Biotechnology From Bench to Market: The Design, Scale-Up and Commercialisation Strategy Development of a Disruptive Bioprocess for Potable Ethanol Production

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by

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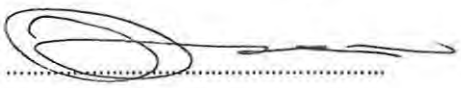
Abstract

The capacity of research institutions to engage in technology transfer activities has important implications on both economic development and technological advancement. This thesis explores the developmental and commercialisation processes involved in the transfer of a potentially disruptive bioprocessing technology for beverage alcohol production. Ethanolic fermentation strategies are of interest due to their global economic importance and their potential to produce clean renewable fuels in the future. Currently used methods are both energetically wasteful and economically inefficient. To this end more effective bioprocessing methods and implementation strategies are required to enable commercially viable decentralised small-scale ethanol production. Perfusion reactors have a number of advantages over batch and other continuous fermentation strategies. This study aimed to develop and study the fermentative efficiency of a perfusion tower bioreactor system at the bench scale, and subsequently through a scale up process to a low level commercial capacity. An HPLC method was developed for the simultaneous quantification of common fermentation analytes; this was used to determine bench scale fermentation efficacies over an operational period. At steady state the ethanol volumetric productivity of the bench scale bioreactor system was 3.40 g. $L^{-1}.h^{-1}$, the average yield of ethanol to consumed sugar was 0.467 g. g^{-1}, with an average sugar conversion percentage of 96%. Results showed that the tower perfusion bioreactor was appropriate for high performance ethyl alcohol fermentations. This reactor design was then scaled up to pilot scale and then commercial scale capacity. Similar efficiencies were achieved with these larger systems. Based on the process performance data obtained, a commercialisation strategy was developed and market performance was projected. It was found that productivity rates per unit volume were favourable, and the bioreactor system was determined to be very cost effective for a decentralised ethanolic beverage manufacturing model.

Declaration

I hereby declare that this dissertation is my own work. It is submitted in fulfilment of the requirements for the degree of MSc in Biotechnology at Rhodes University, Grahamstown, South Africa. It has not previously been submitted for assessment to another University or for another qualification.



Karim Dhanani

Outputs

This thesis has yielded the following outputs:

Submitted Papers:

Dhanani K.C.H., Cambray G.A., Cech A., Boshoff A., Wilhelmi B.S., 2012. Pilot and commercial scale continuous fermentation of mead using immobilised yeast in perfusion tower bioreactors. Biotechnology Letters.

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Other:

- Makana Entrepreneurship Expo: Business Plan Competition - Winner
- Technology Innovation Agency (TIA): National Innovation Competition - Finalist
- Certificate in Bioentrepreneurship (TIA)
- Advanced Certificate in Bioentrepreneurship - Swiss-South African Joint Research Programme (SSAJRP)
- Launch of venture: BrandNew Breweries (PTY) LTD. Manufacture start date 04/12.

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Glossary and Abbreviations

This thesis has a multidisciplinary nature. To avoid confusion the following terms and abbreviations are defined.

Adjunct - this word is employed to describe the addition of 'an adjunct' to a fermentation mixture to achieve an effect.

Angel Investor - High net worth individual who provides capital, and often mentorship, to start-up ventures.

Anticompetitive Behaviour - Distinct from competition; describes practices by businesses (or government) which act to prevent or reduce competition in a given market.

ATP - Adenosine triphosphate.

Axial - Relating to the axis around which the body rotates. E.g., along the spine of a mammal.

Barriers of Entry - Obstacles, such as access to raw materials, access to know-how and access to capital, which act to hinder new companies from entering a given market.

Bioreactor - the containment vessel of any biotechnology based production process

CAMRA - Campaign for real ale.

Commercialisation - To make an activity, process or service commercial.

Capital Equipment - Equipment used to create profits, E.g. a bioreactor.

Capital Expenditure (CAPEX) - Expenditures for creating future profits, typically in the acquisition of assets.

CDIC - Continuous dynamic immobilised cell bioreactor.

CSTR - Continuous stirred tank reactor.

CVP - Cost, volume, profit. The maximisation of profit via optimisation of cost (influences volume).

Debt Finance - Funds used to finance operations that are owed to the creditor and incur interest.

DNS - Dinitrosalicylic Acid.

Equity – Value of shareholders' ownership in a business.

Equity Finance - The injection of funds into a business to finance operation in exchange for ownership; in contrast to debt financing.

Emerging Markets – Nations in which the economy is in a phase of growth, such as India and China.

ESTT - Entrepreneurial separation to transfer technology.

EU – The European Union.

Excise – Tax levied on certain goods such as tobacco and alcohol.

LSE – The London Stock Exchange.

Pervaporation – The separation of fluid mixtures by partial vaporisation across a membrane.

PPB – Capita per Brewery.

PTB – Perfusion Tower Bioreactor.

Genetic Drift – The change in the frequency of a gene variant (or all gene variants) in a given population.

ICR – Immobilised cell reactor.

Intellectual Property (IP) – Copyright, trademarks, patents, trade secrets and designs are all legal constructs which constitute IP. They are intangible assets that may or may not require registration with a body such as an intellectual property office.

Internal Funding – Funding that originates from the companies shareholders/members.

IRR – Internal rate of return. The rate of growth that a project is expected to generate, net present value set to zero (therefore comparative measure).

LSM – Living standards measure.

Mass Transfer – The movement of mass; usually by diffusion across chemical potential gradients.

MLF – Malolactic fermentation.

Perfusion – Flow over or though something. E.g., the flow of an analyte mixture though an agarose gel.

PFK – Phosphofructokinase.

Plug Flow – Model of the flow of fluid through a pipe in which the velocity of the fluid is assumed to be constant at any point along its axis.

Process Economics – Study of the financial implications of process design.

SA/RSA – The Republic of South Africa.

SAB/SABMiller – South African Breweries / SABMiller PLC

Scale-Up – The development of a process from a smaller scale to a larger scale. E.g., from bench scale to industrial scale.

Seed Capital – Initial stage funding for a venture, commonly pre-proof-of-concept.

Specific Gravity – The relative density of a solution compared to pure water. Generally it is measured with a hydrometer and provides a rough quantification of solutes, such as sugars, present.

Superficial Flow – Hypothetical fluid velocity at a given cross sectional area.

Technology Transfer – The transfer of technologies from an originator to a secondary user; especially from research institutions to industry.

TTO – Technology Transfer Office.

UK – The United Kingdom of Great Britain and Northern Ireland.

USA – The United States of America

USO – University Spin-Off

Value Chain – The chain of activities of a company, each of which adds value to the product.

Venture Capital – Capital provided for high risk, early stage start-up companies. VC firms spread risk across a portfolio of investments in which few are expected to succeed.

Volumetric Flow – The volume of fluid that passes though a given surface per unit time.

Chapter 1

Introduction and Review of Literature

1.1 Introduction

1.1.1 Biotechnology and The Scientist's Economic Role

Biotechnology is a variously defined area of the scientific enterprise, which encompasses disciplines as dispersed as the fundamental elucidation of the causes of disease and environmental bioremediation (Waites *et al.* 2001). Contemporary usage of the term usually relates specifically to the development and application of recombinant microorganisms, but more generally biotechnology can be defined as: 'the development and application of biological systems to achieve goals prescribed by humans'. Under this definition, one of the earliest examples of human biotechnology is the practice of agriculture following the Neolithic agricultural revolution, approximately 12, 000 years ago. From here, despite some unfortunate setbacks, humanity has moved steadily towards the mastery of nature through the acquisition of knowledge and has continually sought refuge in biotechnology for survival and betterment (Vaishnav and Demain, 2009). Over the last 60 years, following Watson and Crick's elucidation of the structure of DNA, it has been said that the biological sciences have come of age; joining chemistry and physics as a genuine quantitative science (Kahlil and Collins, 2010). As a result, biological sciences are again at the forefront of human development.

Industrial biotechnology is a subdivision of biotechnology, and can be defined as the application of organisms or biological components in the processing of materials (Shuler and Kargi, 1992; Vaishnav and Demain, 2009). Although biological systems are ordinarily very complex, they obey known physical laws and chemical models and are therefore susceptible to engineering analysis (Towler and Sinnot, 2008). This has brought about a new kind of scientist, a bioprocess engineer, whom combines aspects of the biological sciences with chemical engineering in order to develop industrial biotechnological systems.

