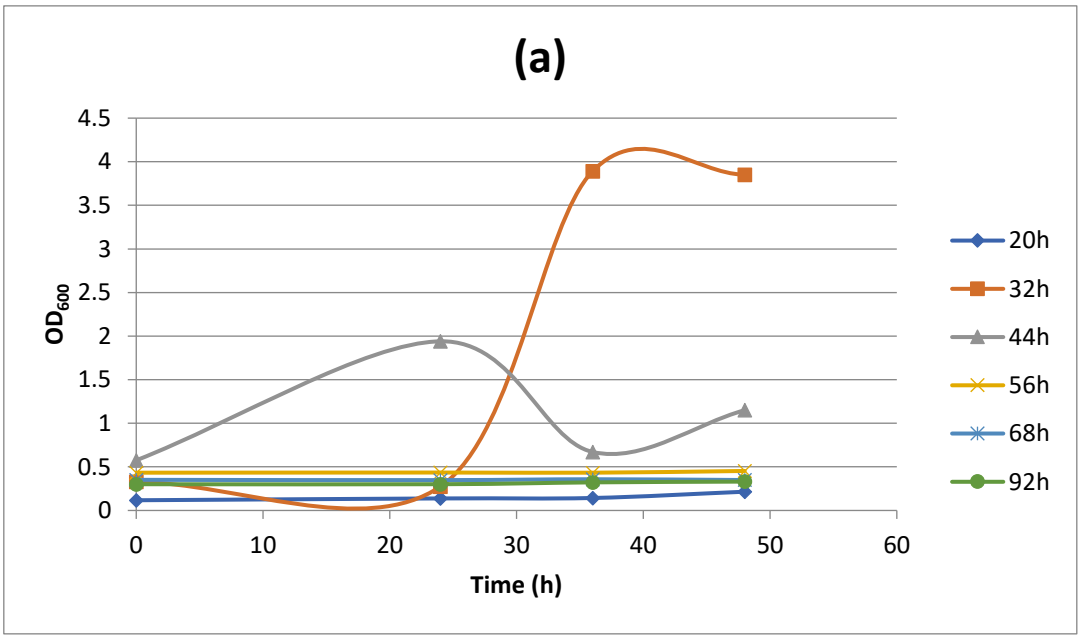


Figure 13 (a) Growth, (b) acetone and (c) butanol of Experiments 5.2(1-8), conducted in 20-mL bottles with varying media as described in Table 12

3.6 Seed study

It was hypothesised that seed age could affect growth in the production environment. This hypothesis was based on the results of the second media study (Experiment 5.2) where only two production bottles grew, both relatively late, indicating that the seeds had likely grown past a viable window. To test this hypothesis, Experiment 6.1 was conducted in 100-L bottles inoculated with the same seed at 12-hour intervals. Runs were not taken to completion, which is defined as no further solvent production; cultures were grown only to the conclusive presence or absence of growth. For the purposes of this project, late growth in production is not a desired result and is counted as a failed run, since a time lag would reduce the commercial viability of the process. Butanol is taken as representative of solvent production. The results of the first experiment (6.1) are shown in Figure 14.



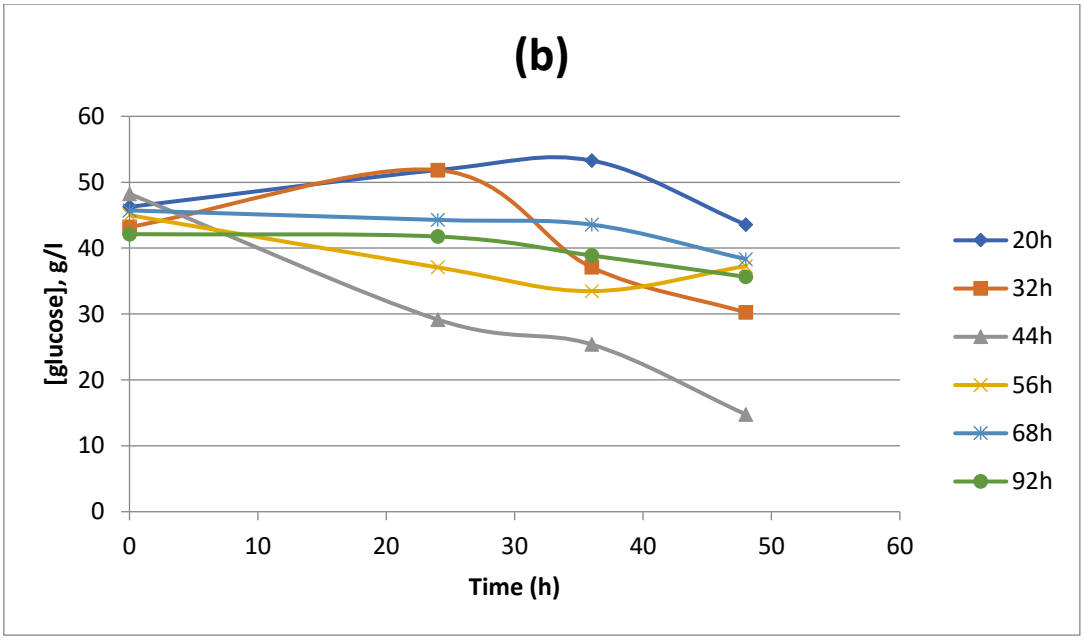


Figure 14 (a) Growth (b) residual glucose in Experiment 6.1, conducted in 100-mL Duran bottles with media inoculated at varying times from the same seed

For Experiment 6.1, production bottle inoculations were extracted from the seed bottle at ages from 20 h – 92 h. The production bottles inoculated with 20-h old seed did not grow visibly in 48 hours. Since the OD_{600} of the seed at inoculation was 0.053, it is likely that the seed had not completed its lag phase of growth yet, and that lag phase continued after the inoculation. In contrast, for the 32- and 44-h inoculations, the seed had visibly grown to an OD of 2.16 and 4.36 respectively. Many bubbles also came out of solution when the inoculation took place. It is known that *C. acetobutylicum* produces gases as it grows, which are sometimes used for gas stripping of solvents during production (Xue et al. 2012). In inoculations at 56-h or older, bubbles were absent.

The study was repeated with a new seed in Experiment 6.2 (Figure 15). In the second run, production cultures from the 20-h inoculation grew, in spite of the seed lag phase apparently not yet being complete (OD_{600} = 0.083). The 44-h inoculation took longer to start growing. Solvent concentrations were analysed on all the runs that grew (Figure 16).

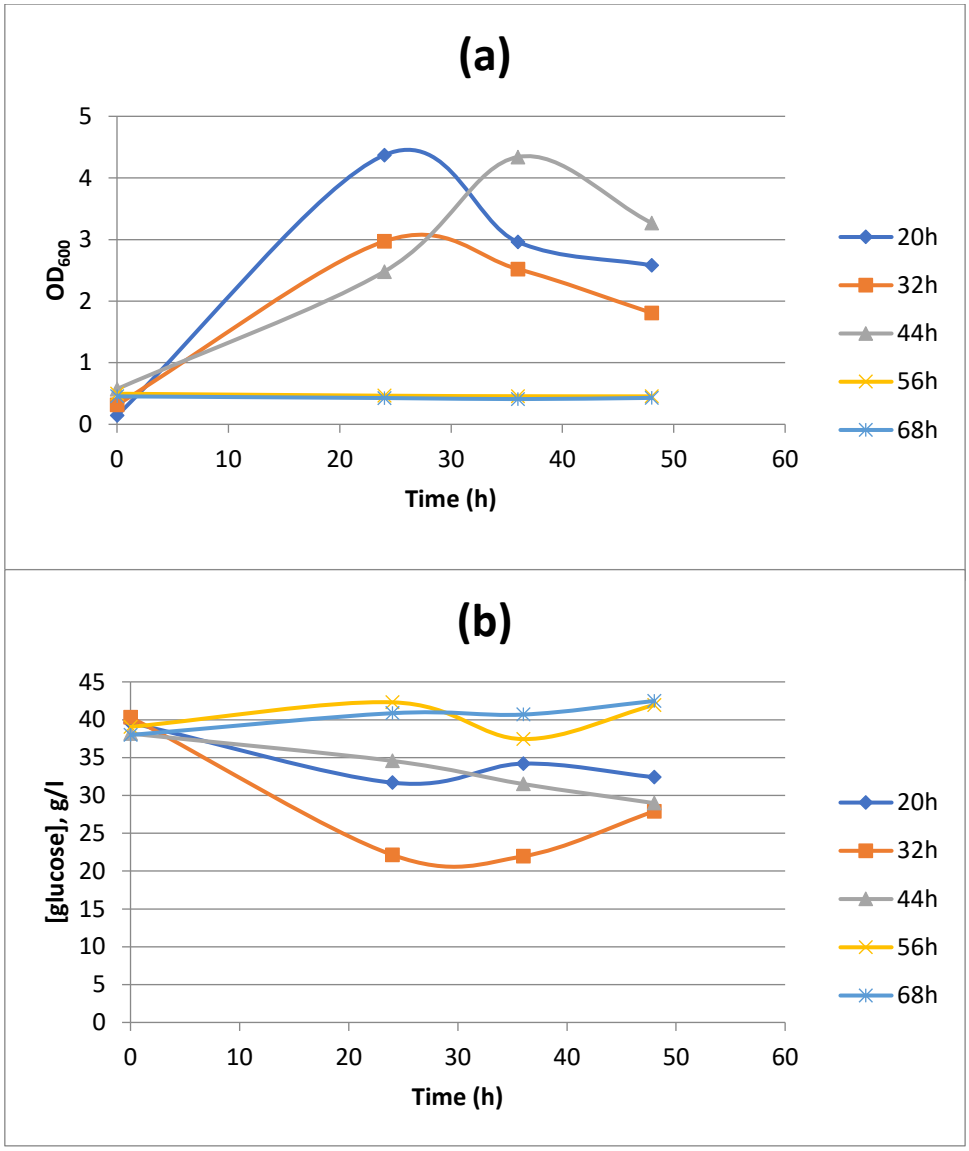


Figure 15 (a) Growth (b) residual glucose in Experiment 6.2, conducted in 100-mL Duran bottles with media inoculated at varying times from the same seed

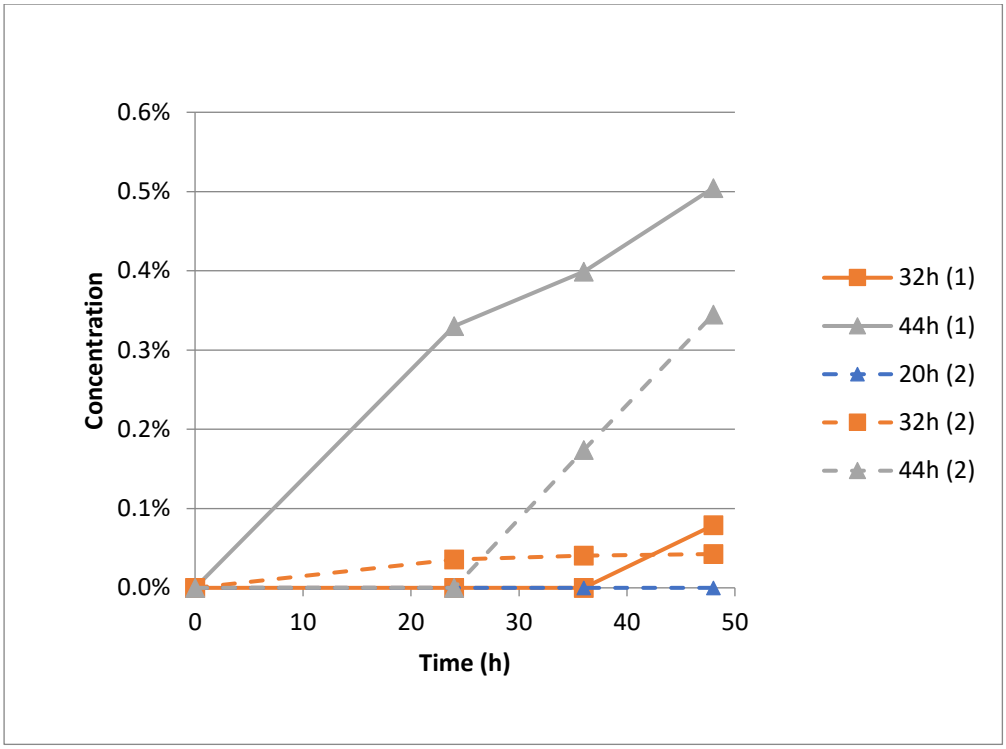
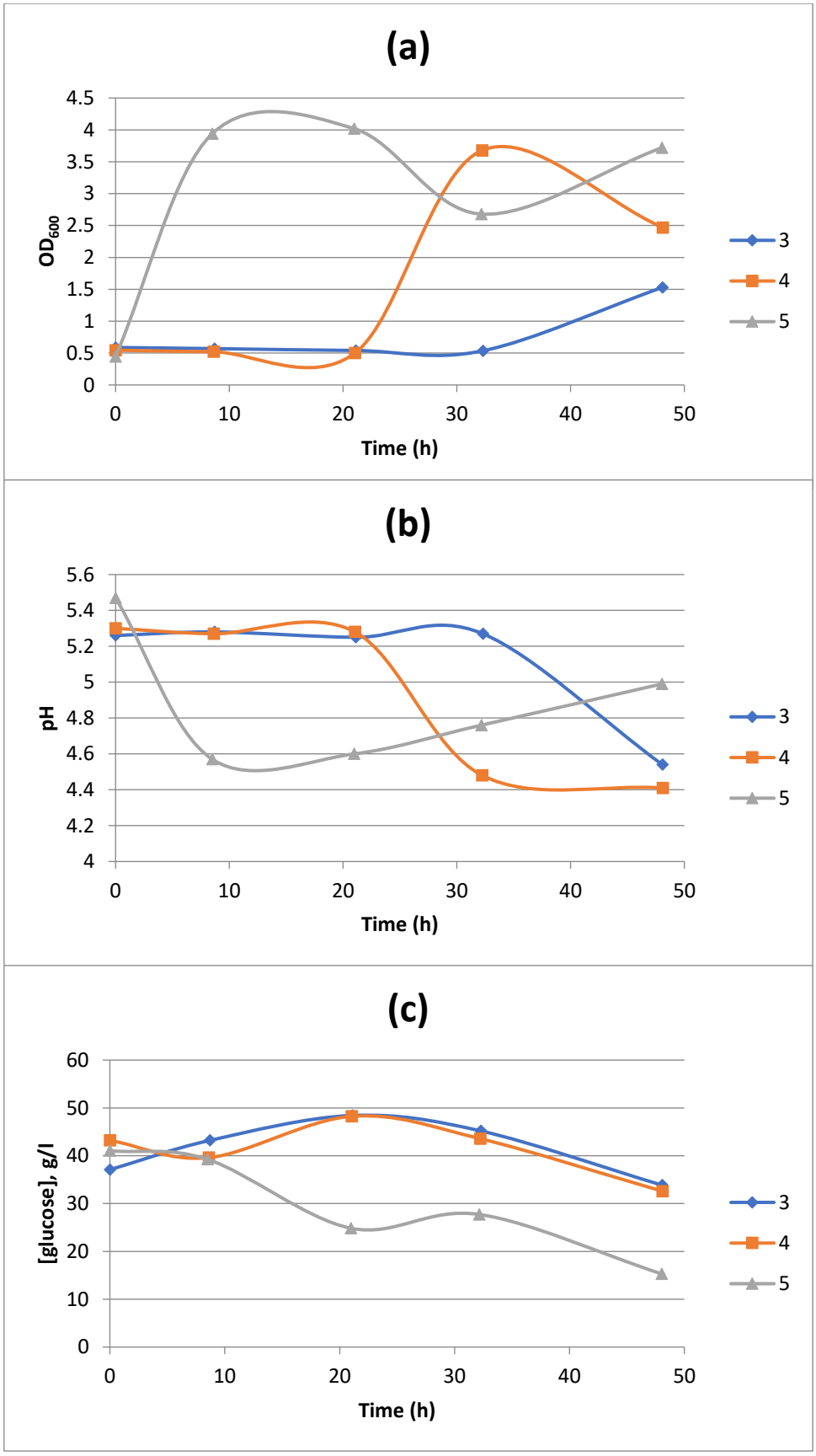


Figure 16 Butanol concentration in the inoculations at different seed ages that grew in production medium

Although 20(2) produced biomass, it did not produce butanol during the time frame of sampling. While the 32-h inoculations appeared to grow better, both in terms of biomass and glucose consumption, this did not correlate with solvent production. In fact, it may even have a negative correlation, as the 32-h inoculations produced less solvents by the end of the experiment despite higher glucose consumption and biomass production.

With the results of part 1, the window for successful inoculation was variable, but tended to lie between 20-44 hours. Confirmation of this hypothesis was sought with three separate but identical seeds grown for 36 hours and then used to inoculate production media bottles. (Figure 17)


(a)
OD600
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
Time (h)
3
4
5
(b)
pH
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
0
10
20
30
40
50
Time (h)
3
4
5
(c)
[glucose], g/l
60
50
40
30
20
10
0
0
10
20
30
40
50
Time (h)
3
4
5

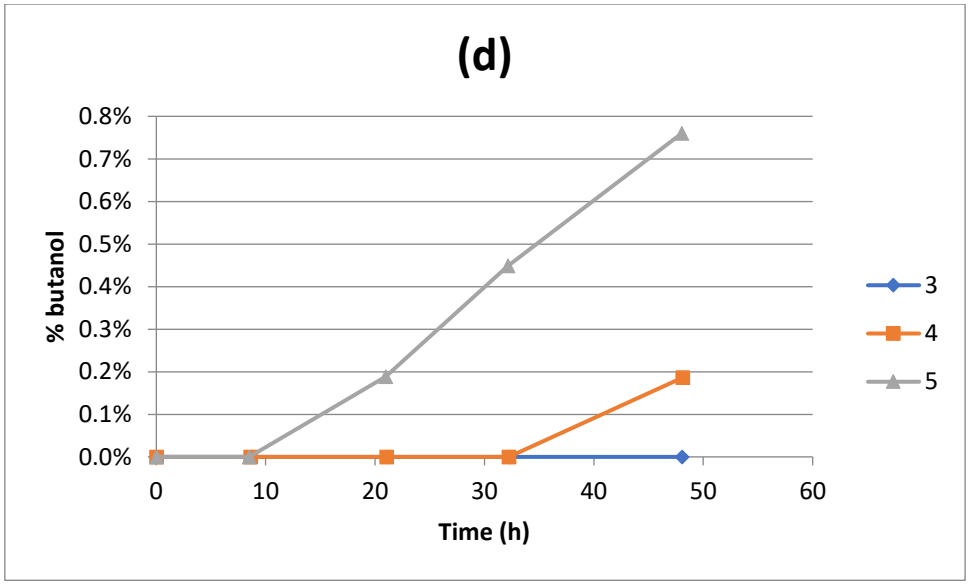


Figure 17 Results of inoculating seeds 3, 4 and 5 into production bottles at 36 hours (a) OD_{600} (b) pH (c) residual glucose (d) butanol concentration

The glucose concentration of seeds 3 and 4 at the time of inoculation (36 h) was in the range of 2 g/L, whereas seed C had 11 g/L (Table 6). Also, the pH of seeds 3 and 4 at this time were about 4.3, whereas seed 5 had a pH of 4.7. These data are consistent with seeds 3 and 4 having grown past the inoculation window, resulting in a slower start to growth in production since most cells in the inoculum were not active, either dead or already sporulated. Solvent production (Figure 17d) supports this hypothesis as well, with butanol production following cell growth. On further examination of the data (Table 13), there are some correlations between production performance and seed culture pH and glucose concentration.

