COMBINED ACETONE, BUTANOL, ETHANOL AND ORGANIC ACID FERMENTATION BY A DEGENERATED STRAIN OF *CLOSTRIDIUM ACETOBUTYLICUM* AND SUBSEQUENT ESTERIFICATION

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Abstract

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Although bacterial ABE (Acetone, Butanol and Ethanol) fermentations have been known for more than 150 years, widespread large-scale ABE fermentations remain absent in today's economy. Main hurdles to overcome include low productivities, strain degeneration and inefficient product recovery techniques. This work proposes and investigates concepts aimed at improving the overall efficiency of the ABE fermentation process using a partially degenerated strain of *Clostridium acetobutylicum* (ATCC 824). In particular, three interrelated concepts were studied in detail.

First, strain degeneration, a process where-by the original solvent-producing strain mutates or is outgrown by a degenerated version incapable of solventogenesis, was investigated. Here, two viable alternatives to circumvent the problem of strain degeneration of solvent producing strains of ATCC 824 are being proposed. They consist of (i) focusing on the precursor of solvents, namely organic acids and (ii) applying a technique of systematic pH control that can trigger the degenerated culture into solventogenesis.

Second, a reactor system consisting of a plug flow reactor (PFR) and two continuously stirred tank reactors (CSTR) was studied. This setup was capable of avoiding strain degeneration while maintaining high final titers and volumetric productivities. The reactor system was continuously operated for 40 days while the effects of dilution rate and substrate composition were investigated. The highest combined product titers for both ABE and organic acids could be achieved when the ABE fermentation was followed by an organic acid fermentation. Here, the final combined product concentration reached 27.7 g/l. The reactor system was modeled using a set of differential equations reflecting the biochemical and physiological behavior of the culture.

Finally, product removal was studied using adsorption onto activated carbon and liquid-liquid extraction. It could be shown that both techniques are capable of recovering equi-molar ratios of organic acids and alcohols into a micro-aqueous system. Subsequent esterification was investigated using a lipase as biocatalyst. Reaction mechanisms and kinetic models for the enzymatic conversion are being proposed.

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1 Introduction and Objectives

Organic solvents such as acetone, 1-butanol and ethanol (ABE) are important chemicals that are produced on a large scale worldwide. They are utilized in a variety of applications in the chemical , food and pharmaceutical industries. In addition, ethanol and butanol have recently received renewed attention for their use as motor fuel and fuel additives. Moreover, an even wider spectrum of applications is possible when used as reactants in standard chemical reaction such as esterification, dehydration and oxidation to form the corresponding esters, alkenes and aldehydes, respectively. With the exception of ethanol, these solvents are currently produced through chemical synthesis from crude oil or natural gas. However, concerns about global warming and recent spikes in oil and gas prices have illustrated the dependence of our modern society on fossil resources and have sparked new interest in the biotechnological production of these solvents [3]. Additionally, chemicals produced by fermentation and those further refined by esterification can be given the title "natural". Because of this, they can be sold at higher price than otherwise chemically derived compounds. "Natural" chemicals are especially desireable in products belonging to the cosmetic-, food-, detergent- and pharmaceutical industry (e.g. fragrances, flavor compounds etc.) [13].

1.1 Historic ABE fermentations and current market considerations

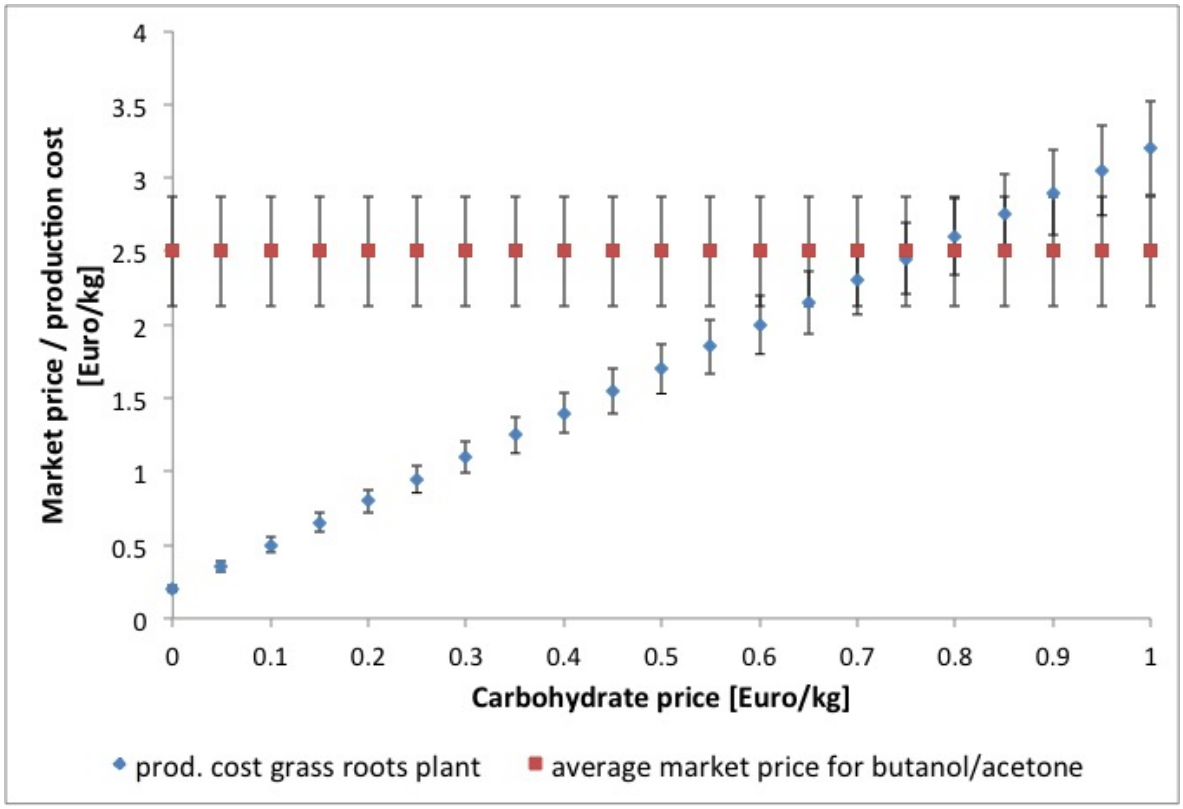
While the first intentional solvent production by mankind dates back to the beginnings of civilization with the yeast fermentation of fruit juice to ethanol containing beverages, solvent producing bacteria, on the other hand, were discovered much more recently. It was the famous French microbiologist Pasteur who discovered these bacteria in 1862. He called the culture "Vibrion butyricum," though it was more likely a mixed rather than a pure

culture [1]. Sauer et al. [2] speculates these organisms to have been similar or identical to *Clostridium butyricum*. Half a century later, when the demand for acetone increased in the midst of World War I, the first large-scale solvent producing factory went into production. Acetone was desperately needed as a bulk chemical for the production of cordite (smokeless ammunition). The culture used in the process was discovered by Weizmann who named it *Clostridium acetobutylicum* [1]. It has been speculated that without the constant supply of acetone, the outcomes of WWI might have been different. Weizmann's accomplishments were greatly acknowledged by the British government and his popularity eventually led to him being installed as the first president of the newly established state of Israel [1].

Solvent producing bacteria from the Clostridia family typically produce three solvents: acetone, butanol and ethanol. Hence, it is often called the ABE-fermentation. The common molar ratios between these chemicals is 3:6:1 [3]. In WWI, with acetone being the most desirable solvent, butanol was simply stored away. However, the automotive industry quickly found a use for butanol by converting it to butyl acetate, a chemical needed for quick drying car lacquers. Other uses, mostly related to the chemical industry, soon emerged. By the late 1950's, fermentation derived solvent production peaked and then quickly declined. By then, it had become financially more attractive to produce solvents using chemical pathways with crude oil as a precursor [3]. Beginning with the first oil crisis in the seventies, interest in solvent fermentation revived among researchers. However, the majority of papers published on this topic are less than ten years old. The reasons for the recent increase in research activity can be attributed to the fact that butanol is a potential bio-fuel and can therefore reduce our dependency on imported oil and decrease our carbon footprint since it uses renewable resources as fermentation substrate. Additionally, recent spikes in oil and gas prices have made potential solvent fermentation plants look more economically feasible. As of today, however, the widespread large scale conversion of biomass to solvents using bacteria remains a relict of the past due to the availability of cheaper petrochemical alternatives. Compared to ethanol fermentations, who have found their way into today's

economy, ABE fermentations are slower and yield only about a tenth of the final titer. Additionally, ABE fermentation require strictly anaerobic and sterile conditions compared to bio-ethanol plants. Economically speaking, this translates to higher cost for equipment and process energy. In the year 2000, Gapes [1] estimated that the break even point for a commercial grass-roots ABE fermentation plant to be at a starch price between 0.05 and 0.09 Euro/kg. At that time, it was concluded that ABE fermentations do not appear economically feasible due to the lack of low cost substrates. Since then, crude oil price have more than quadrupled. Assuming a price range for acetone and butanol between 2.10- 2.90 Euro/kg (143 US ct/lbs as of August 7th 2011, [65]), one can project today's break even point at a starch price between 0.60 and 0.80 Euro/kg (see Figure 1.1). With corn currently trading at about $7.00 per bushel, one can calculate a corn based starch price of about 0.40 $/kg or 0.28 Euro/kg (assuming a 70% w/w starch yield from corn and a dollar value of 1.42 $/Euro). This illustrates that ABE fermentations have become economically feasible in current market environments. However, economic success remains a strong function of crude oil price and the availability of low cost substrates. Exuberant market fluctuations for crude oil and its derivatives, as witnessed in recent years, have the potential of putting commercial ABE fermentations at risk. Any technology that would lower the production cost by increasing volumetric productivity and final product titer as well as the overall yield will therefore be beneficial for future ABE fermentation plants.

Figure 1.1 – Market price of acetone/butanol versus production cost based on a grass-roots ABE production plant with ±10% uncertainty (adapted from Gapes [21]). For the interpretation of the references in color in this and in all other figures, the reader is referred to the electronic version of this dissertation.



1.2 Objectives

It is the objective of this work to propose and investigate concepts aimed at improving the overall efficiency of the fermentation and product recovery steps of ABE fermentations. With respect to the microbial conversion of biomass to solvents and organic acids, two interrelated concepts are studied in detail in Chapter 3. First, strain degeneration, a process whereby the original solventproducing strain mutates or is outgrown by a degenerated version incapable of solventogenesis, is investigated. Secondly, taking advantage of the degenerated strain behavior, a series of continuously operated reactors is studied with the overall goal of improving volumetric productivities while maintaining high final product titers. A kinetic model is developed and characterized in order to predict product composition and titers based on the knowledge of dilution rate, cell mass in the reactor and composition of the influx stream.

Efficient product recovery is essential to the overall economic viability of a fermentation process. Due to low product titers in the fermentation broth of 15 to 25 g/l, the conventional recovery process of distillation is energetically and economically at a disadvantage compared to alternative recovery methods. Chapter 6 investigates product recovery through liquid liquid extraction (LLE) and adsorption on activated carbon with subsequent esterification with n-hexane as a mediator solvent.

2 Review of literature

2.1 General Considerations of ABE Fermentations

Bacterial organisms capable of ABE fermentation generally belong to the Clostridia family. Within this family, it is *Clostridium acetobutylicum* and *Clostridium beijerinckii* that are the most widely studied organisms. Unlike yeast, both species are naturally capable of digesting five and six carbon sugars. Therefore, hemicelluloses from lignocellulosic biomass can also be converted to solvents and organic acids, which increases the overall theoretical yield from biomass [2].

Both species require obligate anaerobic conditions [1]. Hence, cell growth is relatively slow and the desired products have a high reductance degree (e.g. acetone=5.33, butanol=6, ethanol=6.0). However, the high toxicity of 1-butanol, the predominant final metabolite, prevents a high accumulation of products making recovery efforts capital and energy intensive. Traditionally, large scale batch fermentation were used to carry out the bioconversions. As compared to ethanol fermentations, these processes are rather complex and require aseptic and anaerobic conditions of the media and the equipment. In addition to contamination with other microorganism, phage infestations have been reported to have been a major problem in large scale operations [2].

2.1.1 *Clostridium acetobutylicum* ATCC 824: Biochemistry and Physiology

Clostridium acetobutylicum ATCC 824 is a gram positive bacillus that has been found in soil, lake sediment, well water, clam gut and feces of different species [22]. It is a mesophilic bacteria that grows best at temperatures between 36-37ºC. In anaerobic conditions, it is capable of fermenting five and six carbon sugars to a variety of commercially useful products such as acetone, butanol, ethanol and hydrogen as well as the organic acids butyrate and acetate. Flagella provide the organism the capability of chemotaxis with sub-

strates acting as attractants and final metabolites as repellents [23]. The lifespan of ATCC 824 follows a circle comprising of vegetative growth, stationary phase, endospore formation and cell death/cell lysis. Organic acid production is predominately occurring in the vegetative phase, whereas solvent formation peaks in the stationary phase. Endospore formation is typically cued by certain unfavorable environmental conditions such as increasing product accumulation or aerobic conditions. Upon completion of spore formation, the mother cell lyses and thereby releases the endospore. Endospores are capable of enduring great environmental stress and will germinate only once the conditions are favorable again [24].

One of the big advantages of *C. acetobutylicum* is its ability to utilize a broad variety of both five and six carbon sugars as substrates [2]. Six-carbon sugars such as mono-, di-, tri-, and polysaccharides are metabolized through glycolysis yielding 2mol of pyruvate, 2mol of ATP (adenosine triphosphate) and 2mol of NADH (nicotineamide adenine denucleotide) per mole of hexose [2]. Pentoses enter glycolysis after being converted to fructose 6-phosphate and glyceraldehyde 3-phosphate by the transketolase-transaldolase sequence [25, 26, 27]. Pyruvate then undergoes enzymatic cleavage by ferredoxin oxidoreductase in the presence of co-enzyme A (CoA) to yield acetyl-CoA, reduced ferredoxin and carbon dioxide [2]. Acetyl-CoA is the CoA-activated form of an acetate molecule and represents the central intermediate in the biochemical pathway that leads to the formation of organic acids and solvents [2] (see Figures 2.1and 2.2).

In addition to sugars, *A. acetobutylicum* can also metabolize organic acids as co-substrates. Studies undertaken by Hartmanis [28] using C-13-labeled carbon suggest that butyrate and acetate can be directly converted to solvents without the accumulation of intermediates. Wood et al. [29] showed that the majority of butyrate and acetate were converted to 1-butanol whereas about 45% of the acetate was metabolized to acetone and carbon dioxide. This result is important since it opens the possibility of re-using spent fermentation broth in order to increase the overall yield efficiency. Moreover, the water and nutrients in the

spent media can be reutilized as well leading to further cost savings.

Figure 2.1 – Biochemical pathways of *C. acetobutylicum* in acidogenic phase. Reactions which predominate during acidogenesis (a) and solventogenesis (b) of the fermentation are marked by bold arrows. (adapted from Jones et. al [2]). Refer to Table 2.1 for nomenclature.

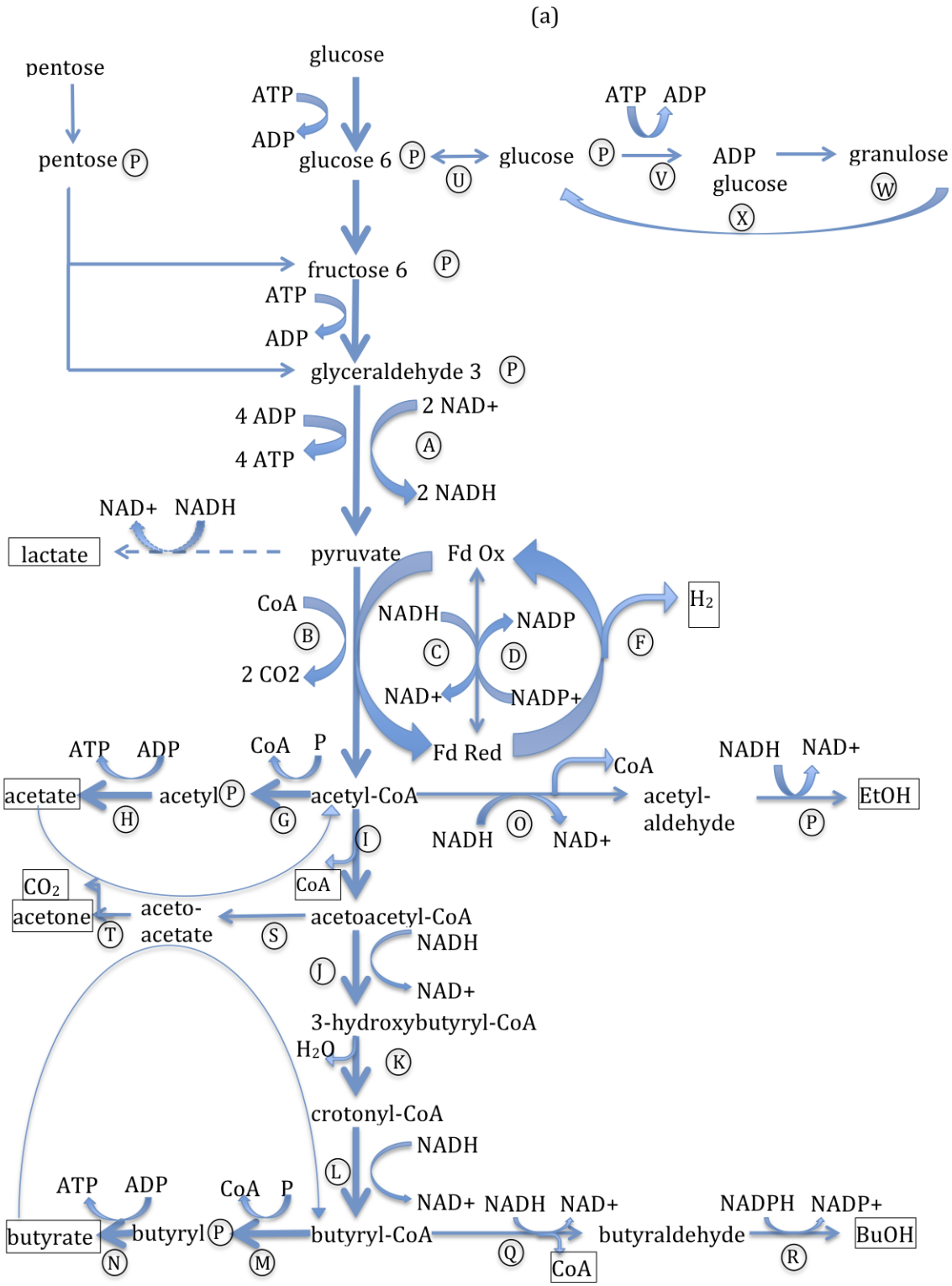


Figure 2.2 – Biochemical pathways in C. acetobutylicum in solventogenic phase (adapted from Jones et. al [2]). Refer to Table 2.1 for nomenclature.

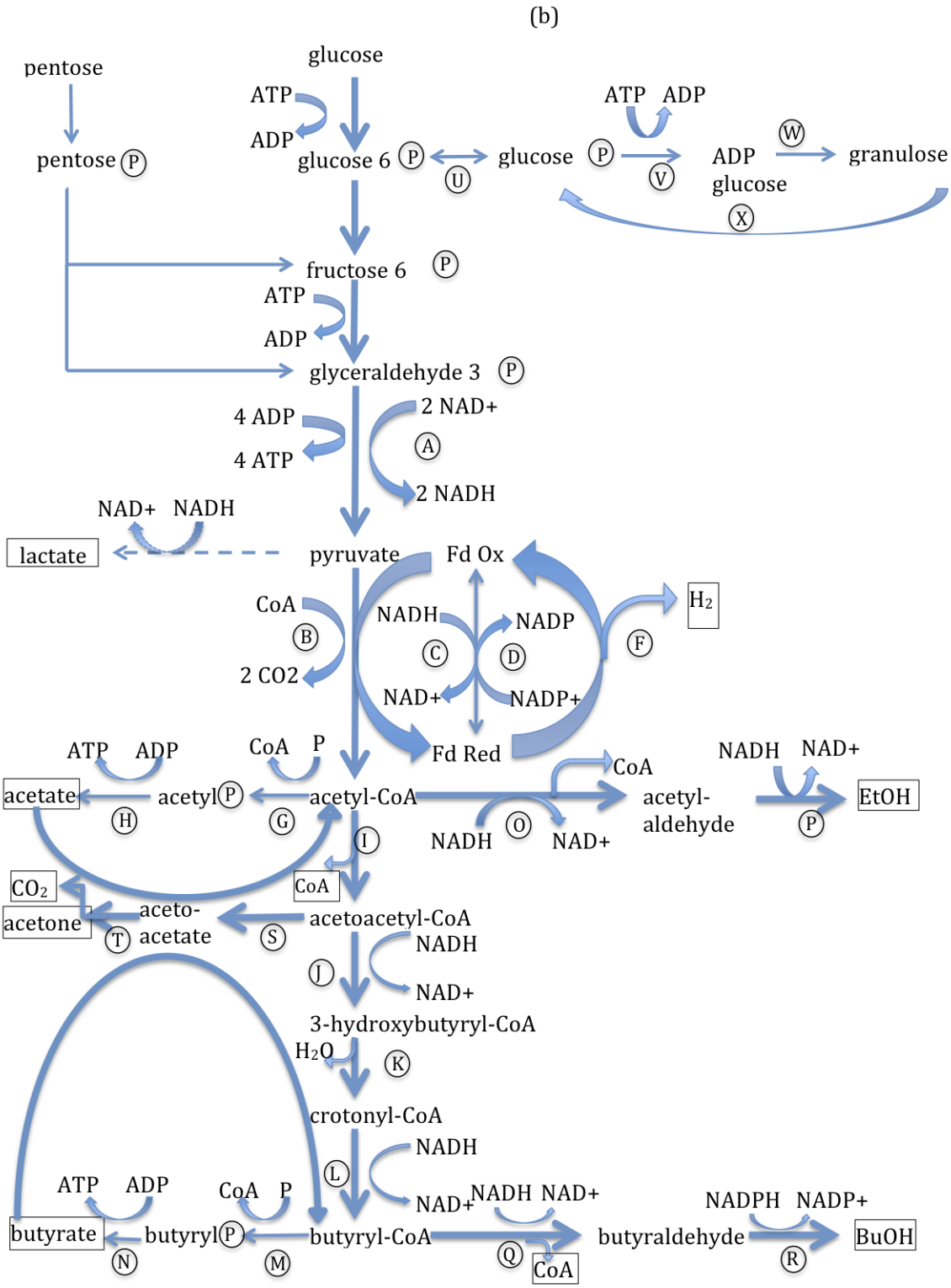


Table 2.1 – Nomenclature for Figures 2.1 and 2.2.

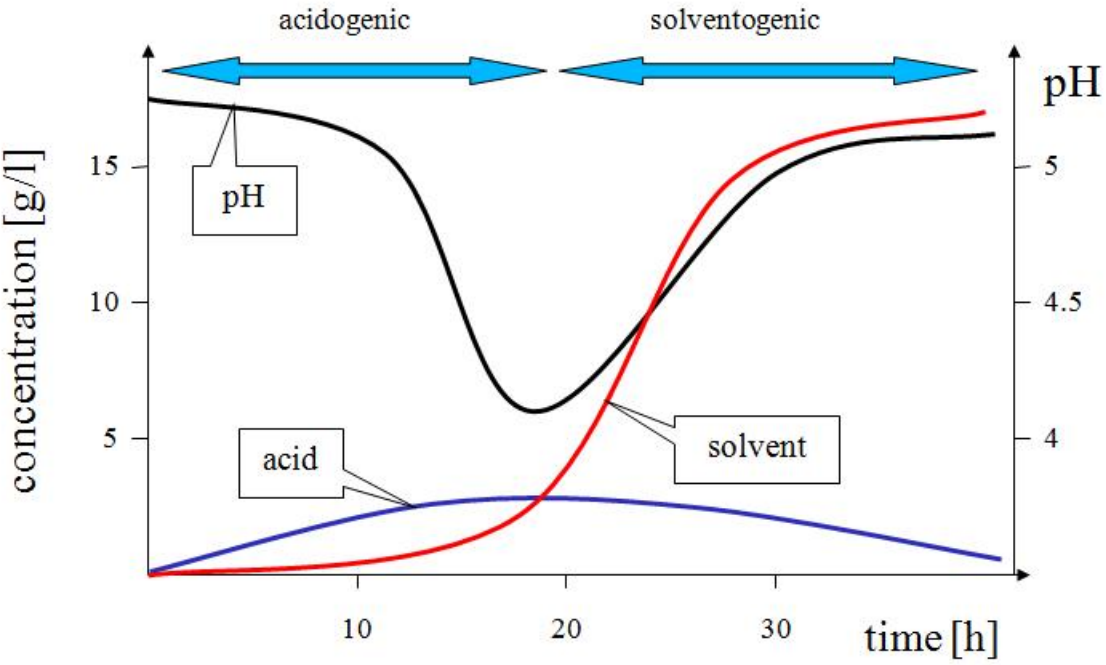
Symbol	Enzyme
A	glyceraldehyde 3-phosphate dehydrogenase
B	pyruvate-ferredoxin oxidoreductase
C	NADH-ferredoxin oxidoreductase
D	NADPH-ferredoxin oxidoreductase
E	NADH rubredoxin oxidoreductase
F	hydrogenase
G	phosphate acetyltransferase (phosphotransacetylase)
H	acetate kinase
i	thiolase (acetyl-CoA acetyltransferase)
J	3-hydroxybutyryl-CoA dehydrogenase
K	crotonase
L	butyryl-CoA dehydrogenase
M	phosphate butyltransferase (phosphotransbutyrylase)
N	butyrate kinase
O	acetaldehyde dehydrogenase
P	ethanol dehydrogenase
Q	butyraldehyde dehydrogenase
R	butanol dehydrogenase
S	acetoacetyl-CoA:acetate/butyrate:CoA transferase
T	acetoacetate decarboxylase
U	phosphoglucomutase
V	ADP-glucose pyrophosphorylase
W	granulose (glycogen) synthase
X	granulose phosphorylase

2.1.2 Batch Fermentation

From the first commercial ABE fermentations at the beginning of the 20th century to the death of the industry in the 1950's, batch fermentations were used to ferment sugars (primarily molasses) into solvents [2]. The sterilized and pH-adjusted media was inoculated through a cascade of seed fermentors using 1-10 vol% inoculums. After an initial lag-phase of one to ten hours, the fermentation first proceeds through the acidogenic phase (see Figure 2.3). Here, substrate is converted to organic acids (acetate and butyrate). Once the pH drops below a certain threshold (typically around 4.1-4.8), a major metabolic shift occurs. The organism uses the previously produced acids as a substrate and converts them into the desired solvents [1]. This is called the solventogenic phase. Final solvents titers are between 15 and 25 g/l and typical solvent productivities range between 0.2 and 0.5 g/l/h [2, 3, 1].