Sener and Saridogan (2011) outline one of the most important principles in modern human society: '*the heart of the long term economic development in all economic growth models is technological change and innovation, and the heart of technological change and innovation is the development of science*'. One of

the key 'pillars' of a competitive economy is an active academic community (Barrell and Pain, 1997), leading knowledge generation activities and effective higher education; especially in the sciences. This is so axiomatic that the number of governments, institutions and individuals that disregard it, or appear not to understand it is truly incredible.

Innovation is defined as the implementation of a new product, process or service (Sener and Saridogan, 2011), not only its conception. In our modern, and currently hard-pressed western free market economy, there is more need than ever for innovation. Unfortunately however, the transfer of technologies to the commercial sector and their application has itself become a problem (Malerba, 2007; Minitulo and Potter, 2011). In highly developed knowledge economies such as Germany, the US and the UK, the challenge of bridging the gap between the research setting and the marketplace has been greatly aided (but not entirely resolved) by the development of a highly competent private biotechnology sector, which is involved in the construction of frameworks for technology transfer (King and Norwack, 2003; McAdam *et al.* 2011). Academia in mature economies is better geared towards the commercialisation of scientific developments; it understands that the scientific enterprise does not fulfil its obligation to society through the generation of knowledge alone. This is because the modern knowledge economy places value on intellectual property; and to sell something, or even to give it away, one must first own it. The scientific enterprise though peer review and publication remains the knowledge validation and dissemination mechanism for fundamental research, but towards the 'applied end' of the scientific continuum recognition through peer reviewed publication holds little value (Owen-Smith, 2001; Jain *et al.* 2009).

In intermediate economies such as South Africa, Chile, Argentina, Mexico and Thailand, industry remains either ignorant of or indifferent to the development of new technologies that have the potential to influence their markets (Marshall, 2004; Salicrup and Fedorkova, 2006). As a framework for facilitating efficient technology transfer is either unavailable or underdeveloped in these economies (Salicrup and Fedorkova, 2006; Kircher, 2011), the role of the academic must shift to compensate; to become an effective innovator the scientist must also become an entrepreneur (Azoulay *et al.* 2007; Jain *et al.* 2009; McAdam *et al.* 2011). This is not only of primary importance for the growth and competitiveness of national economies, but potentially also for the future of fundamental science. The reason for this, as previously mentioned, is that we tend to collectively disregard the critical importance of our academic and research institutions, and this can lead to a steady erosion of state support for these institutions over time as has been recently demonstrated in the United Kingdom (Great Brittan, 2010; Jha, 2011; MacLeod, 2011; Jha and Sample, 2011) and the United States (Atkinson and Stewart,

2011). A clearly demonstrated economic incentive for public investment in scientific research could safeguard human intellectual progress (Azoulay *et al.* 2009).

Biotechnological innovation can be the most powerful and effective tool for the worldwide reduction of poverty, economic development and the advancement of human knowledge (Salicrup and Fedorkova, 2006). Thus, as we have moved from an information society towards a molecular society (Linstone, 2011), it is increasingly important for scientists, particularly in the field of biotechnology, to be competent in both their discipline and in basic entrepreneurial skills (Fontes, 2001; Jain *et al.* 2009).

1.1.2 Market Homogenisation, Disruptive Technologies and The Brewing Industry

A disruptive technology is a new technological innovation, product or service that eventually overturns the existing dominant technology or product on the market (Christensen, 1997; Kostoff *et al.* 2004). Economic studies have shown that where a large expensive technology system exists as a barrier of entry to a market, there is a resulting development of more efficient and less expensive alternatives (Bower and Christensen, 1995; Malerba, 2007; Yu and Hang, 2011). Disruptive technologies can be characterised by the following properties: they enable activities, products and applications that were not previously feasible due to cost or skill level, they impact existing markets and they typically build on existing patterns of consumer behaviour (Kostoff *et al.* 2004; Yu and Hang, 2011).

Market homogenisation is the process by which a market becomes less internally differentiated; this process can either be natural (arising from market or sociological forces) or as a direct result of the commercial activity of one or more companies (Froeb and McCann, 2010). The global beer market is one that has tended strongly towards uniformity due to the activity of large producers, where barriers of entry are created against smaller localised manufacturers and in many cases active anticompetitive behaviour (Bower and Cox, 2010) by larger breweries arising primarily through merger and acquisitions (Mager, 2008; Bower and Cox, 2010). This uniformity has some benefits and some drawbacks for the consumer: benefits include low prices (once an effective monopoly is established, profits can be driven through the optimisation of 'cost-volume-profit', or CVP), product consistency and availability. The disadvantages of market homogenisation typically include the loss of tax revenue (especially in less developed economies, and when companies move revenues across borders) and revenue to local business, loss of local culture and identity, loss of consumer rights, greater environmental impact primarily due to transport, overall lower rates of employment and low quality products (Wood, 1994). Consumers are generally unaware of the downsides of, or alternatives to the

market situation, and because of the high barriers of entry stemming from economies of scale, access to distribution networks, raw materials, trained personnel, and the high cost of required capital equipment; these monopolies typically remain unchallenged.

Recently, first in the UK with the formation of the 'Campaign for Real Ale' (CAMRA) in 1971, and later in US triggered by the Carter administration's repeal of federal regulation dating back to prohibition outlawing the home brewing of beer; informed consumers and entrepreneurs have attempted to reverse the trend of market homogenisation. This has led to the 'microbrewery revolution', during which hundreds of small local breweries have been founded and are successfully challenging the 'mega-breweries' for market share by promoting localism and community identity (Flack, 1997; Schnell and Reese, 2003).

This new trend was met by the biggest brewing companies in the world with two distinct strategies: either supporting the diversification of the market and retaining a smaller share of it (the 'small part of a big pie' strategy), or aggressively acquiring and defending market share (the 'all of the pie' strategy). The company most notable for following the latter strategy was South African Breweries (now SABMiller PLC), and it's strategy has ultimately made it the second biggest brewing company in the world (it has made 26 acquisitions since 1999). It has however developed a record of serious anticompetitive behaviour (Mager, 2008).

A rough marker for homogenisation is the number of people per brewery (PPB) in a country. In the countries with the lowest homogenisation of the brewing industry such as Belgium, the UK and the US, the PPB ranges from 50, 000 to 150, 000. In South Africa, the home country of SAB, the PPB is 1, 687, 000: 16 times higher than that of the UK. Of course comparing South Africa to other countries with European backgrounds in this way is unfair, but even so, this figure is remarkable. SABMiller sells 25, 000, 000 hL per annum to the South African market, this accounts for over 95% of beer sold in South Africa (The Economist Intelligence Unit Limited, 2006). The cider market in SA is divided up between SABMiller, Distell and Brandhouse (Diagio). Distell is the only South African company represented, but Distell itself owns the lion's share of the 955.5 million litre wine industry. For a developing economy, South Africa is unusually difficult for competition in a variety of industries.

Unlike in other countries, breaking the monopolies of SABMiller and Distell is unlikely to be achieved through consumer activism and competition. This is because the government to some extent protects the company by it's passive stance on competition, consumer rights and advertising standards legislation enforcement; for example SAB is allowed to portray itself as the national brewery, even

though the group is listed on the LSE. SAB controls raw materials such as malt and hops, and is unchecked when it uses anticompetitive measures (Mager, 2008). The SA wine industry is even more heavily protected by the state, but South Africa is a country with a long history of state protected monopolies so this should not be surprising. The best way to compete in this environment is through advantages offered by new technologies that offer ways of matching the prices and quality of available products. New production methods must be developed for beverage alcohol production.

Fermentation, as a biotechnology, has remained fundamentally unchanged worldwide since the 6th millennium B.C., our understanding of the processes involved has been greatly improved, and the scale of operations has increased drastically but the basic method of fermentation for the vast majority of the products on the market retain the standard batch fermentation format for production. This is despite the development of numerous alternatives. The reasons for the slow rate of technological change in the brewing industry include a combination of a preference for 'creative accumulation' developments over 'creative destruction' developments (discussed later) and the heavy investment made by producers in specific brands, the characteristics of which must remain unchanged over long periods of time. Coupled with the high cost of capital equipment and the favoured centralised production model (determined by production methodology and resulting economics), the cost of change to the industry would outweigh the benefits in the short term (Nair and Ahlstrom, 2003).

This stance taken by the industry has serious effects on other important considerations irrespective of the technology in use. Firstly the centralised production model increases costs and environmental damage through distribution over long distances. It also adds to the rate of depopulation of rural areas. Heavy investment in brands means a very low brand turnover, and a disproportionate amount of capital is therefore used for marketing. Low research and development budgets and resulting low rates of technology development in the brewing industry have had implications on the rate of development in the bioethanol industry.

