A Study of Butanol Production in a Batch Oscillatory Baffled Bioreactor

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by

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Abstract

As with many bioprocesses, the acetone-butanol-ethanol (ABE) fermentation faces a number of economic drawbacks when compared to the petrochemical route for butanol production. In the 1920s biobutanol was the second largest biotechnology industry, after bioethanol production. However it became difficult to compete against the petrochemical route for reasons including the low product butanol concentration, because of product inhibition resulted in low butanol productivity and due to slow fermentation and low ABE yields. These lead to uneconomical butanol recovery by the conventional method, distillation, due to the high degree of dilution. Recent interest in biobutanol as a biofuel has led to re-examination of ABE fermentation with the aim of improving solvent yield, volumetric productivity and final solvent concentration to reduce the cost of production and thereby produce biobutanol that is cost-competitive with the chemical synthesis butanol.

ABE fermentations were carried out in an intensified plug flow reactor known as the batch oscillatory baffled bioreactor (BOBB). The "BOBB"s were designed and built for this project. The effect of oscillatory flow mixing on ABE fermentation was compared to that of conventional stirred tank reactors (STRs) at power densities in the range 0 to 1.14 Wm^{-3}. The maximum butanol concentration in this range in a BOBB was 34% higher than the STR. Some increase in butanol productivity was also observed: 0.13 $gL^{-1}h^{-1}$ in BOBBs, compared to 0.11 $gL^{-1}h^{-1}$ in the STRs. It can be concluded that at similar power densities, BOBB fermentation shifts to solventogenesis earlier than in STRs, resulting in higher solvent productivity. It is hypothesised that the reason for early solventogenesis in the BOBB was the higher solvent-producing cell concentration, due to the more uniform shear field in the BOBB, so the cell would be less exposed to high shear thereby reducing the risk of cell lysis.

Abstract

Two-stage ABE fermentations in BOBB increased the butanol productivity by up to 37.5% over the one-stage fermentation. Butanol productivity was further increased by 97% when gas stripping was integrated to the two-stage ABE fermentation. While the one-stage fermentation integrated with gas stripping increased the butanol productivity by 69% to 0.12 $gL^{-1}h^{-1}$ (as opposed to 0.071 $gL^{-1}h^{-1}$ in a similar fermentation without gas stripping). A simple model to describe the one-stage at oscillatory Reynolds number (Re_o) 0 and 938, and the two-stage ABE fermentation in BOBB II was developed. The model summarizes the physiological aspects of growth and metabolite synthesis by *Clostridium* GBL1082. The prediction of the models were in good agreement with experimental results incorporating mixing (Re_o 938) and moderately agreed with results from Re_o 0 and the two-stage fermentation.

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Nomenclature and Abbreviations

Symbols		**Unit**
ω	angular frequency of the oscillation	rad s^{-1}
δ	baffle thickness	m
Z	column length	m
ρ	density	kg m^3
ν	liquid kinematic viscosity	m^2 s
f	oscillation frequency	Hz
μ	specific growth rate	h^{-1}
ε_v	power density	W m^{-3}
C_o	orifice coefficient	-
C_p	heat capacity of water	kJ kg^{-1} $^oC^{-1}$
D	column internal diameter	m
D_o	orifice diameter of the baffle plate	m
dT/dt	rate of temperature change	^oC h^{-1}
H	Overall reactor height	m
L	baffle spacing	m
m	mass of water	kg
N	impeller speed	rpm, rps
n	number of baffle plates	-
Q_{growth}	rate of heat generated by cell growth	kJ h^{-1}
Re_n	net Reynolds number	-
Re_o	oscillatory Reynolds number	-
S	fractional open cross-sectional area	-
St	Strouhal number	-
T	temperature	^oC
t	time	h
u	superficial net flow velocity	m s^{-1}
V_L	liquid volume	L
X	cell mass concentration	g L^{-1}
x_o	centre-to-peak amplitude	m

$1/Y_H$	metabolic heat evolved per gram of cell mass produced	kJ g^{-1}

Abbreviations

ABE	acetone, butanol and ethanol
ATP	adenosine triphosphate
BOBB	batch oscillatory baffled bioreactor
CDW	cell dry weight
DNA	deoxyribonucleic acid acid
DNS	dinitrosalicylic acid
GC	gas chromatography
HTR	heat transfer rate
NAD (P)	nicotinamide adenine dinucleotide phosphate
NAD (P)H	reduced form of NAD (P)
NADH	nicotinamide adenine dinucleotide
OBB	oscillatory baffled bioreactor
OBR	oscillatory baffled reactor
OD	optical density
OFμR	oscillatory flow micro reactor
OFR	oscillatory flow reactor
PHA	polyhydroxy alkanoate
pO_2	oxygen partial pressure
PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
RCA	reinforced clostridial agar
RCM	reinforced clostridial medium
rpm	revolutions per minute
rps	revolutions per second
RTD	retention time distribution
STR	stirred tank reactor

Chapter 1 INTRODUCTION

1.1 Research Background

More than a century ago, the production of acetone and butanol via fermentation using the "Weizmann Process" was commercially viable. There was a high demand for acetone, which was used in the manufacture of the explosive cordite during World War 1. At that time, butanol (as a by-product) was stored, as there was no ready use for it (Dürre, 2008). It was in the 1920s that butanol became an important chemical when the dramatic growth in the automobile industry created an urgent need for a solvent in the production of quick-drying lacquers for car manufacturing. Butanol proved to be an excellent solvent, and fermentation using the Weizmann Process became the method of choice for its production (Dürre, 2008). Butanol has also been used in the production of the rubber monomers, butadiene and dimethyl butadiene (Mollah and Stuckey, 1993). Until 1950, almost two-thirds of worldwide butanol demand were met from the fermentation process (Dürre, 2008). The largest plants were located in the United States: for example, Peoria, Illinois had 96 fermenter units with a total capacity of 21,821 m^3. This plant together with the Terre Haute plant (which had 52 fermenter units) produced over 100 tons of solvent per day (Jones and Woods, 1986).

In the 1950s, crude oil became much cheaper. Together with the increasing prices of biobutanol feedstock (mainly molasses) combined with lower sugar contents, this shifted butanol production routes away from biological processes to more efficient chemical processes. By 1960, acetone, butanol and ethanol (ABE) fermentation had virtually ceased in the UK, USA and Japan (Ni and Sun, 2009), followed by Africa and Germany in the 1980s, and finally in China and Russia in the 1990s (Jones and Woods, 1986; Lee *et al.*, 2008b). Since then, butanol has almost exclusively been produced from petrochemicals. The revival of ABE production by fermentation depends on economic conditions, principally the cost relative to the

petrochemical-based processes. The oil price crises in the 1970s and 1999, have revived interest in the ABE fermentation. As shown in Figure 1.1 below, research activities in academia and industry significantly increased in the early 1980s and again in 2000s as a response to the oil crises with effort distributed fairly evenly between various technical aspects fermentation, downstream processing, and research on physiology and genetics of solventogenic clostridia, from 1980 to 1990. In the last decade, scientific publications in clostridial research increased again, generally due to interests in biofuels (Lütke-Eversloh and Bahl, 2011).

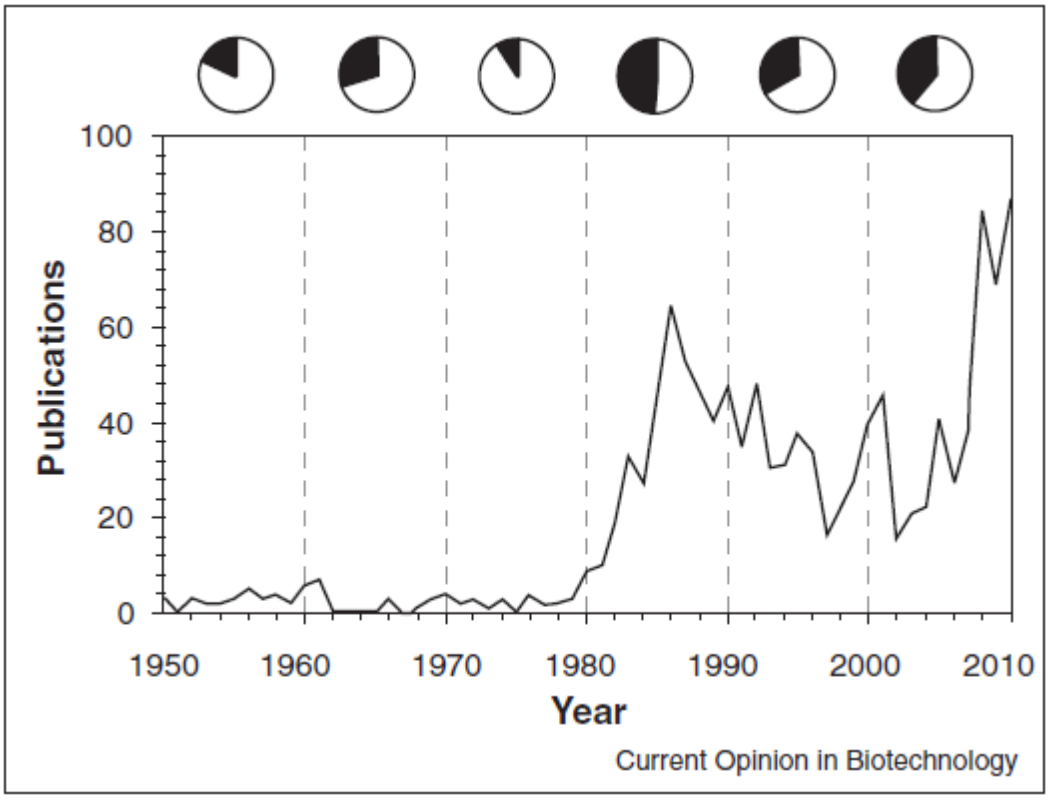


Figure 1.1 Scientific publications on solventogenic clostridia since 1950. The pie charts on top show the ratio between publications on physiology and genetics (white) and those covering topics of fermentation and downstream processing (black) for each decade. (Lütke-Eversloh and Bahl, 2011)

Researchers are working to improve the ABE fermentation process with the aim of reducing the cost of production, so that biobutanol is cost-competitive with chemically synthesised butanol. Recent developments in molecular techniques to

modify solvent-producing clostridia (*i.e. C. acetobutylicum* and *C. beijerinckii*), together with advanced fermentation techniques are leading toward development of integrated ABE fermentations with simultaneous product removal.

1.2 Problem Statement

The global market for biofuels is growing at a rate of 12.9% per annum (Market Publishers, 2010). Biobutanol has significant potential as a "next generation" biofuel due to its superior technical properties compared to bioethanol and biodiesel. Biobutanol has also been a choice as food extractant as chemically derived butanol has a potential of carcinogenic carry over. The major obstacle to bringing this to market is the cost. As with all bioprocess systems, ABE fermentation faces a number of economic drawbacks when compared with its chemical route. Dependency on high cost substrate, low butanol productivity and yield, low butanol concentration due to product inhibition that leads to uneconomical cost of butanol recovery, due to diluted products were among the problems (Qureshi and Blaschek, 2001a). By focusing on driving down the cost of production, it is hoped to the deliver biobutanol that is cost-competitive with chemically synthesis of butanol to market. Recent interest in biobutanol has led to re-examination of ABE fermentation, with the aim of improving solvent yield, volumetric productivity and final solvent concentration hopefully leading to reduced production cost. This can be achieved by combining a successful genetically engineered clostridia strain with a cost-effective feedstock using recent advances in the fermentation techniques (including cell immobilizations, fed batch fermentation and continuous two-stage fermentation) in an efficient bioreactor integrated with low-cost product recovery, which should increase the solvent yield and overall productivity of the ABE fermentation.

Efforts are focused on genetic and metabolic engineering to engineer "second generation" butanol-producing strains. Some of them are:

i. Mutant strain, *C beijerinckii* BA101 produce higher concentrations of butanol over its parent strain *C. beijerinckii* NCIMB 8052 (Qureshi and Blaschek, 2001a)

ii. Gene inactivation in *C. acetobutylicum* ATCC 824 (Green *et al.*, 1996) to disrupt metabolic pathways leading to acetate and butyrate production

iii. Mutant strains of *C. acetobutylicum* ATCC 824 (Matta-El-Ammouri *et al.*, 1986) *C. beijerinckii* NCIMB 8052 cloned with *Neocallimastix patriciacarum's* gene (Lopez-Contreras *et al.*, 2001)

iv. Alternative microbial hosts with artificial metabolic pathway of clostridia (Shen and Liao, 2008; Nielsen *et al.*, 2009)

Others have focussed their interest on media optimization using non-food feedstocks, such as wheat straw, corn stover, barley straw, switchgrass (Qureshi and Ezeji, 2008), and palm oil mill effluent (Somrutai *et al.*, 1996; Ngan *et al.*, 2004; Pang *et al.*, 2004; Hipolito *et al.*, 2008; Takriff *et al.*, 2009). ABE fermentation protocols have also been studied including: varying fermentation technique (batch, fed batch, continuous, continuous with cell recycling and bleeding, continuous with immobilized cell and co-culture fermentation) (Lienhardt *et al.*, 2002; Lee *et al.*, 2008a), novel downstream processing (Ezeji *et al.*, 2007) and integration of the processes *i.e.* pretreatment-fermentation-product recovery (Lienhardt *et al.*, 2002; Qureshi and Maddox, 2005; Qureshi *et al.*, 2007; Fischer *et al.*, 2008). These fermentation strategies had been carried out and resulted in differing degrees of success.

1.3 Research Objectives

The objectives of this research are:

i. Development of a batch oscillatory baffled bioreactor (OBB)

ii. Investigation of the ability of the oscillatory baffle reactor (OBR) as a bioreactor to perform ABE fermentation

iii. Comparison of the effect of different types of mixing (*i.e.* oscillating and stirring) by comparing stirred tank reactors (STRs) and OBBs

iv. Investigation of the effect of variation of oscillatory Reynolds number (Re_o) on batch ABE fermentation.

v. Investigation of the effect of mixing protocols in ABE fermentation on cell growth and solvent production with a view to maximising productivity.

vi. Evaluation of OBR ABE fermentation's integration with simultaneous product recovery (gas stripping) and the effect on the solvent yield and productivity

Chapter 2 LITERATURE SURVEY

2.1 Butanol

Butanol (IUPAC nomenclature 1-butanol) is a colourless, flammable liquid with a banana-like odour. It is an important bulk chemical in various industrial applications. Butanol is a major feedstock for the industrial manufacturing of various chemicals including butyl acrylate, butyl acetate, glycol ethers, and plasticizers (Mata *et al.*, 2010). These chemicals are widely used in water-based coatings, cosmetics, car care products, lacquers, pharmaceuticals, textiles, etc. Butanol is also used as a direct solvent in paints, dyes, varnishes, coatings and for other industrial purposes (Mata *et al.*, 2010). The latest application of butanol is as a transport fuel and it is expected to play a major role in the next generation of biofuels (Dürre, 2008). "Biobutanol", which is butanol derived from fermentation, has been claimed to be "superior biofuel", as it can be blended into standard gasoline similarly to ethanol but with several advantages. These include higher energy content and lower vapour pressure, which make storage and transportation easier. It is also immiscible with water, has a better blending ability with gasoline and diesel fuel, and can be used in conventional internal combustion engines without modification (Dürre, 2008).

Butanol can be produced from petrochemical or biotechnology routes. Chemically, butanol is derived from crude oil via three major routes: oxo synthesis, Reppee synthesis and crotonaldehyde hydrogenation, as shown in Figure 2.1. Oxo synthesis is the main process. It involves two main steps: hydroformylation, followed by hydrogenation. First, CO and H_2 are added to the carbon-carbon double bond of propylene using cobalt, rhodium or ruthenium as catalysts. This produces an aldehyde mixture, which undergoes hydrogenation to produce butanol (Figure 2.1a).

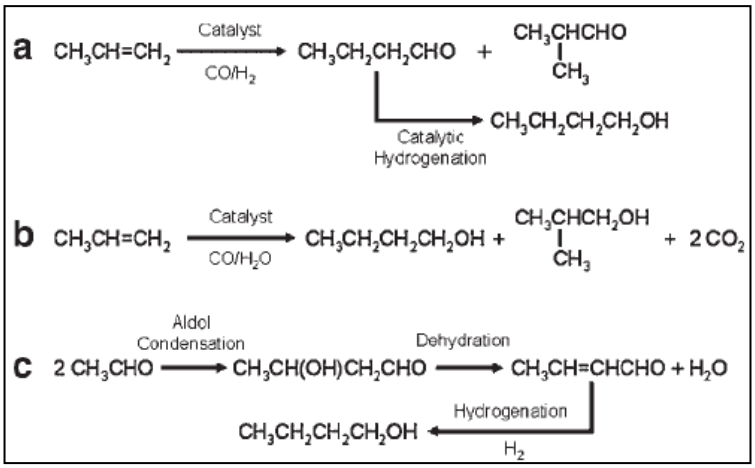


Figure 2.1 Chemical synthesis of butanol: (a) Oxo synthesis, (b) Reppe synthesis, (c) Crotonaldehyde hydrogenation (Lee *et al.*, 2008b)

Different isomeric ratios of butanol can be obtained, depending on reaction conditions of pressure and temperature, and the catalyst used. If hydrogen replaced by steam (as in Figure 2.1b), a mixture of primary butanols can be obtained directly. This is known as Reppe synthesis, which produces butanol directly from alkenes and operated at 100^{o}C and 1.5 x 10^{6} Pa absolute pressures, in the presence of pentacarbonyl iron, butylpyrrolidone and water. This process produces an 88% n-butanol and 12% isobutanol (Chauvel and Lefebvre, 1989). Crotonaldehyde hydrogenation (Figure 2.1c) used to be a common route for petrochemical-derived butanol a few decades ago. The process starts from acetaldehyde and consists of three-step reactions: aldol condensation, dehydration and hydrogenation. This process may again become important in the future as it provides an alternative route from ethanol which can be produced biologically from biomass (Machado, 2010). In this case, acetaldehyde is formed from the dehydrogenation of ethanol and the synthesis proceeds from there (Lee *et al.*, 2008b).

Biologically, butanol is produced from a fermentation known as the acetone, butanol and ethanol (ABE) fermentation. This ABE fermentation can use a wide range of biomass, as well as various sugars (glucose, sucrose and lactose) as substrates. Other than butanol, products include organic acid (lactate, acetate and

butyrate), solvents (acetone and ethanol) and gases (CO_2 and H_2). The wide range of products indicates that this fermentation has a complex metabolic pathway. Today's world demand for butanol has been met mainly via the oxo reaction from propylene. It is estimated that over 4.5 million tonnes of butanol are produced annually, which accounts for a market of 70 million GBP (1.1 billion USD). The market growth rate for butanol is estimated at 3.25% per year (Market Publishers, 2010).

2.2 ABE Fermentation

2.2.1 Microorganisms

The first microorganism used for ABE fermentation was a bacterium, *Clostridium acetobutylicum,* which was first isolated by Weizmann in the 1910s (Jones and Woods, 1986). Other than *C. acetobutylicum*, another three key species have been identified as butanol producers: *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum.* It should be noted that the three species mentioned above were originally designated as *C. acetobutylicum* until the beginning of the 1990s (Dürre, 2008). All species follow anaerobic fermentation with minor differences, such as the type of substrate for optimum solvent production (Dürre, 2008).

Clostridia are rod-shaped, measuring 0.5 – 2 µm in width and up to 30 µm in length (Figure 2.2a). They are Gram-positive bacteria and typically strict anaerobes. Clostridia form robust endospores which are resistant to oxygen, heat, and alcohol. Spores either occur in central, terminal (Figure 2.2b) or subterminal positions, depending on the species. Most clostridia species are motile and have flagella projecting in all directions used for propulsion (Andreesen *et al.*, 1989).

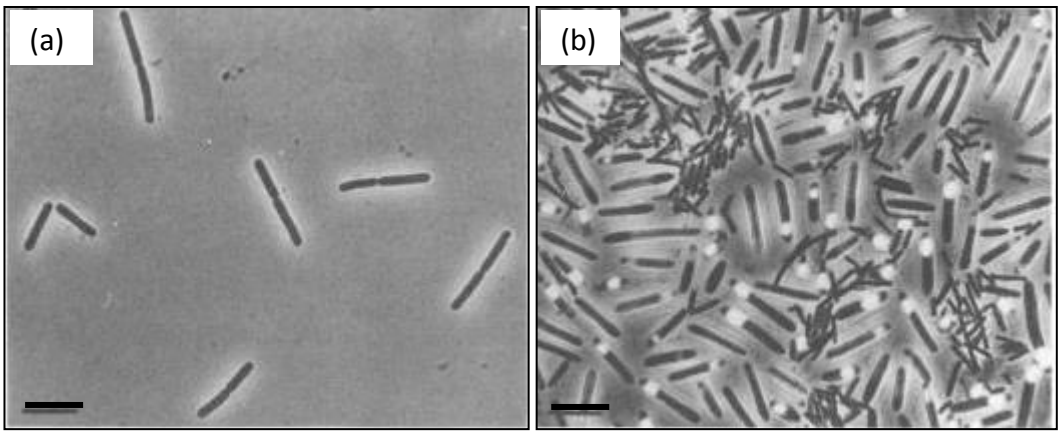


Figure 2.2 Photomicrographs of *C. acetobutylicum* during ABE fermentation. (a) Actively growing phase-dark vegetative rods. (b) Sporulating rods with terminal phase-bright spores. Bar, 10 ,um. (Jones *et al.*, 1982)

Clostridia exhibit varying degrees of intolerance towards oxygen, probably for two reasons:

i. Clostridia lack a defence mechanism against the toxic by-products of oxygen metabolism (superoxide radicals, hydrogen peroxide and hydroxyl radicals), and oxygen interferes with the functioning of vital enzyme systems. The by-products of oxygen metabolism are extremely toxic because they are powerful oxidizing agents and rapidly destroy cellular constituents, such as DNA, phospholipids and other biomolecules. A microorganism must possess enzymes to protect them against toxic oxygen products such as catalase, peroxidases or superoxide dismutase. These have been reported to be absent or at very low levels in most clostridia (Rolfe *et al.*, 1978; Kawasaki *et al.*, 1998).

ii. Molecular oxygen also interferes with NADH-oxidase activities, which causes the intermediary and biosynthetic metabolism of these organisms to suffer from NADH depletion, and thus the collapse of the entire metabolism (Kawasaki *et al.*, 1998).

The choice of strain for use in butanol fermentation, especially in industry, is based on the nature of the raw material used, the ratio of end products required, the need for additional nutrients, and phage resistance (Jones and Woods, 1986). *C. acetobutylicum* (which also includes *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*) has been widely used for the production of solvents (acetone, butanol and ethanol, ABE) in industry since 1940 and has consequently been most extensively studied. The most common ratio for solvent production is ABE in the ratio of 6:3:1 (Jones and Woods, 1986). *C. beijerinckii* has the capability to produce almost the same ratio as *C. acetobutylicum,* but isopropanol is produced instead of acetone, while *C. aurantibutyricum* produces acetone, butanol and isopropanol (George *et al.*, 1983). *C. tetanomorphum* produces approximately equimolar amounts of butanol and ethanol (Jones and Woods, 1986).

2.2.2 Metabolic Pathway of Clostridia

Clostridia undergo two distinct phases of fermentation: “acidogenesis”, followed by “solventogenesis”. The first phase, acidogenesis, is where acid-producing pathways are activated where acetate, butyrate, hydrogen and carbon dioxide are produced as major products. The second phase is solventogenesis in which acids are re-assimilated to produce acetone, butanol and ethanol. It is well known that this biphasic metabolism is closely related to cell growth status (Jones *et al.*, 1982). Acidogenesis occurs during the exponential growth phase and solventogenesis during the late exponential phase. If solventogenesis could be initiated at the early exponential phase, solvent productivity could possibly be increased since the fermentation time would be shortened (Wang *et al.*, 2012). The cause of the metabolic shift has been proposed to be a detoxification mechanism against acidic environments in the broth, as this results in conditions unfavourable for cell growth (Jones and Woods, 1986; Gheshlaghi *et al.*, 2009).

The metabolic pathways for acidogenesis and solventogenesis of *C. acetobutylicum* are shown Figure 2.3. It begins with glucose undergoing glycolysis to produced pyruvate via the Embden-Meyerhof-Parnas pathway. The resulting pyruvate is cleaved to yield acetyl-CoA by the pyruvate:ferredoxin oxidoreductase. Under certain condition, such as sulphate limitation at constantly more neutral pH, lactate can be the major fermentation product (Awang *et al.*, 1988). Acetyl-CoA is further converted to either oxidized products (acetone, acetate or CO_2) or reduced products (butanol, ethanol or butyrate). Butanol is synthesized from a series of intermediates: acetyl-CoA, acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA, butyryl-CoA and butyraldehyde.

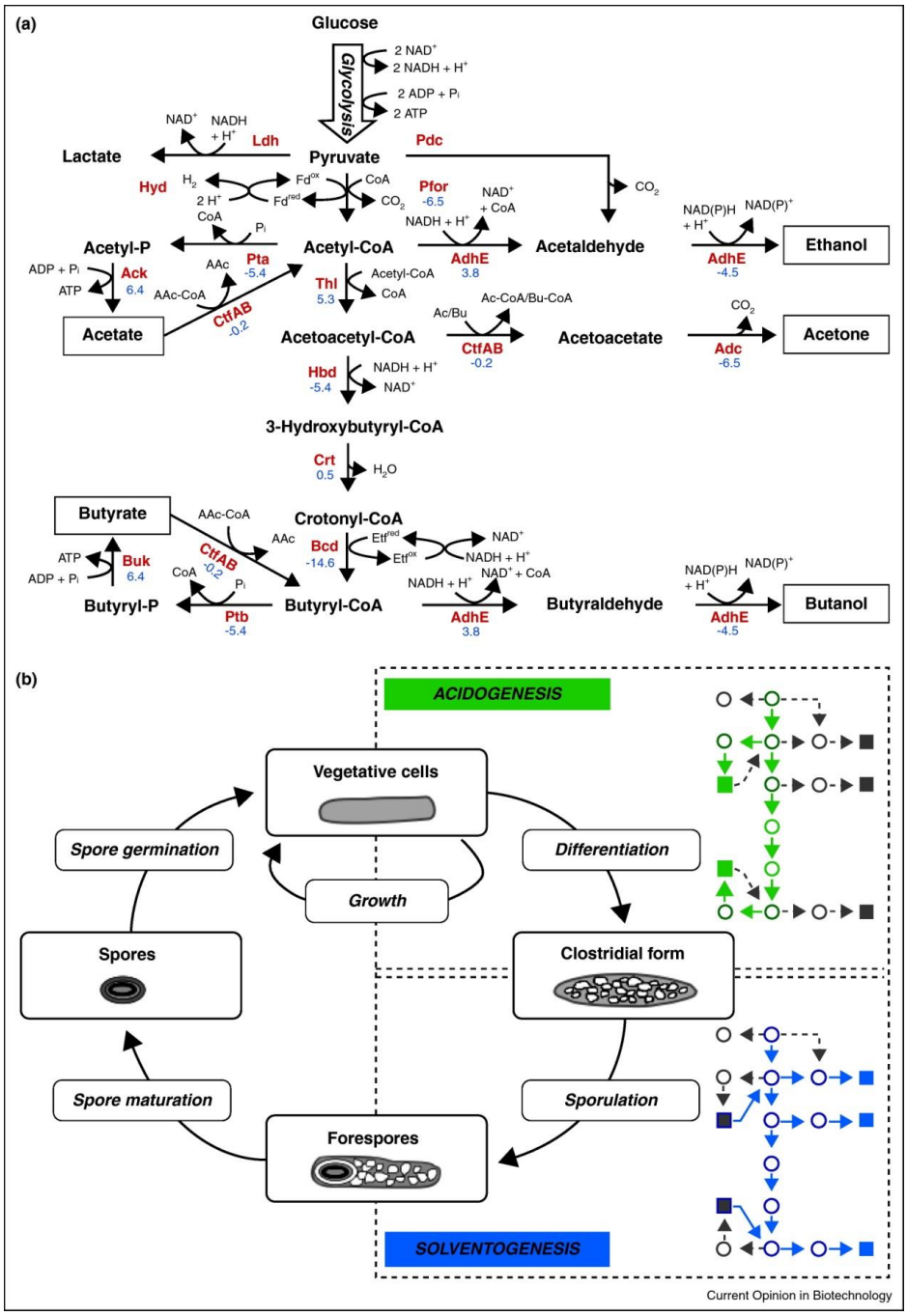


Figure 2.3 Acidogenesis and solventogenesis in *C. acetobutylicum*. (a) Metabolic pathways; (b) their relation to the clostridia life cycle stages (Lütke-Eversloh and Bahl, 2011)

(a) The red letters show the enzymes involved: Ldh: lactate dehydrogenase; Pdc: pyruvate decarboxylase; Hyd: hydrogenase; Pfor: pyruvate:ferredoxin oxidoreductase; Fd: ferredoxin; Pta: phosphotransacetylase; Ack: acetate kinase; AdhE: aldehyde/alcohol dehyrogenase; CtfAB: acetoacetyl-CoA:acyl-CoA transferase; Adc: acetoacetate decarboxylase; Thl: thiolase; Hbd: 3-hydroxybutyryl-CoA dehydrogenase; Crt: crotonase, Bcd: butyryl-CoA dehydrogenase; Etf: electron transfer flavoprotein; Ptb: phosphotransbutyrylase, Buk: butyrate kinase. AAc: acetoacetate; AAc-CoA: acetoacetyl-CoA; Ac/Bu: acetate/butyrate; Ac-CoA/Bu-CoA: acetyl-CoA/butyryl-CoA; ox: oxidized; red: reduced. The blue numbers represent the standard Gibbs energy changes according to Zheng *et. al* (2009).

C. acetobutylicum can utilize a variety of carbohydrates, including pentoses, hexoses, oligosaccharides and polysaccharides (Lütke-Eversloh and Bahl, 2011). Sugar fermentation by clostridia typically causes three different growth phases: first, an exponential growth phase during which the vegetative cell is growing and dividing rapidly producing more cells; second, transition to a stationary growth phase with the re-assimilation of acids and the concomitant formation of solvents; and third, the formation of endospores. After entering the stationary growth phase, cells start to synthesis granulose as intracellular storage compound, and at these stage cells were known as "clostridial form cells" which can be distinguished from vegetative cells, microscopically (Lütke-Eversloh and Bahl, 2011).

Figure 2.3b shows acidogenesis and solventogenesis, referring to the respective growth stages. The major (green or blue) and minor (grey) metabolic fluxes are indicated in the miniature pathways, which represent simplified schemes of the metabolic pathways in Figure 2.3a. Based on the metabolic pathways in the figure, ATP is predominantly formed during acidogenesis and high NAD(P)H levels were proposed to induce solventogenesis (Lütke-Eversloh and Bahl, 2011). Theoretically, it is possible to produce homo-butanol (producing only butanol and some CO_2 and H_2) based on the stoichiometric reaction below:

$$1\ \text{Glucose} \longrightarrow 1\ \text{Butanol} + 2\ CO_2 \quad (\text{yield 2 moles ATP})$$

Equation 2.1

….where two moles of ATP can be provided by glycolysis and the reducing equivalents can be regenerated in the butanol pathway, provided that reduced ferredoxin donates its electrons to $NAD(P)^+$ instead for molecular hydrogen formation. In practice, the used of artificial electron carriers such as methyl viologen and neutral red, gassing with carbon monoxide, or increasing the hydrogen partial pressure have been showed to lead the electron flow to this pathways resulted in high butanol concentration at the expense of acetone concentration (Lütke-

Eversloh and Bahl, 2011). All of these techniques have been demonstrated to reduce the hydrogenase activities thus reducing the molecular hydrogen formation. This has resulted in an electron flow towards butanol formation for the regeneration of the $NAD(P)^+$ pool (Lütke-Eversloh and Bahl, 2011).

2.2.3 Factors Triggering Solventogenesis

During the last two decades, considerable effort has been devoted to understanding the factors that trigger the onset of solventogenesis and respective physiological changes in solventogenic clostridia. It has long been observed that this transition is associated with the induction of solventogenic enzymes and a decrease in the activity of acidogenic enzymes (Jones and Woods, 1986). External pH is a key factor in determining solvent production. This has been confirmed by studies which have reported that cultures maintained in high pH produce mainly acid, while culture maintained at low pH will produce mainly solvents (Häggström, 1985; Jones and Woods, 1986; Awang *et al.*, 1988). It was reported that the switch to solvent production involved the threshold undissociated butyric acid concentration, citing between 6 to 13 mM, when external pH was below 5.0 (Qureshi and Ezeji, 2008). Although it has been demonstrated that a decrease in the pH of broth is important to permit the onset of solvent production, it seems that an attainment of low pH itself is not the trigger (Gottschal and Morris, 1981; Jones and Woods, 1986). Rather, it was concluded that low pH is a requirement for solvent production, combined with relatively high concentration of acetate and butyrate having an additional and more specific “triggering” effect on solvent production (Bahl *et al.*, 1982a; Matta-El-Ammouri *et al.*, 1987).

Another factor shown to regulate the switch from acidogenesis to solventogenesis is the regulator protein SpoOA protein, which is responsible for sporulation, as well as the induction of the genes involved in butanol production (Ravagnani *et al.*, 2000). Their study indicated that SpoOA controls those genes encoding alcohol/aldehyde dehydrogenases that play a major role in butanol

synthesis. Other means that have been reported to trigger solventogenesis include maintaining the internal pH of the bacteria at a higher level than that of the external culture, the use of excess carbon in the fermentation broth, use of a growth-limiting concentration of phosphate, sulphate or iron, maintaining the fermentation temperature between 30^{o}C to 33^{o}C, or a brief exposure to oxygen (Jones and Woods, 1986; Rogers *et al.*, 2006).

2.3 Limitations to Conventional Butanol Fermentation

The problem with conventional batch ABE fermentation is that solvent concentrations rarely exceed 20 gL^{-1}. In fact, a butanol concentration of 13 gL^{-1} will cause cell metabolism to cease due to product inhibition (Jones and Woods, 1986). As a result, concentrated sugar solutions higher than 60 gL^{-1} cannot be used and cell concentrations higher than 3-4 gL^{-1} are rarely achieved (Mariano and Filho, 2012). In addition, reactor productivity in a batch process is low, at between 0.2 – 0.5 gL^{-1} h^{-1}, due to slow fermentation with ABE yield of approximately 0.30 g ABE(g glucose consumed)$^{-1}$, depending on the strain and substrate used (Mariano and Filho, 2012). The maximum theoretical ABE yield has been determined to be 0.399 (gg^{-1}) by Yerushalmi *et al*. (2001) for *C. acetobutylicum* growth on glucose in a batch process. In combination these factors result in the economic disadvantages of ABE fermentation that contributed to the decline in the ABE fermentation industry since the 1960s.

Researchers have identified various factors that affect the economic decline of butanol fermentation (Qureshi and Blaschek, 2001a) which are:

i. Dependency on high cost substrate
ii. Low butanol concentration (<20 gL^{-1}) due to product inhibition which prohibited the use of concentrated substrate
iii. Low butanol productivity due to slow fermentation
iv. Low ABE yields of approximately 30% weight weight^{-1} ($wt.wt^{-1}$)

v. Uneconomical cost of butanol recovery by distillation, due to diluted products
vi. By-product formation
vii. Degeneration of the culture

Of these, the factors with the greatest impact on the economic viability of ABE fermentation were the high costs of the substrate, low productivity and product yield, and high product recovery costs (Dürre, 2008; Green, 2011; Jin *et al.*, 2011). It is important to note that these factors are interconnected, and that by improving or eliminating one factor there can be an increase in productivity. However, greater increases are likely to be achieved if more than one factor can be addressed and applied together. Improvements with respect to these limitations have been grouped into three categories, which are:

i. Strain development
ii. Upstream processing
iii. Downstream processing

2.3.1 Strain development

A problem associated with the conventional ABE fermentation process is the inhibitory effect of the final end products, in particular butanol, on the bacteria (Jones and Woods, 1986; Awang *et al.*, 1988). Although butyric acid is toxic, its concentration always remains low (Mollah and Stuckey, 1993). The feasibility of butanol fermentation depends on strain performance (Lee *et al.*, 2008b). Some researchers have focused on genetic and metabolic engineering in order to engineer second generation strains with the ability to degrade wider ranges of substrates, to produce high butanol yield, where currently the maximum reported yield is between 0.40 and 0.42 ww^{-1} when using *C. beijerinckii* BA101, and there is a high resistance to butanol toxicity. At present, a few hyper-butanol producing strains, which produce higher concentrations of butanol over its parental strain,

have been developed: *C. acetobutylicum* strain 77, *C. acetobutylicum* PJC4BK and *C. beijerinckii* BA101. These strains are able to produce ABE at approximately 24-33 g in a litre of broth under optimized conditions (Matta-El-Ammouri *et al.*, 1986; Green *et al.*, 1996; Parekh *et al.*, 1999; Qureshi and Blaschek, 2001a). Attempts have also been made to eliminate the acetone, ethanol and isopropanol pathways in clostridia, to create an ultimate butanol producer strain (Qureshi and Ezeji, 2008).

Researchers are also investigating the use of alternative microbial hosts as biocatalysts to produce butanol. Lopez *et al.* (2001) have been cloning the *C. beijerinckii* NCIMB 8052 with genes from the fungus *Neocallimastix patriciarum* to increase the clostridia substrate utilization range, thereby enabling the more efficient degradation of celluloses and hemicelluloses. Others have had the artificial metabolic pathway of *C. acetobutylicum* synthesized in different microbial hosts which have higher tolerance towards butanol toxicity, such as *Lactobacillius brevis* (Berezina *et al.*, 2010), *Pseudomonas putida* and *Bacillus subtilis* (Shen and Liao, 2008; Nielsen *et al.*, 2009). These studies yielded various degrees of success, with butanol being synthesized at between 23 – 300 mg L^{-1}.

Some researchers (Atsumi *et al.*, 2008; Inui *et al.*, 2008; Shen and Liao, 2008; Nielsen *et al.*, 2009) have constructed and introduced the recombinant butanol pathways of *C. acetobutylicum* into *Escherichia coli* with butanol titres up to 500 mgL^{-1}. It is likely to be a challenging task to further develop an *E. coli* that can produce butanol above 15 gL^{-1}. A vast amount of information exists on the genetic and physiological characteristic of *E. coli* along with an existing variety of the genetic tools required to carry out host modification. These might be advantageous since *E. coli* can be engineered to be more suitable for butanol production by increasing its tolerance to butanol and minimizing by product formation. In addition, *E. coli* possesses desirable characteristics in terms of becoming an industrial butanol production strain, such as the ability to grow in minimal medium either in aerobic or anaerobic conditions, as well being capable of utilizing a wide range of substrates.

High productivities and yield might be achieved due to its high growth and metabolic rates, and tolerance to high concentrations of substrate and products (Clomburg and Gonzalez, 2010). It is anticipated that there is more research to come on the development of these microorganisms, with the aim of creating a superior butanol strain.

2.3.2 Upstream process

Upstream process studies have focused on finding cheap substrates and improving the fermentation process.

2.3.2.1 Substrate

As the cost of the substrate contributes the most to overall production costs (accounting for up to 79% of the cost) (Green, 2011), the use of cheap substrates such as agriculture waste would be beneficial to the viability of the process. Clostridia have the ability to utilize a wide range of substrates, including hexose and pentose sugar, starch-based crops, lignocelluloses and hydrolysate (Dürre, 2008). Researchers have studied the use of non-food lignocelluloses as substrates for second generation butanol. Qureshi and Blaschek (2000) used corn steep liquor and soy molasses to produce solvents from *C. beijerinckii* BA101. Palm oil mill waste has also been used as a cheap viable substrate following research by Pang *et al.* (2004), Ngan *et al.* (2004), Hipolito *et al.*, (2008) and Takriff *et al.* (2009). Others have used wheat straw and barley straw hydrolysate (Qureshi *et al.*, 2008a; 2010). The problems in using agricultural residues as substrates are the presence of salts and growth inhibitors. Substrate needs to be pre-treated prior to use in fermentation and this will increase the process cost (Qureshi and Ezeji, 2008). Currently, at industrial scale, corn is the major substrate used in China's ABE plant (Ni and Sun, 2009) while sugar cane juice is used in Brazil's plant (Green, 2011).

2.3.2.2 Advanced fermentation process

Batch processes are always preferred in the biotechnology industry due to their simple operation and reduced risks of contamination. ABE fermentation employing *C. beijerinckii* BA101 has been performed in batch reactors ranging in size from 1 to 10 L using cracked corn, cornstarch, maltodextrin and glucose produces between 18 to 33 gL^{-1} ABE with productivity ranging from 0.25 to 0.46 $gL^{-1}h^{-1}$. Low productivity in batch processes is often due to downtime, a long lag phase and product inhibition (Ezeji *et al.*, 2004b). The downtime and long lag phase could be eliminated using a continuous process, and problems with product inhibition could be solved with an *in situ* product removal system. Furthermore, fed-batch techniques can be used to avoid substrate inhibition and to increase cell mass (Lee *et al.*, 2008b).

Fed-batch fermentation is an industrial technique applied to processes with inhibition by high concentration of substrates such as ABE fermentation. For example, Ezeji *et al.* (2004a) concluded that the fed-batch technique resulted in higher ABE productivity than conventional batch process of 1.16 $gL^{-1}h^{-1}$. Continuous fermentation processes have also been investigated, for example by Formanek *et al.*(1997). Here, the use of *C. beijerinckii* BA101 resulted in a solvent productivity rate of 1.74 $gL^{-1}h^{-1}$. However, in a single-stage continuous system, high reactor productivity could be achieved at the expense of lower product concentrations than in the batch system. Because of the instability of solvent levels and the complexity of butanol fermentation, the use of a single-stage continuous system seems impractical on an industrial scale, and instead two- or three-stage continuous systems have been investigated. Stable solvent levels and increases in solvent concentration have been achieved in China's ABE plants with a multi-stage continuous system (Ni and Sun, 2009). This "battery style" fermentation system generally consists of six to eight fermentation tanks of 200-500 m^3 volume capacity per fermenter, where fresh substrate is continuously fed into the first and second tanks together with periodic additions of the seed culture. The fermentation could be steadily operated continuously for around 170 to 480 h (Ni and Sun, 2009).

Immobilized cell reactors and cell recycle reactors have also been applied in continuous fermentation to increase cell concentration and thus increase butanol productivity. Immobilizing *C. beijerinckii* cells on clay brick particles by adsorption resulted in high solvent productivity of 15.8 $gL^{-1}h^{-1}$ (Qureshi *et al.*, 2000). Tashiro *et al.* (2005) investigated continuous fermentation with cell recycling and bleeding, which produced a high cell concentration of 33 gL^{-1} and reactor productivity of 7.55 $gL^{-1}h^{-1}$. The possibility of using co-culture fermentation with other microorganisms such as *Bacillus subtilis* has also been explored. Aerobic organisms with amylolytic activity such as *B. subtilis* have been seen not only to assist clostridia in substrate hydrolysis (in this case of cassava starch), but also consume any available oxygen in the medium and help maintaining anaerobic conditions for clostridial growth (Tran *et al.*, 2010). This might reduce the need to supply reducing agents or continuous N_2 sparging to the medium. The performance of advanced fermentation processes are summarized in Table 2.1.

Table 2.1 Performance of advanced fermentation process with integrated product removal (adapted from Lee *et al.* 2008)

Process	Strain used	Total solvent productivity ($gL^{-1}h^{-1}$)	Yield (gg^{-1})	Solvent conc. (gL^{-1})	Comment	References
Continuous reactor	*C. beijerinckii BA101*	1.74	0.78	15.6	Highest yield	(Formanek *et al.*, 1997)
Continuous immobilized reactor	*C. beijerinckii BA101*	15.8	0.38	7.9	Highest productivity	(Qureshi *et al.*, 2000)
Continuous reactor with cell recycling and bleeding	*C. saccharo perbutylacetonicum N1-4*	7.55	NA	8.6	33 gL^{-1} of cell mass	(Tashiro *et al.*, 2005)
Batch	*C. beijerinckii BA101*	0.34	0.42	24.2	59.8 gL^{-1} of glucose	(Qureshi and Blaschek, 1999)
Batch with pervaporation	*C. beijerinckii BA101*	0.69	0.42	51.5	121.2 gL^{-1} of glucose	(Qureshi and Blaschek, 1999)
Batch with gas stripping	*C. beijerinckii BA101*	0.61	0.47	75.9	161.7 gL^{-1} of glucose	(Ezeji *et al.*, 2003)

Process	Strain used	Total solvent productivity ($gL^{-1}h^{-1}$)	Yield (gg^{-1})	Solvent conc. (gL^{-1})	Comment	References
Batch with perstraction	*C. acetobutylicum P262*	0.21	0.44	136.6	313.3 g of whey lactose	(Qureshi and Maddox, 2005)
Batch with adsorption	*C. acetobutylicum ATCC 824*	0.92	0.32	29.8	92 gL^{-1} of glucose	(Yang *et al.*, 1994)
Batch with liquid-liquid extraction	*C. saccharo perbutylacetonicum N1-4*	0.52	0.38	27.9	Extractant: oleyl alcohol	(Ishizaki *et al.*, 1999)
Batch with liquid-liquid extraction	*C. saccharo perbutylacetonicum N1-4*	0.55	0.40	29.8	Extractant: methylated crude palm oil	(Ishizaki *et al.*, 1999)
Fed-batch with pervaporation	*C. acetobutylicum ATCC 824*	0.18	0.35	155.0	445 gL^{-1} of glucose	(Qureshi *et al.*, 2001)
Fed-batch with gas stripping	*C. beijerinckii BA101*	1.16	0.47	233.0	500 g of glucose	(Ezeji *et al.*, 2004a)
Co-culture fermentation	*B. subtilis WD161* *C. butylicum TISTR 1032*	0.14	NA	9.7	Co-culture of *B. subtilis* and *C. butylicum*	(Tran *et al.*, 2010)

2.3.3 Downstream process development

Downstream process studies have concentrated on finding an economic solvent recovery technique, since distillation, which is the current commercial method, is uneconomical due to low butanol concentrations (Jones and Woods, 1986). *In situ* recovery techniques have been demonstrated to maintain the culture within its butanol tolerance, thereby enabling the use of concentrated substrate and at the same time providing economic benefits for the process (Groot *et al.*, 1992). Overall, with both biological and engineering approaches, the energy efficiency, economics, and environmental performance of the process are expected to be enhanced. Several techniques have been studied, including adsorption, liquid-liquid extraction, pervaporation, reverse osmosis and gas stripping. Table 2.1 also summarizes the advanced ABE fermentation process integrated with various product-recovery systems.

2.3.3.1 Gas stripping

The main problem associated with the conventional ABE fermentation is butanol inhibition. Gas stripping is a simple technique that can be applied for recovering butanol from the fermentation broth and has been demonstrated to increase substrate utilization rate. Gas stripping has several advantages over liquid-liquid extraction and pervaporation , for example:

i. It does not require a separate mixer
ii. the stripping gases are clean and do not interact with the microbes
iii. the product gases of the fermentation can also be used as a stripping gas

Gas stripping can be performed *in situ* (Figure 2.4a) or *ex situ* (Figure 2.4b). *Ex situ* gas stripping uses an external stripping unit which has the advantage of performing the recovery process at higher temperature than the fermentation temperature. This will increase the volatility of butanol, as well as acetone and ethanol, thus improving the gas stripping efficiency.

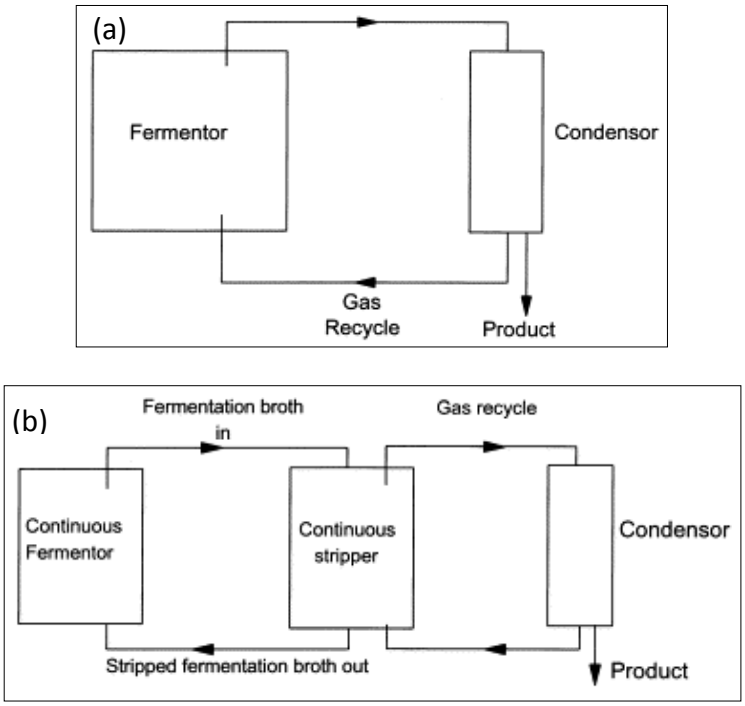


Figure 2.4 Possible reactor configuration for product removal. (a) *in situ* gas stripping. (b) *ex situ* gas stripping (Qureshi and Blaschek, 2001b)

Gas stripping is a simple yet efficient method to recover butanol from the broth (Figure 2.5a). It is easy to set up using simple and cheap apparatus, does not harm the bacteria, selectively removes volatiles (ABE) and reduces butanol toxicity (Qureshi and Blaschek, 2001b). Either nitrogen or the fermentation gases (H_2 and CO_2) are bubbled through the fermentation broth, and subsequently the gas (or gasses) is passed through a condenser. As the gas bubbles through the fermenter, it captures the volatile products (ABE) then condenses in the condenser and is collected in a receiver. Gas stripping reduces product inhibition, allowing for extended fermentation utilizing concentrated sugar at a higher rate and maximising reactor productivity (Qureshi and Ezeji, 2008).