Table 13 Seed conditions at time of inoculation for the runs that grew

Seed age(seed #)	OD	pH	[glucose], g/l
32h(1)	2.16	4.77	13.6
44h(1)	4.36	4.33	2.8
20h(2)	0.083	6.21	13.0
32h(2)	2.19	4.77	11.9
44h(2)	4.06	4.23	4.0
36h(5)	2.81	4.68	12.6

There appears to be a wide range of conditions that may lead to growth in production. However, there are also production runs which were seeded with cultures exhibiting similar properties but which failed to grow. The six production runs which grew were inoculated with seeds between 20-44 h inclusive (Table 13), indicating an upper seed age limit below 56 h (Figure 14). Butanol production is faster the older the seed is when inoculation takes place.

Two key criteria were identified: pH = 4.8 ± 0.7 and [glucose] = 9.6 ± 4.9 g/L. Seeds with values outside these ranges result in variable production growth, as in 44 h (1, 2) and 36 h (4). Run 20 h (2) was an exception with an unusually high pH and low OD, yet grew in production. The aim of this experiment is to produce reliable growth, so only reliable combinations of pH and glucose concentration are taken.

Based on these observations, a production culture will reliably grow if inoculated with a seed culture at pH = 4.8 ± 0.7 and glucose at 9.6 ± 4.9 g/L. Seed cultures with conditions outside these ranges yielded less consistent results (Table 14).

Table 14 Examples of comparable runs which did not grow

Run	OD	pH	[glucose], g/l	Compare with
20 h (1)	0.053	6.13	19.4	20 h (2)
36 h (4)	3.74	4.31	2.5	44 h (1, 2)
68 h (2)	2.95	4.29	2.3	44 h (1, 2)

Since inoculation age did not correlate with successful solvent production, but there were consistent patterns for which seeds grew, another set of seeds (Figure 18) was tested in Experiment 6.4 and inoculation carried out when the pH reached 4.8 ± 0.7.

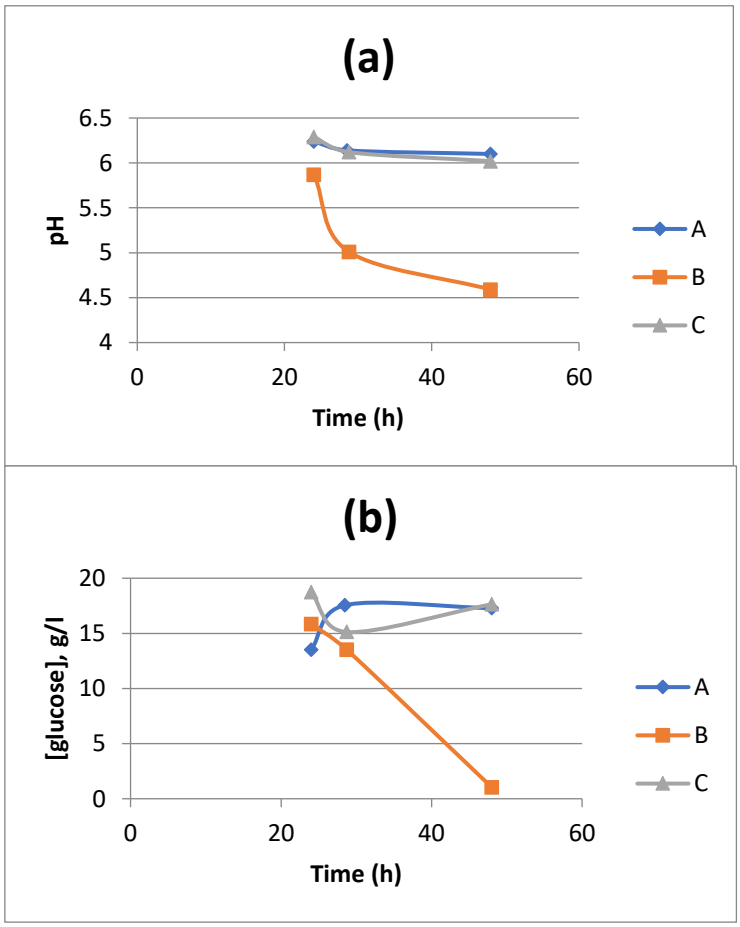


Figure 18 Seed study confirmation test (a) pH of seeds, (b) residual glucose in seeds in Experiment 6.4

One seed of three grew, which was unexpected as all seed cultures inoculated from stock glycerol vials had previously grown. The seed culture that grew was transferred to production once the pH achieved a suitable level at t = 29 h (pH 5.01, glucose 13.5 g/L). Another inoculation was performed at t = 48 h (pH 4.59, glucose 1.0 g/L). Production cultures inoculated from both time points grew well (Figure 19).

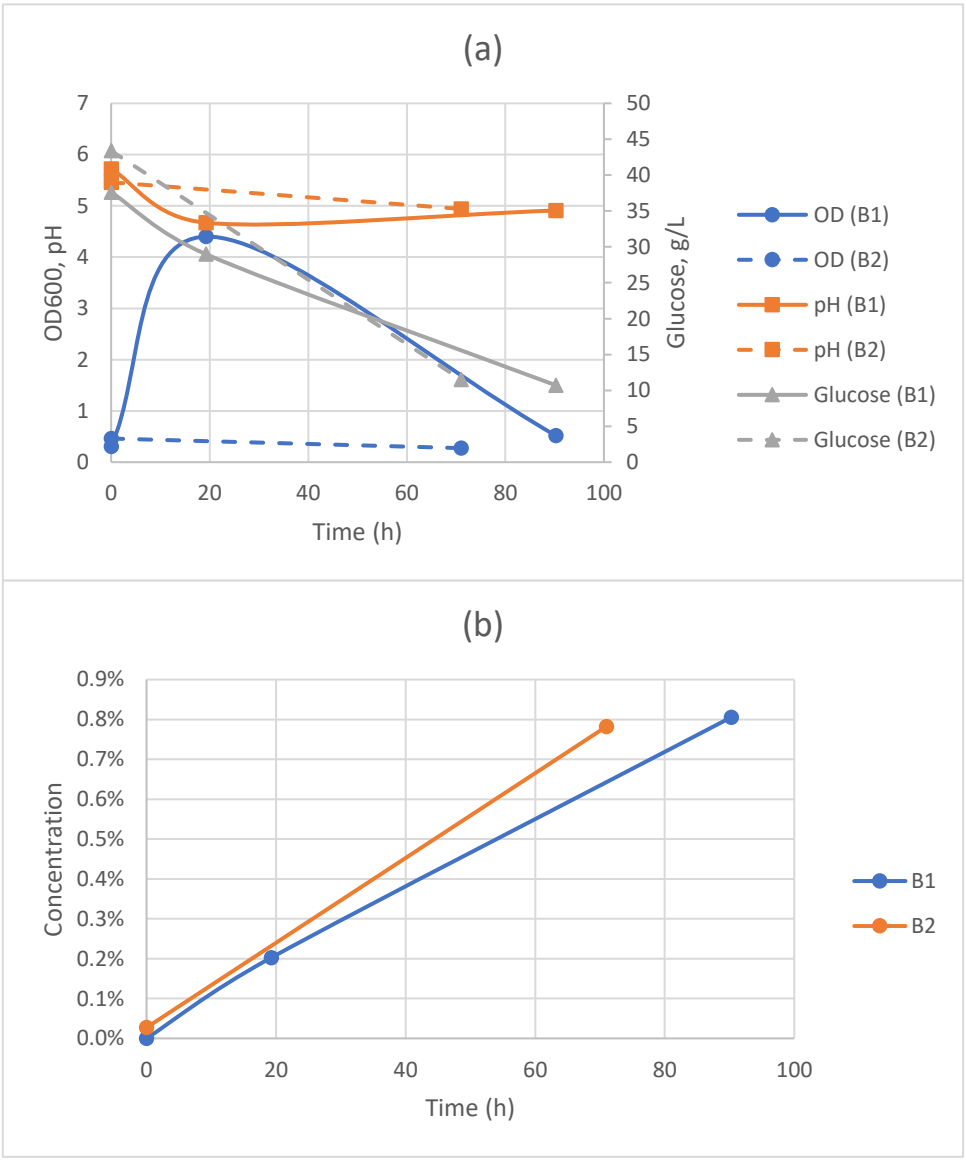


Figure 19 (a) Growth and (b) butanol production results for the production runs inoculated from seed B at 29h (B1) and 48h (B2) in Experiment 6.4. The OD_{600} (B2) graph is accurate – while the seed visibly grew, there were no measurements taken at the time when the OD would have been higher.

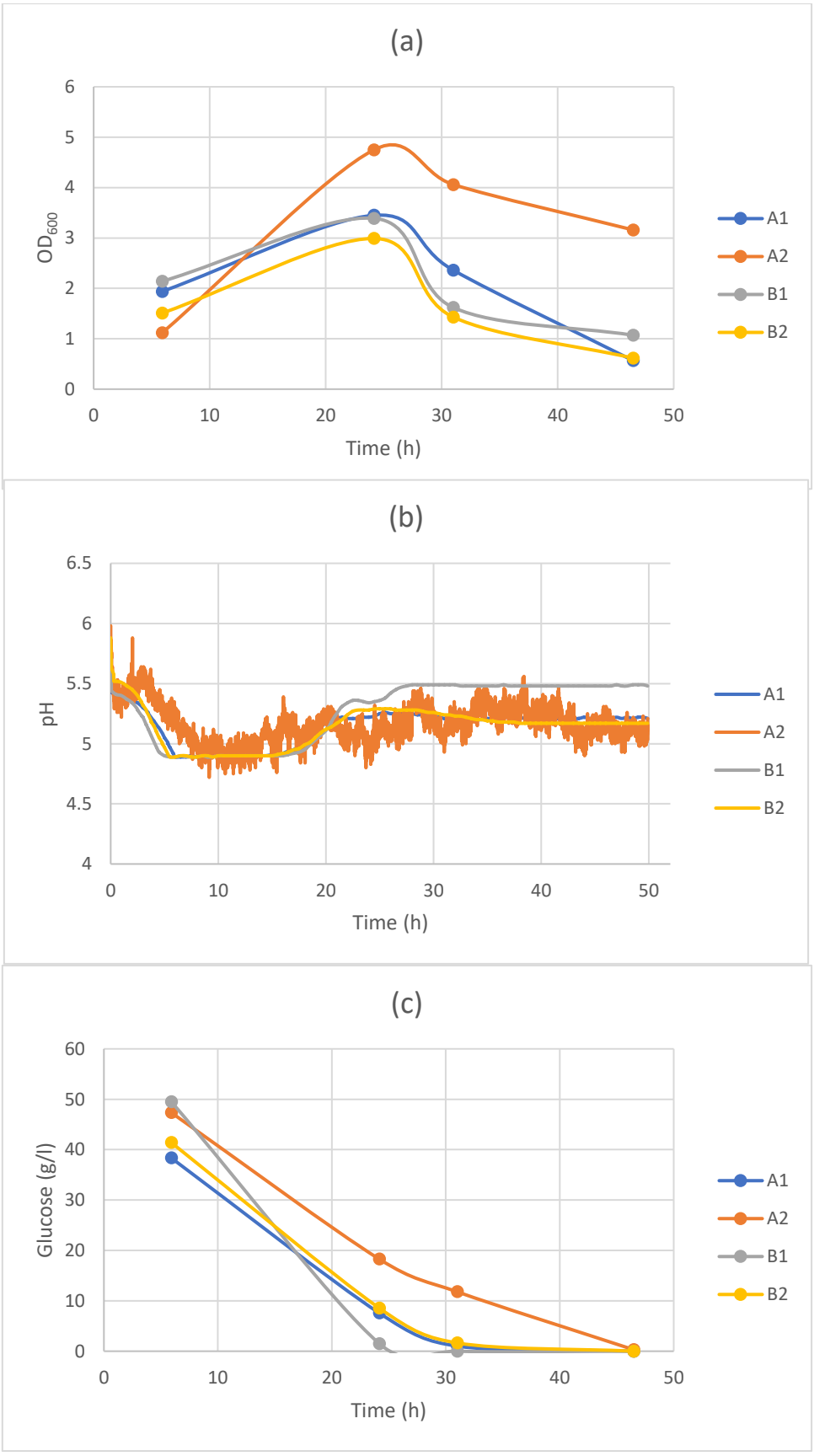
From these experiments, it can be concluded that the appropriate seed age for inoculating production medium was variable (20-44 hours); the upper end of this age range may be better for solvent production but runs the risk of falling outside the target pH and glucose ranges to be productive. The window is also variable enough that choosing a set time point for inoculation is not useful. Instead, testing is conducted on seeds that have visibly grown, to ensure that they remain within the inoculation window.

It is logical that slow growth during the seed culture would make the inoculation window longer, so there is a possibility of using defined medium instead of complex medium for the seeds. In the previous study (media study), seeds did not grow as quickly or as well on defined media, which is why a complex medium was chosen; however, they would reach the defined parameters eventually. Complex medium can still be used for production, and the seed being in defined medium should not have an adverse effect on production.

Experiment 6.5 was designed to determine whether the type of seed medium has any effect on production performance in complex medium, as well as to validate the seed inoculation criteria in bioreactors rather than bottles. Complex and defined media seeds were tested (Table 15). FER_A1 and A2 were inoculated at seed pH 4.18, glucose 4.7 g/L with seeds grown in a complex medium (having unfortunately passed by the target range overnight), while B1 and B2 were inoculated at seed pH 4.42, glucose 12.1 g/L with seeds grown in a defined medium. Figure 20 shows the results of the experiment. There was a minor anomaly in pH control for A2, which can explain the slightly differing result: a noisy signal would result in too much, too little, or wrong timing of base addition, thereby affecting growth. Aside from that process deviation, the fermentations all ran smoothly.

Table 15 Designations for Experiment 6.5 in seed study

Designation	Seed	Seed pH	Seed glucose	Fermenter
6.5 (1)	Complex	4.18	4.7 g/L	FER_A1
6.5 (2)				FER_A2
6.5 (3)	Defined	4.42	12.1 g/L	FER_B1
6.5 (4)				FER_B2


(a)
OD600
Time (h)
A1
A2
B1
B2
(b)
pH
Time (h)
A1
A2
B1
B2
(c)
Glucose (g/l)
Time (h)
A1
A2
B1
B2

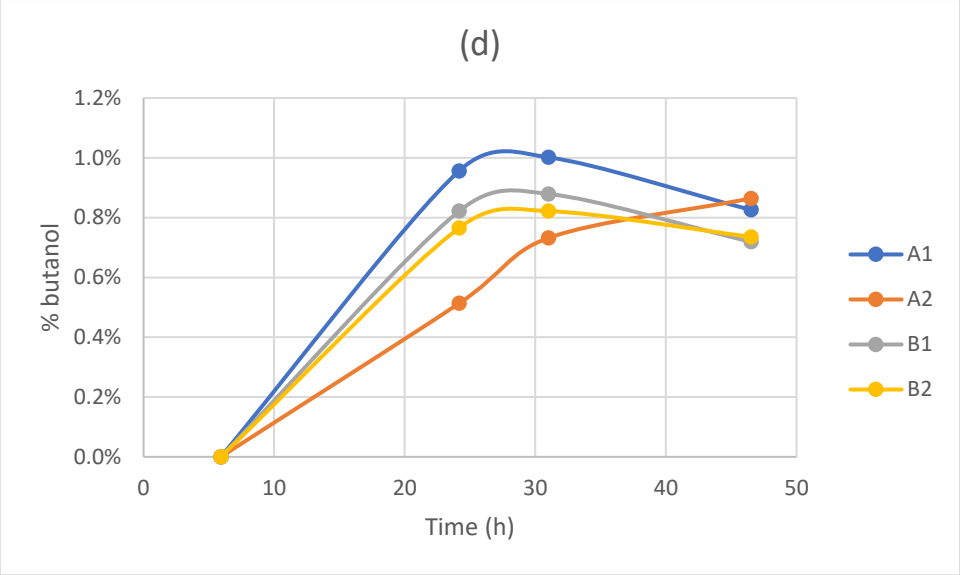


Figure 20 (a) OD_{600} (b) pH (c) glucose concentration (d) butanol production of *C. acetobutylicum* fermentation in batch mode in a 1-L fermenter with complex production medium containing 54 g/L glucose at 37°C, seeded with complex (A1, A2) and defined (B1, B2) seeds meeting the criteria determined in Experiments 6.1-6.4

3.7 Filtered medium

Feed medium for continuous runs, made in 10-L and 20-L bottles, requires a large amount of time (over 4 hours) in the autoclave for a 20-minute run at 121 °C. Besides being inconvenient to scale up further, the effects on the medium of prolonged exposure to heat are not known. Colour is one: before steam sterilisation, the medium is a pale yellow colour; after steam sterilisation, it is brown, with the darkness of the colour directly correlated with time spent in the autoclave. Thus, sterile filtration was tested as a means of sterilising the feed medium before use. (Figure 21) The test was run in batch mode with filtered production medium, using seeds that had been grown in filtered seed medium. Filtered media are in all respects identical to steam-sterilised media except for the means of sterilisation.