A disruptive production technology, which may enable new companies to compete with centralised mega-breweries, would have the following properties:

- Low capital equipment cost per unit production capacity
- Low energy requirements
- Ease of operation
- Low risk of contamination

- High productivity rates
- Versatility
- Low physical footprint
- Low maintenance requirements

In order to develop and successfully scale-up a process with the above properties, a fundamental understanding of the link between the kinetic, hydrodynamic and transport processes in the new bioreactor and their impact on process economics is required (Leib *et al.* 2001). These skills are the traditional purvey of chemical engineers but must now be also be understood by biotechnologists.

1.1.3 Ethanolic Fermentation

The term 'fermentation' is derived from the Latin verb, *fervere*, to boil (Stanbury and Whitaker, 1984), describing the appearance of carbon dioxide evolution during the yeast anaerobic catabolism of carbohydrates. The term fermentation has several meanings. Its biochemical meaning relates to the generation of energy through the microbial catabolism of organic compounds, whereas in industry its meaning is much broader; encompassing processes that produce microbial biomass, cellular components, microbial metabolites and processes that modify compounds added to the fermentation (trans-formation). The common feature of these reactions is that they are all decarboxylation reactions, (Stanbury and Whitaker, 1984, Atkinson and Mavituna, 1991). There are a number of microbial metabolites that have commercial significance including vitamins, polysaccharides, nucleotides, lysine, glutamic acid, acetone, butanol and citric acid. One of the most historically significant and economically important products of a microbial fermentation is ethyl alcohol. Ethanol is the 'active ingredient' in alcoholic beverages and can be used as fuel for internal combustion engines when blended with petroleum (Stanbury and Whitaker, 1984).

Alcoholic (or ethanolic) fermentation is a biological process that has been exploited by humans for many thousands of years (Steinkraus, 1983). Following the revolution of biology by Hooke and van Leeuwenhoek and their cell theory in the mid 1800s, the fungal microorganism *Saccharomyces cerevisiae*, it could be argued, formed the core of the modern field of biotechnology in its early stages. The production of alcoholic beverages dates as far back as the 6th millennium BC, where it appears in the literature of the Egyptian and some of the earliest writings of the Sumerian people of southern Mesopotamia (Nelson, 2004, Steinkraus and Morse, 1966, Eskin, 1990). The earliest chemical evidence

for beer appears in China around 5000 B.C. (McGovern, 2003) and later in the Zargos Mountains in modern day western Iran, around 3500 - 3100 B.C. The production of many of the world's alcoholic beverages, (including wine, beer, mead and cider,) were probably discovered and developed in various locations around the world independently due to their natural spontaneous occurrence. During the 20th century, with the advent of mass-production and the movement of population away from rural areas and into the towns, larger commercial alcoholic beverage production developed (Steinkraus, 1983).

The biochemical process of fermentation is the derivation of energy from organic compounds via the use of an endogenous electron accepter following glycolysis. It differs from cellular respiration in that it does not require the presence of oxygen (exogenous) as an electron acceptor. In both cases the use of an electron acceptor allows substrate-level phosphorylation, and ATP production, to continue (Freeman, 2005).

The balanced reaction may also be summarised as follows:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 + 2\ ATP \qquad (118\ kJ/mol)$$

(Freeman, 2005)

The biochemical fermentation process of microorganisms had been exploited by humans long before the existence of microbes was first confirmed by Robert Hooke in 1665. Beer, wine, mead and cider production all predate the knowledge of the existence of bacteria and fungi that are responsible for the production of ethanol and the flavour compounds characterising a particular beverage (Steinkraus, 1983).

Traditional fermentation systems were predominantly spontaneous (Steinkraus, 1983), and only the introduction of immobilisation materials allowed for predictable results. The microorganisms responsible for many of the characteristics of the resultant beverages were of varying origin (Pretorius, 2000). For example, in traditional winemaking, *Candida* species and *S. cerevisiae* species of yeast were used to contribute to organoleptic properties (Pretorius, 2000).

Solid-state fermentation is typically defined as fermentation involving solids in the near absence or complete absence of free water. However, some configurations demand the presence of enough water to support microbial growth on either the substrate (Pandey, 2003), the carrier surface or some combination of the two via the supply of water vapour to the system (Mitchell *et al.* 2000a). These fermentation systems have the potential to be scaled up (Mitchell *et al.* 2000b) but have little

application in the production of beverages (except in niche processes such as the MLF) or in fuel ethanol, as these fermentations are inherently liquid based.

It is hypothesised that a cost effective fermentation strategy, able to disrupt the beverage alcohol market, can be strategically developed and capitalised upon in an effective manner using a combination of interdisciplinary proficiencies.

1.2 Literature Review

1.2.1 Novel Ethanolic Fermentations

New methods for the production of ethanol are of interest for use in the biofuels industry and in beverage alcohol production (Bayrock and Ingledew, 2001). Bai *et al.* (2008) discussed the comparison between microorganisms commonly used for ethanolic fermentations and concluded that compared with *S. cerevisiae*, the ethanol yield and productivities of *Zymomonas mobilis* were typically higher due to reduced biomass production, and the higher rate of glucose catabolism characteristic of its adapted Entner-Doudoroff pathway (Sprenger, 1996). Lin and Tanaka (1996) concluded that *Z.mobilis* could not be used in the majority of fermentations, however, for a number of reasons: its metabolite profile is unsuitable for the production of most ethanolic beverages, it has a limited range of substrates it is able to catabolise, and the biomass created is not a useful by-product. The sale of *S. cerevisiae* as an animal feed and nutritional supplement can often subsidise the economic costs associated with a given bioprocess (Stanbury and Whitaker, 1984). A review of fermentation technologies for continuous fermentation focusing on cellulosic ethanol production by Brethauer and Wyman (2010) indicated that continuous fermentations are now employed for commercial ethanol production from cane sugar and corn, taking advantage of higher volumetric productivity, reduced labour costs, and reduced vessel down time for cleaning and filling (Sanchez and Cardona, 2008). However, these systems are more susceptible to microbial contamination and in some cases require more sophisticated operating mechanisms and software (Godoy *et al.* 2008).

The success of novel ethanol bioprocesses is typically judged by comparison to basic batch fermentations. Najafpour *et al.* (2004) compared the performance of immobilised cell reactor (ICR) making use of a packed bed (up-flow) of calcium alginate gel entrapped *S. cerevisiae*. Their ICRs maximum production rate was an approximately ten-fold improvement over the batch model standard. Membrane bioreactors make use of membranes to effect a continuous removal of ethanol from the

fermentation broth by pervaporation (Masamitsu *et al.* 2002). O'Brien *et al.* (2000) conducted a preliminary economic analysis of the continuous production of ethanol using pervaporation to concentrate ethanol from fermentation broths, and concluded that when used selectively, the process could be cost-effective. A novel membrane bioreactor for the production of dry wine was characterised by Takaya *et al.* (2002). The yeast growth and ethanol fermentation characteristics were examined and compared to a batch fermentation; cell growth rates were shown to be significantly greater in the continuous membrane bioreactor across a range of dilution rates. Residual sugar concentrations were too high for dry wine production (dry wine post fermentation residual sugar concentration are less than or equal to 4 $g.L^{-1}$) from a single membrane bioreactor, the residual sugar was suitable from a double-vessel membrane bioreactor, however, and the productivity rate was 28 times higher than that a batch fermentation standard. O'Brien and Craig (1996) employed a continuous stirred tank bioreactor in combination to a membrane pervaporation system. The system was able to produce a constant stream of 20-23% (w/w) permeate through the pervaporation unit. Ethanol productivities of 4.9-7.8 $g.L^{-1}.h^{-1}$ were obtained from the system. Nedovic *et al.* (2000) used Ca-alginate gel entrapment to co-immobilise *Saccharomyces bayanus* and *Leuconostoc oenos* in a continuous conical packed bed bioreactor in order to conduct the primary and secondary fermentation steps in cider production simultaneously (NB: cider in this thesis is used in the UK sense of the word; US 'hard cider'). This fermentation setup, it was concluded, permitted faster fermentation rates than traditional configurations, and allowed for greater control over flavour formation.