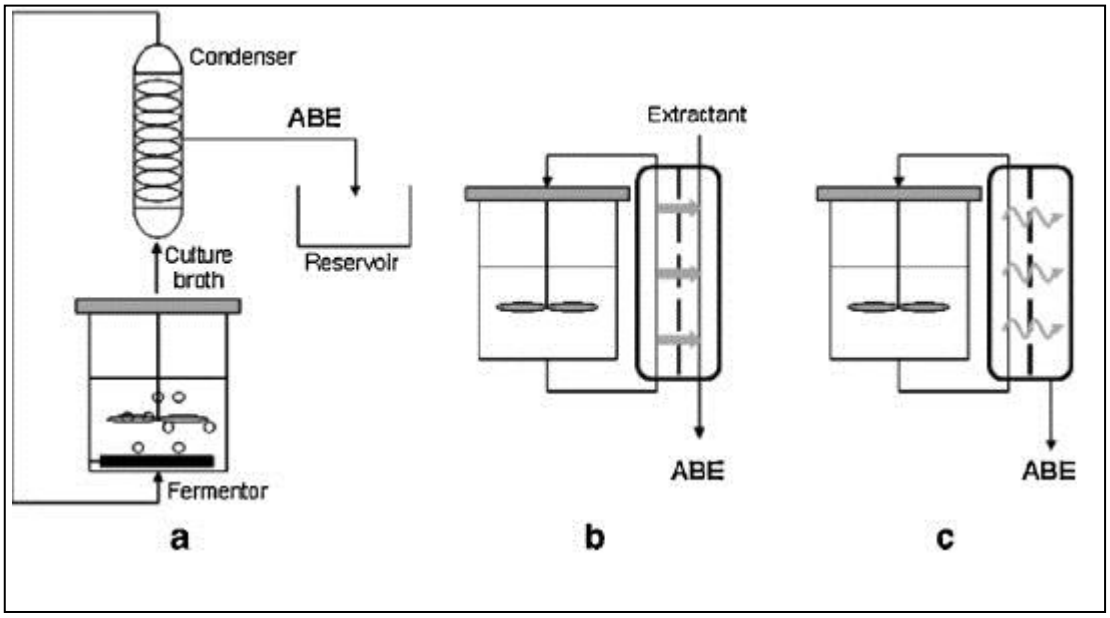


Figure 2.5 Integrated system for fermentation and in situ solvent recovery: fermentation integrated with (a) gas stripping; (b) liquid-liquid extraction; (c) pervaporation (Lee *et al.*, 2008b)

The application of gas stripping in butanol fermentation using *C. acetobutylicum* was first investigated by Ennis *et al.* (1986). In their work, gas stripping increased solvent productivity by 41% with a final solvent concentration of 15.7 gL^{-1} ABE. They also found that gas stripping shifted the fermentation to further acidogenesis due to the low pH (5.8) and low residual lactose, which reduced the

final yield compared with the control. Ezeji *et al.* (2003) applied *in situ* gas stripping to butanol fermentation using *C. beijerinckii* BA101 and reported an improvement of up to 200% in productivity and 118% in yield compared with the non-integrated process. This fermentation produced a total of 75.9 gL^{-1} ABE using 161.7 gL^{-1} glucose with nutrient addition. Most recently, Xue *et al.* (2012) implemented gas stripping with butanol fermentation using *C. acetobutylicum* JB200. Solvent productivity was increased by 32.5%, with a yield similar to that of fermentation without gas stripping. This fermentation produced 172 gL^{-1} ABE by using 474.9 gL^{-1} glucose in six feeding cycles.

2.3.3.2 Liquid-liquid extraction

Liquid-liquid extraction is the mixing of fermentation broth with extractant solvent, taking advantage of the greater affinity of butanol (acetone and ethanol) towards the extractant compared to water. Common extractants used include decanol and oleyl alcohol (Evans and Wang, 1988). Liquid-liquid extraction, however, entails problems with the toxicity of the extractant to the cell, as well as emulsion formation. This can be overcome by separating the fermentation broth from the extractant using a membrane. The membrane will provide surface area for butanol exchange between the two immiscible liquids (extractant and broth); this set-up is called perstraction (Figure 2.5b). Another disadvantage of this technique is the additional cost on the extractant solvent used and the difficulty of separating the butanol from the solvent again.

Ezeji *et al.* (2004b) proved that oleyl alcohol is an effective extractant by showing that *C. beijerinckii* BA101 is not inhibited by the extractant, although the cell was only exposed for a short duration of 24-48 h. Ishizaki *et al.* (1999) used methylated crude palm oil as an extractant, which increased the butanol concentration by 8.9% compared to oleyl alcohol. The use of methylated crude palm oil can be seen to provide an alternative cheap extractant to reduce butanol inhibition in liquid-liquid extraction, especially in palm oil-producing countries.

2.3.3.3 Pervaporation

Pervaporation is a membrane technique with high selectivity, and is used to remove the solvent from the fermentation broth (Figure 2.5c). The solvent diffuses through the membrane as a vapour and is later recovered by condensation. The rest of the broth, including cells, are left in the solution and returned to the reactor. The concentration of solvent across the membrane depends upon the composition and selectivity of the membrane (Ezeji *et al.*, 2004b). Qureshi and Blaschek (1999) performed ABE fermentation with pervaporation using *C. beijerinckii* BA101 and produced 51.5 gL^{-1} ABE from 121.2 gL^{-1} glucose in a batch reactor. Pervaporation was also applied to the fed-batch reactor, resulting in 155 gL^{-1} ABE from 342 gL^{-1} glucose.

The disadvantage of using pervaporation is the need for the membrane itself, which is susceptible to clogging or fouling. The membrane needs to be stable in order to operate for long periods of time whilst having a high degree of selectivity for butanol, acetone and ethanol (Jin *et al.*, 2011). Another issue is the high cost of such membranes, particularly in large-scale operations.

2.3.3.4 Other recovery techniques

There are other recovery techniques available for solvent recovery, such as reverse osmosis and adsorption. These techniques are not prominent in the field of product recovery to reduce inhibition in ABE fermentation. The most common problems with these techniques are the occurrence of clogging from the bacteria and low selectivity for butanol.

It can be concluded that there is still no clearly preferable recovery technique for solvent recovery. So far all of the techniques mention above have advantages and disadvantages, and have only been tested at laboratory scale. The

viability of these techniques at an industrial scale is still unknown. There is also certainly a need for further downstream processing to achieve high levels purity of acetone, butanol and ethanol.

Process integration combining substrate pretreatment, an advanced fermentation process and product recovery would improve the ABE fermentation process. If an alternative product recovery system were to be implemented, it would need to have lower operating costs than the conventional distillation technique. It will also need to match the performance of the conventional process in terms of final purity specifications. Other important factors could relate to the need for little modification to the existing infrastructure, if any (Qureshi and Ezeji, 2008).

2.4 The Oscillatory Baffled Reactor

The oscillatory baffled reactor (OBR) is a unique design of plug flow reactor consisting of a column fitted with a series of baffle plates mounted transverse to the flow and equally spaced. The key feature of OBRs is the interaction of orifice-type baffles and a periodically reversing flow. Typical vortices can be seen in Figure 2.6 at “moderate” oscillation. The fluid accelerates and decelerates as a result of the oscillation, continually forming short-lived vortices due to the interaction with the baffles. The resultant radial and axial velocities are of the same magnitude; which result in relatively uniform mixing in each inter-baffle region, along the length of the column (Jian and Ni, 2003; Ni *et al.*, 2003a). Figure 2.7 shows the effect of different oscillation levels on the fluid flow in the OBR. The vortices can be clearly seen in Figure 2.7a, where the OBR is subjected to moderate oscillatory condition, whereas a turbulent flow can be observed in Figure 2.7b as a result of high oscillation.

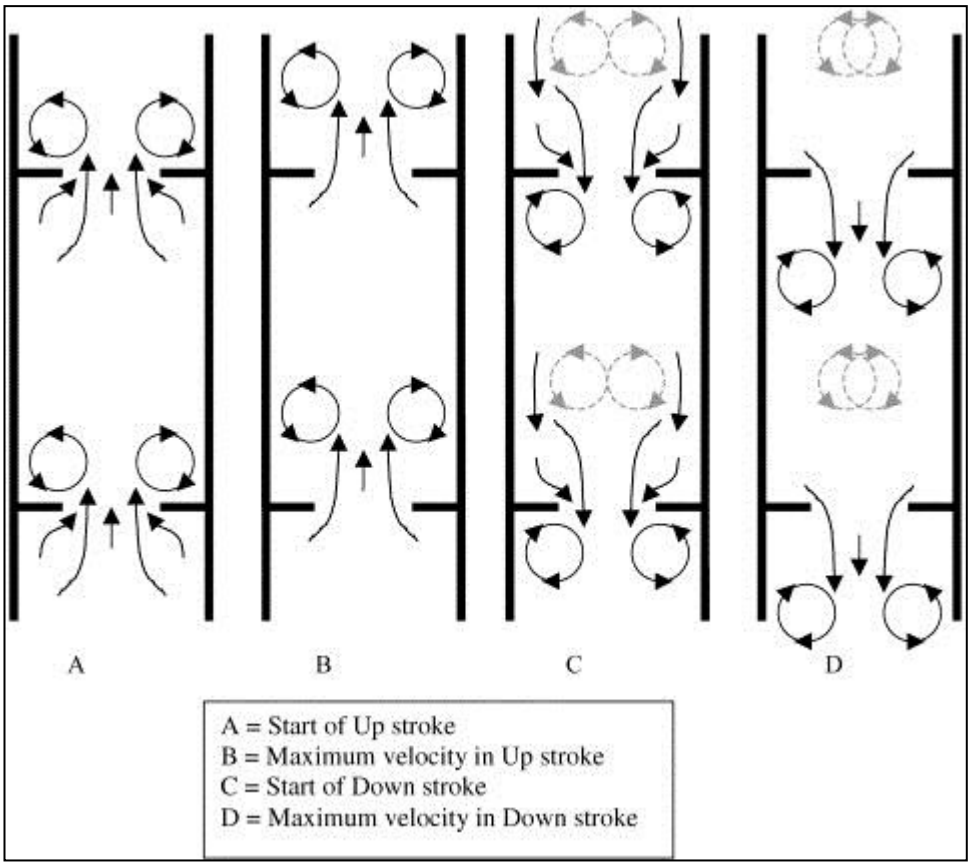


Figure 2.6 The mixing mechanism in an oscillatory baffled reactor (OBR) (Fitch and Ni, 2003)

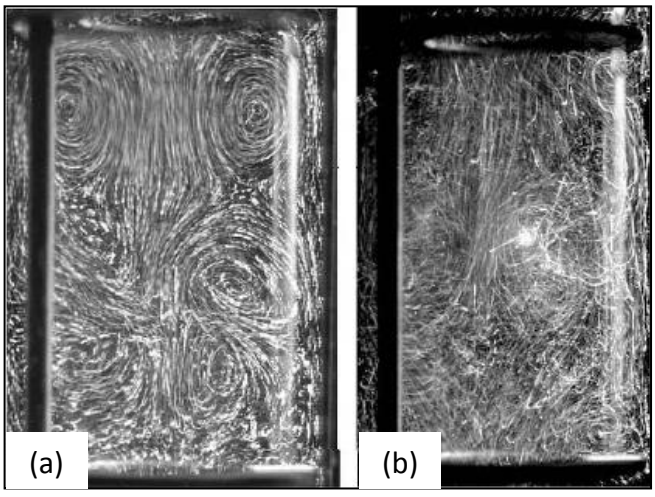


Figure 2.7 Flow pattern obtain in an OBR (a) Low intensity flow at moderate oscillation; (b) Highly energetic flow at high oscillation (Ni *et al.*, 2003b)

The OBR can be operated either horizontally or vertically and in batch or continuous mode. Fluid inside the reactor is oscillated by means of diaphragms, bellows or pistons at one or both ends of the column (Jian and Ni, 2005). A cross sectional diagram of the OBR is shown in Figure 2.8 below.

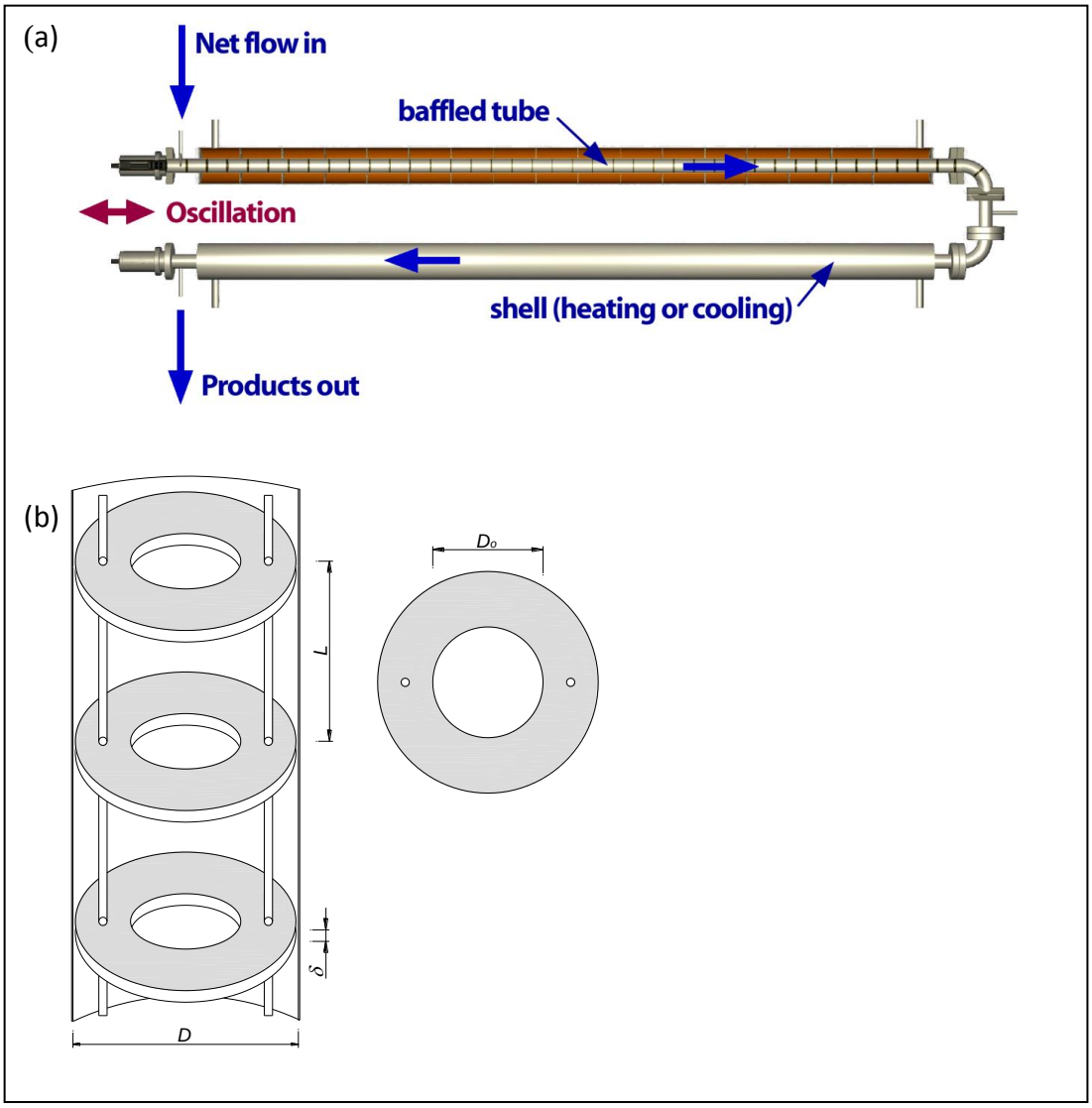


Figure 2.8 An oscillatory baffled reactor (OBR) showing (a) cross section of the reactor; (b) the OBR dimension, where *D* :reactor internal diameter, *L* :baffles spacing, D_o :orifice diameter, δ :baffle thickness .

The baffles are typically spaced at 1-2 times column diameter (*D*) separation, with a spacing of *L* = 1.5*D* being the most common (Brunold *et al.*, 1989). These baffles are

usually orifice plates (sometimes known as 'doughnut' baffles) where the fractional open cross-sectional area, $S = (D_o^2 / D^2)$ is usually in the range 20-35% (Ni *et al.*, 2003b). This is a compromise between minimizing frictional losses and maximizing the mixing effect.

An advantage of this system is that mixing effects are largely decoupled from the mean flow (Stephens and Mackley, 2002), since it is achieved by selection of the flow geometry and oscillation frequency, f, and amplitude, x_o, (Ni *et al.*, 2003a; Ni *et al.*, 2003b), rather than depending on the net flow velocity, as in conventional plug flow reactors. One particular application of OBR is for performing "long" (usually over 10 minutes) reactions in plug flow. A novel design method of continuous flow reactor based on mixing, has been presented by Stonestreet and Harvey (2002). They proposed a general design for continuous oscillatory flow reactors (OFR, which is the same as OBR) which was applied to two industrial case studies to illustrate feasible designs for full scale OFR.

The dynamic nature of oscillatory flow in a baffled tube can be characterized by three dimensionless numbers. Firstly, the oscillatory flow can be described by the oscillatory Reynolds number (Re_o):

$$Re_o = \frac{2\pi f x_o}{v}$$

Equation 2.2

…where x_o is the oscillation amplitude, measured as centre-to-peak (m), and f is the oscillatory frequency (Hz), D is the column internal diameter (m), v is the kinematic viscosity of the liquid (m^2 s). The oscillatory Reynolds number is based on $2\pi f x_o$, which is the maximum oscillatory velocity (ms^{-1}) (Ni *et al.*, 2003b). The second dimensionless number is the Strouhal number (St), defined as:

$$St = \frac{D}{4\pi x_o}$$

Equation 2.3

The Strouhal number relates the amplitude of oscillation to the baffle spacing (which is a multiple of *D*), which indicates the degree of eddy propagation (Ni *et al.*, 2003b). Thirdly, the net flow Reynolds number, (Re_n), defined as:

$$Re_n = \frac{uD}{v}$$

Equation 2.4

…. where *u* is the superficial net flow velocity through the tube (ms^{-1}).

2.4.1 Energy dissipation in OBR

Baird and Stonestreet (1995) estimated the power density of OBRs. They proposed two models: the quasi-steady flow model and the eddy acoustic model. The quasi-steady state model is suitable for predicting power dissipation rates at higher amplitudes and lower frequencies (5 to 30 mm, 0.5 to 2 Hz). The power density for the quasi-steady flow can be expressed as:

$$\varepsilon_v = \frac{2n\rho(\omega x_o)^3(1/S^2-1)}{3\pi C_o^2 Z} \qquad (Wm^{-3})$$

Equation 2.5

…where ε_v is the power density (Wm^{-3}), *n* is the number of baffles, ρ is the liquid density (kg m^{-3}), ω (= $2\pi f$) is the angular frequency of the oscillation (rad s^{-1}), x_o is the centre-to-peak amplitude of oscillation (m), *S* is the fractional open area of baffle, *z* is the column length and C_o is the orifice coefficient usually taken as 0.6. However, through their experimental conditions, this model tends to under-predict the pressure drop and power density in oscillatory flow. Baird and Stonestreet

(1995) proposed an alternative approach to the quasi-steady eddy model on the basis of local eddy turbulence coupled with acoustic behaviour. They advocate the eddy enhancement model, which accurately predicts the power dissipation in oscillatory flow at lower amplitudes (1 to 5 mm) and higher frequencies (5 to 14 Hz). The power density for the eddy enhancement model can be expressed as (Baird and Stonestreet, 1995):

$$\varepsilon_v = 1.5\,\frac{\rho\omega^3 x_o^2 l}{LS} \qquad (\mathrm{Wm^{-3}})$$

Equation 2.6

…where L is the spacing between baffles (m). l is the mixing length (m) which is an adjustable parameter and expected to be of the same order as the orifice diameter, D_o (m).

Previous studies on power dissipation for both unaerated and aerated pulsed columns (equal to oscillatory baffled reactor) (Baird and Garstang, 1967) demonstrate that power density under aeration in a pulsed column equally than that in the absence of gassing. In their experiments, only few data under aeration appeared slightly higher than the unaerated value, but the difference, as they concluded, was not significant. Based on this finding, the effect of aeration was ignored for the power density calculations for the oscillatory baffled reactor.

2.5 OBR as a Bioreactor

The mixing inside the OBR can be precisely controlled by a combination of geometrical and operational parameters, such as orifice diameter, baffle spacing, oscillation frequency and amplitude, providing a wide range of mixing conditions (Ni *et al.*, 2000), from gentle mixing, demonstrating plug flow behaviour, to chaotic mixing (Ni *et al.*, 2002; Jian and Ni, 2005), in which there is greater axial mixing, and consequently a broader residence time distribution (RTD). Flexibility in mixing range

could be an advantage for the use of OBRs as bioreactors. The uniform mixing in OBRs at lower shear stress than stirred tank reactors could be an advantage for biotechnology applications.

An early application of OBR to bioprocesses was reported by Harrison and Mackley (1992), who showed that poly-β-hydroxybutyrate, which is a type of biopolymer, could be produced in a pulsatile reactor from *Alcaligenes eutrophus H16.* They demonstrated the viability of the reactor for the cultivation of rapidly growing, oxygen-demanding microorganisms. Later, Ni *et al.* (1995b) used a oscillatory baffled bioreactor for yeast fermentation and reported the mass transfer of oxygen into yeast re-suspension was 75% higher than in equivalent stirred tank reactors at the same power density, up to 10,000 Wm^{-3}. Table 2.2 below summarises examples of oscillating reactors/bioreactors together with their applications in the biotechnology area.

Table 2.2 Application of OBR in biotechnology area

Designation	Application	References
Pulsatile flow Bioreactor	Production of Poly-*β*-hydroxybutyrate	Harrison and Mackley (1992)
Pulsed baffled tubular photochemical reactor	Degradation of an organic pollutant	Gao *et al.* (2003)
Oscillatory baffled flocculator	Flocculation of bentonite and *Alcaligenes eutrophus*	Ni *et al.* (2001)
Oscillatory flow reactor (continuous mode)	Process intensification of biodiesel production	Harvey *et al.* (2003)
Oscillatory flow bioreactor (batch mode)	Production of solvent (acetone, butanol and ethanol)	Masngut *et al.* (2007)
Oscillatory flow bioreactor (batch mode)	Solvent (acetone, butanol and ethanol) fermentation	Takriff *et al.* (2009)
Oscillatory baffled fermenter	Production of biopolymer (pullulan)	Gaidhani *et al.* (2005)
Oscillatory baffled bioreactor	Production of Polyhydroxyalkanoates	Troeger and Harvey (2009)
Oscillatory flow meso-reactor	Biodiesel reaction screening	Zheng *et al.*(2007)
Oscillatory flow micro-bioreactor	Production of *γ*-decalactone	Reis *et al.* (2006a)
Oscillatory flow micro-bioreactor	Fermentation of *S. cerevisiae*	Reis *et al.* (2006b)

2.5.1 OBR application in biopolymers production

Polyhydroxyalkanoates (PHAs) are biopolymers that are used within the medical and pharmaceutical industry primarily due to biocompatibility and biodegradability (Akaraonye *et al.*, 2010). The production of PHAs from microorganisms is currently not cost effective, because of expensive substrates, low yields and complex downstream processing. Therefore, intensification of the process to achieve higher yields in smaller, more efficient equipment at lower capital costs would be an advantage. OBRs potentially have this advantage to intensify the processes. Troeger and Harvey (2009) performed a batch fermentation of *Pseudomonas putida* KT2442 in an OBR to produce PHAs. They have showed an increase of 56% in cell mass over a comparable STR, at a productivity of 0.035 $gL^{-1}h^{-1}$. This productivity, however was lower than the one reported by Follonier *et al.* (2011), which was 0.170 $gL^{-1}h^{-1}$

achieved in STR under optimized conditions. This significant difference was due to different strain and condition used in both fermentations.

Pullulan is another versatile biopolymer that has been studied. It has a wide range of applications in the coating and packaging industries, as a sizing agent for paper, as a starch replacer in low-calorie food formulations, and in cosmetic emulsions (Gaidhani *et al.*, 2005). *Aureobasidium pullulan*, which produces pullulan, was grown in an OBR and its behaviour in low and uniform shear environments was reported by Gaidhani *et al.* (2005). They compared its growth rate and pattern to those in traditional STRs. A significant increase in the production was achieved when using an OBR rather than an STR, producing 16 gL^{-1} of pullulan with productivity of 0.41 $gL^{-1}h^{-1}$. This represented a 400% improvement in the productivity, compared with the STR. They demonstrated that pullulan synthesis can be initiated earlier (during the microorganism growth cycle) in the OBR, which significantly improved the productivity. Wecker and Onken (1991) have suggested that low shear rate and low dissolved oxygen concentration were essential for achieving the highest yields of pullulan. Furthermore, pullulan fermentation has problems with increasing viscosity of the broth over the fermentation period, which limits mass (oxygen, nutrient), momentum and heat transfer (McNeil and Harvey, 1993), leading to stagnant zones and impeller flooding in the standard STR. The OBR has been shown to be able to reduce this problem with its ability to achieve effective mixing even in viscous environments, thereby improving various fermentation processes.

2.5.2 OBR application in biofuels production

The potential advantages offered by OBRs should be of greatest benefit for “long” processes and shear-sensitive microbiology cultures and can be used for production of various liquid biofuels. Reis *et al* (2006b) used an oscillatory flow micro-bioreactor (OFμB) to grow *Saccharomyces cerevisiae* and produce bioethanol. Experimental results were remarkably promising, with a 214% increase in *S.*

cerevisiae biomass when compared with shake flasks and an 83% increase when compared with stirred tank bioreactor. The culture inside the OFμB required 93.6% less air than an equivalent stirred tank bioreactor, as a result of high k_La value in the OFμB, which makes the aeration very efficient (Reis *et al.*, 2007). Ikwebe and Harvey (2011) found that the OBR improved saccharification of cellulose by 7% over the shake flask as a result of a better mixed hydrolysis environment. This led to a 30% increase in the ethanol productivity in the OBR compared to the shake flask. Furthermore, it should be noted that OBRs are scaleable: what can be achieved at this scale, can probably also be achieved at industrial scale.

Additionally, the OBR may be suitable for fermentations with gas production and product inhibition for example butanol fermentation. Processes which produced gas are suitable to be performed in types of reactor that offer low gas hold up, exhibit non-backmixing or plug flow behaviour (Qureshi and Maddox, 1988). Takriff *et al.* (2009) demonstrated application of OBRs to producing butanol via the acetone, butanol and ethanol (ABE) fermentation from *Clostridium acetobutylicum* using palm oil mill effluent as a substrate (Masngut *et al.*, 2010). ABE fermentation was conducted in a horizontal U-shaped OBR (Figure 2.9, next page). It is reported that, *C. acetobutylicum* produced higher concentrations of butanol in shorter periods of time in the OBR than the STR. Approximately, 1.5 gL^{-1} of butanol was produced within 48 h of fermentation inside OBR, which is almost double the production in the STR, where only 0.8 gL^{-1} of butanol was produced within 72 h of fermentation.

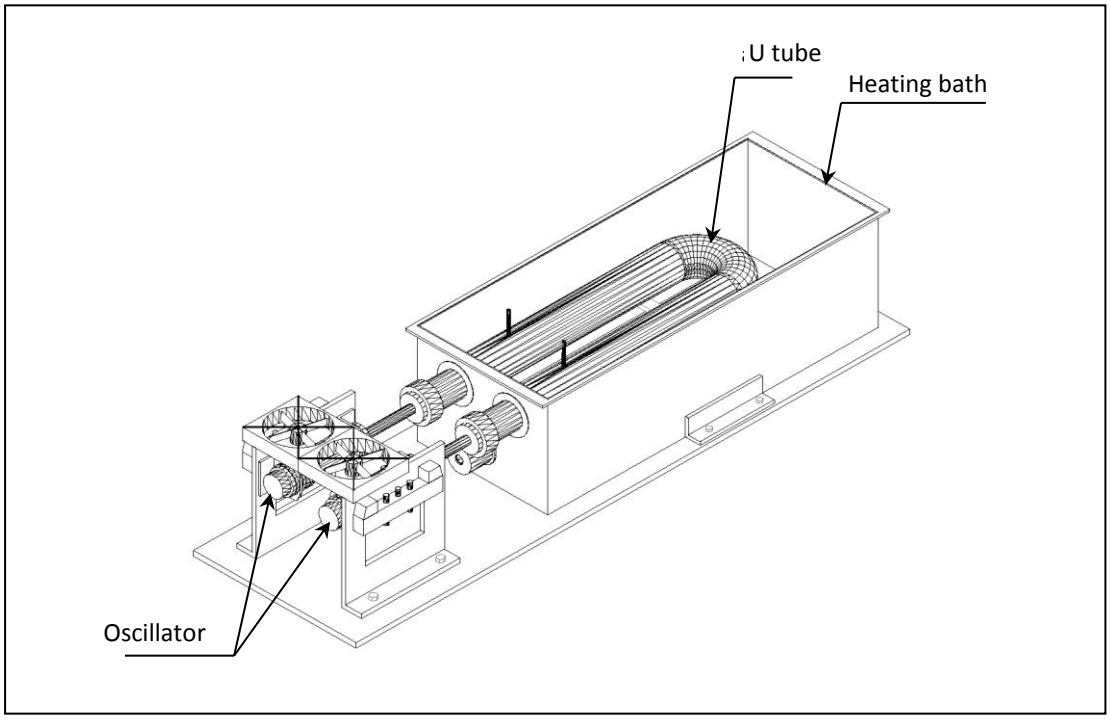


Figure 2.9 OBR used in ABE fermentation from *C. acetobutylicum* (Takriff *et al.* 2009).

The use of palm oil mill effluent as a fermentation substrate in the OBR resulted in a butanol production yield (gram butanol per gram substrate consumed) of 0.44 gg^{-1}, higher than using commercial medium reinforced clostridial medium (RCM) which was 0.17 gg^{-1} performed in the similar reactor (Masngut *et al.*, 2010). While particulate palm oil effluent is rich in carbon (due to the high content of organic and cellulosic matter), it also has 4-5% of total solids, which necessitates energetic mixing to maintain the homogeneity of solid-liquid suspension and efficient mass transfer during fermentation. It was found that OBRs were able to provide more efficient mass transfer than STRs. Palm oil mill effluent was demonstrated to be a viable substrate for ABE fermentation, and the OBR has significant design advantages as an alternative fermentation device when compared to conventional STRs (Masngut *et al.*, 2010).

Harvey *et al.* (2003) first demonstrated continuous production of biodiesel in an OBR. Biodiesel production is not a biological process, unlike bioethanol and biobutanol production, but is based on a biologically derived feedstock. Nonetheless, it can still be advantageously performed in OBRs, as it is a relatively "long" reaction. The reactor used was constructed from 2 x 1.5 m lengths of 0.024 m internal diameter jacketed glass, vertically oriented and connected by an inverted U-bend (Figure 2.10). The total volume was 1.7 L. It was demonstrated that saleable quality (*i.e*. that passed the European biodiesel standard) biodiesel could be produced at 15 minutes residence time when operating at 50^{o}C and in 10 minutes at 60^{o}C.

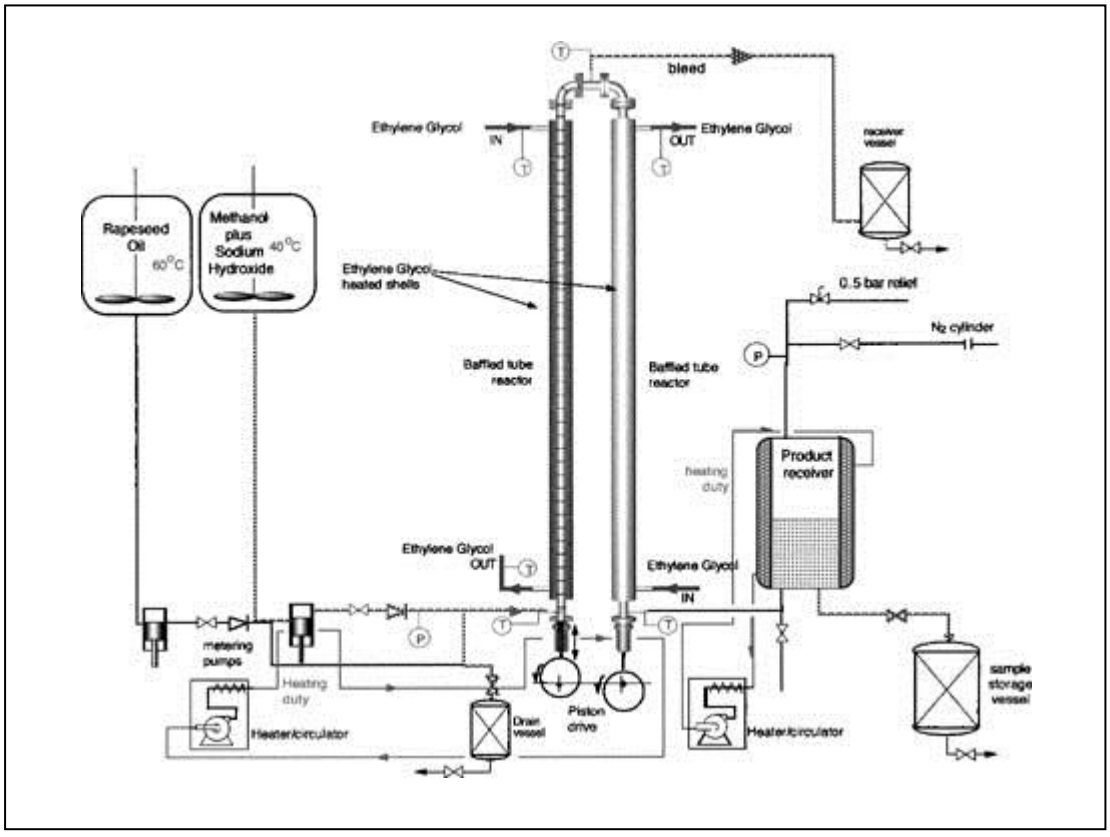


Figure 2.10 Process flow diagram for OBR production of biodiesel (Harvey *et al.*, 2003)

2.5.3 Advantages of OBR as a bioreactor

Various advantages of employing OBRs as bioreactors have been identified. One particular advantage is for screening. Conventional reactors, typically stirred tank reactors and shake-flasks (for smaller scale), are usually chosen for performing this task. However, these reactors are difficult to scale up, as the degree of mixing and heat transfer diminishes with increasing scale, leading to substantial reductions in rate. Furthermore, shake flask systems can be very expensive and lack control of important process variables (such as pH and pO_2). As a result, micro-scale reactors are becoming important high-throughput screening tools in upstream bioprocessing research (Rahman *et al.*, 2009).

Oscillatory flow "meso-bioreactors" could become an important tool in high-throughput screening system, following several studies by Reis *et al.* (2005; 2006a; 2006b). The oscillatory flow meso-bioreactor's design (Figure 2.11) has been shown to offer sterile condition and sufficient heat and mass-transfer rate to facilitate the fermentation of commercially important organisms, *i.e.* the recombinant yeast *Yarrowiya lipolytica*.

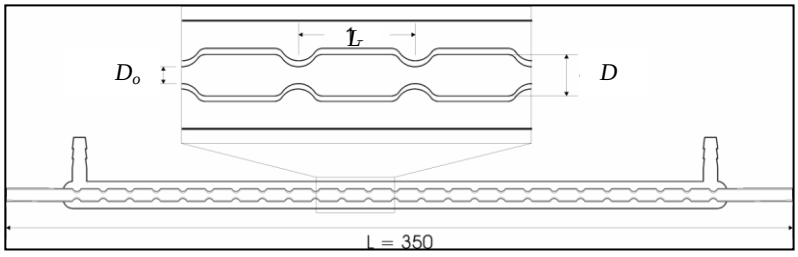


Figure 2.11 Geometry of the oscillatory flow meso-bioreactor (Reis *et al.*, 2006a)

In order to determine its' feasibility, Reis *et al.* (2006a) conducted a fermentation of *Y. lipolytica* to produce γ-decalactone. γ-decalactone is an aroma compound, that is of great interest in the flavour industry. The results showed that, in the oscillatory flow meso-bioreactor, the maximum γ-decalactone concentration was achieved in half the time required by other scaled-down reactors (stirred tank reactor and

shake flask) and exhibited a three-fold increase in the yeast productivity by increasing the oscillation intensity. These findings suggested that novel oscillatory flow meso-bioreactors can be used to control the droplet size, thereby controlling the interfacial areas between the two immiscible liquid phases and the rate of mass transfer between them during the fermentation (Reis *et al.*, 2006a).

Unlike other conventional tubular reactors, where minimum net flow Reynolds numbers must be maintained, mixing inside an OBR is independent of net flow. This allows reactor designs of significantly lower length-to-diameter ratio than equivalent conventional plug flow reactors. This suggests a specific application: converting "long" reactions from batch to continuous mode. As demonstrated by Harvey *et al.* (2003), conversion of vegetable oil to biodiesel was achieved in an OBR at a residence time significantly lower (at least 75% lower) than that of typical batch processes. In addition, with its narrow residence time distribution, OBR has the potential to allow various staged additions and parameter profiles along its length. This is probably beneficial for the ABE fermentation, where multi-stage fermentation processes have proven to improve the solvent volumetric productivity and yield, thus increased the butanol titre (Bahl *et al.*, 1982b; Setlhaku *et al.*, 2012).

2.6 Summary of literature survey

Butanol is an important bulk chemical and is currently produced from propylene oxo synthesis using crude oil as a raw material. However, in the oil price crises in the 1970s, and in 1999, there was considerable interest in research into viable alternative process to produce biobutanol. Biobutanol is produced from the sugar fermentation referred to as the ABE fermentation, after its major products: acetone, butanol and ethanol. Biobutanol is also an attractive biofuel with better properties than bioethanol. All of these reasons mean that ABE fermentation is becoming an attractive fermentation research topic.

Biobutanol production from ABE fermentation was a commercial process in the 1920s to 1960s. This process waned, due to the advent of the more economical petrochemical route. The technical and related commercial challenges for the conventional ABE fermentation have been reviewed in the previous section, and are summarized in Table 2.3 below:

Table 2.3 The challenges and solutions for ABE fermentation (adapted from (Green, 2011))

Challenges	**Solutions**
Dependency on high cost substrate	Transition towards alternative low cost substrate such as wastes and agricultural residues
Low butanol titre (<20 gL^{-1}) due to product and substrate inhibition	Development in microbes with improved butanol titre Relieve substrate inhibition with fed-batch fermentation and product inhibition with in situ product removal
Low butanol productivity increase capital and operating cost	Development of advanced fermentation process such as multi-stage continuous fermentation which will reduce downtime and increase reactor productivity
Low ABE yields of approximately 30% increases substrate cost	Development of microbes with higher butanol yield and/or develop microbes with higher butanol: solvents ratio
Uneconomical cost of butanol recovery by distillation, due to diluted products	Development of methods for in situ butanol removal to alleviate butanol tolerance Development of low energy method for solvent recovery and purification Recovery can also be improve by improving butanol titre
By-product formation	Engineered tailor made microbes to only produce butanol Manipulation of the microbe metabolic pathways (clostridia metabolic pathways)
Degeneration of the culture during continuous fermentation	Development of advanced fermentation process with multi-stages continuous fermentation

Oscillatory baffled reactors (OBRs) may be viable alternative bioreactors for this process. They have several potential advantages over conventional stirred tank reactors (STRs). The OBR can be used to intensify the process. In many other

applications it has been shown to be orders of magnitude shorter than conventional tubular reactors for the same duty. This leads to a much more practical solution to the problem of performing long reactions, such as fermentations, in continuous plug flow mode. OBRs have been demonstrated in various biotechnological processes, including producing biopolymers, such as PHAs and pullulan, and biofuels such as bioethanol, biobutanol and biodiesel. The oscillatory baffled bioreactor's design has been shown to offer sterile conditions and sufficient heat and mass-transfer rate to facilitate the fermentation of commercially important organisms.

Chapter 3 MATERIALS AND METHODOLOGY

This chapter lists all chemicals and biological materials used in this study, followed by the media preparations and their uses. The various methods for maintaining and manipulating the microorganism are also explained. The fermentations set-up in the bottles, BOBBs and STRs are described in this chapter, and all analytical procedures.

3.1 Chemical and Biological Materials

Unless stated otherwise, all chemicals were analytical grade. Ammonium sulphate ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), calcium carbonate ($CaCO_3$), dinitrosalicylic acid, acetone, butanol, ethanol, acetic acid, butyric acid, methanol, sodium sulphite, potassium hydroxide (KOH), hydrochloric acid (HCl), isopropyl alcohol (IPA) and sodium hypochlorite were purchased from Sigma Aldrich Chemicals, UK. Ethylene glycol was purchased from Fluka and sulfuric acid (H_2SO_4) from Merck. Sodium metabisulphite was purchased from Wilkinsons, Newcastle, UK while Virkon® tablets, Presept® tablets and polypropylene glycol 2000 from Fisher Scientific. The biological materials such as glucose, lactose and glycerol are of biological grade from Sigma Aldrich, UK. Yeast extract, nutrient broth and nutrient agar were purchased from Fluka, reinforced clostridial medium (RCM) from Oxoid and agar bacteriological no. 2 from LabM.

3.2 Media Preparation

Three types of media were used in this study, as there were three different applications, as shown in Table 3.1. The nutrient compositions are shown in Table 3.2.

Table 3.1 Types of media used based on its application

Application	Media
Sterilization testing media	Nutrient broth Nutrient agar
Culture maintenance media	Reinforced clostridial medium (RCM) Reinforced clostridial agar (RCA)
Growth media	Complex media

Table 3.2 Nutrient composition in the media used in this study

Nutrient content	Composition (gL^{-1})				
	Nutrient Broth	Nutrient Agar	Reinforced clostridial medium (RCM)	Reinforced clostridial agar (RCA)	Complex media
Meat extract	-	1	10	10	-
Peptone	15	5	5	5	-
Yeast extract	3	2	3	3	5
Sodium chloride	6	5	5	5	-
Agar	-	15	0.5	20.5	-
Glucose	1	-	5	5	50-100
Sodium acetate	-	-	3	3	-
L-cystein hydrochloride	-	-	0.5	0.5	-
Starch	-	-	1	1	-
$(NH_4)_2SO_4$	-	-	-	-	2
$CaCO_3$	-	-	-	-	5

3.2.1 Nutrient agar and nutrient broth

A nutrient agar and nutrient broth were used during sterilization of "BOBB I" as indicators of sterilization effectiveness. The nutrient broth was prepared by dissolving 25 g of nutrient broth powder in a litre of deionised water. The solution was sterilized in the autoclave at 121^{o}C for 15 minutes and properly stored at 4^{o}C until further use.

The nutrient agar was prepared by dissolving 28 g of nutrient agar powder in a litre of deionised water. Solutions were brought to boil whilst being stirred with a magnetic stirrer to ensure homogeneity. Agar solution was sterilized in the autoclave at 121^{o}C for 15 minutes. The sterile agar solution was then aseptically poured into disposable Petri dishes and left to solidify. Agar plates were stored (upside down) at 4^{o}C until further use.

BOBB I was chemically sterilized prior to fermentation to avoid contamination by microorganisms. Five types of chemical solution were prepared in water: 70% isopropyl alcohol, 4% sodium hypochlorite, 5.5% sodium metabisulphite solution, 1% Virkon® solution and 0.25% Presept® solution. The bioreactor was cleaned using soap and water prior to sterilization, followed by soaking in the respective chemical for 12 h at 32^{o}C and oscillated at high oscillation of Re_o 1870. After that, the bioreactor was washed three times with sterilized deionised water to remove chemical residues. Once cleaned, the reactor was tested for maintenance of sterility. A sterile nutrient broth (1500 mL) was aseptically transferred into the bioreactor and incubated at 32^{o}C for 72 h. Within the incubation period, BOBB I was visually observed for colour changes to the media (changes from clear to cloudy indicate contamination). Samples were withdrawn every 24 h and aseptically streaked on the nutrient agar plates for sterility confirmation. Nutrient agar plates were then incubated for 24 hours at 32^{o}C. Plates without microorganism colonies indicated successful sterilization.

3.2.2 Reinforced clostridial agar and media

Reinforced clostridial agar (RCA) was used for pure culture inspection of *Clostridium* GBL1082 and reinforced clostridial medium (RCM) was used for reviving the bacteria and for inoculum development. RCA was prepared by dissolving 38 g of RCM powder with 20 g of agar bacteriological no. 2 in a litre of deionised water. Solutions were boiled whilst being stirred with a magnetic stirrer to ensure homogeneity and sterilized at 121^{o}C for 15 minutes. Sterile agar solution was then

aseptically poured into disposable Petri dish and left to solidify. Agar plates were stored (upside down) at 4^{o}C until further use.

RCM was prepared by dissolving 38 g of RCM powder in a litre of deionised water. Solutions were brought to boil whilst being stirred with a magnetic stirrer to ensure homogeneity. Next, the liquid media were distributed each into 100 mL serum bottles or 250 mL screw cap flask, each was sparged with oxygen-free nitrogen gas, sealed and sterilized at 121^{o}C for 15 minutes.

3.2.3 Complex media

Complex media were used as the growth media for *Clostridium* GBL1082. Complex media was prepared according to the composition in Table 3.2. The media was made up to the required working volume, and the glucose sterilized separately to the other components. Glucose solution was sterilized separately to avoid formation of Maillard reaction products, which will inhibit cell growth (Kim and Lee, 2003). Sterilization occurred within an autoclave at 121°C for 15 minutes. Once cooled to room temperature, glucose solution was aseptically transferred to the media and pH was adjusted to 6.5 using 3 M H_2SO_4 or 5 M KOH.

3.3 Microorganism

The bacterium, *Clostridium* GBL1082*,* used in this ABE fermentation was obtained from Green Biologics Ltd. in freeze-dried form. The bacteria were then maintained and preserved as glycerol stocks in glycerol-lactose solution at -70^{o}C. Glycerol-lactose solution was prepared by mixing 20 mL of glycerol with 10 g of lactose. The mixture was then brought to a final volume of 100 mL with deionised water followed by sterilization at 121^{o}C for 15 minutes.

3.3.1 Glycerol stock preparation

On preparing the glycerol stocks, one vial of freeze dried bacteria was mixed to 90 mL deoxygenated RCM in a serum bottle and incubated at 32^{o}C for 16-20 h to its exponential phase at static condition. The culture will be ready when the pH is between 5.2-5.5 and the culture is observed to be healthy and motile under a microscope at 400x magnification. The glycerol stocks of *Clostridium* GBL1082 were then prepared by mixing an equal part of the grown cultures with the glycerol-lactose solution, under sterile condition. Approximately 1 mL of the mixture was aliquot to each of sterile cryovial tubes and stored at -70^{o}C. The remainder was retained in the sterile tube for further checking under microscope and streaking on RCA plate, after storing at -70°C overnight.

On reviving the glycerol stocks, each cryovial tube (consisting of 1 mL of glycerol stock) was mixed with 90 mL of deoxygenated RCM in a serum bottle under sterile conditions and incubated at static conditions for 72 h at 32^{o}C. This will produced a spore suspension of *Clostridium* GBL1082 for further use in inoculum development.

3.4 Fermentation Protocol

3.4.1 Inoculum development

An inoculum size of 10% from the bioreactor working volume was used in all fermentations as recommended by Alalayah *et al.* (2009). The inoculum was prepared from a *Clostridium* GBL1082 spore suspension. Prior to usage, the spore suspension was heat-shocked by immersing in 90^{o}C water bath for 90 s. After that, 10% v/v (volume of spore suspension per volume of media) was inoculated into sterile deoxygenated RCM aseptically. The mixture was allowed to grow in static conditions for between 16 and 20 h at 32^{o}C until it was ready to be used.