Compared to ethanol fermentations where the final titers and productivities are about 150 g/l and 3 g/l/h, respectively [20], ABE fermentations are slower and do not produce the same high titers. The latter is mostly due to the higher toxicity of butanol as compared to ethanol. Studies have shown that butanol concentrations as low as 5 g/l inhibit the fermentation [2]. Attempts to increase final titers have been made by various researches through chemical mutagenesis, over expression and deletion of genes and adaptation. The highest solvent concentration for modified strains are between 25-30 g/l under ideal conditions without time limitations [3].

Figure 2.3 – Schematic depiction of a batch fermentation with the undegenerated ABE strain. The lowest pH value separates the acidogenic phase from the solventogenic phase.



2.1.3 Continuous Immobilized ABE-Fermentations

In order to increase volumetric productivities, fermentations can be carried out using continuous immobilized cell systems. Here, the culture is either immobilized onto a substrate by adsorption and covalent binding or by cell entrapment. In both cases, the cell motility has been restricted to a defined space. Immobilized cell cultures provide a number of advantages over free cell fermentation including [30]

- high cell concentrations,
- low washout rates,
- re-utilization of cells,
- ability to operate reactor/fermentor similar to a plug-flow-reactor

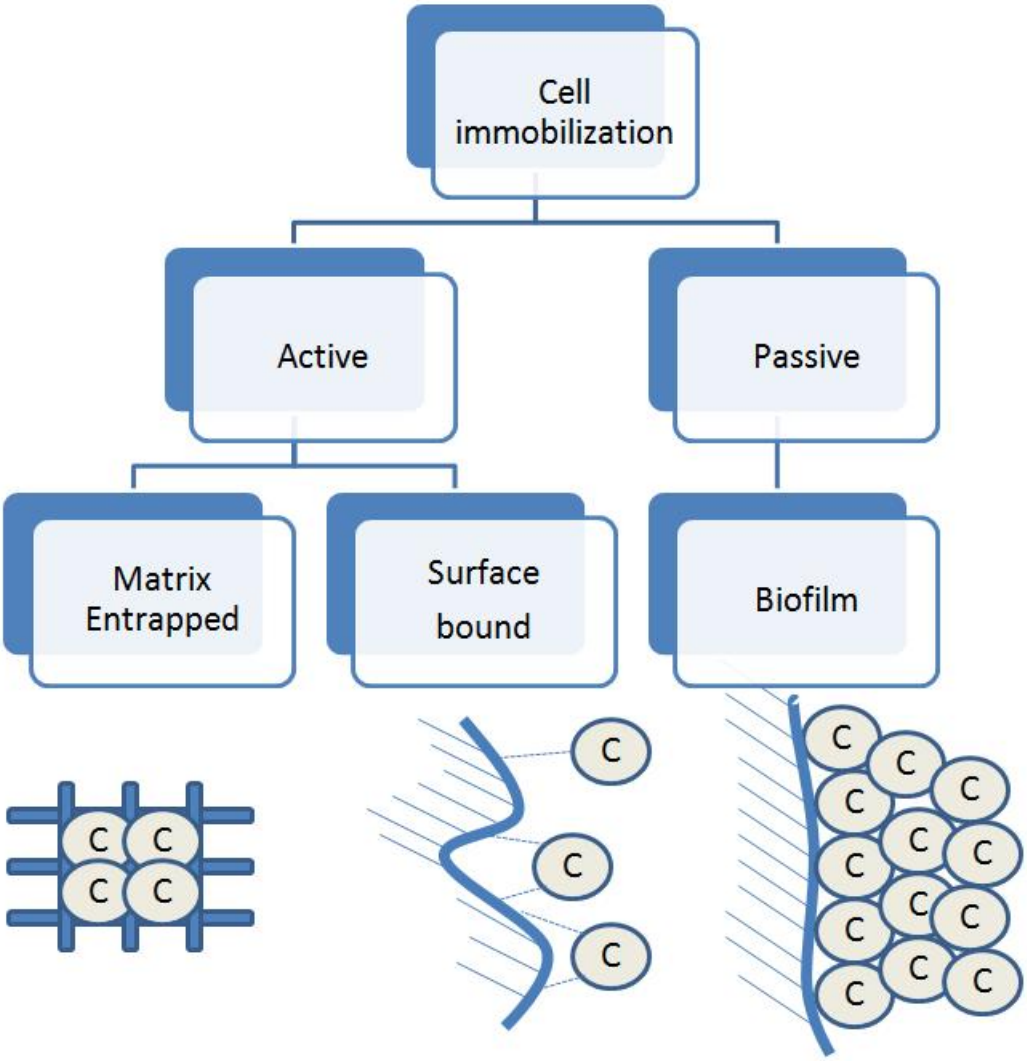
These advantages generally lead to high volumetric productivities and simplified cell- or product recovery due to lower cell concentrations in the effluent.

However, restrictions of immobilized cell systems can occur when the control of environmental conditions is important. Due the heterogeneous character of immobilized cell reactors, environmental conditions such as pH, nutrient and substrate concentrations are difficult to impose throughout the reactor. Further, if the fermentation involves the uptake or the production of gases, the immobilization of cells can be disrupted due to gas/liquid shearing leading to increased cell bleed with subsequently diminished advantages.

Cells can either be immobilized using active or passive techniques. Active immobilization is achieved when cells are entrapped into a porous matrices including polymers, silica gels, porous metal screens and polystyrene [30]. Alternatively, cells can be adsorbed onto an inert support surface utilizing the cells ability to bind to positively charged surfaces through electrostatic forces. Carriers with negative or neutral surface charge can also be utilized for cell adsorption through covalent binding or van der Waals forces [30]. Cell

entrapment offers the advantage of high cell concentrations and low cell bleed, but is more complex and expansive than adsorption [30]. Both techniques have been used successfully with *C. acetobutylicum* [4, 5]. Passive immobilization occurs when multiple layers of cells grow on a solid support surface. These bio-films are common in nature and typically consist of more than one microbial species [30]. Due to the increased thickness of the bio-film, mass transfer limitations can play an important role in the efficiency of such reactor systems. Figure 2.4 depicts an overview of commonly used cell immobilization techniques.

Figure 2.4 – Commonly used immobilization techniques (adapted from [30])



Since batch-ABE fermentations are characterized by low volumetric productivities, numerous immobilization attempts have been undertaken throughout the decades of ABE research. The two most successful experiments were conducted by Largier [4] in 1985 and by Qureshi [7] in 2000. Largier [4] used entrapment in alginate beets and was able to obtain a high final solvent titer of 15.4 g/l at a moderate productivity of 2.0 g/l/h. Qureshi [7] on the other hand obtained high productivities of 15.8 g/l/h at a relatively low solvent titer of 7.9 g/l/h using porous clay brick as adsorption matrix (see Table 2.2). Achieving both high volumetric productivities at high solvent titers has, as of today, not been described in the literature and remains a highly desirable goal.

Table 2.2 – Investigated immobilization techniques and their outcome

Investigator, reference	**Bacterial strain**	**Feedstock**	**Matrix**	**Reactor type**	**Productivity [g/l/h]**	**Solvent concentration [g/l]**	**Solvent recovery**
Largier et al. 1985, [4]	C. acetobotylicum P262	Glucose	Calcium Alginate	Fluidized bed	3.0	15.4	no
Lienhard et al. 2002,[5]	C. beijerinckii BA101	Glucose	Claybrick	plug-flow	10.2	6.8	Pervaporation
Qureshi et al. 2000, [7]	C. beijerinckii BA101	Glucose	Claybrick	plug-flow	15.8	7.9	no
Huang et al. 2004, [6]	C.acetobotylicum ATCC 55025	Glucose	Fibrous bed	plug-flow	4.6	5.1 (BuOH)	no

2.1.4 Culture stability

A phenomenon known as "strain degeneration" describes the tendency of *C. acetobutylicum* (and other solvent producing microorganism belonging to the *Clostridia* family) to partially

or completely loose the ability to produce solvents. The phenomena was first described more than 100 years ago and remains poorly understood [2]. It is believed that with strain degeneration the original solvent-producing strain mutates or is outgrown by a degenerated version incapable of solventogenesis. Therefore, seed cultures are often prepared from spores of solvent producing *Clostridia* through heat activation. However, seeding of large scale batch fermentations (e.g. 5ml to 100ml to 2ltr to 200 ltr to 20,000 ltr) is only possible through the growth of several generations (about 25 if the initial seed contains 1mg dry cell matter and the average cell concentration is 1.5 g dry cell matter per liter of broth) of the original culture and provides therefore a number of opportunities for degeneration. With continuous cultures, there is a constant growth and death of cells. As a result, the degenerative process is most often observed with serial sub-culturing and continuous cultures [9]. The latter is of particular significance since continuous fermentations harbor the potential of increasing volumetric productivities [10]. In summary, strain degeneration constitutes a significant risk to a successful operation of ABE fermentation plants since it can spoil entire batches of media. Next to the labor and energy intensive process of media-reconditioning, it is lost time and fermentation capacity that will affect the economics of a commercial process significantly. Finding techniques that avoid or reduce the effects of culture instability will therefore be crucial to the success of future ABE fermentation plants.

2.2 Down Stream Product Recovery

Historically, steam stripping distillation was used for solvent recovery. However, due to the low product concentration in the fermentation broth, the specific energy demand for distillation is high. To recover one kilogram of 1-butanol, 5789 kcal of energy is required [8]. This represents about two thirds of the combustion energy of butanol. Due to the high toxicity of 1-butanol, it is doubtful that future research will be able to increase final solvent titers to levels significantly higher than 3% .

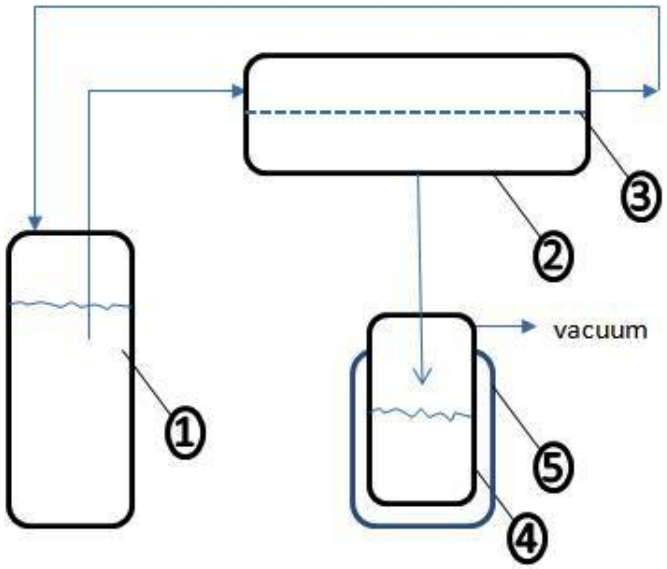
On a positive note, the utilization of the fermentation gas hydrogen as an energy source for distillation seems to be feasible. Given that the average hydrogen yield and the higher heating value are 1.9-2.7 mol H_2/mol glucose (Zang et al. [31]) and 286 kJ/mol, respectively, one can calculate an average hydrogen production of 39.7 mol H_2/kg BuOH assuming a yield of 0.28 g BuOH/g glucose. Hence, the produced hydrogen covers about $\frac{2711kcal}{5789kcal} = 47\%$ of the energy needed for steam stripping distillation. Furthermore, if the stillage is used as influent of an anaerobic digester, the produced methane can also be utilized for steam generation. Adapting Wilkie's [32] data for corn ethanol thin stillage (80 l thin stillage/kg BuOH, COD 20 g/l and a methane yield of 0.25 l/g COD) one can estimate a specific methane production under standard conditions of 400 l/kg BuOH produced. Given the heating value of methane with 889 kJ/mol, a total of 3792 kcal/kg BuOH can be generated. This represents about 65% of the energy needed for steam distillation. In total, the bulk energy demand for the most energy intensive operation of an ABE plant, product recovery by distillation, can be covered by burning the produced hydrogen as well as the methane generated by anaerobic digestion of thin stillage.

However, reducing the energy demand for product recovery can be financially rewarding since hydrogen and methane are valuable commodities themselves. Therefore, the focus of a number of research groups has been on separation techniques involving liquid-liquid extraction, adsorption, pervaporation, gas stripping and reverse osmosis [3]. The most promising trials have been undertaken by Qureshi et al. [8]. They showed that ABE recovery through a cycle of adsorption/thermal desorption on silicalites reduced the energy intensity from 5789 kcal/kg butanol (steam stripping distillation) to 1948 kcal/kg butanol.

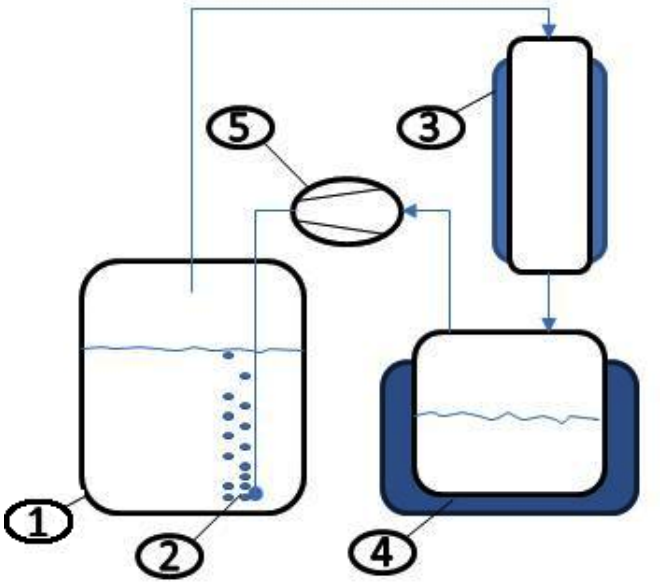
Ideally, product recovery is performed on-line to reduce or remove inhibitory effects of butanol on the fermentation. Techniques that lend themselves well include gas stripping, pervaporation and liquid-liquid extraction [33]. These processes take advantage of the volatility and hydrophobicity of the fermentation products and do not necessarily require prior cell removal or temperatures above the tolerance limit of *C. acetobutylicum*.

Disadvantages include membrane fouling and low specific product fluxes for pervaporation (between 5-10 g BuOH/m^2/h [33]) and high gas flow rates for gas stripping (about 3 l gas/l broth/min [34]). *In situ* extraction requires the none-toxicity of the extractant with respect to cell growth and a small tendency to form emulsions. Distribution coefficients range from 1.5 to 3.0 [2]. Figures 2.5 and 2.6 schematically depict pervaporation, gas stripping and liquid/liquid extraction.

Figure 2.5 – *In situ* product recovery techniques: Pervaporation above, Gas stripping below (adapted from [64])

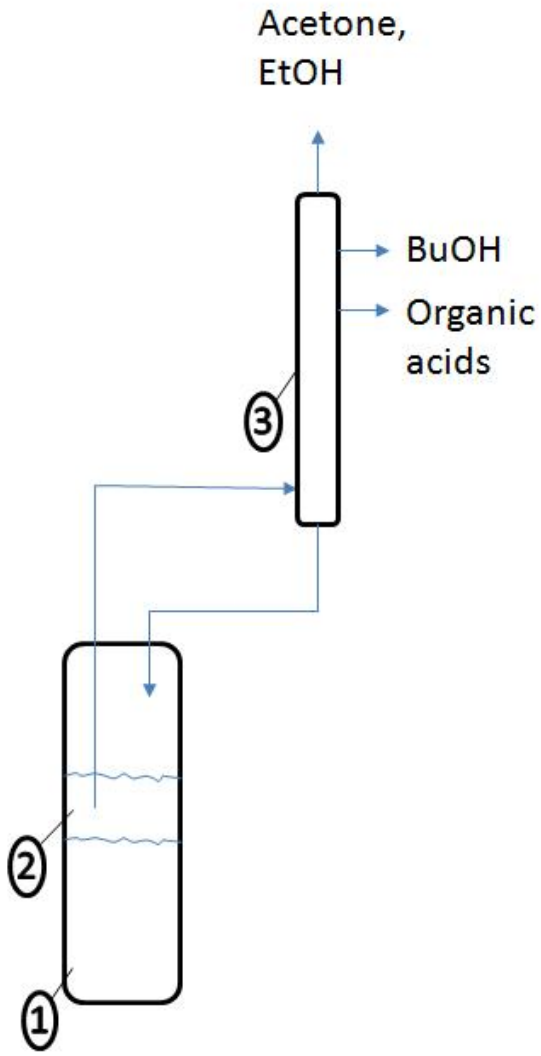


1-Fermentation broth, 2-Pervaporation vessel,
3-Membrane, 4-Cilled retenat collection



1-Fermentation broth, 2-Sparging, 3-Condenser,
4-Chilled condensate, 5-Gas pump

Figure 2.6 – *In situ* product removal through liquid-liquid extraction (adapted from [64])



2.2.1 Esterification

Esters derived from organic acids are commonly used in the cosmetics- and food industry because of their pleasant fragrance and general low toxicity [35]. Currently, the majority of esters are produced through petro-chemical routes using mineral acids, metal-based acids or acidic resins as catalysts [36]. With ABE fermentations, 1-butanol can be produced through bio-conversion of renewable substrates. Moreover, the organic acids acetate and butyrate are produced as precursors to solventogenesis. Therefore, it seems feasible to co-produce and recover both solvents and organic acids, preferentially in an equi-molar ratio for a subsequent esterification reaction. The generated esters butyl butyrate, ethyl butyrate and butyl acetate have low solubilities in water (see Table 2.3) and would therefore easily phase-separate leading to drastically reduced recovery efforts. However, with water being a by-product of esterifications, the achievable equilibrium concentrations and net-reaction rates would be low provided the reaction takes place in an aqueous media without product removal (e.g. through reactive distillation). Therefore, several studies have been undertaken using micro-aqueous media, biphasic- and micellar systems with enzymes as bio-catalysts [35, 36, 37, 38].

	butyl butyrate	ethyl butyrate	butyl acetate
solubility in water at 22ºC in [g/l]	0.52±0.06	3.2±0.3	2.6±0.3

Table 2.3 – Measured solubilities of relevant esters in water at room temperature

Currently, enzymes are commonly used to catalyze commercial reactions. Examples include the production of High Fructose Corn Syrup from corn glucose by glucose isomerase and the enzymatic hydrolyzes of starch molecules to monomeric glucose by amylose and amylopectin in the bio-ethanol industry [11]. Many enzymes are manufactured on large scale by fermentation of overproducing strains of *Bacillus, Aspergillus, Rhizopus* and *Mucor* and have therefore gained commercial significance [12]. Compared to conventional esterification with mineral acids, enzymatically catalyzed esterifications are highly selec-

tive, have high reaction rates at mild conditions and often have a greater final product yield [12]. Disadvantages include restricted process parameters (mostly temperature limited), enzyme inhibition and cost considerations. Lipases consist of a group of enzymes capable of catalyzing both hydrolysis of fatty esters to fatty acids and alcohols as well as the reverse reaction. Both reactions are equilibrium limited. In order to achieve high conversions of the esterification, the reaction medium is often chosen to be a highly non-polar inert solvent such as n-hexane. Here, the resulting reaction product, water, forms a micro-aqueous system (reverse micelles) in the hydrophobic organic solvent, whereas all other species are highly soluble in the solvent. Therefore, the reaction equilibrium is shifted to the formation of the ester. Yields in excess of 90% have been reported [15]. The enzyme, having a hydrophilic and a hydrophobic side, typically resides in the boundary layer between water and the apolar solvent [14].

Biphasic systems involving combined extraction and esterification represent an interesting option for systems operating with and without *in situ* product removal. In both cases, the main fermentation products 1-butanol, acetone, ethanol, butyrate and acetate will extract into the organic phase depending on each components partition coefficient. Organic acids and alcohols can then undergo esterification in the organic phase where a very limited amount of soluble water will push the equilibrium far to the right. In large, the resulting esters will remain in the organic phase due to their low water solubilities (see Table 2.3). In order to take advantage of high conversion temperatures, it seems best to continuously remove, react, recover and recycle the organic phase as depicted in Figure 2.7. Extraction for ABE removal has been investigated by several researchers [42, 43, 44, 45, 46]. Tested non-toxic solvents include alkanes, vegetable oils, esters and some derivatives. Groot et al. [42] compiled a list of tested extractants that is depicted in Table 2.4. In this table, the distribution (or partition-) coefficient refers to the ratio of concentrations of the solute (e.g. butanol) in the organic phase and in the aqueous phase. Selectivity refers to the ratio of distribution coefficients for the desired solute (butanol) and the unwanted compound

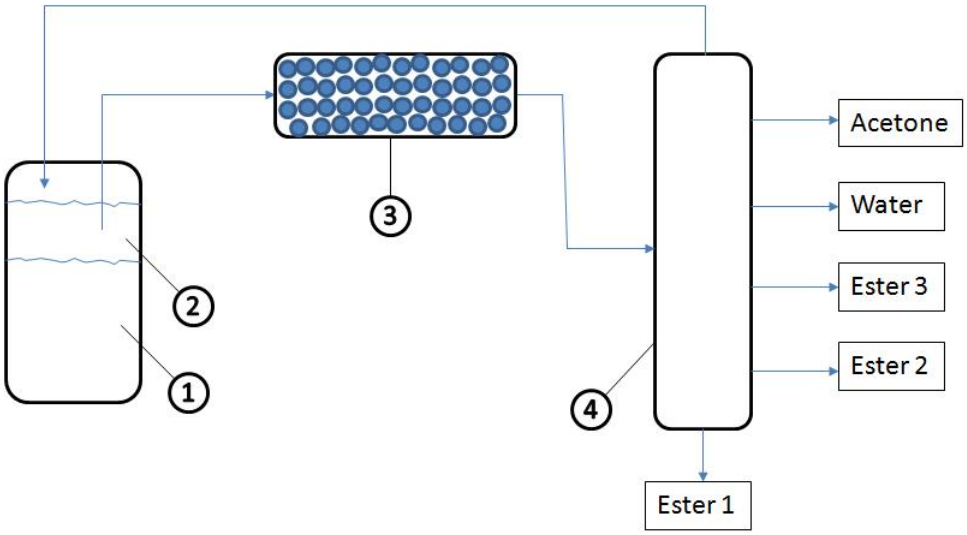
(water).

Generally, the higher the molecular weight and the smaller the solubility in water, the less toxic the solvent is to microorganisms [42]. Liquid alkanes are non-toxic and have low partition coefficients with respect to 1-butanol, but very good selectivity over water. Contrary, vegetable oils and their derivatives have higher partition coefficients but low selectivity. Higher alcohols have been found to be toxic. The most popular extractants in research are hexane, oleol alcohol, oleic acid and castor oil.

Table 2.4 – Investigated extractants for ABE fermentations and their 1-butanol distribution coefficients [kg/kg], selectivities of butanol/water and toxicity to *C. acetobutylicum*; adapted from [42] ^{a} – *own* data, ^{b}- results from [57]

Solvent	Toxicity	Molar mass [g/mol]	Distribution coefficient [kg/kg]	Selectivity towards water [-]
n-hexane	non-toxic	86	0.5	2700
heptane	non-toxic	100	0.5	3300
octane	non-toxic	114	0.3	4100
gasoline	non-toxic	n.a.	0.3	no value
hexanol	toxic	102	12	160
dodecanol	toxic	186	6	140
castor oil	non-toxic	n.a.	2.6	270
soy oil	non-toxic	n.a.	0.7	440
corn oil	non-toxic	n.a.	0.7	440
rapeseed oil	non-toxic	n.a.	0.8	400
butyl acetate	toxic	116	3	no value
hexyl acetate	non-toxic	144	3.6	5
ethyl laurate	non-toxic	214	1.8	7
ethyl oleate	non-toxic	311	1.3	6
oleic acid	non-toxic	283	3.9	6
oleyl alcohol	non-toxic	268.5	2.8	330^{b}
2-octanone^{a}	toxic	128.2	2.4	210

Figure 2.7 – Schematics of a continuous biphasic extraction with subsequent esterification and product removal. 1-Fermentation broth, 2-organic phase, 3-esterification reactor, 4-product and organic phase recovery through rectification



3 Fermentation Characteristics of a Degenerated Strain of ATCC 824

3.1 Batch Fermentation with Undegenerated Strain ATCC 824

3.1.1 Materials and Methods

In order to better understand the fermentation characteristics of the undegenerated strain, a number of batch fermentations were carried out in New Brunswick fermentors (Model number BioFlow 310) with 1l working volume. The autoclaved and oxygen-free media contained a 60 g/l Maltodextrin solution supplemented with 6 g/l yeast extract and 5ppm iron sulfate. The 24-hour-old inoculum consisted of oxygen-free and autoclaved RCM media (Difco) seeded with the original strain of ATCC 824. The fermentors were inoculated using 10ml RCM broth in log-phase. Temperature, pH and agitation were maintained at 36ºC, 5.9 and 200rpm, respectively. Turbidity was measured using absorbence at 595nm. The quantitative relationship between optical density and dry cell weight was established by dry cell weight analysis of samples with known turbidity. Analytical assays were determined by HPLC. The volume of the fermentation products carbon dioxide and hydrogen were measured by water displacement in graduated cylinders assuming a molar ratio of hydrogen to carbon dioxide of 1:1.