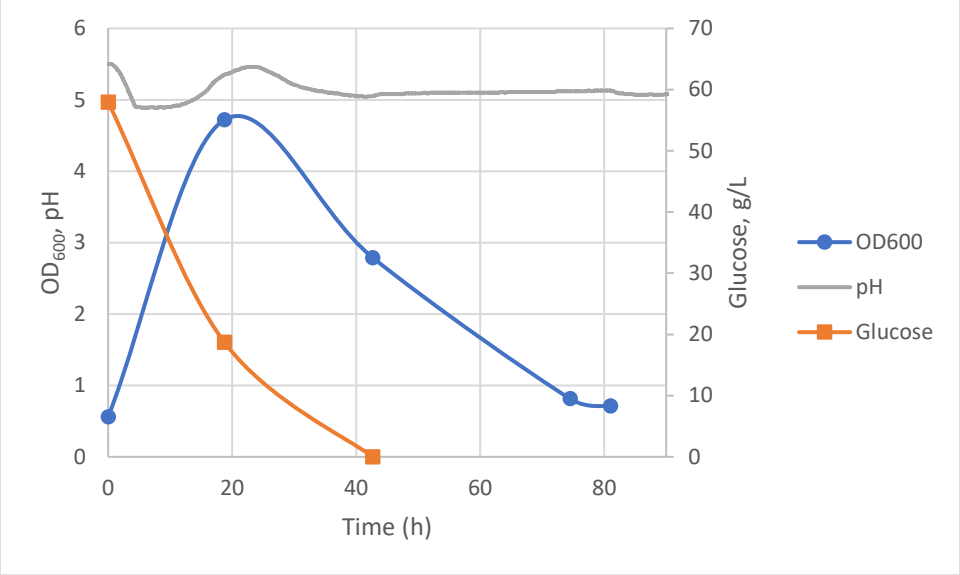


Figure 21 Results of filter sterilisation test, Experiment 7: *C. acetobutylicum* fermentation in batch mode in a 1-L fermenter with filtered complex production medium containing 54 g/L glucose at 37 °C

The fermentation ran smoothly. It was concluded that filtering the medium is an acceptable method of sterilisation, producing results that were comparable with steam sterilisation.

3.8 Summary

The goal of this thesis was to build upon previous work and to construct a continuous system for the production and extraction of ABE in the laboratory. A robust batch fermentation of *C. acetobutylicum* was necessary as a prerequisite to developing a continuous fermentation process. Conditions testing was carried out to establish the required experimental conditions for a reliable and robust batch fermentation process.

In the laboratory, the 20-L fermenter that was used in the previous work was too large for a continuous fermentation. At 20-L scale, even a dilution rate of 0.2 h^{-1} required almost 100-L of feed medium a day, all of which would require sterilisation before and after followed by disposal. Continuous fermentations of *C. acetobutylicum* can reach up to 0.85 h^{-1} dilution rate with cell recycling, or 0.26 h^{-1} without cell recycling. Besides the impractical volumes for laboratory-scale work, there was a lack of equipment available to conduct a

continuous fermentation at that scale. Thus, the experiment was scaled down to run in 1-L fermenters.

It was established that the batch fermentation could run at 1-L scale. With the smaller volume, a gas stripping effect was observed. However, the total impact of gas stripping on solvent concentrations was difficult to quantify, and the escaped gases were impossible to recover with the current experimental setup. Instead, consistent experimental conditions were used across all experiments. It was assumed that evaporation across experiments would be equivalent, and therefore that the results of experiments would be comparable.

Due to the potential for acid crash, it was determined that the system should be pH controlled. It was established that the PID settings needed to be conservative, as the buffered system had a nonlinear control response. Controlling pH at 5.0 with either 3% aqueous ammonia or 1 M sodium hydroxide produced good results, though some literature indicated that 4.3 is optimal (Hubert Bahl et al. 1982). The pH set-point of 5.0 was chosen as several acid-crash fermentations had reached a final crash value at 4.5, and pH 5.0 was still low enough to obtain good solvent production results (Maddox et al. 2000; Monot, Engasser, and Petitdemange 1984). Acid addition was not necessary and may be counterproductive if applied too early, as it acidifies the broth to the point where *C. acetobutylicum* cannot grow, which opens up potential avenues for failure in case of unexpected events. As seen in Experiments 2 and 3 (scale-down and pH control), the use of pH control affects neither the speed of growth nor the final solvent production levels, which is in agreement with the literature for pH control at and below 5.0 (Monot, Engasser, and Petitdemange 1984).

Inconsistent growth was observed during the scale-down and pH control experiments, and therefore the decision was made to investigate the fermentation medium. A defined medium had previously been used (Monot et al. 1982). It was established that the presence of complex components in the medium (yeast extract or tryptone) affected the growth rate and final density, but not the occurrence of it. The sample size was not large enough to conclusively determine the effect of complex components on solvent production. In the literature, a 3:1 tryptone:yeast extract mixture was optimal for butanol production (Al-Shorgani et al. 2016). The effect of adding any complex component was visible in that extra defined seed medium had to be added to production to reach the same initial OD, whereas differences between media with different complex components were not apparent. Therefore,

5 g/L yeast extract was added to the seed and production media in all further experiments. This concentration was chosen because of its prevalence in the literature (Jang, Malaviya, and Lee 2013; Li et al. 2011), and because it preserved the benefits of adding complex components with minimal cost increase.

The condition of the seed culture upon inoculation to production medium appeared to be crucial. When seed culture age was first investigated, the time window for inoculating *C. acetobutylicum* production medium from the seed culture was variable. Therefore, choosing a fixed seed age for inoculation is unreliable. Rather than relying on culture age, the pH and residual glucose in seed cultures were found to be better criteria for inoculation of production medium. These criteria were demonstrated to be reliable in batch culture, and subsequent production runs were much more consistent in growth.

Sterile filtration was shown, in batch production, to be as effective as steam-sterilisation for the sterilisation of media for production. Filtered medium was subsequently used for the feed in continuous fermentations. Bioreactors already containing production medium, which was used to start the fermentation before changing to continuous operation, continued to be autoclaved.

By incorporating all these results, a robust batch process was developed. The final batch process, conducted in 1-L bioreactors, was determined to be reliable. The medium was the same as previously described (Monot et al. 1982), with the addition of 5 g/L yeast extract. Fermentations were inoculated with seed cultures that had reached the pH and glucose concentrations thresholds of 4.8 ± 0.7 and 9.6 ± 4.9 g/L respectively, and pH control was done at 5.0 with a base (1 M NaOH) but no acid.

4 Continuous fermentation

4.1 Reproduction of previous results

The objective of this experiment was to test a working continuous fermentation setup with cell recycling, as presented in the literature (Tashiro et al. 2005). In the published report, the researchers implemented 100% cell recycling after continuous mode was switched on until OD_{600} had reached 20, and then utilised cell bleeding to maintain a constant biomass.

A ceramic membrane filter with pore size 0.2 µm was used for cell recycling (details in section 2.1). A working volume of 500 mL was chosen, with an intended dilution rate of 0.2 h^{-1}. The fermentation ran for 3 days (Fig. 16). Solvent production was not analysed in this experiment.

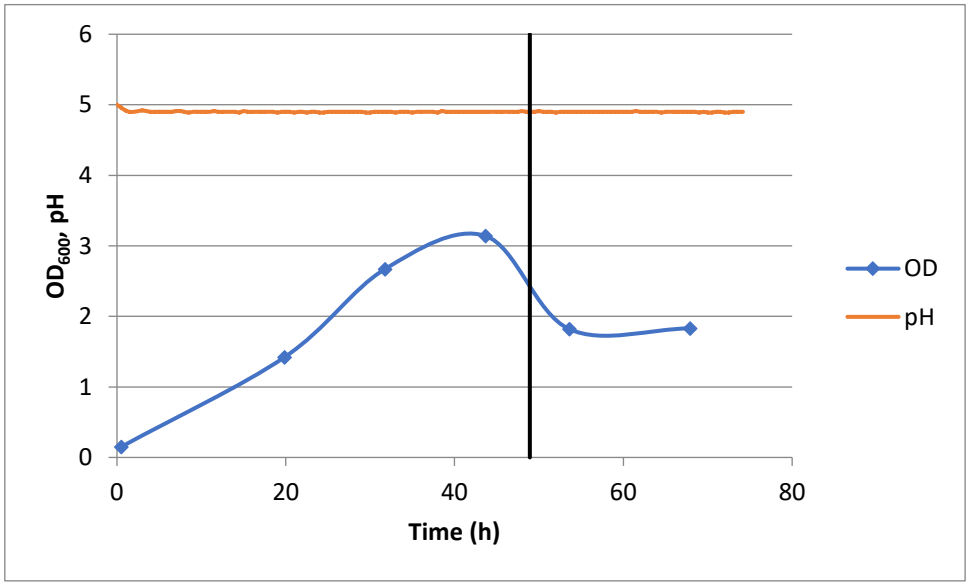


Figure 22 Growth curve in Experiment 8, *C. acetobutylicum* fermentation in a 1-L fermenter (500-mL working volume) with production medium containing 54 g/L glucose at 37 °C and pH 5. The system was changed to continuous mode at hour 48, as indicated by the vertical line, with feed containing 60 g/L glucose.

Several problems were encountered over the course of this experiment. It had previously been determined that the optimal time to change to continuous mode would be at the time of the first pH peak, as this corresponded with the highest rate of butanol production

(pH begins to increase when acids are consumed for solvent production.) Despite the previous results (Appendix A, scale-down, pH control experiments) which indicated that the first pH peak would be reached between 21 and 30 hours, it was not reached it even at 48 hours. Similarly, growth was lower than previously observed, with an OD of 3.2 vs 10 or 18 (Figure 7, Figure 9). Continuous mode was implemented at 48 h to evaluate the suitability of the cell recycling set-up.

Upon initiation of 100% cell recycling, additional problems were encountered. The necessary permeate throughput (450 mL/hour) to replicate published results could not be achieved with this filtration module, as transmembrane pressure was insufficient. To increase transmembrane pressure, several modifications were made to the process. The recirculation pump rate was doubled, a peristaltic pump was used to attempt to draw permeate through the membrane, and a back-pressure valve was added on the retentate outlet of the filtration module. This resulted in the electronic shutdown of the extraction pump on several occasions, as it could not sustain the required pressure and overheated. Another major constraint on the system was the diameter of the return port for recirculated medium, which was < 2 mm in diameter and limited the retentate flow rate.

The highest sustainable permeate flow rate achieved was approximately 40 mL/hour, corresponding to a 0.08 h^{-1} dilution rate. Thus, the addition of feed was initiated at the same rate of flow (40 mL/hour) in order to maintain a constant volume in the bioreactor. However, the extraction pump failed overnight at approximately 59 hours. With no exiting flow, fermentation volume almost doubled to 950 mL and the experiment was terminated at 70 hours.

There was a drop in OD_{600} after the system was set to run in continuous mode at hour 48 (Figure 22). This was most likely due to an increase in the total bioreactor volume due to the addition of feed without the removal of permeate. Total biomass continued to increase slowly, as seen at hour 69 when the OD was similar despite a doubling of the fermentation broth volume (Figure 23). The addition of nutrients from the feed addition likely contributed to this.

The difference in growth rate between this fermentation and the previous ones (20-L, scale-down, and pH control testing) may be due to experimental variance. It is also possible that the higher inoculation rate was responsible for the sluggish growth in this fermentation

relative to previous ones – the seed was the same, 100-mL, but a 20% inoculation rate was used to bring the total working volume up to 500-mL. Additionally, the seed medium was several months old and that may have contributed to a suboptimal culture condition; or the feed medium, which had been autoclaved in 20-L bottles, had been adversely affected by the longer time required in the autoclave. Unfortunately, due to the nature of this microorganism and the limited number of samples taken, growth rates cannot be estimated quantitatively, only comparatively. The time taken for this experiment to reach the first pH peak was at least twice that of previous experiments, therefore growth rate is at most half of what it was.

A more suitable filter was sought (details in Appendix B) and the Sartocon Slice TFF was chosen. The experiment was repeated with the new filter (Fig. 17).

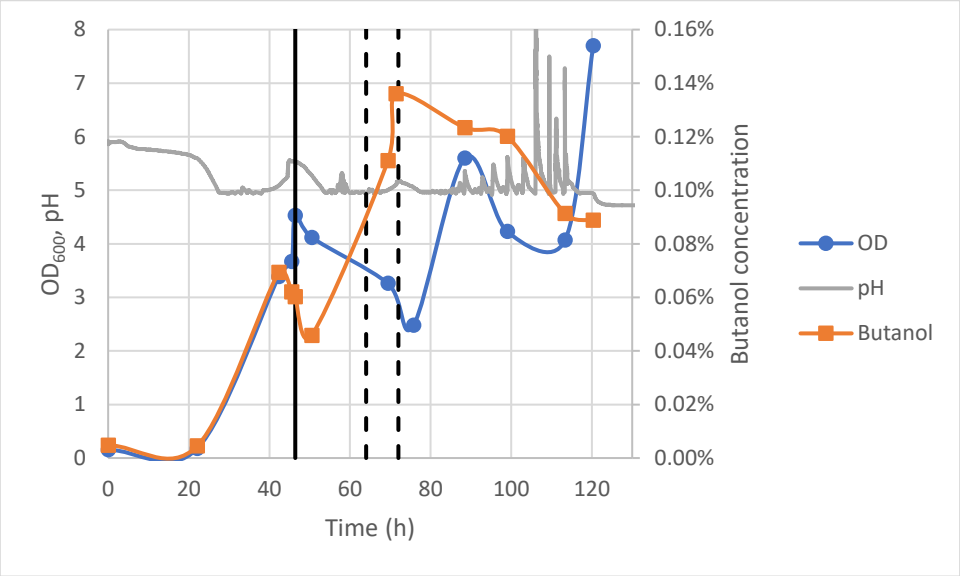


Figure 23 Optical density, pH and butanol concentration of *C. acetobutylicum* fermentation, in a 1-L fermenter with production medium containing 54 g/L glucose at 37 °C. The system was changed to continuous mode with cell recycling at hour 47, as indicated by the vertical line, with feed containing 60 g/L glucose. After filter failure, the system ran in batch mode between hours 64 and 71, between the dotted vertical lines, before transitioning to a continuous system without cell recycling at 71 hours.

Initially, the working volume of the fermentation was 1 L. A pH peak was observed at 45 hours. In this interval, since antifoam had not been used initially, there was sufficient

foaming to cause a minor foam-out, thus reducing the working volume to ca. 800 mL. Biomass had reached OD_{600} = 4.5 when the switch to continuous mode was carried out at hour 47, with an initial dilution rate of 0.2 h^{-1}, and the fermentation volume was reduced to ca. 500 mL, according to plan.

At approximately hour 61 (overnight), the Slice TFF failed due to the accumulation of slime on the membrane, causing the working volume to increase to ca. 800 mL in the hours following. It was found that the cause of the failure was that the fermentation had become highly viscous and the TFF membranes had become clogged with slime, stopping permeate flow. The Slice TFF was removed from the system, which was then modified to be a continuous fermentation without cell recycling. During continuous operation without cell recycling, the fermentation grew steadily more viscous, with increasing stringy clumps of slimy material. The slime interfered with pump uptake, which meant that less broth was being pumped out of the system. The feed pump rate was changed to 80% of the extraction pump rate, from 100%, while the extraction pump rate remained unchanged. This arrangement adequately maintained the level in the reactor.

While the system was running in continuous mode without recycle (hour 71-120), the OD remained reasonably constant. However, this measurement was not necessarily reflective of biomass, since many cells may have become immobilized in the slime. When the stirrer rate was increased to 600 RPM to break it up at the end of the fermentation run (hour 120), OD increased to 7. This is likely a more accurate measurement of total biomass, slime being largely transparent. The slime also interfered with mixing in the bioreactor, as evidenced by the pH spikes that began to occur shortly after the transition back to continuous mode at hour 71. When base was added for pH control, it failed to mix well, which led to surplus base addition.

The slime may have been a polysaccharide, which *C. acetobutylicum* is known to produce sometimes. Polysaccharide production is known to be affected by the carbon:nitrogen ratio in the medium (Sutherland 1990). While the medium used in this fermentation had a C:N ratio in line with previous work, pH control had usually been done with sodium hydroxide (NaOH) instead of ammonia (NH_3) (Tashiro et al. 2005). Further runs were done using NaOH for pH control.

4.2 Continuous runs without cell recycling

Five additional continuous fermentations were done without cell recycle. Table 16 summarises the successful continuous runs. Figure 24 shows the butanol concentration in each run.

Table 16 Details of continuous runs. Arrows indicate an increasing or decreasing trend observed in place of a steady state value, blanks indicate that a steady state was never reached in that variable.

Run	Transition to continuous operation (h)	Dilution rate (h^{-1})	Steady state		
			Time achieved (h)	Glucose (g/L)	OD_{600}
10.1	15	0.12	100	10	12
10.2	16	0.2	-	↓	↑
10.3	15	0.16	60	13	-
10.4	15	0.16	40	15	-
10.5	18	0.16	43	15	↑

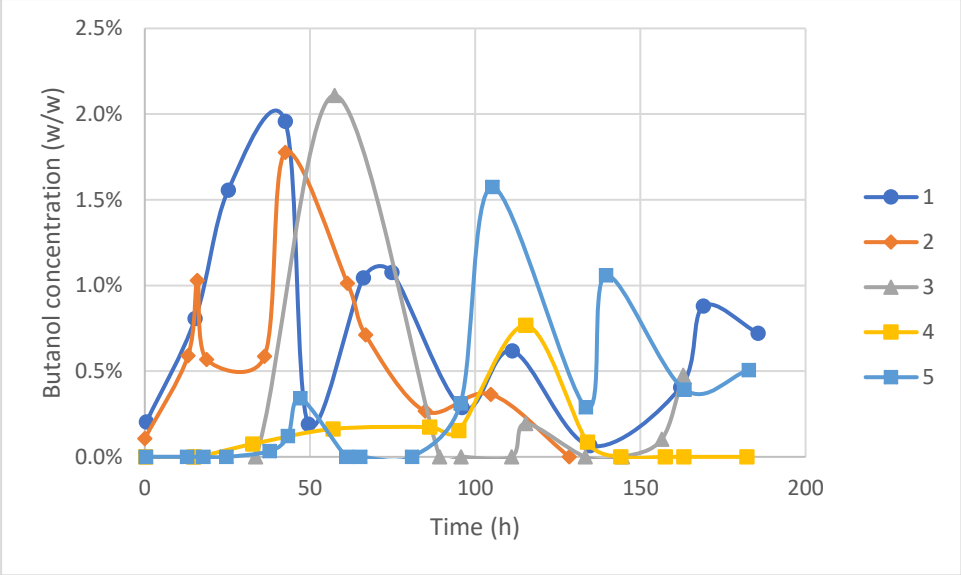


Figure 24 Butanol concentrations in continuous runs, Experiment 10, in 1-L fermenters with production medium containing 54 g/L glucose at 37 °C and feed medium containing 60 g/L glucose, controlled at pH 5

The butanol production was cyclical. The minimum time between peaks was 20 hours, and the maximum was 60 hours. Certainly, it never appeared to reach steady state in any of the five fermentations conducted, except maybe a zero steady state in run 4. A maximum recorded concentration of 2% butanol was reached, which had not been achieved in the 1-L fermenters in batch mode. There may be a partial explanation in that the samples from these runs were not processed for solvent analysis until well after they were taken, due to GC issues. However, it is more likely that this butanol concentration is due to glucose consumption for solvent production in this part of the continuous cycle, whereas in a batch run much of it would have been converted to biomass.