The inoculum was deemed to be ready to be used in fermentation, when it had grown and met the following conditions:

i. Numerous bubbles (CO_2) had been produced, indicating active respiration by the growing cells, as shown in Figure 3.1
ii. Under a microscope at 400x magnification rod-shaped motile bacteria were present, and other bacterial morphologies were absent
iii. The optical density at 600 nm gave a reading between 1.5 and 2.0
iv. The pH was in the range 5.2-5.5

Figure 3.1 Grown inoculum developed in (a) 100 mL serum bottles and (b) 250 mL screw capped conical flask

3.4.2 Batch fermentation in bottle

Acetone, butanol and ethanol (ABE) fermentations were conducted in 100 mL serum bottles and 2000 mL screw-capped Schott bottles with working volumes of 90 mL and 1500 mL respectively, as shown in Figure 3.2. Growth media was prepared according to Table 3.2 (complex media) and put into each bottle. Prior to inoculation, each bottle were sparged with oxygen-free nitrogen to create an anaerobic atmosphere. Each bottle was then inoculated with 10% v v^{-1} (volume of inoculum per volume of media) of actively growing inoculum developed earlier. Fermentations were preceded at 32^{o}C without agitation until growth ceased. Over the course of the fermentation, 3 mL samples were taken intermittently for analytical procedure i.e. off-line pH reading, optical density measurement, sugar and products (solvents and acids) determination. Optical density readings were taken at 600 nm wavelength using a Jenway 6705 UV-Vis Spectrophotometer. Fermentations were performed with the same conditions at 100 rpm agitation.

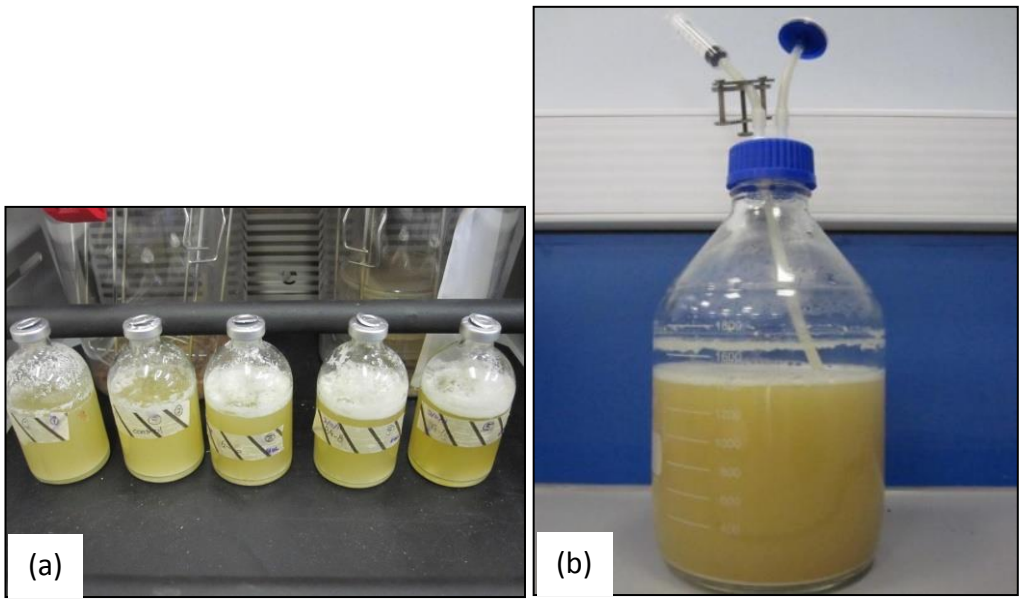


Figure 3.2 Fermentation of *Clostridium* GBL1082 in (a) 100 mL serum bottle and (b) 2000 mL screw-capped Schott bottle

3.4.3 Batch fermentation in bioreactor

Three types of bioreactor were used in this study: the "batch oscillatory baffle bioreactor I" (BOBB I), the "batch oscillatory baffled bioreactor II" (BOBB II) and a stirred tank reactor (STR). Fermentation in BOBB I and STR were carried out at 1.5 L working volume while in BOBB II either 1.5 L or 1.0 L. A comparison of the physical dimensions of all three bioreactors is shown in Table 3.3.

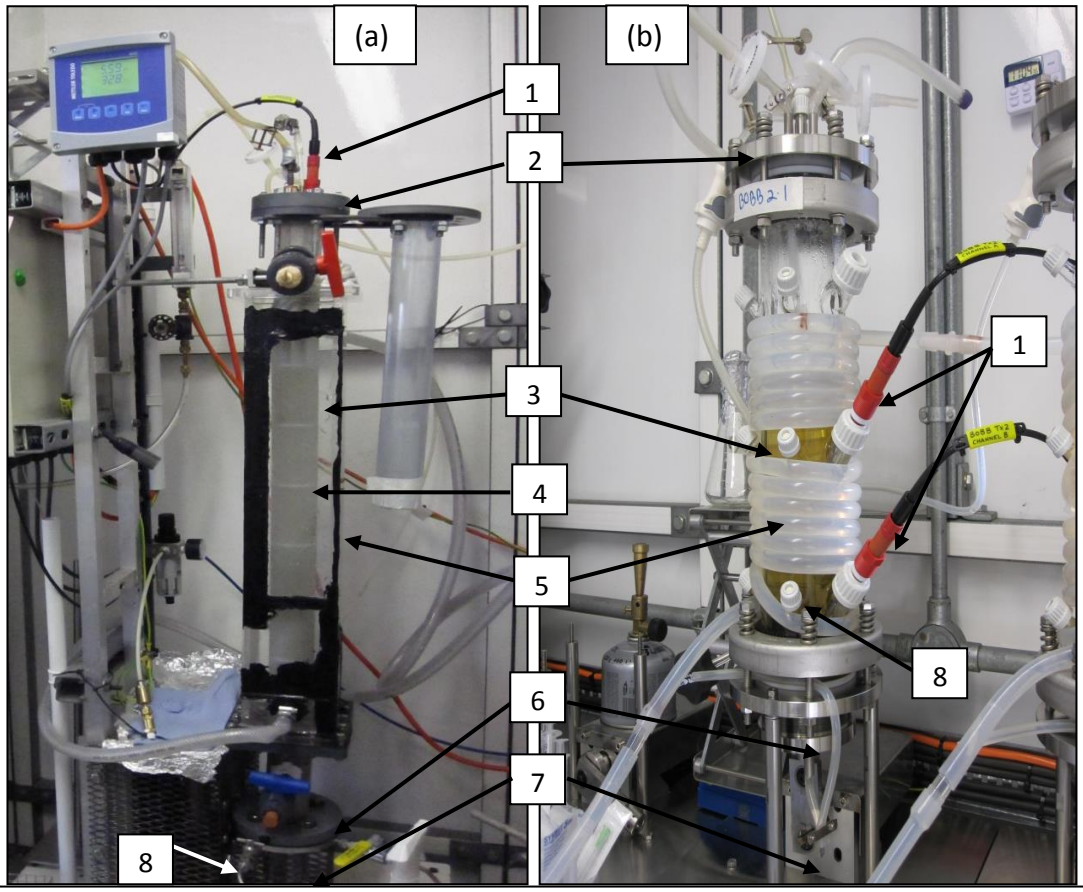
Table 3.3 Physical comparison of the bioreactors

Specification	BOBB I	BOBB II	STR
Mixing mechanism	Liquid oscillation from the base	Liquid oscillation from the base	Liquid agitation
Column internal diameter (*D*)	50 mm	80 mm	105 mm
N_2 sparger type	Single sparger	Single sparger	L-shaped sparger
Stirrer type	NA	NA	6-blade Rushton turbine
Stirrer diameter	NA	NA	45 mm
Baffle orifice diameter (D_o)	19 mm	40 mm	NA
Baffles spacing (*L*)	95 mm	120 mm	NA
Baffle plate dimension (width X height)	NA	NA	15 mm x 140 mm
Overall reactor height	1005 mm	456 mm	290 mm
Overall volume	2 L	2 L	2 L
Working volume height (*H*)	764 mm	300 mm (1.5 L) 220 mm (1.0 L)	156 mm
Working volume	1.5 L	1.5 @ 1.0 L	1.5 L
Aspect ratio (H/D) working volume	20.1	5.7	1.9
No. of baffles	9 orifice baffles	4 orifice baffles	3 rectangular baffles

3.4.3.1 Fermentation set-up in BOBB I

The BOBB I was run at 1.5 L working volume including 150 mL inoculum (10% v/v). The bioreactor was chemically sterilized with 1% Virkon solution for 12 h at 32^{o}C and oscillated at Re_o 1870. After that, the reactor was washed three times with sterilized deionised water to remove chemical residues. The pH electrode was sterilized separately in the autoclave and fitted to the bioreactor once it was sterile. Sterile complex media and glucose solution (made up to 1350 mL) were then aseptically transferred to the bioreactor. Oxygen-free nitrogen was sparged through for 2 h at 1 vvm to create an anaerobic atmosphere. The pH of the media was adjusted to 6.5 with 3M H_2SO_4 prior to inoculation (approximately 0.8 mL of acid was needed). The pH and temperature in the bioreactor was monitored using pH electrode with temperature compensation (Mettler Toledo) and the reading was recorded using PicoLog data logger and software (Pico Technology). The BOBB I set-up is shown in Figure 3.3a (next page).

An initial sample was taken, and then the inoculum was added to the reactor. At this point, the nitrogen gas was switched from sparging to sweep over the headspace of the bioreactor until the fermentation produced its own gases (CO_2 and H_2). Over the course of the fermentation, 3 mL samples were taken at appropriate intervals for analytical procedures. Fermentations proceeded without oscillation at 32^{o}C until growth ceased. Fermentations were performed with the same conditions at Re_o 1870.



(1) pH/redox electrode
(2) Top plate
(3) Column
(4) Baffle plate
(5) Heating jacket (BOBB I) /Heating coil (BOBB II)
(6) Bottom plate (BOBB I: membrane / BOBB II: bellows)
(7) Oscillation system
(8) Sampling port

Figure 3.3 BOBB fermentation set-up: (a) BOBB I (b) BOBB II

3.4.3.2 Fermentation set-up in BOBB II

The BOBB II was run at 1.5 L or 1.0 L working volume (including 10% vv^{-1} inoculum). Complex media was made up for a total of working volume, with the glucose sterilized separately to the other components, to a volume of 200 mL. All other components were sterilized in the bioreactor. Three pH/redox probes were fixed along the length of the bioreactor column before sterilization take place. Sterilization was performed using an autoclave at 121°C for 15 minutes. After

sterilization and cooling to 32^{o}C, glucose solution was added to the bioreactor. Oxygen-free nitrogen was sparged through the reactor at 1 vvm to create an anaerobic atmosphere. A silicone tube through which heating fluid was pumped was wrapped around the bioreactor to maintain a temperature of 32°C for all fermentations. The pH of the media was adjusted to 6.5 with 3M H_2SO_4 prior to inoculation (approximately 0.8 mL of acid needed). Thermocouples monitored the temperature using a PicoLog data logger and software (Pico Technology). This programme also recorded the pH and redox profile of the fermentation.

An initial sample was taken, and then the inoculum was added to the reactor. At this point, the nitrogen gas was switched from sparging to sweep over the head space of the bioreactor until the fermentation produced its own gases products (CO_2 and H_2). Over the course of the fermentation, 3 mL samples were taken at appropriate intervals for analytical procedures. Fermentations were performed without oscillation at 32^{o}C until growth ceased and repeated at Re_o 470, 938 and 1870. BOBB II fermentation set-up is shown in Figure 3.3b.

3.4.3.3 Fermentation set-up in stirred tank reactor

A diagram of the stirred tank reactor (STR) used in this study is shown in Figure 3.4. It is a 2.0 L jacketed reactor with an internal diameter of 105 mm, and is supplied by Applikon Biotechnology. Three baffle plates were placed perpendicularly to the vessel diameter to increase mixing efficiency. Fluid mixing inside the STR was provided by a 6-blade Rushton turbine. An *ez-Control* Bio Controller (Applikon Biotechnology) was used for measurement and control of process variables (pH, temperature, dissolve oxygen, level and stirrer speed).

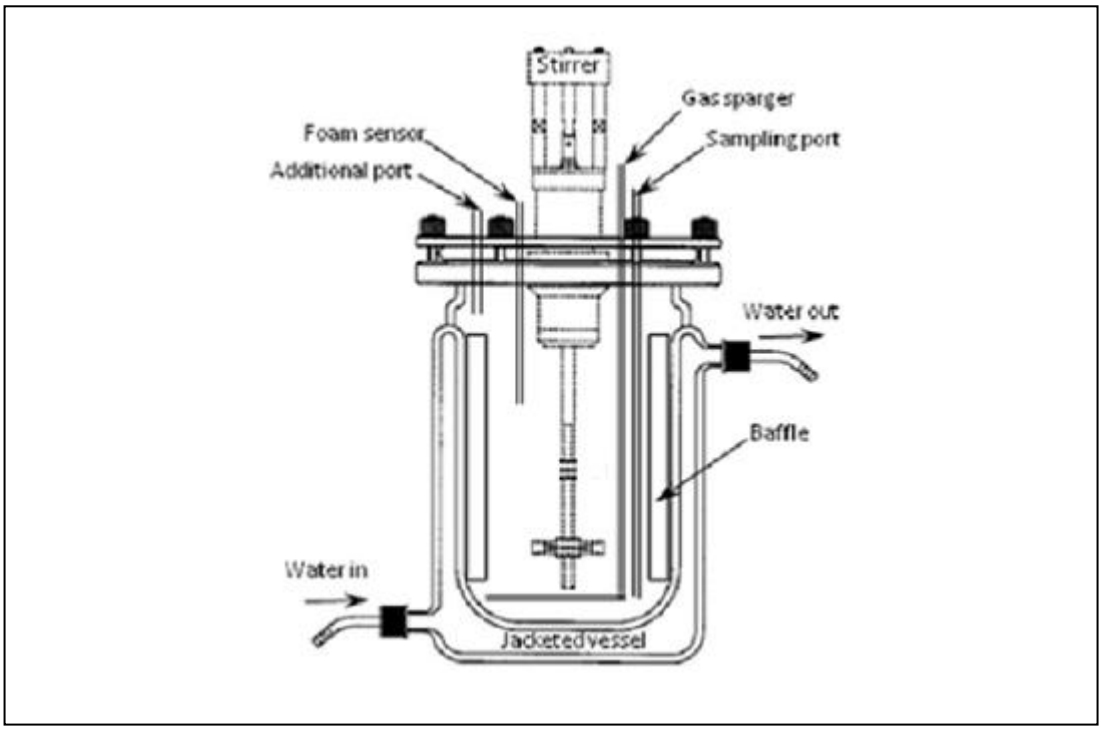


Figure 3.4 Stirred tank reactor (STR) used in the ABE fermentation

The STR was run at 1.5 L working volume including 10% vv^{-1} inoculum. Complex media was made up for the total working volume, with the glucose sterilized separately to the other components, in a 200 mL volume. All other components were sterilized in the reactor. Sterilization occurred within an autoclave at 121°C for 15 minutes. After sterilization, followed by cooling to 32^{o}C, glucose solution was added to the reactor. Oxygen-free nitrogen was sparged through at 1 vvm to create an anaerobic atmosphere. The thermo circulator on *ez-Control* was activated to maintain the temperature at 32^{o}C. The pH of the media was adjusted to 6.5 with 3M H_2SO_4 prior to inoculation (approximately 0.8 mL of acid needed).

An initial sample was taken, and then the inoculum was added to the reactor. At this point, the nitrogen gas was switched from sparging to sweep over the head space of the reactor until the fermentation produced its own gases (CO_2 and H_2).

Over the course of the fermentation, 3 mL samples were taken at appropriate interval for analytical procedures. Fermentations proceeded without agitation at 32^{o}C until growth ceased and were repeated for a stirrer speed of 18, 35, 70 rpm.

3.4.4 Two-stage ABE fermentation

BOBB II is prepared at 1 L working volume according to section 3.4.3.2. Two stage fermentations were perform as: first stage without oscillation and the second stage with oscillation or vice-versa according to the matrix below:

Table 3.4 Matrix for the one-stage and two-stage ABE fermentation in BOBB II

A (control)	**B (One-stage)**
without oscillation (0)	Oscillation at Re_o 938 (938)
C (Two-stage)	**D (Two-stage)**
Stage 1: without oscillation Stage 2: oscillation at Re_o 938 Switch time*: i) 12 h (0-938 12 h) ii) 24 h (0-938 24 h)	Stage 1: oscillation at Re_o 938 Stage 2: without oscillation Switch time*: 24 h (938-0 24 h)

* Switch time is the time when switching from stage 1 to 2

3.4.5 Gas stripping experiment

Gas stripping experiments were performed in BOBB II with 1 L working volume. BOBB II was prepared as in section 3.4.3.2. Two-stage fermentation in BOBB II was performed as in Table 3.4C following switching time after 12 h fermentation. All ports on the head plate were closed, leaving only one inlet and one exhaust. The inlet supplied the stripping gas (O_2-free N_2) and the exhaust was attached to a condenser (36 cm height x 4 cm width). The condenser was connected to a collector, which was a conical flask with a side arm. The side arm was fitted with silicon tubing to act as a gas exhaust. The collector was kept at 0^{o}C in an iced box. A schematic diagram of the set-up is shown in the Figure 3.5 (next page).

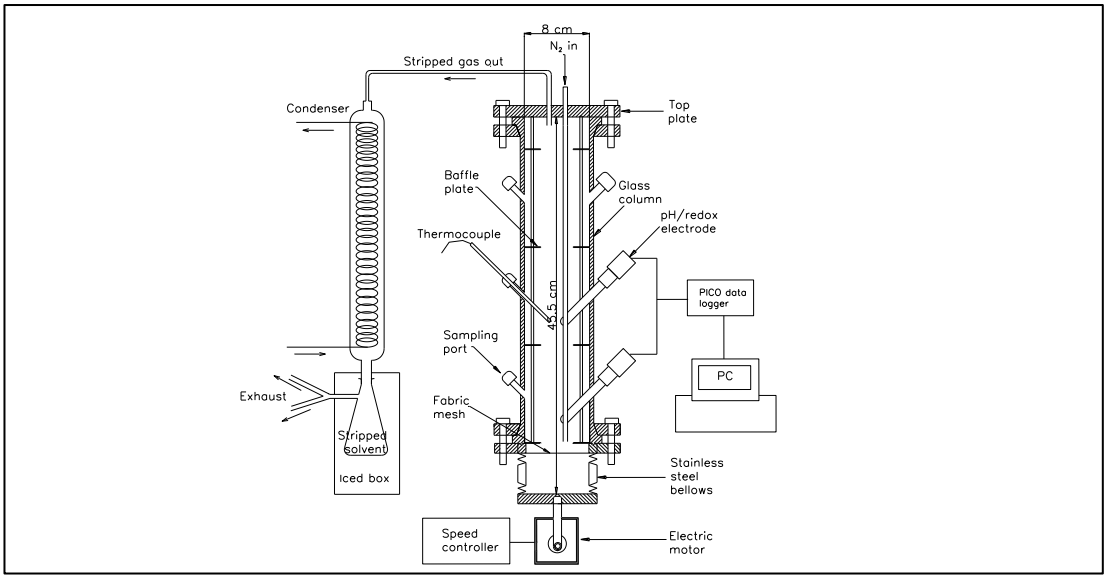


Figure 3.5 A schematic diagram of butanol production in BOBB II integrated with *in situ* recovery by gas stripping.

Batch fermentations were allowed to proceed for 48 h, after which gas-stripping was applied at three different intervals. The first interval was from 48-54 h fermentation, second interval from 72-78 h and the third interval from 96-102 h. During gas-stripping, stringent anaerobic conditions were maintained using oxygen-free nitrogen gas, flowing at 2.0 L min^{-1}. The ABE vapours were condensed using a condenser operating at -18 ±1^{o}C. In order to cool to this temperature, 60% (vv^{-1}) ethylene glycol was circulated at temperature of -25 ±1^{o}C through the condenser. The temperature in the bioreactor was maintained at 32 ±2^{o}C. There was no pH control. Antifoam (polypropylene glycol 2000) was added manually as required. Two sets of BOBB II were run in parallel, where one acted as the control (ABE fermentation without gas stripping).

3.5 Analytical Procedure

The fermentation samples were centrifuged prior to analytical procedure at 13,300 rpm in accuSpin Micro 17 centrifuge (Fisher Scientific) for 5 min. The clear supernatant liquid was transferred to a clean Eppendorf tube and stored at -4^{o}C for analysis, which was carried out within seven days. The bottom sediment consisting mainly of cell mass was used for visual checking of cell morphology under the microscope.

3.5.1 Cell concentration

Cell concentration was determined by the cell dry weight method. A correlation between the cell dry weight (CDW) and optical density at 600 nm (OD_{600}) needed to be established beforehand. First, the optical density of sample (fermentation broth) was determined by measuring at 600 nm using UV/visible spectrophotometer (Jenway 6705). Next, 2.0 mL from the same sample was centrifuged at 13,300 for 5 min and the pellet was washed once with PBS buffer and leave to dry at 80^{o}C for 48h. CDW of corresponding OD_{600} was determined by weighing the cell mass afterwards. A correlation between the CDW and the OD_{600} was developed, that is CDW (gL^{-1}) = (0.407 × OD_{600}) + 0.0014. Cell concentration at CDW basis can be determined from this correlation.

3.5.2 Cell morphology

In order to visually check the cell under microscope, sample (fermentation broth) was mixed together with Lugol's iodine at equal ratio and left for 30 s. Cells were observed under microscope at 90x magnification. Cells with granulose (clostridial forms cell) were red in colour under the microscope. Lugols' Iodine was prepared according to the method in Appendix B.

3.5.3 Residual glucose concentration

Residual glucose concentration in the fermentation broth was determined using the method of Miller (1959), using 1% dinitrosalicylic acid (DNS) reagent. The DNS method involves 3,5-dinitrosalicylic acid being reduced and establishing an equivalence between its reduced form, 3-amino-5-nitrosalycylic acid, with the sugar present in the mixture. Here, 1% DNS solution was prepared by dissolving 10 g of 3,5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulfite, 10 g of NaOH and 200 g of potassium sodium tartarate together in 1 L of deionised water.

Standard solutions of anhydrous D-glucose containing 0.5–3 g glucose L^{-1} in deionised water were prepared and l mL of each standard solution was mixed with 3 mL DNS reagent. The mixture was incubated at 90^{o}C for 10 min to develop the red-brown colour. Once cooled to room temperature, the mixture absorbance was measured at 540 nm, using a Jenway UV/visible spectrophotometer. A blank (deionised water) was incubated with the reagent and used for zero adjustment of the spectrophotometer. A glucose concentration standard curve was developed against the absorbance reading.

The sample's supernatant liquids were diluted appropriately, so that its sugar concentration was within the standard curve. One mL of diluted sample was mixed with 3 mL DNS reagent and incubated at 90^{o}C for 10 min. After the mixture cooled to room temperature, the absorbance was measured at 540 nm. The glucose concentration in the sample was determined from the standard glucose curve.

3.5.4 Solvents and organic acids determination

The concentration of acetone, butanol, ethanol, acetic acid and butyric acid were determined by gas chromatography (GC) (5890; Hewlett Packard, USA). An internal standard method was used in which 10 gL^{-1} methanol (internal standard) was mixed at 1 to 1 ratio with the sample's supernatant liquid. Two microliters of the mixture

were injected into a stainless steel column (2 mm by 2 m) packed with Porapak Q, 50/80 mesh. The column temperature was held at 150^{o}C for 20 min, programmed at 15^{o}C min^{-1} to 180^{o}C with a 15 min final hold. The injector and detector temperature, were both set at 250^{o}C. A flame ionization detector (FID) was used for signal detection with helium as a carrier gas.

3.5.5 Calculation of the fermentation performance

The ABE fermentation performances were calculated based on the specific growth rate, solvent productivity, solvent yield and specific solvent productivity. All formulas were shown in Appendix D

Chapter 4 DESIGN AND FABRICATION OF A BATCH OSCILLATORY BAFFLED BIOREACTOR

There were two types of batch oscillatory baffled bioreactor (BOBB) used in this study, known as "BOBB I" and "BOBB II". BOBB I was a "legacy" reactor, used for previous research into biological reactions in OBRs. It was initially used for performing the acetone, butanol and ethanol (ABE) fermentation, but was unable to maintain sterility throughout fermentation as it could not be maintained as completely sterile. Hence, the decision was taken to design and build an entirely new design of batch oscillatory bioreactor "BOBB II", specifically for ABE fermentation. The author was fully responsible for the design and build of the new reactor.

4.1 The "BOBB I" Design

BOBB I was mainly constructed from various plastics, with dimensions listed in Table 4.1 below.

Table 4.1 Detailed specifications of the BOBB I

BOBB I specification	Dimensions
Mixing mechanism	Linear pulsing fluid from the base
Column internal diameter (D)	50 mm
Orifice diameter (D_o)	19 mm
Baffles spacing (L)	95 mm
Overall height (H)	1020 mm
Overall volume	2000 mL
Working volume	1500 mL
No. of baffles (n)	9

The column was Perspex with wall thickness of ~10 mm, and the top and bottom plate were PVC. A set of orifice plate baffles, made from polyethylene, was used, with baffle free areas (*S*) of 14.4% and baffle thickness (δ) of 3 mm. Each baffle plate was arranged at uniform spacing (*L*) of 95 mm. A linear piston acting as an oscillator was located at the base of the column. Oscillation frequencies of up to 1.7 Hz, and amplitudes in the range 0.5 to 3.5 mm (centre-to-peak) can be obtained in the reactor. Even though the piston was able to provide up to 35 mm centre-to-peak amplitude, the oscillation amplitude was restricted by the silicone membrane that was placed between the bottom plate and the piston surface to prevent leakage of biofluid. A diagram of BOBB I is shown in Figure 4.1 (next page).

A combination pH electrode (Mettler Toledo) was used to measure pH and redox balance during fermentations. The electrode automatically temperature-compensates in the range 1^{o}C to 140^{o}C. Prior to the fermentation, the pH electrode was calibrated using a two-point calibration method at pH 4 and pH 7. Then it was sterilized separately in the autoclave at 121^{o}C for 15 min. The BOBB I was chemically sterilized prior to fermentation, as the ABE fermentation required strict contamination-free environment.

A number of ABE fermentations were performed earlier in BOBB I, where several problems were identified:

i. BOBB I was unable to maintain sterility throughout fermentation as it can not be made completely sterile
ii. The reactor could not be autoclaved as it was made of plastic. This also restricted the use of strong chemical for *in situ* chemical disinfection
iii. There were limitations to the pH probe location and sampling port, as they could only be inserted from the top of the reactor.
iv. The reactor was over a metre long and was fixed in one place, which made it hard to clean

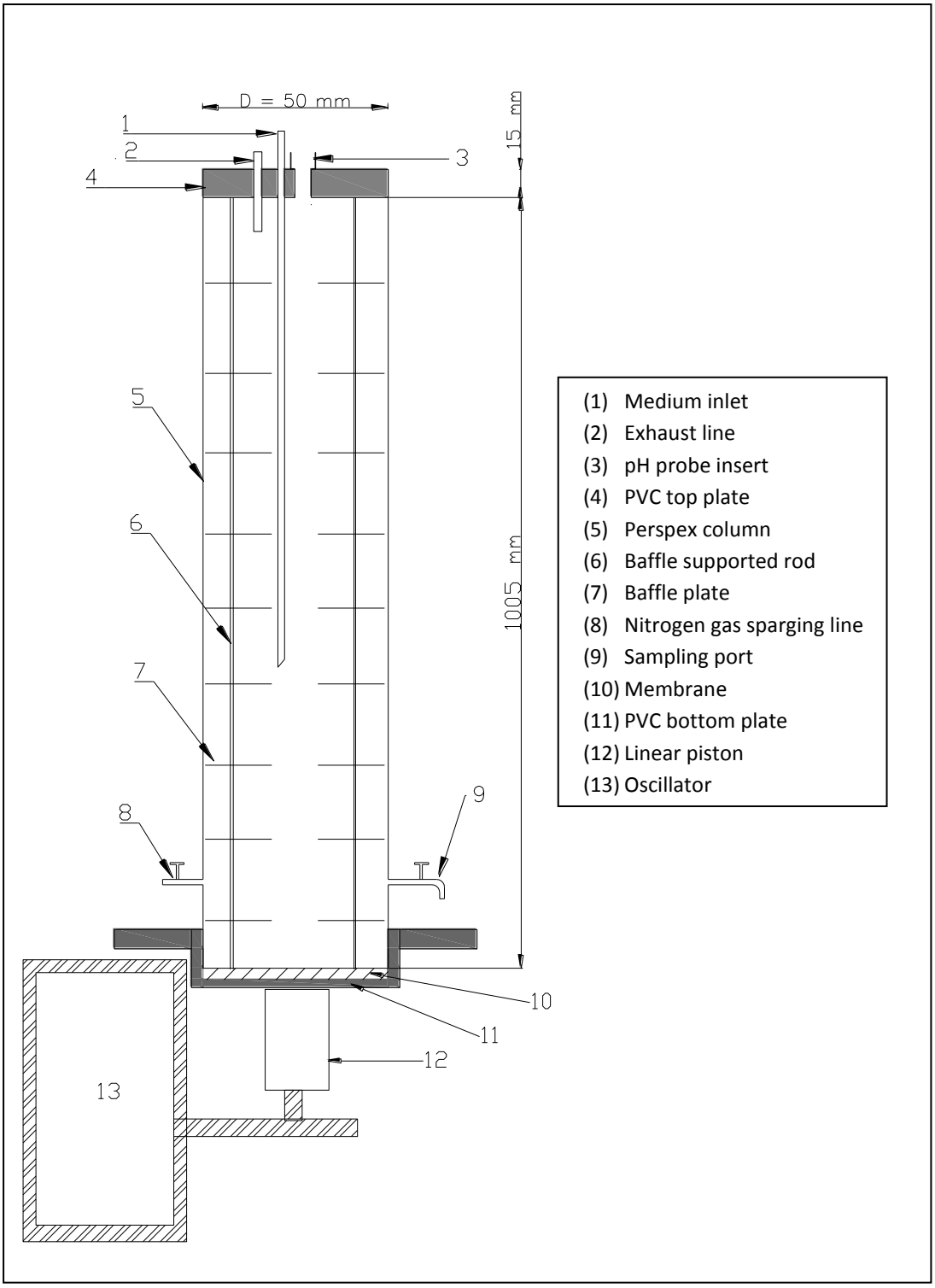


Figure 4.1 Diagram of the BOBB I

4.2 BOBB II Design Criteria

BOBB II was designed and built to overcome the practical shortcomings of BOBB I, and was specifically designed only for biological applications. A partnership between The School of Chemical Engineering and Advanced Materials (CEAM) of Newcastle University, Green Biologics Ltd. and WH Partnership Ltd. via a Technology Strategy Board (TSB) aided the development of BOBB II. A collaborative project grant between these partners was successfully sought to fund the development and exploitation of novel oscillatory baffled bioreactors (OBBs) for the acetone, butanol and ethanol (ABE) fermentation. The objective of this partnership was to demonstrate significant improvement in ABE fermentation productivity over conventional anaerobic fermentation in stirred tank reactors and non-agitated vessels. The reactor design, development, procurement and fabrication were entirely conducted at Newcastle University.

A number of design criteria were listed for designing BOBB II, based on the requirement to perform ABE fermentation as shown in Table 4.2 (next page).

Table 4.2 Design criteria for BOBB II

No.	Design criteria	Reasons
1.	The bioreactor must have the following dimensions: less than 490 mm diameter less than 700 mm height	To fit into existing autoclave in the CEAM Biotechnology Laboratory, Newcastle University (UK) which has a cylindrical compartment with diameter of 490 mm and height of 700 mm.
2.	The reactor column should be made from cylindrical glass.	Glass column, large diameter: both to make cleaning relatively easy.
3.	The reactor column had to be made from a single-wall glass column, instead of a jacketed column	Attaching several ports along the column length on a single wall glass would be easier than the jacketed column
4.	The top plate should have sufficient ventilation line openings. In addition, other lines were also needed for the addition of anti-foam, medium, inoculum, acid and/or alkali and gas sparging line	Ventilation line to prevent gas build-up in the bioreactor. Gas sparging line to sparge oxygen-free nitrogen gas to create anaerobic atmosphere inside the bioreactor. Other line for a variety of uses.
5.	The ports for redox, pH and temperature sensors will be fitted at one side of the column with an internal diameter enough to fit a standard pH/redox electrode	One sided electrode ports ensure their safety during transferring, autoclaving and setting-up the bioreactor. Contrast to BOBB I with only one port for sensor, which was at the top
6.	There should be at least three sampling ports along the column length	Sample can be taken from various places along the column
7.	The temperature of the bioreactor could be maintained by silicone tube coiled around the glass column and connected to an external circulated water bath	Simple set-up
8.	Baffle train should be placed where it cannot block the sensor electrode's way	Risk to break the electrode by the baffle train
9.	Suitable bottom plate which will endure stress from repeated oscillation and autoclaving	Bottom plate is prone to damage due to repeated oscillation. A suitable bottom plate will ensure continuous oscillation and to prevent contamination

Based on the criteria listed in Table 4.2 above, BOBB II was designed and built, and Table 4.3 (next page) shows the improvement made in comparison with BOBB I, while Figure 4.2 shows the configuration of BOBB I and the new BOBB II.

Table 4.3 Comparison between old BOBB I with the new developed BOBB II

Problems	BOBB I	BOBB II
Limited opening on the top plate	The top plate was made from PVC with only three openings *i.e.* probes insert, medium addition and exhaust line. Repeated autoclaving will eventually damage the opening fitting.	More openings were welded onto the stainless steel top plate. Stainless steel was used, ensuring durability for repeated autoclaving
Durability of the reactor materials	Most of the parts were made of plastic, PVC and polycarbonate. This materials cannot stand heat sterilization and were even unsuitable for strong chemical disinfection e.g. 5% sodium hypochlorite	Most of the parts were glass and stainless steel, hence were suitable for sterilization by heat or strong chemical disinfection
Sterilization of the reactor	BOBB I consist of a metre-long column made from Perspex, surrounded by square water jacket and fixed permanently to the oscillator unit. This design cannot be sterilised by heat. Chemical disinfection was performed *in situ*	Shorter column, so as to fit inside the autoclave. Detachable oscillator unit for easy movement and cleaning. The whole reactor unit, except for the oscillator motor, fits inside a standard (490 mm diameter and 700 mm high) autoclave, so the entire unit, with or without media inside can be sterilised in one step.
Few sampling port	There was only one sampling port placed at the bottom part of the column. This limits the sampling point to be taken	BOBB II was built with three sampling ports along the length of the column. Samples can therefore be taken from various points along the column length.
Monitoring and recording system	Equipped with online data reading with an offline data recording throughout fermentation time. Limited to one probe usage which can only read the combination of these two: pH, redox potential, temperature.	Equipped with online monitoring with a real-time data reading and recording throughout fermentation time. There were three probes insertion for pH and redox potential and three thermo wells for thermocouples.
Membrane oscillation system-leaking problem	The column was separated from the bottom plate by a silicone membrane. Leakage occurred due to ruptured membrane caused by repeated oscillation.	New stainless steel bellows replaced the membrane and also acts as the bottom plate. The design of the bellows allowed prolonged oscillation and ensured sterility throughout fermentation time.

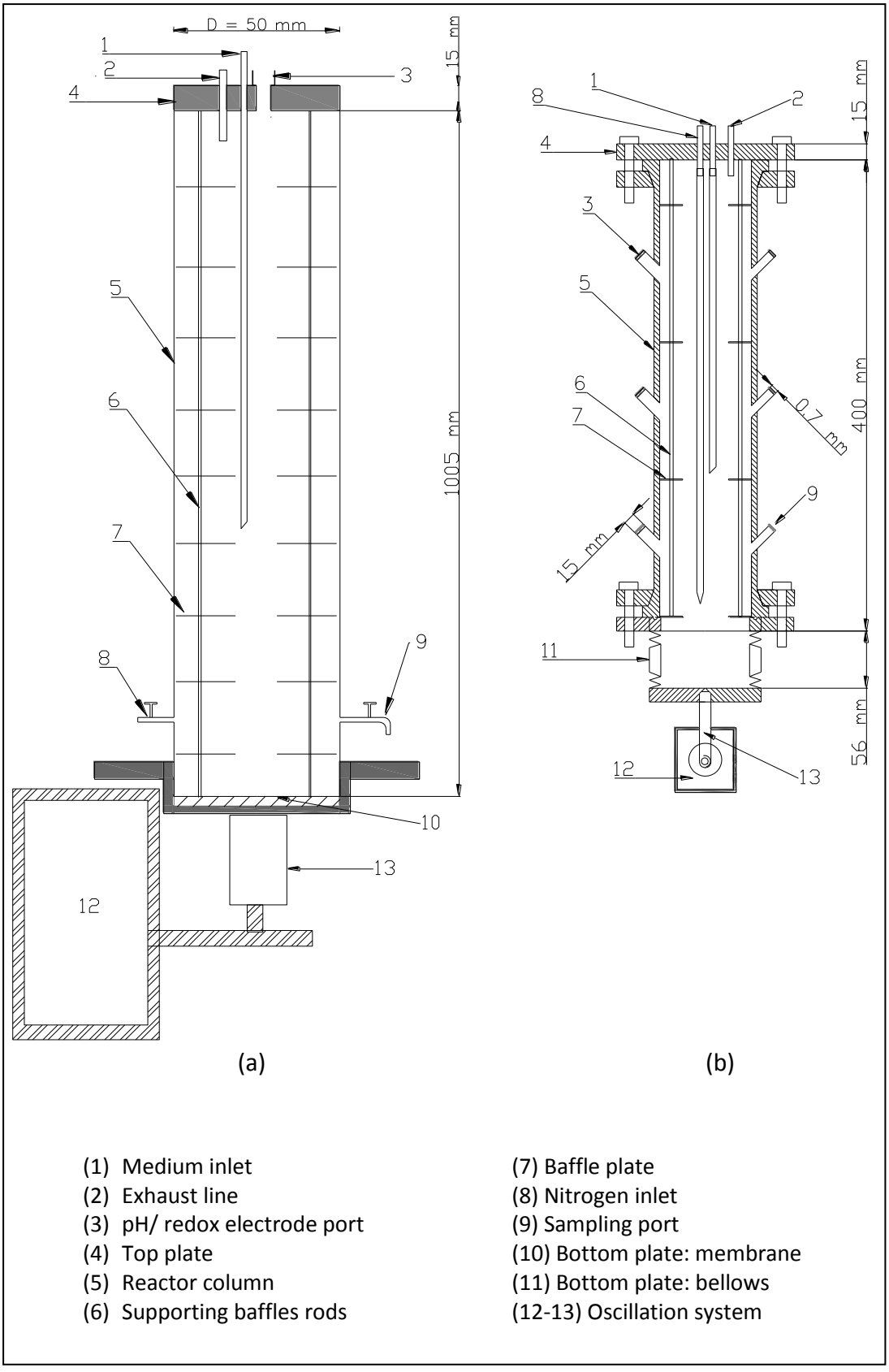


Figure 4.2 Diagram of (a) BOBB I (b) BOBB II

4.3 BOBB II Design Description

This section describes the new BOBB II in more detail. Description will be divided into five parts:

i. Reactor column
ii. Top plate
iii. Baffles
iv. Bottom plate
v. Oscillation system

4.3.1 Reactor column

The reactor column was made from Pyrex glass supplied by De Dietrich Process Systems (Germany). As shown in Figure 4.3 (next page), the glass column has an internal diameter of 80 mm and length of 400 mm. The glass column was fitted with stainless steel flanges at both ends, to mate with the top and bottom plates. The glass column was divided into three equal sections in which each section has a set of three ports along the circumference (one for pH electrode and the other two as sampling ports or thermo well). The idea of having three equivalent sections along the column is to allow determination of whether there will be any pH and concentration gradients while the reactor in operation. Attachment of the ports was carried out by Multi-Lab Ltd (UK), who were required, because the glass was too thick for standard glass-blowing techniques.

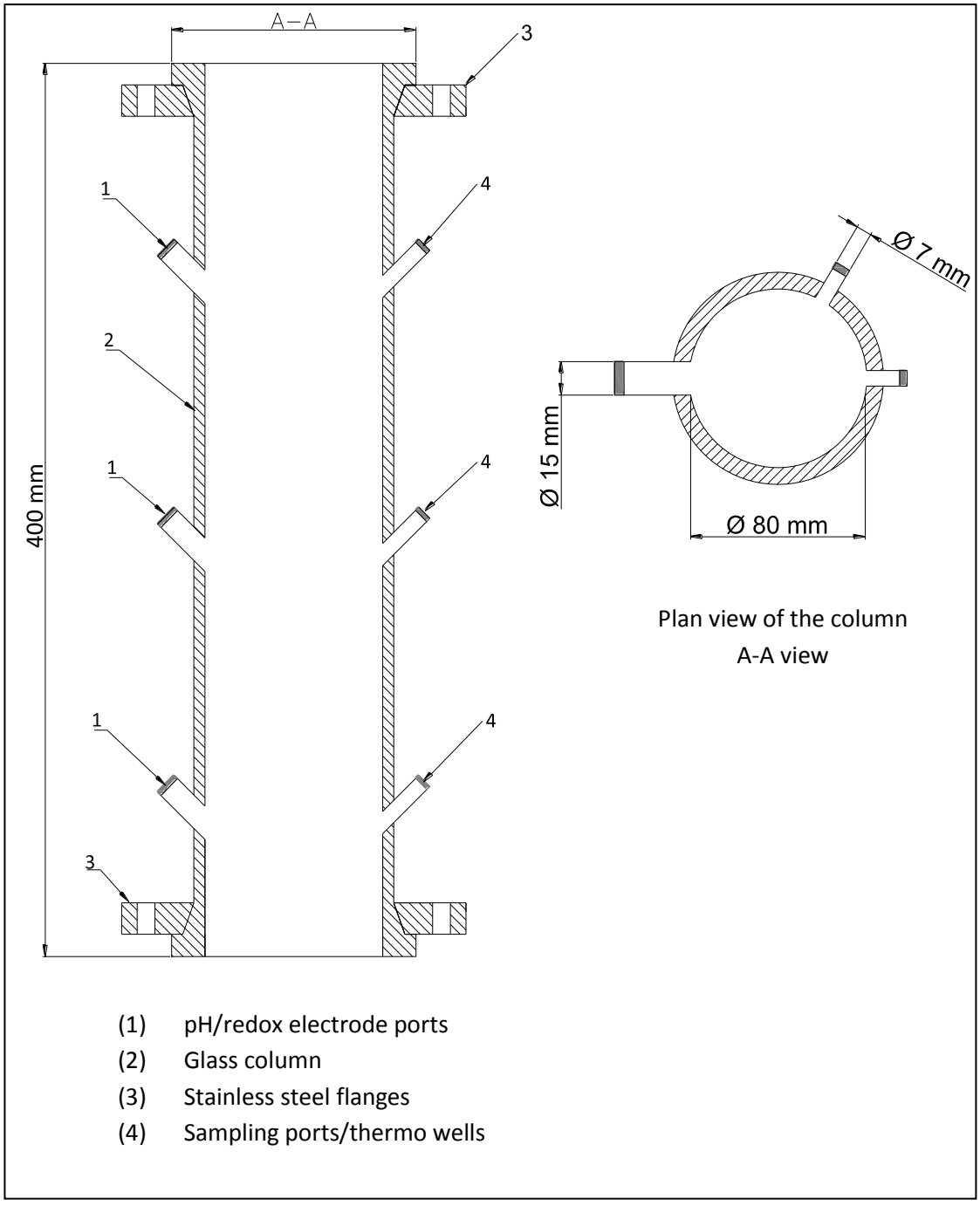
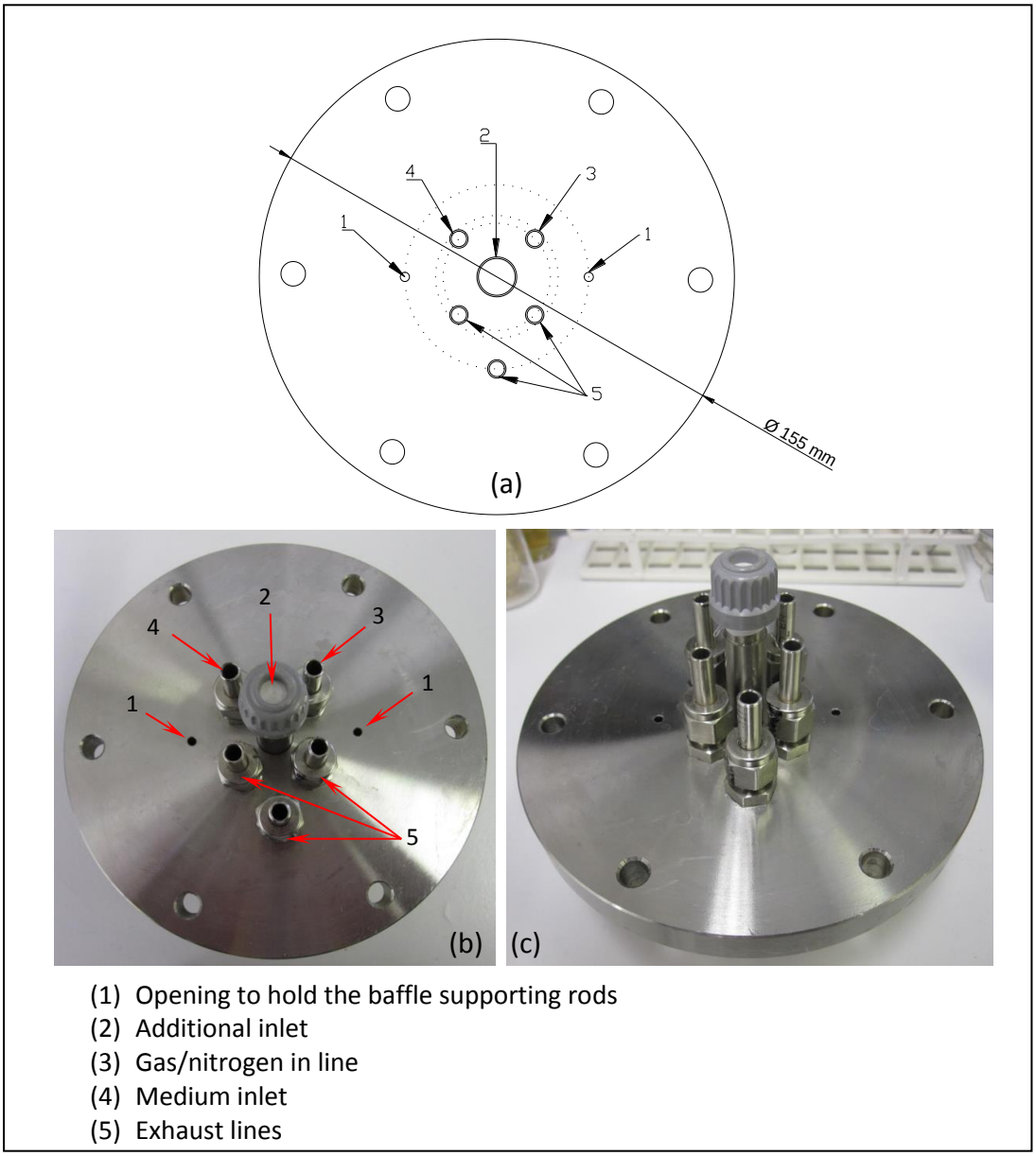


Figure 4.3 The reactor column

4.3.2 Top Plate

The top plates were made from 155 mm OD 304 grade stainless steel discs of 15 mm thickness. They were designed to have several openings for: exhaust line to prevent pressure built up in the reactor; antifoam feed line; medium and inoculum addition line. The top plate has an additional two small holes of diameter 4 mm to hold the baffle supported rods. The top plate will be connected to the glass column using the column stainless steel flange with PTFE gasket in between to prevent leakage. Figure 4.4 below shows the top plate configuration.



(1) Opening to hold the baffle supporting rods
(2) Additional inlet
(3) Gas/nitrogen in line
(4) Medium inlet
(5) Exhaust lines

Figure 4.4 BOBB II top plate (a) diagram of the top plate; (b) top view; (c) side view

4.3.3 Baffle train

The orifice-type baffles used in this design were fabricated from 304 grade stainless steel. The baffle plate thickness, δ, was 1 mm, after Ni *et al.* (1998), to favour the generation of vortices. If a thicker baffle plate were used, e.g. greater than 3 mm in thickness, this would increase the required Re_o for vortex-shedding. An orifice diameter of 40 mm was used, as it gave a baffle-free area ratio (S) of 0.25. Four baffle plates were arranged uniform distances apart, with two stainless steel rods of 3 mm diameter as supported rods. The distance between each baffle plate was 1.5 times the column internal diameter (120 mm), as suggested by Brunold *et al.* (1989). This is to achieve uniform mixing within the provided range of amplitude. Figure 4.5 below, shows the baffle train design.