3.1.2 Growth Kinetics

Maximum cell growth for a typical substrate concentration of 60 g/l was measured by plotting the logarithmic cell concentration over time for the first nine hours after inoculation at 36°C. The basis for this experiment is the correlation between the net specific growth rate μ_{net} , rate of change of cell dry mass $\frac{dX}{dt}$ and cell dry mass X as follows

$$\mu_{net} = \frac{dX}{dt}\frac{1}{X} \tag{1}$$

where μ_{net} is the difference between gross specific growth rate μ_g and specific death rate μ_d.

$$\mu_{net} = \mu_g - \mu_d \tag{2}$$

Integrating equation 1 for constant specific growth yields

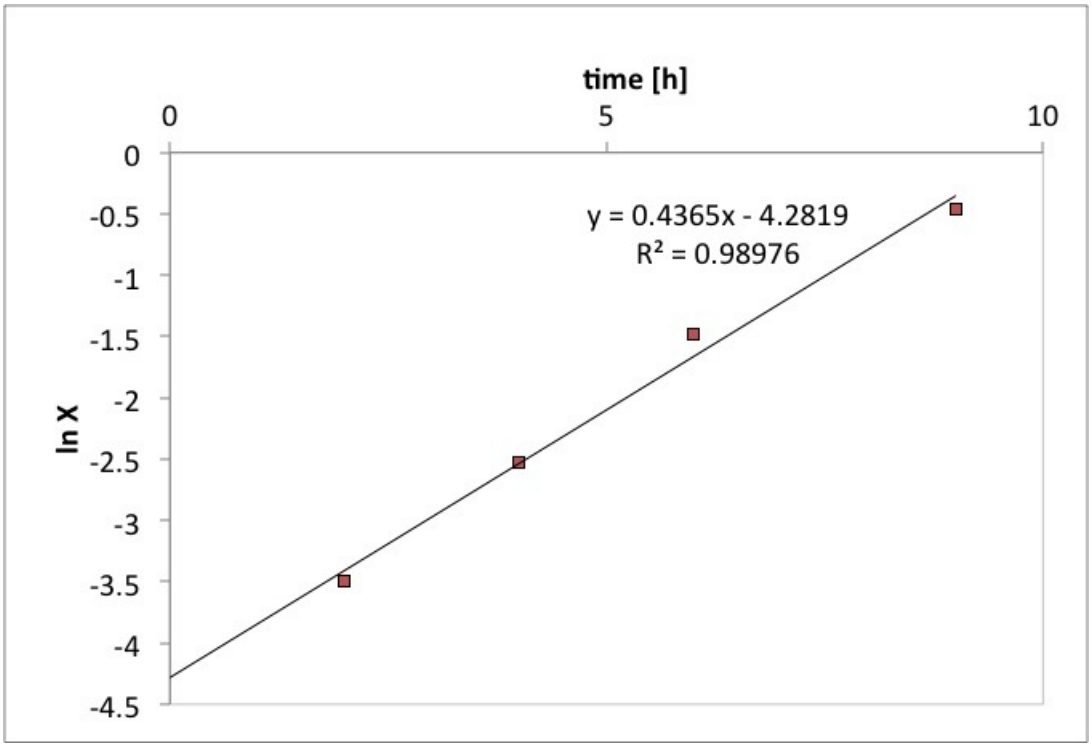
$$X = X_0 exp(\mu_{net}(t - t_0)) \tag{3}$$

Equation 3 can be further rearranged to

$$lnX = \mu_{net}(t - t_0) + ln(X_0) \tag{4}$$

where the resulting slope, when lnX is plotted over $(t - t_0)$, equals the net specific growth rate (see Figure 3.1). Linear fitting of the data points yielded an average maximum specific growth rate of 0.44 h^{-1}. This translates to an average doubling time of approximately 100min and falls within the range of growth rates reported for yeast (*Saccharomyces cerevisiae*) cultured at 30ºC [20].

Figure 3.1 – Exponential growth phase of the undegenerated strain of ATCC 824 at 36 ºC plotted as natural logarithm of cell concentration X over fermentation time.



3.1.3 Electron and Mass Balance

In order to quantify all involved compounds and account for the available electrons in substrate and products, an electron balance for a typical batch fermentation was established (see Table 3.1). The electron balance closes, if the amount of electrons provided by the substrate(s) matches the amount of electrons of the product(s) within a reasonable amount of error (typically ±5%). The available electrons for each species can be calculated as follows:

$$available\,electrons = n_C \cdot \gamma \tag{5}$$

Here, n_C is the number of C-moles of each species and γ is the reductance degree. C-moles differ from "standard" moles in that the amount of moles of each species is multiplied by the number of carbon atoms in its structure. If the compound does not contain any carbon molecules (e.g. hydrogen), each C-mole refers to one "standard" mole. The reductance degree of a compound can be evaluated by the following equation:

$$\gamma = \frac{\sum(\#atoms\,of\,element\,i) \cdot (\#e^- per\,atom)}{\#carbon\,atoms\,in\,molecule} \tag{6}$$

The number of available electrons per atom results from the valence of the element (e.g. carbon: 4 e^-, hydrogen: 1e^-, oxygen: 2e^-). Generally, the higher the degree of reductance, the higher the specific energy content.

When the number of available electrons of the utilized glucose (8.8) is compared to the sum of available electrons of all fermentation products (8.62), one finds the balance to close within 2%. Consequently, all major metabolites and catabolites are accounted for. Also from Table 3.1 one can infer a solvent yield of $Y_{ABE} = 0.3$ and a combined ABE- and organic acid (o.a.) yield of $Y_{ABE+o.a.} = 0.4$.

Table 3.1 – Electron balance on an ABE fermentation with undegenerated strain of ATCC 824 grown at 36°C

Compound	reacted/ produced	n	C-mol	Reduc tance degree	Available electrons
	[g]	[mol]	[C-mol]	[-]	[-]
glucose	65.7	0.365	2.2	4	8.8
acetic acid	4.7	0.078	0.16	4	0.64
butyric acid	2.4	0.027	0.11	5	0.55
acetone	5.7	0.1	0.3	5.33	1.6
1-butanol	12.6	0.17	0.68	6	4.08
ethanol	1.1	0.024	0.05	6	0.3
hydrogen	1.2	0.6	0.6	2	1.2
cells	1.5	-	0.06	4.2	0.25

3.2 Degenerated Strain Behavior

With strain degeneration, the original solvent-producing strain mutates or is outgrown by a degenerated version incapable of solventogenesis. This genetic instability can occur when the plasmid containing the solventogenic pathway is altered or not passed on to one of the daughter cells. The resulting mutant cell can channel more energy and nutrients to cell growth and often outcompetes plasmid containing cells [12]. This results in the partial or complete loss of solventogenic behavior. Consequently, serial sub-culturing and continuous cultures are most susceptible to strain degeneration [9]. In order to study degenerated strain behavior, a degenerated strain of *Clostridium acetobutylicum* (ATCC 824) was isolated from a continuous culture exhibiting degenerative behavior.

3.2.1 Materials and Methods

The degenerated culture was harvested from a failed immobilization attempt on calcium carbonate pellets after three days of operation with little solvent accumulation. Table 3.2 lists the conditions at day three of operation with broth recycling.

Note that the inlet and outlet streams were nearly identical in composition, indicating

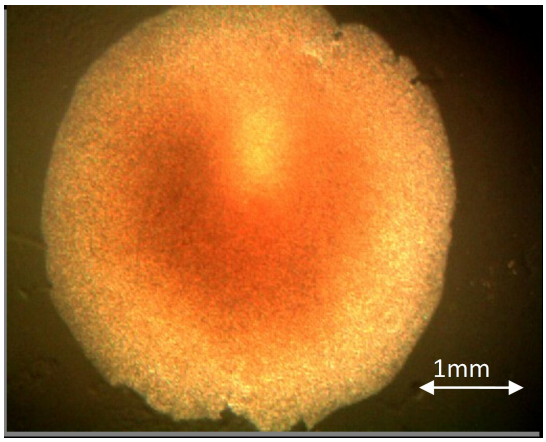
Table 3.2 – Conditions under which the degenerated strain of ATCC 824 was harvested

	Substrate	**Acetate**	**Butyrate**	**BuOH**	**pH**	**dilution time**
	[g/l]					1/h
in	46	4.3	6.9	0.7	4.5	0.14
out	45	4.3	7.0	0.7	4.5	0.14

that significant solventogenesis had not occurred despite the presence of ample quantities of substrate, organic acids and low pH. The harvested organism was grown anaerobically in an autoclaved DifcoTM Reinforced Clostridial Medium (RCM) at 36ºC for 24 hours. After appropriate dilution in RCM, the harvested culture was plated on oxygen-free solidified RCM (with 15 g/l agar added) and grown at 36 degrees Celsius.

The plating of the harvested degenerated culture resulted in the growth of colonies with brown centers, no outgrowths and a smooth outer perimeter (see Figure 3.2).

Figure 3.2 – Magnified image of a colony of the degenerated strain of ATCC 824 plated on solidified RCM after 72h of growth at 36°C



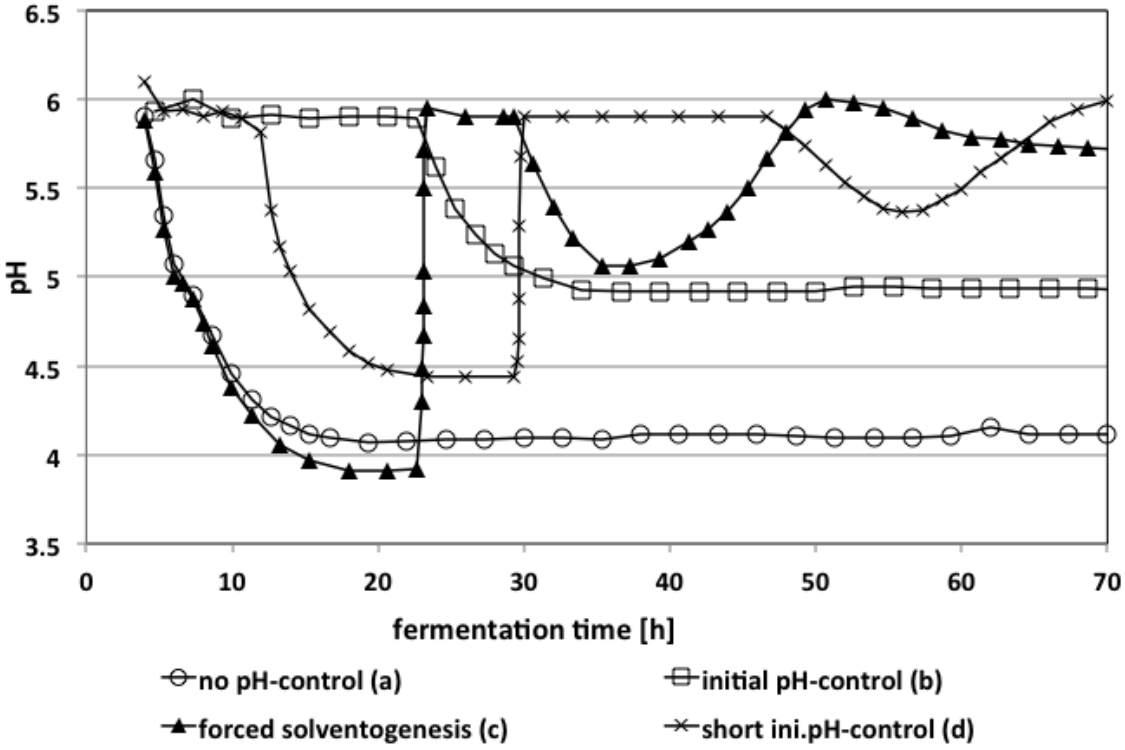
Adler et al. [13] designated colonies of this nature with type III and attributed them with intermittent solvent production on a low level (< 6.5 g/l Butanol) if the culture produced spores. Final butanol concentrations between 0.2 and 2.2 g/l were observed if no sporulation occurred. In this investigation, liquid cultures of the harvested strain grown in batch mode produced final butanol titers of less than 2 g per liter at a final pH of 3.9. The formation of endospores could not be observed.

Fermentations involving the degenerated culture were started by propagating colonies of the plated culture in serum bottles containing 50ml autoclaved and oxygen-free RCM media (Difco). The fermentations were carried out in fermentors (New Brunswick Sci., Model BioFlo 310) containing one liter of autoclaved and oxygen-free media with the following composition: 70 g/l Maltodextrin, 6 g/l yeast extract and 5ppm ironsulfate. The fermentations were started with 10ml of vigorously growing seed cultures and temperature-controlled at 36ºC. The pH was adjusted automatically by dosing 5M sodium hydroxide solution. Analytical assays were performed using HPLC.

3.2.2 Forced Solventogenesis Through Systematic pH-control

Regaining of solventogenic behavior can be observed, when the pH of liquid cultures is allowed to level out at around pH=4 initially. Although no metabolic shift to solventogenesis was observed at this point, the fermentation produced high levels of solvent when the pH was adjusted to near neutral levels to allow for further build up of organic acids and cell growth followed by a subsequent cessation of pH-control as depicted in Figure 3.3.

Figure 3.3 – Forced solventogenesis through systematic pH-control illustrated by means of pH trend. Conditions: degenerated strain of ATCC 824 grown at 36ºC in pH-controlled fermentors with 1l working volume



It should be noted that if the initial pH drop was omitted, fermentations did not enter solventogenesis despite an ample supply of organic acids, substrate and stressful conditions due to a low pH. Final solvent titers of type III degeneratives with forced solventogenesis were between 15 and 20 g/l and compared to those reported by Adler et al. [16] for type I colonies associated with vigorous, solvent-producing and spore forming cultures. To further rule out acid crash as the reason for non-solventogenic behavior, the pH of the fermentation was briefly held constant initially to allow for moderate acid built-up (d). The neutralized organic acids act as a buffer system so that the pH platoes at a higher level and an acid crash can be avoided. Again, with cessation of pH-control, the culture did not enter solventogenesis despite favorable conditions. Further, upon raising the pH back to 5.9, fermentational activity resumed without delay testifying to the culture's overall viability and absence of an acid crash. Forced solventogenesis could still be initiated by a subsequent cessation of pH-control yielding a total solvent concentration of 19.4 g/l (see Table 3.3).

Table 3.3 – Final product- and substrate titers in [g/l] of fermentation broth involving the degenerated strain with and without forced solventogenesis through systematic pH-control.
(a) without pH-control; (b) initial pH-control; (c) forced solventogenesis; (d) short initial pH-control followed by forced solventogenesis

	Substrate	**Acetate**	**Butyrate**	**Solvents**
	[g/l]			
a	44.6	2.4	3.6	1.7
b	26.3	5.7	13.3	1.5
c	8.4	4.7	2.4	19.4
d	3.6	6.3	9.4	15.1

3.2.3 Genetic Characterization of Degenerated Strain

In order to characterize the genetic changes occurring before and after forced solventogenesis, real-time qPCR was used to determine the presence of the plasmid pSOL1 that carries

the ABE formation genes [9]. Cells of *C. acetobutylicum* that were subjected to forced solventogenesis were harvested at the pH-minimum of 3.9 (Figure 3.3) as well as 12 hours after the second pH-minimum at a total ABE concentration of 13.9 g/l and pH=5.8. DNA was extracted using SurePrepTM Soil DNA Isolation Kit (Fisher Scientific) and by following the manufactures instructions. DNA content was measured with NanoDrop's ND1000. Real time qPCR was carried out using a procedure described by Ireland et al. [17] using equal amounts of DNA (20ng samples). The primers used were synthesized by Invitrogen according to a sequence published by Lee et al. [18]. The primeres were targeting plasmid DNA and read as follows (5' to 3'): CGAATTCTTCTGACTGGTGGCTAT (F-primer) and TTTGAAATCATCGTAACTCCCAAGT (R-primer).

Table 3.4 – Real-time PCR sample conditions and results. Samples were taken before and after forced solventogenesis

		Sample 1: before forced solventogenesis	Sample 2: after forced solventogenesis
pH	[-]	3.9	5.8
Solvent concentration in broth	[g/l]	0.5	13.9
Sample amount fermentation broth	[ml]	0.4	0.4
Amount of purified DNA for real time qPCR	[ng]	20	20
Crossing amplification threshold at cycle number	[-]	16.7	17.0

As Table 3.4 illustrates, both samples crossed the amplification threshold approximately at the same cycle number. This is typical for samples containing the same number of of cells carrying the pSOL1 plasmid sequence. It can therefore be concluded that (i) The degenerated strain still possesses the genetic ability to produce solvents; (ii) Forced solventogenesis did not effect the presence of pSOL1. This leaves to conclude that the solventogenic pathways for the investigated degenerated strain are intact but their triggering mechanisms have changed (see Figure 3.3).

3.2.4 Organic Acid Production with the Degenerated Strain

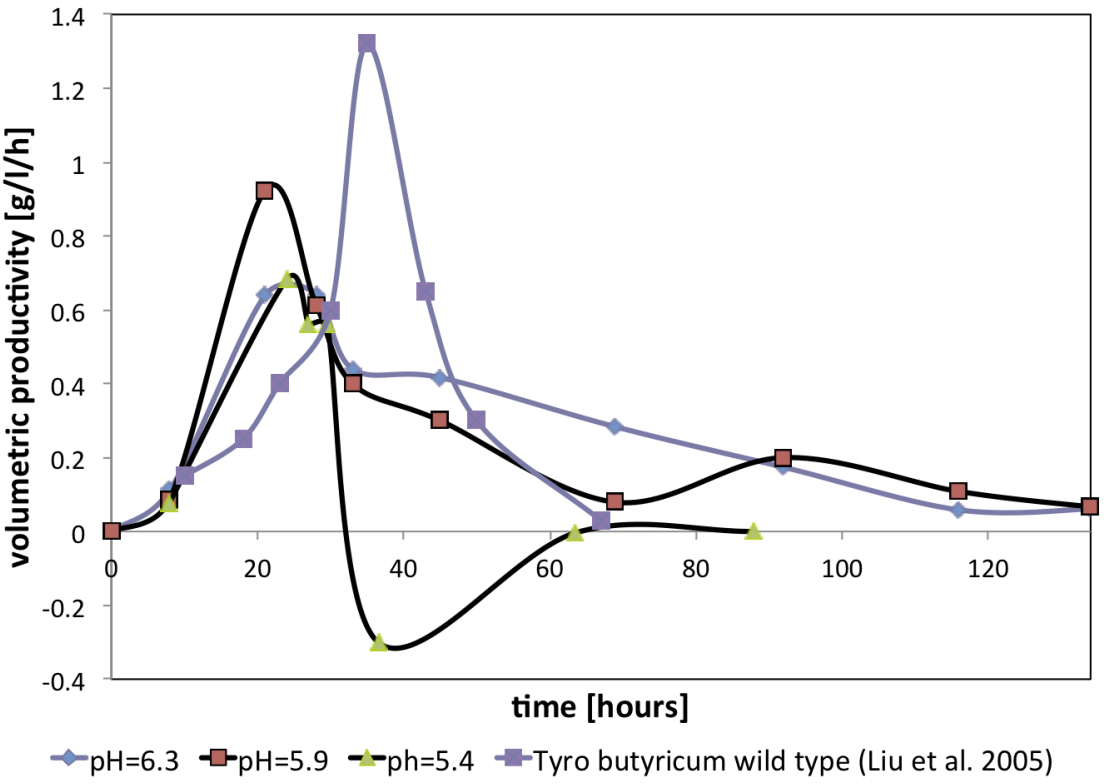
If the pH of the fermentation broth is held constant at pH=6.3, the degenerated culture primarily produced the precursors of said solvents, acetate and butyrate (see Table 3.5).

Table 3.5 – Final titers and yields of the degenerated strain at different pH values compared to *C. tyrobutyricum* (wildtype). Conditions: degenerated strain of ATCC 824 grown at 36ºC in pH-controlled fermentors with 1l working volume

	acetate/ butyrate	Solvents	Organic acid yield	Organic acid yield
	[g/l]		[weight organic acid/weight of Maltodextrin]	[weight organic acid/weight of glucose equivalent]
pH=5.4	4.2/7.6	15.0	0.2	0.22
pH=5.9	9.4/23.5	6.3	0.37	0.41
pH=6.3	9.0/25.2	0.3	0.49	0.54
C. tyrobutyricum, pH=6.0, (Liu et al. [19])	3.6/20.5	0	0.4	0.4

However, at lower pH values (pH=5.9 and pH=5.4) a partial reoccurrence of solventogenic behavior could be observed starting at total acid concentrations of greater than 15 and 12 g/l, respectively. This shift in metabolic pathway did not coincide with a reoccurrence of motility or endospore formation. At pH=5.4, between 30 and 65 hours of fermentation time, solventogenesis was prevalent over acid formation as indicated by a negative volumetric productivity. With respect to organic acid production, the volumetric productivity peaked earlier at a lower level when compared to values found in the literature reported for *C. tyrobutyricum* (wild type) [19], a bacterium incapable of solventogenesis with acetate and butyrate as final metabolites (see Figure 3.4). At pH=6.3, the degenerated strain produced higher final acid titers with a higher overall organic acid yield of 0.49 [w/w] or 0.54 [w/w glucose equivalent].

Figure 3.4 – Volumetric productivities for organic acids (acetate and butyrate) with varying pH values compared to *C. tyrobutyricum* (wild type). Conditions: degenerated strain of ATCC 824 grown at 36ºC in pH-controlled fermentors with 1l working volume



3.3 Conclusions

Two viable alternatives to circumvent the problem of strain degeneration of solvent producing strains of ATCC 824 present themselves. The first is to focus on the precursor of solvents, namely organic acids, while the second involves applying a technique of systematic pH control that can trigger the degenerated culture of type III into solventogenesis. Depending on the degree of degeneration, either or both techniques can still be applied when strain degeneration has prevented a fermentation from proceeding regularly, that is, without systematic pH-control. Thereby, costly re-conditioning through sterilization or disposal of fermentation media can be avoided. While it is assumed that the technique to

shift from solvents to organic acids as main products can be applied to the vast majority of degenerated ABE strains, further research is needed to study the behavior of different degenerated cultures when subjected to the technique of systematic pH-control. It is further imaginable to combine both techniques (organic acid fermentation followed by forced solventogenesis or vise versa) in such a way that the final fermentation broth contains both organic acids and solvents with an overall higher titer. Chapter 4 will further explore this possibility by investigating a continuous reactor system consisting of a plug flow reactor (PFR) followed by two chemostats.

4 Continuous Fermentation with a Degenerated Strain of ATCC 824

Continuously operated fermentations have the advantage of eliminating the time-consuming steps of cleaning, filling and seeding of batch reactors. Moreover, processes downstream from the fermentation vessel can be more easily operated at a constant flow rate without the need of additional holding tanks. If the fermentation is inhibited, continuously operated reactors provide selection pressure which can result in a better adapted microbial culture with higher tolerance level and/or increased volumetric productivities. A sub-type of continuously operated reactors are immobilized cell reactors. Among these, the culture is either immobilized onto a substrate by adsorption and covalent bonding or by cell entrapment. Due to much higher achievable cell densities, continuously operated immobilized cell reactors can have volumetric productivities that are a multitude of those reported for batch cultures. Disadvantages include possible mass transport limitations and potential activity loss due to immobilization [2]. A specific problem which arises frequently with *Clostridial* ABE fermentations is the degeneration of the original strain to a mutant with drastically decreased ABE production capabilities [2]. However, taking advantage of systematic pH-

control to trigger forced solventogenesis, it seems feasible to design a stable reactor system capable of both high volumetric productivities and final titers.

4.1 Materials and Methods

A degenerated strain of ATCC 824 was used throughout the experiment. The culture was harvested according to a procedure described in section 3.2.1 and propaged in RCM for 20 hours before inoculation.

The experimental setup is depicted in Figure 4.1. In addition to two CSTRs, the setup consisted of a tubular reactor wrapped around CSTR 1. This PFR consisted of a two meter long silicon tubing (Masterflex® 96410-82) with an inner diameter of $\frac{1}{2}$ inch. Within the tubing run an equally long thread composed of a polyester core and a cotton mantle with an outer diameter of $\frac{1}{4}$ of an inch (cloth line from hardware store). The staple fibers contained in the cotton mantle of the thread serve as immobilization support for the culture. The cross-section of the PFR is depicted in Figure 4.2. In order to maintain the temperature in the tubular reactor close to the temperature set in CSTR 1, the tubular reactor was insulated with fiber glass.

Figure 4.1 – Experimental setup for continuous ABE and organic acid fermentation; P1 through P4 indicate sampling locations. P-1 and P-2 are located after 1m and 2m of PFR reactor length, respectively.