Oscillatory behaviour has been previously demonstrated in *C. acetobutylicum* continuous cultures (Clarke, Hansford, and Jones 1988; Grupe and Gottschalk 1992). The potential explanation provided is as follows: acidogenic and solventogenic cells coexist in a continuous fermentation. Acidogenic cells, with a higher specific growth rate, are favoured by continuous culture conditions compared to solventogenic cells. This leads to an increase in acid concentration in the culture, during which time solvents are washed out and not replaced. On reaching the acidity threshold, solventogenesis is initiated, and solvents are produced at a

higher rate than they are removed; however, solventogenic cells have a lower specific growth rate and are washed out at higher rates than acidogenic cells, and the cycle begins anew (Clarke, Hansford, and Jones 1988). Figure 25 shows the correlations between caustic addition (indicative of acid production) and solvent concentration.

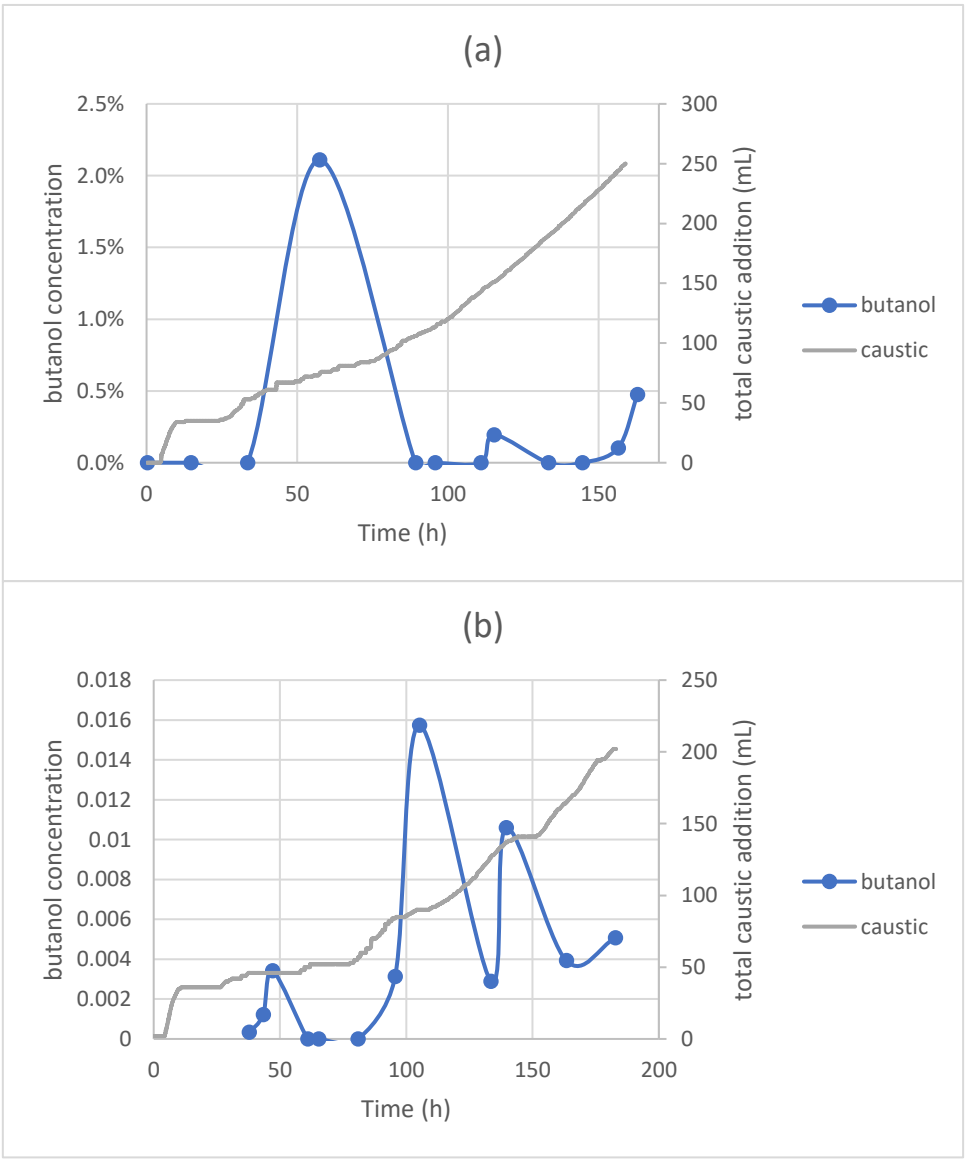


Figure 25 Correlation of caustic addition and solvent concentration in 1-L continuous fermentations (a) 10.3 (b) 10.5

Figure 26 shows the fermenter data from the first two successful runs, which were done at different feed flow rates to study the effect of dilution rate on these fermentations.

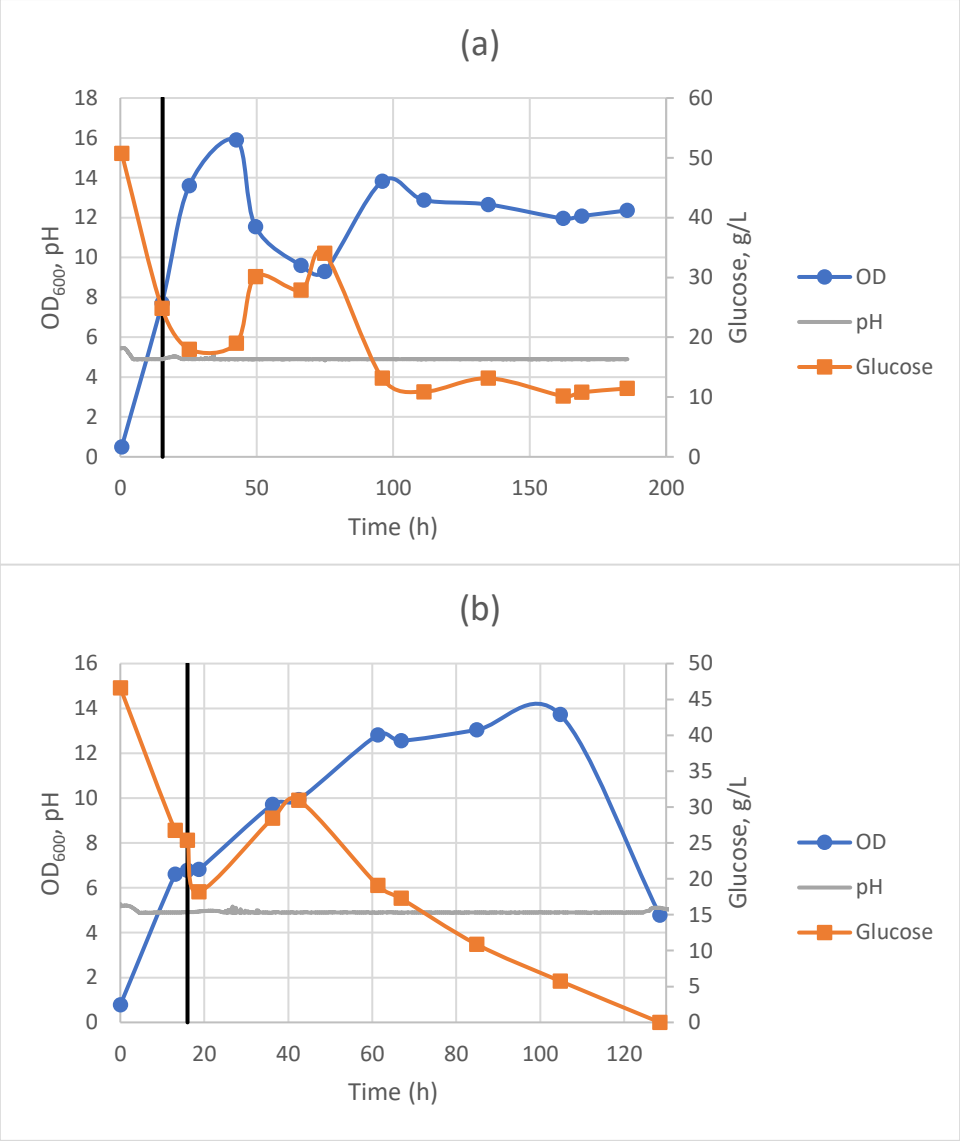


Figure 26 Continuous runs (a) 10.1, at 0.12 h^{-1} dilution rate and (b) 10.2, at 0.2 h^{-1} dilution rate, in 1-L fermenters with production medium containing 54 g/L glucose at 37 °C and feed medium containing 60 g/L glucose, with pH control above 5. Transition to continuous mode is indicated by the vertical lines.

In run 10.1, OD_{600} peaked at 16 and eventually settled to a steady state at about 12. Glucose steady state was about 10 g/L. The steady state values for both OD and glucose were reached at 100 hours into a 185 hour run.

In run 10.2, with a higher dilution rate, it is unclear whether a true steady state was ever reached; the run ended at 128 hours when the feed medium ran out overnight (~ 117 h) and the reactor finished going through the end stages of an equivalent-volume batch run. OD_{600} went through a slow but steady increase from 6.6 at 13 hours, 12.5 at 66 hours to 13.7 at 104 hours. Glucose generally decreased over time, but between 13 and 66 hours all readings were between 17-30 g/L.

An intermediate dilution rate was chosen for run 10.3. Results are shown in Figure 27.

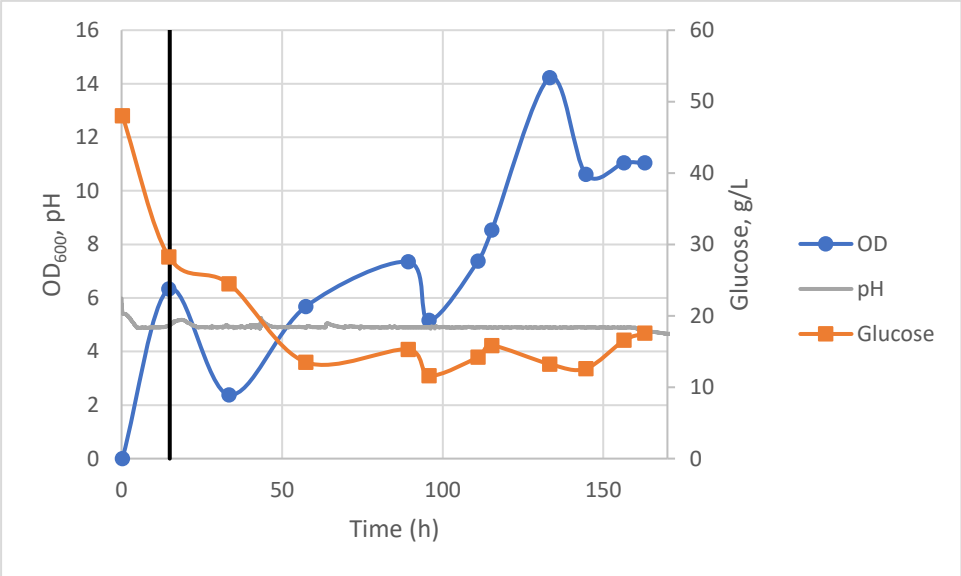


Figure 27 Run 10.3 at 0.16 h^{-1} dilution rate, in a 1-L fermenter with production medium containing 54 g/L glucose at 37°C and feed medium containing 60 g/L glucose, with pH control above 5. Transition to continuous mode is indicated by the vertical line.

In this run, OD_{600} increased erratically to a maximum of 14 without appearing to reach a true steady state. Glucose reached steady state between 12-15 g/L, starting from approximately 60 hours until the end of the run at 162 hours. The OD increase between 100-140 hours, without a change in glucose consumption, indicates that the glucose was now being used to produce solely biomass where previously it been used for both biomass and end product (either acid or solvent). This is borne out by the butanol concentration which dropped to 0 shortly before hour 100 (Figure 24).

All runs showed some evidence of polymer production in an early part of the run before reaching steady state, and steady state was only reached after it dissipated. Figure 28 shows the results from the next runs (10.4 and 10.5), which were done with filtered feed medium.

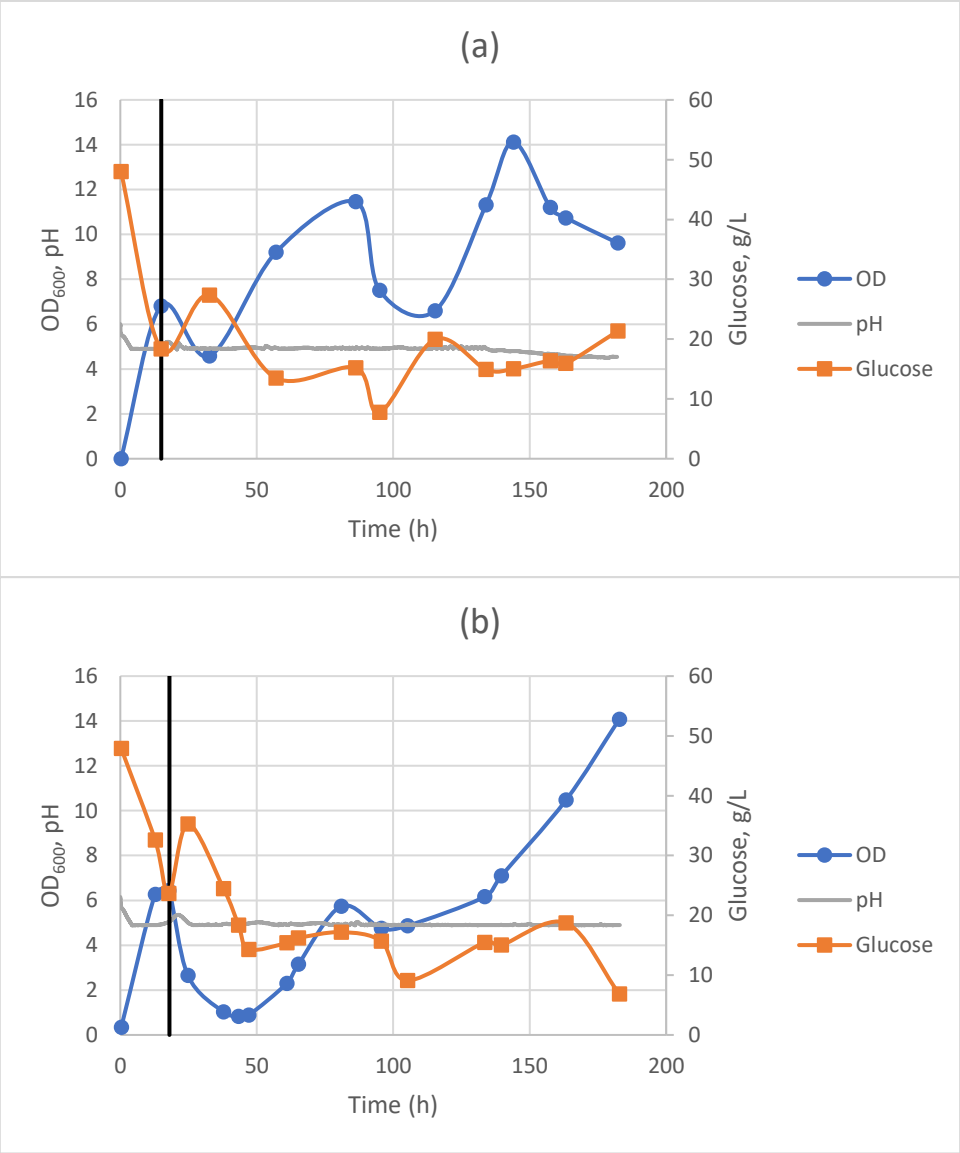


Figure 28 Runs (a) 10.4 and (b) 10.5, both done at 0.16 h^{-1}, in 1-L fermenters with production medium containing 54 g/L glucose at 37°C and filtered feed medium containing 60 g/L glucose, with pH control above 5. Transition to continuous mode is indicated by the vertical lines.

Experiment 10.4 ran smoothly. OD peaked at 14 but varied between 6 and 12 after hour 40 in the 180 hour run. Glucose reached an approximate steady state of about 15 g/L at about 40 hours. Polysaccharides dissipated between hours 86 and 95, roughly coinciding with a drop in glucose level before it returned to the same steady levels as before.

Experiment 10.5 ran less smoothly throughout the 180+ hours. Polysaccharides remained, at varying levels, throughout most of the run. OD_{600}, instead of levelling off, increased at a steady rate throughout incubation. Glucose seemed to reach a steady state around hour 43, with ups and downs that may correspond with polysaccharide levels. Curiously, there was one point late in the run (~140 h) where significant foaming was observed; foaming is usually observed early in a batch run, or in a continuous run before steady state is achieved. During foaming and after foaming subsided, polysaccharides were found to be absent. Solvent data do not reflect two distinct steady states with a changeover at this time. Instead, the butanol trend shows the same cyclical characteristics at relatively high concentrations as in the other continuous runs, beginning at about 100 h where it was low and erratic before. This discrepancy in timing may be caused by the preferential consumption of acids and/or glucose before polysaccharides for solvent production.