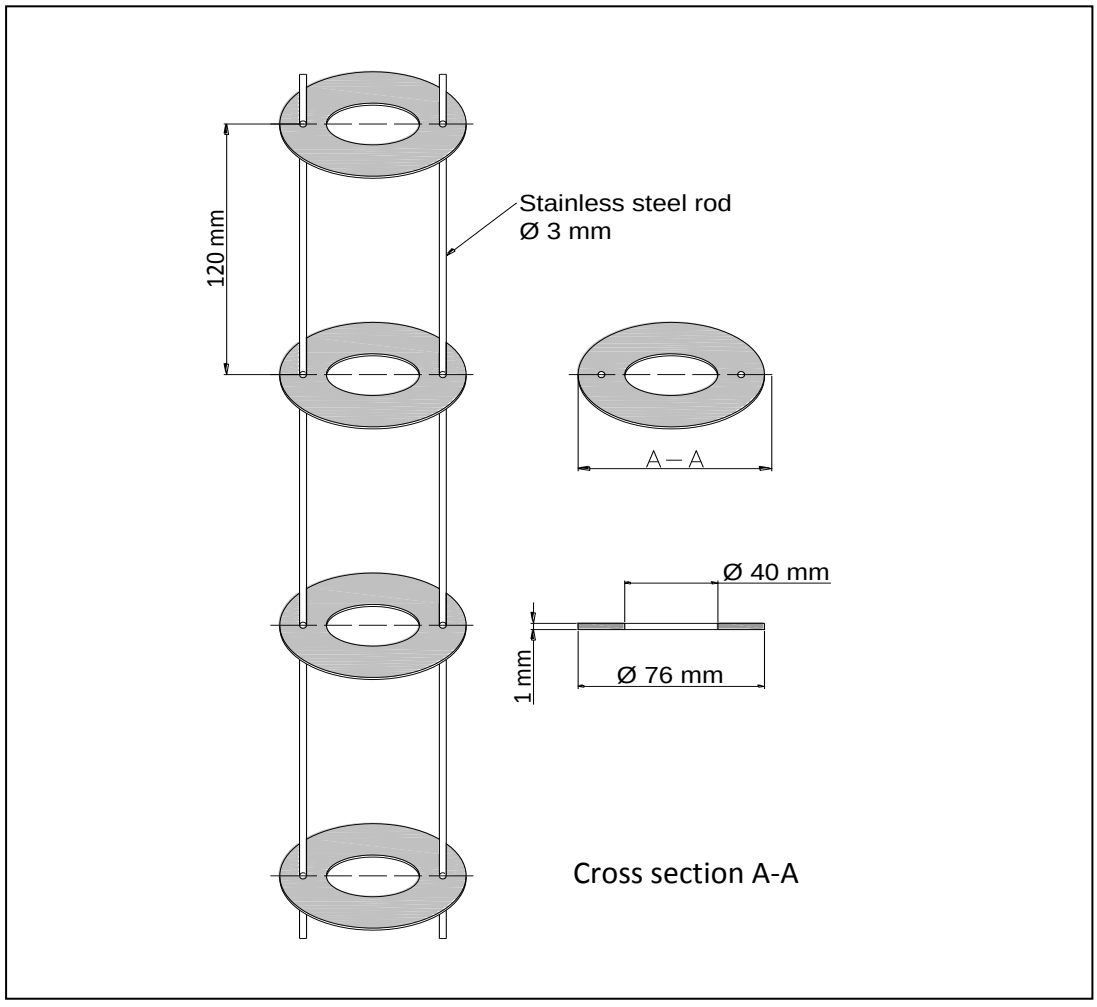


Figure 4.5 The baffle train design

4.3.4 Bottom plate and bellows

The bottom plate was part of a stainless steel bellows, which was able to be compressed and released repeatedly to create liquid pulsation inside BOBB II. The bellows assembly is of a "nested" design, made by Palatine Precision Ltd. (UK). "Nested" design has a very good resistance to pressure in the compressed state with low spring rate characteristics. It is ideally suited for long stroke applications such as in fermentation process, whilst maintaining a short free length. The bellows internal diameter was 78.6 mm, and the thickness of the corrugated wall was 0.2 mm. The maximum stroke allowed for the bellows was 20 mm, meaning that the bellows could in principle provide peak-to-peak amplitude of 20 mm. However, in practice, only a small fraction of this range was used, to prevent deposit built up and fatigue to the bellows. The force required to compress the bellows element was 8 Nmm^{-1}. The bellows was joined to the bottom of the reactor column by a stainless steel flange with PTFE gasket placed in between two flanges (bellows and column flanges) to prevent leakage. Figure 4.6 below shows the bellows design.

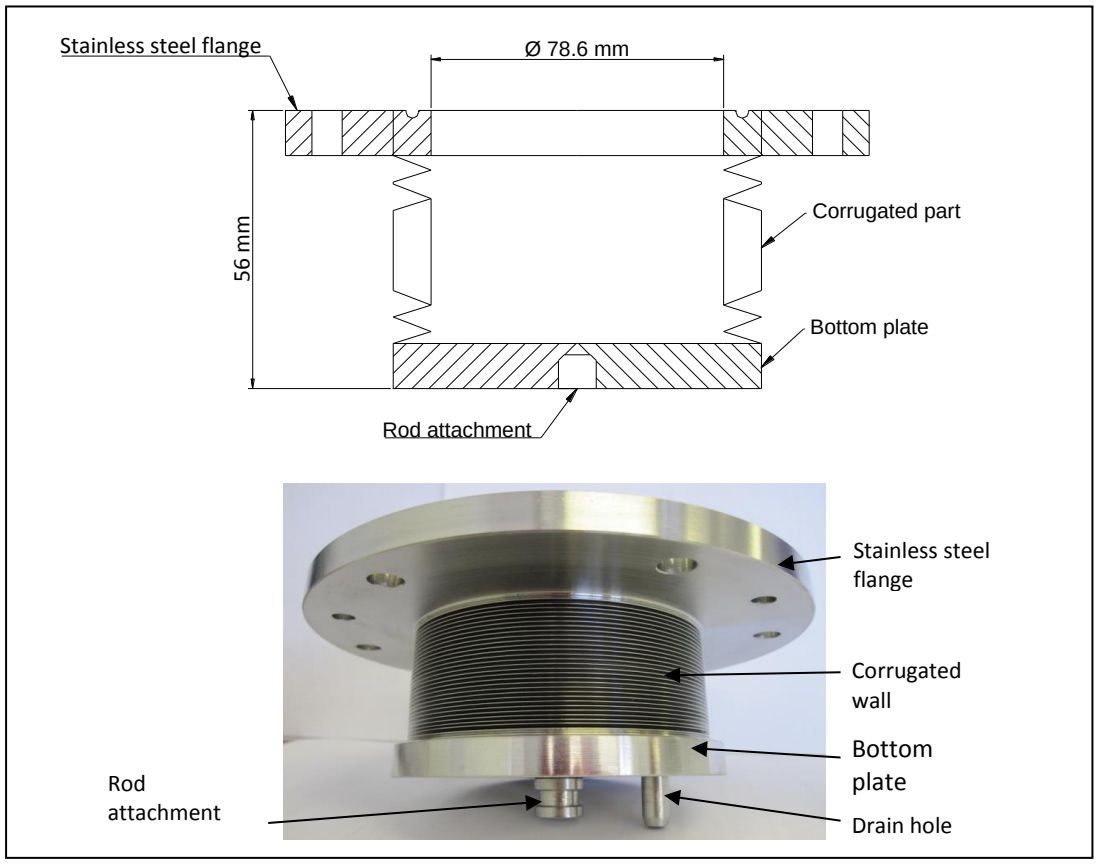


Figure 4.6 Bellows design

4.3.5 Oscillation system

The oscillation system consists of a bellows, fly-arm, crank and electric motor. The system was connected to the reactor by linking the bottom part of the bellows to the fly-arm via a rod attachment. This connection is detachable. An electric motor drives the oscillation and the speed of the motor provides oscillation frequencies between 0.2 – 1.0 Hz. The eccentric distance between the fly arm and the crank of the motor generates oscillation amplitudes of 2 mm and 4 mm (centre-to-peak). Figure 4.7 below shows the oscillation system connected to the reactor.

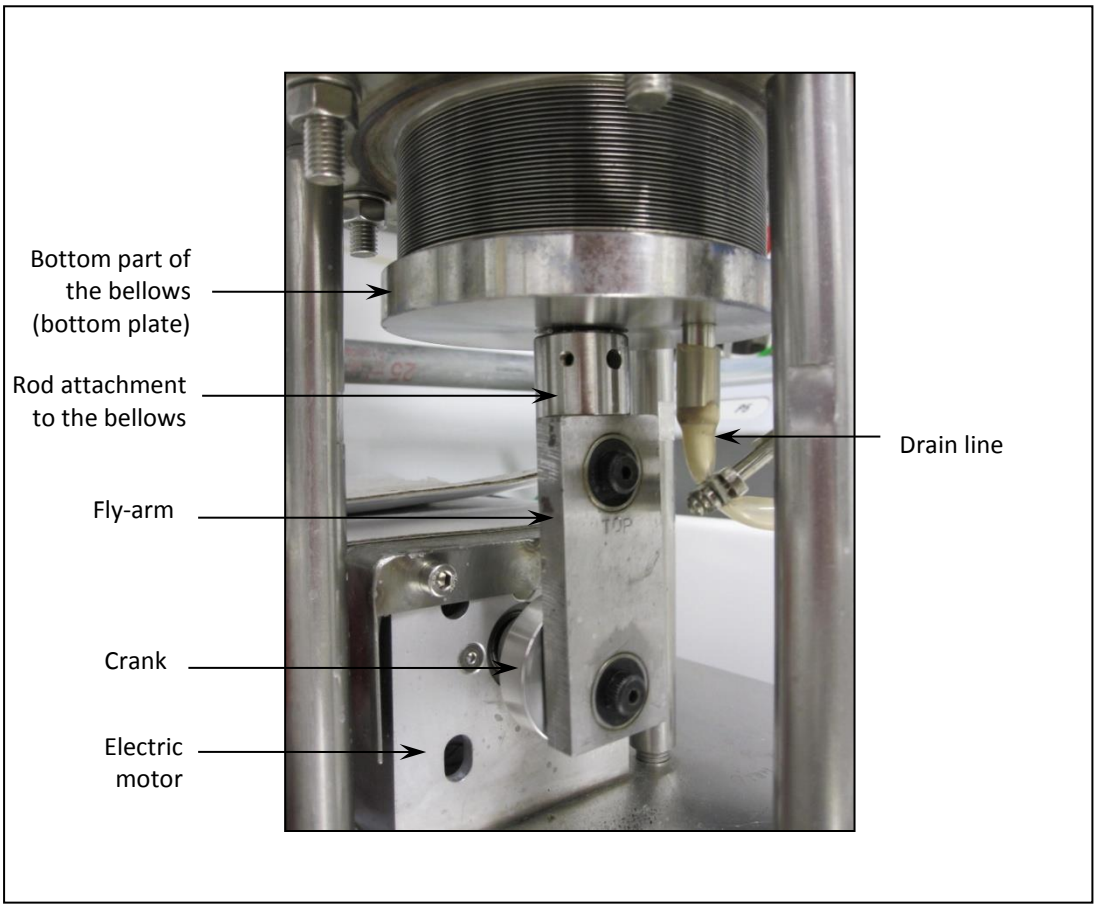


Figure 4.7 The oscillation system

4.4 BOBB II Characterization as a Bioreactor

BOBB II was successfully design and fabricated exclusively for biology reaction. Upon completion of the design, the reactor was tested for sterility hold and was characterized for bioreactor. These tests were used to gain understanding of the reactor capabilities before starting the fermentation run. A wet test approach was used as suggested by Hayda *et al.* (2010). These include the test for evaporation during sterilization, evaporation during media hold and heat removal characteristic.

4.4.1 Evaporation during sterilization

BOBB II was designed to be sterilised together with the media inside in a standard autoclave. The autoclave cycle included heating up to 123^{o}C, holding time for 30 min at 1 atm, and cooling down to 50^{o}C. Evaporation and condensation might occur during the cycle. It is desirable to characterize (or eliminate) the change in the reactor weight during sterilization to ensure the target starting medium concentration is achieved. If significant evaporation and condensation occurs, an adjustment to the amount of water during media preparation should be considered to compensate for the water loss or gain.

BOBB II was filled with 1.5 L deionised water, weighted before and after sterilization cycle. Table 4.4 below show the results of the test.

Table 4.4 Percentage of weight loss or gain after autoclaving BOBB II

	% weight lost/gain
BOBB II a	0.0
BOBB II b	0.0
BOBB II c	0.0

From the results, there was no weight loss or gain in BOBB II during autoclaving. Therefore, it was concluded that the media can be prepared without any

adjustment in the amount of water used. That is, to start with a litre of media before inoculation, one can prepared the media using exactly one litre of deionised water.

4.4.2 Evaporation during media hold

The sterilized media must be held for a period of time before inoculation to allow schedule flexibility during fermentation and to ensure robust process. Evaporation may occur during this hold period if the medium was oscillated and nitrogen was applied to the reactor. Determining the evaporation rate is essential for achieving the correct medium concentration at the time of inoculation. To compensate for evaporation rate, additional water can be added during medium preparation; however this require a fixed hold time for every batch. Alternately, adjustments to the gas flow rate can be made to minimize evaporative losses.

BOBB II, filled with 1.5 L water was held under the fermentation condition (32^{o}C, 1 vvm air, oscillated at Re_o 1870) at various Re_o. The reactor weight was continually monitored for 120 h and the data were used to calculate the evaporation rate. Weight was plotted against holding time and the slope was used as an estimate for evaporative loss occurring during media hold. Result is shown in Figure 4.8 (next page).

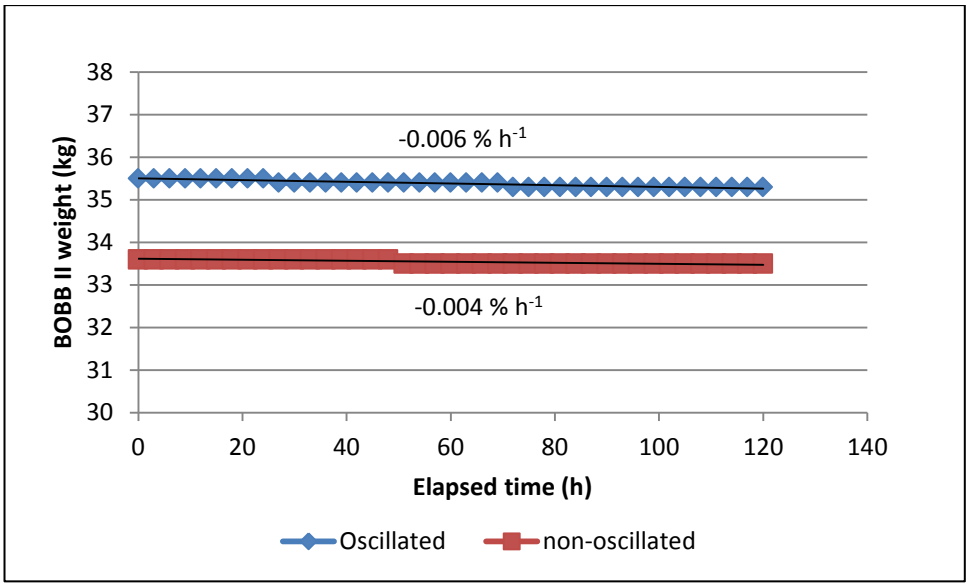


Figure 4.8 BOBB II evaporative losses. Oscillated BOBB II was performed at Re_o 1870. All experiments were performed with BOBB II filled with 1.5 L deionised water and hold for 120 h at 32°C sparged with 1 vvm air

The result demonstrated zero need for any water adjustment to achieve the target media concentration. Over 120 h, the reactor weight dropped by approximately between 0.004% and 0.006 % h^{-1} of starting weight (Figure 4.8) for non-oscillated and oscillated reactor, respectively. This is below the calibrated tolerance of the 96 h fermentation (0.2 % h^{-1}).

4.4.3 Heat removal characteristic

On maintaining constant temperature, the reactor must have sufficient heat removal capacity to remove metabolic heat generated by cells and mechanical heat generated during oscillation. The heat transfer rate (HTR) can be calculated using the equation below:

$$HTR = mC_p \frac{dT}{dt}$$

Equation 4.1

…where *m* is the mass of water in the reactor (kg), C_p is the heat capacity of water at 32^{o}C (4.179 kJ kg^{-1} ^{o}C^{-1}), *T* is the water temperature in the reactor (^{o}C), *t* is time (h) and *dT/dt* is the rate of temperature change determine from the slope of water temperature versus time curve.

BOBB II was filled to different target weights and heated to approximately 75^{o}C. The temperature set point was changed to 10^{o}C and oscillation was initiated. The rate of temperature change (*dT/dt*) was estimated from the slope of the linear portion of the temperature-versus-time curve from 36^{o}C to 28^{o}C. This range covers the process temperature set point of 32^{o}C. The HTR was then calculated using Equation 4.1 at different Re_o.

In BOBB II ABE fermentation, heat was generated mainly from cell growth and oscillator. Heat generated from cell growth can be calculated using Equation 4.2:

$$Q_{growth} = V_L \, \mu \, X \, \frac{1}{Y_H} \quad (kJ \; h^{-1})$$

Equation 4.2

…where Q_{growth} is rate of heat generated by cell growth (kJ h^{-1}), V_L is the liquid volume (L), μ is a specific growth rate (h^{-1}), X is a cell mass concentration (g L^{-1}) and $1/Y_H$ is the metabolic heat evolved per gram of cell mass produced (kJ g^{-1}) taken as 0.072 kJ g^{-1} for glucose substrate (Shuler and Kargi, 2002). The rate of heat generated by the cell growth calculated using Equation 4.2 approximately 7.96 kJ h^{-1}. Heat generated from oscillation was estimated from the power density of the highest oscillation (Re_o 1870) where total power input is equivalent to heat output. It was estimated to be approximately 0.59 kJ for 96 h oscillation operated with liquid volume of 1.5 L. This heat is an insignificant contributor to the overall heat load, thus the HTR required for BOBB II is estimated as 7.96 kJ h^{-1}.

The calculated HTR using Equation 4.1 for BOBB II at different Re_o are shown in Figure 4.9 below. The calculated HTR varied slightly between Re_os with values varied between 7.88 and 10.9 kJ h^{-1}; therefore BOBB II had sufficient HTR capacity to support the process need of 7.96 kJ h^{-1}.The maximum heat transfer rate was observed at Re_o 470 with 10.94 kJ h^{-1}.

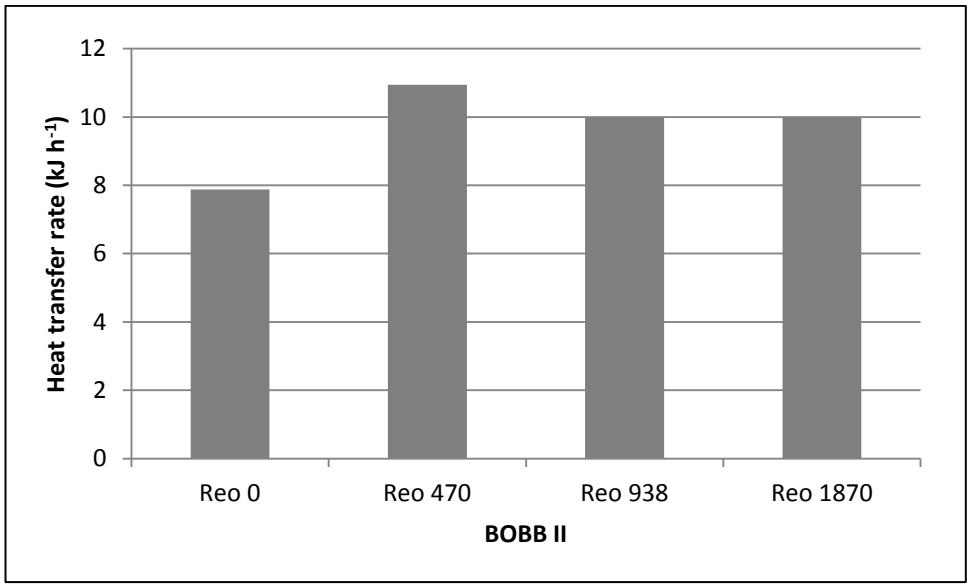


Figure 4.9 Heat transfer rate in BOBB II at different Re_o

Chapter 5 RESULTS AND DISCUSSION

5.1 Chemical Sterilization of "BOBB I"

ABE fermentation was carried out in BOBB I to determine its viability as a bioreactor to produce solvent (acetone, butanol and ethanol). Before the fermentation could be performed, BOBB I had to be sterilized to ensure only *Clostridium* GBL1082 grew in the bioreactor. *In situ* chemical sterilization was chosen, as BOBB I construction was fixed on its own base, and fabricated mainly from Perspex, so it could not fit into a standard autoclave and could not withstand high temperatures. Several chemicals were evaluated which were: 70% ($v.v^{-1}$) isopropyl alcohol, 4% ($v.v^{-1}$) sodium hypochlorite, 5.5% ($w.v^{-1}$) sodium metabisulphite solution, 1% ($w.v^{-1}$) Virkon® solution, and 0.25% ($w.v^{-1}$) Presept® solution. The results of the experiments are shown in Table 5.1 below.

Table 5.1 BOBB I *in situ* chemical sterilization results

Chemicals	Microorganism colonies form after 72 h incubation period?	Conclusion
70 % isopropyl alcohol	Yes	Not an effective sterilant
5.5% sodium metabisulphite solution	Yes	Not an effective sterilant
4% sodium hypochlorite	No	Sterile, but corroded the metal bolts used to join the top-plate to the column, and reduced the clarity of the Perspex column
0.25% Presept® solution	No	Sterile, but dissolved the silicone sealant used on the piston to prevent leakage, and reduced the clarity of the Perspex column
1% Virkon® solution	No	Sterile

Successful sterilization was achieved using sodium hypochlorite, Virkon® and Presept®, as no microorganism colonies appeared on agar with samples taken from each sterilization cycle by these chemicals. The medium (nutrient broth) inside BOBB I also remained clear over the 72 h incubation period. However, chlorine in the sodium hypochlorite and Presept® solution damaged parts of BOBB I during the soaking process. From the experiment, it was concluded that 1% Virkon® solution should be used to sterilize BOBB I prior to fermentation, and 0.25% Presept® solution can be used in BOBB I for cleaning after fermentation as the final wash. These two chemicals were chosen based on this result, and because it was relatively simple to prepare these solutions.

Sterilization is a process that destroys or eliminates all forms of microbial life (Rutala *et al.*, 2008). Here the term is intended to convey an absolute meaning: a thing is either sterile or not (over a given period in certain condition). Here in BOBB I, it was hard to maintain a sterile condition for a long period because of the reactor‘s design and fabrication. It could not withstand the chemical sterilants, which eventually damaged the bioreactor in long-term usage. Despite this problem, several ABE fermentations were carried out successfully in BOBB I, and it demonstrated the ability of oscillatory flow reactor to perform anaerobic ABE fermentations.

5.2 Batch Fermentation in BOBB I and Schott Bottles

A series of ABE fermentations using *Clostridium* GBL1082 were conducted in BOBB I with comparison to the Schott bottle fermentations to evaluate its ability to perform anaerobic fermentations, as summarized in Table 5.2. The cultivation protocol and conditions are detailed in section 3.2.1.

Table 5.2 Matrix of batch ABE fermentation in BOBB I and Schott bottle

	Oscillation/agitation	No oscillation/no agitation
Baffle	(a) Oscillation with baffle	(b) No oscillation with baffle
No baffle	(c) Oscillation without baffle	(d) No oscillation without baffle
Schott bottles	(e) 1500 mL working volume agitated at 100 rpm	(f) 1500 mL working volume without agitation

BOBB I was oscillated at Re_o 1870 at amplitude of 3.5 mm (centre-to-peak). Results from the fermentation are shown in Figure 5.1. Typically, microorganism cell growth can be divided into five growth phases: lag, exponential, deceleration, stationary and death phase (Shuler and Kargi, 2002). As the fermentation proceeded in BOBB I with oscillation and baffle, cell growth was observed (Figure 5.1a). There was no lag phase and cell concentration increased exponentially until it reached a maximum OD_{600} of 9.8 within 27 h, before entering stationary phase, followed by the death phase. The same trend was observed in Figure 5.1c(oscillation without baffles). However, the exponential growth phase quickly became stationary after just 20 h and the maximum OD_{600} achieved was only 5.9, 60% lower than that achieved in BOBB I with oscillation and baffle. When there was no oscillation in BOBB I, a long lag phase (more than 20 h) was observed regardless of the presence of baffles (Figure 5.1b and d). A low growth rate was also observed in both Schott bottle fermentation with agitation (Figure 5.1e) and without agitation (Figure 5.1f). A significantly longer lag phase (24 h) was observed in the Schott bottle without agitation than the agitated Schott bottle. The agitated Schott bottle fermentation exhibited a maximum OD_{600} of 1.45 (Figure 5.1e) which was 85% lower than the maximum achieved in BOBB I.

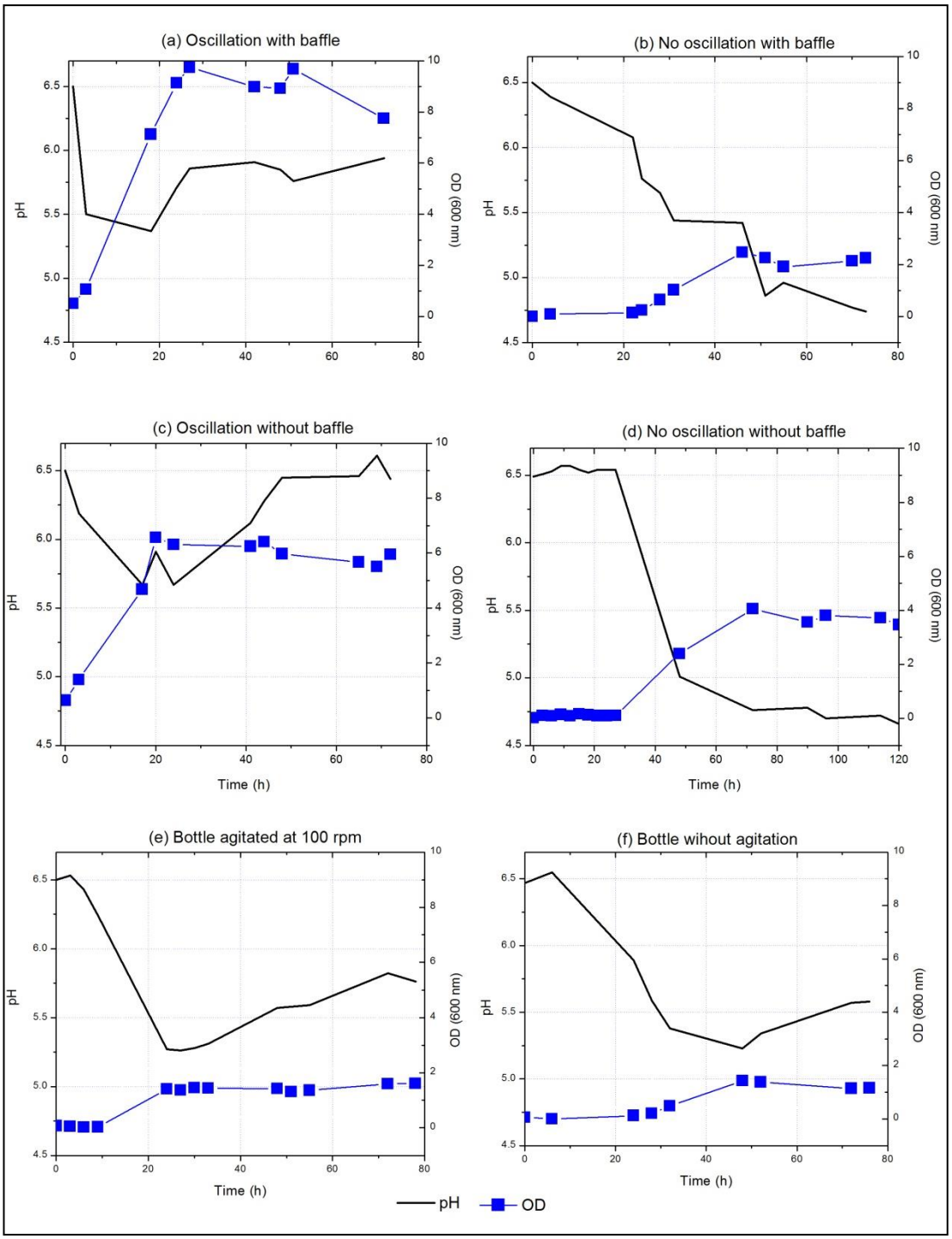


Figure 5.1 *Clostridium* GBL1082 growth and pH profile in BOBB I and Schott bottles.

In a commercial fermentation plant, a long lag phase is undesirable as it will reduce the reactor productivity (Shuler and Kargi, 2002). It has been proven here within the range of studied conditions that *Clostridium* GBL1082 can be grown in BOBB I. Furthermore, the interaction of liquid oscillation with the baffles, producing "oscillatory flow mixing" encouraged cell growth by initiated an early exponential phase, thereby producing high cell concentrations rapidly.

Solvent production based on the highest productivity from each of fermentation is tabulated in Table 5.3 below.

Table 5.3 Solvent production in BOBB I and bottles

BOBB I	(a) osc. with baffle	(b) no osc. with baffle	(c) osc. without baffle	(d) no osc. without baffle	(e) Bottle agitated (100 rpm)	(f) Bottle no agitation
Acetone (gL^{-1})	6.59	1.30	4.03	0.51	2.52	0.83
Butanol (gL^{-1})	11.89	5.51	6.62	0.79	6.99	4.92
Ethanol (gL^{-1})	0.35	0.04	0.04	0.01	0.11	0.07
Total ABE (gL^{-1})	18.83	6.88	10.52	1.31	9.62	5.83
Maximum OD_{600}	9.75	2.47	6.56	4.05	1.60	1.43
Solvent productivity ($gL^{-1}h^{-1}$)	0.37	0.14	0.22	0.03	0.12	0.08

osc. = oscillation

As expected, fermentation in BOBB 1 with oscillation and with baffle (Table 5.3a) had the highest reactor productivity of 0.37 $gL^{-1}h^{-1}$, with a total solvent concentration produced of 18.83 gL^{-1} (6.59 gL^{-1} acetone, 11.89 gL^{-1} butanol and 0.35 gL^{-1} ethanol). In ABE fermentation, high cell concentration is preferred, as it will increase the productivity (Qureshi *et al.*, 2000) by improving the reaction rate thereby producing high product concentrations more rapidly. Here, cell concentration was measured by OD_{600}. Between Schott bottle fermentation and BOBB I, it was found that BOBB I produced higher solvent concentrations at higher

productivity than the Schott bottle fermentation. ABE fermentation in the agitated Schott bottle produced a total of 9.62 gL^{-1} of solvents, which represented half of what had been produced in BOBB I, with only one-third the productivity of BOBB I.

Due to the shortcomings of the BOBB I design for bioreaction purposes, it was hard to maintain sterility throughout the course of the fermentation, over time, as the reactor was prone to contamination and experienced material degradation over time. BOBB I was built mostly from polyvinyl chloride (PVC) that cannot be sterilised using autoclave and chemically degraded over time. From here on, it was decided to build a second generation of oscillatory flow reactor known as “BOBB II”.

5.3 ABE Fermentation in BOBBs II and STR vs. Power Density

ABE fermentations were carried out in BOBB II to evaluate its performance. Batch fermentations of ABE were performed in reactors of the BOBB II design and the STRs at increasing power density with 1.5 L working volume as summarized in Table 5.4. The cultivation protocol and conditions are detailed in section 3.4.

Table 5.4 Matrix of batch fermentation in BOBB II and the STR

Power density (Wm^{-3})	Bioreactors BOBB II / STR	
0	BOBB Re_o 0	STR 0 rpm
0.02	BOBB Re_o 470	STR 18 rpm
0.14	BOBB Re_o 938	STR 35 rpm
1.14	BOBB *Reo* 1870	STR 70 rpm

Operation of BOBB II at Re_o 1870 is the maximum that can be achieved using the current oscillator system. This will give a power density of 1.14 Wm^{-3} calculated using Equation 2.5 in Chapter 2, while the power density of the STR was calculated using an equation in Ni *et al.* (1995a). All calculations can be seen in Appendix A.

Initially, two pH/redox probes were placed at the middle and bottom of the column to show that the system is well mixed. From the initial experiment showed that the pH reading was fairly the same between these two probes showing that the liquid in BOBB II was well mixed as shown in Figure 5.2. It was decided that for upcoming experiment run, only one pH/redox probe is needed, which placed at the middle of the column.

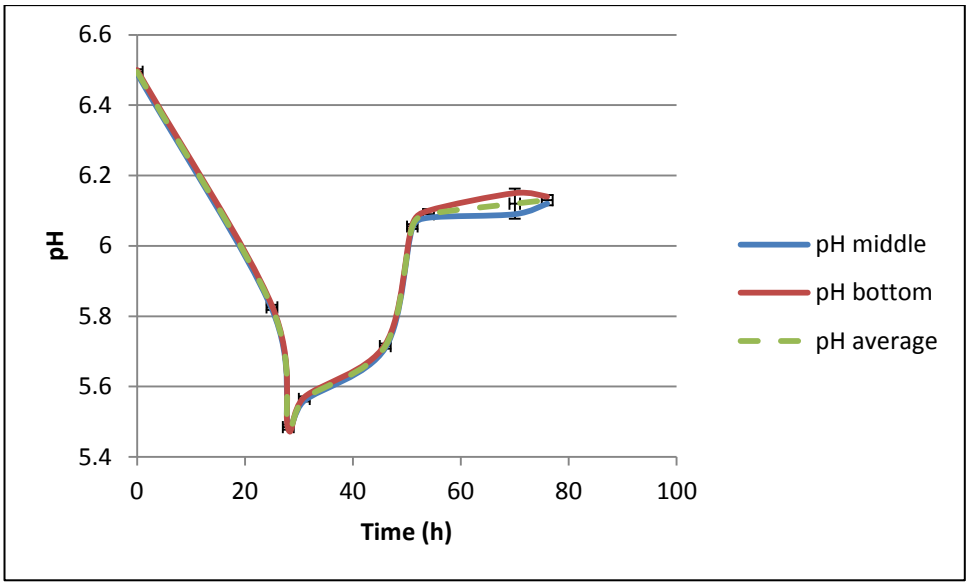


Figure 5.2 pH reading from two probes placed at the middle and bottom of BOBB II column. BOBB II was oscillated at Re_o 1870

5.3.1 ABE fermentation in BOBB II at increasing power density

The ABE fermentation profiles in BOBB II at different oscillatory flow Reynolds number (Re_o) are shown in Figure 5.3. The fermentation can be divided into two distinct phases: acidogenesis and solventogenesis. The grey band in Figure 5.3 marks the solvent shift (butanol concentration > 0.1 gL^{-1}). Acetate and butyrate were produced during the acidogenesis, while acetone, butanol and ethanol were produced during solventogenesis. Over the first 12 h of the fermentation, in all fermentations, acids were rapidly produced, causing the pH to decrease from the initial set-point of 6.5. Cell concentration increased exponentially during acidogenesis before reaching its stationary phase at ~24 h, except for Re_o = 0 (Figure 5.3a), where no stationary phase was observed. An important trend observed here was that the total solvent concentration decreased with increasing Re_o, with the maximum solvent concentration obtained at Re_o 0 with 11 gL^{-1} (Figure 5.3a) at 96 h. On the other hand, the total organic acid produced during acidogenesis was directly proportional to the Re_o where the maximum acid concentration produced, at the highest Re_o (1870), was triple the lowest Re_o (Re_o 0) (Figure 5.3d). Another important trend observed here was the solvent production rate.

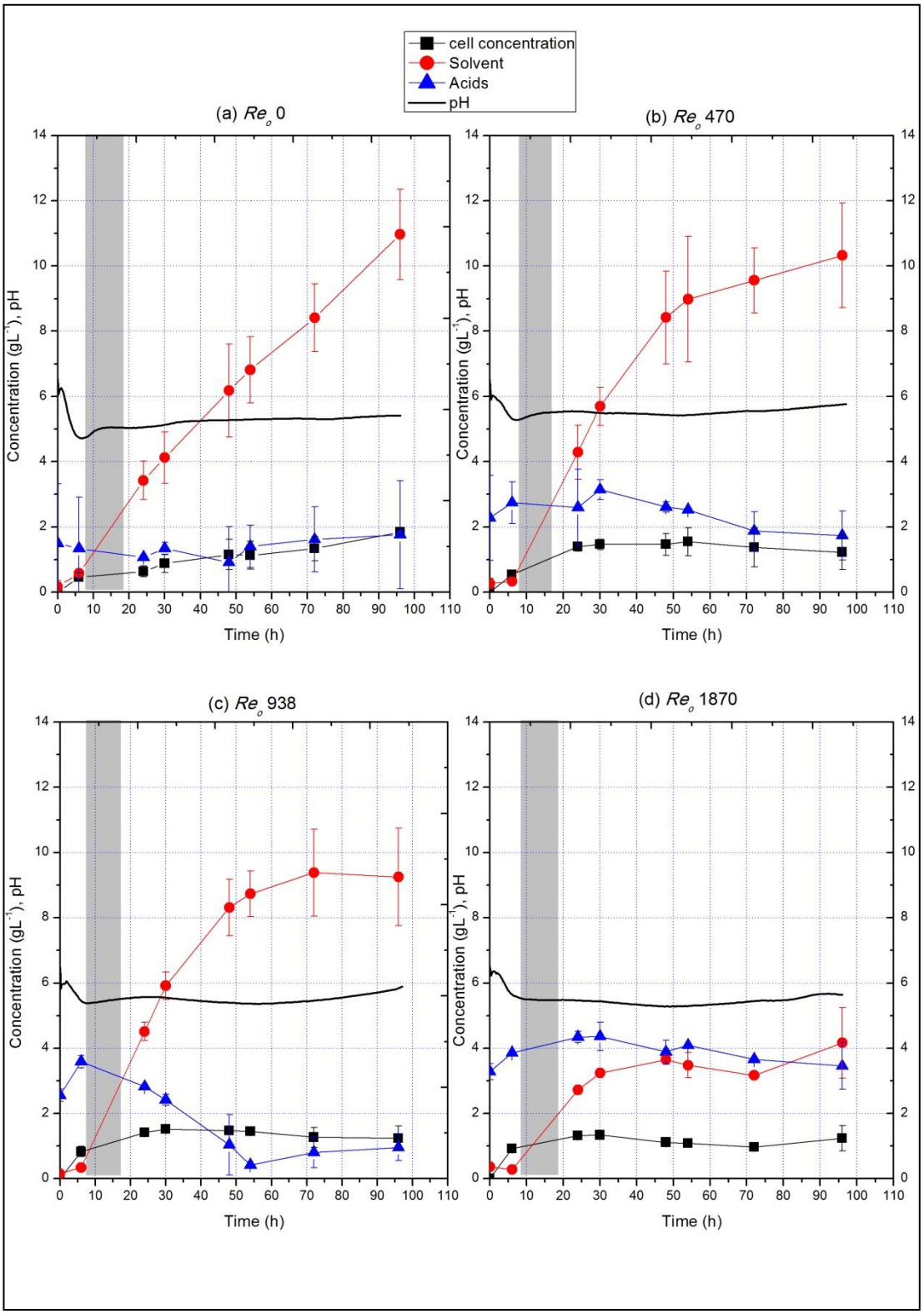


Figure 5.3 Batch ABE fermentation profile in BOBB II at various oscillatory Reynolds number (Re_o). The grey band marks the solvent shift (butanol concentration > 0.1 gL^{-1}). Error bars represent standard deviation from the mean of duplicate experiments

As the Re_o increased, the time for the solvent to reach its maximum concentration was shortened, which resulted in increased solvent productivity as Re_o increased.

Data on ABE fermentations in BOBB II at an increasing power densities from 0 to 1.14 Wm^{-3} are presented in Table 5.5 (next page) at 30 h fermentation time. The 30 h fermentation time is shown as it was typically where the solvent productivity was the highest in all BOBB II fermentations. From Table 5.5, the total ABE concentration increased as the power density increased within the studied range, except at 1.14 Wm^{-3}. Solvent concentration increased by 43% from 4.12 gL^{-1} ABE produced at Re_o 0 when the oscillation increased to 5.91 gL^{-1} at Re_o 938. This gave the highest volumetric solvent productivity of 0.20 g ABE $L^{-1}h^{-1}$ achieved at Re_o 938 with a yield of 0.28 g ABE (g glucose consumed)$^{-1}$. The fermentation at Re_o 938 produced a total of 5.91 gL^{-1} ABE where: 1.92 gL^{-1} acetone, 3.90 gL^{-1} butanol and 0.09 gL^{-1} ethanol. At Re_o 938, about 41% of the glucose provided was consumed to produce cells, organic acids and solvents. In addition to the increase in the solvent productivity, the specific growth rate of *Clostridium* GBL1082 also increased with increasing Re_o, to its maximum value of 0.288 h^{-1} at Re_o 938.

Table 5.5 performance of ABE fermentation in BOBB II at various power densities

Power density (Wm^{-3})	0	0.02	0.14	1.14
Oscillatory Reynolds number (Re_o)	BOBB II Re_o 0	BOBB II Re_o 470	BOBB II Re_o 938	BOBB II Re_o 1870
Fermentation time (h)	30	30	30	30
Acetone (gL^{-1})	1.42±0.28	1.76±0.05	1.92±0.15	0.85±0.19
Butanol (gL^{-1})	2.63±0.49	3.83±0.51	3.90±0.27	2.31±0.62
Ethanol (gL^{-1})	0.08±0.022	0.10±0.03	0.09±0.007	0.08±0.15
Acetate (gL^{-1})	0.78±0.88	3.06±0.19	2.30±0.17	3.57±0.07
Butyrate (gL^{-1})	0.26±0.28	0.08±0.11	0.12±0.002	0.80±0.65
Total ABE (gL^{-1})	4.12	5.69	5.91	3.24
A:B:E ratio	18:33:1	18:38:1	21:43:1	11:29:1
Total acid (gL^{-1})	1.35	3.15	2.42	4.36
Cells (gL^{-1})	0.88±0.27	1.46±0.16	1.52±0.10	1.34±0.15
Specific growth rate, μ (h^{-1})	0.0154	0.0525	0.288	0.285
Initial glucose (gL^{-1})	54.7±0.73	48.5±0.73	51.3±0.84	48.1±5.84
Glucose utilized (%)	31%	45%	41%	23%
ABE productivity ($gL^{-1}h^{-1}$)	0.14	0.19	0.20	0.11
ABE yield [g ABE(g glucose consumed)$^{-1}$]	0.25	0.26	0.28	0.29

On the other hand organic acid concentrations were highest at the highest Re_o (1870) suggesting an inefficient conversion of organic acids to solvents during solventogenesis. This was probably caused by unfavourable growth conditions due to high mixing intensity rather than product toxicity. It is unlikely to be due to product toxicity, as all metabolic activity including glucose uptake, acid production and solvent production would cease (Maddox *et al.*, 2000), since butanol concentration at Re_o 1870 was only 2.31 gL^{-1} which is well below the toxicity level. The butanol toxicity level in the ABE fermentation as reported by Jones and Woods (1989) is between 12 and 16 gL^{-1}.

Figure 5.4, shows the butanol productivity in BOBB II at different oscillatory Reynolds number (Re_o).

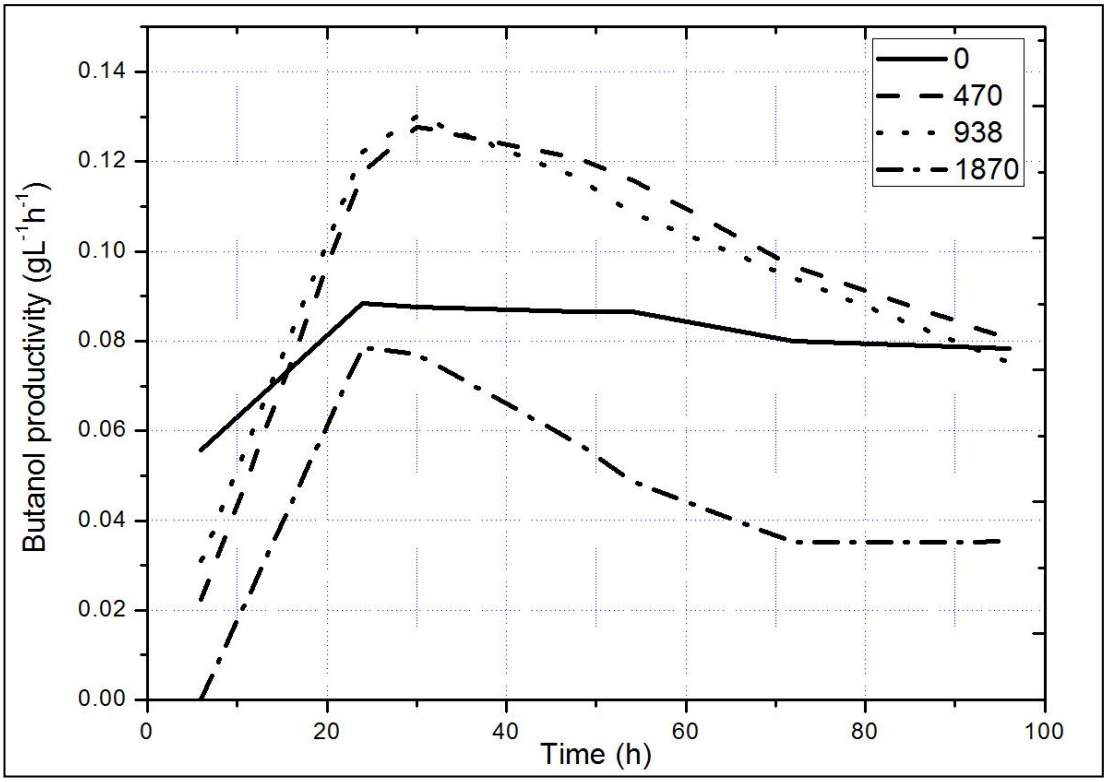


Figure 5.4 Butanol productivity in BOBB II at different Re_o. Butanol productivities were calculated as a function of time from butanol concentration data taken during *Clostridium* GBL1082 fermentation in BOBB II.

This agrees with the mixing effect reported by Yerushalmi and Volesky (1985) for *C. acetobutylicum,* where an increase in mixing rate increased the specific rates of solvent production. Figure 5.4 also shows that a well-defined time period exists during which butanol production was at a maximum when BOBB II was under oscillation. Higher productivity of butanol was observed at Re_o 470 and 938 than at Re_o 0 and 1870. This effect is caused by the enhancement of the extracellular mass transfer of the nutrients and metabolites in oscillated BOBBs II which reflected by increased in biomass concentration. However, the lowest butanol productivity was observed at the highest Re_o (1870) within the studied range. This was probably caused by unfavourable growth conditions for the cell as a result of vigorous mixing rather than product toxicity. Yerushalmi and Votruba (1985) reported that intensive mixing could hinder solvent production and even terminated the fermentation. Vigorous mixing may damage the cell wall and/or membrane of the microorganism

at certain times during the fermentation. The enzyme system responsible for converting butyrate to butanol seems to be associated with the cell membrane, thus damage to the cell membrane could change the enzyme activities (Welsh and Veliky, 1984). This may resulted in hindering the solvents production or even completely stop the fermentation.

Reduction in butanol productivity was observed for all Re_o as the fermentation progressed. At the point of maximum butanol productivity for Re_o 938, for example, the glucose concentration in the media was 29.6 gL^{-1}. The butanol concentration at that point was only 3.90 gL^{-1} which, again, makes it unlikely that butanol toxicity caused the decline in productivity. It was pointed out by Yerushalmi and Volesky (1985) that increasing the agitation to 410 rpm resulted in a decrease in the final solvent concentration and, when further increase to 560 rpm, led to complete loss of solvent production. They concluded that low net production of the solvents could be caused by their accumulation at intracellular level. Increased level of these products, particularly butyrate and butanol, will thus result in inhibition of the cellular metabolism.

5.3.2 ABE fermentation in STR at increasing power density

Figure 5.5 (next page) shows the ABE fermentation profile in the STR as a function of agitation rate. During acidogenesis, the fermentation produced organic acids (acetate and butyrate) which reduced the pH culture to approximately pH 5.0. *Clostridium* GBL1082 grew exponentially during this phase before reaching the stationary phases. The highest solvent concentration was obtained at 18 rpm with 12.4 gL^{-1} (Figure 5.5b).