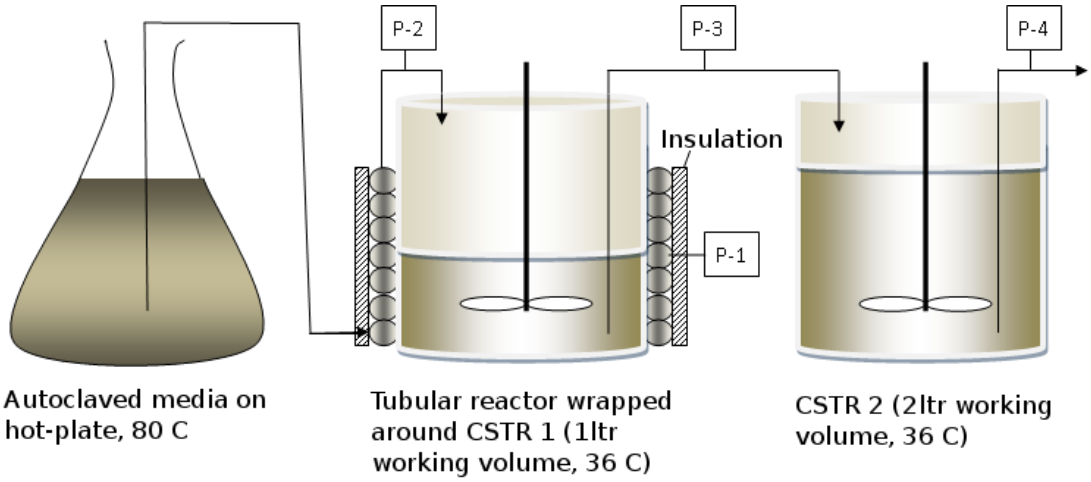
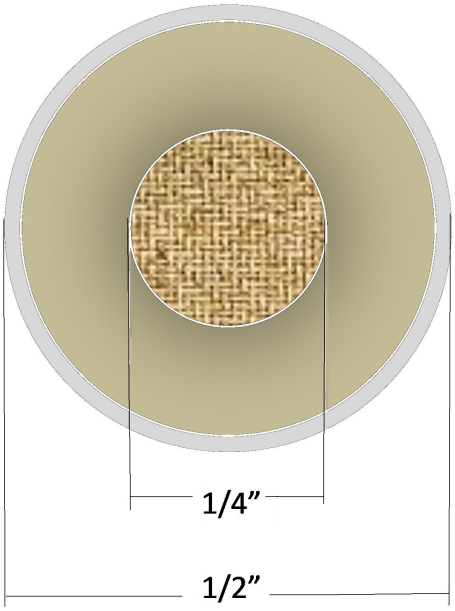


Figure 4.2 – Cross section of the tubular reactor: silicon tubing with cotton thread serving as bacterial support structure



The PFR was fed with autoclaved and oxygen-free media containing substrate and nutrients through a peristaltic pump. A typical media composition would be 70 g/l Maltodextrin, 6 g/l yeast extract and 5ppm ironsulfate. The fermentation broth then entered the first CSTR with one liter working volume. This reactor was pH-controlled with 5M sodium hydroxide. The broth was then pumped into CSTR 2 with two liter working volume by a second peristaltic pump for further bio-conversions without pH control. Probing occurred at the half point (1m) and at the end of the tubular reactor (2m) as well as in CSTR 1 and CSTR 2.

Start-up was effected by seeding of CSTR 1 with 10ml of RCM inoculum. After 15 hours of growth in CSTR 1 at pH=5.9, the fermentation broth was recycled through the tubular PFR for four hours. This was followed by 'normal' continuous operation by pumping autoclaved and oxygen-free media into the inlet of the tubular reactor.

The dilution rate in the PFR was determined by injecting 1ml autoclaved arabinose into the reactor inlet as a tracer. The effluent concentration of arabinose was measured in five minute intervals.

All analytical assays were performed using High Performance Liquid Chromatography (HPLC).

4.2 Results and Discussion

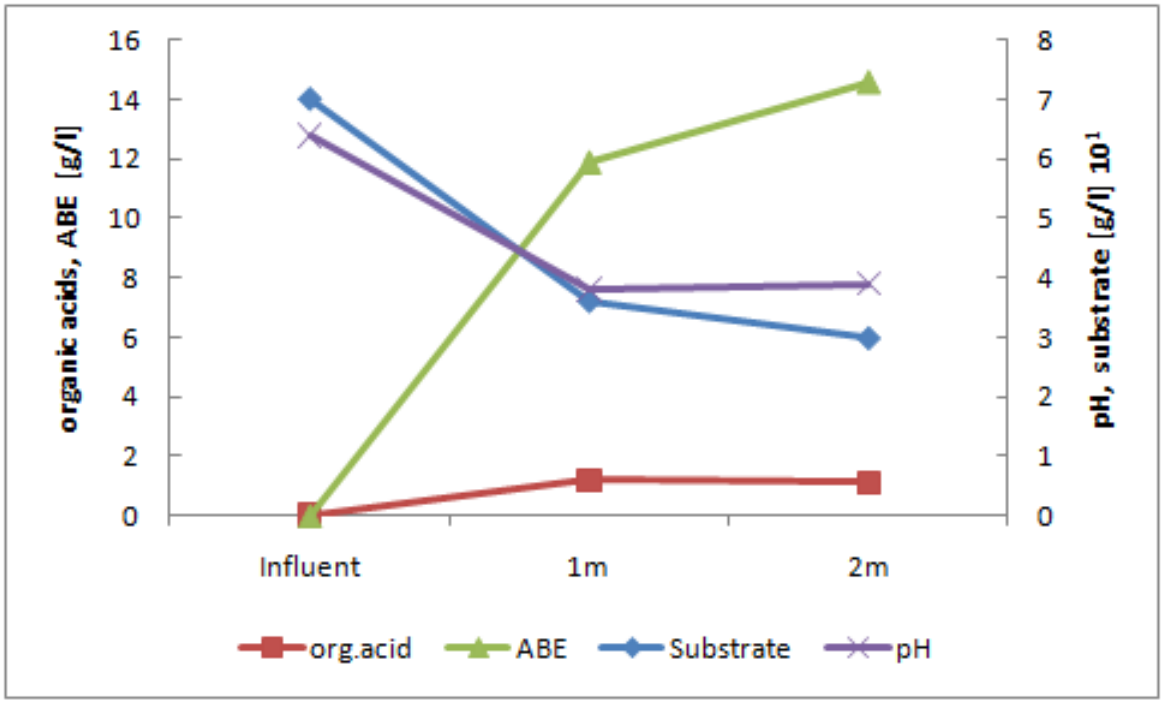
The reactor system was continuously operated for over 40 days. During that time various operating conditions were tested. In particular, the influence of the pH-set point in CSTR 1, dilution rate, and xylose consumption were investigated.

4.2.1 Solventogenesis in the Tubular Reactor

Originally, the PFR was thought to provide the culture with the necessary initial pH-drop in order to perform forced solventogenesis in CSTR 2 after pH-adjustment to near neutral level in CSTR 2. However, it could be observed that significant solventogenesis had already

occurred in the PFR (see Figure 4.3).

Figure 4.3 – Concentration profile in tubular reactor at a dilution rate of 0.86 h^{-1}after three weeks of operation with Maltodextrin as substrate



At a feed rate of 68 ml/h, an average retention time of 70 minutes could be inferred (data not shown). The effective reactor volume at this condition is therefore 79.3ml. This is significantly less than the theoretical volume that can be calculated by the difference of the void volume of the silicone tubing and the effective thread volume as follows:

$$V_{PRF,effective} = V_{void,tubing} - \varepsilon V_{thread} \tag{7}$$

The porosity of the thread is represented by ε and was measured to be 50 percent. This yields a theoretical reactor volume of 221ml. The difference between theoretical reactor volume and inferred reactor volume can be explained by the accumulation of fermentation gases, hydrogen and carbon dioxide, in the tubular reactor.

If a linear relationship between dilution rate and location along the tubular reactor is assumed, one can calculate a volumetric solvent productivity of 20.4 and 12.6 g/l/h after one and two meter reactor length, respectively. Correspondingly, the substrate consumption rates are 57.9 and 46.5 g/l/h and the overall solvent yields are 35.2 and 36.5 % (w/w) or 39.1 and 40.5 (w/w glucose equivalent), respectively. These values are on the high end of specific volumetric production rates reported in the literature (see Table 2.2). Moreover, the final titers exceed those reported in the literature and are on the same level with values announced by Largier [4]. However, Largier's setup consisted of an immobilized cell reactor with broth recycling and doesn't fall into the same category as the reactor investigated in this report. Additionally, Lagier's fermentor operated at volumetric productivities that were a fourth of this tubular reactor's.

The pH of the fermentation broth quickly falls to levels around pH=4 and increases only slightly over the reactor length. The continuously low pH in the reactor is indicative of a coexistence of cells in both acidogenic- and solventogenic phase. Moreover, acid and solvent production must occur at the same rate. It can further be hypothesized that the low pH prevents the culture from further degeneration due to a high selection pressure for strains capable of surviving and growing at low pH values. Thus, strains lacking the genetic pathways for solventogenesis or those that have otherwise compromised solventogenic behavior exhibit slower growth rates at low pH values and are therefore outgrown by the investigated degenerated culture of *C. acetobutylicum*.

4.2.2 Influence of pH-set Point in CSTR 1

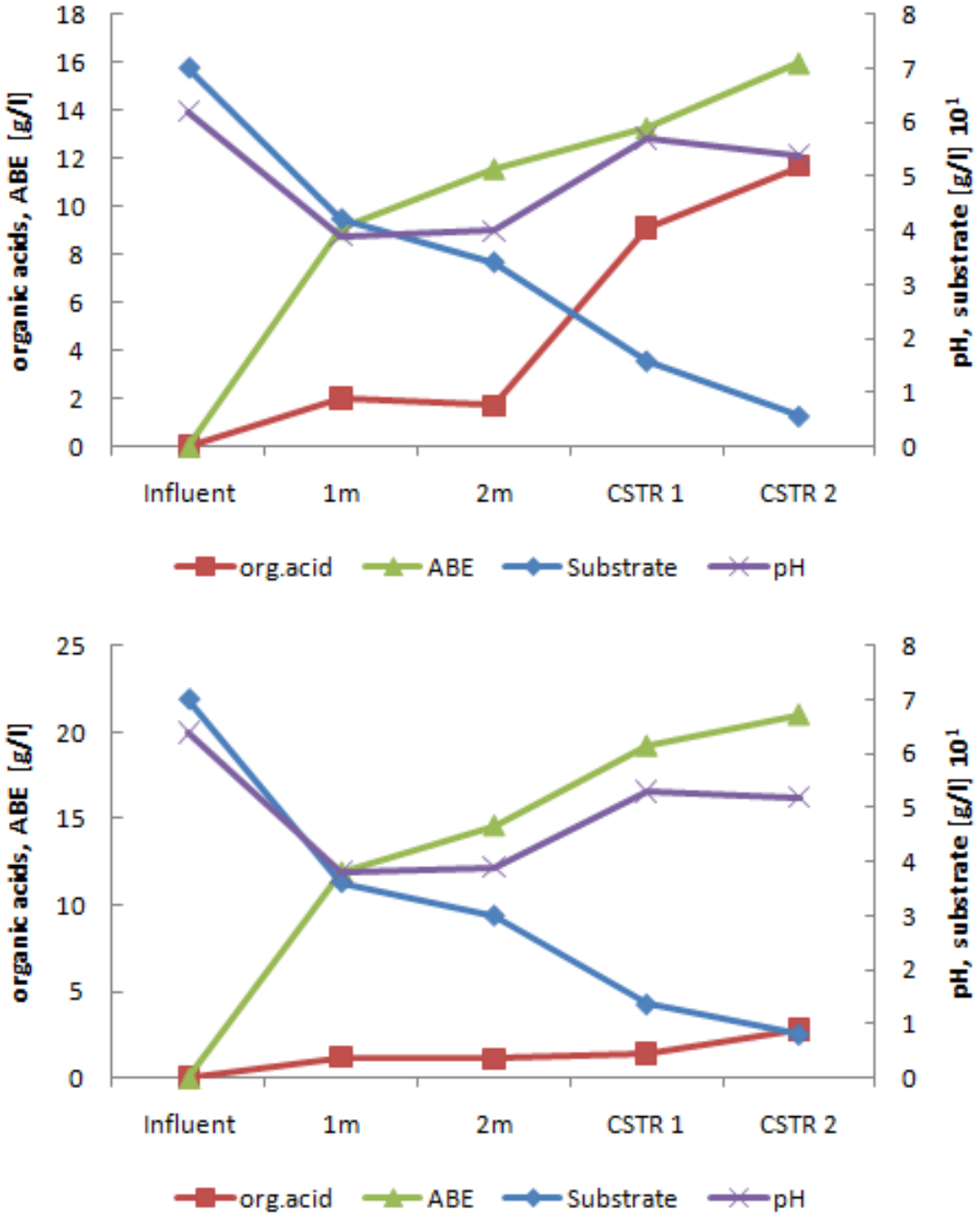
As indicated earlier, it is possible to follow the ABE fermentation in the PFR by a period of constant pH at higher levels to allow for additional organic acid built-up. The combined titers for ABE and organic acids would allow for a more efficient product removal, especially when acids and solvents are recovered by the same technique at the same time.

In order to shift the metabolic pathway from solventogenesis back to acidogenesis, the

pH in CSTR 1 can be adjusted. Figure 4.4 compares the outcome of pH set points of 5.3 and 5.7. At pH=5.3, solventogenesis is still prevalent and final organic acid titers are 2.7 g/l. In contrast, final acid titers reach 11.7 g/l when the pH in CSTR 1 is kept at 5.7 with a ratio of butyrate to acetate of 2.1 (w/w).

In the case of successive ABE and organic acid production, the total product concentration reaches 27.7 g/l at a yield of 43% (w/w) or 47.7% (w/w of glucose equivalent).

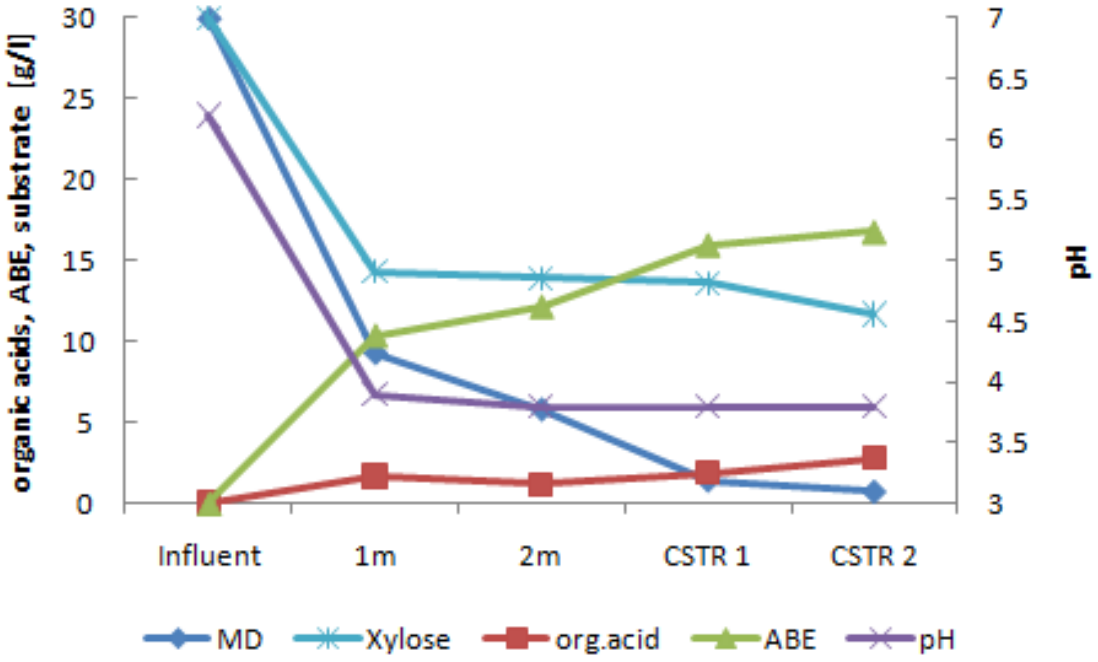
Figure 4.4 – Effect of pH in CSTR 1: Top - pH=5.7, bottom - pH=5.3, dilution rates for PFR (1m and 2m), CSTR 1 and CSTR 2 are 0.86, 0.068 and 0.034 h^{-1}, respectively.



4.2.3 Xylose Consumption

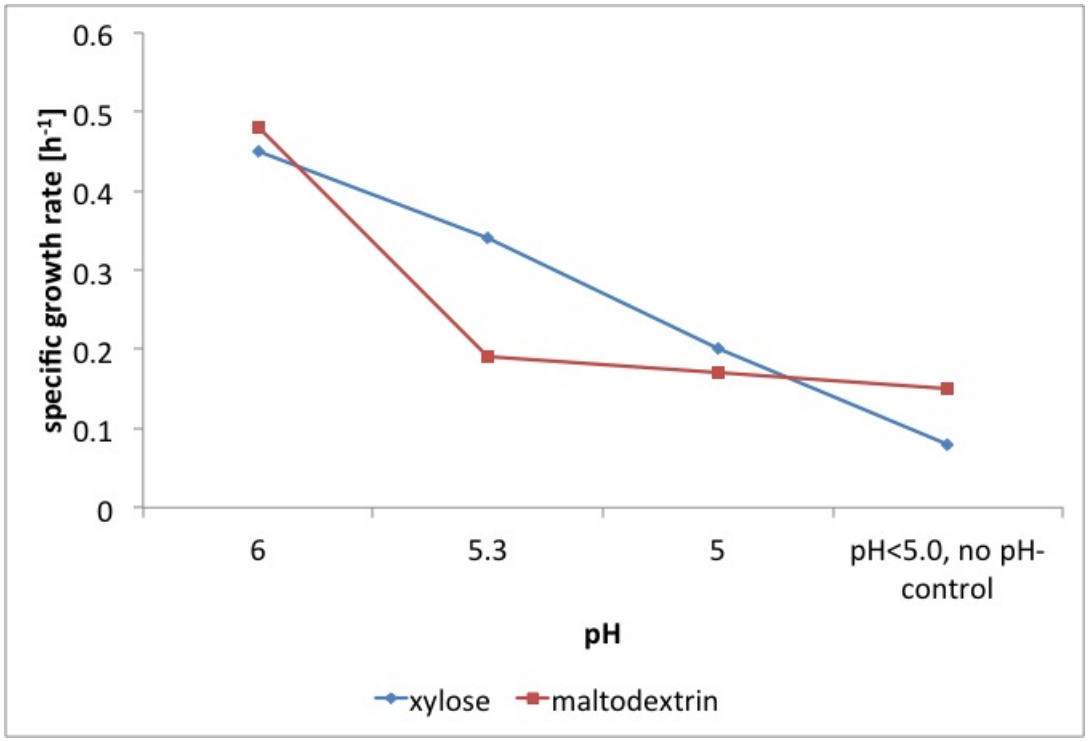
One of the benefits of bacterial fermentations with *Clostridium acetobutylicum* is its ability to naturally digest five carbon sugars [2]. The most abundant subunit in hemicellulose is D+(-) xylose and was therefore chosen exemplary as the sole or complimentary substrate. Figure 4.5 depicts the values of key parameters in the reactor system.

Figure 4.5 – Substrate uptake when the initial concentrations of xylose a Maltodextrin are equal (30 g/l). The reactor system consists of a PFR with two probing ports after 1m and 2m, CSTR 1 and CSTR 2 with dilution rates of 0.86, 0.068 and 0.034 h^{-1}, respectively.



Interestingly, there is only a small bias towards Maltodextrin over xylose in the beginning of the tubular reactor. However, from probe point one forward, Maltodextrin is consumed preferentially by a wide margin. In fact, the xylose concentration only decreased by 14% between the first probe point and CSTR 2, whereas the Maltodextrin concentration decreases by 92%. Since xylose uptake correlates well with the decrease in pH, it can be hypothesized that xylose consumption is strongly pH dependent. To test this theory, a set of batch fermentations at varying pH values using xylose or Maltodextrin as a substrate were carried out. Figure 4.6 depicts the specific growth rate μ over the pH of the fermentation broth with xylose and Maltodextrin as substrates. While cell growth for Maltodextrin consuming bacteria declines more quickly from pH 6.0 to 5.3, xylose consuming bacteria exhibit slower growth when the pH is below 5.0. Since the pH in the tubular reactor falls very quickly to levels around 4.0, bacteria utilizing six carbon sugars have a growth advantage over those consuming five carbon sugars.

Figure 4.6 – Measured specific growth rates of the degenerated culture of ATCC 824 at 36°C with varying broth-pH and type of substrate (xylose and Maltodextrin).



4.3 Conclusions

It could be shown that a partially degenerated strain of *Clostridium acetobutylicum* ATCC 824 can be used in a continuous reactor system consisting of a tubular reactor and two CSTR in series for an extended period of time. Furthermore, the tubular reactor excelled at producing high final solvent titers (14.6 g/l) at high volumetric productivities (12.6 g/l/h). To my knowledge, the combination of high solvent titer coupled with high ABE production rates has not been achieved at this level before.

Combined solvent and organic acid production was possible, when the pH in CSTR 1 was raised to levels where the culture shifts its metabolic pathway back to acidogenesis. Applying this technique, a total product concentration of 27.7 g/l at a yield of 43% (w/w) or 47.7% (w/w of glucose equivalent) could be achieved.

Xylose uptake was demonstrated by supplementing the media with the five carbon sugar.

However, xylose consumption quickly decreased over the length of the tubular reactor. Batch fermentations with Maltodextrin and xylose indicated that bacteria utilizing six carbon sugars have a growth advantage over those consuming five carbon sugars in acidic environments (pH below 5.0).

5 Mathematical Modeling of the Reactor System

Kinetic models for growth and metabolism of microorganism provide the potential to gain knowledge and predict the behavior of a microbial system and it's environment (e.g. the bio reactor) without engaging in time consuming physical experiments. The usability of the model will depend on the underlying assumptions and the accuracy of the model parameters. The ABE fermentation with its five final products (three solvents and as well as hydrogen and carbon dioxide) and two metabolic intermediates (acetic and butyric acid) represents a challenging system to model. Votruba et al. first described a mathematical model of a batch acetone-butanol fermentation using elucidated kinetic relationships between substrate consumption, growth rate and product formation [47]. Batch-process experimental data as well as biochemical pathways were used to gain and validate a kinetic model that describes the batch fermentation of *C. acetobutylicum* growing on dextrose to a high degree of accuracy. The model consists of 9 interrelated differential equations relating the rate of change of cell matter, the dimensionless concentration of RNA, the intermediates acetate and butyrate as well as the final metabolites to the time dependent concentrations of cell matter, substrate, intermediates and final metabolites. Due to the high accuracy and flexibility of the kinetic batch-model, it was subsequently adapted to portray the reactor system consisting of a tubular bio reactor (PFR) and two consecutive CSTR reactors as described in chapter 4 (see Figure 4.1).

The mathematical model that reflects the biochemical and physiological behavior of the culture in its environment is based on the following assumptions [47]:

- Nutrients are not limited
- Glucose is below inhibitory levels (0-70 g/l)
- Concentrations for butanol, acetone and ethanol do not exceed [g/l] 13, 6.5 and 1.5, respectively.

5.1 Batch Fermentation without pH-Control

In order to accurately describe the lag phase during start up, the model uses the physiological state of the culture. This concept dates back to theoretical considerations of Malek and Votruba [48, 49] and uses the dimensionless intracellular RNA concentration y as a marker of the cultures viability as depicted in equation 8:

$$y = \frac{RNA}{RNA_{min}} \tag{8}$$

RNA_{min} is the intracellular RNA concentration when the specific growth rate $\mu = 0$. Now the specific growth rate can be expressed as a function of the physiological state so that

$$\mu = \lambda\,(y-1) \tag{9}$$

where λ is a constant that is identical and equal to $\lambda = 0.56$ for most bacterial cultures as demonstrated by Harder and Roels [51]. The rate of change of the product of the physiological marker y with biomass concentration X with respect to time can be expressed as

$$\frac{d(yX)}{dt} = \mu(S,B)\,yX \tag{10}$$

where the function $\mu(S,B)$ symbolizes the dependency of the specific growth rate on substrate concentration S and the inhibitory product butanol B. Rearranging equation 10 and incorporation of equation 9 will yield

$$\frac{dy}{dt} = y[k_1\mu(S,B) - 0.56(y-1)] \tag{11}$$

Cultures in stationary phase are characterized by $y = 1$ whereas declining and increasing viability are represented by $y < 1$ and $y > 1$, respectively. The knowledge of the actual amount of intracellular RNA is not necessary [47]. The function $\mu(S,B)$ from equation 11 can be determined from experimental data by assuming a linear relationship for $\mu(S)$ and a hyperbolic correlation for the product inhibition by the product B. The Yerusalimski-Monod relationship [52] for product inhibited growth combines both requirements so that the following equation can be derived where K_I is the inhibition constant :

$$\frac{dy}{dt} = y\left[k_1 S\frac{K_I}{K_I + B} - 0.56(y-1)\right] \tag{12}$$

The rate of change of biomass over time can be expressed as

$$\frac{dX}{dt} = \mu X - k_2 BX \tag{13}$$

Substituting equation 9 for the specific growth rate yields

$$\frac{dX}{dt} = 0.56(y-1)X - k_2 BX \tag{14}$$

where the first term on the right hand side represents bacterial growth taking the physiological state of the culture into account. The second term stands for cell decay due to butanol toxicity.

Additional differential mass balances for substrate S, butyrate BA, acetate AA, butanol B, acetone A, ethanol E and the fermentation gases carbon dioxide CO_2 and hydrogen H_2 were developed by Votruba et al.[47] and are listed in Table 5.2.

The numerical coefficients in equations 17-1 and 18-1 result from stoichiometric conversion ratios of the molecular weight of butanol/butyric acid and acetone/acetic acids,

respectively. The differential mass balance for butanol (equation 17-1) reflects the initial delay of butanol production due to insufficient accumulation of butyric acid with the second term on the right hand side. Initially, the rate of change of butyrate accumulation is high so that $\frac{dB}{dt}$ is suppressed. Once the butyrate accumulation slows down and the pH levels out, equation 15-1 will be dominated by the production term represented by k_7SX.