4.3 Summary

Two attempts were made at reproducing systems in the literature, specifically, continuous fermentations with cell recycling. In the first attempt at cell recycling using a ceramic membrane, the growth during the initial batch phase was unusually slow. This is likely due to unoptimized fermentation conditions. Despite the slow growth observed during batch phase, cell recycling was nevertheless attempted. The size of the return port on the bioreactor posed a mechanical problem, but only to this particular lab-scale fermentation – the narrow diameter of the port placed an upper limit on retentate flow rate. There were also issues around filtration, with the ceramic filter not letting sufficient permeate through despite the pore size being adequate in theory. On further investigation, it became clear that an inappropriate filter had been chosen (Dyer 2016). The required flow rate for that filter was 1.4 litres per second (Appendix B), which could not be generated in the laboratory with current equipment or a fermentation that had a 500-mL working volume.

Slow growth was demonstrated in the second attempt at continuous operation as well, which used a Sartorius Stedim tangential flow filtration (TFF) system equipped with a 0.2 µm

Slice membrane. Cell recycling in this fermentation failed due to slime clogging up the TFF, so the filter was removed and the continuous fermentation proceeded without cell recycling. Slime build-up continued, with the broth becoming more viscous to the point that it was causing mixing issues, before the fermentation ended. This slime was hypothesised to be polysaccharides. While the appearance of polysaccharides was expected, their persistence was not, and this phenomenon may interfere with future production.

Given the failure of two filtration approaches and the limited project time, cell recycling as a strategy was not pursued further. The cell recycling challenges identified in this project could be costly in commercial production in terms of the cost of equipment, resources, and time to address the filtration difficulties, and would also make the entire process more complex. Should the slime problem be solvable, cell recycling could be seriously considered as it greatly increases the throughput of the system.

Besides the two attempted fermentations with cell recycling, a total of five continuous fermentations were done without cell recycling. The first three runs were done with steam sterilised media and the other two with filtered media.

The dilution rate for the last two continuous fermentations was chosen based on the performance of run 10.3, which had the maximum glucose consumption rate at steady state. There was still remaining glucose in the output stream of the continuous fermentation; there would also have been acetic and butyric acid, polysaccharides, and biomass. While full conversion of glucose to solvents is not possible due to biomass production, the current level of conversion is also not ideal, as the exit streams in these experiments contained 15-20% residual glucose as well as acids. In the literature, up to 94% of substrate has been converted to butanol, though at a lower dilution rate (Li et al. 2011).

The current level of conversion, in fact, seems to be oscillatory in nature, a known phenomenon with proposed explanations (Clarke, Hansford, and Jones 1988; Grupe and Gottschalk 1992). This poses a potential problem for the commercial separation of solvents, as an inconsistent feed stream would necessarily enter a distillation tower at the wrong level at least some of the time, increasing heat duty and thus reducing commercial viability. To resolve this issue, the feed stream to the distillation tower would be averaged over time; this would dampen if not eliminate oscillations.

These fermentations took between 15-18 hours to reach the changeover to continuous mode. After that, the time taken to reach a steady state in glucose level was a minimum of 24 hours (3.9 fermenter volumes), if one was reached. In the literature, continuous systems reached steady state after 2.4-3.1 fermenter volumes, taking less total volumes but more time as dilution rate decreased (Napoli et al. 2009; Li et al. 2011). This is in accordance with mixing principles (Nauman and Buffham 1983). Interestingly, in Experiment 10.5, there appeared to be two steady states. In the first, polysaccharides were produced; at about 140 hours, the fermentation underwent a visible physical change. Much of the gelatinous polysaccharide mass dissolved, and foaming took place again before the fermentation reached another apparent steady state without polysaccharides. The cause of the changeover is not known.

Polysaccharides have been a recurring problem in the continuous fermentations. In batch mode, they are consumed for solvent production, so that they are at best only a nuisance during the middle part of the fermentation. However, in continuous mode, polysaccharides pose a real problem to both mixing and filtration. They interfere with free mixing of the fermenter, as observed in pH spikes, so that nutrients and pH control solutions are not well mixed and the fermentation becomes non-homogeneous. Then, also, the large polysaccharide molecules cannot pass through filter pores designed for bacteria, instead clogging them up and fouling membranes. Finally, like the acid intermediate products, polysaccharides produced impact the final yield of a process if not consumed.

Quantitative sampling of polysaccharides is difficult, due to their mucous nature and the fact that polysaccharides are often heterogeneous. Further complicating the issue, different Clostridium species may produce different polysaccharides, both intra- and extra-cellular, of which few have been characterised (Reysenbach et al. 1986). (This particular *C. acetobutylicum* exopolysaccharide has not.) Samples rarely contain a representative amount of polysaccharide as it tends to clump together and is distributed non-uniformly. Measurements of other variables may also not be representative, due to the mixing issues described above. Thus, these considerations are all qualitative rather than quantitative.

5 Supercritical extraction

In this thesis, supercritical extraction was investigated as a means of extracting butanol from *C. acetobutylicum* fermentation broth. The goal of this section was to flesh out work done previously (Moreno et al. 2012), as well as to evaluate its suitability for integration into a continuous process. In the previous work, butanol concentration in the extract ranged from 73-95%, with feed solutions from 0.5% to 5%. As the solvent concentration in fermentation broth generally does not exceed 1.2 wt% in a batch fermentation with wild-type strain, or up to 3% with currently available genetically engineered strains, the decision was made to maintain a constant conservative estimate of feed solvents, while focusing on optimising extraction conditions. Test solutions containing 1% butanol and 0.5% acetone (v/v) were used for all experiments except the reproduction of previous results. The raw data used for charts in this section may be found in Appendix D.

5.1 Reproduction of previous results

Two attempts were made to reproduce prior results using centrifuged fermentation broth, at 100 bar and 40 C. A total of five extraction runs were done (Appendix A). The residual ethanol contamination from cleaning the equipment made it impossible to measure ethanol extraction. The concentration of butanol in the extract was approximately double the feed concentration, at 1.6% from 0.8%, or 1.0% from 0.5%. The prior results, which had extract concentrations of 73% and above, could not be replicated. It may be that the previous results were unusually good, or it may have been an effect of low solvent concentrations in the feed.

5.2 Conditions testing

In this section, the effects of solvent and feed flow rates, S/F ratios, and operating temperatures were tested. Also tested was the process change of raising temperature after supercritical extraction was carried out (diagram in section 2.2).

5.2.1 Temperature

Previous results suggested that running the extraction under near-critical conditions at 20 °C produced better results than at 40 °C (Montanes 2016). Four operating temperatures were tested at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2. (Figure 29)

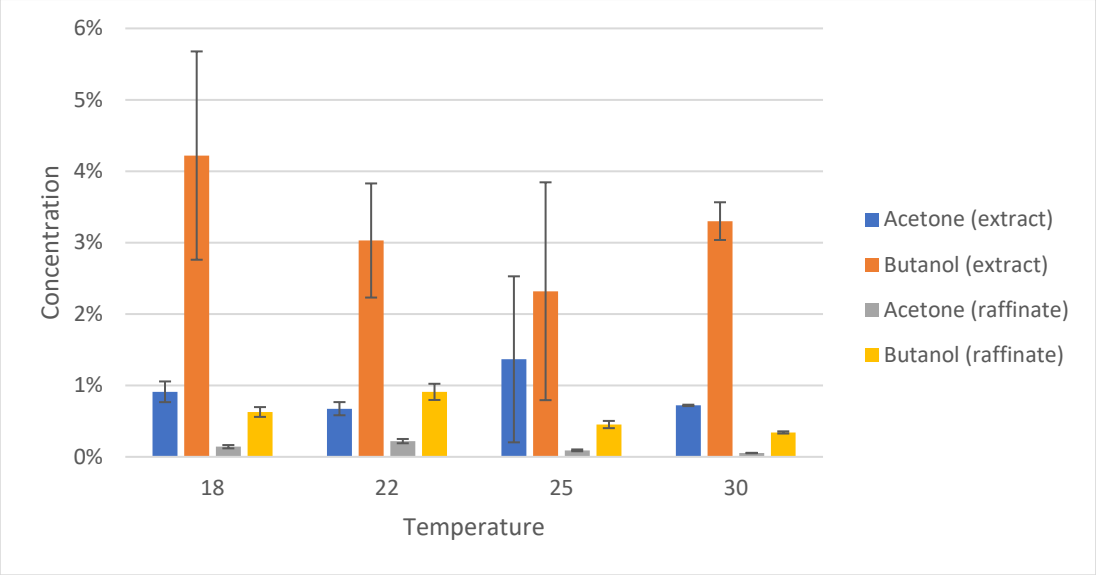


Figure 29 Results of operating temperature testing at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2

There was a lot of variance in results, but a U-shaped curve appears to be present. The extraction was much more difficult to operate at lower temperatures, because of frequent pipe blockage due to freezing. At 30 ºC, the freezing effect was almost non-existent and these data were more consistent. As both the extract and raffinate butanol concentrations were lower in the run at 25 °C than at 22 °C, it seems possible that there is a different factor at work, likely to do with the freezing itself.

5.2.2 Solvent and feed flow rates

For each of these experiments, four combinations of solvent and feed flow rates were tested with S/F ratios ranging from 1-2.5, with feed flow rates ranging from 4-12 g/min and solvent flow rates from 6-15 g/min, at 20 °C and 100 bar (Figure 30).

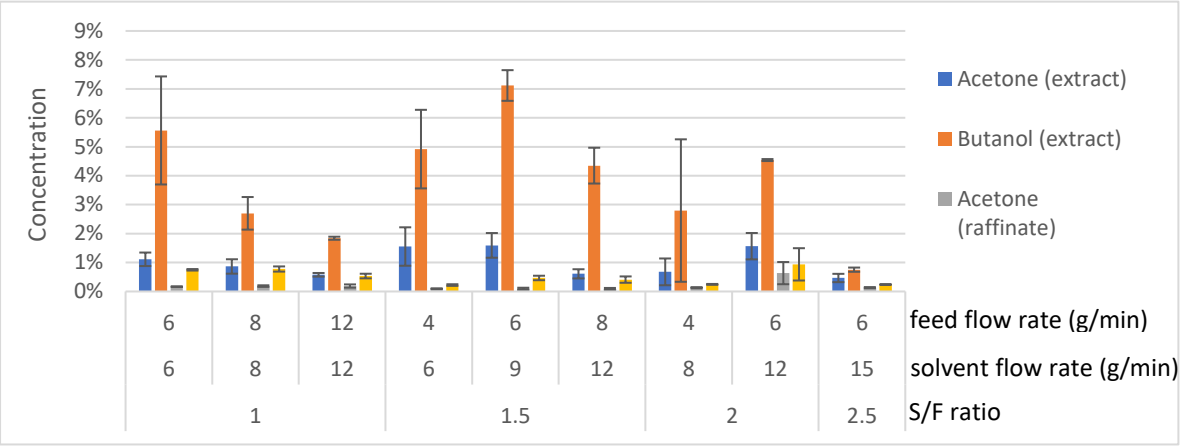


Figure 30 Results of flow rate and S/F ratio testing at 20 °C and 100 bar

There was a correlation between S/F ratio and extract concentration. At the lower S/F ratio of 1, the concentration of butanol in the extract stream was consistently lower than at the higher ratio of 1.5, for each feed flow rate. Between experiments conducted at the same S/F ratio, the results from a lower flow rate were much better. The optimal feed flow rate appears to be 6 g/min across all S/F ratios. The extraction results for acetone were unremarkable. Given the low starting concentration of 0.5%, this is not unexpected despite the higher partition coefficient of acetone.

The extraction was much more difficult to operate at higher CO_2 flow rates, because of frequent pipe blockage due to freezing. There is a very sharp decline in extract concentration at 2.5 S/F ratio. This may indicate that the relationship between S/F ratio and extraction efficiency is not linear but instead bell-shaped. However, since the raffinate concentration did not increase accordingly, there may be other explanations, such as the freezing pipes.

5.2.3 Making extraction easier with temperature

In these experiments, after passing through the contactor at 20 °C, the exit streams (extract and raffinate) were raised to a higher temperature by another water bath for collection, as described previously (section 2.2). This was intended to solve the freezing problem encountered in temperature testing.

Two combinations of solvent and feed flow rates were tested at 100 bar and 1 or 1.5 S/F ratio, with 6 g/min feed and 6 or 9 g/min CO_2. (Figure 31) Figure 32 compares the butanol extraction from this experiment with the results in Figure 30, which were obtained under the same conditions except for the temperature increase before collection.

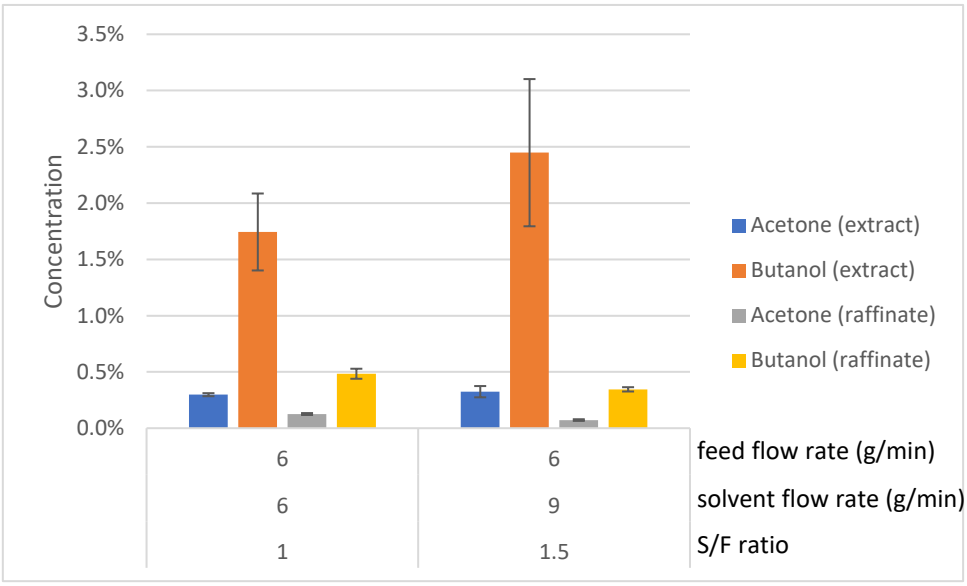


Figure 31 Results of S/F ratio testing at 20 °C and 100 bar, with an increase in collection temperature to 45 °C

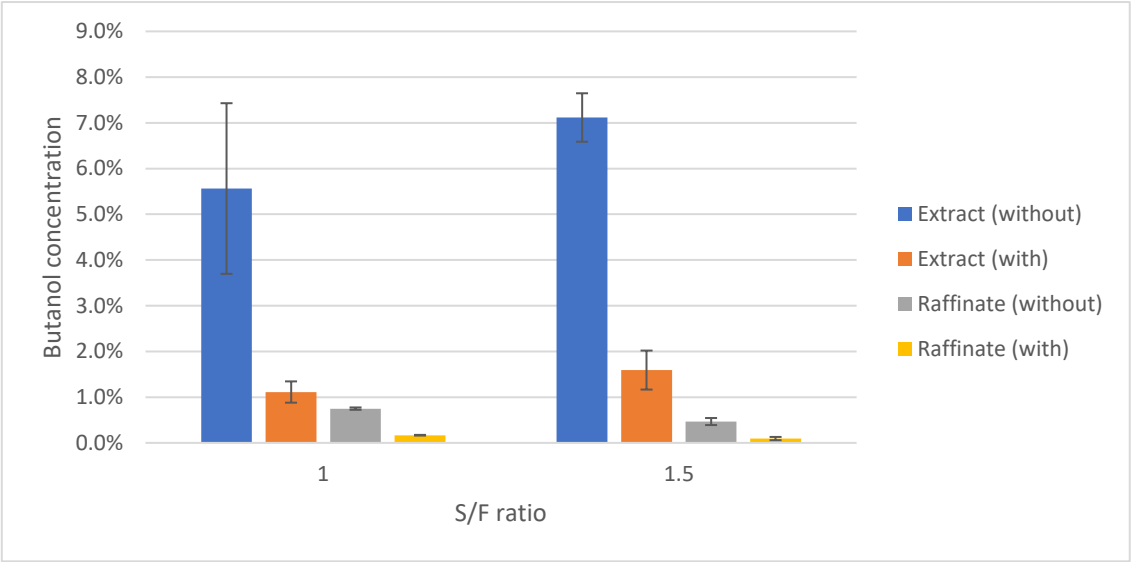


Figure 32 Comparison of S/F ratio testing results at 20 °C and 100 bar, with and without increase in collection temperature to 45 °C

The extraction was much easier to operate at low contactor temperatures, since the exit streams were raised in temperature to prevent freezing. However, there appears to be a negative impact on extract concentration. As both the extract and raffinate butanol concentrations were lower in the run with collection at increased temperature than without, it seems likely that the previously mentioned different factor related to freezing is at work here also.

Different operating temperatures were also tested with the exit stream temperature increase. (Figure 33) These experiments were conducted at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2. Figure 34 compares the butanol extraction from this experiment with the results in Figure 29, which were obtained under the same conditions except for the temperature increase before collection.