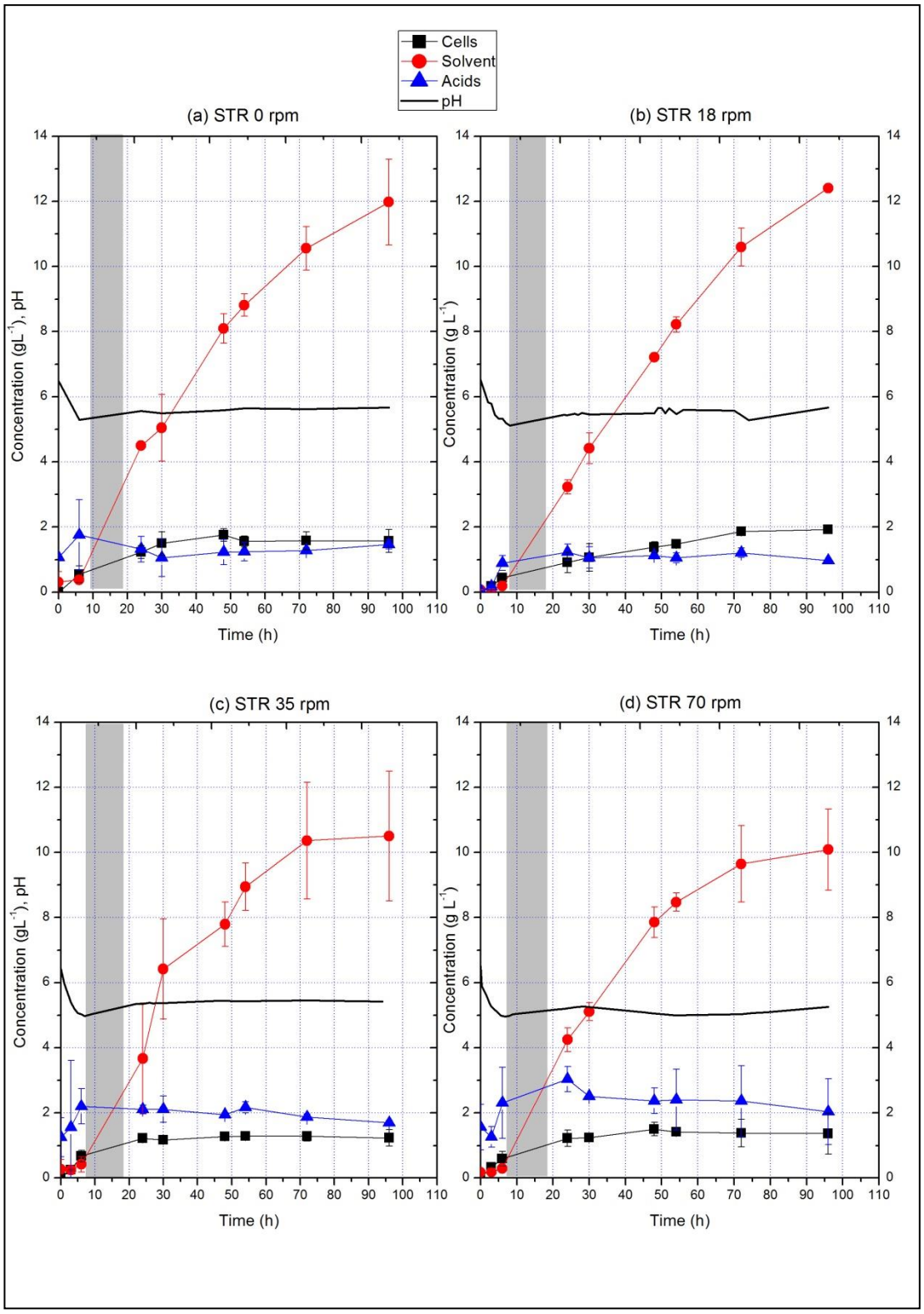


Figure 5.5 Batch ABE fermentation profile in STR at various agitation rate. The grey band marks the solvent shift (butanol concentration > 0.1 gL^{-1}). Error bars represent standard deviation from the mean of duplicate experiments

It can be observed here that at the higher agitation rates of 35 rpm and 70 rpm (Figure 5.5c and d), the total organic acid concentrations were almost doubled those at the lower agitation rate (0 and 18 rpm, Figure 5.5a and b). This was due to inefficient conversion of acids into solvents, resulting in an accumulation of acids in the culture broth.

In an anaerobic fermentation, agitation is mainly required for maintaining a homogenous solid-liquid suspension and to ensure good mass transfer in (nutrients) and out (metabolites) of the clostridia cell (Yerushalmi and Volesky, 1985). From the study by Yerushalmi and Volesky (1985) in a STR with a ~7 times larger working volume than in this study, an increase in the rate of agitation from 190 rpm to 410 rpm was shown to reduce the time required for the completion of *C. acetobutylicum* fermentation. However, further increases to 560 rpm generally had completely stopped the fermentation. The maximum agitation rate used in this study was about one-third (70 rpm) of their minimum rate.

The performance of ABE fermentations in the STR at different agitation rate is shown in Table 5.6. These results are based on the highest ABE productivity achieved in each of the fermentation. From the table, it is clear that the solvent concentrations increased as the agitation rate increased, except at 1.14 Wm^{-3}. The solvent concentration was the highest at 35 rpm with 6.42 gL^{-1} ABE, 2.67 gL^{-1} acetone, 3.49 gL^{-1} butanol and 0.25 gL^{-1} ethanol. A significant trend that can be seen from the table is that the percentage of glucose utilization decreased as the agitation rate increased, with the maximum of 40% glucose consumption at 0 agitation rate implies that the cells are more focussed on solvent production at higher agitation. This led to higher solvent yields in the STR than in BOBB II, with the highest yield obtained at 35 rpm with 0.47 g ABE (g glucose)$^{-1}$ at a solvent production rate of 0.21 g ABE $L^{-1}h^{-1}$.

Table 5.6 The performance of ABE fermentation in the STR at various agitation rates

Power density (Wm^{-3})	0	0.02	0.14	1.14
Agitation rate	STR 0 rpm	STR 18 rpm	STR 35 rpm	STR 70 rpm
Fermentation time (h)	30	30	30	30
Acetone (gL^{-1})	1.59±0.25	1.42±0.16	2.67±1.20	1.75±0.032
Butanol (gL^{-1})	3.25±0.67	2.85±0.25	3.49±0.24	3.23±0.23
Ethanol (gL^{-1})	0.21±0.10	0.15±0.06	0.25±0.09	0.13±0.013
Acetate (gL^{-1})	1.05±0.58	1.01±0.26	2.03±0.40	2.34±0.28
Butyrate (gL^{-1})	0±0	0.05±0.08	0.09±0.005	0.17±0.23
Total ABE (gL^{-1})	5.04	4.42	6.42	5.11
A:B:E ratio	8:15:1	9:19:1	11:14:1	13:25:1
Total acid (gL^{-1})	2.42	1.06	2.11	2.51
Cells (gL^{-1})	1.49±0.36	1.06±0.42	1.17±0.10	1.24±0.12
Specific growth rate, μ (h^{-1})	0.0279	0.0368	0.282	0.282
Initial glucose (gL^{-1})	55.0±6.67	48.7±0.28	49.0±0.56	47.1±2.79
Glucose utilized (%)	40%	31%	29%	26%
ABE productivity ($gL^{-1}h^{-1}$)	0.17	0.15	0.21	0.17
ABE yield [g ABE (g glucose consumed) $^{-1}$]	0.25	0.30	0.47	0.43

5.3.3 Comparisons between BOBB II and STR

Comparisons of the ABE fermentation performance in the BOBB II and the STR as a function of the power density are shown in Figure 5.6 (next page). From the graphs, it can be seen that the fermentation rate was a function of power density for both BOBB II and the STR. The specific growth rate (μ) increased in both bioreactors as the power density increased (Figure 5.6a). It can be seen that an increase in mixing rate (indicated by an increase in power density) improved the clostridia specific growth rate, with BOBB II supporting the highest specific growth rate of 0.288 h^{-1} at a power density of 0.14 Wm^{-3}. As for the glucose consumption rate, fermentation in BOBB II resulted in glucose consumption of 31-63% higher than the STR (Figure 5.6b).

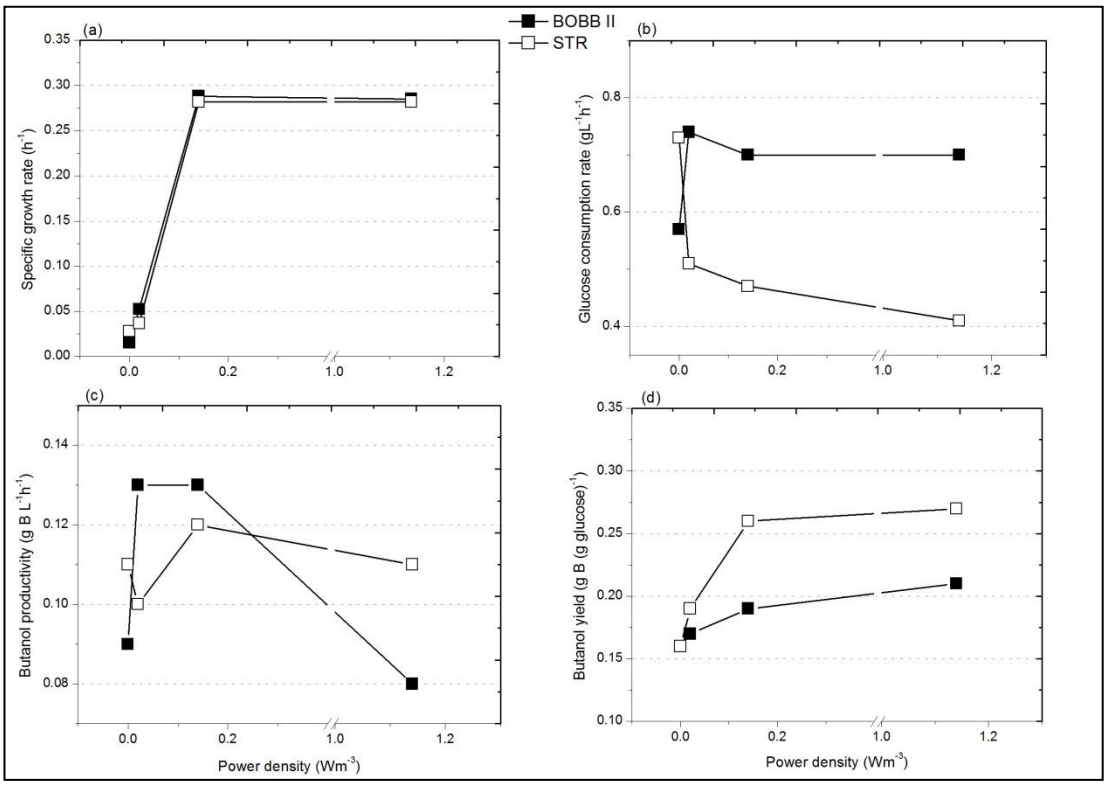


Figure 5.6 Comparison of the ABE fermentation in BOBB II and the STR. (a) Specific growth rate; (b) Glucose consumption rate; (c) Butanol productivity; (d) Butanol yield based on glucose consumed. Error bars represent standard deviation from the mean of duplicate experiments

Butanol productivity was significantly increased to the maximum value of 0.13 $gL^{-1}h^{-1}$ in BOBB II, as the power density increased, but decreased sharply at the highest power density (Figure 5.6c) to 0.08 $gL^{-1}h^{-1}$. Meanwhile in the STR, the butanol productivity remains fairly constant between 0.10 and 0.12 $gL^{-1}h^{-1}$ (Figure 5.6c). With regards to the high glucose consumption rate in BOBB II at higher power densities (Figure 5.6b), it seems that it was not used for butanol production but rather used for the cell and acids formation, as the butanol yield was 22% lower than that in the STR (Figure 5.6d). Consequently, the highest butanol yield was achieved in the STR with 0.27 g butanol $(g\ glucose)^{-1}$, obtained at the highest power density within the studied range.

The high glucose consumption rate in BOBB II was associated with the high cell growth and high acid production during acidogenesis as shown in Figure 5.7 below.

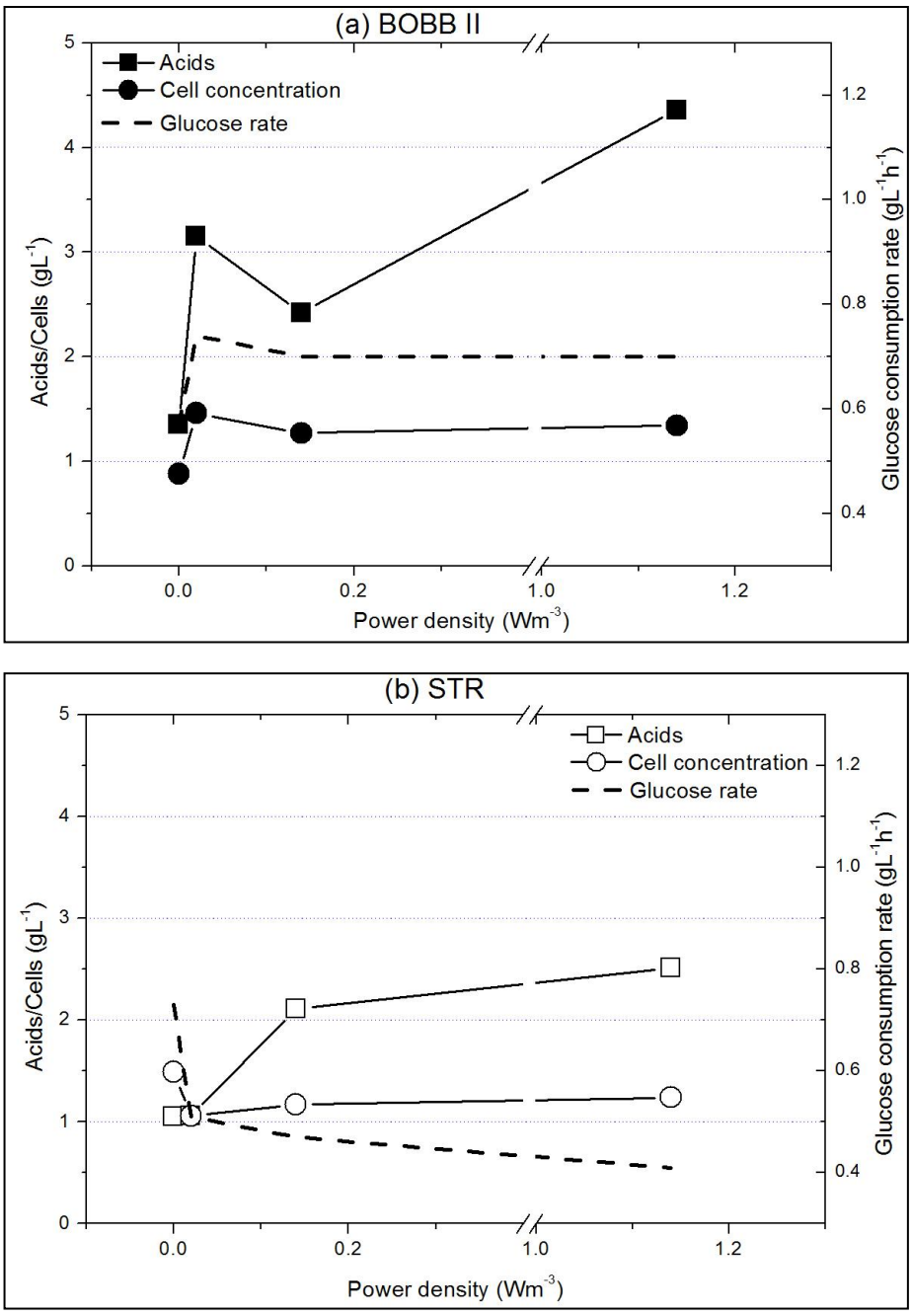


Figure 5.7 Acid and cell production, and glucose consumption rate in (a) BOBB II and (b) the STR

From Figure 5.7a, it can be observed that in BOBB II the glucose consumption rate was approximately 0.7 $gL^{-1}h^{-1}$ at power densities between 0.02 and 1.14 Wm^{-3}. The high glucose consumption rate was observed to coincide with high concentration of the cell (about 1.3 gL^{-1}) and organic acids (more than 2.4 gL^{-1}). Different scenarios were observed in the STR fermentation, where the glucose consumption rate was significantly lower than in the BOBB II. The glucose consumption rate sharply decreased between 0 and 0.02 Wm^{-3}, and gradually decreased afterwards. Lower acid production was also observed in the STR during acidogenesis than in BOBB II (less than 2.5 gL^{-1}).

These observations in BOBB II agree with those by Yerushalmi and Volesky (1985) where they revealed that the specific consumption rate of the substrate (g glucose $gcell^{-1}$ h^{-1}) increased with the increasing cell specific growth rate. The cell utilized the substrate more efficiently when its physiological activity, reflected by its specific growth rate, increased. They showed increasing the mixing intensity from 340 rpm to 410 rpm, accelerated microbial activity, resulting in increased specific growth rate. In contrast with the STR fermentation in this study, even though the specific growth rate increased increasing mixing intensity, the glucose consumption rate was lower than in BOBB II at similar power density. Based on Figure 5.7 it can be concluded that more glucose was consumed in BOBB II than in the STR to produce more acid, suggesting higher glucose consumption rate in BOBB II than the STR.

Figure 5.8 (next page) shows the comparison of solvent production per unit energy between BOBB II and the STR. The highest specific solvent production was achieved in BOBB II at power density of 0.02 Wm^{-3}, with 12 g ABE $(kWh)^{-1}$. This is 60% higher than the maximum achieved in the STR at the same power density.

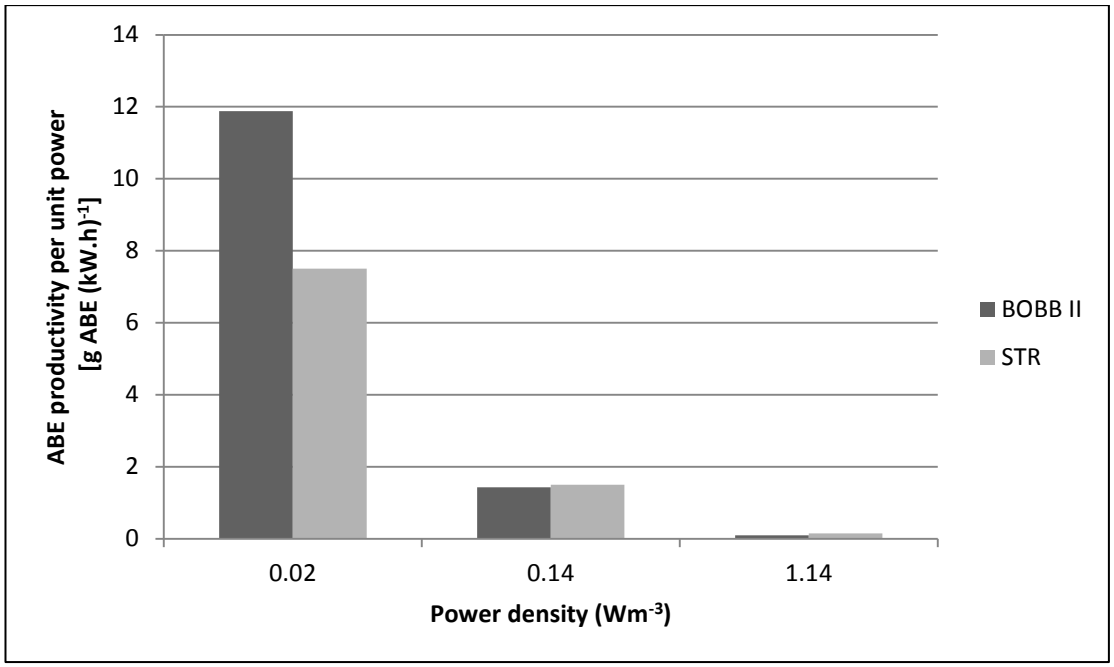


Figure 5.8 Solvent production per unit energy with respect to BOBB II and STR at various power densities.

Increasing the power density further resulted in a significant decrease in solvent concentration in both reactors, to below 2 g ABE $(kWh)^{-1}$, representing an 84% decrease from the maximum concentration produced in BOBB II at higher power density. Further decreases in the solvent concentration were observed as the power density was increased to 1.14 Wm^{-3}, to approximately only 0.1 g ABE$(kWh)^{-1}$ produced.

Higher solvent concentration per unit energy in BOBB II fermentation would mean less energy used for the same amount of ABE produced than in the conventional stirred vessel. This would have an impact on the ABE production cost particularly at larger production scales. It is an advantage of the OBBs that they are scalable: productivity at this laboratory scale can be reproduced at the industrial scale (Smith and Mackley, 2006). The same cannot be said for conventional stirred vessels, in which the reaction time increases with scale up, as good mixing and efficient heat and mass transfer becomes increasingly difficult to achieve and it is

typically only achieved at the expense of substantially higher energy usage than in OBBs.

5.3.4 ABE fermentation in BOBB II and the STR at 0 Wm^{-3}

The results shown in Figure 5.9 below, represent the ABE fermentations in different bioreactors at power densities of 0 Wm^{-3} *i.e*. without mixing. Data presented here is a repeat of previous data, represented differently. Figure 5.9a and b show the batch ABE fermentation profile in BOBB II and STR, respectively over a 96 h fermentation run. Both fermentations were carried out simultaneously using the same batch of inoculum at the same initial pH of 6.5 and temperature of 32^{o}C. The time course of the fermentation run can be divided into two distinct phases, acidogenesis and solventogenesis: the grey band indicates acidogenesis.

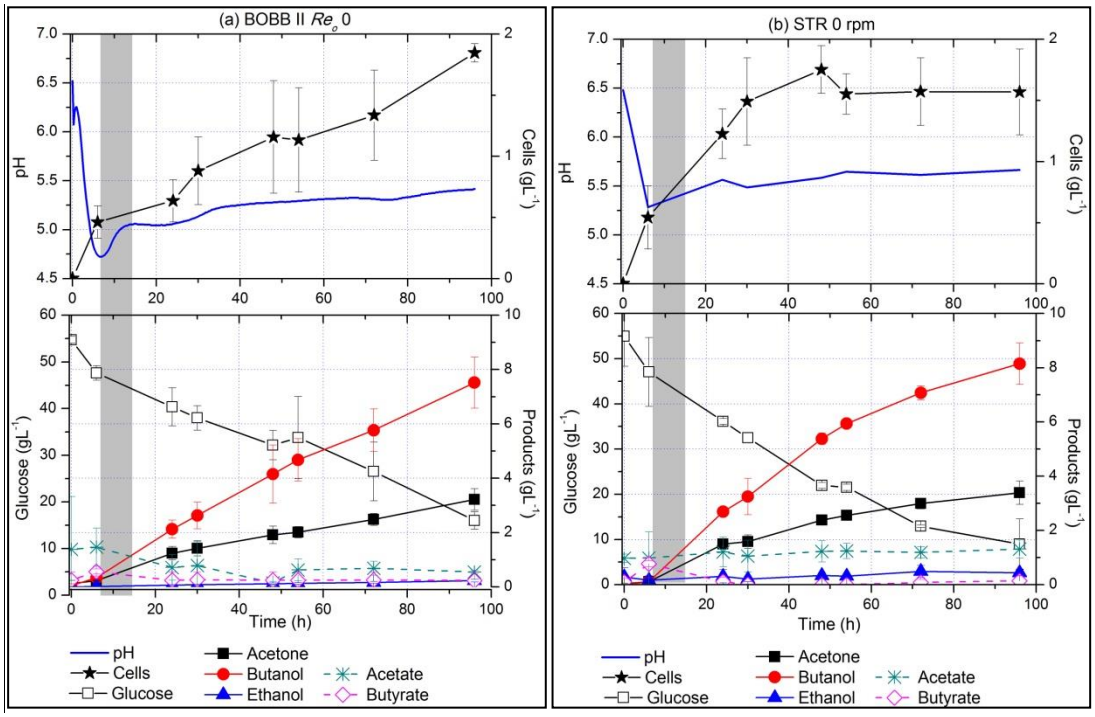


Figure 5.9 ABE fermentation profile at 0 Wm^{-3} in (a) BOBB II and (b) STR. The grey band marks the solvent shift (butanol >0.1 gL^{-1})

It has been anticipated that any significant discrepancies between the bioreactors would be mainly due to the difference in bioreactor configuration, as there was no mechanical mixing involved during the fermentation. In BOBB II (Figure 5.9a), *Clostridium* GBL1082 cells grew at a specific growth rate of 0.02 h^{-1} and there was no evidence of a stationary growth phase or a death phase being achieved. The maximum cell concentration in BOBB II was 1.85 gL^{-1} at 96h fermentation time. In the STR (Figure 5.9b), cells grew exponentially until 48 h at higher specific growth rate (0.0279 h^{-1}) than in the BOBB II. The maximum cell concentration achieved in the STR was 1.75 gL^{-1}. Acidogenesis occurred for the first 12 h, where organic acids (acetate and butyrate) were produced and the pH decreased from 6.5 to 4.7 and 5.3 in BOBB II and the STR, respectively. During acidogenesis, BOBB II produced a maximum of 1.49 gL^{-1} of total organic acids (Figure 5.9a), while the STR produced 1.75 gL^{-1} acids (Figure 5.9b). Greater acid concentration in the STR (1.75 gL^{-1})than in BOBB II (1.35 gL^{-1}) during acid production phase was believed to induce early solventogenesis in the STR, as time to reach acid threshold for phase shifting was shorter than in BOBB II. This was proved by higher solvent productivity in the STR than in BOBB II at corresponding fermentation time (30 h shown in Table 5.7).

Table 5.7 (next page) shows various data for the ABE fermentations in BOBB II and the STR at 0 Wm^{-3} at the highest ABE productivity achieved within the course of the fermentation. The total solvent produced in BOBB II was 4.12 gL^{-1} made up of 1.42 g/L acetone, 2.63 g/L butanol and 0.08 g/L ethanol (Table 5.7). In the STR, the total solvent produced was 5.04 gL^{-1} with 1.59 gL^{-1} acetone, 3.25 gL^{-1} butanol and 0.21 gL^{-1} ethanol. At that time, the residual glucose concentration in BOBB II and STR were about 38 gL^{-1} and 33 gL^{-1}, respectively. The STR exhibited higher total volumetric solvent productivity (0.17 g ABE $L^{-1}h^{-1}$), with butanol productivity of 0.12 g butanol $L^{-1}h^{-1}$. This represents 25% higher butanol productivity than in BOBB II. Both bioreactors exhibited similar solvent yields based on glucose consumed which was 0.25 g ABE (g glucose)$^{-1}$ with butanol yield of 0.17 g butanol (g glucose)$^{-1}$.

Solvent producing clostridia follow metabolic pathways such that acid is produced earlier followed by switching to solvent production (Jones and Woods, 1986).

Table 5.7 ABE fermentations in BOBB II and STR at 0 Wm^{-3} and 30 h fermentation

Parameter	Bioreactors	
	BOBB II Re_o **0**	**STR 0 rpm**
Fermentation time (h)	30	30
Acetone (gL^{-1})	1.42±0.28	1.59±0.25
Butanol (gL^{-1})	2.63±0.49	3.25±0.67
Ethanol (gL^{-1})	0.08±0.02	0.21±0.10
Acetate (gL^{-1})	0.78±0.88	1.05±0.58
Butyrate (gL^{-1})	0.26±0.28	0±0
Total ABE (gL^{-1})	4.12	5.04
Total acid (gL^{-1})	1.35	1.05
Cell concentration (gL^{-1})	0.88±0.27	1.49±0.36
Specific growth rate (h^{-1})	0.0154	0.0279
Initial glucose (gL^{-1})	54.7±0.73	55.0±6.67
Residual glucose (gL^{-1})	38.0±2.62	32.5±1.12
ABE productivity ($gL^{-1}h^{-1}$)	0.14	0.17
ABE yield [g ABE (g glucose consumed) $^{-1}$]	0.25	0.25
Butanol productivity ($gL^{-1}h^{-1}$)	0.09	0.16
Butanol yield [g B (g glucose consumed) $^{-1}$]	0.16	0.11

In commercial ABE fermentations, for example at National Chemical Products, South Africa, no mechanical mixing is involved. It is claimed that sufficient mixing can be achieved by gas evolution during clostridia metabolism (Maddox, 1989). However, there is always a problem if a large tank is used, as stagnant or death zones might exist within the vessel in a poorly agitated STR. Insufficient mass transfer of the nutrients in and out of the microbial cell might limit cell growth, thereby reducing the bioreactor productivity. In addition, limited heat transfer will cause temperature gradients within the vessel, which would have an adverse effect on cell growth and the ability to tolerate butanol (Baer *et al.,* (1987). Baer *et al*.'s

studies (Baer *et al.*, 1987) on the effects of butanol concentration and growth temperature on the membrane composition and fluidity of *C. acetobutylicum* found that since both butanol and higher temperature (they used 42^{o}C) both increase the cell membrane fluidization, a combination of these would be expected to have an even greater negative effect on the clostridia cell. On the other hand, lower growth temperatures within their studied range (22^{o}C and 37^{o}C) would be expected to counteract the fluidizing effect of butanol on the membrane, since lower growth temperatures tend to solidify the membrane bi-layer and improve the ability of the cell to tolerate butanol toxicity (Baer *et al.*, 1987). They concluded that, by maintaining the optimum clostridia growth temperature throughout the bioreactors, the toxic effect of butanol may be offset.

As there was no mixing involved in the ABE fermentation in BOBB II and the STR, significant fluctuation in the temperature profile was observed during the course of the fermentation time, especially in BOBB II as shown in Figure 5.10 below.

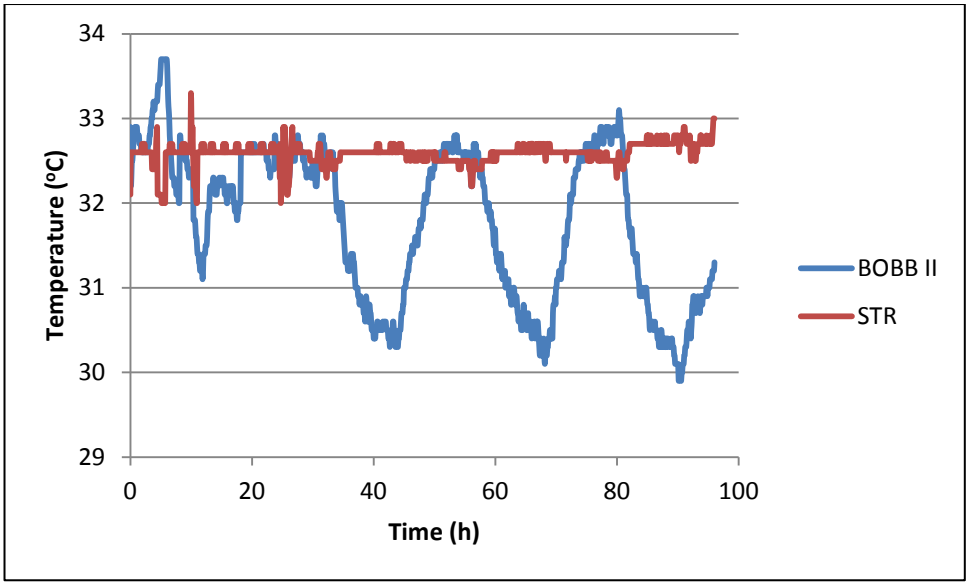


Figure 5.10 Temperature profile during ABE fermentation in BOBB II and the STR without mixing

Unlike the STR that had a well-developed temperature control, BOBB II, as the first iteration of a reactor design manufactured in-house, had a less developed design, and lacked proper temperature control. The temperature was manually controlled by changing the water bath temperature supplying hot water to the silicone tube that coiled around the BOBB II glass column. From Figure 5.10, a temperature drop was observed at three intervals (36-48 h, 60-72 h and 84-96 h), corresponding to night-time. A subsequent set of experiments were performed with mixing in both bioreactors (BOBB II and the STR) to evaluate the effect of mixing on heat and mass transfer on the cell growth and solvent production, generally to improve the solvent productivity in the ABE fermentation.

5.3.5 ABE fermentation in BOBB II and the STR at 0.02 Wm^{-3}

Figure 5.11 shows the results from ABE fermentation in BOBB II and the STR at a power density of 0.02 Wm^{-3}. The fermentations were carried out at the same initial pH of 6.5 and a temperature of 32^{o}C. Data presented here is a repeat of previous data, represented differently. Cell growth, pH, residual glucose, solvent and acid concentrations were monitored during the fermentation. Cell growth in BOBB II and the STR exhibited exponential rapid increase with almost no lag phase (Figure 5.11). The specific growth rate in BOBB II (0.053 h^{-1}) was higher than the STR (0.040 h^{-1}). Consequently, the time for growth in BOBB II to reach stationary phase was only 24 h, compared to 72 h in the STR.

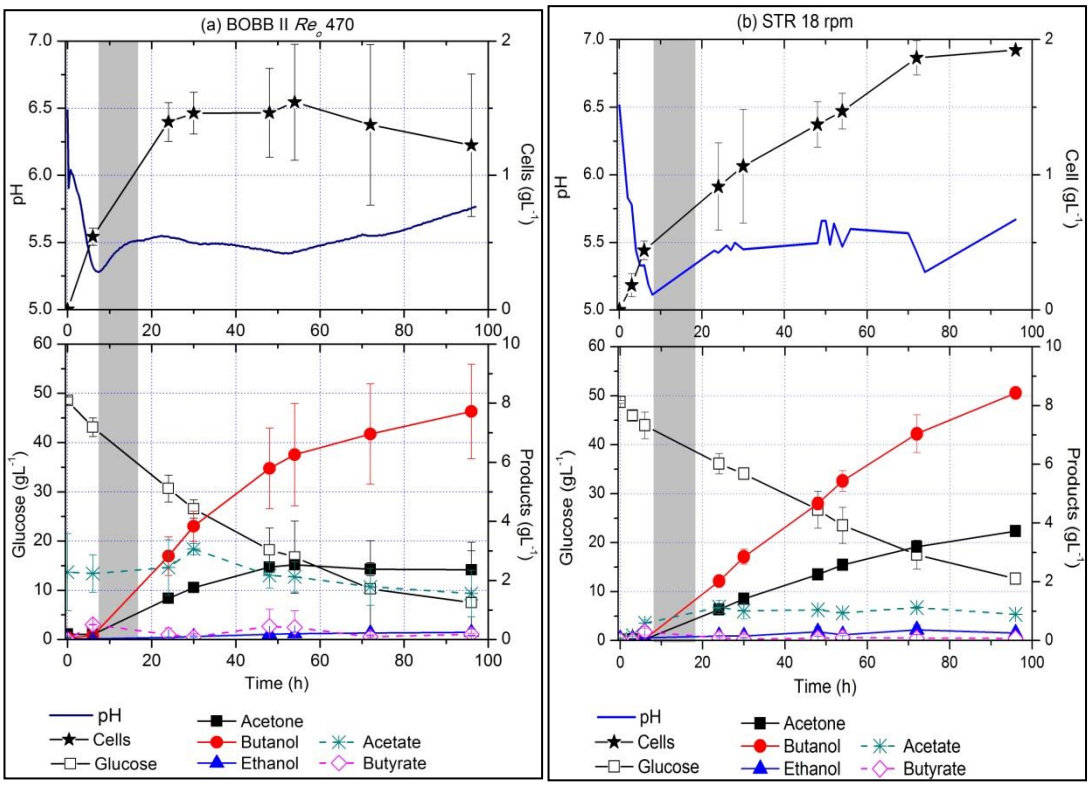


Figure 5.11 ABE fermentation profile at 0.02 Wm^{-3} in (a) BOBB II and (b) STR. The grey band marks the solvent shift (butanol >0.1 gL^{-1}). Error bars represent standard deviation from the mean of duplicate experiments

During the first 6 h, the pH of the culture in the BOBB II rapidly fell from 6.5 to 5.3 in response to exponential growth and the production of organic acids (acetate and butyrate, Figure 5.11a). As solventogenesis began at ~6h, the pH increased, as a result of the conversion of acids to solvents (Jones and Woods, 1986). The pH range stabilised in the range 5.4 to 5.6 after 24 h. The greatest increase in solvent production was in the butanol concentration between 6 and 72 h. This coincided with the pH readjustment, stabilization of cell concentration and a marked reduction in residual glucose. Total glucose, which was initially present at 49 gL^{-1} at the start of BOBB II fermentation, was reduced to 7.5 gL^{-1} after 96 h. This represents 85% utilization of glucose during this fermentation.

The pH drop in the STR was steeper than that in BOBB II (Figure 5.11b). The lowest pH of 5.1 at 8 h coincided with the onset of solvent production. Interestingly,

cell growth increased monotonically until 72 h, before reaching the stationary phase, some 48h later than in the BOBB II. The final solvent concentration in the STR was 12.41 gL^{-1}, 20% higher than that produced in BOBB II (10.32 gL^{-1}). The residual glucose in the STR was 12.6 gL^{-1} after 96 h, representing 74% utilization.

Table 5.8 represents the results of ABE fermentation in BOBB II and the STR based on the highest solvent productivity achieved in both reactors, which was at 30 h. Based on Table 5.8, solvents were produced at a high productivity of 0.19 $gL^{-1}h^{-1}$ in BOBB II, leading to 5.69 gL^{-1} of total solvent, where 3.83 gL^{-1} was butanol, 1.76 gL^{-1} acetone and 0.10 gL^{-1} ethanol. Solvent productivity in the STR was 21% lower (0.15 $gL^{-1}h^{-1}$) than in BOBB II, produced a total of 4.42 gL^{-1} solvent with 2.85 gL^{-1} butanol, 1.42 gL^{-1} acetone and 0.15 gL^{-1} ethanol. High cell and acid concentrations were observed in BOBB II with 1.46 gL^{-1} cells and 3.15 gL^{-1} acids, 51% and 197% higher than that in the STR, respectively (1.06 gL^{-1} cells and 1.06 gL^{-1} acids). However, a low solvent yield was observed in BOBB II, with 0.26 g ABE (g glucose)$^{-1}$ and 0.17 g butanol (g glucose)$^{-1}$**,** whereas in the STR the yields were 0.30 g ABE (g glucose)$^{-1}$ and 0.19 g butanol (g glucose)$^{-1}$. The decrease in solvent yield resulted from the increased glucose utilization required for cell growth and acid production, rather for the solvent (Qureshi *et al.*, 2000).

Table 5.8 ABE fermentation in BOBB II and STR at 0.02 Wm^{-3} and 30 h fermentation

Parameter	Bioreactors	
	BOBB II Re_o **470**	**STR 18 rpm**
Fermentation time (h)	30	30
Acetone (gL^{-1})	1.76±0.05	1.42±0.16
Butanol (gL^{-1})	3.83±0.51	2.85±0.25
Ethanol (gL^{-1})	0.10±0.03	0.15±0.06
Acetate (gL^{-1})	3.06±0.19	1.01±0.26
Butyrate (gL^{-1})	0.08±0.11	0.05±0.08
Total ABE (gL^{-1})	5.69	4.42
Total acid (gL^{-1})	3.15	1.06
Cells (gL^{-1})	1.46±0.16	1.06±0.42
Specific growth rate (h^{-1})	0.0525	0.0368
Initial glucose (gL^{-1})	48.5±0.73	48.7±0.28
Residual glucose (gL^{-1})	26.5±1.89	34.1±1.19
ABE productivity ($gL^{-1}h^{-1}$)	0.19	0.15
ABE yield [g ABE (g glucose consumed) $^{-1}$]	0.26	0.30
Butanol productivity ($gL^{-1}h^{-1}$)	0.13	0.10
Butanol yield [g B (g glucose consumed) $^{-1}$]	0.17	0.19

Solvent-producing clostridia follow metabolic pathways in which acid is produced first followed by switching to solvent production (Jones and Woods, 1986). It appears that the timing and magnitude of the switch depends on the pH and composition of the growth medium, as well as on the relative concentration of acetate and butyrate (Bahl *et al.*, 1982a; Matta-El-Ammouri *et al.*, 1987). The batch ABE fermentations were performed in BOBB II and the STR at a power density of 0.02 Wm^{-3} and the results indicated that BOBB II was more suitable for cell growth and production of butanol than the STR. Higher specific growth rate and higher final stationary-phase cell population in BOBB II resulted in a greater amount of butanol being produced more quickly than in the STR. It was demonstrated here, that attainment of low pH is a requirement for solvent production, with relatively high

concentrations of acetate and butyrate triggering solventogenesis as demonstrated in the ABE fermentation in BOBB II. This result agreed with the findings of Gottschal and Morris (1981), Bahl *et al.* (1982a) and Matta-El-Amouri *et al*. (1987), who concluded that the combination of low pH of the culture broth and sufficiently high concentration of acetate or butyrate, alone or together, have a more specific "triggering" effect on solvent production.

Higher acid concentrations in BOBB II earlier in the fermentation (~2.5 gL^{-1} between 0 h and 6 h) than in the STR suggested that the organic acids, acetate and butyrate are produced sooner in the fermentation. The more rapid onset of acid formation may be a major factor in the improved production of the secondary metabolite, butanol, during solventogenesis. Previous studies concluded that high concentrations of acetate and butyrate increased the rate of metabolic transition, the amount of solvent produced and the percentage of sugar consumption (Fond *et al.*, 1985), and altered the solvent ratio (Martin *et al.*, 1983; Matta-El-Ammouri *et al.*, 1987). Earlier commencement of butanol synthesis in BOBB II than the STR has important economic implications for the ABE fermentation.

5.3.6 ABE fermentation in BOBB II and the STR at 0.14 Wm^{-3}

The ABE fermentation profile in both bioreactors is shown in Figure 5.12 below. Fermentations were performed at similar power density of 0.14 Wm^{-3}, with initial pH of 6.5 and temperature of 32°C.

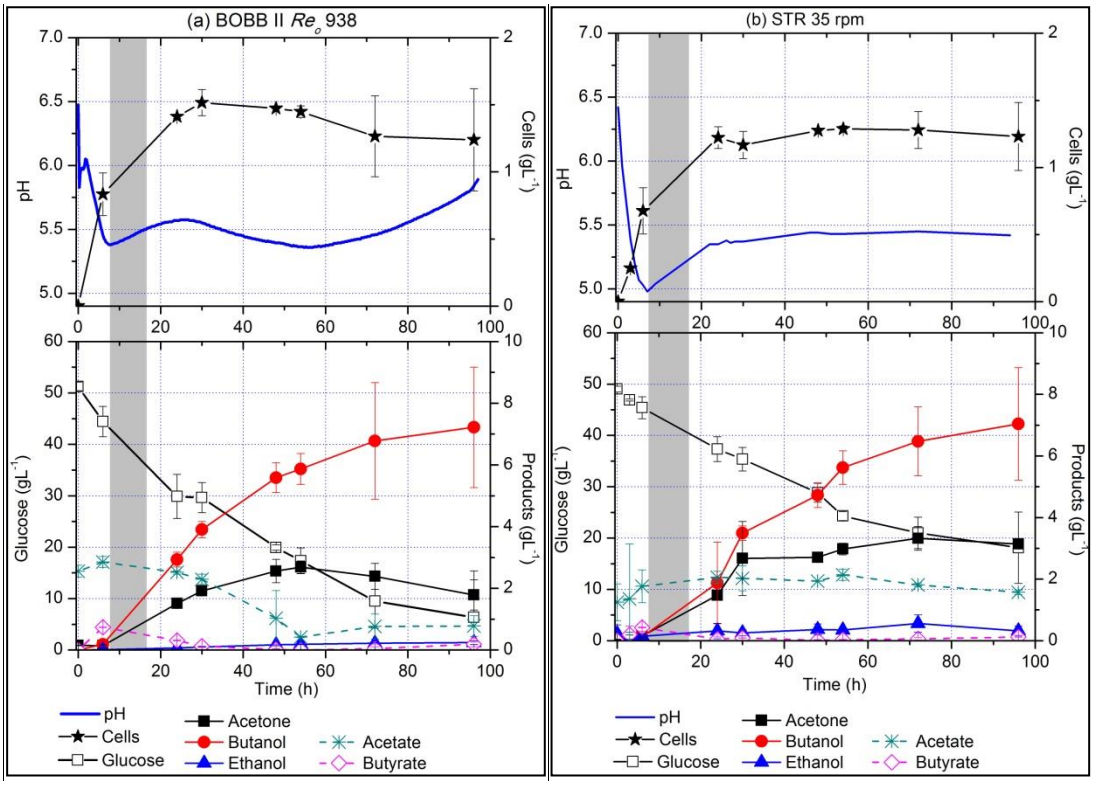


Figure 5.12 ABE fermentation profile at 0.14 Wm^{-3} in (a) BOBB II and (b) STR. The grey band marks the solvent shift (butanol >0.1 gL^{-1}). Error bars represent standard deviation from the mean of duplicate experiments

Cell growth, pH, residual glucose, solvent and acid concentrations were monitored during the fermentation. Cell growth in BOBB II and the STR were exponential and rapid with almost no lag phase (Figure 5.12). The specific growth rate in BOBB II and the STR was comparable (~0.28 h^{-1}). However, the time for growth in BOBB II to reach stationary phase was slightly longer (30 h), compared to 24 h in the STR.

During the first 6 h, the pH of the culture in the BOBB II fell rapidly from 6.5 to 5.4 in response to exponential growth and the production of organic acids (acetate and butyrate, Figure 5.12a). An increased in the pH caused by the conversion of the acids produced earlier was observed after ~6 h of fermentation which coincided with the beginning of solvent production. Within 6 to 72 h of fermentation, a significant increase in the solvent concentration corresponded with the pH readjustment, stabilization of cell concentration and a marked reduction in residual glucose. Total glucose, which was initially present at 51.3 gL^{-1} at the start of BOBB II fermentation, was reduced to 6.4 gL^{-1} after 96 h. This represents 88% utilization of glucose during this fermentation in BOBB II.

The pH drop was steeper in the STR than that in BOBB II (Figure 5.12b). The lowest pH of 4.98 at 7 h coincided with the initiation of solvent production. The final solvent concentration in the STR was 10.5 gL^{-1}, as opposed in BOBB II (9.2 gL^{-1}). The residual glucose in the STR was 18.1 gL^{-1} after 96 h, representing 63% utilization which was lower than the percentage of glucose consumption in BOBB II. Interestingly, it was observed in BOBB II that the acetone concentration started to decrease after 54 h (Figure 5.12a), after reaching the maximum acetone concentration (2.69 gL^{-1}). The acetone concentration decreased to 1.78 gL^{-1} at the end of fermentation, representing a 34% reduction in concentration. It may be that the mode of mixing was responsible for this condition. As acetone is the most volatile solvent among other products (butanol and ethanol), it was the most easily released from the fermentation broth. The agitation of the free surface by the vortex formed during the upward stroke of the oscillation may have accentuated this. Over the course of the fermentation, a relatively small amount of acetone (~1 gL^{-1}) was lost to the bioreactor headspace.

Table 5.9 (next page) shows the fermentation results based on the highest solvent productivity which occurs at 30 h, in the STR and BOBB II.

Table 5.9 ABE fermentation in BOBB II and STR at 0.14 Wm^{-3} and 30 h fermentation

Parameter	Bioreactors	
	BOBB II Re_o **938**	**STR 35 rpm**
Fermentation time (h)	30	30
Acetone (gL^{-1})	1.92±0.15	2.67±1.20
Butanol (gL^{-1})	3.90±0.27	3.49±0.24
Ethanol (gL^{-1})	0.09±0.007	0.25±0.09
Acetate (gL^{-1})	2.30±0.17	2.03±0.40
Butyrate (gL^{-1})	0.12±0.002	0.09±0.005
Total ABE (gL^{-1})	5.91	6.42
Total acid (gL^{-1})	2.42	2.11
Cells (gL^{-1})	1.52±0.10	1.17±0.10
Specific growth rate (h^{-1})	0.288	0.282
Initial glucose (gL^{-1})	51.3±0.84	49.0±0.56
Residual glucose (gL^{-1})	29.6±2.93	35.4±2.30
ABE productivity ($gL^{-1}h^{-1}$)	0.20	0.21
ABE yield [g ABE (g glucose consumed) $^{-1}$]	0.28	0.47
Butanol productivity ($gL^{-1}h^{-1}$)	0.13	0.12
Butanol yield [g B (g glucose consumed) $^{-1}$]	0.19	0.26

Based on the Table 5.9 above, the butanol in BOBB II was 3.90 gL^{-1}, followed by acetone and ethanol of 1.92 gL^{-1} and 0.09 gL^{-1}, respectively. This gave a total solvent produced in BOBB II of 5.91 gL^{-1}. In the STR, the culture produced 2.67 gL^{-1} acetone which is 28% higher than that in BOBB II, 3.49 gL^{-1} butanol and 0.25 gL^{-1} ethanol, resulting in a total ABE concentration of 6.42 gL^{-1}, slightly higher than in BOBB II.