5.2 Batch Fermentation with pH-Control in Organic Acid Mode

The simulation of a batch fermentation for organic production is not possible with the set of coupled differential equations described in chapter 5.1, since the accumulation of butyrate and acetate will trigger the production of solvents regardless of the pH. Therefore, the equations describing a batch ABE fermentation without pH-control need to be adjusted to reflect negligible solvent production when the pH is held constant at levels above pH=6.0. Consequently, it is assumed that there is

- no solvent production
- accumulating organic acids alone will inhibit cell growth and product formation
- glucose is below inhibitory levels
- nutrients are in sufficient supply

As a result, equations 11-2 through 16-2 now contain the combined organic acid concentration OA in g/l as inhibitory component. Furthermore, the acid consumption terms of equations 15-2 and 16-2 were dropped and solvent production set to zero. If the fermentation broth already contains solvents (e.g. it comes from the initial PFR), an additional inhibitory effect due to the butanol can be expected. Hence, equations 11-3, 13-3, 15-3 and 16-3 contain an complimentary inhibitory term reflecting the toxicity of butanol.

Table 5.2 lists the values of the parameters used in the simulation and Figures 5.1 to 5.3 depict the accumulation and consumption of products and substrates. Each run was started with initial conditions listed in Table 5.1. Experimental values are indicated by markers whereas calculated results are drawn with a solid line. Figures 5.1, 5.2 and 5.3 illustrate model predictions and experimental data for batch fermentations without pH-control (undegenerated strain), with pH-control in organic acid mode (degenerated strain) and with pH-control in organic acid mode with solvents present (degenerated strain), respectively. In general, there is good agreement between model and experimental results. Larger deviations occur when the biomass concentration is calculated. While initial cell growth is predicted within a small margin of error, the maximum concentration of dry cell mass is generally overpredicted.

Table 5.1 – Initial conditions for simulations. Y - dimensionless intracellular RNA concentration [-]. All other capital letters represent concentrations of [g/l]: X - cell dry mass, S - substrate, BA - butyric acid, AA - acetic acid, B - butanol, A - acetone, E - ethanol

	Y	X	S	BA	AA	B	A	E	CO_2	H_2
without pH-control	1	0.02	60	0	0	0	0	0	0	0
with pH control in organic acid mode	1	0.02	60	0	0	0	0	0	0	0
with pH control in organic acid mode and solvents present	1	1.3	60	0.8	0.4	10	5	1	0	0
PFR	1	0.02	70	0	0	0	0	0	0	0

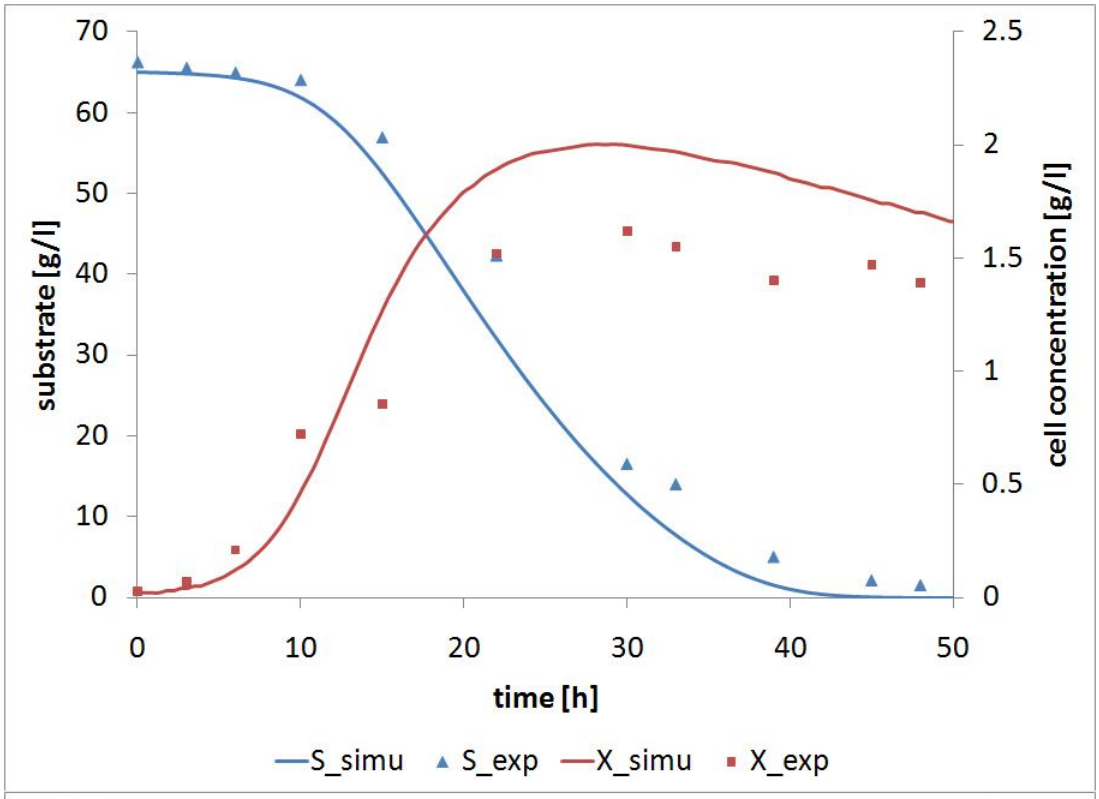
	without pH control	with pH control in organic acid mode	with pH control in organic acid mode and solvents present	Eq.#
$\frac{dy}{dt}$	$= y\left[k_1 S \frac{K_I}{K_I+B} - 0.56(y-1)\right]$	$= y\left[k_1 S \frac{K_I}{K_I+BA+AA} - 0.56(y-1)\right]$	$= y\left[k_1 S \frac{K_I}{K_I+OA}\frac{K_B}{K_B+B} - 0.56(y-1)\right]$	11-1, 11-2, 11-3
$\frac{dx}{dt}$	$= 0.56(y-1)X - k_2 B \cdot X$	$= 0.56(y-1)X - k_2 \cdot BA \cdot X \cdot AA$	$= 0.56(y-1)X - k_2 BA \cdot AA \cdot X \cdot B$	13-1, 13-2, 13-3
$\frac{dS}{dt}$	$= -k_3 S \cdot X - k_4 \frac{S}{K_S+S} X$	$= -k_3 S X - k_4 \frac{S}{K_S+S} X$	$= -k_3 S X - k_4 \frac{S}{K_S+S} X$	14-1, 14-2, 14-3
$\frac{dBA}{dt}$	$= k_5 S \frac{K_I}{K_I+B} X - k_6 \frac{BA}{K_{BA}+BA} X$	$= k_5 \frac{S}{K_S+S}\frac{K_I}{K_I+BA+AA} X$	$= k_5 \frac{S}{K_S+S}\frac{K_I}{K_I+BA+AA}\frac{K_B}{K_B+B} X$	15-1, 15-2, 15-3
$\frac{dAA}{dt}$	$= k_8 \frac{S}{K_S+S}\frac{K_I}{K_I+B} X - k_9 \frac{AA}{K_{AA}+AA}\frac{S}{K_S+S} X$	$= k_8 \frac{S}{K_S+S}\frac{K_I}{K_I+BA+AA} X$	$= k_8 \frac{S}{K_S+S}\frac{K_I}{K_I+BA+AA}\frac{K_B}{K_B+B} X$	16-1, 16-2, 16-3
$\frac{dB}{dt}$	$= k_7 S\, X - 0.841 \frac{dBA}{dt}$	0	0	17-1
$\frac{dA}{dt}$	$= k_{10} \frac{S}{K_S+S} X + 0.484 \frac{dAA}{dt}$	0	0	18-1
$\frac{dE}{dt}$	$= k_{11} \frac{S}{K_S+S} X$	0	0	19-1
$\frac{dCO_2}{dt}$	$= k_{12} \frac{S}{K_S+S} X$	$= k_{12} \frac{S}{K_S+S} X$	$= k_{12} \frac{S}{K_S+S} X$	20-1, 20-2, 20-3
$\frac{dH_2}{dt}$	$= k_{13} \frac{S}{K_S+S} X + k_{14} S\, X$	$= k_{13} \frac{S}{K_S+S} X + k_{14} S \cdot X$	$= k_{13} \frac{S}{K_S+S} X + k_{14} S \cdot X$	21-1, 21-2, 21-3

Table 5.2 – Set of differential equations describing a batch fermentation under various conditions

Table 5.2 – Parameter values for specified conditions

	batch without pH control	batch with pH control in organic acid mode	batch with pH control in o. a. mode and solvents present
k_1	0.006	0.01	0.0055
k_2	0.0008	0.00075	0.001
k_3	0.0255	0.04	0.02
k_4	0.7664	0.7664	0.25
k_5	0.01	6.5	9.0
k_6	0.117	n.a.	n.a.
k_7	0.0113	n.a.	n.a.
k_8	0.715	2.3	3.7
k_9	0.1350	n.a.	n.a.
k_{10}	0.2	n.a.	n.a.
k_{11}	0.0258	n.a.	n.a.
k_{12}	0.6139	0.6139	0.6139
k_{13}	0.0185	0.0185	0.0185
k_{14}	0.00013	0.00013	0.00013
K_I	0.833	1.8	3.1
K_S	3	15	5
K_{BA}	2	n.a.	n.a.
K_{AA}	0.5	n.a.	n.a.
K_B	n.a.	n.a.	1.3

Figure 5.1 – Batch simulation without pH-control. Solid lines represent calculated values whereas markers indicate experimental results. Experimental conditions: undegenerated strain of ATCC 824 grown at 36ºC in fermentor with 1l working volume



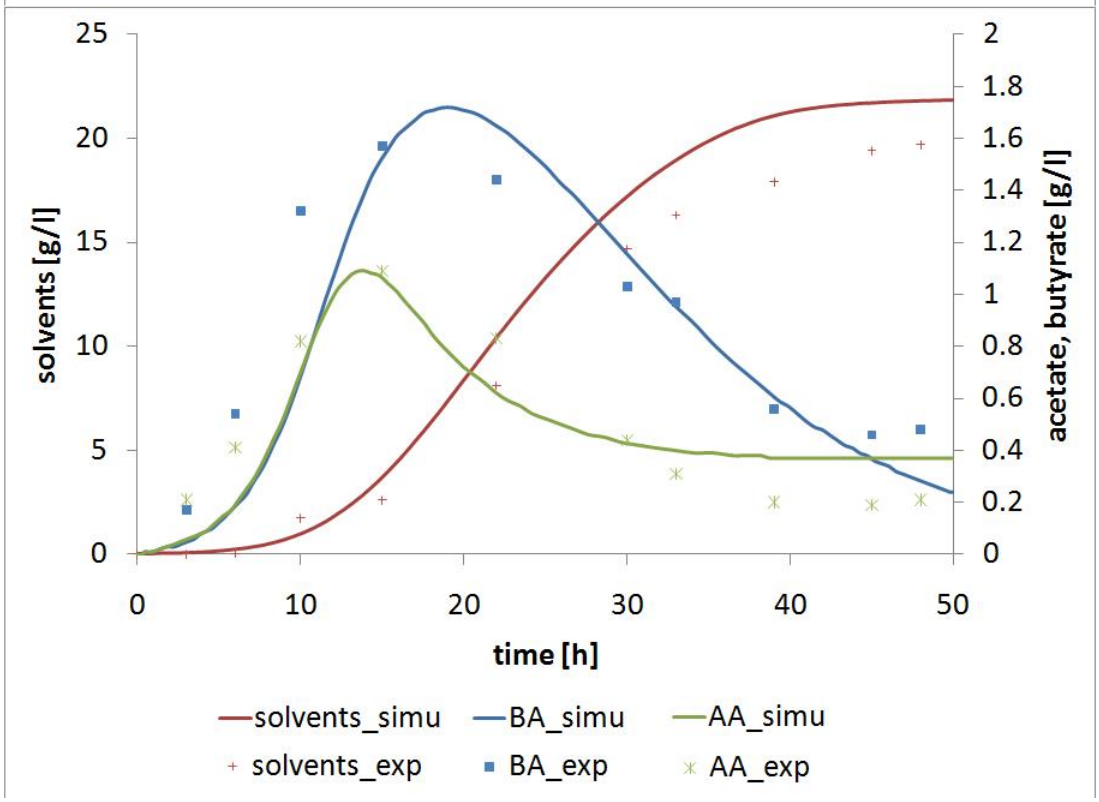
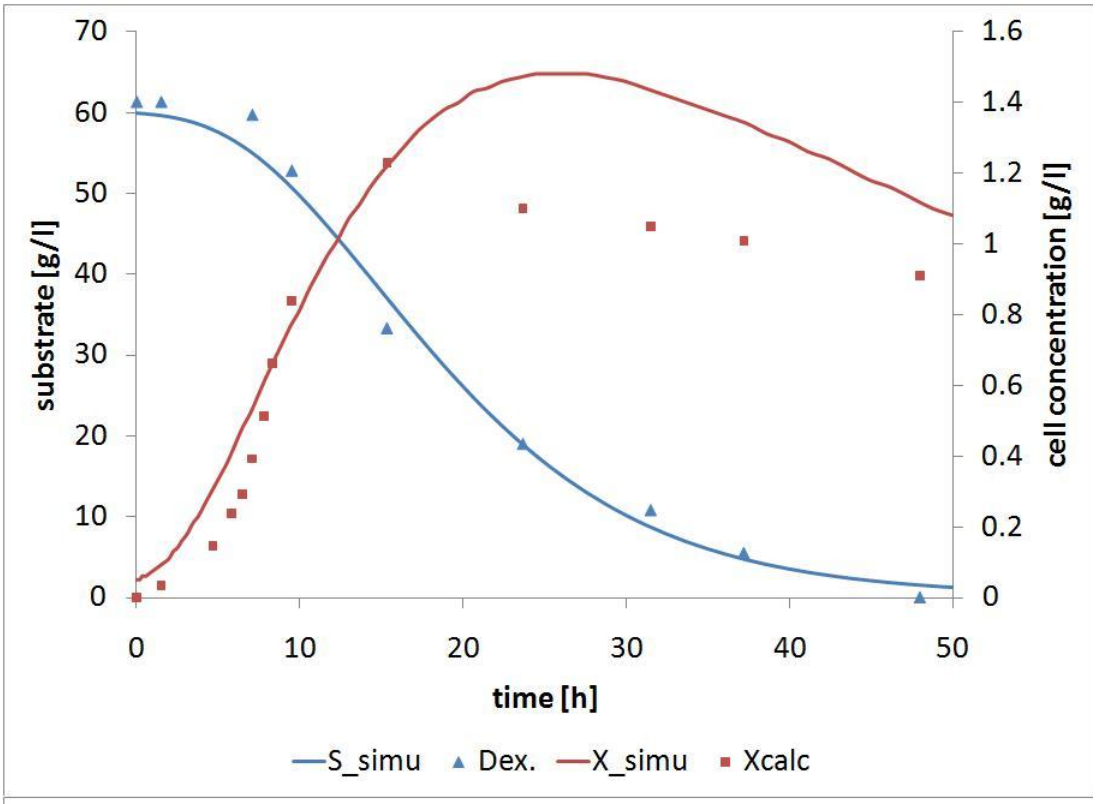


Figure 5.2 – Batch simulation with pH-control in organic acid mode. Solid lines represent calculated values whereas markers indicate experimental results. Experimental conditions: degenerated strain of ATCC 824 grown at 36ºC in pH-controlled fermentor with 1l working volume



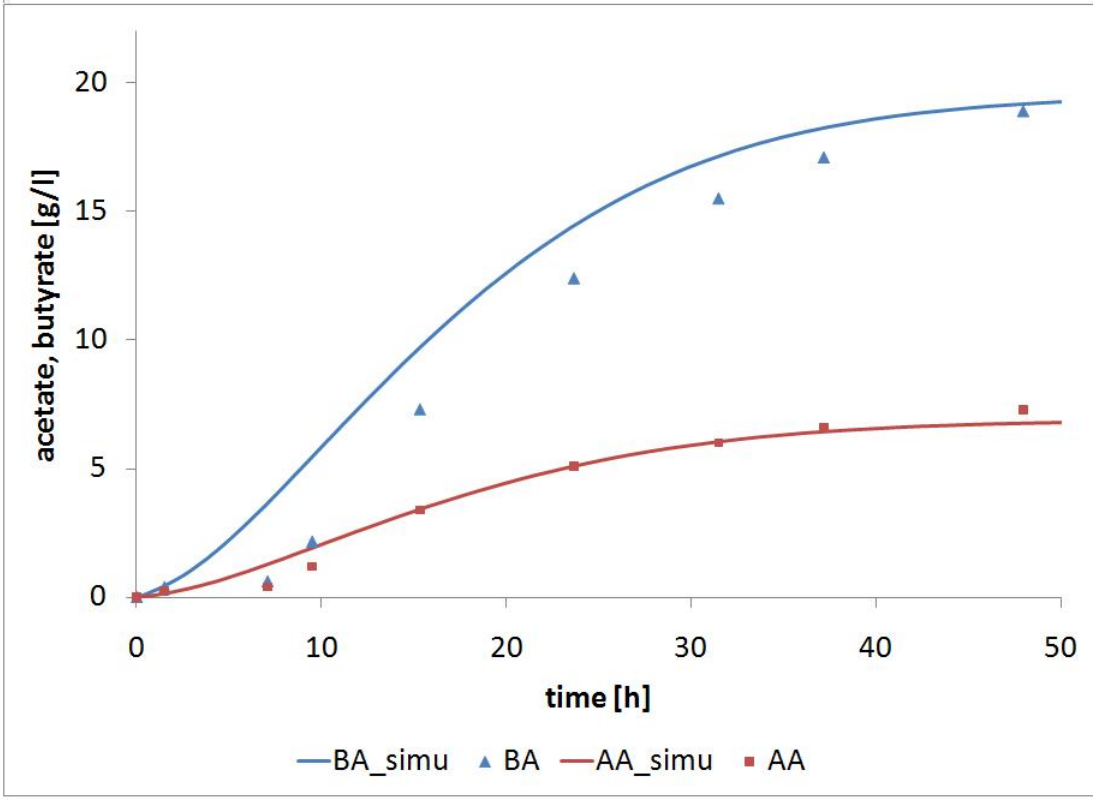
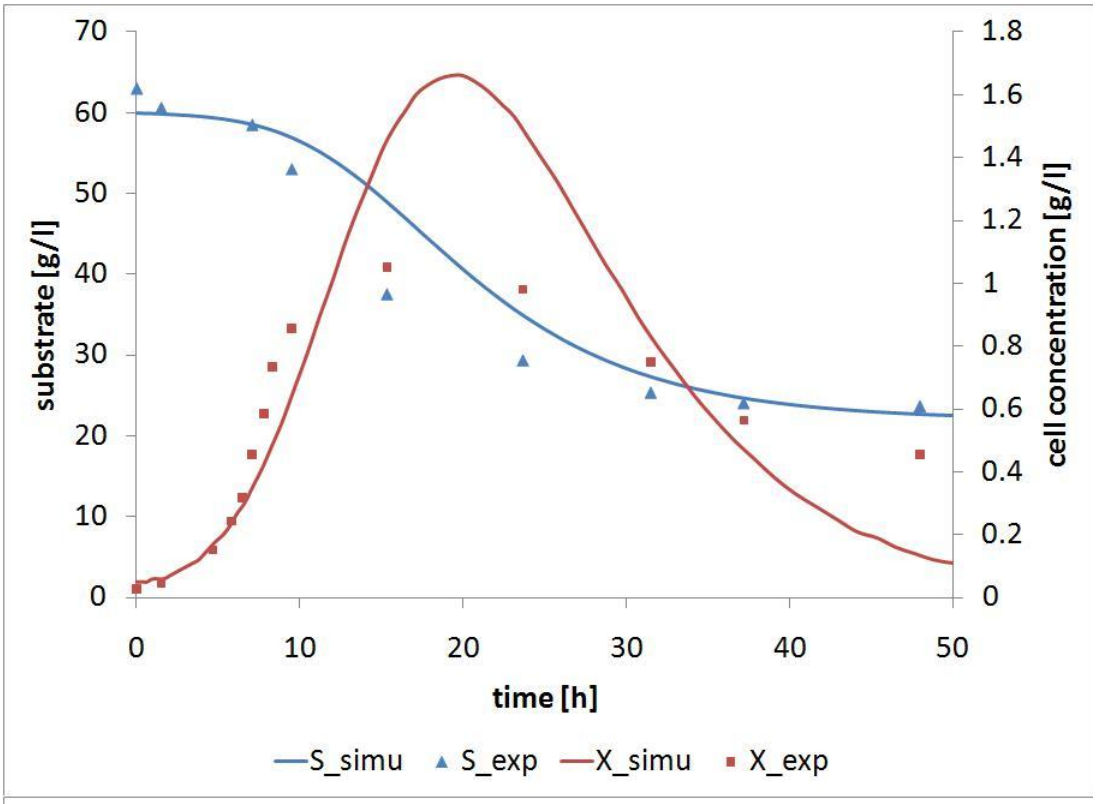
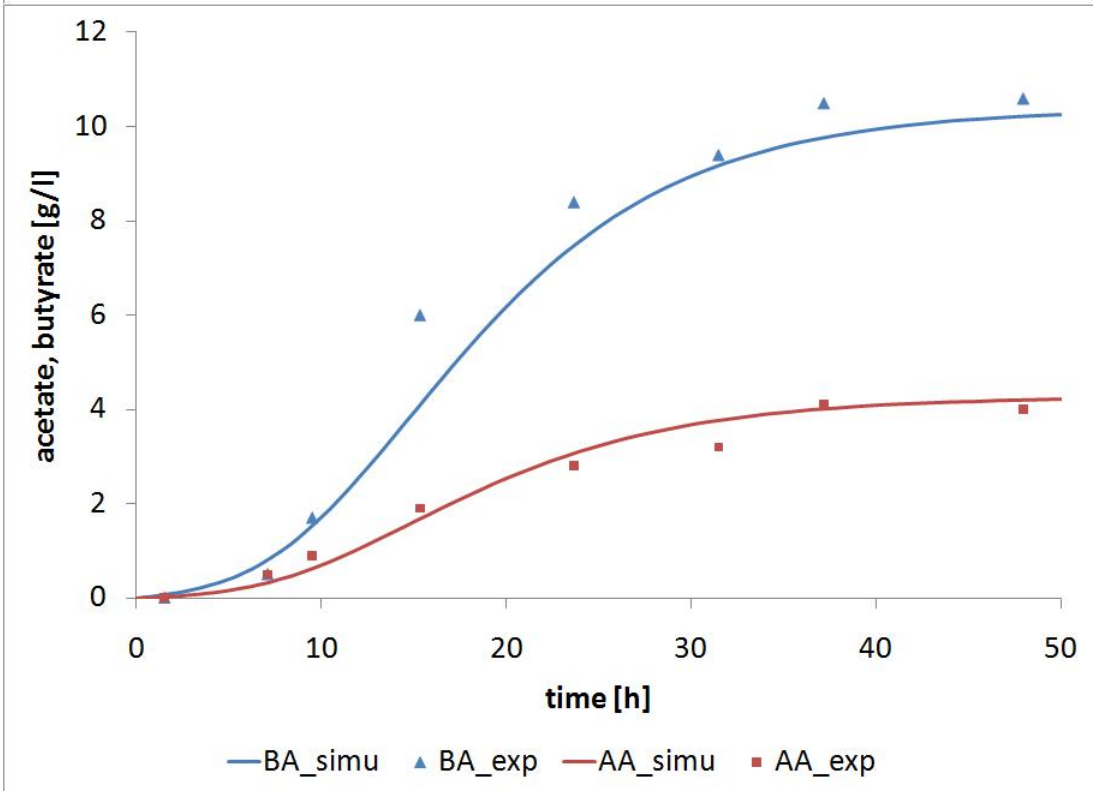


Figure 5.3 – Batch simulation with pH-control in organic acid mode and 10 g/l butanol present at all times. Solid lines represent calculated values whereas markers indicate experimental results. Experimental conditions: degenerated strain of ATCC 824 grown at 36ºC in pH-controlled fermentor with 1l working volume





5.3 Continuous Fermentation in a PFR followed by two CSTR

In order to simulate a biological PFR, the left hand side of equations 11-1 through 21-1 need to be adapted to account for the continuous variation of the concentration of both reactants and product along the cylindrical axis of the bio reactor. Consequently, the rate of reaction will vary axially as well. Equation 15 represents the differential form of the change in molar flow rate of species j F_j per differential reactor volume dV:

$$\frac{dF_j}{dV} = r_i \tag{15}$$

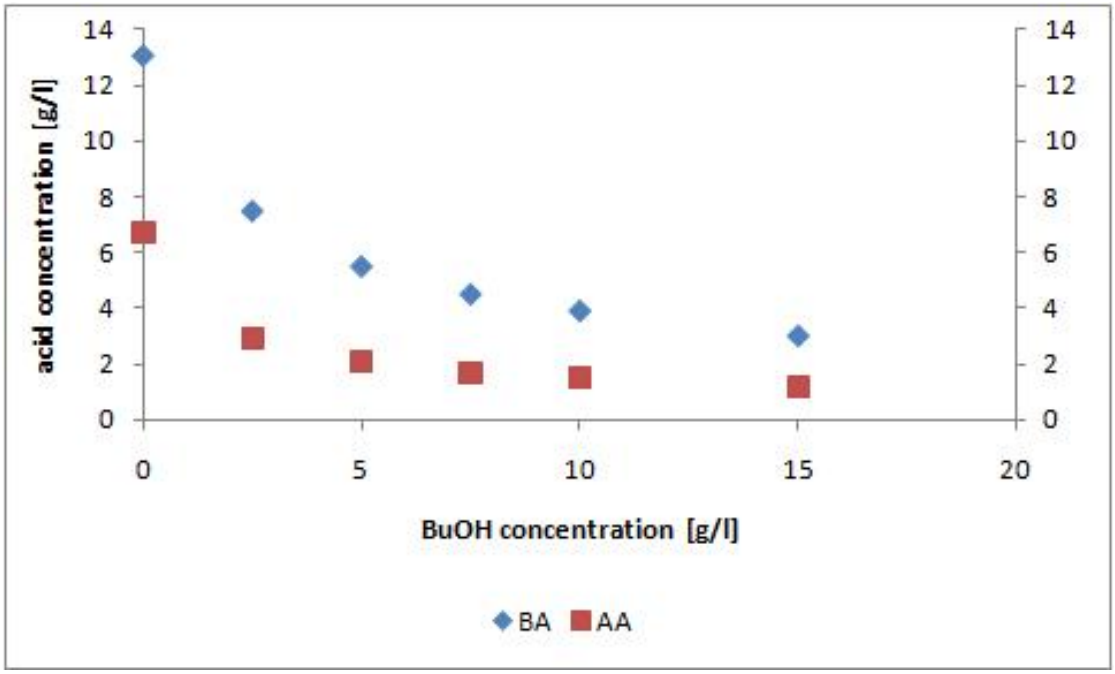
r_j is the reaction rate which can be set equal to expressions listed in Table 5.2 for the batch reactor without pH-control.