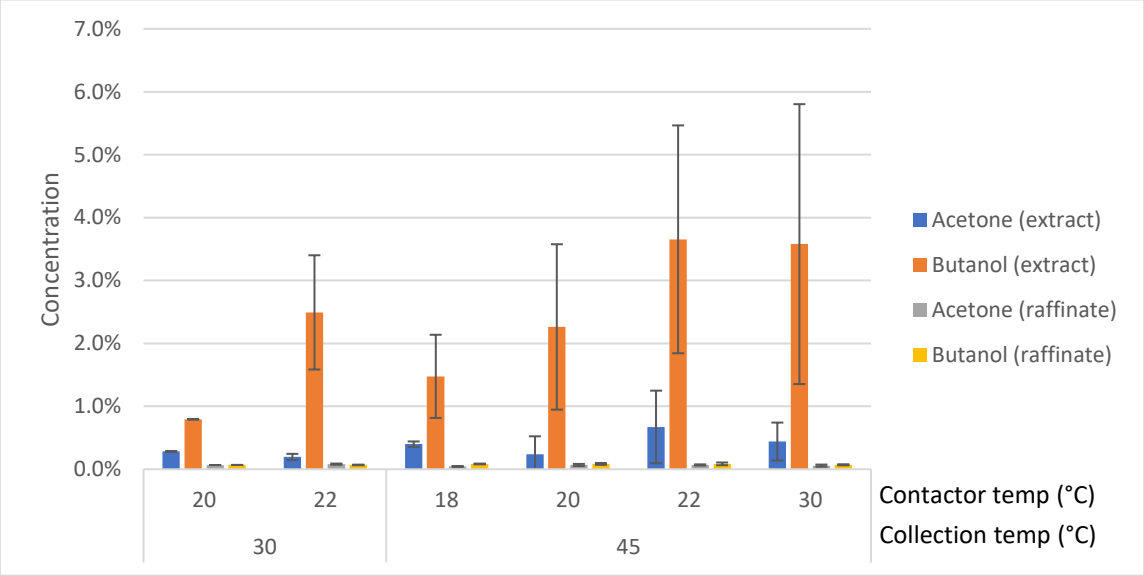


Figure 33 Results of operating temperature testing at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2, with increase in collection temperature

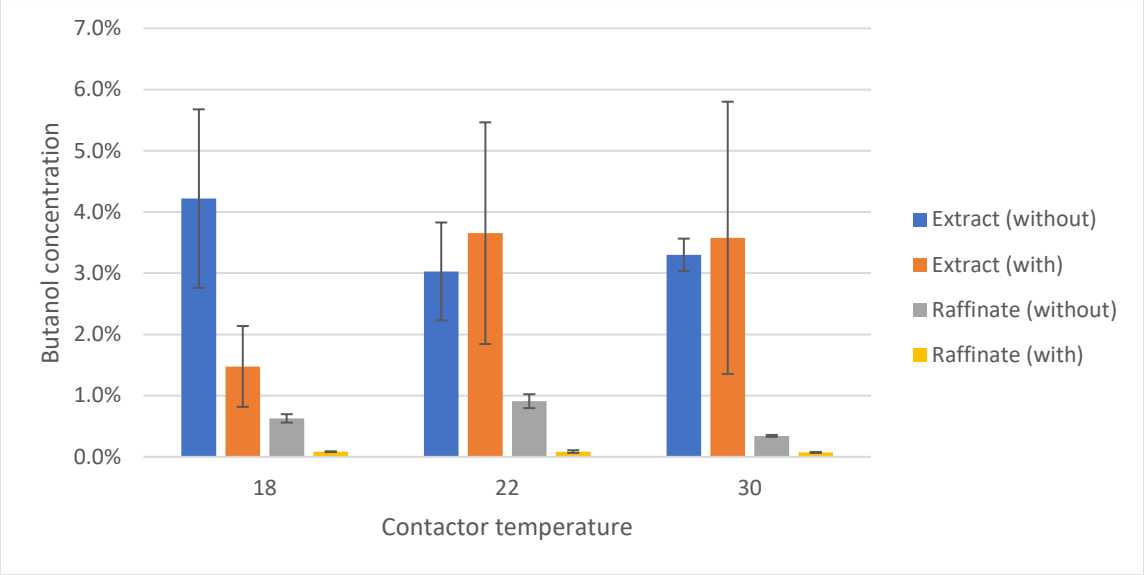


Figure 34 Comparison of temperature testing results at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2, with and without increase in collection temperature

Extract concentrations continued to be wildly inconsistent, as evidenced by the error bars in Figure 33 and Figure 34, though raffinate concentrations became very consistent at 0.07%-0.1%, or 7-10% of feed concentration.

5.3 Confirmation of assumptions

Given the inconsistency in results, the decision was made to re-examine assumptions under which the experiments were conducted. Solvent evaporation was tested, and confirmation of steady state timing was also sought.

5.3.1 Solvent evaporation

It was hypothesised that, during the long time taken to collect samples manually, solvents could have been evaporating out of the collection vessels, especially as the streams were discharged at pressure and were frequently misty. To evaluate the possibility of solvent evaporation at this stage of the process, one set of runs during temperature testing had additional samples taken in a burst method, in which the extract valve (refer Figure 5 in section 2.2) was opened quickly and then closed immediately to minimise the effect of evaporation in this collection. The burst method of collection resulted in two additional data

points. (Table 17) It was found that solvent evaporation, if present, was not the only contributor to inconsistency in results.

Table 17 Burst data: extract concentration at 25 ^{o}C, 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2

Acetone		Butanol	
Burst	Normal	Burst	Normal
0.87%	0.20%	2.00%	0.79%
0.63%	2.53%	2.48%	3.84%

5.3.2 Steady state testing

Passing five contactor volumes through the reactor to reach steady state before collecting samples should have been sufficient, as this had been used in the previous work (Moreno, Tallon, and Catchpole 2014), but the assumption had not been examined in this work. It was theorised that one reason for the inconsistency in extract concentrations was that they may have been affected by previous runs, so that a longer period was required to reach steady state. Some runs in the operating and collection temperature experiments (Figure 33) had been rearranged, to determine whether they were affecting each other. If they were, then a pattern should have been discernible in Figure 35.

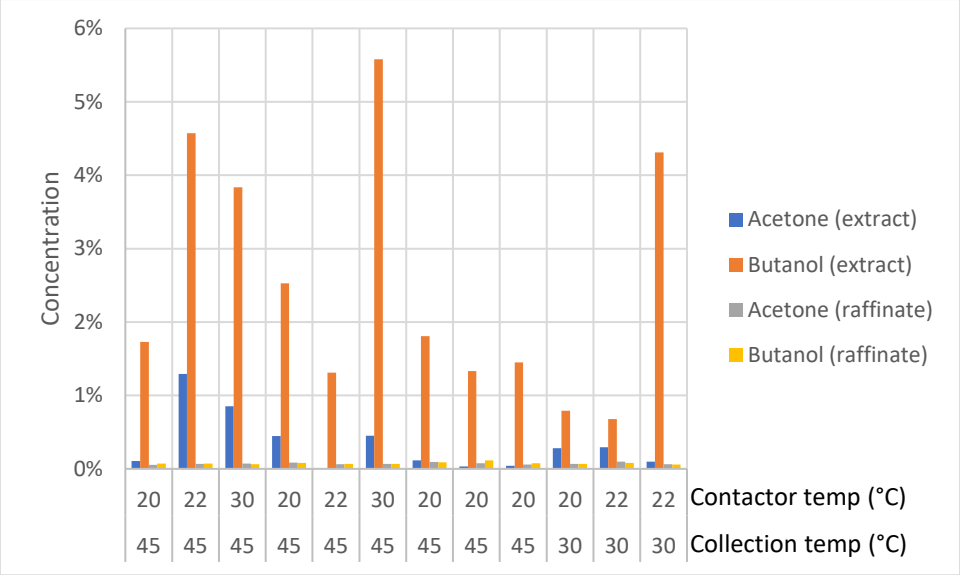


Figure 35 Results of steady state testing with mixed order of runs. Runs are shown in chronological order from left to right, done at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2, with increase in collection temperature

Freezing was observed in the runs with collector temperature at 30 °C. No pattern could be found with higher-temperature runs affecting the results of the following lower-temperature runs or vice versa. Extract results were still inconsistent, but it was concluded that this inconsistency was not due to order of operations.

The assumptions made appeared to be reasonable. Inconsistency in results made it difficult to draw any conclusions from the data regarding optimising conditions for supercritical extraction, but a temperature increase for ease of collection appeared to be viable.

5.4 Summary

In the previous experiment, which used feed concentrations from 0.5-5%, butanol concentrations in the extract were generally above 70% and reaching a maximum of 93.5% (Moreno et al. 2012). However, in this thesis, the prior results were not replicable. Results were highly variable. Acetone extraction was also consistently suboptimal, which poses a major problem to the viability of commercial supercritical extraction in this case. While ethanol was not measured in this project, the partition coefficients suggest that a similar

argument would apply even more strongly. (See section 1.9 for details.) For a process resulting in industrial bulk chemicals such as ABE, yield and recovery are very important. Variability in results, which could affect them, is highly undesirable.

Solvent and feed flow rates, and S/F ratio, were investigated. A S/F ratio of 1.5 was better than 1 and not much different from 2, but efficiency dropped sharply when a ratio of 2.5 was tested. Lower flow rates gave better results. This is in contrast with the prior work, which made use of a S/F ratio of 3.

Temperature testing was inconclusive, but ease of operation was found to be a major issue at lower temperatures, as the collector had a tendency to freeze. Raising the temperature of the exit streams before collection resolved the issue, but was unable to provide more conclusive results in the extract. It was theorised and disproven that steady state for each run had not been reached. Instead, it may be that small variances in feed concentration are responsible for the large variances in extraction results. Feed concentration is known to be critical to extract concentration. Any small imperfection in mixing, or other perturbation in the feed line, would be magnified in extraction.

Across all experiments conducted without raising the temperature of the exit streams, relatively high concentrations of butanol remained in the raffinate, compared to the starting concentration. After introducing the increase in temperature, the raffinate concentrations settled down to lower values, though still between 7-10% of feed concentration.

The end result of the investigations is that supercritical CO_2 extraction of ABE from fermentation broth is an avenue of research not worth pursuing further. At current solvent production levels, the concentration of solvents in the broth is not high enough for good recovery. In this set of experiments, the maximum concentration of the extract was 7.58%, which requires further separation to be useful. Also, in these experiments, supercritical extraction left a high proportion of solvents in the raffinate, compared to the feed (between 7-10% of feed concentration). Supercritical extraction is cost-prohibitive and better suited for high-value organic products, whereas simple solvents like ABE do not suit it (Rosa and Meireles 2005). This is despite the market for butanol being relatively tight (Grand View Research, Inc. 2015).

It has been shown that feed composition is critical for the extraction of butanol. When butanol concentration in the feed is greater than 1.6%, concentrations above 50% are obtainable in the extract; with feed concentrations above 2% butanol, extract concentrations reach 80% (Montanes 2016). While the above conditions, such as they are, have been established as ideal for extraction at the feed concentrations examined in this thesis, supercritical extraction should not be revisited for solvent extraction from ABE fermentation until solvents can be consistently produced above 2% of fermentation broth.

6 Further exploration

6.1 The two-stage reactor

A solution to the problem of incomplete fermentation has been explored already – the two-stage reactor (Figure 36) (Hubert Bahl, Andersch, and Gottschalk 1982; Bankar et al. 2012). In the first stage, exactly the same process takes place as in these fermentations (batch fermentation followed by transition to continuous mode). The outlet stream, however, is not the final product. Instead, it goes into another reactor, in which the fermentation continues, and which may be run under different conditions or with an additional feed inlet (Kayaalp 2013). As many stages may be added as are necessary to run the fermentation to completion, that is, all feedstock has been converted either to biomass or to solvents. There are examples of this approach at commercial scale, notably in plants in China (Chiao and Sun 2007; Ni and Sun 2009).

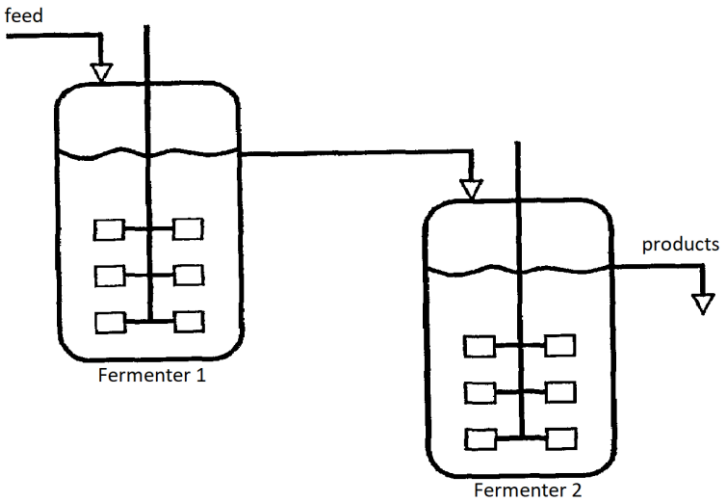


Figure 36 A two-stage reactor

A two-stage reactor would also resolve the polysaccharide problem somewhat, as it is a subset of the incomplete fermentation issue. As observed both in the work described in this thesis and by others, polysaccharides are consumed during solvent production; by allowing a

fermentation to run to completion, they would be naturally removed from the fermentation. The final broth can presumably be filtered without issue.

By allowing the fermentation to run to completion in multiple stages, oscillations in solvent production will also be reduced or eliminated, assuming the offered explanation of alternating acidogenesis and solventogenesis dominance is correct. During phases where acidogenesis predominates, downstream stages will allow time for solvent production to take place. Broth in which solventogenesis dominated in early stages will spend longer than strictly necessary in the fermenters, but that is inevitable.

Based on the results of the experiments conducted during conditions testing, a stable continuous fermentation process without cell recycling was developed. With refinement of process parameters to determine ideal conditions and increase efficiency, especially in the area of reducing or eliminating polysaccharides, this single-stage continuous process may be a good initial step for a multi-stage reactor system similar to the commercial ABE fermentation process used in China. Cell recycling for increased productivity is a consideration, especially if the polysaccharide issue can be resolved, but may not be feasible on a commercial scale with a multi-stage process.

6.2 Polysaccharides

A study of polysaccharide production by *C. acetobutylicum* should be conducted. Experimental results in this thesis have shown that it is possible to achieve a steady state without exopolysaccharides, but it is unclear how it can be done consistently. A solution had been proposed to handle the issue of polysaccharide production, by ignoring it and letting it dissipate on its own in a second stage fermentation as it is consumed for solvent production. This addresses one of the immediate problems at hand, which is the effect of polysaccharide slime on filters, but does not address root causes or the effect of polysaccharides on mixing while they are present.

The exopolysaccharide(s) should be characterised, first of all, and its place on the metabolic pathway identified. Different conditions should be tested to establish its conditions of production, and more importantly, conditions of non-production. Medium composition is frequently a factor in exopolysaccharide production, so careful control of stoichiometric ratios is key to reducing exopolysaccharide production in *C. acetobutylicum* fermentations.

This is especially true as the presence of exopolysaccharides appears necessary to the smooth running of the fermentation (Haggstrom and Forberg 1986).

However, commercial substrates are not always consistent over time, so that level of control may not be reasonable. Instead, perhaps other conditions can be found that minimise polysaccharide production, or else an additive can be found that causes inhibition. (Methyl viologen has been studied relatively widely (Grupe and Gottschalk 1992).) Due to the regulatory pathways in common with sporulation and solvent production, genetic engineering to prevent exopolysaccharide production promises to be difficult, so easier alternatives should be sought if possible.

6.3 Cell recycling

In this project, cell recycling was not successful with the available lab-scale filtration equipment, due to the production of polysaccharides. If a solution can be found to control polysaccharide production, cell recycling fermentations for this process should be studied. As biomass separations is generally a required step when cell suspensions are used for fermentation, high-density cultures achieved by recycling allow greater throughput while conducting filtration which would have to be done anyway.

A major issue with cell recycling, especially from a commercial viewpoint, is the long start-up time requirement for cell growth to high density (Tashiro et al. 2005). The reward, however, is much higher dilution rates, increasing productivity greatly.

Cell recycling and multi-stage fermentations have previously been combined (Chaabane et al. 2006; Chang et al. 2014). Recirculation pumps are needed for each stage in which cell recycling takes place, which is associated with high energy cost. Recycled cells are also not "passed down" to the next stage, partly defeating the purpose of a multi-stage reactor for this system, though it makes sense for fed-batch systems in which final-stage cells are recycled to the first stage.

This thesis proposes a semi-recycle multi-stage system for *C. acetobutylicum* fermentation, as shown in Figure 37. One or several continuous, potentially multi-stage fermentations feed into the last fermenter, which is attached to a cross-flow filtration unit (advantageous compared to dead-end filtration) for biomass separation. The permeate goes on

to separations, while the retentate is returned to the bioreactor (with a bleed stream). In theory, this system has low energy requirement as there is only one recirculation pump. The cell-recycle is carried out on the last stage, so that membrane fouling due to polysaccharides is less of a consideration, as by this time they are more likely to already have been consumed. Since the volume feeding into the last-stage reactor is large, start-up time for the recycle system is reduced. Study is required to characterise and evaluate this reactor system.

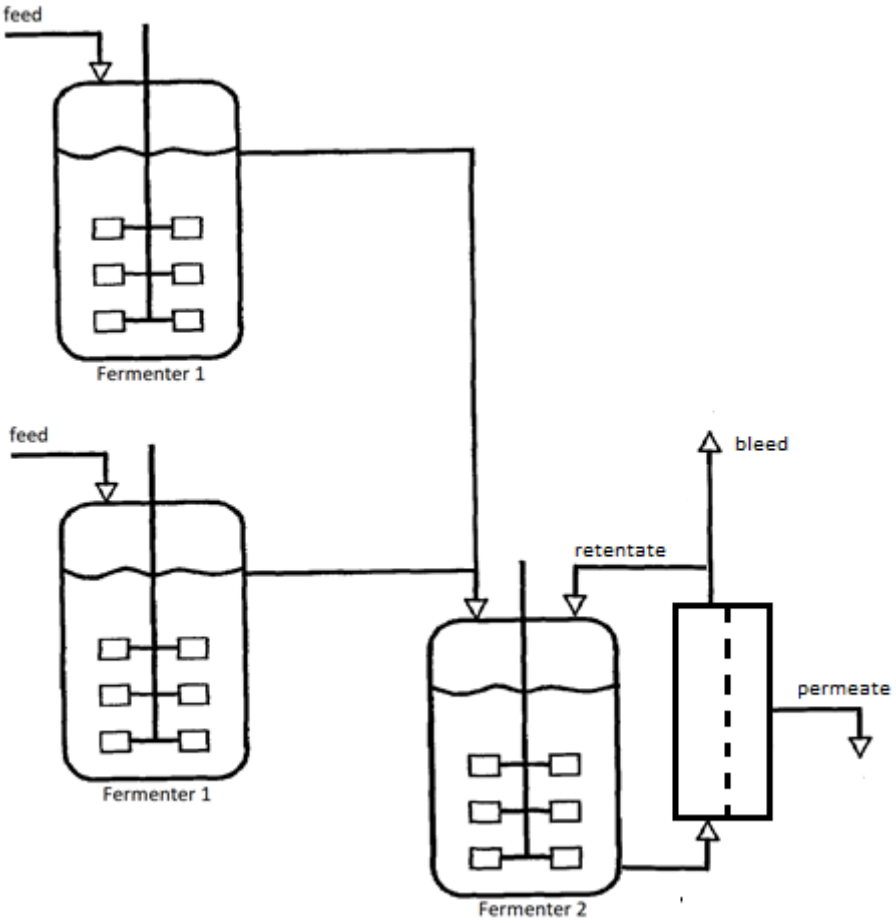


Figure 37 Proposed cell recycling in multi-stage fermentation. The several multi-stage fermentations feeding into the final reactor may be combined into a larger fermentation with higher throughput to achieve the same result.