Corresponding to a high cell concentration (1.52 gL^{-1}) and high organic acid concentrations (2.42 gL^{-1}) in BOBB II, almost half of the glucose provided was utilized with the remaining 29.6 gL^{-1} left in the culture broth, giving a butanol yield of 0.13 g butanol (g glucose)$^{-1}$ produced at a rate of 0.14 g butanol $L^{-1}h^{-1}$. On the other hand only 29% of the original 49 gL^{-1} of glucose provided was consumed in

the STR to produced 1.17 gL^{-1} cells and 2.11 gL^{-1} organic acids. As a consequence of lower sugar consumption, the STR exhibited a higher butanol yield with 0.26 g butanol (g glucose)$^{-1}$, but at lower productivity (0.12 g butanol $L^{-1}h^{-1}$) than in the BOBB II. In addition, the total solvent productivity in BOBB II was slightly lower than the STR with 0.20 g $L^{-1}h^{-1}$ in BOBB II and 0.21 g $L^{-1}h^{-1}$ in the STR. The productivity achieved at this power density of 0.14 Wm^{-3} was the highest within the range studied here (0-1.14 Wm^{-3}). Higher solvent productivity was probably due to early initiation of solventogenesis, eventually accumulating more solvent earlier in both bioreactors. An increase in the mixing intensity accelerated the microbial activities and resulted in the increased growth rate of the cell. This entailed a greater production rate of the acids and solvents than the fermentation at lower power density.

5.3.7 ABE fermentation in BOBB II and the STR at 1.14 Wm^{-3}

This ABE fermentation was performed at the highest power density that could be achieved with the available equipment. The ABE fermentation profile is shown in Figure 5.13 below.

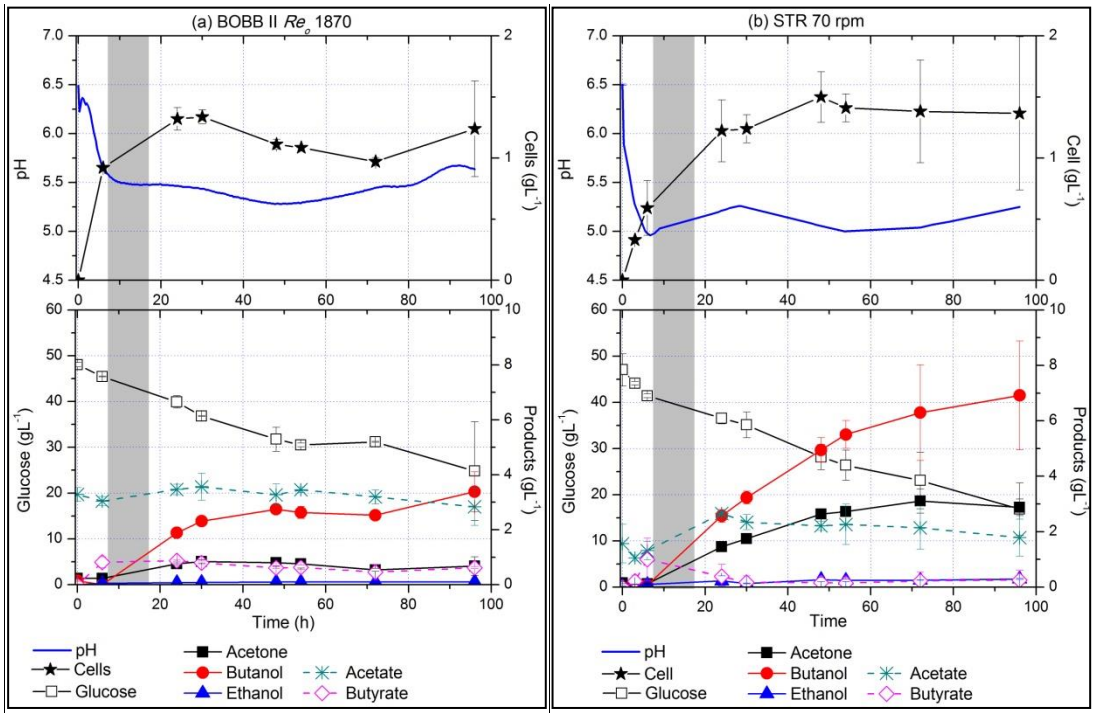


Figure 5.13 ABE fermentation profile at 1.14 Wm^{-3} in (a) BOBB II and (b) STR. The grey band marks the solvent shift (butanol >0.1 gL^{-1}). Error bars represent standard deviation from the mean of duplicate experiments

The fermentations were carried out at the same initial pH of 6.5 and temperature of 32^{o}C. Cell growth, pH, residual glucose, solvents and acid concentrations were monitored during the fermentation. Cell growth in BOBB II and the STR were exponentially rapid with no lag phase. The specific growth rate in BOBB II was slightly higher (0.285 h^{-1}) than the STR (0.282 h^{-1}

It can be observed from Figure 5.13a, in BOBB II, that most of the glucose was consumed to produce organic acids and cells rather than solvent, at an average

rate of 0.3 g glucose L^{-1} h^{-1}. A constant acid concentration (about 3.5 gL^{-1}) throughout the fermentation course was observed with low solvent production. The results above were from duplicate runs at the same condition. Similar results were observed for each BOBB II runs at this power density, where it is likely that the fermentation experienced acid crash. Acid crash occurs due to excess acid being produced, exceeding the threshold of switching phases, but without significant switch to solventogenesis (Maddox *et al.*, 2000). This, causes cessation of glucose uptake, acid production and solvents production. About 72 h after cessation, metabolic activity recommenced. Subsequently, glucose was again utilized and the ABE concentration started to increase. This is a phenomenon called "recommencement" after an acid crash (Maddox *et al.*, 2000). It has been suggested that acid crash was unlikely to be due to failure of switching from acidogenesis to solventogenesis but, due to rapid termination of solventogenesis after the switching (Maddox *et al.*, 2000). This is true for this BOBB II fermentation, as the fermentation switched from acidogenesis to solventogenesis but rapidly ceased afterwards due to unfavourable conditions. This implies the termination of solvent and acid production and the cessation of glucose uptake after 48 h of the fermentation illustrated by a constant concentration of respective substance in Figure 5.13a.

Interestingly, in the STR, the fermentation proceeded normally with acidogenesis followed by solventogenesis, where the total solvent produced was 10.05 gL^{-1} (2.88 gL^{-1} acetone, 76.92 gL^{-1} butanol and 0.25 gL^{-1} ethanol, Figure 5.13b) at 96 h of the fermentation. Glucose consumption was 33% higher than in BOBB II, on average at 0.4 g glucose $L^{-1}h^{-1}$, with high residual glucose of 16.9 gL^{-1} at the end of the fermentation.

Data from the ABE fermentation in the BOBB II and the STR, at 30 h fermentation, where the highest productivity achieved in the fermentation, is shown in Table 5.10 (next page).

Table 5.10 ABE fermentation in BOBB II and STR at 1.14 Wm^{-3} and 30 h fermentation

Parameter	Bioreactors	
	BOBB II Re_o **1870**	**STR 70 rpm**
Fermentation time (h)	30	30
Acetone (gL^{-1})	0.85±0.19	1.75±0.03
Butanol (gL^{-1})	2.31±0.62	3.23±0.23
Ethanol (gL^{-1})	0.08±0.15	0.13±0.013
Acetate (gL^{-1})	3.57±0.07	2.34±0.28
Butyrate (gL^{-1})	0.80±0.65	0.17±0.23
Total ABE (gL^{-1})	3.24	5.11
Total acid (gL^{-1})	4.36	2.51
Cells (gL^{-1})	1.34±0.15	1.24±0.12
Specific growth rate (h^{-1})	0.285	0.282
Initial glucose (gL^{-1})	48.1±5.84	47.1±3.49
Residual glucose (gL^{-1})	36.8±0.63	35.1±2.79
ABE productivity ($gL^{-1}h^{-1}$)	0.11	0.17
ABE yield [g ABE (g glucose consumed) $^{-1}$]	0.29	0.43
Butanol productivity ($gL^{-1}h^{-1}$)	0.08	0.11
Butanol yield [g B (g glucose consumed) $^{-1}$]	0.21	0.27

The fermentation in BOBB II produced a total of 3.24 gL^{-1} solvent consisting of 2.31 gL^{-1} butanol, 0.85 gL^{-1} acetone and 0.08 gL^{-1} ethanol. High organic acid concentrations (4.36 gL^{-1}) achieved in the culture broth after 30 h of fermentation. While in the STR, a total of 5.11 gL^{-1} solvents were produced of which 1.75 gL^{-1} was acetone, 3.23 gL^{-1} butanol and 0.13 gL^{-1} ethanol with lower total organic acids (2.51 gL^{-1}) than in BOBB II. Due to acid crash fermentation in BOBB II, the solvent productivity was only 0.11 g ABE $L^{-1}h^{-1}$ with the butanol productivity was 0.08 g butanol $L^{-1}h^{-1}$. The solvent productivity in the STR was 55% higher (0.17 g ABE $L^{-1}h^{-1}$) and butanol productivity was 38% higher (0.11 g butanol $L^{-1}h^{-1}$) than in BOBB II. Despite acid crash fermentation in BOBB II, a high yield was obtained in both bioreactors with 0.43 g ABE (g glucose)$^{-1}$ and 0.29 g ABE (g glucose)$^{-1}$ for the STR and BOBB II, respectively.

5.3.8 Acid crash fermentation in BOBB II at 1.14 Wm^{-3}

ABE fermentation in BOBB II at Re_o 1870 with the power density of 1.14 Wm^{-3} underwent acid crash fermentation. According to Maddox *et al*. (2000), a possible cause of the acid crash is the high concentration of the undissociated form of the acids (acetate and butyrate), which accumulates in the culture broth. This form of acid is more toxic than its dissociated forms and solventogenesis is believed to be a detoxification process (Jones and Woods, 1986; Gheshlaghi *et al.*, 2009). High concentrations of the undissociated form of acetate and butyrate are likely to be related to the acid production rate during early stage of the fermentation. Thus, if acids were produced faster than they can be utilized, they would accumulate to toxic levels before they can be removed by solventogenesis. Figure 5.14 below, represented the difference in an acid crash fermentation (Figure 5.14a) and a good solventogenic fermentation (Figure 5.14b) in BOBB II.

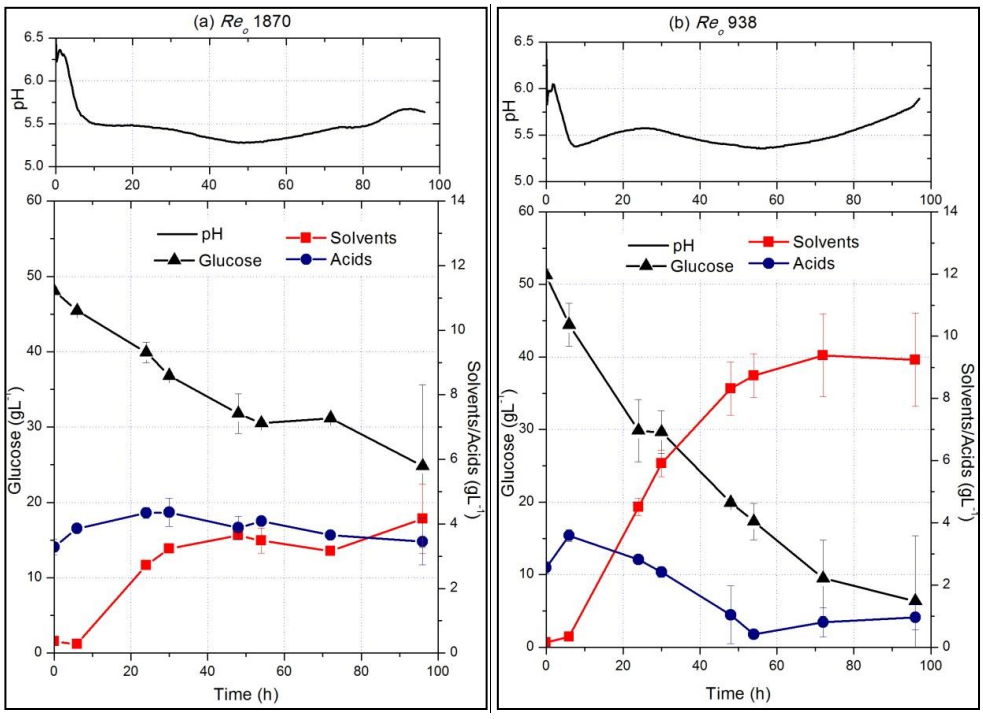


Figure 5.14 Fermentation profile in relation to the pH, glucose and solvent concentration in BOBB II at (a) Re_o 1870 and (b) Re_o 938. Error bars represent standard deviation from the mean of duplicate experiments

In Figure 5.14a, organic acids were produced to the highest concentration of 4.4 gL^{-1} during the course of 24 h fermentation. Although the fermentation had switched to solventogenesis after that, the solvent production ceased after 48 h. From this point, the acid concentration remained at a toxic level for this fermentation. Thus, the glucose consumption ceased and pH of the culture remains low around pH 5.0 leading to low total solvent production, around 3.6 gL^{-1}. Whereas in Figure 5.14b, organic acid produced earlier (between 0 and 6 h) were utilized concomitantly with glucose to produce solvent during solventogenesis. Glucose was almost fully consumed by the end of the fermentation and the pH of the culture increased from 5.4 to 5.9 during solventogenesis.

Maddox *et al.* (2000) suggested several techniques to prevent acid crash by trying to minimize the undissociated form of the organic acid production rate, which it is more toxic than its dissociated form. A pH-controlled fermentation or use of lower fermentation temperature can minimise the likelihood of acid crash. The application of temperature ramping during fermentation can also prevent acid crash. Based on the observations of these results, it was decided that a protocol involving no oscillation during acidogenesis, to increase the rate of cell growth and acid production, with oscillation commencing before the acid concentration reached the toxic level, but after the solventogenesis switch level. From then, “two-stage” ABE fermentations were carried out to evaluate the effectiveness of the proposed strategy to avoid acid crash in BOBB II.

5.4 Two-stage ABE Fermentation in BOBB II

It has been observed, that the ABE fermentation in BOBB II at Re_o 938 resulted in the highest solvent productivity (0.20 $gL^{-1}h^{-1}$). However, low total ABE concentration was produced at this productivity (5.91 gL^{-1}), lower than that achieved in the STR at similar productivity (6.42 gL^{-1}). Furthermore, the BOBB II fermentation productivity at Re_o 938 decreased over the course of the fermentation: at 96 h the solvent productivity had decreased to only 0.13 $gL^{-1}h^{-1}$ with a total of ABE concentration 9.38 gL^{-1}. The same was observed with the fermentation at Re_o 0 where a total of ABE concentration of 10.97 gL^{-1} was produced at the same duration which gave the productivity of 0.11 $gL^{-1}h^{-1}$. It can be concluded here that a one-stage fermentation in BOBB II either produced a high solvent concentration at low productivity or low solvent concentration at high productivity. From this observation, it was anticipated that by performing the fermentation in two stages, *i.e.* a first stage without oscillation and the second stage with oscillation or vice-versa, the solvent productivity as well as the solvent concentration could be increased. The fermentations based on the two-stage strategy were carried out as summarised in Table 5.11:

Table 5.11 Matrix for the one-stage and two-stage ABE fermentation in BOBB II

A (control)	**B (One-stage)**
without oscillation (0)	Oscillation at Re_o 938 (938)
C (Two-stage)	**D (Two-stage)**
Stage 1: without oscillation Stage 2: oscillation at Re_o 938 Switch time*: i) 12 h (0-938 12 h) ii) 24 h (0-938 24 h)	Stage 1: oscillation at Re_o 938 Stage 2: without oscillation Switch time*: 24 h (938-0 24 h)

* Switch time is the time when switching from stage one to two

Switching time of 12 h was chosen as it was the phase shifting time taken from the one-stage fermentation at Re_o 938, then 24 h was determine by doubling the switching time to observe any effect to the fermentation. All fermentations were

performed at 1 L working volume at 32°C. Cell growth from above fermentations is presented in Figure 5.15. Some discrepancies between these results and those performed earlier (in section 5.3) were due to the use of lower working volume (1 L vs. 1.5 L), and the use of a new batch of clostridia freeze dry stock. The fermentation results were compared to the control fermentation at Re_o 0.

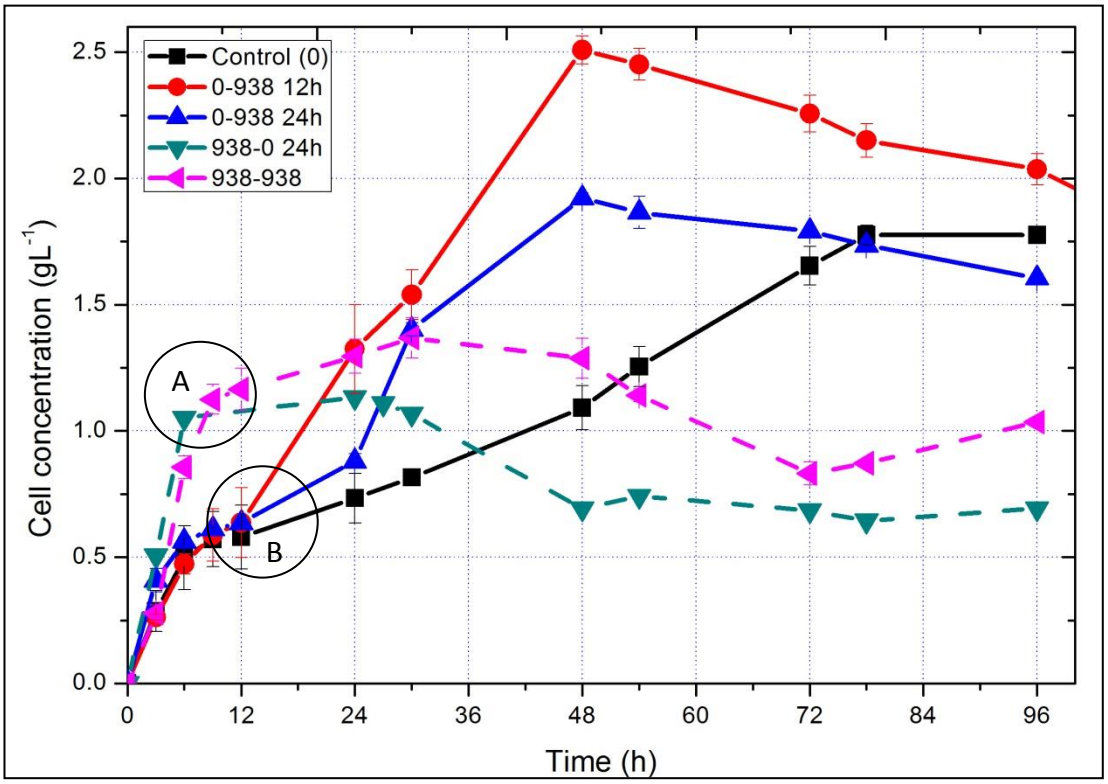


Figure 5.15 Cell concentration based on cell dry weight (CDW) at various fermentation strategy, *i.e* one-stage and two-stage fermentation in BOBB II at 1 L working volume. Error bars represent standard deviation from the mean of duplicate experiments

Based on Figure 5.15 above, an important observation can be made that the cell growth trend can be divided into two groups:

- Group A: rapid exponential phase but growth ceases realatively early
- Group B: slower initially, but with a prolonged exponential phase

The specific growth rates for Group A, which consist of fermentation with early oscillation (938 and 938-0 24 h), were higher (0.2 h^{-1}) than that in Group B (control and late oscillation: 0-938 12 & 24 h) which was 0.1 h^{-1}. However, Group A fermentation exhibited a short exponential growth phase which ceased after six hours, while the Group B fermentations exhibited exponential phase that was typically eight times longer. This resulted in higher cell concentrations in the Group B fermentation. The highest cell concentration was observed in Group B two-stage fermentation at 0-938 12 h (*i.e* the oscillation started after 12 h of fermentation) with 2.5 gL^{-1} cells obtained at 48 h. Group A two-stage fermentation, where oscillation commenced at the start of the fermentation and stopped at 24 h (938-0 24 h), had the lowest cell concentration (1.13 gL^{-1}) with cell growth ceasing after the oscillation stopped. In group B two-stage (0-938 12 h and 24 h) cell growth ceased approximately 48 h after fermentation, which was 24 h longer than that in Group A. It is clear that ABE fermentation performed in two-stage fermentation with oscillation starting either after 12 or 24 h (Group B) produced higher cell concentrations, which is generally desirable in the ABE fermentation, as it will improve the reaction rate and therefore solvent productivity (Qureshi *et al.*, 2000). Figure 5.16 illustrates the profiles of the extracellular acetate and butyrate.

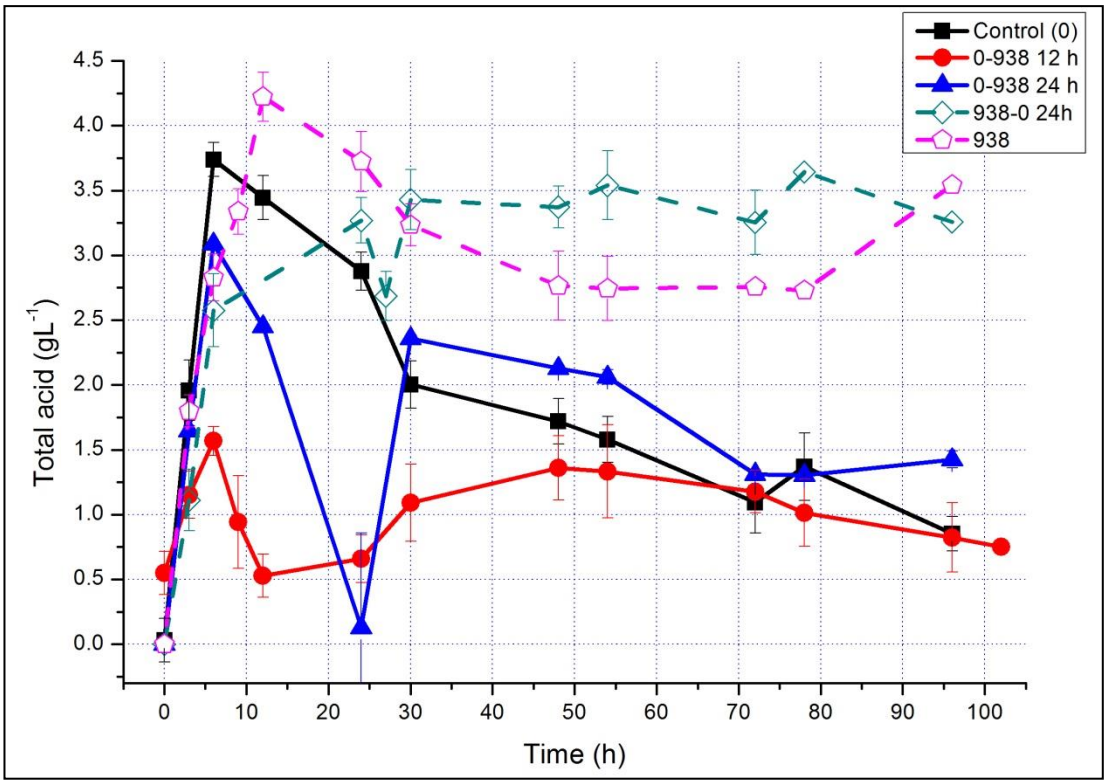


Figure 5.16 Organic acid production trend in one-stage and two-stage fermentation compared to the control fermentation. Error bars represent standard deviation from the mean of duplicate experiments

A sudden drop in acid concentration can be observed when oscillation commenced in the two-stage fermentations (0-938 12 and 24 h, and 938-0 24 h). Eventually the acid concentration increased again, but not to its initial concentration as some of the acid was utilized to produce solvent. Yerushalmi and Volesky (1985) reported that an increase in mixing will increase the overall rate of primary metabolism, which implies higher cell and acid production in the 938 and 938-0 24 h during the early stages of the fermentation. Although the net production of the solvents was very low at that stage, more solvents may have accumulated in the cell walls during that time, due to the imbalance between the rate of production and the outflux of the acids and solvents. High levels of these products, particularly butyrate and butanol result in inhibition of the cellular metabolism (Yerushalmi and Volesky, 1985). The acid production in the two-stage fermentation 938-0 24 h recommenced 24 h after oscillation stopped as the fermentation gained a “second wind”, and acid concentration started to increase again. However this is not reflected in the cell

growth profile (shown earlier in Figure 5.15) as the cell concentration at that period of time (48 to 96 h) was stationary.

The solvent production profiles in the two-stage and one-stage fermentations were compared to the control (0), as shown in Figure 5.17 (next page). The two-stage fermentation (0-938 12 h) produced the highest solvent concentration. In all fermentations, acetone and ethanol production can be observed after 3 h of inoculation. During the first 24 h, concentrations were generally similar to one another, although the concentration started to deviate afterwards. Acetone concentration reached a higher concentration earlier in two-stage fermentation (0-938) than in the control after 30 h fermentation. For example at 54 h fermentation time, the acetone concentration in 0-938 12 h, 24 h and control were 2.8 gL^{-1}, 2.5 gL^{-1} and 1.8 gL^{-1}, respectively. This represents acetone productivities of 0.052 $gL^{-1}h^{-1}$, 0.046 $gL^{-1}h^{-1}$ and 0.033 $gL^{-1}h^{-1}$, respectively. Similar trends were also observed for butanol and ethanol production. On the other hand in the one-stage fermentation (938) and two-stage fermentation (938-0), the solvent production was lower than the control for acetone and butanol production. Interestingly, higher ethanol concentrations were observed in this fermentation than in the control fermentation.

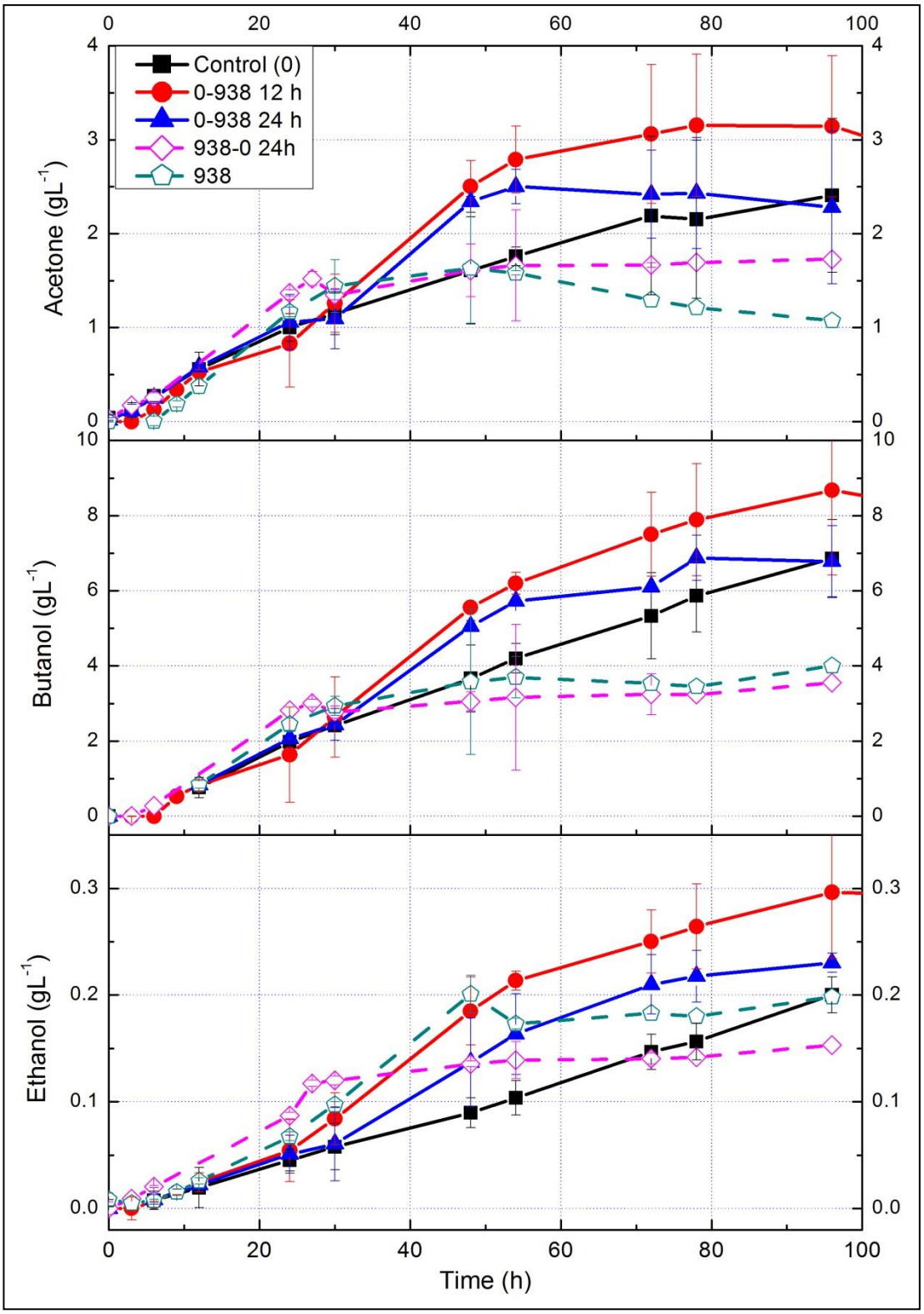


Figure 5.17 ABE production in two-stage and one-stage fermentation in comparison with the control fermentation. Error bars represent standard deviation from the mean of duplicate experiments

The aim of performing a two-stage ABE fermentation was to produce high cell and acid concentration during the first stage. Then when the acid level has reached what is believed to be the threshold for phase shifting, the second stage would be commenced. This was intended to trigger the solventogenesis earlier than in the control fermentation, since mixing was incorporated earlier or later during the operation of the reactor, depending on the two-stage strategy tested. Mixing in an anaerobic fermentation is mainly to maintain solid-liquid suspension (solid buffer) in the medium and to enhance mass transfer in (substrate) and out (products) of the cell (Yerushalmi and Volesky, 1985). Homogenous solid buffer suspension in the medium helped to cushion the pH drop during acidogenesis. As can be seen from the results, the commencement of late mixing in the two-stage ABE fermentation enhanced the cell growth and increased product concentration, which is reflected in significantly higher productivities compared to the control experiments, as shown in Figure 5.15 (cell production) and Figure 5.17 (solvent production).

The highest solvent concentrations in each fermentation and their respective productivities are compared between one-stage, two-stage and control fermentations in Table 5.12. Two-stage fermentation was observed to improve solvent productivity by up to 28% over the control fermentation, with oscillation commencing at 12 h, resulting in a slightly higher ABE productivity than in the fermentations with oscillations commencing at 24 h. This two-stage fermentation also improved the total solvent concentration to 12 gL^{-1}: 21% higher than the control and 24 h (0-938 24h), and 122% higher than the one-stage fermentation at Re_o 938 (5.46 gL^{-1}). On the other hand, the two-stage fermentation with early oscillation (938-0 24 h) achieved half of the solvent productivity compared to the control and one-stage fermentation at Re_o 938.

Table 5.12 Comparison between solvent productions in one-stage and two-stage fermentation with control fermentation

Parameter	Control Re_o 0	Two-stage 0-938 12 h	Two-stage 0-938 24 h	Two-stage 938-0 24 h	One-stage Re_o 938
Fermentation time (h)	96	96	78	96	54
Cell concentration (gL^{-1})	1.78±0.03	2.04±0.063	1.74±0.02	0.69±0.001	1.14±0.03
Acetone (gL^{-1})	2.4±0.82	3.1±0.75	2.4±0.81	1.7±0.02	1.6±0.02
Butanol (gL^{-1})	6.9±1.04	8.7±2.25	6.9±0.95	3.6±0.01	3.7±0.55
Ethanol (gL^{-1})	0.20±0.02	0.30±0.062	0.22±0.009	0.15±0.006	0.17±0.005
Total ABE (gL^{-1})	9.5	12	9.5	5.4	5.5
A:B:E ratio	12:35:1	10:29:1	11:31:1	11:24:1	9:22:1
Total Acids (gL^{-1})	0.85	0.82	1.43	3.26	2.74
Acetone productivity ($gL^{-1}h^{-1}$)	0.025	0.033	0.031	0.018	0.029
Butanol productivity ($gL^{-1}h^{-1}$)	0.072	0.090	0.088	0.037	0.069
Ethanol productivity ($gL^{-1}h^{-1}$)	0.0021	0.0031	0.0028	0.0016	0.0032
ABE productivity (g ABE $L^{-1}h^{-1}$)	0.099	0.13	0.12	0.057	0.10
Specific butanol productivity (g B g $cell^{-1}$ h^{-1})	0.0404	0.0441	0.0506	0.0536	0.0605

By stopping the oscillation after 24 h of fermentation, the total solvent concentration was reduced compared to in the control fermentations by up 43%, respectively. In addition, the butanol and ethanol concentrations were reduced by 48% and 25%, respectively, than in the control fermentation. Lower solvent concentration in this two-stage fermentation could be attributed to lower cell concentration than in the control fermentation (Figure 5.15). Interestingly, the specific butanol productivity was comparable in all fermentation (between 0.04 and 0.06 g butanol g $cell^{-1}$ h^{-1}) with the highest achieved in the one-stage fermentation at Re_o 938.

5.4.1 Solvent production and cell morphology changes

It is important to note that phase switching in these ABE fermentations (acidogenesis to solventogenesis) is closely related to cell morphology changes. Solvent production has been correlated with a cellular morphology change and the appearance of clostridial form cell (Jones *et al.*, 1982). During the whole course of the ABE fermentation in BOBB II, clostridia cells' morphologies can be divided into two forms:

i. The acid producer cell, known as a "vegetative cell". This is rod-shaped
ii. The solvent producer cell known as the "Clostridial form cell". This is cigar-shaped with granulose in the cytoplasm

During sporulation, cells swell markedly and store granulose, a polysaccharide-based material that serves as a carbon and energy source during solventogenesis (Gholizadeh, 2009). This transforms the cell from a vegetative cell to the "clostridial form" cell. Both of the cell morphologies can be seen in Figure 5.18. The "clostridial form" cells were red in colour, as the granulose inside the cell reacted with iodine, which acted as the cell colorant and resulted in the red colour. The vegetative cells are the narrow rod-shaped, uncoloured cells.

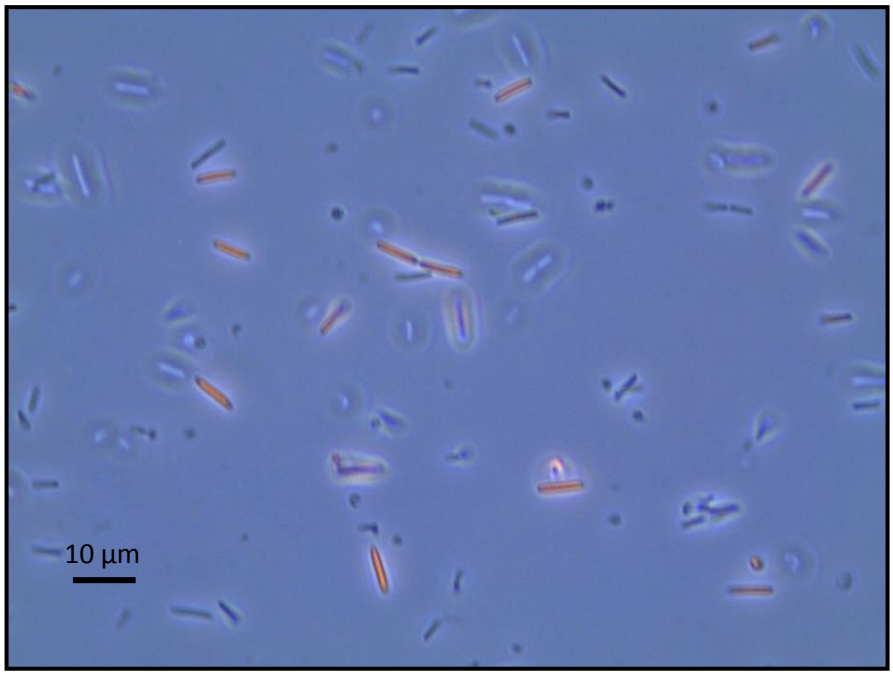


Figure 5.18 *Clostridium* GBL1082 morphology in glucose medium under light microscope at 1000x magnification. Picture was taken from two-stage 0-938 12 h at 48 h fermentation

Figure 5.19 (next page) show the percentage and concentration of vegetative and clostridial form cells between one-stage and two-stage fermentations in comparison with the control fermentation at 48 h. Vegetative and clostridial form cell concentrations were calculated using an Equation 5.1:

$$\text{Veg or Clost. form cell} = (\%\ \text{of veg.@ clost.forms cell}) \times (\text{total cell conc.}) \quad gL^{-1}$$

Equation 5.1

…where veg. is vegetative cell and clost. form is clostridial form cell.

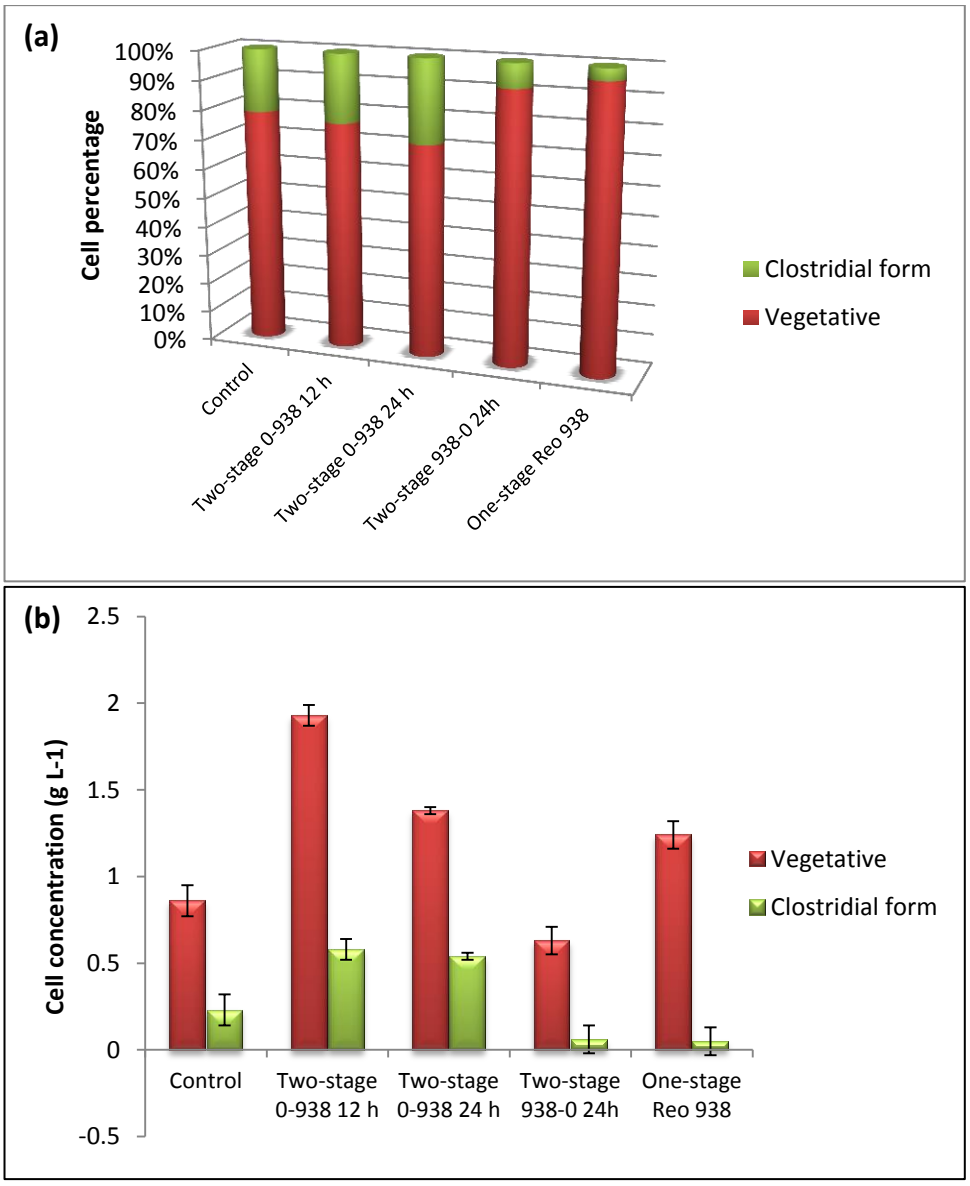


Figure 5.19 (a) The percentage and (b) the concentration of vegetative and "clostridial form" cell in one-stage and two-stage fermentation in comparison with the control fermentation at 48 h

Based on Figure 5.19 above, two-stage fermentation with late oscillation (0-938 12 and 24 h) resulted in a higher percentage of "clostridial form" cells (more than 20% of total cells concentration) than that in the one-stage (Re_o 938) and two-stage fermentation with early oscillation (938-0 24 h) which contained ~ 10% of total cells concentration. High cell concentration in two-stage 12 h fermentation (1.93 gL^{-1}) resulted in the highest clostridial form cell concentration. It appears that high cell concentration with a high percentage of clostridial form cell resulted in the highest

solvent concentration. The cell distribution in one-stage, two-stage and the control fermentation over the course of 96 h time is shown in Figure 5.20.

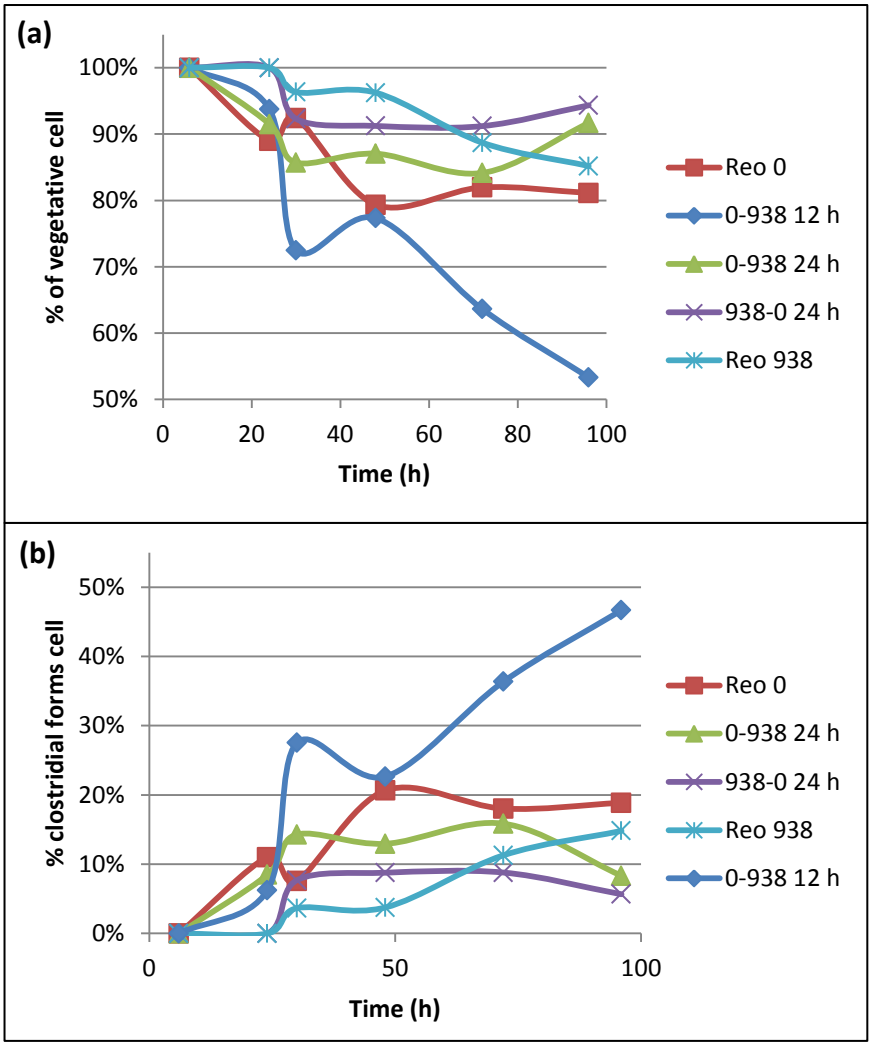


Figure 5.20 Cell type distribution in the ABE fermentation at various fermentation strategy in BOBB II. a) Percentage of vegetative cell type; b) Percentage of clostridial form cell type. The total of the respective percentage in (a) and (b) is equal to 100%

Generally, as the fermentation proceeded, the percentage of "clostridial form" cells increased as more vegetative cells underwent the differentiation process becoming clostridial forms. The differentiation process became apparent when the growth conditions were unsuitable for the cell growth (because of high acid concentration). This is the point when vegetative cells started to build a storage material called granulose, thus becoming the clostridial form cells. The clostridial form cells were

closely related to the solventogenesis, as their existence was directly correlated with the solvent production (Jones *et al.*, 1982). The two-stage fermentation (0-938 12 and 24 h), had a higher percentage of clostridial form cells compared to of the one-stage (Re_o 938) and two-stage fermentation with early oscillation (938-0 24 h) after 24 h of fermentation. That is to say a non-oscillation environment during an early hour of the ABE fermentation provided suitable condition for vegetative cells to grow and multiply their number, thus when oscillation started, they began the differentiation process of becoming clostridial forms cells. This was not the case in the early oscillation condition (938 and 938-0 24 h), although these fermentations produced a high concentration of cells early in the fermentation, most of these cells did not undergo differentiation into clostridial form cells: instead they were lysed.

Figure 5.21 qualitatively shows the lyse cell in the early oscillation fermentation condition. This was likely to have been caused by early mixing that negatively affected the young vegetative cells. Intracellular accumulation of butanol and butyrate is also a good candidate for inhibition of cellular metabolism. As the overall primary metabolism increased during mixing, this implies a greater production rate of acids and solvents. Though high net solvent production was not observed, perhaps these products were accumulated in the cell's cytoplasm due to lower permeability of the cell membrane thus creating a transient imbalance between the rate of the production and the outflux of these products (Yerushalmi and Volesky, 1985).

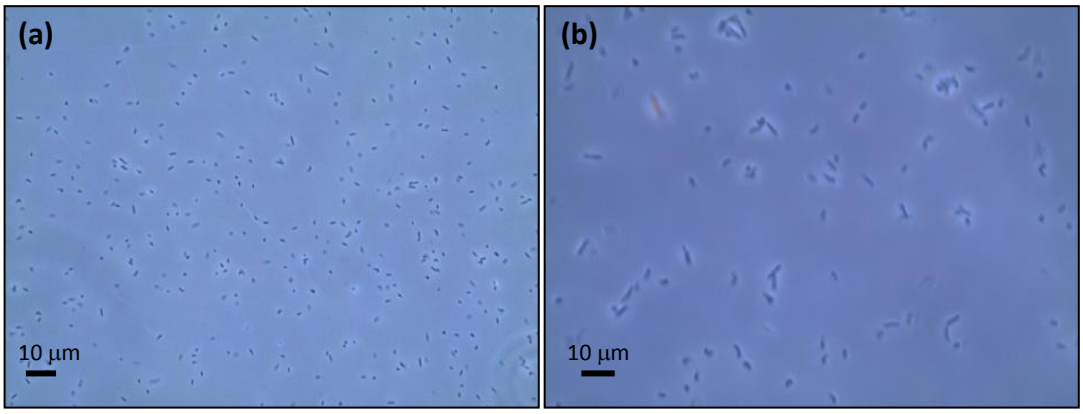


Figure 5.21 Cell lysed under light microscope at 400x magnification in (a) two-stage *Re_o* 938-0 fermentation at 54 h and (b) one-stage *Re_o* 938 fermentation at 72 h

5.5 Temperature Control in BOBB II

It has been observed recently that during the fermentation in BOBB II, the temperature profile during ABE fermentations fluctuated from the set point of 32^{o}C due to simple manual temperature control. The temperature fluctuated with the ambient temperature changes (day or night time) and the degree of mixing in the BOBB II. In response to this problem, an automatic temperature control was developed in BOBB II using a water bath external temperature loop. A Pt 100 thermocouple connected to the water bath was used to measure BOBB II temperature. Whenever BOBB II temperature deviated from the set point of 32^{o}C, a signal was sent to the water bath. The water bath automatically heated or cooled the water flowing in the coil around BOBB II glass column accordingly to maintain a uniform temperature throughout the fermentation course.

Figure 5.22 (next page) shows the temperature profile in the two-stage fermentation in BOBB II, with manual and automatic temperature control. From Figure 5.22 a and c, with manual temperature control, there were several intervals of significant temperature drops, occuring during the night time as highlighted by the ellipse. When an automatic temperature control was applied to both fermentation conditions, a stable temperature around the set point can be observed (Figure 5.22 b and d). In addition, by oscillating the BOBB II a better temperature control was achieved than without oscillation. The temperature profile under the automated temperature controlled fermentations (Figure 5.22 b and d) was fluctuated before an oscillation started and the fluctuation diminished after the commencement of an oscillation.