A CSTR on the other hand operates under well mixed conditions so that there is no variation in concentration throughout the reactor. At steady state, the molar flow rate of species j can be expressed as

$$F_j = r_j V + F_{j0} \tag{16}$$

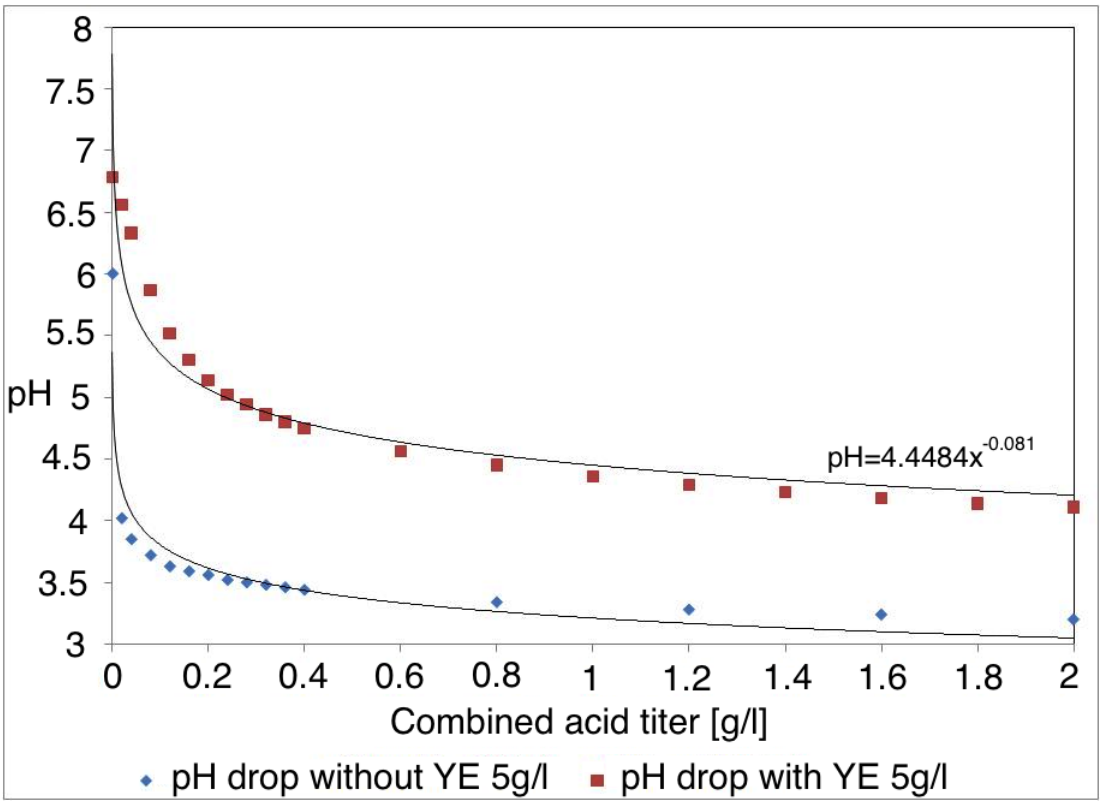
where F_{j0} is the inlet concentration of a compound. If the right hand side of equations 11-3 through 21-3 is inserted for r_j, flow rate expressions at steady state can be derived. The resulting system of related none-linear equation can be solved numerically. Exemplary, the system was solved for various butanol inlet concentrations as depicted in Figure 5.4. As expected, the rate of acid production falls rapidly with increasing butanol concentration.

Figure 5.4 – Calculated organic acid effluent concentration in second CSTR with varying BuOH inlet concentrations (BA butyric acid, AA acetic acid). Conditions: V=2 l, D=0.04 h^{-1},$S_0 = 20$ g/l, $BA_0 = 6.8$ g/l, $AA_0 = 2.2$ g/l, $X_0 = 2$ g/l, $Y_0 = 1$



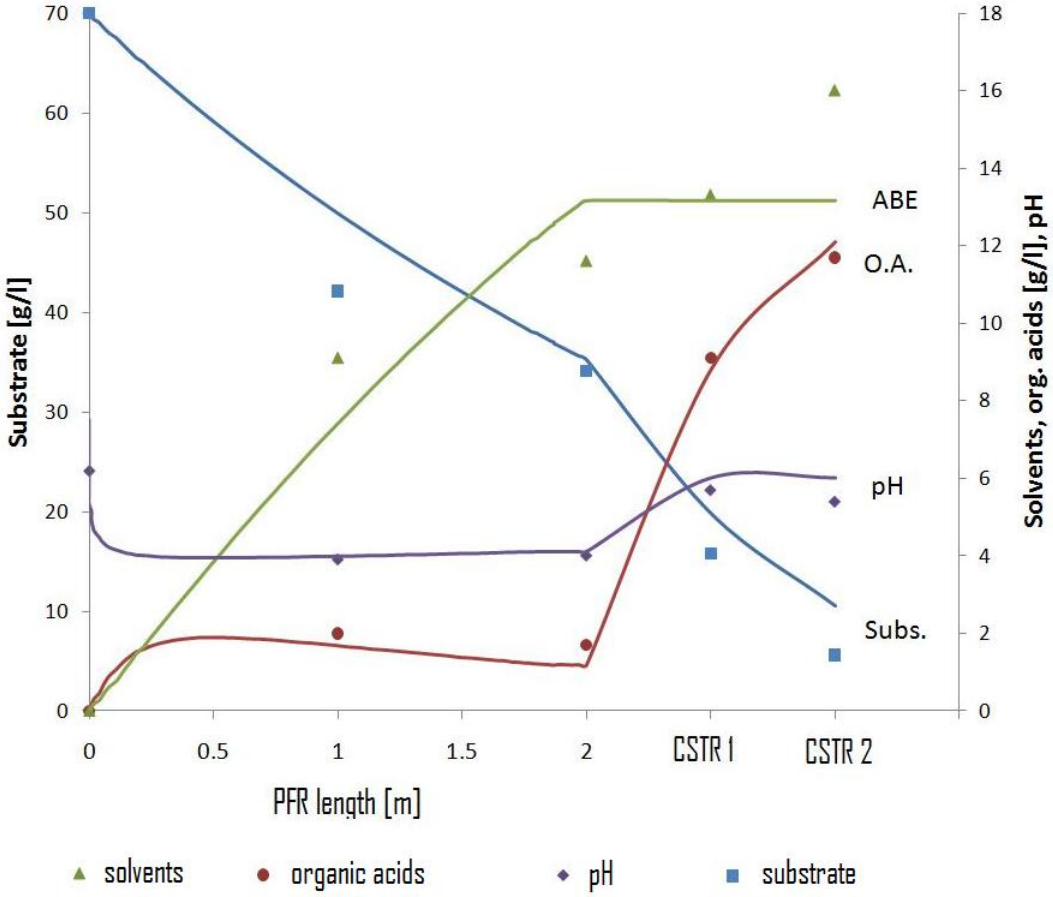
Although the model doesn't require the calculation of the actual pH values to simulate a fermentation, pH values are often measured during experiments and can be used to monitor the progress of a fermentation. During a simulation, the pH can be inferred from the knowledge of organic acid titers and the buffering capacity of the media. The buffering capacity results from organic compounds contained in yeast extract and other complex nutrient sources such as corn steep liquor (CSL), that act as conjugated acid/base pairs when interacting with the produced acids [53]. In order to accurately relate acid concentration to pH, a set of experiments was conducted to relate the concentration of acetate and butyrate to the pH of water with and without 5 g/l yeast extract. Figure 5.5 depicts the experimental results and the equation used to model the relationship.

Figure 5.5 – Effect of 5 g/l yeast extract on the buffering capacity of deionized water. The pH of diionized water with and without the addition of yeast extract was measured after incremental and equimolar amounts acedic- and butyric acid were added



Finally, the described expressions and relations can be used to model the behavior of a PFR bio reactor followed by two CSTR. The initial conditions of the PFR are listed in Table 5.1. Cell dry matter in the reactor was determined experimentally after the continuous fermentation had been concluded. For the chemostats, negligible solventogenesis (pH>6.0) and a biomass concentration of 3 g/l were assumed. The elevated cell concentration was determined experimentally and is a result of flocculated biomass (pellets of very high cell density) leaving the PFR. Figure 5.6 compares experimental results with those gained by the simulation. Overall, the simulation is in good agreement with the experimental results. Deviations occur mostly with respect to solvent titers. The model under predicts ABE accumulation in the first half of the PFR and over predicts solvent titers in the second half of the tubular reactor. Also, the model does not account for solventogenesis in the chemostats as observed experimentally. Consequently, the predicted substrate concentration in the chemostats is higher than determined experimentally.

Figure 5.6 – Simulated reactor system (solid lines) and experimental results (markers) in comparison. Experimental conditions: degenerated strain of ATCC 824 grown at 36ºC. CSTR 1: 1l working volume, pH-controlled at pH=5.7; CSTR 2: 2l working volume, no pH-control. Initial conditions for the PFR-simulation are listed in Table 5.1. Effluent concentrations of upstream reactors were used as initial conditions for downstream reactors.



6 Product Recovery

The efficiency of product recovery plays an important role in the overall economic feasibility of a potential ABE/organic acid fermentation process. Since the combined ABE and organic acid fermentation can yield titers for alcohols and organic acids in equi-molar ratios (see Figure 5.6: 1-butanol 0.15 mM, butyrate 0.09mM and acetate 0.06 mM) an esterification reaction seems advantageous to recover alcohols and organic acids simultaneously. The resulting esters represent valuable commodities for the cosmetics- and food industry [35] and can also be used as diesel fuel additives for combustion engines. As pointed out in chapter 2.2.1, water is one of the products of an esterification reaction. Therefore, by removing water from the system, an increase of both net reaction rates and yields can be expected. In this work, this is accomplished by one of two methods: (i) Adsorption of products from the aqueous fermentation broth onto activated carbon and (ii) extraction of products from the fermentation broth by a none-polar solvent. Subsequent esterification is investigated using a lipase as bio catalyst.

6.1 Adsorption onto Activated Carbon

Ever since the century-old discovery that carbon could be used as an adsorbent, it has found numerous applications throughout various industries. Examples include the purification of drinking water, decolorization of liquids, waste water treatment, odor removal as well as adsorbent in gas masks and other safety related products [54]. Activated carbon inherits its merits from a large surface area (250-1500 m^2/g, [54]) and due to the fact that it is much less costly than ion-exchange resins and other materials used for adsorption [55]. Moreover, it can be regenerated with steam, and the desorbed components may be recovered and marketed [55]. Depending on the application, the surface properties of activated carbon can be modified by treatment with oxidizers such as ozone or sodium hydroxide to yield a higher surface acidity [56].

6.1.1 Materials and Methods

The activated carbon used for this study was supplied by Jacobi (AquaSorb®CS) and it's properties are listed in Table 6.1. Adsorption isotherms where determined by using artificial fermentation broth of varying pH at 30ºC. The pH of the liquid was adjusted with 5M NaOH. The adsorption was carried out in closed 20ml glass vessels that contained known amounts of synthetic broth and activated carbon. Prior to use, the 'virgin' activated carbon was vacuum desorbed at 10 mTorr for 24 hours. Mixing and temperature were controlled by placing the vessels horizontally in an incubator/shaker set at 100 rpm shaking speed. Adsorption q_i of each species was determined by equation 17

$$q_i = \frac{(C_{i,0} - C_i)V_0}{m_{ac}} \tag{17}$$

where $C_{i,0}$ and C_i are the initial and final concentrations of a species i in the liquid phase, V_0 is the liquid volume and m_{ac} is the mass of activated carbon. The composition of the initial synthetic fermentation broth is listed in Table 6.2

A series of experiments involving adsorption and back extraction with hexane was undertaken using actual fermentation broth at room temperature. For the adsorption step, three liters of centrifuged fermentation broth (for initial properties see Table 6.2) were continuously pumped at 90ml/min through a column (0.5 l) filled with activated carbon (283.1g initially). During adsorption, the pH of the broth was held constant at pH=4.35 using a 50% solution of lactic acid. After adsorption, the packed column was blown out with compressed air to remove water. Further drying was accomplished by heating the column to 65°C and passing air through it at 3 l/min for 15h. Back extraction was accomplished by pumping hexane through the heated (60°C) activated carbon at a flow rate of 5 ml/min for 200min. After back extraction, excess hexane was vacuum desorbed at a temperature of 90°C for 12h. This cycle was repeated three times using the same initial fermentation broth and activated carbon.

Table 6.1 – Properties of the activated carbon

iodine number	moisture content	total ash content	surface area (BET)	density	particle size	original plant material
>1000 mg/g	<5%	<4%	1050 m^2/g	0.51g/ml	0.425-1.7mm	coconut shell

Table 6.2 – Composition of synthetic and actual fermentation broth

[g/l]	acetate	butyrate	acetone	butanol	ethanol
synthetic broth	4.8	15	6	13.5	1.4
actual broth	4.2	8.1	2.7	8.1	1.6

6.1.2 Results and Discussion

Adsorption Figure 6.1 depicts the adsorption of combined solvents and acids depending on the initial pH of the synthetic broth and the equilibrium concentration. Organic acid adsorption strongly correlates with pH. This indicates that only the protonated form of the acid molecule is adsorbed. Compared to butyric acid, acidic acid exhibits a much lower affinity for the sorbate due to its higher polarity (see Figure 6.2). The highest combined acid adsorption occurs at pH values between 2.7 and 3.58. At this point, solvent adsorption is at a minimum due to a limited number of adsorption sites according to Langmuirs's adsorption model for single sites. Equi molar ratios for adsorbed organic acids and alcohols can be found according to Figure 6.3 at values for

$$\Upsilon = \frac{m_{ac}}{m_{(BA+AA)_{ini}}} \tag{18}$$

between 2 and 10 for pH values between 4.45 and 2.7, respectively. Consequently, given a final acid concentration and a corresponding pH, the amount of activated carbon can be estimated where equi molar adsorption occurs. Virgin activated carbon exhibits its highest adsorption capacity of approximately 25% (w/w) at a combined solvent and organic acid

equilibrium concentration of 35 g/l whereas practically more relevant adsorption capacities average around 15% at combined equilibrium concentrations between 6 and 8 g/l. The remaining acids and solvents at low equilibrium concentrations mostly comprise of acetate, acetone and ethanol due to their more polar character.

Figure 6.1 – Adsorption of organic acids (combined acetic- and butyric acid) and solvents onto activated carbon at various pH values at 22ºC.

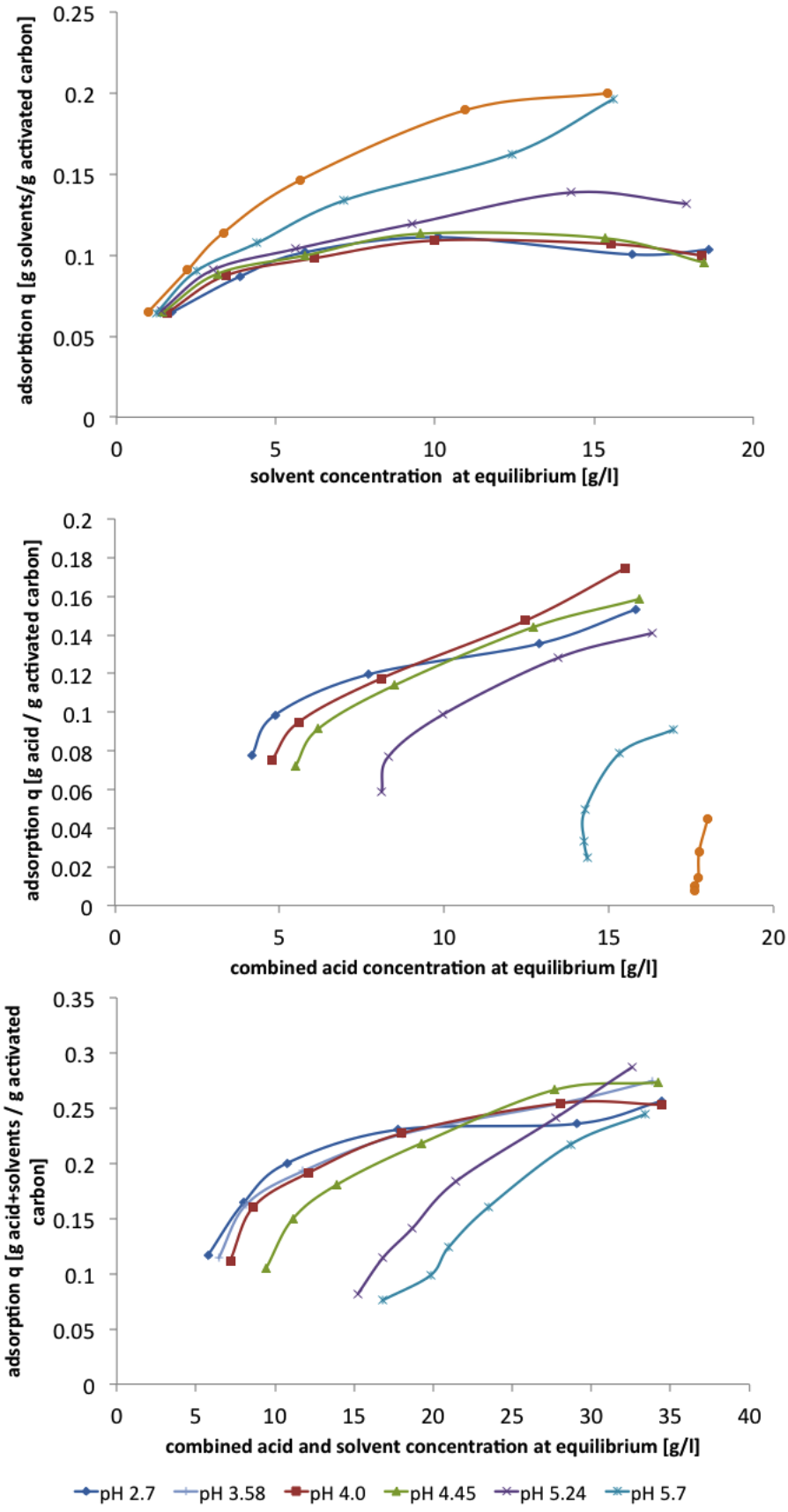


Figure 6.2 – Adsorption of butyric- and acetic acid on activated carbon from synthetic fermentation broth at various pH-values at 22°C.

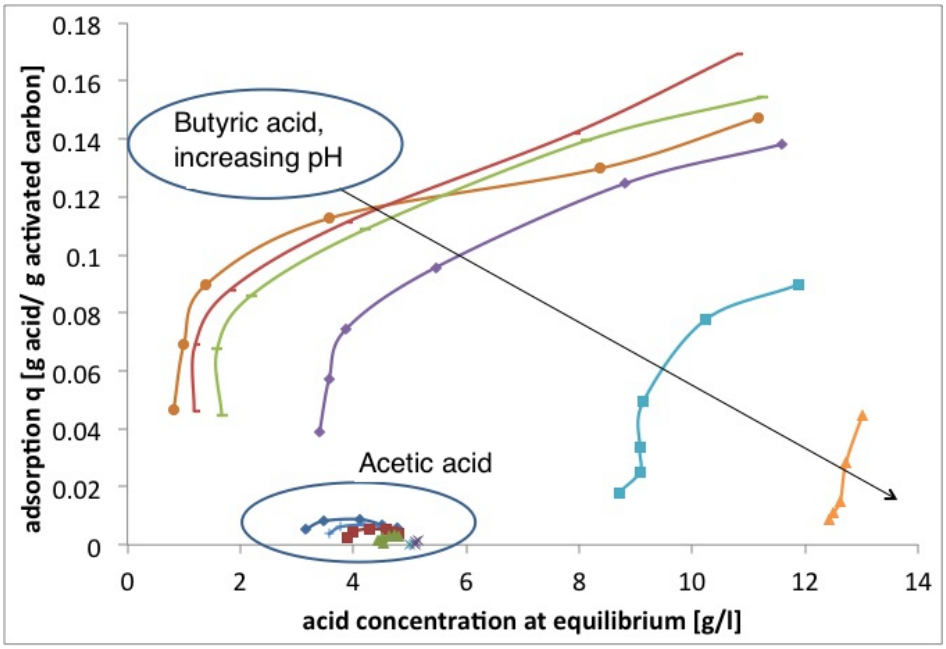
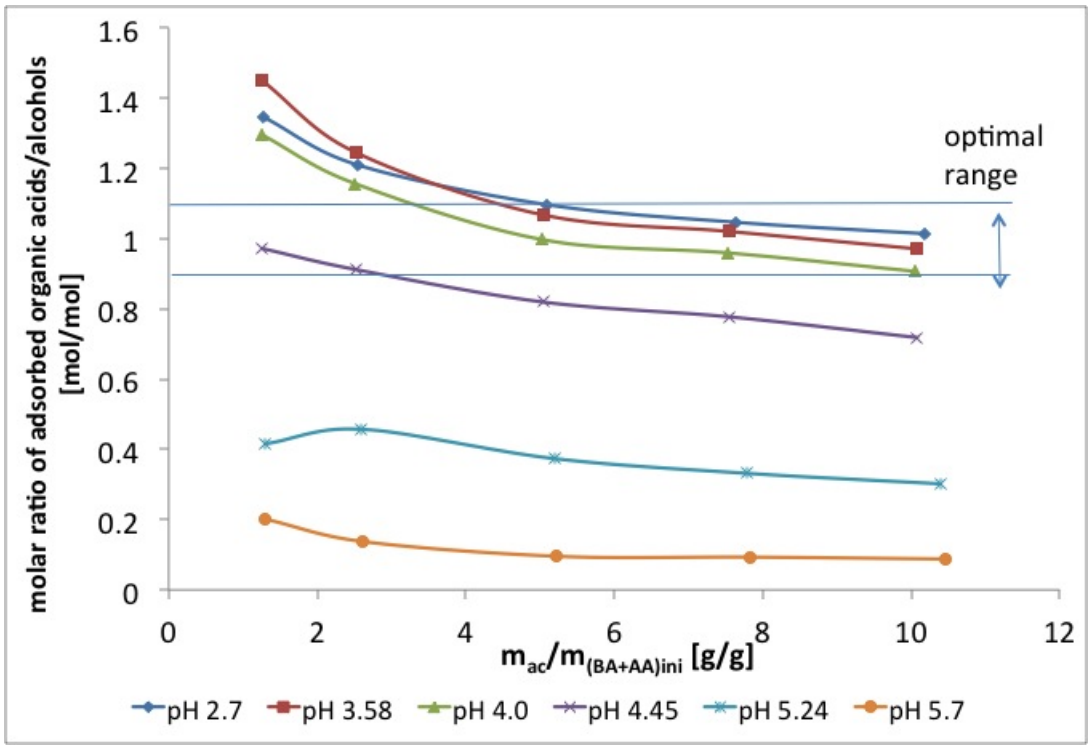


Figure 6.3 – Molar ratios of adsorbed acids to alcohols under various conditions. $\frac{m_{ac}}{m_{(BA+AA)ini}}$ represents the mass ratio of activated carbon to the combined amount of organic acids present in the media



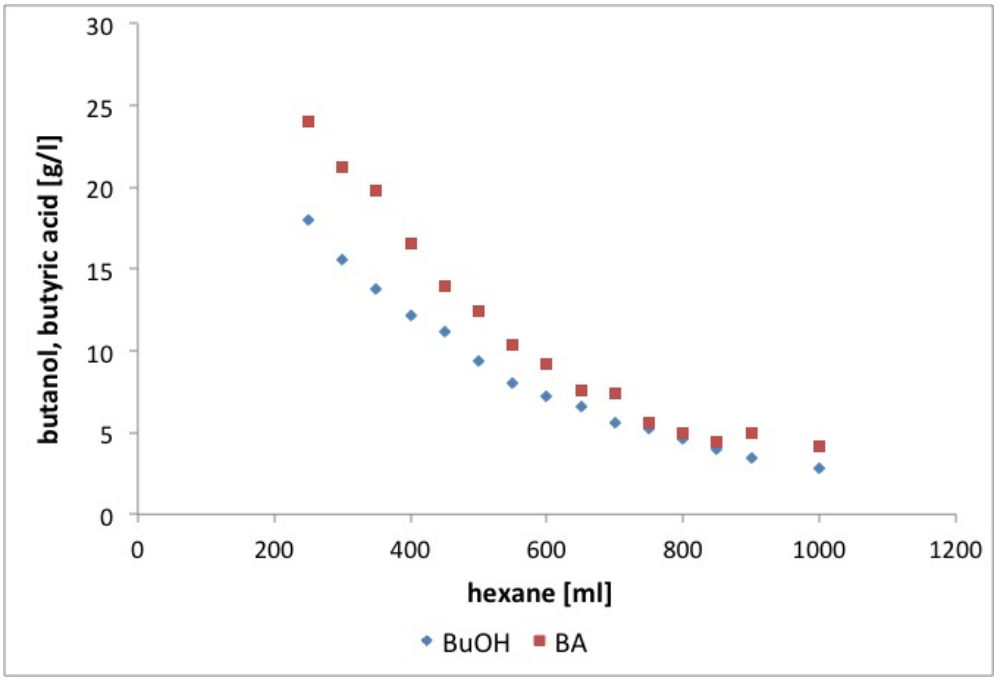
Back Extraction Table 6.3 lists key parameter that describe the success of back extraction over the course of three cycles using the same activated carbon. It becomes evident that the achievable final titers for butanol and butyric acid in hexane are rather low (between 8.25 - 11 g/l for butanol and 10.6 - 11.1 for butyric acid). Although initial extraction rates are quite high, the product concentrations fall quickly (see Figure 6.4). If back extraction is followed by esterification, the final butyl butyrate titer would be just under 20 g/l. The adsorption capacity of activated carbon diminishes from 15.4% initially to 9.9% in the third cycle. This indicates that either the total BET number has decreased (e.g. through blocked or collapsed pores) or that a certain percentage of the surface area is still occupied by products or the solvent. While the answer probably lies in between, there is evidence to suggest that product desorption by back extraction followed by heat and vacuum treatment was incomplete: the final titer of butyric acid in hexane (after back extraction with 1l hexane) is almost identical after each cycle despite decreased adsorption capacity of activated carbon. This indicates that an increasing percentage of surface area is occupied by butyric acid. A more complete recovery of internal surface area could be achieved by intensifying the vacuum/heat desorption step.