6.4 Solvent extraction

Supercritical extraction was unsuitable for solvent extraction at the concentrations studied in this thesis, due to low recovery rates not justifying the cost. With the proposed

multi-stage fermentation, solvent concentration in the feed may reach high enough concentrations that supercritical exploration becomes viable, and studies should be undertaken at that time with the achievable feed concentration.

Besides SCE, liquid-liquid extraction (LLE) and other methods of ABE extraction have been studied and modelled (Huang, Ramaswamy, and Liu 2014). Economic and environmental trade-offs are very much in evidence (Sánchez-Ramírez et al. 2015). Further study and investigation of promising methods with a view to potential commercialisation is recommended.

6.5 Proposal for ABE plant in New Zealand

An ABE production plant may be built in New Zealand, following the Chinese method. Specifically, a multi-stage fermentation should be used, which allows for complete fermentation to produce the maximum amount of solvents. Regular seed culture inoculations in the first stage(s) have successfully prevented culture degeneration and can be used in this facility (Ni and Sun 2009). Ideally, one would visit the existing ABE plants in China to get an idea of how operations are conducted.

The process is best optimised by minimising the running costs of the separation process (Sánchez-Ramírez et al. 2015). While in-situ extraction has been much studied, it is not necessarily recommended. In general, the more unit operations are combined (fermentation and separations, to name two), the less robust the process becomes; to maximise production and ease of maintenance, fermentation and separation process streams should be kept separate as much as possible. However, heat integration is desirable. Nor does this principle preclude the addition of extraction operations (e.g. by gas stripping using produced gases) in between stages of a multi-stage fermentation (Bankar et al. 2012). **Error! R eference source not found.** shows a possible process diagram.

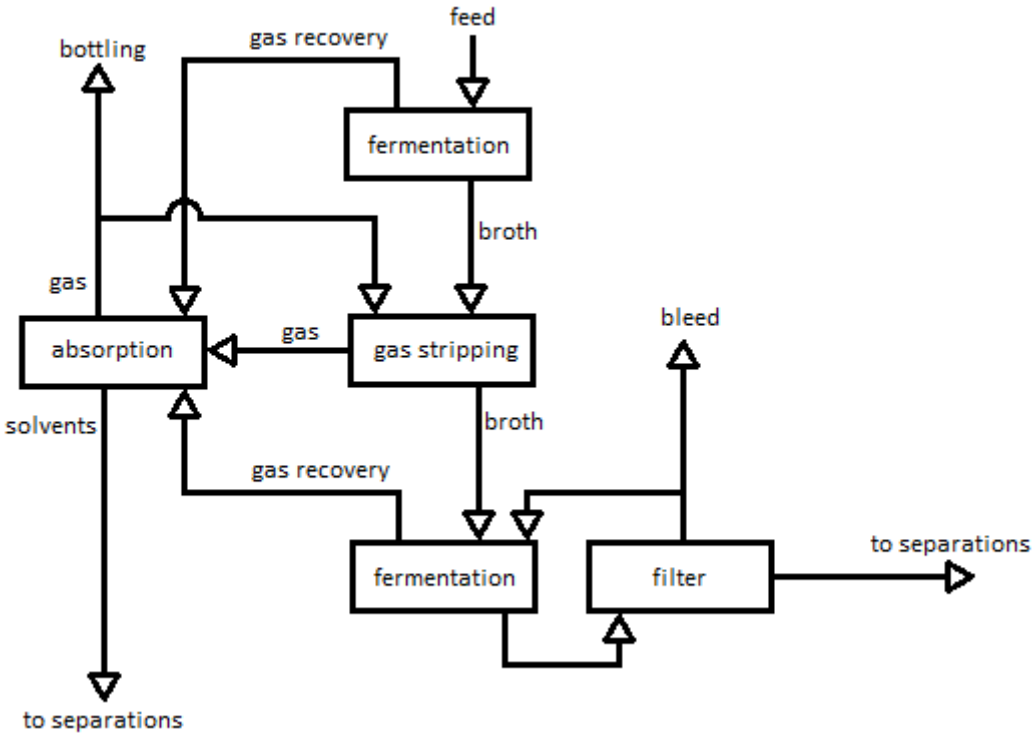


Figure 38 A simplified process diagram for commercial production of ABE

Initially, distillation could be used while an alternative method for separation of solvents from the broth is investigated and scaled up. After investigation is complete, the operation may switch over to the alternative separation method for solvent removal, with distillation to separate the solvents from each other. A major question that needs answering in this stage is: If the alternative separation method produces contaminants in the sludge (primarily composed of cells and wastewater), how does it affect the potential for further use of the sludge? This may happen with, for example, an extractant used for LLE before separation of biomass. Both animal feed and further fermentation have been discussed as potential uses, which places limits on toxicity (Zverlov et al. 2006; Ni and Sun 2009). Otherwise, the efficiency of separation would have to be so great as to outweigh the loss of a revenue stream and pay for the cost of waste disposal, while still being commercially competitive; this is quite unlikely.

A strain of *Clostridium sp.* needs to be found, which is optimised for the local conditions and feedstock (Ni and Sun 2009). Preferably it would be hyper-butanol producing so that higher solvent concentrations can be achieved in the final fermentation broth, improving the economics of separations (N. Qureshi and Blaschek 2001). Resistance to bacteriophages is ideal to prevent process disruptions. Genetic engineering may be conducted to knock out the genes for acetone and/or ethanol production, thus simplifying the separation process and reducing process cost while increasing production of butanol (Nasib Qureshi, Hodge, and Vertès 2014).

For feedstock, the plant could use whey permeate, preferably demineralised (Maddox, Qureshi, and Roberts-Thomson 1995). This is to take advantage of New Zealand's dairy industry, which produces large amounts of whey as a by-product of cheese or casein production. For good growth, additional complex components were required in whey permeate (Kanchanatawee 1991), which can be expensive. Instead of commercial yeast extract, replacements should be investigated (Parekh, Formanek, and Blaschek 1999)(Sánchez and Gutiérrez 2010). The final products are ABE, H_2 and CO_2, wastewater, and biomass. Biomass can possibly be treated and used for animal fodder, which has seasonal demand in New Zealand. Wastewater processing is well studied and should not be an issue.

7 Conclusion

As part of the search for viable alternatives to petroleum processing, biological processes to produce solvents are being investigated. The aim of this project was to develop a continuous system for the fermentation of *C. acetobutylicum* to produce ABE, and its subsequent separation by supercritical CO_2 extraction. A reliable batch process was operated at 1-L scale, at pH 5.0 using either NaOH or NH_3(aq) for pH control, using a medium which included yeast extract as a complex component. This process required the inoculation of the production medium with seeds which met the criteria of pH = 4.8 ± 0.7 and [glucose] = 9.6 ± 4.9 g/L. The batch process resulted in 0.5% butanol at the end of the fermentation, after gas stripping effects (compared to a 20-L fermentation producing 0.8%, and 1.2% in the literature, using the same strain). As gas will be recycled in a final commercial process, the stripping effect is acceptable, and even desirable as it reduces solvent toxicity in the process. Nonetheless, it would be ideal to use a strain that produces higher solvent concentrations – genetic engineering has produced strains yielding up to 3% w/v butanol.

Following on from the batch fermentation, a 1-L continuous process was developed with 600-mL working volume, which was stable if unsatisfactory at a 0.16 h^{-1} dilution rate, and made use of sterile filtration instead of steam sterilisation for the sterilisation of the feed medium. The continuous fermentation resulted in cyclical butanol production between 0% and 2%. Polysaccharide slime was a recurring issue, affecting mixing in the fermenters, and preventing cell recycling from succeeding in this project by fouling membranes.

A two-stage reactor is proposed as a better solution for continuous fermentation, as it allows for complete fermentation, thus bypassing the issues around incomplete fermentation. With a little refinement to determine ideal conditions, the continuous fermentations in the current work would function exceedingly well as the first stage in a multi-stage system with commercial potential. Extraction will have to be carried out by other methods, but determining them is outside the scope of this work.

In contrast with fermentative successes, supercritical extraction was found to be unsuitable for solvent extraction, as it does not provide good solvent recovery. The ideal S/F ratio appeared to be 1.5 under these circumstances, with extraction at 20 °C and raising the temperature to 45 °C for easier collection of the output. But regardless of S/F ratio or

temperature, butanol concentrations in the extract reached a maximum of 7.65% (5.58% in runs with the temperature raised for easier collection) and were highly variable. The results were worse for acetone and theoretically would be even worse for ethanol. As the aim of exploring alternative solvent recovery processes is to avoid separating azeotropes by distillation, this result is not sufficiently good to pursue further study at this time.

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9 Appendix A: Reproduction of previous results

Objective

The objective of this experiment was to reproduce the fermentation and extraction results detailed in previous work (Moreno et al. 2012). The fermentation portion was designed to establish baseline growth curve, fermentation profile, and solvent production at 20-L scale.

Methods

CRM was used, as described in section 2.1. Seed medium was prepared in 1-L Duran bottles. The seed culture was inoculated with 0.15% glycerol stock of IRL 542 and grown at 37 °C in an anaerobic chamber, agitated at 50 RPM in an incubator. The fermentation was conducted in a 30-L fermenter (FER100, working volume 20-L) with a 7.5% inoculation rate (1.5 L of seed culture). Temperature was controlled at 37 °C, with stirring at 50 RPM, and flow of N_2 at 0.8 LPM to maintain dissolved oxygen (DO) at 0%. pH control was not used in this experiment. Samples were taken twice a day in the mornings and evenings on weekdays, and once a day on weekends, then stored at -20 °C until processed for solvent analysis.

The fermentation broth from this experiment was centrifuged at 7333 RCF for 45 minutes, and the supernatant used as the feed for the first three supercritical extraction runs. This feed contained approximately 0.8% butanol, 0.5% acetone and 0.1% ethanol. The fermentation broth from the next experiment (section 3.1, scale down, FER_A1) was centrifuged similarly and used for the other supercritical extraction runs described here.

Supercritical extraction was conducted as described in section 2.2. The feed flow rate was 6 g/min and solvent flow rate was 15 g/min. All runs were conducted at 100 bar and 40 °C, except for run 3 which took place at 20 °C. Samples were analysed as described in section 2.3.

Results and discussion: fermentation

The fermentation ran for 7 days. Figure 39 shows the overall results of the experiment, while Figure 40 shows the total amount of each solvent produced over the course of the experiment.

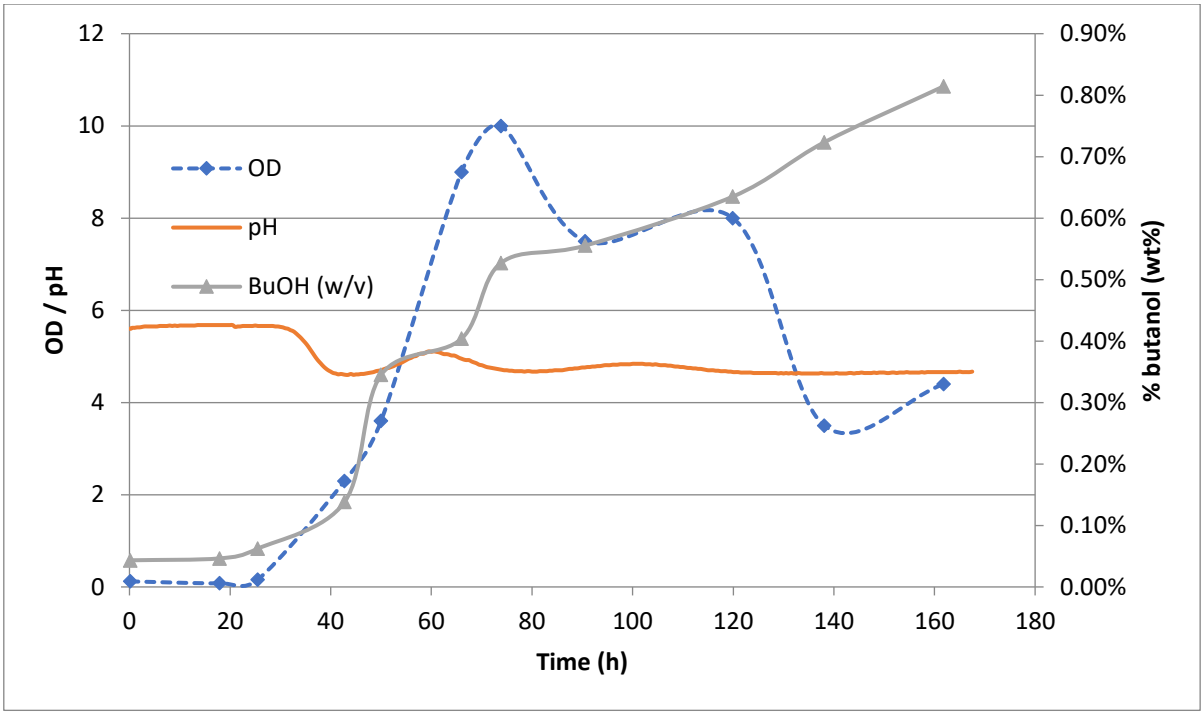

Figure 39 Growth curve, pH profile and butanol production in Experiment 1

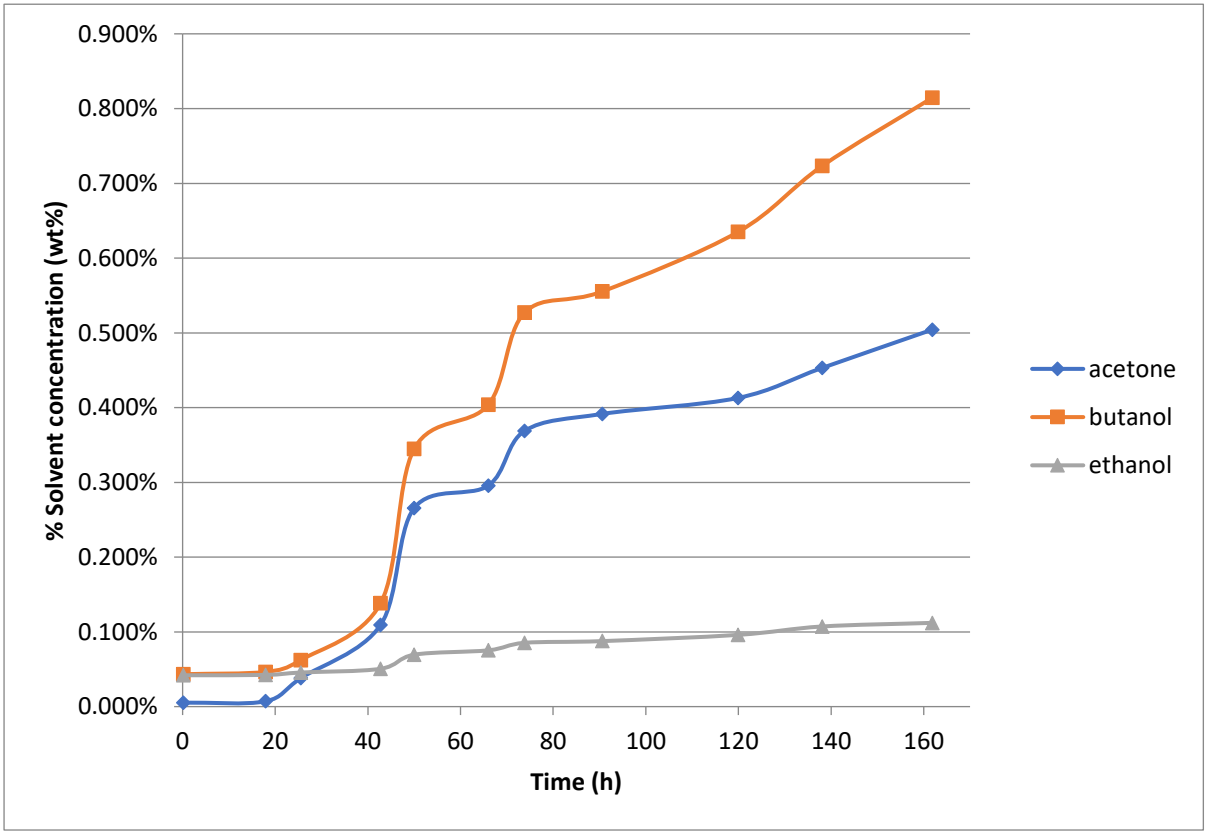

Figure 40 Solvent production in Experiment 1

The experiment ran smoothly. Biomass peaked at OD_{600} = 10 on day 3 (Figure 1) and then declined over the course of the rest of the fermentation. Solvent production (Figure 2) was lower than expected, with 0.8% final butanol concentration, as opposed to 1.2% which was reported before (Moreno et al. 2012). The differences between this fermentation and the previously reported one may be due to experimental variance: because this was the first fermentation run as part of this project, it is possible that the precise conditions of the previous fermentation were not achieved. Possibilities include different sources of raw materials, slight variations in sterilisation conditions (particularly time taken to reach sterilisation temperature), and analytical error. The variable rate of solvent production through the 90.5 h sample may be due to sample preparation error, e.g. dilution, or simple variance as only one sample was taken at each data point.

Ultimate solvent production fell short of previous reports. Nothing unexpected occurred during the fermentation or with the analyses. Additional fermentation runs would reveal whether the results obtained in this experiment are representative of a baseline.

Results and discussion: supercritical extraction

Figure 41 shows the overall result of the three extraction runs. The concentration of butanol in the extract was double the feed concentration for the runs done at 40 °C, at 1.6% from 0.8%. This is quite a bad result, and does not agree with the literature (Moreno et al. 2012; Moreno, Tallon, and Catchpole 2014); one possible reason is that the starting feed concentration is lower than in the papers. Also, previous data suggest that running the extraction at 20 °C produces better results than at 40 °C (Montanes 2016). In this experiment, it was not seen; however, only one extraction run was conducted at 20 °C here, so experimental variance must be considered. Also, the extract flow rate was much lower at 20°C, which increases the possibility that solvent evaporation during the course of the experiment was significant.