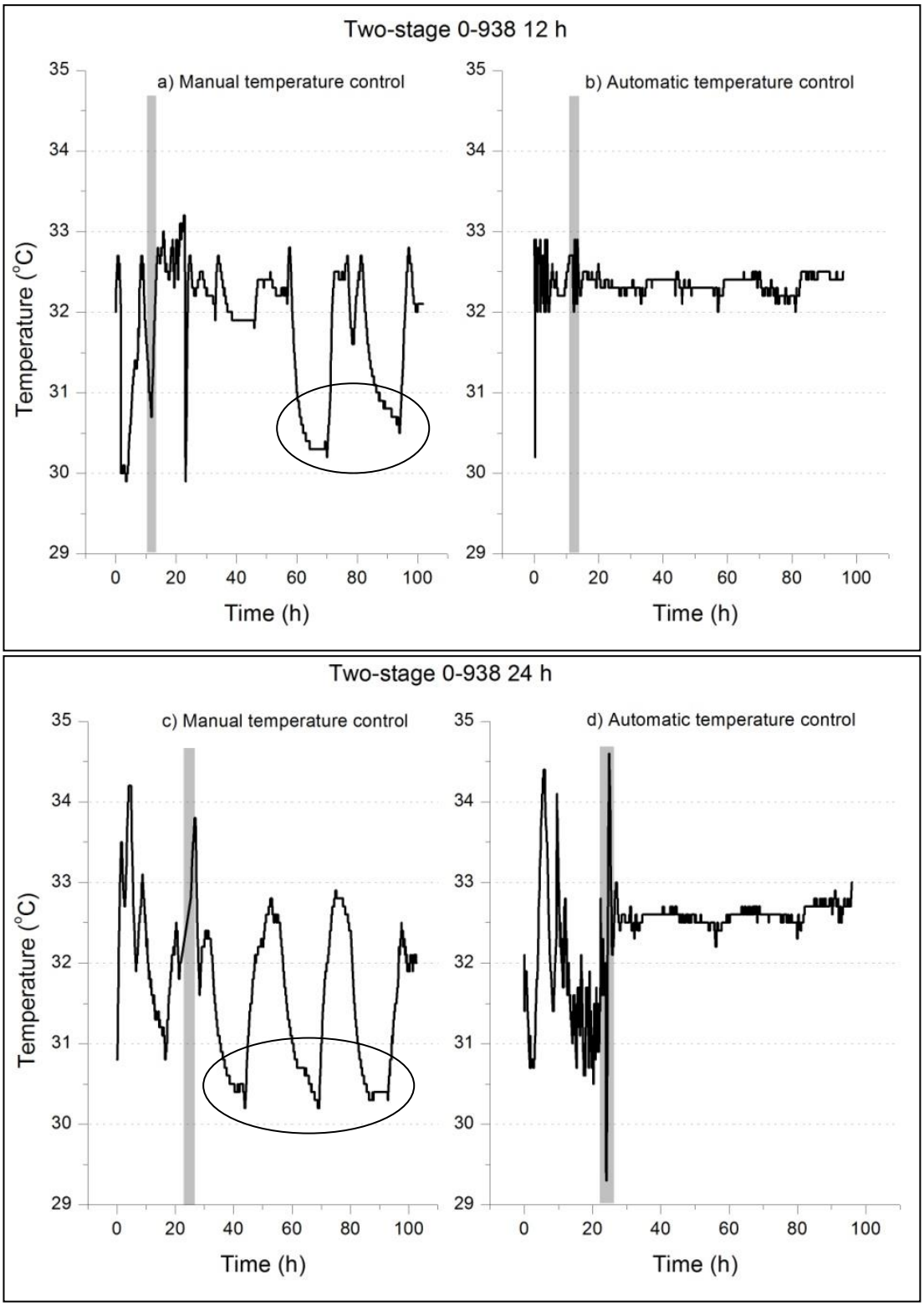


Figure 5.22 The ABE fermentation temperature profile in two-stage fermentation in BOBB II with manual and automatic temperature control. Grey band indicates when the oscillation started.

To evaluate the effects of temperature fluctuation on the ABE fermentation performance, serum bottle fermentations were carried out at the minimum and maximum temperature achieved during previous BOBB II fermentation. Duplicate fermentations in 100 mL serum bottles were carried out with 90 mL working volume at static condition at minimum temperature of 28^{o}C and maximum temperature of 37^{o}C with 32^{o}C as a control. Results of the fermentations can be seen in Figure 5.23 (next page). Cell concentrations were similar during the first 9 h of the fermentation at all temperatures (Figure 5.23a). At the end of the ABE fermentation, an optimum temperature of 32^{o}C (control fermentation) produced the highest cell concentration of 1.2 gL^{-1}. On the other hand, at 37^{o}C, cell concentration was only half that achieved at 32^{o}C with early growth inhibition.

At high temperature the cell activities were high (enzymes speed up the metabolism and cell rapidly increased in size), but at a certain temperature, enzymes begin to denature and cellular growth ceases (Shuler and Kargi, 2002). Conversely, at low temperature *i.e.* below 28^{o}C, enzymes cannot mediate properly in the chemical reaction resulting in slow growth. This temperature effect on the cell concentration can be seen clearly in Figure 5.23b. Prior to inoculation, cell growth at 32^{o}C and 37^{o}C proceeded similarly, while at 28^{o}C was slightly slower. The fermentation at 37^{o}C started to slow down and halted after 9 h. In the meantime, fermentation at 28^{o}C is accelerated and cell grew exponentially to reach similar level with the 32^{o}C fermentation. After 9 h of the fermentation, cell concentration at 28^{o}C reached levels between those of fermentations at 32^{o}C and 37^{o}C. It can be concluded here that cell growth halted earlier at high temperature of 37^{o}C than at 28^{o}C and 32^{o}C, where an optimum temperature of 32^{o}C produced the highest cell concentration.

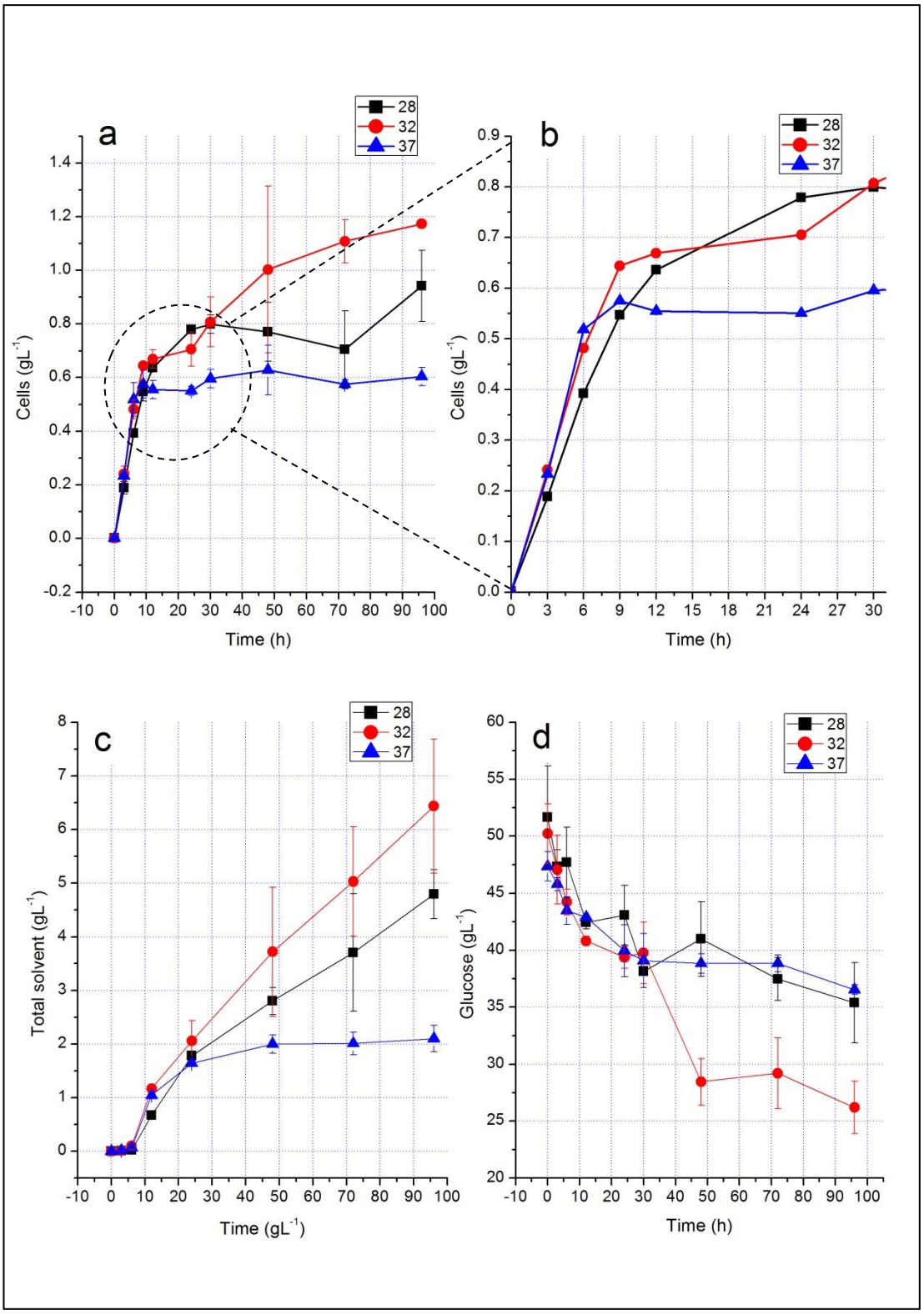


Figure 5.23 ABE fermentations in 100 mL serum bottles. Fermentations were carried out at 28°C, 32°C and 37°C at static condition using the same media as in BOBB II fermentation, complex growth media. Error bars represent standard deviation from the mean of duplicate experiments

Corresponding to high cell concentrations at the 32^{o}C fermentation, a high total solvent concentration was achieved with 6.4 gL^{-1} at 96 h giving a total solvent productivity of 0.07 gL^{-1}h^{-1} (Figure 5.23c). This was followed by fermentation at 28^{o}C with 4.8 gL^{-1} and productivity of 0.05 gL^{-1}h^{-1}. The lowest solvent concentration was achieved in the fermentation at 37^{o}C where the concentration was 2.1 gL^{-1} and productivity of 0.02 gL^{-1}h^{-1}. As a result of high cell concentration and high solvent concentration at 32^{o}C fermentation, about half of 50.2 gL^{-1} glucose provided was consumed, where there was 26.2 gL^{-1} of glucose left in the fermentation medium (Figure 5.23d). This will give a solvent yield at 32^{o}C as 0.27 g ABE (g glucose consumed)$^{-1}$. Fermentation at 28^{o}C and 37^{o}C had a similar glucose consumption trend where at the end of the fermentation, about 32% from 51.7 gL^{-1} glucose and 23% from 47.3 gL^{-1} glucose were consumed, respectively. This gave the solvent yield at 28^{o}C and 37^{o}C as 0.29 g ABE(g glucose consumed)$^{-1}$ and 0.19 g ABE(g glucose consumed)$^{-1}$, respectively.

It can be concluded from the serum bottle fermentation results, that unstable fermentation temperature in the BOBB II might affect the cell growth and thus solvent production. Decreases in the solvent concentration up to 67% to those in an optimum condition (32^{o}C) was observed if grew at temperature of 37^{o}C. As can be seen in the previous Figure 5.22 a and c, the BOBB II temperature fluctuated between 30-34^{o}C in the first 24 h of the fermentation. As this temperature fluctuation might have a significant effect on the product synthesis; each of the product profiles in the manually controlled fermentation was compared to the one with the automatic temperature controller and the linear regression (R^2) was calculated. R^2 values above 0.9 were considered to reflect no significant difference between respective profiles. Result of the R^2 calculation can be seen in Table 5.13 (next page).

Table 5.13 Comparison of R^2 values for product profiles between manual temperature and automatic temperature control fermentation in BOBB II

Products	Cell	Acetone	Butanol	Ethanol	Acetate	Butyrate
0-938 12 h	0.68	0.93	0.92	0.99	0.97	1.00
0-938 24 h	0.91	0.77	0.97	0.98	0.94	0.77

As most of the R^2 values were above 0.9 excepted for cell concentration at two-stage 0-938 12 h fermentation, acetone and butyrate concentration at 0-938 24 h, it can be concluded that the temperature fluctuation during ABE fermentation in BOBB II with manual temperature controller only has a minor effect on the cell growth, acetone and butyrate, and no significant effect on other products (butanol, ethanol and acetate).

5.6 Biobutanol Production with *In Situ* Gas Stripping

5.6.1 Characterization of the gas stripping process

To study application of the gas stripping to the ABE fermentation, the BOBB II fist needed to be characterized for its gas stripping mass transfer coefficient defined as $k_s a$, where the subscript s represents the solvent being stripped from the solution. The method of calculating $k_s a$ relates to the two film theory used to describe how mass transfer across a phase boundary works (Coulson *et al.*). The mass transfer is limited by the liquid side mass transfer; therefore the starting equation for determining $k_s a$ is as same as that of determining the $k_L a$, which is:

$$\frac{dC_s}{dt} = k_s a\ (C_s^* - C_s)$$

Equation 5.2

Since the stripping gas was not recycled, it can be assumed that the influent gas is free from solvent, therefore C_s^* is zero. So Equation 5.2 becomes:

$$\frac{dC_s}{dt} = -k_s a\ C_s$$

Equation 5.3

Integrating Equation 5.3 with the limits:

$$t = 0, C_s = \ C_{s_o} \text{ and } \ t = t, C_s = C_s$$

Equation 5.4

$$\int_{C_{s_o}}^{C_s} \frac{dC_s}{C_s} = \int_0^t -k_s a\ dt$$

Equation 5.5

$$ln(C_s) - ln(C_{s_o}) = -k_s a\ t$$

Equation 5.6

$$ln(C_s) = -k_s a\ t + ln(C_{s_o})$$

Equation 5.7

Plotting Equation 5.7 gives a straight line with a gradient of $k_s a$.

5.6.2 $k_s a$ experimental results

The experiment was conducted in collaboration with a Masters-level research project student. The experimental works were performed by the student under the author's close supervision. The $k_s a$ for each solvent (acetone, butanol and ethanol) was determined from the rate of change of concentration in the BOBB II. Using a model ABE system at a ratio of acetone:butanol:ethanol of 3:6:1, a dynamic gassing out technique was performed at several nitrogen gas flow rate levels, each lasted for 6 h. Figure 5.24 shows the calculated $k_s a$ based on reactor concentration at Re_o 1870. Example of the linear relationship between $ln\ C_s$ vs time is shown in Appendix F.

Based on the Figure 5.24 it can be seen that the change in the nitrogen flow rate slightly affected the $k_s a$. The more pronounced changes in the $k_s a$ of the acetone are likely to be due to acetone being more volatile than butanol and ethanol. Based on that principle, it is expected that the $k_s a$ for the ethanol will be higher than the butanol, but it was not the case here, as the two values were very close. The $k_s a$ for butanol and ethanol was fairly constant for the nitrogen flow rate considered. These results were confirmed by plotting the $k_s a$ using the change in solvents in the condensate, where the $k_s a$ should be approximately the same as in reactor, as shown in Figure 5.25.

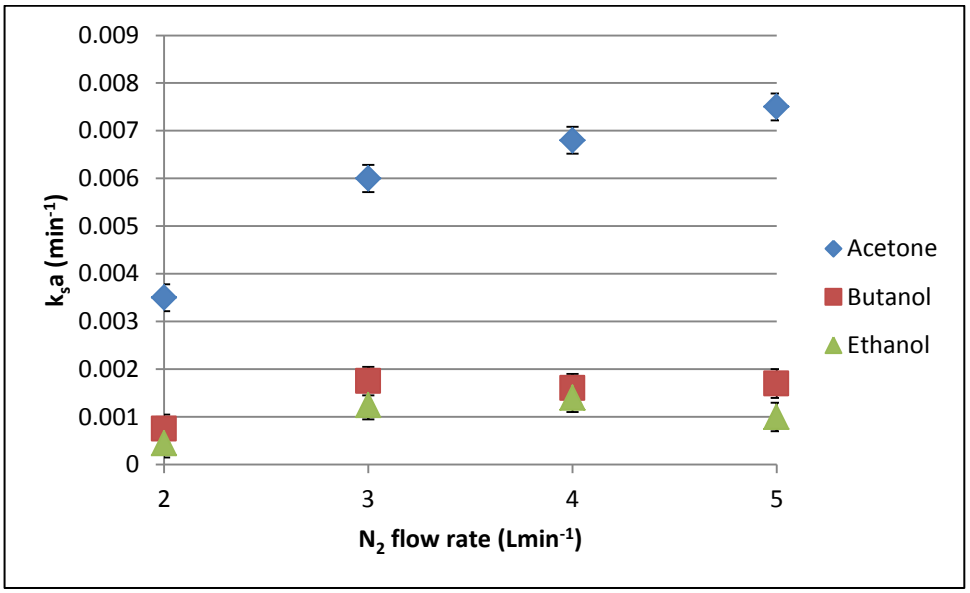


Figure 5.24 Calculated k_sa based on the reactor concentration at Re_o 1870. Error bars represent standard deviation from the mean of duplicate experiments

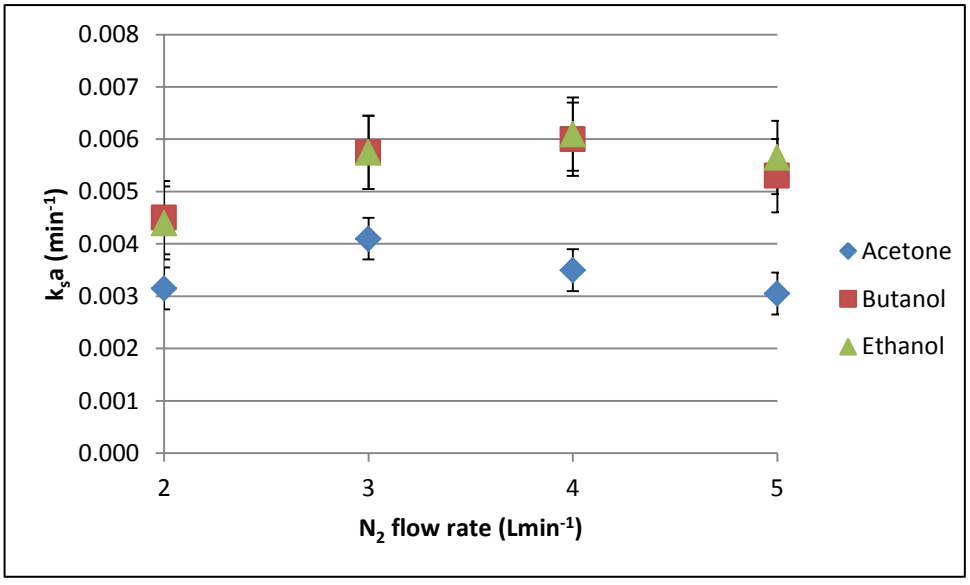


Figure 5.25 Calculated k_sa based on the solvent present in the condensate at Re_o 1870. Error bars represent standard deviation from the mean of duplicate experiments

In Figure 5.25 above, it can be seen again that k_sa for butanol and ethanol can be assumed constant over the flow rates considered. Although this time the k_sa for acetone can also be considered constant. The average k_sa for reactor and condensate for each solvent was compared in Table 5.14.

Table 5.14 Comparison of calculated k_sa based on reactor and condensate

Solvent	k_sa from reactor (min^{-1})	k_sa from condensate (min^{-1})
Acetone	0.0048	0.0028
Butanol	0.0011	0.0044
Ethanol	0.00082	0.0044

The differences in value between k_sa based on reactor and condensate were believed due to the inefficiency of the condenser. Solvents lost to the reactor head space and through the tube connection to the condenser also contribute to the differences. The lower k_sa value for the acetone in condensate than the reactor could be attributed to the acetone being more volatile therefore not completely condensing out of the gas, again due to the inefficiency of the condenser. Table 5.14 also suggests that the k_sa for butanol and ethanol was fairly similar, whereas acetone is different given that both butanol and ethanol are alcohols exhibiting similar properties, as opposed to acetone which is a ketone. The k_sa from the reactor was used in the calculation as it was less prone to error.

The effect of the oscillatory Reynolds number (Re_o) on the k_sa also been considered. Considering Re_o of 0 and 1870, it was calculated that Re_o had no effect on the k_sa as shown in Figure 5.26 (next page). From these results, 2 $Lmin^{-1}$ was chosen as the stripping gas flow rate to be used for ABE fermentation.

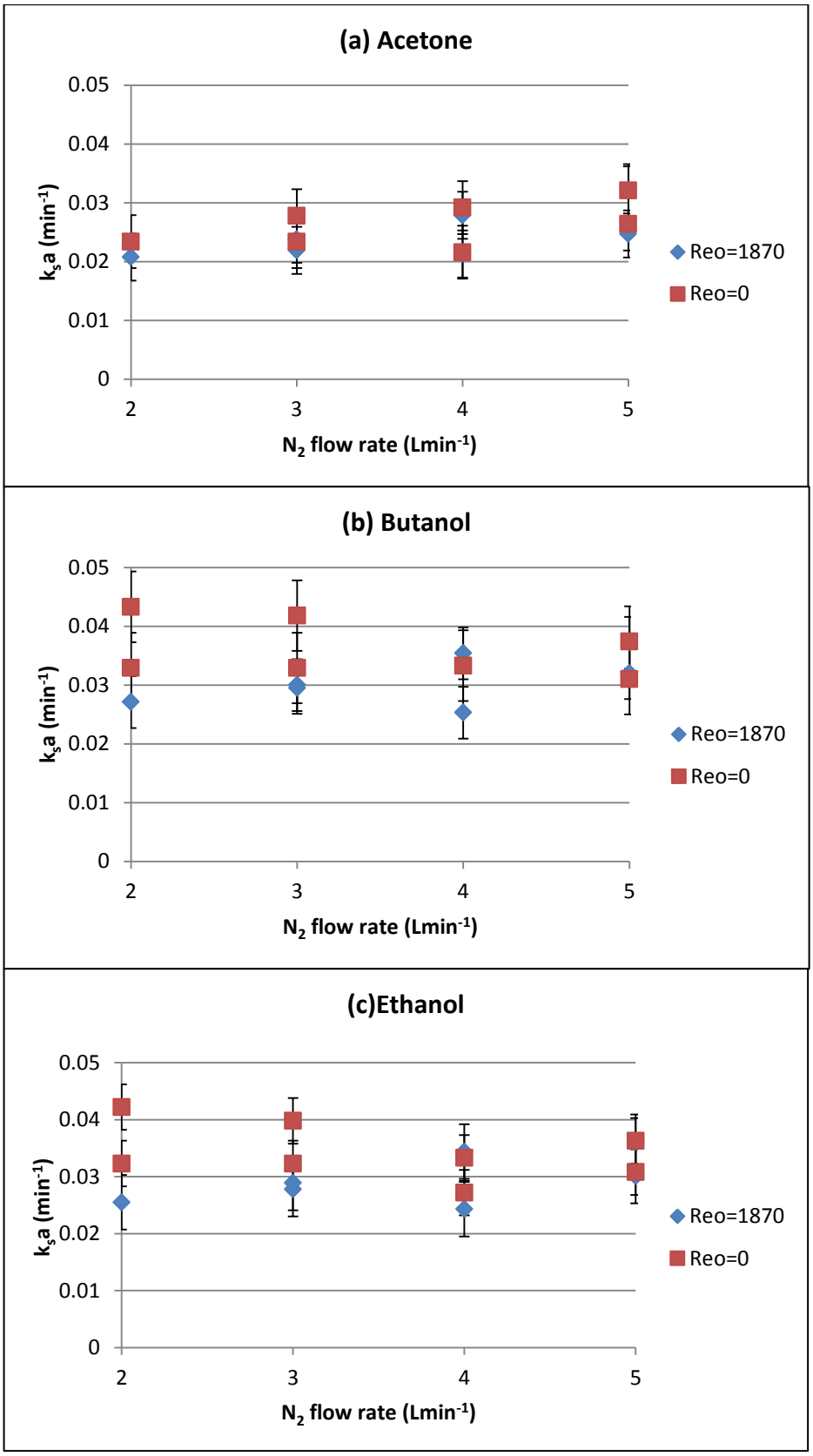


Figure 5.26 Calculated k_sa based on the reactor concentration at different Re_o. Error bars represent standard deviation from the mean of duplicate experiments

5.6.3 2-stage ABE fermentation integrated with gas stripping

The intrinsic problem with the ABE fermentation is that the butanol toxicity inhibits the cell growth and limits the maximum of total ABE that can be produced to approximately 20 gL^{-1} (Qureshi and Blaschek, 2001a). Previous studies have investigated *in situ* gas stripping as a method to relieve the butanol toxicity with huge success on increasing fermentation productivity (Maddox *et al.*, 1995; Ezeji *et al.*, 2003; Qureshi *et al.*, 2008b; Mariano *et al.*, 2011). It is hope by performing the *in-situ* gas stripping in this study, will improve the solvent productivity and glucose consumption and consequently improving the product yield. A two-stage ABE fermentation, 0-938 12 h was carried out in BOBB II with *in situ* gas stripping performed at three intervals, each lasting for 6 h. Fermentation was allowed to proceed for 48 h before gas stripping started. The control fermentation was carried out without gas stripping with all other conditions the same as that for the fermentation with *in situ* gas stripping. These two fermentations were carried out simultaneously with the same inoculum for each reactor. Figure 5.27 and Figure 5.28 show the solvent and acid production that occurs in the control fermentation and that with *in situ* gas stripping respectively.

Figure 5.27 shows a fermentation profile for the batch two-stage ABE fermentation without gas stripping. The fermentation experienced a gradual increase in solvent concentration over 96 h, after an initial lag phase where the rate of the solvent production significantly increased after 6 h of fermentation. Figure 5.28 shows the fermentation profile with gas stripping where the grey areas represent the three gas stripping intervals (gas stripping 1: GS 1, gas stripping 2: GS 2 and gas stripping 3: GS 3). During these intervals it is obvious that there was a decrease in the solvent concentrations in the reactor. Figure 5.28 indicates that a reduction in solvents, predominantly acetone and butanol, is evident. It can be seen that during the first interval of gas stripping which was between 48 to 54 h, the acetone concentration had a larger decrease than butanol and ethanol with approximately 78.1% of acetone, 27.1% of butanol and 14.2% of ethanol being stripped out of the reactor.

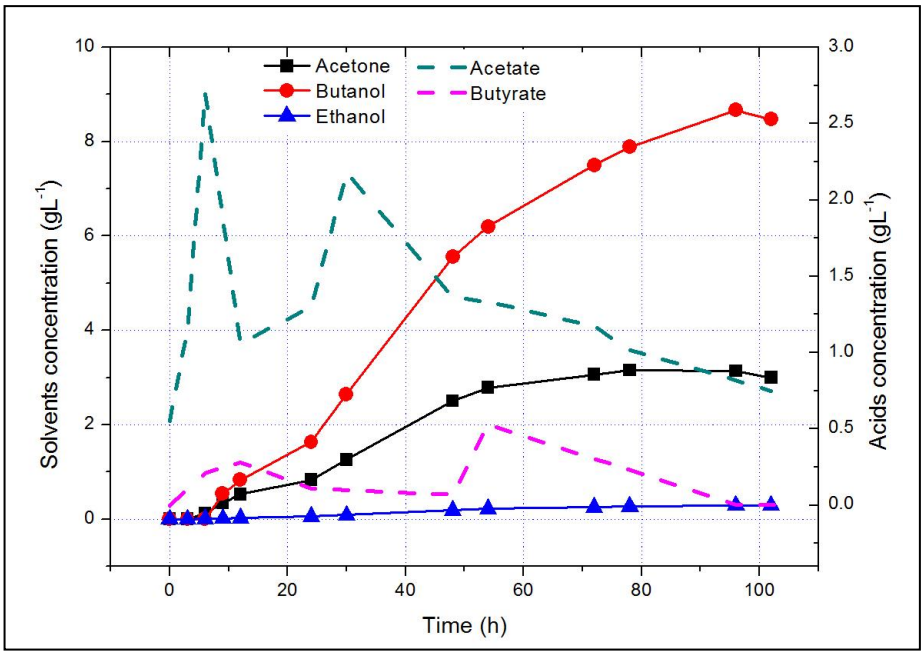


Figure 5.27 Solvent and acid profile of ABE fermentation without gas stripping. Fermentation condition: 2-stage fermentation (0-938 12 h), working volume 1 L, initial pH 6.5, temperature 32°C

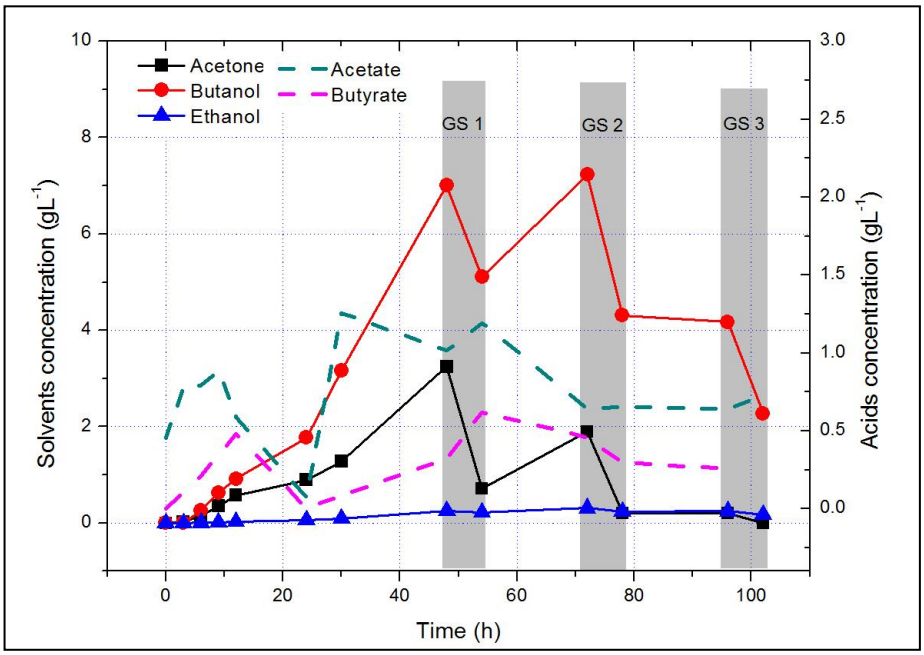


Figure 5.28 Solvent and acid profile of ABE fermentation with *in situ* gas stripping. Fermentation condition: 2-stage fermentation (0-938 12 h), working volume 1 L, initial pH 6.5, temperature 32°C. Gas stripping condition: Gas stripping starts after 48 h at three stripping intervals, stripping gas flows at 2 $Lmin^{-1}$, temperature of the cooling liquid to the condenser is -25°C

Once the first interval of gas stripping had concluded, the fermentation effectively gained a second wind and the butanol concentration in the reactor began to increase to higher than that before the gas stripping. During the second interval of gas stripping, the percentage of acetone, butanol and ethanol being stripped out from the reactor was higher than the first one with 88.9% of acetone, 40.4% of butanol and 24.2% of ethanol. Interestingly, once the second interval of gas stripping had stopped, there was no solvent produced in the reactor as depicted by a plateau line between 78 to 96 h in Figure 5.28. The third interval of gas stripping resulted in further decreases in the solvent concentrations in the reactor where all of the acetone was being stripped out left only butanol and ethanol. During the first stripping interval (GS 1), the cell concentration increased from 3.6 gL^{-1} to 4.1 gL^{-1}, with a simultaneous increase in both acetate and butyrate concentration. Perhaps this is due to the removal of solvents, which can have an inhibitory effect on cell growth and acid production. Another possible reason could be a consequence of the mixing engendered by the aeration.

Solvent was not produced after 78 h of the fermentation since all of the glucose in the reactor had been consumed as shown in Figure 5.29.

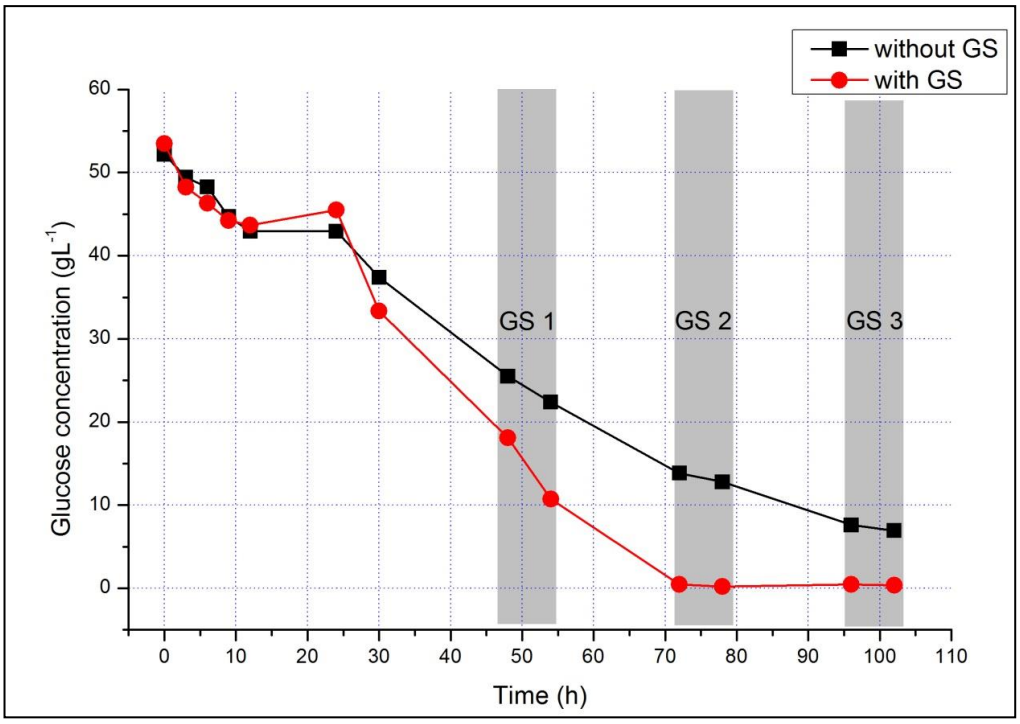


Figure 5.29 Glucose concentration profile in two-stage ABE fermentation with *in situ* gas stripping vs. without gas stripping

The glucose consumption within the two reactors is shown in Figure 5.29. It is important to note that the rate of glucose consumption was greater once the gas stripping had started as seen by the steeper gradients in GS 1: 1.23 $gL^{-1}h^{-1}$ in fermentation with gas stripping compared to 0.517 $gL^{-1}h^{-1}$ without gas stripping. This is probably due to the reduction in inhibition from the butanol, therefore more glucose is able to be consumed (Mollah and Stuckey, 1993; Qureshi and Blaschek, 2001b; Ezeji *et al.*, 2005). Another important observation to note is that by 72 h, all of the glucose in the reactor was fully consumed during the fermentation with gas stripping as opposed to the control where about 7 gL^{-1} of glucose left in the reactor at the end of the fermentation. This is why there was no solvent produced after 78 h in the gas stripping fermentation (as shown earlier in Figure 5.28). This was further exhibited by a significant decreased in the cell concentration soon after the first interval gas stripping stopped, as shown in Figure 5.30.

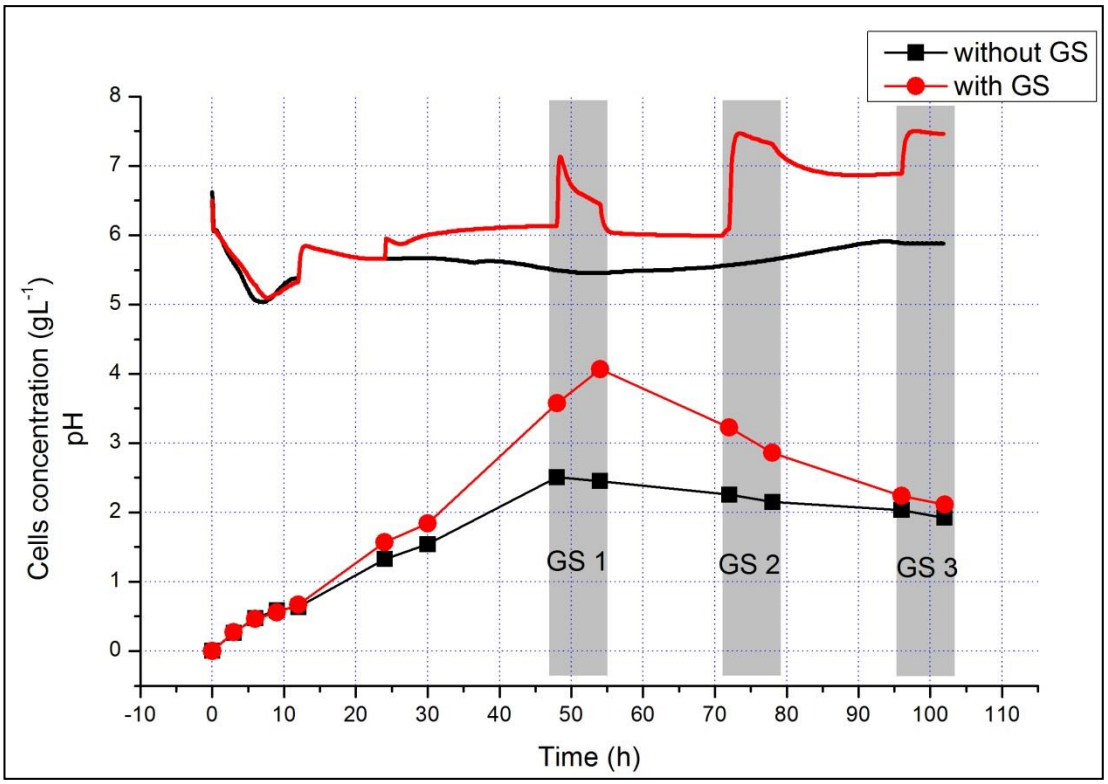


Figure 5.30 Cell concentration profile and pH profile in two-stage ABE fermentation with *in situ* gas stripping

The cell concentration profile in Figure 5.30 shows a definitive increase once the gas stripping started. A significant difference can be seen as the cell concentration was fairly consistent between the two reactors before the gas stripping started. It is interesting to note that the cell concentration increased significantly in the fermentation with gas stripping over control fermentation even before it started. The second gas stripping interval (GS 2) did not have the same effect on the cell growth as the first one, as all the glucose had been consumed, resulting in the cessation of cell growth. The pH between 0 and 30 h fermentation follows a similar trend in that it was consistent between the two reactors until the stripping commenced (Figure 5.30). At this point the pH increased significantly where the increase was due to the conversion of acids to the solvents and/or due to the additional mixing occurring in the reactor (by the N_2 sparging) effectively re-suspending the calcium carbonate which would have settled out in the reactor before that. The calcium carbonate would have reacted with some of the acids

produced and acted as a pH buffer for the fermentation medium. Prior to the second interval of gas stripping, the pH did not return to its initial value (before the gas stripping started) which indicated that no more acids were being produced as glucose was finished at that time. This pH increased was not that pronounced in the fermentation without gas stripping.

Table 5.15 (next page) summarizes the one- and two-stage ABE fermentation with and without *in situ* gas stripping. Solvent concentration in the gas stripping fermentation was the total concentration of solvent in the reactor and stripped solvent. Based on Table 5.15, the two-stage control fermentation with the medium initially contains 52.2 gL^{-1} glucose, about 3.1 gL^{-1} acetone, 8.7 gL^{-1} butanol and 0.29 gL^{-1} ethanol were produced when the fermentation ceased at 96 h with about 7.6 gL^{-1} glucose remains in the fermentation medium. The product yields based on glucose consumed were (gg^{-1}): acetone 0.07, butanol 0.19, ethanol 0.007 and total ABE 0.27. One-stage control fermentation initially contains 44.5 gL^{-1} glucose, producing 2.4 gL^{-1} acetone, 6.9 gL^{-1} butanol and 0.2 gL^{-1} ethanol with 16.7 gL^{-1} glucose remains in the fermentation medium at 96 h.

When gas stripping was integrated with one- and two-stage ABE fermentation, the fermentation was completed in 78 h, when all of the glucose was completely consumed in the two-stage fermentation and about 81% glucose consumed in the one-stage fermentation. With an initial glucose concentration of 53.3 gL^{-1} in the two-stage fermentation with gas stripping, about 4.3 gL^{-1} acetone, 10.7 gL^{-1} butanol and 0.37 gL^{-1} ethanol were produced within 78 h of the fermentation. The overall products yield based on glucose consumed were (gg^{-1}): acetone 0.08, butanol 0.20, ethanol 0.007 and total ABE 0.29. This fermentation was 56-67% faster than that similar fermentation without gas stripping which leads to higher butanol productivity of 0.14 $gL^{-1}h^{-1}$ (vs. 0.09 $gL^{-1}h^{-1}$) and total ABE productivity of 0.20 $gL^{-1}h^{-1}$ (vs. 0.13 $gL^{-1}h^{-1}$) than the respective control fermentation.

Table 5.15 Data of one- and two-stage fermentation without and with *in situ* gas stripping. One-stage fermentation was performed at Re_o 0 and two-stage fermentation was performed as 0-938 12 h fermentation

Parameter	2-stage without gas stripping (control)	2-stage with gas stripping	1-stage without gas stripping (control)	1-stage with gas stripping
Fermentation time (h)	96	78	96	78
Glucose consumed (gL^{-1})	44.6	53.3	27.8	34.4
% glucose consumed	85.4	100	62.4	81.2
Glucose consumption rate ($gL^{-1}h^{-1}$)	0.46	0.68	0.23	0.44
Acetone (gL^{-1})*	3.14	4.26	2.41	3.37
Butanol (gL^{-1})*	8.67	10.65	6.86	9.19
Ethanol (gL^{-1})*	0.29	0.37	0.20	0.24
Acetate (gL^{-1})*	0.82	0.66	0.85	0.89
Butyrate (gL^{-1})*	0.004	0.30	0	0
Total ABE (gL^{-1})	12.1	15.2	9.46	12.8
A:B:E ratio	11:30:1	12:29:1	12:34:1	14:38:1
Total acid (gL^{-1})	0.82	0.96	0.85	0.89
Cell dry weight (gL^{-1})	2.04	2.86	1.78	3.77
Acetone productivity (g A $L^{-1}h^{-1}$)	0.033	0.055	0.025	0.043
Butanol productivity (g B $L^{-1}h^{-1}$)	0.090	0.14	0.071	0.12
Ethanol productivity (g E $L^{-1}h^{-1}$)	0.0030	0.0047	0.0021	0.0031
ABE productivity (g ABE $L^{-1}h^{-1}$)	0.13	0.20	0.099	0.16
Acetone yield [g A (g glucose)$^{-1}$]	0.070	0.080	0.087	0.098
Butanol yield [g B (g glucose)$^{-1}$]	0.19	0.20	0.25	0.27
Ethanol yield [g E (g glucose)$^{-1}$]	0.0065	0.0069	0.0072	0.0071
ABE yield [g ABE (g glucose)$^{-1}$]	0.27	0.29	0.34	0.37
Specific butanol productivity [g B (g biomass)$^{-1}$ h^{-1}]	0.044	0.049	0.040	0.032

Parameter	2-stage without gas stripping (control)	2-stage with gas stripping	1-stage without gas stripping (control)	1-stage with gas stripping
Specific butanol yield [g B (g biomass)$^{-1}$ (g glucose)$^{-1}$]	0.093	0.070	0.14	0.072

*Concentrations are totals of product in the reactor and stripped

Performing gas stripping to one-stage fermentation also increased the butanol productivity by 69% to 0.12 $gL^{-1}h^{-1}$ (as opposed to 0.071 $gL^{-1}h^{-1}$ in similar fermentation without gas stripping).

In comparison with the control fermentations, the fermentation with gas stripping showed an 18 h reduction in fermentation time (from 96 h to 78 h) accompanied by total consumption of glucose (as opposed to 85.4% and 62.4% consumption in control fermentations) respectively, in the two-stage and one-stage fermentation due to increase concentration of cell (from 2.04 gL^{-1} to 2.86 gL^{-1} in the two-stage and 1.78 gL^{-1} to 3.77 gL^{-1} in the one-stage) and decreased butanol toxicity to the culture. Clearly, removing butanol by gas stripping not only alleviated butanol toxicity and increased the fermentation rate, but also increased the butanol yield.

When compared the fermentation with gas stripping in the two-stage and one-stage, clearly the two-stage fermentation had better specific butanol productivity (0.049 g B (g biomass)$^{-1}$ h^{-1}) than the one-stage (0.032 g B (g biomass)$^{-1}$ h^{-1}). It was observed that performing gas stripping to the one-stage fermentation reduced the specific butanol productivity by 20% to 0.032 g B (g biomass)$^{-1}$ h^{-1} (as opposed to 0.04 g B (g biomass)$^{-1}$ h^{-1} in the control fermentation). Improvement in butanol productivity and specific butanol productivity can be observed when performing gas stripping to the two-stage ABE fermentation as opposed to the one-stage fermentation with gas stripping.

5.6.4 Effects of gas stripping on ABE fermentation

Gas stripping only removes volatile compounds, mainly ABE and a very small amount of acids. The removal of butanol from the fermentation medium alleviated the butanol toxicity to the cells thus allows the fermentation to consume more substrate at a higher rate with higher ABE productivity. Compared to the fermentation without gas stripping, butanol and ABE productivities increased 55.5% and 53.8%, respectively, in this study. Similar improvements in butanol and ABE productivities have also been reported by others as shown in Table 5.16.

Table 5.16 Comparisons of batch ABE fermentation with and without *in situ* gas stripping

	ABE titre (gL^{-1})			Productivity ($gL^{-1}h^{-1}$)		Yield (gg^{-1})		
	Reactor	Condensate						
***Clostridium* strain**	**Butanol in reactor**	**Butanol**	**ABE**	**Butanol**	**ABE**	**Butanol**	**ABE**	**Ref.**
C. acetobutylicum P262	2.0-7.2 (5.5)	–	–	0.20 (0.05)	0.32 (0.07)	0.22 (0.17)	0.35 (0.26)	Maddox *et al.* (1995)
C. beijerinckii BA101	2.0-8.5 (11.9)	–	42.7-82.5	0.42 (0.20)	0.61 (0.29)	0.27 (0.27)	0.40 (0.40)	Ezeji *et al.* (2003)
C. acetobutylicum P262	1.0-6.0 (7.4)	10-46	18-67	– (0.16)	0.31 (0.27)	– (0.29)	0.41 (0.47)	Qureshi et *al.* (2008b)
C. beijerinckii P260	1.8-6.5 (11.8)	13.3	16.2	0.23 (0.20)	0.28 (0.26)	0.19 (0.26)	0.22 (0.35)	Mariano *et al.* (2011)[a]
Clostridium GBL1082	2.3-7.2 (8.7)	13.1-19.2	13.9-21.7	0.14 (0.09)	0.20 (0.13)	0.20 (0.19)	0.29 (0.27)	This study

Values in the parenthesis were from experiments without gas stripping

[a] Vacuum was applied for 6 h to remove ABE vapour

Generally, fermentation with integrated gas stripping, increases reactor productivity significantly and more substrate can be converted to ABE. The differences in the

productivity and yield were down to different strain and media used in the fermentation. *C. beijerinckii* BA101 is known as a "hyper-butanol producer", and has exhibited butanol productivities of over 0.4 $gL^{-1}h^{-1}$ under optimized condition (Qureshi and Blaschek, 2001a). These are the highest in the highest so far. Gas stripping usually has a butanol selectivity of 4-30.5 (Qureshi and Blaschek, 2001b), depending on the operating conditions, and is able to produce a condensate with much concentrated ABE than that in the reactor. It should be noted, however, that in some of the previous studies (Qureshi *et al.*, 2008b; Mariano *et al.*, 2011), gas stripping reduced butanol and ABE yield by as much as 39% (Table 5.16). The decrease in the yield was possibly caused by ABE loss through the connecting tube or/and changes in metabolic activities of cells towards acid production (Mariano *et al.*, 2011).

Gas stripping also removes a significant amount of water from the reactor, thus causing the accumulation of salts, which could affect cell growth and solvent production negatively. Maddox *et al.* (1995) reported that 50% of inhibition towards growth rate with salt concentration range 15-20 gL^{-1} for both sodium chloride and magnesium chloride. In contrast to the insignificant effect on organic acid production, high salt concentration could shift the fermentation from being solventogenic to acidogenic. In this study, only a small amount of water was removed from the reactor in the three gas stripping intervals: 4.3 mL, 7.8 mL and 9.8 mL, respectively, which should not have a significant effect on the fermentation as the final fermentation medium in the reactor was 830 mL as opposed to the one without gas stripping which was 860 mL.