Note that butyl butyrate is already being formed during back-extraction. At the last cycle, about 5% of butanol and butyrate are being converted to the corresponding ester. It is assumed that the auto-catalytic effects of butyric acid is largely responsible for this phenomena.

Table 6.3 – Summary of three consecutive adsorption/desorption cycles with activated carbon and actual fermentation broth using hexane as a back-extractant. See the Materials and Methods section 6.1.1 for experimental details.
*after back extraction and heat/vacuum desorption
**recovered by back-extraction only

	1st cycle	2nd cycle	3rd cycle
m^*_{ac}[g]	291.2	292.8	294.1
activated carbon loading [%]	15.4	10.5	9.9
$C_{BuOH,hexane}$[g/l] / % recovered**	11.0 / 56	10.5 / 55	8.25 / 51
$C_{BA,hexane}$[g/l] / % recovered**	10.6 / 57	11.6 / 67	11.1 / 68
$C_{A,hexane}$[g/l] / % recovered**	1.65 / 80	0.95 / 57	0.69 / 60
$C_{AA,hexane}$[g/l] / % recovered**	1.0 / 36	0.76 / 59	0.39 / 22
$C_{E,hexane}$[g/l] / % recovered**	0.6 / 68	0.21 / 19	0.1 / 15
$C_{BB,hexane}$[g/l]	0.6	0.8	1.0

Figure 6.4 – Back extraction with hexane at 5 ml/min, 60°C, third cycle



6.2 Liquid-Liquid Extraction

On-line liquid-liquid extraction (LLE) of fermentation products from the aqueous phase of an ABE fermentation is considered a viable strategy to reduce both product inhibition and costs associated with downstream product removal [57]. A number of solvents have been evaluated with regard to toxicity, partition coefficient and selectivity for 1-butanol extraction (see Table 2.4). Ideally, the solvent should be none-toxic to the microorganisms and have a high partition coefficient and selectivity for the desired products. In order to allow for a high product yield when extraction is followed by an esterification, the emphasis should be on a high selectivity. Additionally, the solvent should be readily available, inexpensive, exhibit a low tendency to form emulsions and allow for easy product separation. Moreover, if LLE is combined with esterification, the solvent should not participate or inhibit the reaction. The number of solvents that meet these criteria is very short. Indeed, from the compounds listed in Table 2.4, only alkanes exhibit the desired properties. If the condition of culture toxicity is relaxed (on-line product removal not possible, reusing of spent broth only after removal of toxic extractant), long-chained ketones such as 2-octanone are better alternatives due to their high partition coefficients and selectivities. Surprisingly, these long-chained ketones have not been investigated yet despite their good performance as ABE and organic acid extractants. Many authors investigating LLE for ABE removal report their findings in comparison to oleyl alcohol due to its non-toxic properties and relatively high partition coefficient and selectivity of 2.8 and 330, respectively. In addition to oleyl alcohol, Table 6.4 compares the properties of other popular and novel solvents for ABE recovery with 2-octanone.

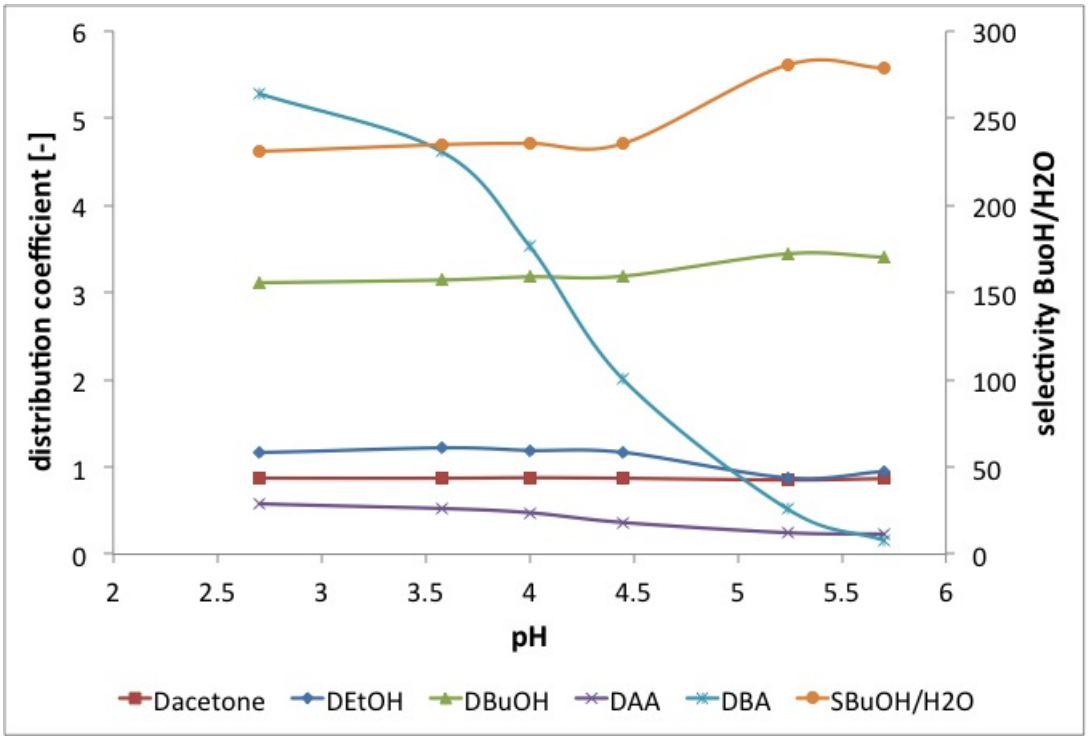
Since ketones do not participate in esterification reactions, 2-octanone was tested as solvent for combined ABE and organic acid extraction. Figure 6.5 illustrates the dependency of the distribution coefficient and selectivity on the pH of the model fermentation broth (for initial concentrations see Table 6.2). While the distribution coefficients of the solvents are largely unaffected by pH, organic acid extraction is strongly pH dependent. Moreover,

Table 6.4 – Solvent properties in comparison. D - distribution coefficient. Selectivities are calculated with respect to butanol and water

properties at 20ºC/1atm	Oleyl alcohol [57, 42]	mesitylene [57]	n-hexane[42]	2-octanone [own data]
D BuOH	2.8	0.76	0.5	2.4
D acetone	0.34	0.43	0.2	0.8
D ethanol	0.28	0.03	0.04	0.4
selectivity wrt. BuOH/H_2O	330	1650	2700	208
solubility of water in solvent [g/l]	11.4	4.6	0.03	11.1
solubility of solvent in water [g/l]	0.02	0.05	0.013	0.5
boiling point [ºC]	330-360	165	69	173-175

at pH values below 4.0 butyric acid exhibits a higher affinity for the solvent phase than butanol does. Consequently, if the goal is to achieve equi-molar ratios of alcohols and acids in the solvent phase, lower acid titers in the aqueous phase will suffice. Hence, the fermentation can be carried out at a level that is less inhibitory to the microorganisms. In addition, both the partition coefficient and selectivity for butanol increase by approximately 30% compared to single component extraction as indicated in Tables 6.4 and 2.4. This can be explained by an enrichment effect of similar functional groups in the solvent phase due to the combined extraction of ABE and organic acids. In other words, the organic phase's affinity for butanol increases with the simultaneous extraction of organic acids. This is an advantage over adsorption onto activated carbon, where the affinity for butanol decreases with the adsorption of organic acids.

Figure 6.5 – Distribution coefficients (D) and selectivity (S) with respect to BuOH/H_2O for LLE of synthetic media with 2-octanone at 22°C



6.3 Enzymatic Esterification

Compared to conventional esterification with mineral acids or solid acid catalysts, enzymatically catalyzed esterifications are highly selective, have high reaction rates at mild conditions and often have greater final product yields [12]. Disadvantages include restricted process parameter (mostly temperature limited), enzyme inhibition and cost considerations. Lipases consist of a group of enzymes capable of catalyzing both hydrolysis of fatty esters to fatty acids and alcohols as well as the reverse reaction. Both reactions are equilibrium limited. In order to achieve high conversions of the esterification, the reaction medium is often chosen to be a highly non-polar inert solvent such as n-Hexane. When used, the resulting reaction product (water) forms a micro-aqueous system (reverse micelles) in the hydrophobic organic solvent, whereas all other species are highly soluble in the solvent. Therefore, the reaction equilibrium is shifted to the formation of the ester. Yields in excess of 90% have been reported [15]. The enzyme, having a hydrophilic and a hydrophobic side,

typically resides in the boundary layer between water and apolar solvent [14].

Ideally, the hydrophobic solvent has the following properties: (i) it does not inhibit the enzyme, (ii) it has a favorable partition coefficient for the involved species with regard to water, (iii) it enables efficient product recovery (ester/solvent separation) and (iiii) it is not cost prohibitive. A literature review yielded alkanes with chain length of 6 to 8 as possible candidates [15, 11, 42, see Table 3]. n-Hexane, being a widely used and inexpensive none-polar solvent that meets the criteria listed above, was chosen to be the mediator solvent. Furthermore, since reasonably high reactant titers can only be achieved if product recovery is performed using adsorption (see chapters 6.1 and 6.2), enzymatic esterification will be investigated with the presence of the adsorbent activated carbon.

6.3.1 Materials and Methods

Enzymatic esterifications were carried out using a liquid form of a Lipase from *Rhizomucor miehei* (Palatase®20000L from Novozymes). According to the manufacturer, the activity of the lipase is 20,000 U/g where U corresponds to the moles of substrate converted per unit of time. Unless stated otherwise, the reactions took place in 250ml round bottom shake flasks with glass stoppers in an incubator/shaker set to 200rpm. The enzyme was added after combining all involved compounds and once the temperature had reached equilibrium (typically 45min). Analytical data was obtained using gas chromatography (Shimadzu GC-17A with a 30m Restek® column with 0.18mm inner diameter).

6.3.2 Results and Discussion

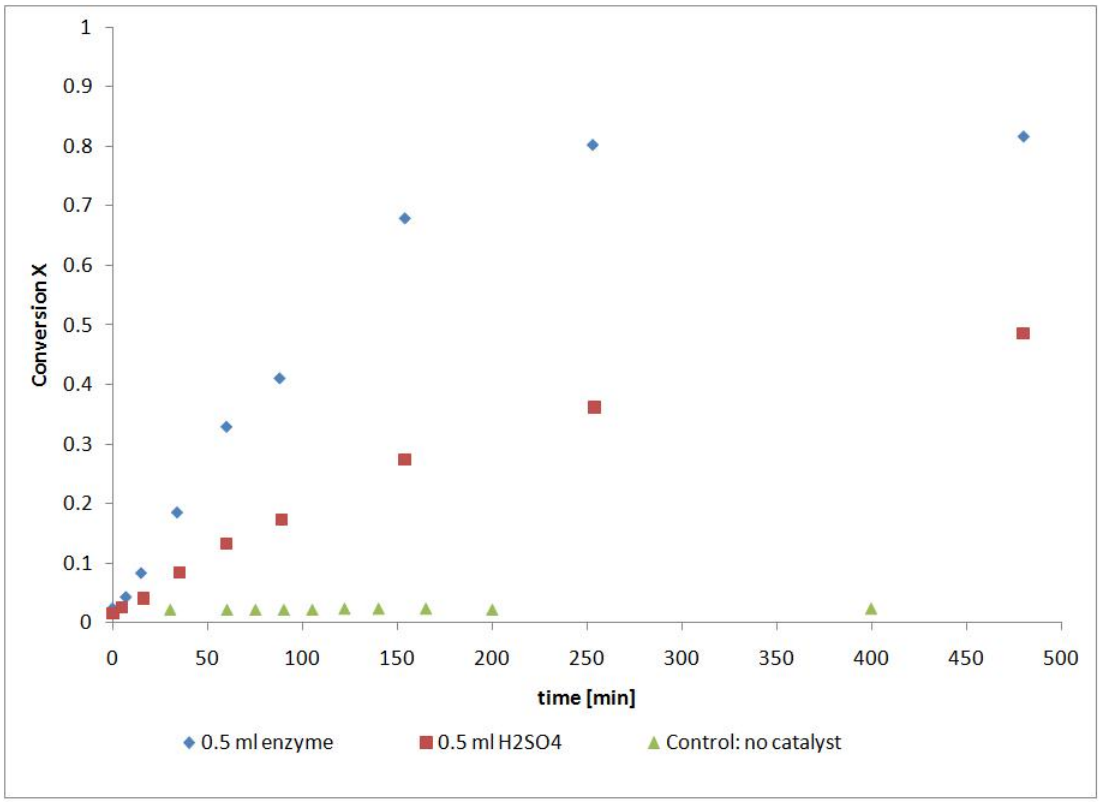
Figure 6.6 quantitatively compares an enzymatically catalyzed esterification with a reaction catalyzed by concentrated sulfuric acid. Throughout the experiment, the enzymatically catalyzed conversion outperforms both in reaction rate and final product titer. The measurable butyl butyrate yield for the biologically catalyzed reaction platoes after 300min at about 82%. The actual yield, however, is believed to be higher since an unknown amount of ester

is assumed to have bound to the activated carbon. Experiments aimed at determining the adsorbed amount of ester have not been undertaken at this point and remain the subject of future research.

In order to compare both catalysts directly, the turnover number (TON) was calculated. For an enzyme, the TON is defined as the number of substrate molecules an enzyme can convert per time. From Figure 6.6 one can determine the initial rate to be 1.54 $\frac{\mu mol}{s}$ at an enzyme concentration of 0.5ml. Noel et al. [60] determined the molecular weight of the lipase to be 30kDa. At a protein content of 8.7% by weight [61], the moles of enzymes contained in 0.5ml enzyme solution can be calculated to be 1.22 μmol, so that $TON_{enzyme} = 1.3s^{-1}$. This value is in the same as range as $k_{cat} = TON_{enzyme} = 3s^{-1}$ reported by [62] for a lipase catalyzed esterification of lauric acid with dilaurin in cyclohexane. The turnover number for the sulfuric acid catalyzed esterification can be calculated similarly by dividing the initial rate of substrate consumption (0.62 $\frac{\mu mol}{s}$) by the number of active sites. The number of active sites is assumed to be equal to the number of hydronium ions released when sulfuric acid reacts with water so that $TON_{H_2SO_4} = 1.1 \cdot 10^{-4}s^{-1}$. The large difference between the turnover numbers is believed to be caused by the difference in location of the catalyst. Sulfuric acid is a hydrophilic substance that phase separates from the media. Consequently, the reaction rate is limited by mass transfer due to a relatively small contact area between catalyst and solvent. Contrary, the enzyme exists in the boundary layers between polar and apolar compounds [14]. These boundary layers can not only exist between water and solvent but also between the surface area of the activated carbon (hydrophobic side) and the adsorbed substrates or reaction products (hydrophilic side). This brings reactants and catalyst into close proximity on the surface area of the activated carbon and results to a significantly higher turnover number. If the activated carbon is not present in the reaction mixture, the initial rate for the biologically catalyzed reaction drops to 0.16 $\frac{\mu mol}{s}$ which translates to $TON_{enzyme,no\,a.c.} = 0.13s^{-1}$ (data not shown).

Enzymatically catalyzed esterifications involving two substrates usually follow a ping

Figure 6.6 – Esterification of equi-molar amounts of 1-butanol with butyric acid (18.5mmol each) adsorbed on 15g activated carbon in 50ml hexane at 50°C



pong bi-bi mechanism [63].

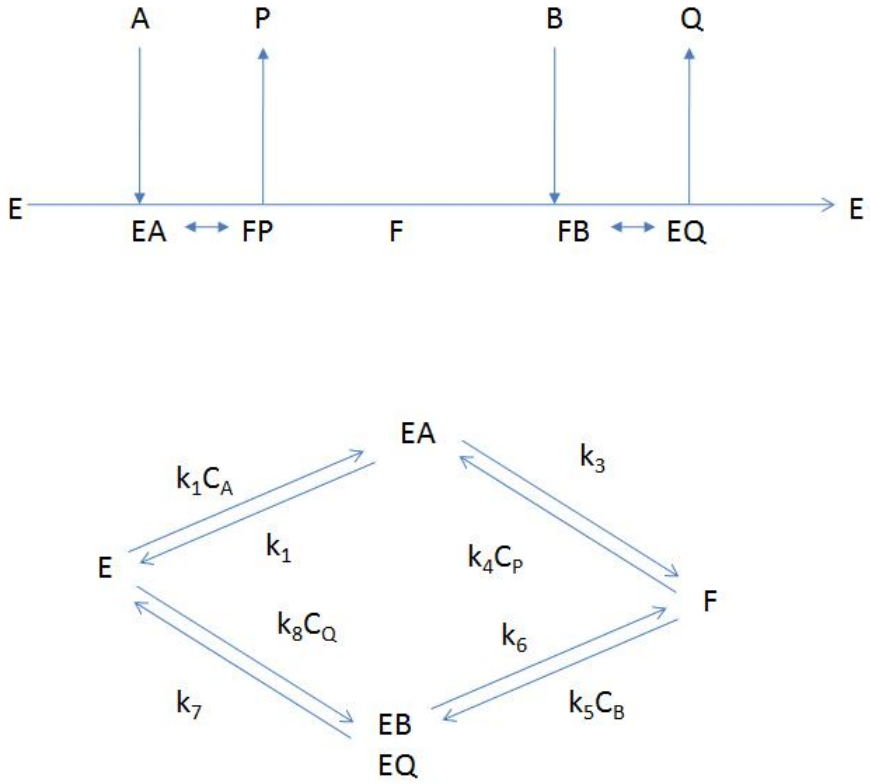
The schematics of the mechanism are depicted in Figure 6.7 where A and B represent the substrates, P and Q the products and E and F the enzyme. The rate equation, taking all kinetic constants into account and dropping P and Q (initial rates, product concentrations are negligible), can be written as

$$v = \frac{k_1 k_3 k_5 k_7 C_A C_B C_E}{k_1 k_3 (k_6 + k_7) C_A + k_5 k_7 (k_2 + k_3) C_B + k_1 k_5 (k_3 + k_7) C_A C_B} \tag{19}$$

where A represents the acid and B the alcohol [59]. The above equation can be simplified by combing constants so that

$$v = \frac{V_{max} C_A C_B}{K_b C_A + K_a C_B + C_A C_B} \tag{20}$$

Figure 6.7 – Two substrate ping pong bi-bi reaction mechanism: A and B substrates, P and Q products, E and F enzyme, $k_1$through k_8 rate constants. Adapted from [59]



where V_{max} is the maximum esterification rate, K_a and K_b are the Mechaelis-Menton constants for the acid (n-butyric acid) and the alcohol (1-butanol), respectively. The reciprocal of the above equation is

$$\frac{1}{v} = \frac{K_b}{V_{max}}\frac{1}{C_B} + \frac{1}{V_{max}}\left[1 + \frac{K_a}{C_A}\right] \tag{21}$$

and can be used to determine the kinetic constants experimentally. Plotting $\frac{1}{v}$ over $\frac{1}{C_B}$ results in three lines with similar slopes, which are independent of the butyrate concentration so that an average $\frac{K_b}{V_{max}} = 141\frac{ml_{enzyme}min}{l}$ can be determined (see Figure 6.8). By plotting y-axis intercepts of Figure 6.8 against the reciprocal of the butyrate concentration, a secondary plot (Figure 6.9) can be derived where the slope corresponds to $\frac{K_a}{V_{max}} = 351\frac{ml_{enzyme}min}{l}$

and the intercept equals $\frac{1}{V_{max}} = 4160\frac{ml_{enzyme}min}{mol}$. The final values that could be determined are as follows: $V_{max} = 0.24\frac{mmol}{ml_{enzyme}min}$, $K_a = 79\frac{mmol}{l}$ and $K_b = 34\frac{mmol}{l}$. V_{max} can be converted to a turnover number as outlined earlier in this section. This yields a maximum turnover number of $TON_{max,enzyme} = 3.3s^{-1}$.