A continuous membrane bioreactor with cell recycling was used to determine the effects of cell recycling on ethanol productivity by Kargupta *et al.* (1998). The results showed that the elimination of cell washout allows the membrane bioreactor to operate at high dilution rates and increased the ethanolic productivity at a given pervaporation factor compared with systems without a cell separator. An up-flow packed bed continuous bioreactor with Ca-alginate gel entrapped *S. cerevisiae* was used by Roca *et al.* (1996) to study the effects of feed flow pulsation on the performance of the ethanolic fermentation. Using the optimal pulsation characteristics the system's performance could be enhanced by up to 18%. It was also demonstrated that if the pulsation frequency was not correctly synchronised with gas production in the packed bed, unfavourable vertical mixing occurred. A similar up-flow packed bed bioreactor was studied by Kumar *et al.* (2011) using a thermotolerant yeast strain (*Kluyveromyces* species) immobilised on bagasse chips to produce ethanol. High ethanolic productivity rates were obtained for high and low substrate concentrations at different dilution rates. Continuous ethanol fermentation using a gravity fed (down-flow) packed bed bioreactor with yeast cells immobilised on sugar-cane stalks was investigated by de Vasconcelos *et al.* (2004). Using molasses as the bioreactor

substrate, the system showed operational stability for 60 days at a high dilution rate (3.00 h^{-1}) with good ethanol yields and conversion efficiencies at a dilution rate of 0.83 h^{-1}.

Simon *et al.* (1996) compared the organoleptic properties of ciders manufactured using a continuous fermentation making use of immobilised *Saccharomyces bayanus* cells, and in free cell batch fermentation. The volatile compound profiles from both fermentation set-ups were comparable, and the authors determined that the continuous fermentation model could be transposed to other fruit wine production processes. Models for the examination of biomass growth and fermentation characteristics in batch reactors have also been developed using an alternative bioreactor configuration by Clement *et al.* (2011). They used multistage chemostat fermentation bioreactors, which mimicked batch fermentations at different stages, and each stage was defined by its glucose uptake rate. It was shown that the configuration making use of more reactors were better able to reproduce the conditions within batch fermentations, and this was determined to be a useful tool for gaining insights into yeast metabolism during ethanolic fermentations.

Other bioreactor configurations are occasionally used in experimental work; Lebeau *et al.* (1997) used co-immobilised *S. cerevisiae* and *Candida shehatae* cells in a two-chambered bioreactor, in which one half of the bioreactor contained substrate, and the other half contained a substrate-free mineral solution. Separating the two halves were two microporous membranes enclosing an agar layer containing the microorganisms. This bioreactor was used to test the fermentation efficiency of the co-immobilised biomass under different oxygenation conditions. Jia *et al.* (1997) conducted an experiment to test the fermentation performance of yeast in the presence of organic oxygen vectors using an airlift bioreactor. The fermentation of a variety of substrates by two different yeast strains (*S. cerevisiae* and *Picha stipitis*) in a continuous dynamic immobilised cell bioreactor (CDIC) was investigated by Parekh and Wayman (1987). The CDIC is characterised by its structure: a cylinder, slightly inclined above horizontal, packed with porous fibre discs mounted on a central shaft. The whole reactor was rotated at 40 rpm for the duration of operation. This novel bioreactor configuration achieved some success but was found to have low operational stability, with a half-life on productivity rates of only 15 days.

1.2.2 Bioprocess Design

The theory of the design and scale up of bioprocesses is sparsely covered in the scientific literature; this is likely due to the fact that the engineering techniques involved are traditionally the purview of engineers and are available primarily to the private sector. There are, however, numerous textbooks

covering the subject such as Shuler and Kargi (1992), Atkinson and Mavituna (1991), Demain and Davies (1999).

The kinetics of ethanolic fermentation are characterised by strong product inhibition (Bai *et al.* 2008). *In situ* removal of ethanol is an effective way to minimise this effect, which allows for improved fermentation rates and productivities (Roffler *et al.* 1984). The removal of ethanol from the biomass' environment can be accomplished either in the macro scale using methods such as pervaporation, or on the micro scale using perfusion type bioreactor configurations such as a packed bed (Leib *et al.* 2001). As previously discussed, the combination of pervaporation and fermentation has been studied (O'Brien and Craig, 1996; O'Brien *et al.* 2000). The flux of currently available pervaporation membranes is not sufficient to meet the large ethanol production capacities of current industrial ethanol processes, and in addition, issues such as membrane fouling must be addressed (Bai *et al.* 2008). Taylor *et al.* (2000) evaluated a small pilot plant in which ethanol fermentation was coupled with a stripping column and condenser. Ethanol was stripped by the recycled CO_2, and the ethanol enriched gas was passed through the condenser where the ethanol was absorbed by the circulated dilute ethanol condensate. The resulting drop in ethanol concentration inside the fermenter allowed for significantly improved fermentation rates. Due to their relative complexity, these macro scale ethanol removal strategies are not economically feasible at this time.

Micro scale bioreaction engineering strategies that alleviate ethanol inhibition through decreasing the back-mixing in the fermentation system are the only current viable alternative. Batch bioreactors and plug/tubular flow bioreactors (in which a concentration gradient in reactants exist across the axial dimension) are equivalent in theory because their time-averaged product inhibition levels are similar (Levenspiel, 1999; Leib *et al.* 2001), when metabolic regulation adaptations are taken into consideration, however, the plug flow model is more attractive.

A key disadvantage of batch-operated bioreactors is their long operational downtime required for mash adding, broth harvesting, cleaning and sterilisation. This downtime means that their effective productivity rates are much lower than predicted by their process kinetics (Bothast and Schlicher, 2005). One method proposed to overcome this problem is the use of several continuously stirred tank reactors (CSTR) in series, which reduces product inhibition by reducing the overall back-mixing, without the need for extended downtime (Levenspiel, 1999). Plug flow bioreactors with high superficial flow rates are vastly superior to this configuration, as in theory a plug flow reactor with no axial mixing acts (in effect) as a long series of CSTR reactors with small environment increments along its length. In addition to this, unlike in a CSTR series, there is no need to balance improved fermentation kinetics

with capital investment. In reality though, this strategy is difficult to achieve because plug flow bioreactors are difficult to design correctly (Madson and Monceaux, 1999).

De la Roza *et al.* (2003) evaluated the production of ethanol and ethyl acetate during the scale up of cider fermentation from laboratory to industrial scale. Fermentation metabolites were monitored during cider fermentations carried out at laboratory, semi-pilot, pilot and industrial scales. Simple kinetic models were developed and these were successfully applied to simulate the evolution of the fermentation metabolites found in the experimental results. Bideaux *et al.* (2006) demonstrated the minimisation of glycerol production in a fed-batch ethanolic fermentation using a metabolic model as a prediction tool. The fermentation strategy was formulated to reduce the surplus formation of NADH, which is responsible for glycerol production during ethanolic fermentations, and was shown to be effective.

The performance of a bioreactor can also be influenced by cell separation based on the affinity of cells to gas bubbles (deSousa *et al.* 2003). The interrelationship between the fermentation media and yeast cell wall hydrophobicity is difficult to predict, however the apparent hydrophobicity at the cell wall-medium interface can be manipulated with changes to temperature and supernatant modification with buffer systems and organic solvent addition (deSousa *et al.* 2003).

Typically the scale-up of a bioprocess involves the development of kinetic models in an attempt to predict the behaviour of reactors of varying sizes. Birol *et al.* (1998) describe the development of mathematical descriptions of ethanolic fermentation by immobilised *S. cerevisiae* in a stirred batch system. Their models were validated using data collected from an inclined reactor with nutrient circulation.

Successful bioreactor design and scale-up requires a fundamental understanding of the link between the kinetic, hydrodynamic and transport processes in a bioreactor and their impact on process economics (Leib *et al.* 2001). These skills are the traditional purview of chemical engineers, and as such remain inaccessible to the majority of small and medium scale beverage alcohol producers. Coupled with the strong tendency of large-scale beverage manufacturers to avoid new techniques and methods, this results in bioreactor design and scale-up remaining an impassable knowledge chasm fraught with pitfalls and dangers that has laid claim to many attempts to improve production technologies, including many unpublished near misses from industry (Leib *et al.* 2001).

Additional global considerations such as interventions to improve safety, measurement techniques for biological and chemical parameters during fermentation, up/down-stream processing , the integration of procedures both 'up' and 'down' the value/supply chain are factors that require attention in the design of a bioprocess. Ingham and Schoeller (2002) studied the acceptability of a multi-step apple cider safety system for the destruction of *Escherichia coli*. It was determined that although the multi-step system provided sufficient lethality under certain circumstances, it was unreliable and had inherent lower consumer acceptability, being an inferior alternative to pasteurisation. The safety of cider was also studied by Gentry and Roberts (2005), the efficacy of a continuous flow microwave pasteurisation system was evaluated through *E. coli* load reduction.