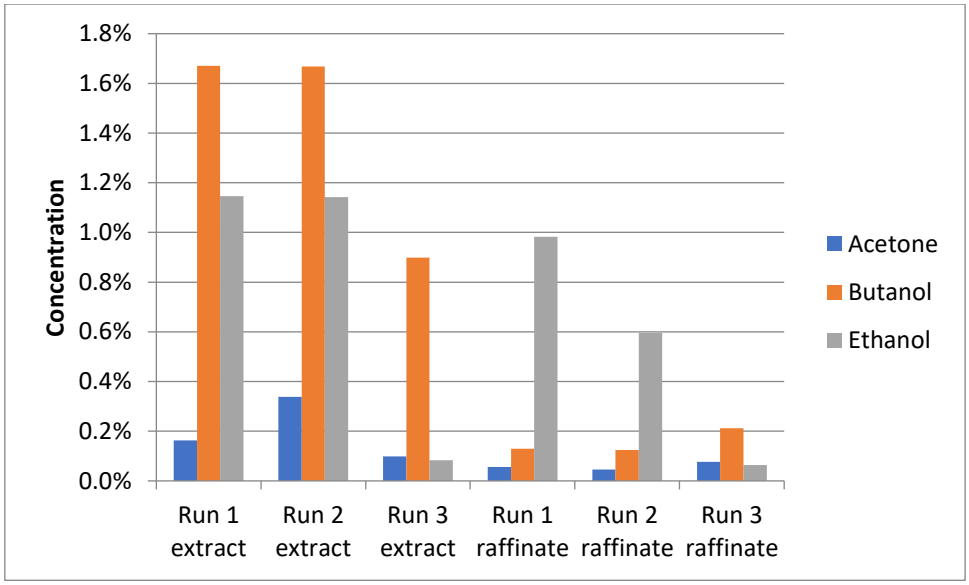

Figure 41 ABE concentrations in extract and raffinate from 3 SCE runs

Of particular note is the ethanol concentration in both extract and raffinate: this was close to 1.0% despite being only 0.1% in the feed. On further consideration, the pipes were cleaned with ethanol before the experiment, so it is likely that some of the measured ethanol is comprised of residual ethanol from the cleaning instead of extraction. This means that 15-20 minutes of continuous operation is not sufficient to flush ethanol out of the system. Figure 42 shows the overall result of the two extraction runs in the second attempt. Ethanol concentration is not shown due to contamination from the cleaning system.

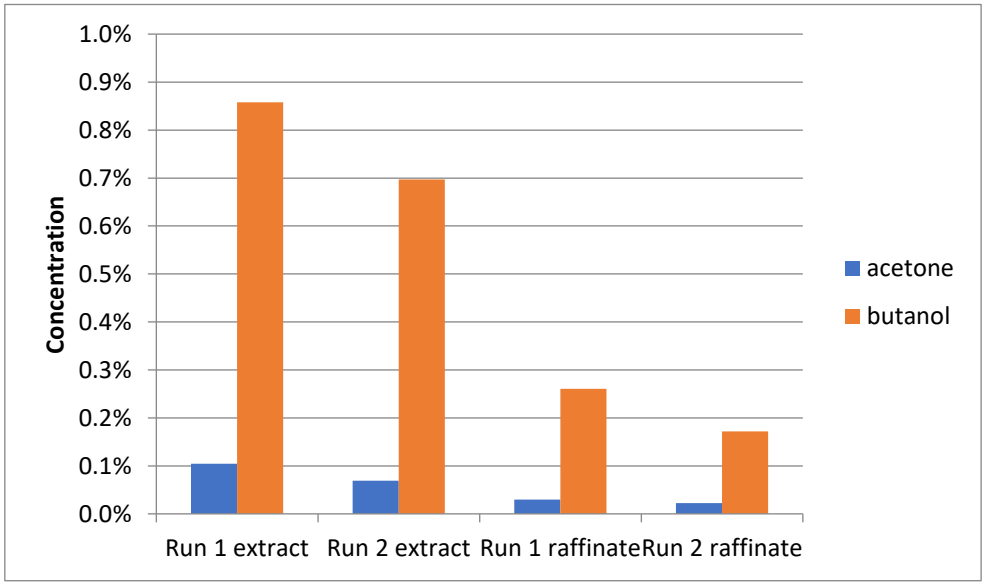

Figure 42 Extraction results in the second attempt at reproducing previous results

The concentration of butanol in the extract was double the feed concentration again, at 0.86% from 0.43%. This is quite a bad result in terms of absolute concentration, but it agrees with the data from the previous experiment. Again, ethanol contamination was evident, as the fermentation broth contained 0.04% and the exit streams from supercritical extraction all contained a minimum of 1.5% ethanol.

It may have been that the previous results were unusually good, or it may have been an effect of low solvent concentrations in the feed; regardless, it was decided that investigating the effect of experimental conditions on extraction rate would be more productive than continued attempts to reproduce prior results.

Conclusion

The objectives of this experiment were not achieved as we were unable to reproduce the previous results. For these experiments, as long as the tubes are cleaned with ethanol, it does not make sense to measure ethanol concentration. Since ethanol is not produced in significant quantities in fermentation, nor extracted in significant quantities through supercritical means, it is likely safe to disregard it for the moment.

10 Appendix B: Filtration testing

Objective

To establish a method of filtration and cell recycling which is able to meet the demands of the ABE fermentation system with a permeate throughput of 450 ml/hour.

Methods

The strain used was *Bacillus subtilis*, grown in tryptic soy broth (TSB) which contained, per litre, 3.0 g soy peptone, 17 g peptone (casein), 2.5 g K_2HPO_4, 2.5g glucose and 2.5 g NaCl. *E.coli* from another researcher's experiments was also used in part 1, grown in modified Terrific broth which contained, per liter, 12 g tryptone, 24 g yeast extract, 10 g NaCl, 6 g K_2HPO_4, 1.2 g KH_2PO_4, and 5 g glucose. The fermentation broth was centrifuged and cells were resuspended in feed medium, which was described in section 2.1.

In part 1, the hollow fibre filtration modules were used, which were ceramic membranes with pore sizes 0.1 and 0.02 µm. The pump used was a Cole-Parmer digital gear pump, and in this experiment, the pump speed was set to a constant "150 mL/min" (not representative of true pump speed because of a different pump head installed post-manufacture, but providing a constant driving force). In part 2, the Sartocon Slice TFF unit was used. The TFF unit can be run under constant flow rate or constant transmembrane pressure settings.

Tests were run with water first, then with a cell suspension. The flow of permeate was measured in a measuring cylinder for a specific time, as determined by a stopwatch. The exact time spent for the measurement depended on the flow rate.

Optical density (OD_{600}) measurements were performed on a model 83059-15 spectrophotometer from Cole-Parmer. Samples were diluted with PBS where necessary, so that the final absorbance reading was between 0.1 and 1.0. OD_{600} was calculated by multiplying the absorbance reading by the dilution factor.

Results and discussion

In the first test, with the 0.1 μm ceramic membrane, a *B. subtilis* culture was used. The OD of the suspension was 11.6. The data were lost, but results were consistent with those obtained in the fermentation experiment. In the next test, with the 0.02 μm membrane, *E.coli* was used. (Table 18) The OD of the suspension was 11.2.

Table 18 Flux through 0.02 μm ceramic membrane

Settings	OD	Stream	Volume	Time	Flux
No back pressure	(water)	Permeate	35.5 mL	5 min	426 mL/h
		Retentate	460 mL	1 min	27600 mL/h
Back pressure	(water)	Permeate	160 mL	3min	3200 mL/h
		Retentate	180 mL	1 min	10800 mL/h
More back pressure	(water)	Permeate	160 mL	3 min	3200 mL/h
		Retentate	82 mL	3 min	1640 mL/h
Cell suspension, no back pressure	11.2	Permeate	6.5 mL	3 min	130 mL/h
Cell suspension, back pressure	11.2	Permeate	5.0 mL	4min	75 mL/h
No back pressure	(water)	Permeate	18 mL	5 min	216 mL/h
		Retentate	42 mL	5 sec	30240 mL/h
Back pressure	(water)	Permeate	36 mL	1 min	2160 mL/h

The experiment with the cell suspension and back pressure turned out a lower flux than the one without back pressure. This is contrary to expectations, but understandable as the experiment without back pressure was the first time the membrane was used. Thus, membrane fouling is to be expected. This is further shown in the last experiments with water, where the flow rate of permeate did not reach the levels it had previously, in spite of membrane cleaning.

It is completely unexplained how the membrane with the smaller pore size has a much better flux. The current working hypothesis is that the two modules were mislabelled; as custom orders, this is within the realm of possibility. Alternatively, the membrane pores may have been of the exact size to become blocked by cells.

Consultation with an experienced process engineer revealed that 4 m/s is the optimal flow velocity through those membranes (Dyer 2016). With an internal diameter of 6.7 mm (measured), this translates to a flow rate of 1409.5 mL per second, which is not achievable in a system with 500 mL working volume. Thus, part 2 of the experiment was conducted with a different filter system, with results shown in Table 19. *B. subtilis* was used, with an initial OD of 5.3.

Table 19 Flux through Sartorius Slice TFF unit (0.2 µm membrane)

Settings	OD	Volume permeate	Time	Flow rate
Constant flow “40 mL/min”, manual back pressure adjust to 1.0	(water)	126 mL	20 s	22680 mL/h
Constant flow “40 mL/min”, manual back pressure adjust to 1.0-1.2	~5.3 at start of experiment	76 mL	30 s	9120 mL/h

Constant TM pressure 1.00	~10	44 mL	30 s	5280 mL/h
Constant TM pressure 2.00	>10 (probably about 15-20)	37 mL	30 s	4440 mL/h

The experiment ran smoothly. It is not known why the 0.1 µm ceramic filter has much lower flow rate than the 0.02 µm one. Since these were custom jobs, it is possible that there had been a mix-up. Regardless, neither was able to provide the required flow rate of 450 ml/h permeate with cell broth, so an alternative method was found which did provide it.

It was not possible to test the filtration system at the cell densities mentioned in the literature of up to 100 g/L dry cell weight, but more membranes can be added to expand the capacity of the Slice TFF in case the current setup turns out to be insufficient at that cell concentration (Tashiro et al. 2005).

Conclusion

Overall, the objectives of this experiment were achieved, and a viable filtration and cell recycling system found. It is possible to test whether the ceramic filters were mislabeled, by using a molecule about 100 kDa in size. This is not relevant to the project since these filters cannot be used here, but it may be useful for another project.

11 Appendix C: Testing of other strains

Objective

To determine if better results could be achieved by changing the strain of *C. acetobutylicum* used

Materials and methods

The strains used in part 1 were *Clostridium acetobutylicum* ATCC 824, strain IRL 542, and DSMZ 6228 which had arrived in a freeze-dried state. In part 2, DSMZ 792, which also came in a freeze-dried state, was added. DSMZ 6228 and DSMZ 792 were obtained from DSMZ.

Clostridial reactor medium (CRM), as described in section 2, was used. Reinforced clostridial medium (RCM), pre-packaged from Becton Dickinson, was also used in part 2 to test it against the standard solution used previously. Seed medium was prepared in 100-mL Duran bottles. In addition to bottled media, plates were prepared by adding 1.5% agar.

IRL 542: The seed culture was inoculated with 0.15% glycerol stock of IRL 542 and grown at 37 °C in an anaerobic chamber, agitated at 50 RPM in an incubator. The fermentation was conducted in a 250-mL conical flask which was inoculated to OD = 0.118 and bubbled through it to reach 0% dissolved oxygen (DO). Temperature was controlled at 37 °C, with agitation at 50 RPM. pH control was not used in this experiment. Samples were taken at t= x and y hours, then stored at -20 °C until processed for solvent analysis.

DSMZ 6228 and 792 were rehydrated as follows: The glass vial in which the samples came was broken per instructions, and N_2 was piped over it briefly. Seed medium (or RCM) was added to the samples to rehydrate them, the mixture was added to a bottle of seed medium, and N_2 bubbled through the bottle. This was grown at 37 °C in an anaerobic chamber, agitated at 50 RPM in an incubator.

Results and discussion

IRL 542 grew in all conditions. DSMZ 6228 failed to grow.

The experiment was repeated with the addition of DSMZ 792, plating some of the new samples in addition to growing in bottles. One bottle each of CRM and RCM was used for each strain, in addition to CRM and RCM plates.

After 6 days, IRL 542 had grown in all conditions. DSMZ 6228 failed to grow. DSMZ 792 only grew on the CRM plate. The decision was made to take the fuzzy particulate matter from the bottom of the bottles where it had settled and grow it in 15-mL vials (sparged with N_2), along with plating some.

None of the vials showed any evidence of growth. In addition, DSMZ 792 from the plate failed to grow when transferred to bottles of either CRM or RCM.

Conclusion

It was decided to continue with development of the system with the original strain, as a more productive use of time.

12 Appendix D: Supercritical extraction raw data

Table 20 Result of extraction runs conducted at different temperatures at 100 bar and 1.5 S/F ratio with 6 g/min feed and 9 g/min CO_2 (data used in Figure 1)

Temperature	Concentration in extract		Concentration in raffinate	
	Acetone	Butanol	Acetone	Butanol
18	0.77%	2.76%	0.12%	0.56%
18	1.06%	5.68%	0.17%	0.70%
22	0.77%	3.83%	0.25%	1.02%
22	0.58%	2.23%	0.19%	0.80%
25	0.20%	0.79%	0.10%	0.50%
25	2.53%	3.84%	0.08%	0.40%
30	0.73%	3.04%	0.05%	0.33%
30	0.71%	3.57%	0.06%	0.36%

Table 21 Acetone and butanol concentrations (wt%) in extract and raffinate from S/F ratio testing (data used in Figure 2)

Feed flow rate (g/min)	Solvent flow rate (g/min)	S/F ratio	Concentration in extract		Concentration in raffinate	
			Acetone	Butanol	Acetone	Butanol
6	6	1	0.88%	3.70%	0.17%	0.77%

6	6	1	1.34%	7.43%	0.16%	0.72%
8	8	1	1.11%	3.26%	0.21%	0.87%
8	8	1	0.62%	2.14%	0.16%	0.69%
6	9	1.5	2.02%	6.59%	0.06%	0.39%
6	9	1.5	1.17%	7.65%	0.13%	0.54%
8	12	1.5	0.45%	3.73%	0.05%	0.30%
8	12	1.5	0.77%	4.97%	0.12%	0.52%
12	12	1	0.51%	1.79%	0.24%	0.61%
12	12	1	0.64%	1.89%	0.13%	0.45%
4	6	1.5	0.89%	3.56%	0.11%	0.25%
4	6	1.5	2.22%	6.28%	0.08%	0.19%
4	8	2	1.14%	5.26%	0.11%	0.23%
4	8	2	0.21%	0.34%	0.15%	0.26%
6	12	2	2.02%	4.57%	0.25%	0.38%
6	12	2	1.11%	4.51%	1.02%	1.50%
6	15	2.5	0.61%	0.68%	0.15%	0.24%
6	15	2.5	0.32%	0.83%	0.12%	0.24%

Table 22 S/F ratio testing with temperature raise after extraction (data used in Figure 3)

Feed flow rate (g/min)	Solvent flow rate (g/min)	S/F ratio	Concentration in extract		Concentration in raffinate	
			Acetone	Butanol	Acetone	Butanol
6	9	1.5	0.37%	3.10%	0.06%	0.33%
6	9	1.5	0.27%	1.79%	0.08%	0.36%
6	6	1	0.31%	2.09%	0.13%	0.53%
6	6	1	0.28%	1.40%	0.12%	0.44%

Table 23 Butanol concentration in extract, with and without temperature raise after extraction (Fig. 4)

Feed flow rate (g/min)	Solvent flow rate (g/min)	S/F ratio	Concentration in extract		Concentration in raffinate	
			With	Without	With	Without
6	9	1.5	3.10%	6.59%	0.33%	0.39%
6	9	1.5	1.79%	7.65%	0.36%	0.54%
6	6	1	2.09%	3.70%	0.53%	0.77%
6	6	1	1.40%	7.43%	0.44%	0.72%

Table 24 Operating temperature testing with collection temperature raise, (data used in Fig. 5)

Temperature	Concentration in extract		Concentration in raffinate	
	Acetone	Butanol	Acetone	Butanol
18	0.36%	0.82%	0.04%	0.09%
18	0.44%	2.14%	0.05%	0.08%
20	0.13%	1.88%	0.06%	0.08%
20	0.80%	5.11%	0.05%	0.08%
22	0.99%	5.48%	0.06%	0.09%
22	0.40%	3.25%	0.08%	0.11%
30	0.31%	4.48%	0.04%	0.08%
30	0.15%	0.42%	0.05%	0.07%

Table 25 Operating temperature results comparison with and without temperature raise (data used in Fig 6)

Temperature	Butanol concentration in extract		Butanol concentration in raffinate	
	Without	With	Without	With
18	2.76%	0.82%	0.56%	0.09%
18	5.68%	2.14%	0.70%	0.08%
22	3.83%	5.48%	1.02%	0.09%
22	2.23%	3.25%	0.80%	0.11%
30	3.04%	4.48%	0.33%	0.08%
30	3.57%	0.42%	0.36%	0.07%

Table 26 Operating temperature testing with temperature raise and changed order of runs (data used in Figures 5 and 7)

T contactor	T collection	Extract		Raffinate	
		Acetone	Butanol	Acetone	Butanol
20	45	0.11%	1.73%	0.06%	0.07%
22	45	1.29%	4.57%	0.07%	0.07%
30	45	0.85%	3.84%	0.07%	0.06%
20	45	0.45%	2.53%	0.08%	0.08%
22	45	0.01%	1.31%	0.06%	0.07%
30	45	0.45%	5.58%	0.07%	0.07%
20	45	0.11%	1.81%	0.09%	0.09%
20	45	0.03%	1.33%	0.08%	0.12%
20	45	0.04%	1.45%	0.06%	0.08%
20	30	0.28%	0.79%	0.07%	0.07%
22	30	0.29%	0.68%	0.10%	0.08%
22	30	0.10%	4.31%	0.06%	0.06%