5.7 Kinetics and Modelling of the ABE Fermentation at Different Oscillatory Reynolds number (Re_o)

The dynamic of a typical batch acetone-butanol-ethanol (ABE) fermentation can be divided into two distinctive phases: (i) active growth of the cell and acid synthesis, and (ii) conversion of acid to solvent (ABE). The active growth of the cell are accompanied by the accumulation of intermediates, acetate and butyrate, which extend to the "acid break" when the pH of the culture increasing. Conversion of acid and remaining substrate into solvent follows the acid break. It has been shown that butanol and acetone were synthesized from butyrate and acetate, respectively (Jones and Woods, 1986).

The objective of the modelling is to have a better interpretation of the kinetics of batch ABE fermentation by *Clostridium* GBL1082 using glucose substrate at increasing Re_os (from Re_o 0 to 938). Further to that, it was intended that the model be used to predict and verify the two-stage ABE fermentation. The proposed model may allow better understanding of the one-stage and two-stage ABE fermentation process and may be used in optimization and development of the control strategy of these fermentations.

5.7.1 Formulation of the model

Development of biologically interpretable kinetic relationships between the fermentation variables was based on the information known from metabolic pathways of *C. acetobutylicum*. This information, which was introduced earlier in chapter three, established that acetone, butanol, ethanol, biomass, CO_2 and H_2 are the end products of the metabolism while butyrate and acetate are the intermediate compounds connected with the growth kinetics. The development of the kinetic model reflecting the experimental data was based on the following assumption:

i. No mixing limitation
ii. No process limitation by nitrogen source
iii. Glucose is the only limiting substrate in the batch fermentation

In a batch bioreactor, the rate of microbial growth is characterized by the specific growth rate of the bacteria (Shuler and Kargi, 2002), defined as:

$$\mu = \frac{1}{X}\frac{dX}{dt}$$

Equation 5.8

….where μ is the specific growth rate (1/h), X is the cell concentration (g/L) and t is time (h). Parameter μ generally related to the substrate concentration with the well known Monod equation (Özilgen, 1988):

$$\mu = \mu_{max}\frac{S}{K_S + S}$$

Equation 5.9

….where μ_{max} is the maximum specific growth rate, K_s is a saturated coefficient constant and S is the substrate concentration. The batch growth curve can be predicted by combining Equation 5.8 and Equation 5.9, yields:

$$\frac{dX}{dt} = \mu_{max}\frac{S}{K_S + S}X$$

Equation 5.10

The relationship between microbial growth yield and substrate consumption is:

$$X - X_o = Y_{X/S}(S_o - S)$$

Equation 5.11

…where X_o and S_o are initials values and $Y_{X/S}$ is the cell mass yield based on the limiting nutrient (glucose). Substituting for S in Equation 5.10 to Equation 5.11 yields:

$$\frac{dX}{dt} = \frac{\mu_{max}\left(Y_{X/S}S_o + X_0 - X\right)}{\left(K_s Y_{X/S} + Y_{X/s}S_o + X_o - X\right)} X$$

Equation 5.12

The proposed kinetic model for cell growth rate assumed that the microbial activity was inhibited by butanol. Thus, Equation 5.12 will become:

$$\frac{dX}{dt} = \frac{\mu_{max}\left(Y_{X/S}S_o + X_0 - X\right)}{\left(K_s Y_{X/S} + Y_{X/s}S_o + X_o - X\right)} X - \ k_d \, X \, B$$

Equation 5.13

...where k_d is the first-order death rate constant and B is the butanol concentration (Shuler and Kargi, 2002).

The relationship between the specific rates of substrate consumption and the substrate concentration are following a differential equation consist of a linear relationship and the Monod equation (Votruba *et al.*, 1985) as shown below:

$$\frac{dS}{dt} = -\left(k_1 S + \ k_2 \frac{S}{K_S + \ S} \right) X$$

Equation 5.14

The kinetics of butanol and butyrate are related as there is a mutual interaction between the syntheses of these two products as in the metabolic pathway of *C. acetobutylicum*. In line with this agreement, the expression of a differential mass balance of butyrate accumulation should be considered from two reaction lines. The first one: butyrate is produced from glucose with simultaneously inhibition by butanol. The second reaction line is reflecting the conversion of butyrate to butanol, shown as:

$$\frac{dBA}{dt} = \left(k_3 S \frac{K_I}{K_I + B} - k_4 \frac{BA}{K_{BA} + BA}\right) X$$

Equation 5.15

The terms K_I and K_{BA} are inhibition and saturation constants, respectively, for the two reactions. Next, a subsystem consisting of butanol and butyrate was considered for the analysis of butanol kinetics. The differential mass balance for butanol system, which enables to simulate the initial delay in the butanol production and accumulation caused by intermediate accumulation of butyrate, can be expressed as:

$$\frac{dB}{dT} = k_5 S X - 0.841 \frac{dBA}{dt}$$

Equation 5.16

Coefficient 0.841 resulted from the stoichiometric conversion treated as a ratio of molecular weights of butanol to butyrate.

Butanol also has a similar controlling effect on acetate production. Acetate production is associated with the culture growth as the case is with butyrate, but, have lower conversion rate than butyrate. The differential mass balance for acetate can be written as:

$$\frac{dAA}{dt} = \left(k_6 \frac{S}{K_S + S} \frac{K_I}{K_I + B} - k_7 \frac{AA}{AA + K_{AA}} \frac{S}{K_S + S}\right) X$$

Equation 5.17

The first term on the right-hand side of Equation 5.17 represents the production of acetate from glucose simultaneously inhibited by butanol. The second term describes the conversion of acetate into acetone.

The Monod-type function can be used for simulation of the acetone and ethanol production rate:

$$\frac{dA}{dt} = k_8 \frac{S}{K_S + S} X$$

Equation 5.18

$$\frac{dE}{dt} = k_9 \frac{S}{K_S + S} X$$

Equation 5.19

5.7.2 Validation of the model

The kinetic parameters of k_1 to k_9, k_d, k_{AA}, k_{BB} and K_I were taken from Votruba *et al.* (1985) and the rest were obtained from batch fermentations in BOBB II at Re_o 0 and 938. Values of the kinetic parameters are shown in Table 5.17.

Table 5.17 Values of kinetic parameters for BOBB II fermentation at Re_o 0 and Re_o 938

Kinetic parameters	Values	Kinetic parameters	Values
μ_m	0.5 (1.5)	k_2	0.06764
$Y_{X/S}$	0.05 (0.03)	k_3	0.003 (0.0037)
K_s	24.72	k_4	0.2170 (0.1170)
K_d	0.005 (0.05)	k_5	0.003
K_{AA}	0.5	k_6	0.01
K_{BA}	0.5	k_7	0.000018 (0.000025)
K_I	0.833	k_8	0.08
k_1	0.006 (0.0155)	k_9	0.0048

Values in parentheses are for Re_o 938. Both Re_os use the same value if no parentheses indicates

μ_m represent the maximum specific growth rate of the bacteria at Re_o 0 and 938. $Y_{x/s}$ represents the bacteria cell yield based on substrate. K_s represent substrate concentration to reach half of μ_m.

The kinetic models (Equation 5.17 to Equation 5.19) were solved in MatLab® using the differential equation solver ODE45 (Appendix E). The fitness of model prediction to experimental data was statistically determined using pair sample t-test of OriginPro 8 with a significant probability of 5%. That is to say the model prediction data was not significantly different from the experimental data if the P-value was more than 0.05.

5.7.3 The fitness of the model to fermentation data

Example of the fitness of the model to the experiment data for cell and glucose concentration in BOBB II ABE fermentation at Re_o 938 is shown in Figure 5.31.

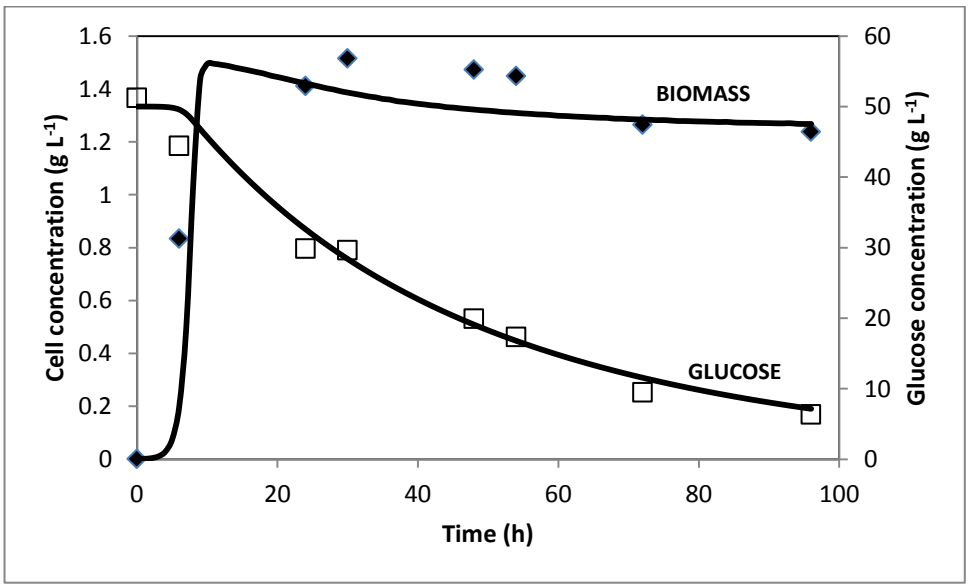


Figure 5.31 Comparison of experimental data (symbol) and model prediction (solid line) for biomass and glucose concentration during ABE fermentation by *Clostridium* GBL1082 in BOBB II at Re_o 938

The model satisfactorily predicted the cell growth trend and glucose consumption during the ABE fermentation with p-value of 0.0817 and 0.549 for biomass and glucose concentration, respectively. It is well known that high concentration of

butanol accumulated in culture broth inhibit cell growth, and consequently cause cell death (Jones and Woods, 1986; Baer *et al.*, 1987). As shown in Figure 5.31, the growth of *Clostridium* GBL1082 slowed down as the fermentation proceeded and eventually stopped when the butanol concentration reached a value of 4 gL^{-1}. Beyond this value, the decrease in the cell concentration occurred at a rate of 0.05 h^{-1} (K_d).

For BOBB II fermentation at Re_o 0, the model poorly predicted the cell growth and glucose consumption, as shown in Figure 5.32 with P-value of 0.610 and 0.356, respectively.

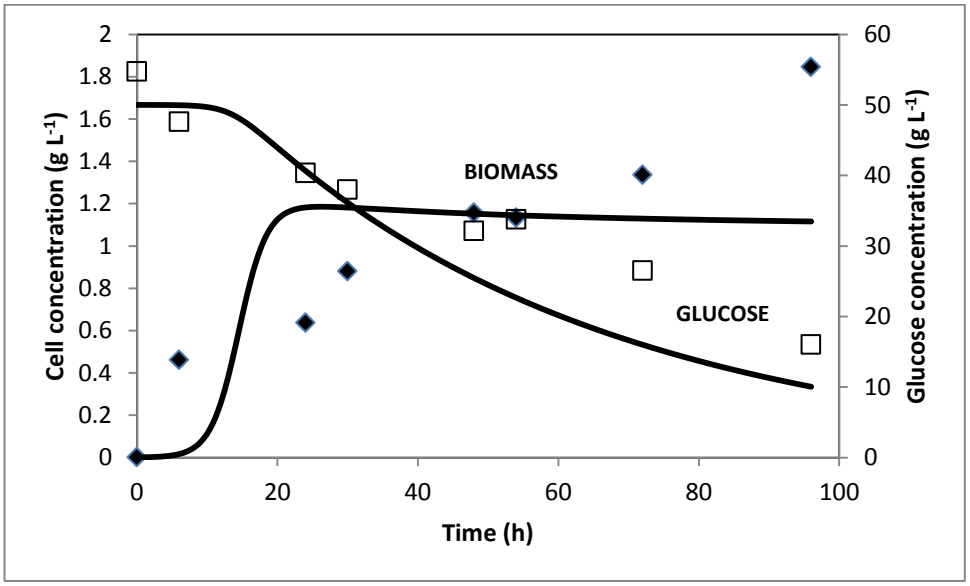


Figure 5.32 Comparison of experimental data (symbol) and model prediction (solid line) for biomass and glucose concentration during ABE fermentation by *Clostridium* GBL1082 in BOBB II at Re_o 0

The cell growth trend in BOBB II at Re_o 0 did not follow the typical sigmoid shape, thus the model failed to describe the growth trend as the model assumes a sigmoidal shape for a batch growth curve. Clearly there were phenomena that cannot be captured by the model for the fermentation without mixing in BOBB II. Cell concentration increased exponentially without stationary phase observed until

the end of the fermentation. On the other hand, the specific growth rate (μ) and glucose consumption rate (k_1) in Re_o 0 was lower by 3-fold and 2.5-fold, respectively compared to Re_o 938. The model predicted higher growth rate with higher glucose consumption but at the expense of higher death rate when mixing incorporated in the fermentation, which were exhibited in the experimental fermentation data. It should be noted that glucose also been consumed during products formation, and butanol accumulation in the broth does contributed to cell growth inhibition.

The figures below show the model prediction for intermediate products (acetate and butyrate) and key end products (acetone, butanol and ethanol) with respect to the experimental value in BOBB II fermentation at Re_o 938 (Figure 5.33) and Re_o 0 (Figure 5.34).

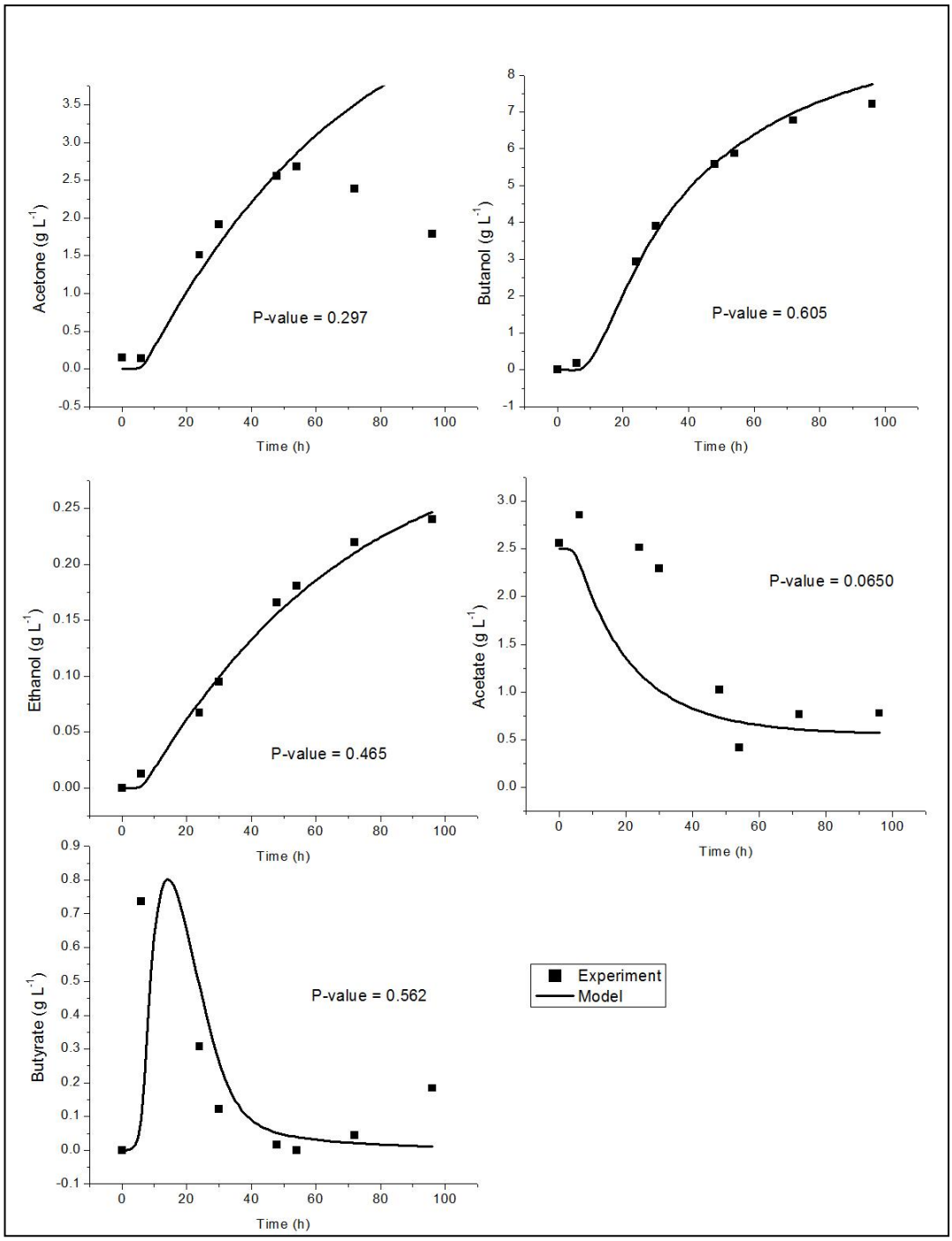


Figure 5.33 Experimental data (symbol) and model prediction (solid line) for product concentration during ABE fermentation by *Clostridium* GBL1082 in BOBB II at Re_o 938

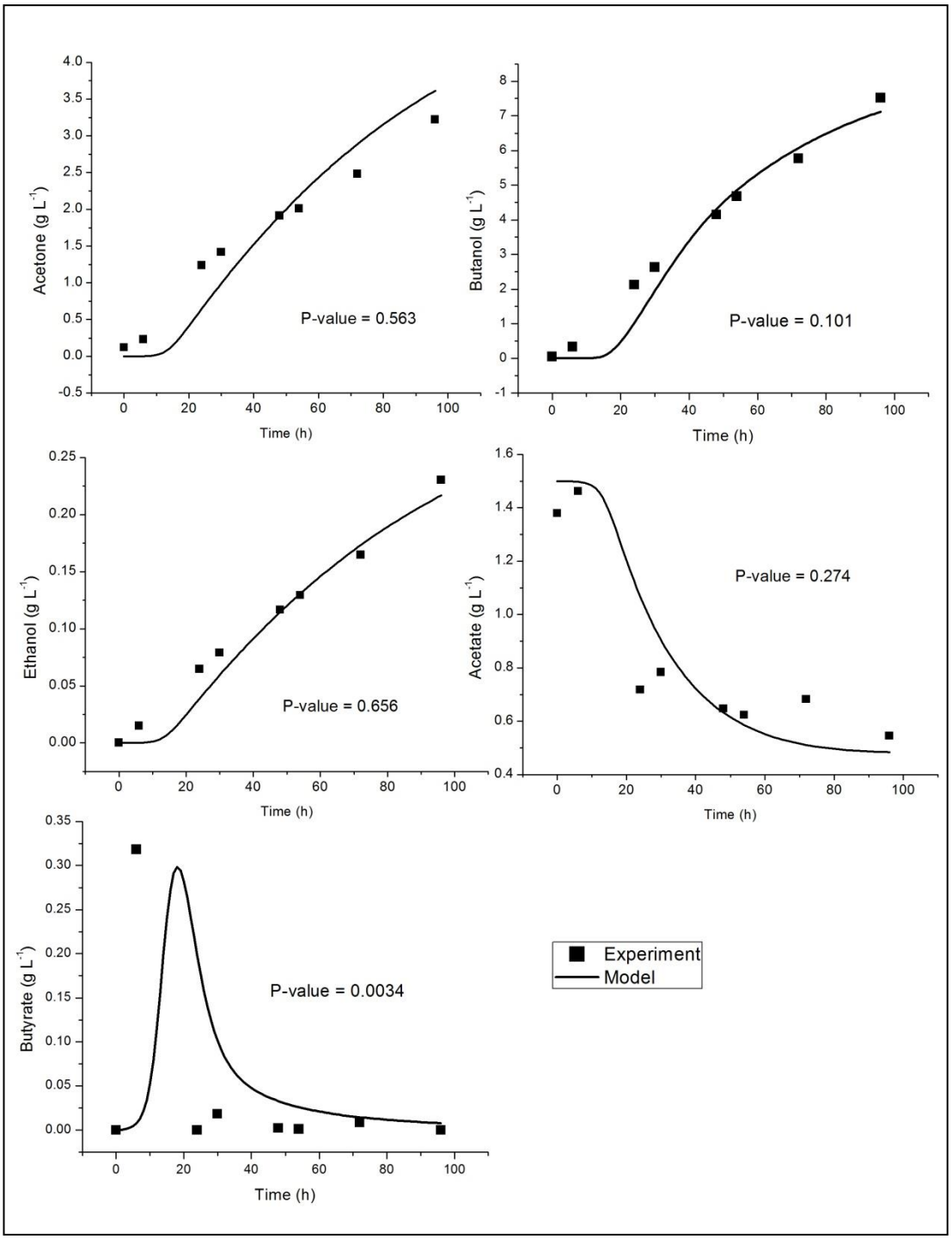


Figure 5.34 Experimental data (symbol) and model prediction (solid line) for product concentration during ABE fermentation by *Clostridium* GBL1082 in BOBB II at Re_o 0

The model prediction fitted to the experimental data for fermentation with mixing (Re_o 938). *Clostridium* GBL 1082 produced butanol as a major fermentation end-product with acetone and ethanol as by-products. At Re_o 938, the model over predicted the acetone concentration after 48 h, where the concentration supposed to decrease as a result of the oscillation (which released ~1 gL^{-1} of acetone to the bioreactor headspace). On the other hands, since the model assumes no mixing limitation to the process, the model prediction fitted poorly to the experimental data in Re_o 0. Clearly, there are some phenomena in Re_o 0 fermentation that are not been described by the proposed model.

Fond *et al.* (1985) suggested that solvent production started when acid concentration reached a certain level. In the present study these effects were covered in the model, such that, when certain amounts of acids and solvent accumulated, the growth curve became concave down and at this point, butanol, acetone and ethanol production by *Clostridium* GBL1082 started. In agreement with the metabolic pathway, the proposed models considered that butyrate and acetate were intermediate products which were respectively reduced to butanol and acetone.

The proposed model was used to predict the behaviour of the two-stage ABE fermentation of 0-938 switching at 12 h with a low degree of success when fitted to the experimental data as shown in Figure 5.35. The model fairly predicted the behaviour before the switching, where no mixing involved in the fermentation. Further after switching, the predicted data was over the experimental data for solvent production.

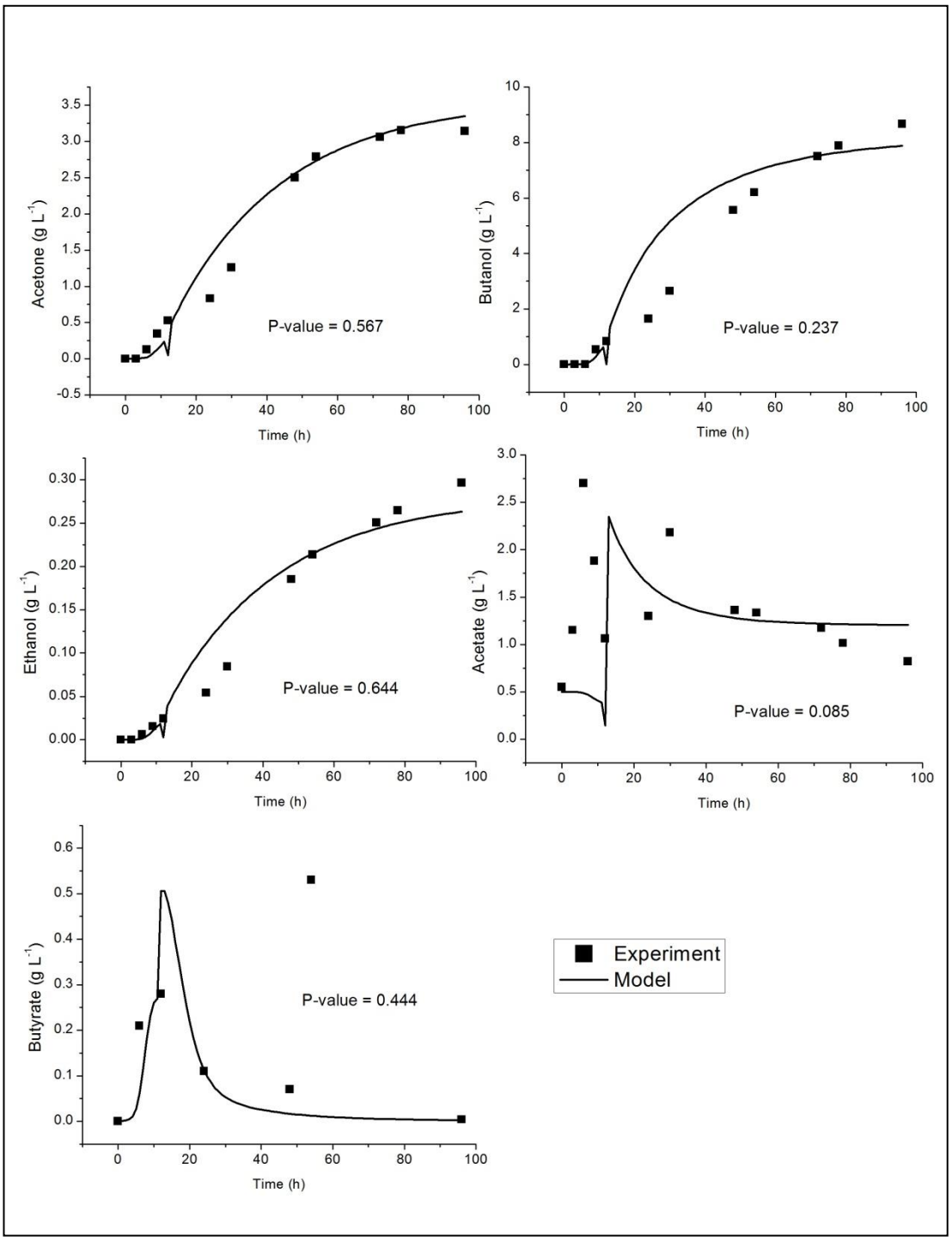


Figure 5.35 Experimental data (symbol) and model prediction (solid line) for product concentration during two-stage ABE fermentation by *Clostridium* GBL1082 in BOBB II at Re_o 0 switching to Re_o 938 after 12 h

The rate constants used to predict the two-stage model were then re-apply to the Re_o 0 and 938 fermentation, and the predicted data was compared to its previous best fit model as shown in Figure 5.36.

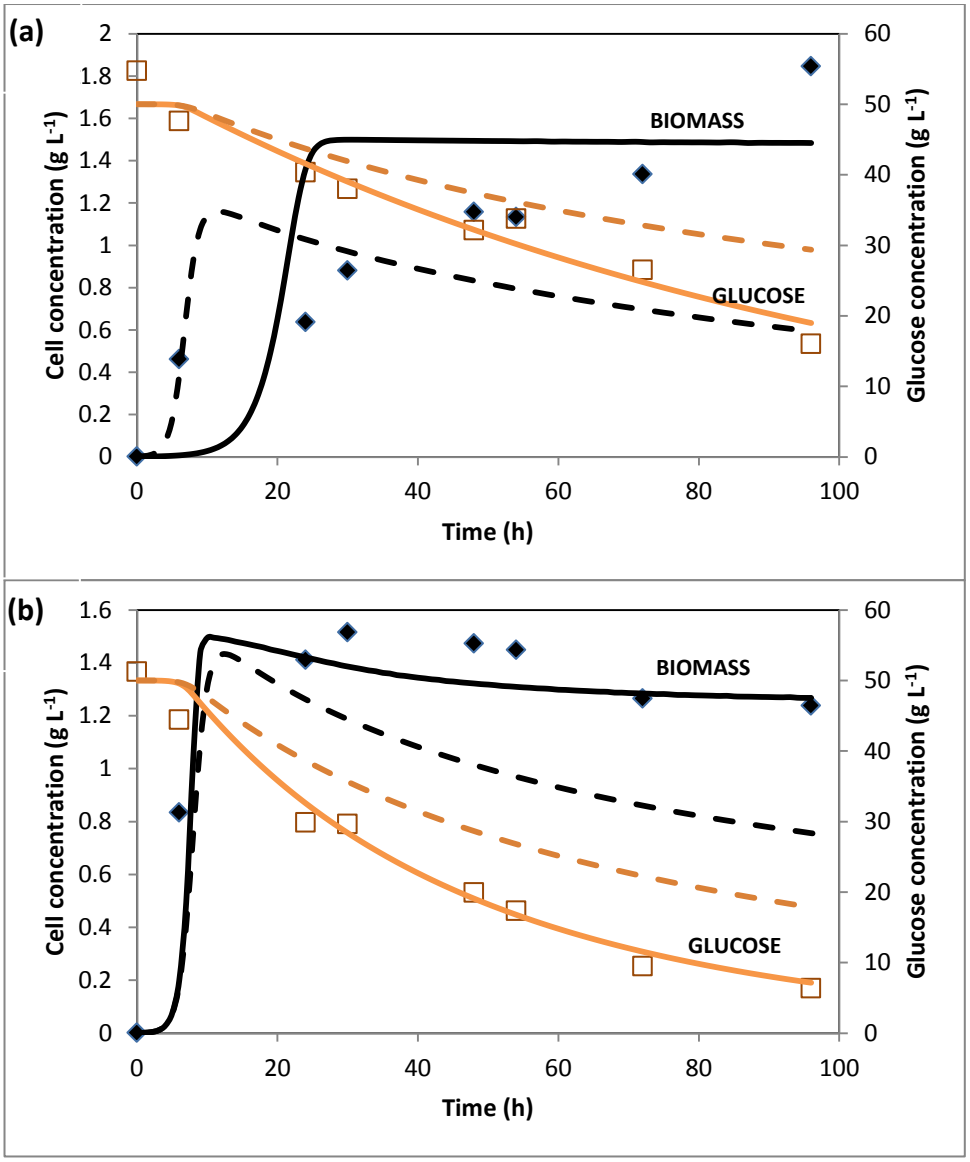


Figure 5.36 Model prediction in (a) Re_o 0 and (b) Re_o 938 using rate constants from two-stage model. New predicted data (dashed line), best predicted data (solid line) and experimental data (symbol).

From Figure 5.36, rate constants from the two-stage fermentation were unable to match the ABE fermentation behaviour in both Re_os (as shown by the dashed line). As the model used in this study was a simple systematic and Monod equation, these

equations was not adequate to describe the fermentation behaviour accurately. Terms that describe the transport process of the nutrient in and out of cell, and physical culture parameters such as pH, temperature and redox potential, could potentially improving the model fitness. A holistic modelling approach, incorporating the biochemical pathways and culture physiological state marker would better reflect the ABE fermentation. However, as this study was heavily experiment-based, there was not adequate time to expand the proposed model to accurately describe the ABE fermentation in BOBB II.

Chapter 6 CONCLUSION AND FURTHER WORK

6.1 Conclusion

6.1.1 Development of a new batch oscillatory baffled bioreactor design

Chemical sterilization of a pre-existing batch oscillatory baffled reactor, "BOBB I", was successfully achieved using a commercial sterilant, Virkon®. Following sterilization, several successful ABE fermentations were performed. Significantly higher productivity was achieved in BOBB I (0.37 $gL^{-1}h^{-1}$) than in comparable Schott bottle fermentations at similar working volumes. However, the reactor was constructed from polyvinyl chloride (PVC). These materials were unsuitable as they were eventually degraded by the chemical sterilant, leading to material failure. Hence, a second generation of this reactor, "BOBB II", was designed and built.

"BOBB II" is a novel design of autoclavable batch Oscillatory Baffled Bioreactor. This "new generation" of OBBs represents an improvement over previous designs, as they were not explicitly built for biotechnology applications. The BOBB II is a 2L bioreactor, constructed from glass and stainless steel only, so that it can withstand repeated autoclaving. It was designed such that a section containing the reactor vessel itself fitted inside a standard autoclave available within CEAM which has a cylindrical compartment with diameter of 490 mm and height of 700 mm. This section, containing media, could then be satisfactorily autoclaved. It was also designed so that it could be fitted with a variety of monitoring instrumentation: temperature, pH and redox electrode, to record various profiles during fermentations. A later addition to the reactor instrumentation was an automatic temperature controller, ensuring precise temperature control during the fermentation course. For a sterilization cycle at 121^{o}C for 15 minutes, BOBB II was successfully sterilized in the autoclave with or without the medium without damaging any parts of the reactor. The BOBB II was

successfully used in numerous experiments to characterize butanol fermentation at oscillatory Reynolds numbers (Re_o) in the range 0 to 1870.

6.1.2 A Comparison of ABE fermentation in BOBB II and the STR

ABE fermentations performed in BOBB II and the STR at an increasing power density (0 to 1.14 Wm^{-3}) increased the specific growth rate and specific solvent production rate. This effect was attributed to the enhancement of the extracellular mass transfer of the nutrients and metabolites as a result of enhanced mixing, as power density increased. However at the highest mixing rate, BOBB II fermentation underwent an acid crash, resulting in a premature inhibition of solvent production (down to 3.24 gL^{-1}, 21.4% lower than that without oscillation). At the highest mixing rate in BOBB II, before the acid break point, the high mixing rate resulted in increase the primary metabolic rate (specific growth rate and acid production rate). The acid crash occurred when an excess of acid production took place without a significant switch to the solventogenesis. In terms of energy consumption, BOBB II produced up to 12 g ABE $(kW.h)^{-1}$, which was 60% higher than the STR.

BOBB II fermentation was compared to that in a conventional STR at power densities in the range 0 to 1.14 Wm^{-3}. The butanol concentration in BOBB II was higher than in the STR, except at the lowest and highest power densities: 0 and 1.14 Wm^{-3}. It was concluded that at the lowest power density (0 Wm^{-3}), higher butanol concentration in the STR than BOBB II was due to the geometry of the reactor. While at the highest power density (1.14 Wm^{-3}), the oscillation mixing in BOBB II enhancing the cell growth and acids production, to the point that the conversion of these acid to the solvent slower than the acid production, thus leads to acid crash. Generally, fermentation in BOBB II increased the butanol concentration. For example, at a power density of 0.14 Wm^{-3}, at a fermentation time of 30 h, the butanol concentration in BOBB II was 3.9 gL^{-1}, while in the STR was 3.5 gL^{-1}, equating to a higher butanol productivity of 0.13 $gL^{-1}h^{-1}$ in BOBB II compared to 0.11 $gL^{-1}h^{-1}$ in the STR. It can be concluded that at similar power densities, BOBB II fermentation

shifts to solventogenesis earlier than in the STR, resulted in higher solvent productivity. BOBB II exhibited a higher maximum cell concentration (1.52 gL^{-1}) than the STR (1.29 gL^{-1}), at similar power density. The higher solvent-producing cell population in BOBB II resulted in a greater amount of butanol being produced more quickly than in the STR.

6.1.3 Two-stage ABE fermentation in BOBB II

In the two-stage fermentation, where at the first stage the fermentation was left without mixing for 12 h and after that oscillation was applied at Re_o 938 in the second stage or vice versa. This two-stage fermentation increased the butanol productivity by up to 37.5% over the one-stage fermentation. Delaying the onset of oscillatory mixing by 12 h, as in the two-stage fermentation, increased the cell concentration and reduced the time to achieve the acid concentration threshold for phase shifting (from acidogenesis to solventogenesis), thus higher butanol concentrations were achieved in less time than in one-stage fermentation. Analysis of the cell morphology at 48 h, showed that the concentration of clostridial form of the cells was higher in the two-stage fermentation than the one-stage.

6.1.4 ABE fermentation with *in situ* gas stripping

When *in situ* gas stripping was incorporated into the one-stage and two-stage ABE fermentation, a significant increase in butanol was observed:

- Butanol concentration increased to 10.4 gL^{-1}: a 22.7% improvement over that without gas stripping
- Butanol productivity increased by 55.6% when gas stripping was integrated to the two-stage ABE fermentation.
- 100% glucose consumption was exhibited in the two-stage fermentation with gas stripping, whereas without gas stripping had consumed only 75.5% of provided glucose at similar fermentation time (78 h)

Overall, cell and butanol concentration, and butanol productivity and yield increased when gas stripping was applied to the one-stage and two-stage ABE fermentation, as the limiting effects of butanol toxicity was relieved *in situ* through gas stripping process.

6.1.5 Modelling of ABE fermentation in BOBB II

A mathematical model of the batch ABE fermentation process presented in this work consists of a set of differential equations representing bioreactor mass balance for the biomass, substrate, intermediate products and key final products. The model is capable of reflecting the growth of *Clostridium* GBL1082, sugar consumption and the production of all metabolites (acetone, butanol, ethanol, acetate and butyrate) in one stage BOBB II fermentation at Re_o 938. However, the model failed to predict the fermentation at Re_o 0 and the two-stage fermentation (0-938 12 h).

6.2 Further Work

It is recommended that further work should be undertaken in these areas:

i. **Improvements to the design of BOBB II.** Heating should be provided by a heating element embedded in the baffle plates, or by use of a jacketed column. A robust automated pH and temperature controller will add more flexibility to the reactor, as more BOBB II characterization experiments as a fermenter could be performed using these features.

ii. **Continuous operation.** Because of the complex nature of the ABE fermentation (biphasic fermentation, cell degeneration and decreased solvent yield over time), batch mode was always the main choice to carry out this fermentation. Two-stage batch ABE fermentation has been demonstrated to produce higher butanol concentration, thereby increasing productivity and yield compared to single stage. Next, it is suggest performing this ABE fermentation in the continuous mode. The niche application of the OBB is the conversion of long batch processes to continuous processing whilst maintaining plug flow residence time distribution (RTD) characteristics (Harvey *et al.*, 2003). Performing the ABE fermentation in the OBB at continuous mode could offer more advantages of shorter downtime, simpler automatic operation and usually higher productivity over batch fermentation. Continuous immobilized cell or free cell system could also be used as they offer considerably higher reactor productivities than batch. Cell recycling could be adopted in continuous free cell system to retain high cell concentration inside the OBB.

iii. **Improvements to the integrated product removal via gas stripping.** This is especially important if two-stage or continuous ABE fermentations are to be implemented. Using simultaneous product-recovery techniques will allow the use of concentrated sugar solution as substrate (this will have an impact on the reactor size) and product inhibition to the cell growth can be relieved *in-situ*. Here in this study, gas stripping was performed *in situ* to the two-stage ABE fermentation resulted in 100% sugar consumption with an

improvement on the butanol productivity and yield up to 56% and 5.3%, respectively, compared to that without gas stripping. Further improvement on the gas stripping recovery needs to be achieved to determine the maximum sugar concentration that can be used in the fermentation. This would be expected to improve the butanol yield and productivity significantly, if higher concentration of sugar can be used. Improvement on the condenser should also be considered so that all stripped solvent can be captured.

iv. **Improvements to the modelling.** Improvement of the developed model by incorporating other terms such as loss of the acetone vapour to the head space by the oscillation, mixing terms describing the transport process of the nutrient in and out of cell, and physical culture parameters such as pH, temperature and redox potential. In the future a model incorporating the biochemical pathways and culture physiological state marker is required to better reflecting the ABE fermentation behaviour. This will expected to better reflect what actually happens in the fermentation. A computational fluid dynamic (CFD) model can be introduced to study the fluid mechanic of the oscillation and the resultant effect of shear stress or degree of mixing on the cell.

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APPENDIX

Appendix A Reactor power density calculation

Power density for BOBB II

$$\boldsymbol{\varepsilon_v} = \frac{2n\rho(\omega x_o)^3(1/S^2-1)}{3\pi C_o^2 Z} \quad (\mathrm{Wm^{-3}})$$

Power density for STR

$$\boldsymbol{\varepsilon_v} = \frac{\rho * N^3 * D_S^5 * P_o}{V} \quad (\mathrm{Wm^{-3}})$$

where:

Symbols	Name	Value	Unit
n	Number of baffle	3	-
ρ	Liquid density	995	$\mathrm{kgm^{-3}}$
ω	Angular frequency ($2\pi f$)	-	-
f	Oscillation frequency	0, 0.25, 0.47, 0.93	$\mathrm{s^{-1}}$
x_o	Oscillation amplitude	0.004	m
S	Fractional open area of baffle	0.25	-
C_o	Orifice coefficient	0.6	-
Z	BOBB II liquid height	0.3	m
N	STR agitation speed	0, 0.3, 0.58, 1.16	rps
D_s	Stirrer diameter	0.045	m
P_o	Stirrer power number	6	-
V	STR working volume	0.0015	m

Results:

BOBB II	STR	Power density
f ($\mathrm{s^{-1}}$)	N (rps)	ε_v ($\mathrm{Wm^{-3}}$)
0.25	0.30	0.02
0.47	0.58	0.14

0.93	1.16	1.14

Appendix B Lugol's Iodin preparation

The 5% solution consists of 5 g (wt.v^{-1}) iodine (I_2) and 10 g (wt.v^{-1}) potassium iodide (KI) mixed in distilled water to a 100 mL volume.

Appendix C Error calculation

Systematic errors are errors that are introduced by an accuracy (as of observation and measurement) inherent in the system. Such errors are calculated from scale resolution of the measuring device and usually taken as half of the scale resolution.

Random errors are errors in measurement that lead to measurable values being inconsistent when repeated measures of a constant attribute or quantity are taken.

Example of error calculation:

BOBB II ABE fermentation at Re_o 0

1. **Cell dry weight (CDW)**

Error type	**Devices/instruments**	**Error calculation**	**Error value**
Systematic	100-1000 µL pipette	0.001/2	±0.0005
	20-200 µL pipette	0.0001/2	±0.00005
	UV-spectrophotometer	0.001/2	±0.0005
Random	CDW	standard error	±0.27

Total systematic error = ±0.0015 and total random error = ±0.27. Since systematic error was significantly lower than the random error, the systematic error was not included in all cell dry weight calculated values.

2. Butanol concentration

Error type	**Devices/instruments**	**Error calculation**	**Error value**
Systematic	100-1000 µL pipette	0.001/2	±0.0005 mL
	20-200 µL pipette	0.0001/2	±0.000005 mL
	micro-syringe	0.0001/2	±0.000005 mL
	GC Digital flow meter	0.01/2	± 0.005 mL min^{-1}
Random	Butanol calibration curve	standard error	±0.01
	Butanol concentration	standard error	±0.6

Total systematic error = ± 0.0056 and total random error = ±0.61. Since systematic error was significantly lower than the random error, the systematic error was not included in all butanol concentration calculated values. This also applies for other metabolite concentration: acetone, ethanol, acetate, butyrate and glucose.

Appendix D Calculation of ABE fermentation performance.

D-1 Specific growth rate (μ)

Specific growth rate can be calculated using following equation:

$$\mu = \frac{ln\left(\frac{CDW_2}{CDW_1}\right)}{t_2 - t_1} \qquad (h^{-1})$$

…where CDW is a cell dry weight (gL^{-1}) and t is fermentation time (h)

D-2 Solvent productivity

Solvent productivity can be calculated using following equation:

$$\frac{Solvent\ concentration\ (gL^{-1})}{time\ (h)} \qquad (gL^{-1}h^{-1})$$

D-3 Solvent yield

Solvent yield can be calculated using following equation:

$$\frac{Solvent\ concentration\ (gL^{-1})}{Glucose\ consumed\ (gL^{-1})} \qquad (g_{solvent}\ {g_{glucose}}^{-1})$$

D-4 Specific solvent productivity

Specific solvent productivity can be calculated using following equation:

$$\frac{Solvent\ concentration\ (gL^{-1})}{cell\ concentration\ (gL^{-1})\ \times\ time\ (h)} \qquad (g_{solvent}\ {g_{cell}}^{-1}\ h^{-1})$$

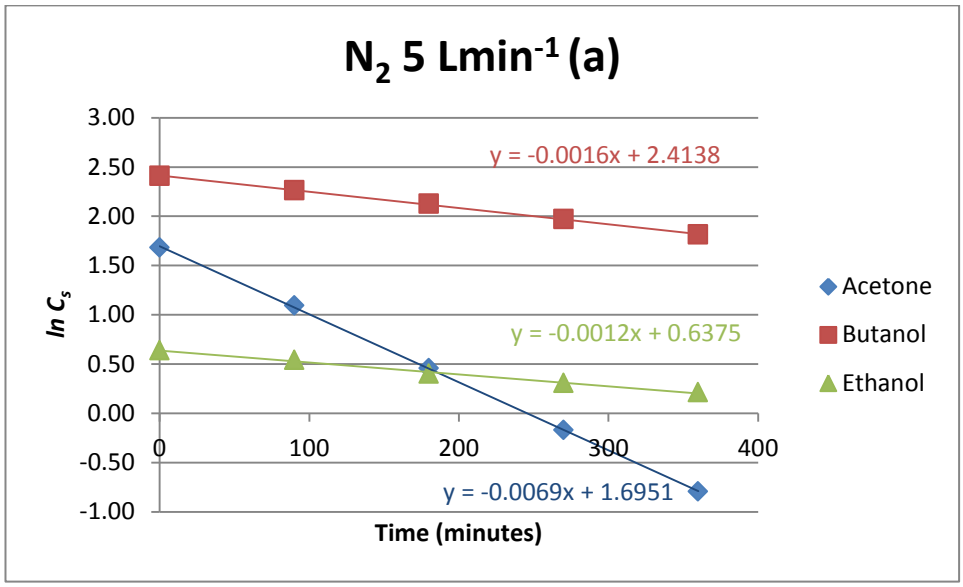
Appendix E MatLab® coding

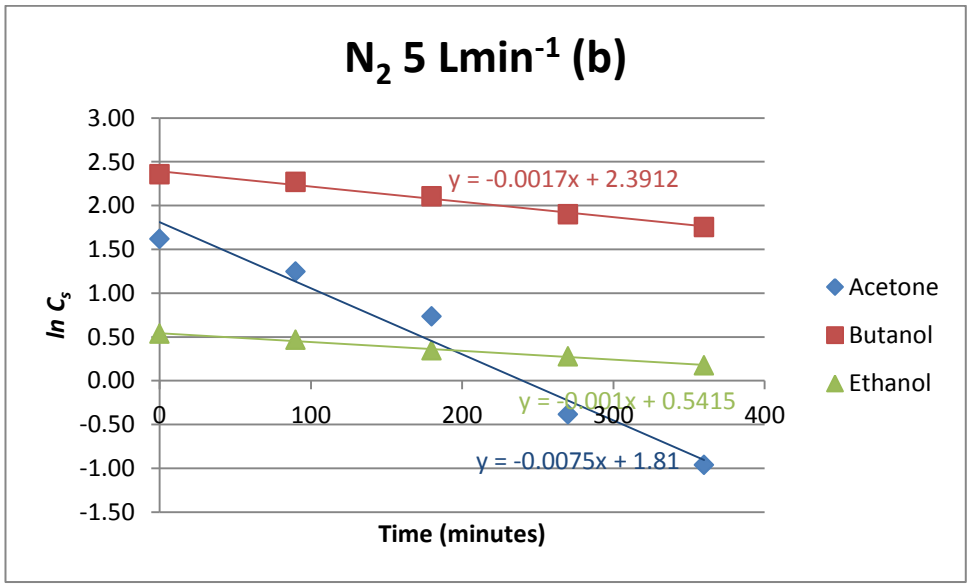
```
function cp=clostmassNas2 (t,c)
global miu So Y Xo Xm Ks Kd k1 k2 k3 k4 k5 k6 k7 k8 k9 Ki Kaa Kba

%modified Votruba modelling
c1=( (miu*(So*Y + Xo-c(1)))/(Ks*Y + So*Y +Xo-c(1)) )*c(1) -
Kd*c(1)*c(4) ;%biomass X
c1= miu* ( 1-c(1)/Xm)*c(1) - Kd*c(1)*c(4);
c2= -k1*c(2)*c(1) - k2*c(1)*c(2)/(Ks+c(2));%Substrate concentration
c3=k3*c(2)*( Ki/(Ki+c(4)) )*c(1) - k4*( c(3)/(Kba+c(3)) ) *c(1);%BA
production
c4=k5*c(2)*c(1) - 0.841*c3;% Butanol production
c5=( k6*( c(2)/(Ks+c(2)) ) * c(1) ) - k7*( c(5)/(Kaa+c(5)) ) *
( c(2)*(Ks+c(2)) )*c(1);%AA MODIFIED production
c6=k8*c(2)*c(1)/(Ks+c(2));% - 0.484*c5;% Acetone production
c7=k9*c(2)*c(1)/(Ks+c(2));% Ethanol production

cp=[c1;c2;c3;c4;c5;c6;c7];
```

Appendix F Example plots of ks_a determination based on the reactor concentration at Re_o 1870 at nitrogen flow rate of 5 $Lmin^{-1}$





Appendix G Publication

Nasratun Masngut, Adam P Harvey and Joseph Ikwebe. (2010) 'Potential Uses of Oscillatory Baffled Reactors for Biofuel Production', Biofuels, 1(4), pp. 605-619