1.2.3 Biomass Immobilisation

In order to obtain high performance continuous fermentation systems, the immobilisation of the biocatalyst is necessary (Navratil *et al.* 2001; Kourkoutas *et al.* 2004). Kierstan and Bucke (1977) developed the idea of immobilised whole cells from the older application of immobilised enzymes. The use of immobilised cells and specifically their application to ethanolic fermentations have been extensively reviewed (Klein and Ziehr, 1990; Scott, 1987; Kourkoutas *et al.* 2004; Branyik *et al.* 2005 Verbelen *et al.* 2006). In general immobilised cells are more effective for processes in which the goal is the production of secondary metabolites, rather than the production of primary metabolites associated with biomass growth as in ethanolic fermentations (Mijnbeek *et al.* 1992a). The rates of ethanolic fermentation in yeast cell populations that are not increasing are much lower than those of the populations during growth because the accumulation of ATP inhibits the activity of PFK (phosphofructokinase) in the glycolysis pathway (Ingledew, 1999). The reason high productivity rates are obtained using immobilised cells in bioreactors is the high cell densities able to be achieved through cell immobilisation. If a limited amount of growth is enabled, the productivity rates are enhanced.

The primary objective of using attached cells, rather than free cells, within a bioreactor is to improve the productivity of the system (McGhee *et al.* 1982; Kourkoutas *et al.* 2004). This has the effect of reducing the capital cost per unit production capacity. This effect is small in real economic terms, as the material cost of fermenters becomes less significant at larger scales. At a small scale however, this effect is large as access to capital is a major barrier of entry into the industry. There is another, perhaps more important effect of cell immobilisation on bioreactor operation; when aggregated yeast cells are better able to withstand unfavourable conditions in the fermentation environment such as high ethanol concentrations (Desimone *et al.* 2002; Hu *et al.* 2005).

Gel entrapment is a very common method of immobilising cells in a bioreactor (Norton and D'Amore, 1994; Simon *et al.* 1996; Roca *et al.* 1996; Nedovic *et al.* 2000; Jamai *et al.* 2001; Saraydin *et al.* 2002; Oztop *et al.* 2003; Najafpour *et al.* 2004; Decamps *et a*l. 2004). Natural or synthetic polymeric hydrogels such as Ca-alginate, agar, polyurethane, polystyrene and polyvinyl acrylamide can all be used for cell entrapment (Ramakrishna and Prakasham, 1999). High biomass concentrations can be obtained within a bioreactor using gel entrapment, but there is little use of this immobilisation method above bench-scale experiments because of mass transfer limitations of the polymeric materials and of biomass within them. The gels also break down due to the physical growth of the biomass over time, thus the bioreactor has restricted long term operational stability. Other porous matrices such as foam, chitosan and fibrous materials can also be used to entrap cells. If the cells are incubated with the porous material, they diffuse into it and become immobilised due to the presence of other cells (Baron and Willaert, 2004). Parekh and Wayman (1987) used the containment of cells within a porous material to carry out ethanolic fermentations in their CDIC bioreactor. Many of these gels are expensive, and therefore the immobilisation of yeast cells with the use of gel entrapment is not suitable for commercial ethanol production because the additional cost of the consumption of the supporting materials makes this immobilisation strategy economically unacceptable (Bai *et al.* 2008).

The immobilisation of yeast cells onto a solid carrier is commonly used in the production of beverage alcohol as the process is natural and does not require any potentially harmful inducers (Branyik *et al.* 2004). The adhesion between the cell and carrier is typically conferred by weak forces such electrostatic, acid/base and van der Waals interactions (Oliveira 1997). Kumar *et al.* (2011), de Vasconcelos *et al.* (2004), Kourkoutas *et al.* 2003 and Farmakis *et al.* (2007) report the application of natural adsorption as an immobilisation mechanism for ethanolic fermentations.

The containment of cells behind a barrier such as a microporous membrane is another potential method of immobilising cells within a bioreactor. This type of immobilization is most suited when a cell free product is required, or when high molecular weight products need to be separated from the effluent (Verbelen *et al.* 2006). Inherent problems of this technique are mass transfer limitations and possible membrane fouling caused by cell growth (Lebeau *et al.* 1998). This type of immobilisation can offer high productivity rates due to reduced cell growth rates, but the inhibitive cost of high performance membranes required for non-limiting mass transfer rates means that this method of immobilising cells is of limited use in ethanol production. Despite this, several studies have investigated their use for this application (Kargupta *et al.* 1998, O'Brien *et al.* 2000).

Other yeast cell immobilisation methods that have been investigated include the use of hydroxyapatite (HAP) ceramics (as used in bio-implants) with cells attaching through adsorption. This has been used in wastewater treatment applications (Rapoport *et al.* 2011). Adhesion to chrysotile (a fibrous magnesium silicate) by yeast cells was demonstrated by Wendhausen *et al.* (2001) for use in the production of fuel ethanol. In continuous fermentation in a packed bed reactor configuration, the immobilised yeast achieved significantly higher rates and yields than free cell systems.

Scott and O'Reilly (1996) used a sponge-like material to co-immobilise *S. cerevisiae* and *L. plantarum* cells for the fermentation and partial maturation of cider. It was found that when media was circulated through a side loop packed with the sponge-like material preloaded with the biocatalysts, both the fermentation rate and flavour development of the product were enhanced. Modified stainless steel wire spheres have also been demonstrated as a carrier for yeast cell immobilisation in ethanolic fermentations (Bekers *et al.* 1999).

The self-immobilisation of yeast cells through adsorption and flocculation can effectively overcome many of the drawbacks of other immobilisation methods (Chen *et al.* 1994; Hu *et al.* 2005). Natural cell aggregation is an attractive method of immobilising cells within a bioreactor. This process in yeast is called flocculation.

1.2.4 Yeast Flocculation

Yeast flocculation can also play an important role in the immobilisation of cells in biological processes (Branyik *et al.* 2005). Flocculation is a reversible, asexual, calcium dependent process in which cells adhere to each other through lectin-like proteins on the yeast cell wall, which selectively bind mannose residues present on the cell walls of adjacent yeast cells (Bidard *et al.* 1995). Yeast flocculation is a process that depends on the expression of several genes, including *FLO1*, *FLO5*, *FLO8* and *Lg-FLO1* whilst other genes of the same family, such as *FLO11*, confer adhesion to inert substrates and the formation of biofilms (Guo *et al.* 2000). Flocculation of yeast and related phenomena are the subject of multiple reviews (Vestrepen *et al.* 2003; Vestrepen and Kilis, 2006; Bauer *et al.* 2010; Goossens and Willaert 2010;).

Due to their relative density, yeast flocs typically sediment within fluid medium, providing a natural method of cell immobilisation. Flocculation as an immobilisation method is cheap and easy to achieve under the correct conditions. The factors influencing the flocculation behaviour of yeast strains include

nutrient concentrations, agitation of the bioreactor, Ca^{2+} concentration, pH, temperature, and yeast handling and storage conditions prior to fermentation (Verstrepen *et al.* 2003; Chang *et al.* 2005; Sampermans *et al.* 2005; Verbelen *et al.* 2006). The fermentation broth content of sugars, importantly glucose, sucrose and mannose, nitrogen compounds and ethanol content all heavily influence the flocculation of yeast cells (Chang *et al.* 2005; Verstrepen *et al.* 2004). Most importantly, flocculation behaviour is strain specific, with yeast strains typically divided into flo1-type (flo1 based) or newflo type (Lg-flo1 based) flocculation. (Stratford, 1989; Kobayashi *et* al. 1998; Jin and Speers 1998; Van Mulders *et al.* 2009). Flo1-type yeast flocculation is constitutive and only sensitive to mannose, whereas newflo-type flocculation occurs in the stationary phase and can be inhibited by mannose, glucose, sucrose and maltose (Stratford and Assinder 1991).

Fundamental studies of the genetics and mechanisms involved in yeast flocculation is well covered in the literature, however some of the key publications include Bidard *et al.* (1995), with the discovery that the *FLO1* gene involved in flocculation encodes a cell surface protein. The link between the cell surface distribution of the Flop protein, and its local availability on the level of yeast flocculation was determined by Bony *et al.* (1998). Liu *et al.* (1996) found that the standard laboratory *S. cerevisiae* S288C strain lost the ability form pseudohyphae when nitrogen starved as wild type *S. cerevisiae* strains do. It was determined that the defect was the result of a nonsense mutation of the *FLO8* gene. The genetic and external regulation of the *FLO* gene family was investigated by Halme *et al.* (2004). It was found that the variation in both genetic and epigenetic regulation of these genes explained the observed heterogeneity of cell-surface proteins of a population of yeast clones.