Figure 6.8 – Double reciprocal plot of the initial esterification rate v at varying butanol concentrations. Conditions: 50°C, 50ml hexane, 200rpm shake speed, 0.5ml enzyme, varying concentrations of butyric acid

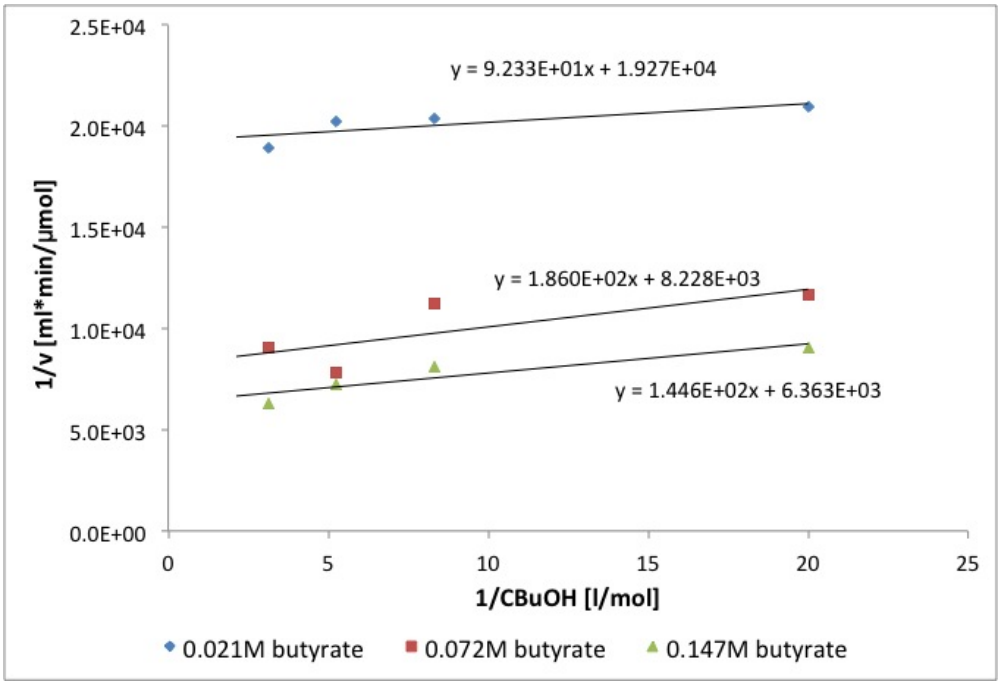
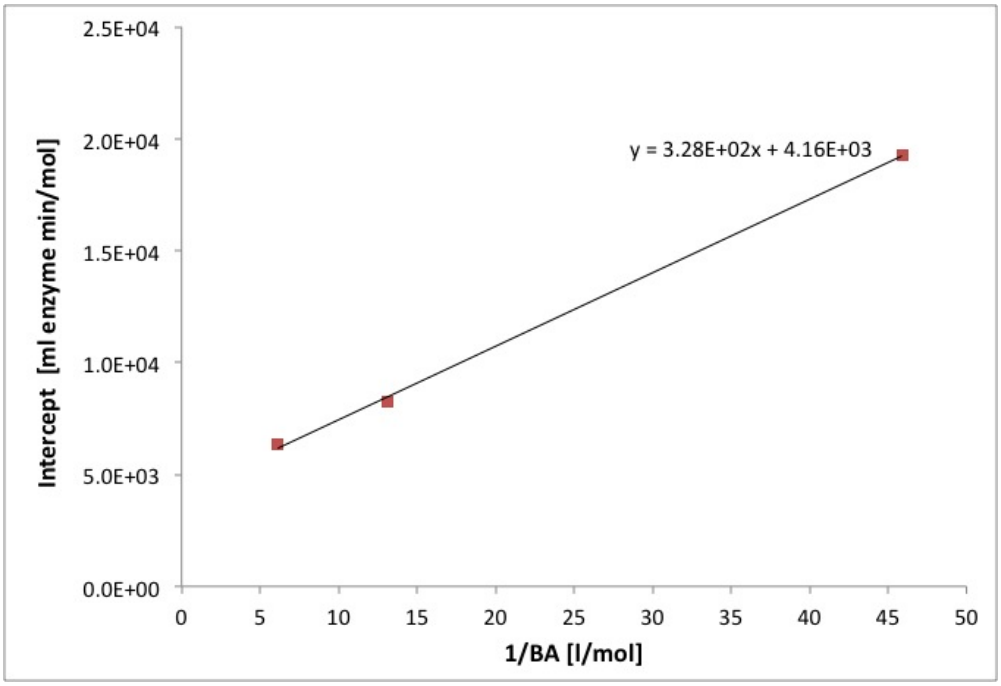
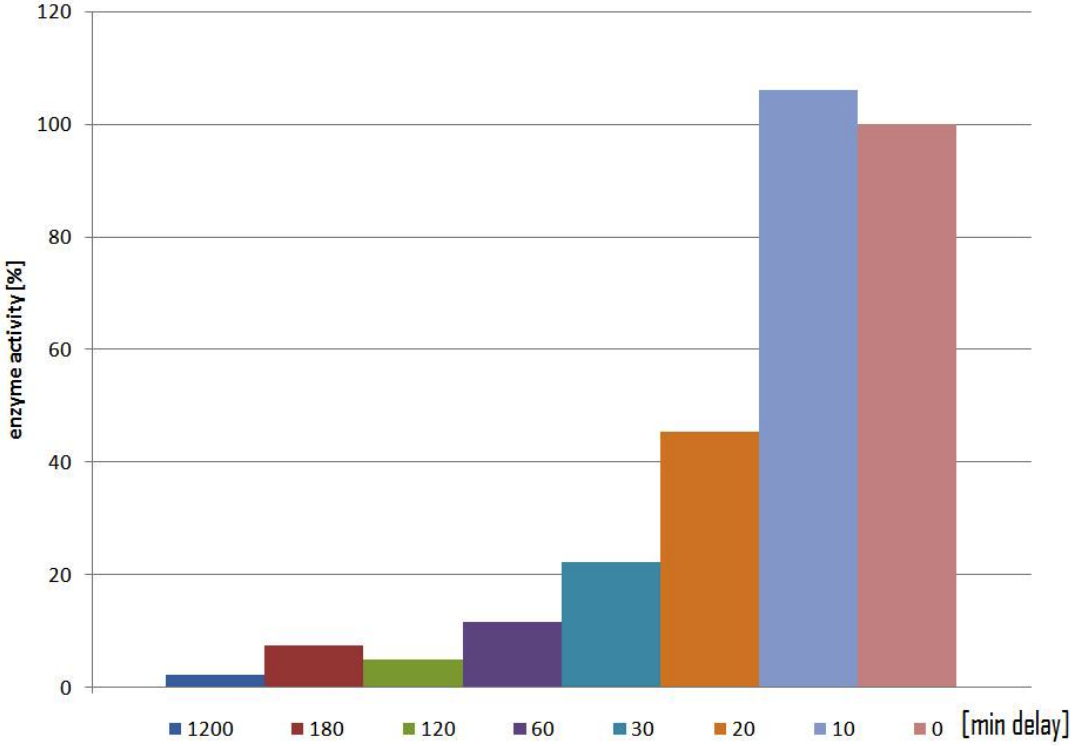


Figure 6.9 – Intercepts of Figure 6.8 against the reciprocal of the butyric acid concentration



Enzyme Stability Although the mass production of enzymes using genetically modified organism has lowered their price considerably [12], overall process economics would benefit from enzyme reuseability. The enzyme stability for the reaction conditions outlined in the methods section has been examined by delaying the addition of substrate to a mixture of 15 g activated carbon, 50ml hexane and 0.5ml enzyme at 50°C. Figure 6.10 shows that a delay of more than 10min results in a drastically diminished enzyme activity. This is surprising since the observed reaction rate without a delay in substrate addition does not decrease significantly between 10 and 100min (see Figure 6.6). It seems that the enzyme is more stable when it is allowed to interact with the substrate. When adsorption is followed by enzymatic esterification, the substrate is already bound to the activated carbon so that enzyme degradation is inhibited until the reaction runs low on substrates. Therefore, in an effort to maintain the substrate concentration at a suitable level, continuous or fed-batch operation seems feasible to avoid a premature enzyme degradation.

Figure 6.10 – Initial enzyme activity after delaying substrate addition (0.02mol of each butyric acid and butanol) to a mixture of 15 g activated carbon, 50ml hexane and 0.5ml enzyme at 50°C



7 General Conclusions and Recommendations for Future Work

7.1 General Conclusions

The objective of this work was to propose and investigate concepts aimed at improving the overall efficiency of the fermentation and product recovery steps of ABE fermentations.

It could be shown that a partially degenerated strain of *Clostridium acetobutylicum* ATCC 824 can be used in a continuous reactor system consisting of a tubular reactor and two CSTR in series for an extended period of time (40 days) without further degeneration. The tubular reactor excelled at producing high final solvent titers (14.6 g/l) at high volumetric productivities (12.6 g/l/h). The combination of high solvent titer coupled with high ABE production rates has not been achieved at this level before. Combined solvent and organic acid production was possible, when the pH in the first chemostat was raised to levels where the culture shifts its metabolic pathway back to acidogenesis. Applying this technique, a total product concentration of 27.7 g/l at a yield of 47.7% (w/w of glucose equivalent) could be achieved. A mathematical model consisting of a set of coupled differential equations was successfully used to describe each of the consecutive reactors.

The fermentation products could be recovered using adsorption onto activated carbon and liquid-liquid extraction. Long-chained ketones such as 2-octanone were identified as good alternatives to standard extractants such as oleyl alcohol due to their higher partition coefficients and selectivities. Depending on the pH of the aqueous broth, both techniques were capable of transferring equimolar amounts of organic acids and alcohols into a micro-aqueous solvent system where subsequent esterification is practical. Over the course of three cycles consisting of adsorption, drying, back-extraction with hexane and final desorption, the activated carbon lost about one third of its adsorption capacity. Average product titers in hexane after back-extraction were approximately 9.9 and 11.1 g/l for 1-butanol and

n-butyric acid, respectively. Enzymatic esterification with the enzyme Palatase®20000L from Novozymes proofed to be advantageous. Due to a very limited solubility of the reaction product water in the mediator solvent hexane, the esterification equilibrium is pushed far to the formation of products so that yields in excess of 82% are feasible. Additionally, the derived esters can be given the attribute of having been produced naturally. Enzyme stability for the duration of one batch reaction with a final titer of butyl butyrate of 45 g/l and a yield in excess of 82% after 240min could be demonstrated.

7.2 Recommendations for Future Work

Bioreactor and Feedstock Due to their extended operating live, continuously operated bioreactors are often more susceptible to contamination than their batch counterparts. However, during the course of 40 days, the tubular reactor described in chapter 4 did not show signs of bacterial contamination. An intentionally contaminated feed stream at with varying bacterial loads could give valuable insides to the mechanisms and to the extend of resistance to contamination.

Immobilization of biomass in the tubular reactor was largely supported by the presence of a cotton/polyester thread. Scaled up versions of this bioreactor will in all likelihood rely on a more advanced bacterial support structure and material. Imaginable alternatives include tubes with an inner bacterial support lining. This configuration would allow for a more uniform and standardized production of the bioreactor. Finding the optimal material and structure of the lining should be the subject of future studies.

Large scale fermentations commonly rely on less processed forms of carbohydrates than Maltodextrin or pure xylose. Prospective work should therefore include a variety of substrate solutions including feedstocks that are currently being used in commercial ethanol fermentation plants and those that are of lignocellulosic origin.

Product Recovery Liquid-liquid extraction with long-chained ketones has shown to hold a lot of potential. However, culture toxicity of the investigated ketone (2-octanone) prohibits on-line product removal. Future studies should therefore test similar compounds with varying chain length and position of the functional group with respect to partition coefficients, selectivities and culture toxicity. Additionally, the culture could be adapted to increasing levels of possible extractants.

Although it could be shown that enzymatic esterification with hexane as a mediator solvent is possible, other solvents might allow for a larger enzyme stability. Possible candidates should include longer chained alkanes and long-chained ketones.

Esterification When the natural origin of the desired esters is of little significance, solid acid catalysis should be investigated. Compared to traditional homogeneous catalysts such as mineral acids, solid acid catalysts are less toxic and corrosive and easily separted from the reaction media. Additionally, solid acid catalysts often tolerate higher temperatures than lipases which translates to higher reaction rates. The solid nature of these catalysts makes them ideal for use in packed bed reactors.

APPENDIX

8 Appendix

Raw data used in the preparation of figures.

Table 8.1 – Raw data of Figure 3.1: Exponential growth phase of the undegenerated strain of ATCC 824 at 36 ºC

time [h]	optical density at 595nm	cell dry weight X [g/l]	ln(X)
0	0	0	-
2	0.08	0.0304	-3.49
4	0.21	0.0798	-2.52
6	0.6	0.228	-1.47
9	1.66	0.6308	-0.46

Table 8.2 – Raw data of Figure 3.3: Conditions: degenerated strain of ATCC 824 grown at 36°C in pH-controlled fermentors with 1l working volume with varying forms of pH-control

0	6.31	6.31	6.37	6.46
4	5.9	5.9	5.88	6.1
6	5.07	5.9	5	5.9
8	4.79	5.9	4.74	5.9
10	4.46	5.9	4.38	5.9
12	4.26	5.9	4.16	5.82
14	4.16	5.9	4.02	5.03

Table 8.2 (cont'd)

	pH			
time [h]	no pH-control (a)	initial pH-control (b)	forced solven-togenesis (c)	short initial pH-control (d)
16	4.11	5.9	3.95	4.75
18	4.08	5.9	3.91	4.58
20	4.07	5.9	3.91	4.49
22	4.08	5.9	3.92	4.45
23	4.08	5.78	4.3	4.44
23.08	4.08	5.74	4.84	4.44
23.13	4.08	54.71	5.5	4.44
24	4.09	5.62	5.9	4.44
26	4.09	5.31	5.9	4.44
28	4.09	5.13	5.9	4.44
29.3	4.1	5.06	5.9	4.44
29.6	4.1	5.04	5.85	4.53
29.65	4.1	5.04	5.83	4.88
30	4.1	5.03	5.81	5.9

Table 8.2 (cont'd)

	pH			
time [h]	no pH-control (a)	initial pH-control (b)	forced solven-togenesis (c)	short initial pH-control (d)
32	4.1	4.97	5.4	5.9
34	4.11	4.93	5.16	5.9
36	4.09	4.93	5.05	5.9
38	4.12	4.92	5.07	5.9
40	4.12	4.92	5.13	5.9
42	4.12	4.92	5.23	5.9
44	4.12	4.92	5.37	5.9
46	4.12	4.92	5.59	5.9
48	4.12	4.92	5.82	5.83
50	4.11	4.92	5.98	5.69
52	4.11	4.94	5.99	5.53
54	4.11	4.96	5.96	5.41
56	4.1	4.95	5.91	5.37
58	4.1	4.94	5.85	5.4

Table 8.2 (cont'd)

	pH			
time [h]	no pH-control (a)	initial pH-control (b)	forced solven-togenesis (c)	short initial pH-control (d)
60	4.11	4.93	5.8	5.49
62	4.15	4.96	5.79	5.64
64	4.13	4.94	5.76	5.75
66	4.13	4.94	5.75	5.87
68	4.12	4.93	5.73	5.94
70	4.12	4.93	5.73	5.99
72	4.12	4.93	5.72	5.99
74	4.1	4.95	5.71	5.95

Table 8.3 – Raw data for Figure 3.4. Conditions: degenerated strain of ATCC 824 grown at 36^{o}C in pH-controlled fermentors with 1l working volume.

pH=6.3	0	0	0	0
	8	0.2	0.7	0.11
	21	2.9	6.3	0.64
	28	4.1	9.6	0.64

Table 8.3 (cont'd)

	time [h]	AA [g/l]	BA [g/l]	productivity [g/l/h]
	33	4.4	11.5	0.44
	45	5.6	15.3	0.42
	69	7.3	20.4	0.28
	92	8.3	23.4	0.17
	116	8.7	24.4	0.06
	134	9	25.2	0.06
pH=5.9	0	0	0	0
	8	0.3	0.4	0.09
	21	3.6	9.1	0.92
	28	4.3	12.7	0.61
	33	4.6	14.4	0.4
	45	5.3	17.3	0.3
	69	6.4	18.1	0.08
	92	7.8	21.3	0.2
	116	8.9	22.8	0.11
	134	9.4	23.5	0.07

Table 8.3 (cont'd)

	time [h]	AA [g/l]	BA [g/l]	productivity [g/l/h]
pH= 5.4	0	0	0	0
	8	0.2	0.4	0.08
	24	2.9	8	0.68
	27	3	9.6	0.56
	29.5	3.9	10.1	0.56
	36.5	4	7.9	-0.3
	63.5	4.1	7.7	0
	88	4.2	7.6	0
C. tyrobutyricum, wildtype, pH=6.0	0	0	0	0
	10	0.5	1	0.15
	18	1.5	2	0.25
	23	2	3.5	0.4
	30	3.2	6.5	0.6
	35	3.3	13	1.32
	43	3.5	18	0.65

Table 8.3 (cont'd)

	time [h]	AA [g/l]	BA [g/l]	productivity [g/l/h]
	50	3.6	20	0.3
	67	3.6	20.5	0.03

Table 8.4 – Raw data for Figures 4.3 and 4.4. Dilution times for PFR, CSTR1 and CSTR2 are, 0.86, 0.068 and 0.034 h^{-1}. Substrate: matlodextrin. Data obtained after steady state had been reached.

[g/l]	$pH_{CSTR1=5.3}$				$pH_{CSTR1=5.7}$			
	Substrate	org. acid	ABE	pH	Substrate	org.acid	ABE	pH
Influent	70	0	0	6.4	70	0	0	6.2
1m	36.2	1.2	11.9	3.8	42.1	2	9.1	3.9
2m	30	1.1	14.6	3.9	34.1	1.7	11.6	4
CSTR 1	13.6	1.4	19.2	5.3	15.8	9.1	13.3	5.7
CSTR 2	8	2.8	21	5.2	5.6	11.7	16	5.4

Table 8.5 – Raw data for Figure 4.5. Dilution times for PFR, CSTR1 and CSTR2 are, 0.86, 0.068 and 0.034 h^{-1}. Data obtained after steady state had been reached.

[g/l]	maltodextrin	xylose	org.acid	ABE	pH
Influent	30	30	0	0	6.2
1m	9.3	14.3	1.7	10.4	3.9
2m	5.8	13.9	1.2	12.2	3.8
CSTR 1	1.4	13.6	1.9	16	3.8
CSTR 2	0.7	11.7	2.8	16.8	3.8

Table 8.6 – Raw data of Figure 4.6: Measured specific growth rates of the degenerated culture of ATCC 824 at 36°C with varying broth-pH and type of substrate (xylose and Maltodextrin)

	specific growth rate $\mu[h^{-1}]$	
pH	maltodextrin	xylose
6	0.48	0.45
5.3	0.19	0.34
5	0.17	0.2
no pH control	0.15	0.08

Table 8.7 – Raw data of Figure 5.1. Experimental conditions: undegenerated strain of ATCC 824 grown at 36°C in fermentor with 1l working volume without pH control

time	S	AA	BA	BuOH	Ace	EtOH	solvents	X
[h]	[g/l]							
0	66.3	0	0	0	0	0	0	0
3	65.6	0.21	0.17	0	0	0	0	0.07
6	65	0.41	0.54	0.05	0	0	0.05	0.21
10	64.1	0.82	1.32	1.09	0.52	0.13	1.74	0.72
15	57	1.09	1.57	1.56	0.77	0.28	2.61	0.87
22	42.4	0.83	1.44	4.9	2.6	0.6	8.1	1.52
30	16.6	0.44	1.03	8.7	4.9	1.1	14.7	1.62
33	14.1	0.31	0.97	9.9	5.3	1.1	16.3	1.55
39	5.1	0.2	0.56	11	5.7	1.2	17.9	1.4
45	2.2	0.19	0.46	11.9	6.2	1.3	19.4	1.47
48	1.6	0.21	0.48	12.3	6.1	1.3	19.7	1.39

Table 8.8 – Raw data of Figure 5.1. Experimental conditions: undegenerated strain of ATCC 824 grown at 36^{o}C in fermentor with 1l working volume at constant pH=5.7

time	S	AA	BA	BuOH	X
[h]	[g/l]				
0	61.4	0	0	0	0
1.5	61.4	0.24	0.39	0	0.035224
7.1	59.8	0.4	0.61	0	0.39
9.5	52.9	1.2	2.16	0	0.84
15.3	33.4	3.4	7.3	0.15	1.23
23.7	19.1	5.1	12.4	0.3	1.1
31.5	10.9	6	15.5	0.4	1.05
37.2	5.6	6.6	17.1	0.6	1.01
48	0.1	7.3	18.9	0.8	0.91

Table 8.9 – Raw data of Figure 5.3. Experimental conditions: undegenerated strain of ATCC 824 grown at 36^{o}C in fermentor with 1l working volume at constant pH=5.7 and 10 g/l BuOH present

time	S	AA	BA	BuOH	X
[h]	[g/l]				
0	63	0	0	0	0
1.5	60.6	0	0	9.4	0.05
7.1	58.5	0.5	0.5	8.6	0.46
9.5	53	0.9	1.7	8.7	0.86
15.3	37.5	1.9	6	9.4	1.05
23.7	29.3	2.8	8.4	9.7	0.98
31.5	25.3	3.2	9.4	9.6	0.75
37.2	24	4.1	10.5	9.4	0.56
48	23.6	4	10.6	9.7	0.46

Table 8.10 – Raw data for Figure 5.4. Calculated organic acid effluent concentration (BA butyric acid, AA acetic acid) in second CSTR with varying BuOH inlet concentrations. Conditions: V=2 l, D=0.04 h^{-1},$S_0 = 20$ g/l, $BA_0 = 6.8$ g/l, $AA_0 = 2.2$ g/l, $X_0 = 2$ g/l, $Y_0 = 1$

BuOH	BA	AA
[g/l]		
0	13.4	7.2
2.5	11.1	4.5
5	9.8	3.7
7.5	9.1	3.3
10	8.6	2.7

Table 8.11 – Raw experimental data for Figure 5.6. Experimental conditions: degenerated strain of ATCC 824 grown at 36^{o}C. CSTR 1: 1l working volume, pH-controlled at pH=5.7; CSTR 2: 2l working volume, no pH-control. Initial conditions for the PFR-simulation are listed in Table 5.1. Effluent concentrations of upstream reactors were used as initial conditions for downstream reactors.

	substrate	organic acids	solvents	pH
	[g/l]			[-]
influent	70	0	0	6.3
1m	42.1	2.0	9.1	3.9
2m	34.1	1.7	11.6	4.0
CSTR1	15.8	9.1	13.3	5.7
CSTR2	5.6	11.7	16.0	5.4

Table 8.12 – Raw data for Figures 6.1, 6.2 and 6.3. Experiments involved 10ml artificial fermentation broth (composition see Table 6.2) at varying pH and at varying amounts of virgin activated carbon. Adsorption occured at room temperature and broth composition/pH was measured after 15h.

pH_{ini}=2.7						
0	2.7	4.79	14.85	6.25	1.34	13.61
0.25	3.26	4.64	11.17	6.08	1.3	11.24
0.5	3.59	4.51	8.36	6.12	1.49	8.57
1	4.06	4.11	3.58	4.51	1.26	4.33
1.5	4.58	3.47	1.39	3.22	1.12	1.59
2	5	3.15	1	2.15	0.94	0.77
3	5.66	3.21	0.82	0.84	0.66	0.24
pH_{ini}=3.58						
0	3.58	4.81	15.03	5.93	1.34	13.62
0.25	3.8	4.68	10.8	5.99	1.33	11.07
0.5	3.99	4.55	7.92	5.65	1.34	8.56
1	4.35	4.2	3.89	4.47	1.27	4.25
1.5	4.73	3.77	1.82	3.27	1.16	1.76
2	5.25	3.57	1.2	1.78	0.95	0.69

Table 8.12 (cont'd)

[g]	[-]	[g/l]				
m_{ac}	pH_{final}	AA	BA	Ace	EtOH	BuOH
3	5.91	3.68	1.18	0.73	0.64	0.21
pH_{ini}=4.45						
0	4.45	4.81	15.03	6.14	1.34	13.71
0.25	4.53	4.73	11.58	5.93	1.33	10.64
0.5	4.71	4.64	8.8	5.18	1.33	7.75
1	5.06	4.5	5.46	4.46	1.26	3.55
1.5	5.51	4.43	3.87	3.12	1.12	1.38
2	6.09	4.53	3.57	1.6	0.9	0.52
3	6.72	4.64	3.4	0.63	0.62	0.16
pH_{ini}=5.24						
0	5.24	5.14	14.11	6.17	1.9	12.46
0.25	5.3	5.1	11.87	5.93	1.92	7.77
0.5	5.6	5.09	10.23	5.32	1.89	5.2
1	6.18	5.14	9.14	3.8	1.61	1.74
1.5	6.77	5.18	9.07	2.35	1.36	0.7

Table 8.12 (cont'd)

[g]	[-]	[g/l]				
m_{ac}	pH_{final}	AA	BA	Ace	EtOH	BuOH
2	7.64	5.27	9.07	1.14	1.07	0.29
3	8.03	5.3	8.7	0.5	0.69	0.05
pH_{ini}=5.7						
0	5.7	5	14.13	6.01	1.87	12.54
0.25	5.95	5	13.01	5.65	1.85	7.92
0.5	6.35	5.02	12.72	4.92	1.77	4.26
1	7.19	5.1	12.62	3.11	1.46	1.21
1.5	7.92	5.13	12.49	1.63	1.24	0.48
2	8.7	5.19	12.41	1.06	0.96	0.21
3	9.44	5.3	10.51	0.32	0.63	0.03

Table 8.13 – Raw data for Figure 6.4. Back extraction with hexane at 5 ml/min, 60°C, third cycle

hexane	BuOH	BA
[ml]	[g/l]	
200	12.3	0.7
250	18	24
300	15.6	21.2
350	13.8	19.8
400	12.2	16.6
450	11.2	14
500	9.4	12.4
550	8	10.4
600	7.2	9.2
650	6.6	7.6
700	5.6	7.4
750	5.2	5.6
800	4.6	5
850	4	4.4
900	3.4	5

Table 8.14 – Raw data for Figure 6.5. Distribution coefficients (D) and selectivity (S) with respect to BuOH/H_2O for LLE of synthetic media (composition see Table 6.2) with 2-octanone at 22°C

pH	D-acetone	D-EtOH	D-BuOH	D-AA	D-BA	D-H_2O	S-BuOH/H_2O
2.7	0.87	1.16	3.11	0.57	5.29	0.0135	231
3.58	0.87	1.22	3.14	0.52	4.61	0.0134	235
4	0.88	1.19	3.18	0.48	3.53	0.0135	235
4.45	0.87	1.17	3.19	0.36	2.00	0.0135	235
5.24	0.85	0.88	3.44	0.25	0.52	0.0123	281
5.7	0.87	0.95	3.41	0.23	0.16	0.0122	279

Table 8.15 – Raw data for Figure 6.6. Esterification of equi-molar amounts of 1-butanol with butyric acid (18.5mmol each) adsorbed on 15g activated carbon in 50ml hexane at 50°C. BB-butyl butyrate concentration, X-conversion

	with 0.5ml enzyme		with 0.5ml H_2SO_4	
t	BB	X	BB	X
[min]	[g/l]	[-]	[g/l]	[-]
0	1.93	0.025	1.3	0.015
7	3.44	0.044	2.2	0.026
15	6.6	0.084	3.5	0.041
34	14.6	0.186	7.2	0.085
60	25.9	0.330	11.3	0.133
88	32.3	0.411	14.7	0.173
154	53.4	0.680	23.2	0.273
253	63.1	0.803	30.7	0.362
480	64.2	0.817	41.2	0.486

Table 8.16 – Raw data for Figure 6.8. Initial esterification rate v at varying butanol concentrations and varying amounts of butyric acid. Conditions: 50°C, 50ml hexane, 200rpm shake speed, 0.5ml enzyme. BB-butylbutyrate, BA-butyric acid

	0.021M BA	0.072M BA	0.147M BA
$1/C_{BuOH}$	$1/v_{BB}$	$1/v_{BB}$	$1/v_{BB}$
[l/mol]	$[min \cdot ml_{enzyme}/\mu mol]$	$[min \cdot ml_{enzyme}/\mu mol]$	$[min \cdot ml_{enzyme}/\mu mol]$
20	20959	11682	9060
8.3	20358	11207	8146
5.3	20253	7821	7258
3.1	18911	9031	6298

Table 8.17 – Raw data for Figure 6.9. Intercepts of Figure 6.8 plotted against the reciprocal of the butyric acid (BA) concentration

intersept from Figure 6.8	1/BA
	[1/mol]
1.93E+04	45.9
8.23E+03	13.1
6.36E+03	6.1

Table 8.18 – Raw data for Figure 6.10. Initial enzyme activity after delaying substrate addition (0.02mol of each butyric acid and butanol) to a mixture of 15 g activated carbon, 50ml hexane and 0.5ml enzyme at 50°C

delay	initial rate	remaining activity
[min]	[g/l/min]	[%]
0	0.124	100
10	0.132	106
20	0.057	46
30	0.027	22
60	0.015	12
120	0.006	5
180	0.009	8
1500	0.003	2

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