Smukalla *et al.* (2008) discovered that FLO1 is one of a very few known 'green beard' genes (genes that direct cooperation towards carriers of the same gene). Yeast flocculation is therefore a model for the evolution of cooperative behavior. Yeast flocculation has been shown to improve tolerance to multiple stresses including antimicrobials, ethanol and physical stresses (Palkova, 2006; Hall-Stoodley *et al.* 2004; Hu *et al.* 2005; d'Enfert, 2006; Reynolds, 2006).

The flocculation ability of yeast is of great importance for the brewing industry (Bauer *et al.* 2010; Vestrepen *et al.* 2003), as it provides the primary biomass removal method in fermentations. Strong and complete flocculation behaviour is therefore a desirable characteristic of yeast strains used in ethanolic fermentations. In batch fermentations it is key that the yeast does not flocculate before full attenuation of the fermentation broth, which is the cause of 'hung' fermentations, which can result in the development of off-flavours (Verstrepen *et al.* 2003). The economic implications that improved flocculation behaviour could have on existing bioprocessing systems has led to a number of recent investigations into the genetic modification of yeast to improve its biotechnological applications. Wang

et al. (2008) transformed *S. cerevisiae* ZWA46 with a plasmid expression vector (pYX212) containing the *FLO1* gene, conferring strong and stable flocculation ability. Wang (2009) conducted a similar experiment, transforming the industrial Angel yeast strain with the *S. cerevisiae* W303-1A *FLO1* gene, the transformant exhibited strong flocculation behaviour. The genetic modification of the industrial *S. cerevisiae* strain YSF5 was undertaken to obtain newflo-type characteristics by Wang *et al.* (2008). The yeast was transformed with an expression cassette containing the *FLONS* gene downstream of the alcohol dehydrogenase II (ADH2) gene; the transformants tested exhibited a 1.5 to 2.3-fold increase in flocculation ability over the original strain. When flocculation in the presence of key sugars was tested newflo flocculation type was confirmed. Choi *et al.* (2010) constructed a hybrid yeast strain (CHF0321) through protoplast fusion between a non-flocculent, highly fermentative *S. cerevisiae* CHY1011, and the flocculent, weakly fermentative *S. bayanus* KCCM12633. The hybrid strain was compared to the parental strains in batch fermentations; the hybrid exhibited the highest flocculation level and demonstrated higher fermentative capacity than the parental strain.

Zhao and Bai (2009) reviewed the recent uses of yeast flocculation in fuel ethanol production, and conclude that the use of flocculating yeast in continuous culture has several technical and economic advantages over many support immobilised systems and free cell systems for ethanolic fermentation. Yeast flocculation has been shown to have an influence on the immobilisation of yeast cells on carrier surfaces, with strongly flocculent strains forming biofilm-like attachments more readily than non-flocculent strains (Linko *et al.* 1998; Van Iersel *et al.* 1998).

A variety of bioreactor configurations have been developed that make use of flocculation immobilised cells including an airlift reactor (Bu'lock *et al.* 1984), packed bed reactors (Gong and Chen, 1984; Jones *et al.* 1984; Admassu and Korus, 1985; Kuriyama *et al.* 1993) and fluidised bed bioreactors (Kida *et al.* 1989). Royston (1966) also developed a packed bed (flocculated yeast), fluidised bed hybrid, which is commonly cited as the archetypal continuous tower bioreactor (Atkinson and Mavituna, 1991; Stanbury and Whitaker, 1984).

1.2.5 Fermentation Performance

The fermentation performance in a given bioreactor can be optimised by influencing the conditions within the bioreactor, and achieve conditions which favourably influence the microorganisms' metabolism (Wang and Hatzimankatis, 2006). Another approach is to change the metabolic characteristics of the biocatalyst itself (Matsumoto *et al.* 2004; Blieck *et al.* 2007). Several mechanisms involving the kinetics of some fermentation metabolites have been proposed and tested (Richard, 2003; Madsen *et al.* 2005). Perhaps the most important of these is the activity of phosphofructokinase (PFK), which undergoes allosteric regulation. Substrate inhibition of PFK by ATP was identified as the primary source of glycolytic dynamic behaviour in yeast cells by Ghosh and Chance (1964). This allosteric regulation of PFK is the key control in the dynamic regulation of the glycolytic pathway in *S. cerevisiae* (Yuan *et al.* 1990; Boiteux *et al.* 1975). Although there has been much progress in discovering the mechanisms of yeast cell metabolism, links between scientific insights and the practical aspects of fermentation are rare. This makes the process design for ethanolic fermentations dependent to a large extent on know-how rather than on a fundamental scientific understanding of yeast metabolism (Bai *et al.* 2008). Wang and Hatzimankatis (2006) attempted to resolve some of the uncertainty inherent in 'first principles' kinetic modelling for the purposes of fermentation design; A mathematical framework in which the parameter uncertainty inherent in kinetic modelling was addressed using the Monte Carlo method (approximation of a quantity through a simplified, representative calculation (Gell-Man, 1994)). Their model is used to describe the practical implications of feedback constraints of chemostat fermentation models and how the clustering of compartmentalised carbon pathways reveals functional coupling of metabolic reactions.

The availability of nitrogen within fermentation broths is a key consideration in ethanol production; Mendes-Ferreira *et al.* (2007) monitored the transcriptional responses of *S. cerevisiae* PYCC4072 during ethanolic fermentations under different nitrogen concentrations. Under nitrogen limiting conditions several genes involved in oxidative glucose metabolism were induced, including a significant number of mitochondrial associated genes typically associated with the yeast cell's response to glucose starvation. Salmon (1989) demonstrated that under nitrogen limiting conditions, the arrest of protein synthesis and the associated loss of transmembrane sugar transport accounted for part of the inhibition of fermentations.

Genetic engineering of yeast in order to achieve a variety of effects has also been studied. Akada *et al.* (1999) constructed a recombinant saké yeast strain containing bacterial drug resistance genes. The study demonstrated the construction of a recombinant yeast strain without extraneous DNA sequences

using a novel two-step protocol developed for this purpose. Recombinant *S. cerevisiae* encoding the *Thermus thermophiles xylA* gene was used to catabolise xylose during ethanolic fermentations by Walfridsson *et al.* (1996). It was found that the recombinant strain was able to express an active xylose isomerase enzyme. Wiedemann and Boles (2008) described how wild type *S. cerevisiae* strains cannot utilise pentose sugars present in bioethanol fermentations from plant biomass feed stocks, except after heterologous expression of metabolic pathways from other organisms. Recombinant *S. cerevisiae* expressing codon-optimised bacterial genes from *Bacillus subtilis* were used to produce ethanol from L-arabinose and the yeast showed greatly improved conversion rates of L-arabinose over the wild type strain (Weidemann and Boles, 2008).

Matsumoto *et al.* (2004) used expression plasmids containing artificially synthesised random sequences to transform known *S. cerevisiae* strains (*S. cerevisiae* MT8-1 and IFO10150). The resulting combinational yeast libraries contained approximately 200 strains with ethanol-tolerance exceeding that of the parental strain. The ten clones exhibiting the highest ethanol tolerance were selected and the translated peptides were examined. Three peptides were identified as hydrophobic and four were predicted to be transmembrane peptides and the three others were determined to be more hydrophilic (Matsumoto *et al.* 2004). The *recA* gene, which encodes a protein involved in an array of DNA metabolic processes including efficient post-replication repair of genetic material was cloned from *E .coli*, and expressed in *S. cerevisiae* by Cernakova *et al.* (1991). The expression of this gene in yeast might limit the level of genetic drift over time experienced by a population of yeast in a bioreactor system.

A different strategy of obtaining genetic variants with favourable characteristics was undertaken by Blieck *et al.* (2007). Cells of the lager yeast strain *S. cerevisiae* CMBS33 were subjected to differing levels of UV radiation, and the resulting pool of genetic mutants were subjected to consecutive rounds of high gravity fermentation. Two variants that exhibited higher rates of fermentation under the high gravity initial conditions, which achieved more complete attenuation of available sugars and a greater degree of ethanol tolerance, thus demonstrating that the use of selection pressures allows for the isolation of superior yeast strains for a given purpose. Microarray analysis showed several genes with altered expression may have been responsible for the mutants' superior performance.

Yeast cells exposed to ethanol adjust their intracellular metabolism accordingly, this process is known as ethanol inhibition, under which yeast cell growth and ethanol production is inhibited. Fluctuation in yeast activity is triggered by the delayed response of yeast cells to ethanol inhibition (D'Amore and Stewart, 1987; D'Amore *et al.* 1988). The effects that ethanol has on the yeast cell include the inhibition of key enzymes in the glycolytic pathway such as hexokinase and alcohol dehydrogenase (ADH).

Ethanol also affects nutrient uptake and membrane potential by decreasing the activity of the plasma membrane ATPase (Casey and Ingledew, 1986; Larue *et al.* 1984). Some unsaturated fatty acids such as palmitoleic acid and oleic acid are key membrane components, whose presence increases the fluidity of the plasma membranes, counteracting the loss in fluidity experienced by membranes in the presence of ethanol. The levels of these two unsaturated fatty acids are higher in more ethanol tolerant strains (You *et al.* 2003). A small amount of oxygen is required for yeast cells to synthesise these unsaturated fatty acids under anaerobic fermentation conditions (Ryu *et al.* 1984). The trans-membrane proton flow that drives the secondary transport of nutrients is impaired through the dissipation of the proton gradient in the presence of ethanol, this effect is linked to both an increase in plasma membrane permeability (Pascual *et al.* 1988) and the inhibition of the proton-pumping plasma membrane ATPase activity (Salguerio *et al.* 1988). Cartwright *et al.* (1987) showed that the plasma membrane ATPase is key to ethanol tolerance as ATPase activity allows yeast to maintain their intracellular physiological pH; H^+ ions produced during fermentation need to be continuously removed from the cell via the proton motive force driven by the ATPase.

1.2.6 Beverage Production

Beverage alcohol production has been a long-term subject of intensive study worldwide (Okabe *et al.* 1994). This thesis will involve the primary (ethanolic) fermentation of only two beverages; apple cider and mead. These beverages were selected in part for practical reasons regarding the acquisition of an adequate supply of substrate, and because of the availability of funding for research into specific aspects of the production of these beverages. Both beverages are also the subjects of current upward trends in the beverage alcohol market (Daya and Steenkamp, 2005; Berry, 2009). Continuous fermentation is most readily applicable for processes that make use of substrates that are able to be stored for periods of time without a significant degradation in quality, this is axiomatic, as without this property, simultaneous processing of the total substrate volume is the only viable model. Honey, as a hyperosmotic solution is able to be stored for considerable lengths of time (20+ years) without microbial contamination, although commonly undergoes crystallisation if stored for a number of days (Krell 1996). This process is easily reversible, however and does not cause any subsequent complications or problems (Krell, 1996). Mead production therefore, is a suitable candidate for continuous processing as honey is the only readily accessible and storable natural source of fermentable sugars (Krell, 1996). Apple must traditionally may not be stored for any significant length of time before the initiation of spontaneous fermentation of available sugars by wild yeast associated with fruit skins. Modern processing techniques developed for the fruit juice industry however, have

enabled both the enzymatic depolymerisation of all available sugars in apple pulp, and the concentration of fermentable sugars to 700 $g.L^{-1}$ with acceptable quality and flavour. Popular beverages such as 'Appletiser™' are reconstituted from these concentrates before packaging due to added consistency of product, the ability to store raw materials and reduced transport costs to the site of packaging.

1.2.6.1 Mead Production

Mead is probably the oldest of the fermented beverages produced and consumed by humans, this is due to the fact that it is the most likely to occur spontaneously in nature (Gayre, 1948; Acton and Duncan, 1984). As the global population of humans increased, so did the relative scarcity of honey (Gayre, 1948), and eventually the honey as a source of fermentable sugars became economically unsustainable and cheaper sugar sources became preferred. Mead is currently undergoing a revival, across North America and Europe due to renewed interest in apiculture following recent popular interest in 'colony collapse disorder', and for this reason the production of mead using efficient bioprocessing techniques is of interest.

Mead ethanolic fermentations observed by Steinkraus and Morse (1966) were generally very slow, hence the development of a nutrient and vitamin solution which could be added to new mead batches to encourage fermentation. This increased the rate of fermentation of mead allowing it to be produced in weeks rather than months. The fermentation of mead can also been hastened by agitation to improve mass transfer between yeast and the must (Steinkraus and Morse 1966).

The currently available literature is focused on improving the time consuming nature of mead fermentations. Roldan *et al.* (2011) attempted to alleviate honeys low nutrient content with the addition of varying concentrations of pollen to the must ranging from 10 to 50 $g.L^{-1}$. The results demonstrated that pollen improved fermentation rates, ethanol yields, and the organoleptic characteristics of the mead produced. An increase in the volatile organic compound levels in products was also observed. A study was conducted by Mendez-Ferreira *et al.* (2010) who optimised must formulation using a number of additives including nitrogen supplementation and the addition of organic acids. It was found that the addition of diammonium phosphate increased the fermentation rate but did not ensure complete attenuation of available sugars, from which the authors concluded that factors other than nitrogen availability could account for reduced yeast activity in mead fermentations. Observed levels of volatile aromatic compounds were also higher in nitrogen-supplemented

fermentations (Mendez-Ferreira *et al.* 2010). Navratil *et al.* (2001) used immobilised cells in order to reduce mead primary fermentation times.

Pereira *et al.* (2009) evaluated the capacity of a variety of local *S. cerevisiae* strains isolated from wild honey to survive the adverse stress conditions found in mead fermentations. The isolated yeast strains were evaluated in terms of their respective fermentation performance under ethanol, SO_2 and osmotic stress. The results indicate that wild yeast performed similarly to the commercial control strains used.

Nagodawithana and Steinkraus co-authored two papers investigating the effect of certain parameters on the viability of *S. cerevisiae* during ethanolic fermentations using mead fermentation as a model (Nagodawithana *et al.* 1974; Nagodawithana and Steinkraus, 1976).

1.2.6.2 Cider Production

Cider is an alcoholic beverage made from the fermented juice of apples (and sometimes pears) (Nedovic *et al.* 2000). (Cider made only with pear juice is known as *perry* or pear cider). Both cider and wine production involve two successive biological fermentations (del Campo *et al.* 2008, Simon *et al.* 1996). The first is the primary, or ethanolic, fermentation and the second is malolactic fermentation (MLF), or maturation (Herrero *et al.* 2006). Traditionally both fermentations are carried out spontaneously in batch bioreactor configurations. This process can be relatively slow, with fermentation taking up to three weeks and the maturation up to six months (Agouridis *et al.* 2008, Scott and O'Reilly, 1996).

MLF is the bioconversion of malic acid to lactic acid, and is critical in the production of high quality wines and ciders (Sanchez *et al.* 2010; Herrero *et al.* 2001). This fermentation is typically carried out by lactic acid bacteria (LAB), of the species *Oenococcus oeni* and various *Lactobacillus* species, and contributes to the flavour complexity of the beverage by producing compounds such as acetaldehyde, acetic acid, ethyl acetate, ethyl lactate, diacetyl, acetoin and 2,3-butanediol (Maicas *et al.* 2001). The malolactic fermentation also reduces the acidity of the product and stabilizes it due to the bacteriostatic effect of lactic acid (Nedovic *et al.* 2000).

Cider production may either be spontaneous or controlled (Valles *et al.* 2007), and either 'natural' or 'sparkling'. In Northern European markets natural ciders that have not undergone a post-fermentation carbonation are preferred (Blanco-Gomis *et al.* 2009), whereas in the majority of markets highly carbonated ciders are preferred (reminiscent of the beers available in these homogenous markets).

Sparkling ciders are either artificially carbonated or carbonated naturally by fermentation in the bottle, which is termed the 'champenoise' method after the French for the champagne method (méthode champenoise) (Valles *et al.* 2008). The technology used in the cider production bioprocess plays a major role in the taste components experienced in the final product (Mangas *et al.* 1994). Of particular interest are the foaming properties of the product. Hydrophobic proteins contribute to form and stabilise foams (Blanco-Gomis *et al.* 2010). Volatile and other sensory characteristics of ciders are made up of alcohols, esters, carbonyls, acetyls, ethers, lactones, hydrocarbons, and halogenated compounds (Williams *et al.* 1980). These defining characteristics of cider products are affected by a number of factors, including the fermentation process, maturation, processing and storage (Madrera *et al.* 2008).

During the primary (ethanolic) fermentation the main sources of variability in taste influencing compounds are the microorganisms responsible for the bioconversion of carbohydrates. Most large cider producers make use of *S. cerevisiae* due to its high alcohol tolerance and greater efficiency in conversion to ethyl alcohol specifically (Xu *et al.* 2006). These starter cultures eliminate contamination from other microbial species which would otherwise add flavour complexity as with spontaneous fermentations. Valles *et al.* (2008) reported the isolation of 54 individual *Saccharomyces* yeast species in a single cider undergoing a spontaneous natural carbonation. The addition of complexity in controlled fermentations of cider has been attempted with the use of *Hanseniaspora valbyensis* yeast in mixed culture with *Saccharomyces* species; although the complexity increase was achieved, it was accompanied by a substantial drop in ethanol production (Xu *et al.* 2006).

The effects of different fermentations on the fatty acid composition of ciders has also been investigated as they constitute the precursors of volatile compounds and so play an important role in the sensory qualities of ciders (Madrera *et al.* 2010). The production of higher alcohols during cider fermentation has also been investigated as they have been found to contribute to a pleasant taste within certain concentration boundaries (Vidrih and Hribar, 1999). Spoilage is a problem for many alcoholic beverages, cider typically undergoes 'framboise', or cider-sickness, which is caused by contamination with *Zymomonas mobilis* (Coton *et al.* 2006).

Nedovic *et al.* (2000) made use of Ca-alginate gel entrapment to co-immobilise *S. bayanus* and *Leuconostoc oenos* in a continuous conical packed bed bioreactor in order to simultaneously perform the primary and secondary fermentation steps in cider production. This fermentation setup, the researchers concluded, permitted faster fermentation rates than traditional configurations, and allowed for greater control over the flavour formation. Immobilised cells were also used by Scott and O'Reilly (1996) in cider production; the co-immobilisation of *S. cerevisiae* and *L. plantarum* through adsorption

to a sponge-like material was also used to simultaneously perform both the primary and secondary fermentations, with similar conclusions regarding fermentation rates and flavour compound development. Simon *et al.* (1996) investigated a bioreactor configuration comprising an immobilised *S. bayanus* packed bed bioreactor coupled to an immobilised *L. oenos* packed bed bioreactor in series which again compared favourably with traditional production methods. To date, only limited attempts in applying immobilised cell technologies to cider manufacture at commercial scale have been undertaken (Colagrande *et al.* 1994; Divies *et al.* 1994). This is likely due to a combination of high market 'barriers of entry' and a staunch resistance to new technologies in non-homogenous (traditional/decentralised) markets.

1.2.7 Fermentation Metabolite Analysis

Qualitative and quantitative knowledge of a variety of compounds including organic acids, sugars and alcohols is key to a number of scenarios involving any number of natural matrices, including fruit, honey and fermentation broths (Molnar-Perl, 1999). In the field of fermentation research, quantitative data describing the concentrations of a number of sugars, organic acids and alcohols is key to the determination of reaction models and performance data, and also to commercial application of the systems developed due to considerations such as consistency and excise. Many modern fermentation facilities now feature in-line analysis of fermentation metabolites and in some cases real-time data from fermentation vessels and other components (Danielsson, 1991).

In the laboratory there are a number of analytical techniques available for the quantification of the analytes of interest, these may include a range of techniques from first principle techniques such as hydrometric density measurement and ebulliometry, through spectrophotometric assays, such as the Somogyi-Nelson and DNS assays, through to infra-red spectroscopy and sophisticated chromatographic methods with an array of sample preparation and detection methods. In general, chromatographic methods are the customary methods used in the analysis of food samples (Bovanova and Brandsteterova, 2000).

Ion-exchange high-performance liquid chromatography is the most commonly used method for the analysis of sugar and alcohol compounds in fermentation products as it allows the simultaneous separate quantification of most compounds of interest in a single run, allowing for high throughput analysis with the loss of only very small volumes of the sample (Bonn, 1985; Molnar-Perl, 1999). A review by Bovanova and Brandsteterova (2000) details the different strategies used in the HPLC

analysis of complex samples, however rapid fermentations with low complexity substrates tend not to contain levels of secondary metabolites that might interfere with the simultaneous quantification of the key performance linked fermentation metabolites. A wide selection of commercial analytical columns and standardised protocols are available for this purpose. Bonn (1985) achieved the simultaneous separation and quantification of several carbohydrates, alcohols and diethylene glycol using ion-exchange columns.

1.2.8 Technology Transfer

Yu and Hang (2011) discussed the strategy of developing technology candidates of disruptive innovations and demonstrated four research and development (R&D) strategies that have been used to create successful disruptive technologies in the past. These were miniaturisation, simplification, augmentation and exploitation for another purpose. These strategies can be used alone or in combination to develop effective disruptive technologies and the authors indicate that although conscious use of these strategies would help more companies to develop disruptive innovators, they are not intended to be prescriptive, and no single strategy is necessarily 'superior' to another.

The transfer of knowledge arising from research to industry is often more difficult than it would first appear (Fontes, 2005). This is due to the complex, systematic, context-related, tacit and person-embodied nature of knowledge (Pavitt, 1990). In addition to this, the differences in purpose and scope between academia and industry make the transfer process a potentially complex undertaking. Given the inherent cultural and language barriers between these two different contexts there is a need for 'translators' (Fontes, 2005).

A case study from the aviation industry showed that governmental policy plays a major role in dictating the dynamics of technology transfer, especially across borders (King and Norwak, 2003). The study suggested that many companies, industries and even nations may suffer from what the authors describe as the 'not-invented-here' syndrome, which can severely impact the adoption of new technologies and ultimately prevent the evolution of industry. This effect may play a role in emerging economies and act to delay economic development. One policy implemented in the United States is the Entrepreneurial separation to transfer technology program (ESTT), which is aimed at strengthening the technology transfer from the laboratory to industry (Minutolo and Potter, 2011). ESTT exists primarily to increase the ability of the scientist to successfully commercialise technology developed whilst under employment with a public or private laboratory through risk reduction. For example separating the

scientist/potential entrepreneur from areas of development that may conflict with the activity of the research laboratory, allowing labs to continue development without compromising technology commercialisation and *visa versa*. Technology transfer offices (TTOs) are a formal method for carrying research to the market, which do not necessarily make use of any of the original scientists with the technical competency pertinent to the innovation, as this is not always requisite. Siegel *et al.* (2004) highlight the importance of competent university management of intellectual property developed under its purview for TTOs to be able to play a meaningful role in enabling technology transfer. This is still a stumbling block for many academic institutions in emerging economies (Salicrup and Fedorkova, 2006). University spin-offs (USOs) (spin-outs in US literature) by comparison, are an alternative to this model, in which the original team take an executive role in the formation, and sometimes even the management of the new company.

University tech spin-off companies allow for the direct transformation of scientific and technological knowledge originating in research institutions into economic value, and can also play a useful agency-type role aiding in the understanding, application and dissemination of knowledge produced in research institutions (Perez and Sanchez, 2003). For this reason the creation of USOs is a major policy objective of governments and universities (Wright *et al.* 2006). It was found by Fontes (2005) that new technology intensive companies have been extensively involved in the transformation process, and as an effect of this, enjoy a number of advantages in emerging and established fields. The advantages are even greater for these spin-off companies in industries where science and technology are proximate, such as biotechnology and electronics companies. Within these contexts, an early involvement in the knowledge development relating to a technology and access to complementary information often create a competitive advantage over established players in the given industry (Perez and Sanchez, 2003).

Pries and Guild (2011) considered characteristics of 42 new innovations developed within academic settings and related these to the business models used in the commercialisation process. The study confirmed correlations between the level of intellectual property associated with and licencing agreements and other limited rights transfer to existing companies for successful commercialisation, the results also suggested that where commercial uncertainty is a factor with a new technology, the greater the chance of success exited with the creation of a new entity to produce the goods or services, or with total transfer of IP rights, as opposed to an arrangement involving allowing access to others.

Finance is a catalyst of the wealth creation associated with technology transfer, and in the vast majority of cases, is a prerequisite for the creation of university spin-off ventures (Wright *et al.* 2006). This is why an established financial sector stands with a strong scientific academia as a pillar of a competitive

economy (Sener and Saridogan, 2011). Despite this, due to the high risk associated typically associated with entrepreneurial ventures in general, and compounded by the commercial uncertainty of novel technologies, access to capital is a major impediment to the creation of USO companies. Venture capital (VC) firms position themselves to invest in high risk – high return ventures but have come under criticism for reluctance to invest in high-tech early stage university spin-offs (Lockett *et al.* 2002). The reasons for this may include poorly developed methods for valuation of these early stage ventures and their IP (Wright *et al.* 2006), the assembly of unsuitable entrepreneurial teams and other examples of the poor understanding of the requirements of external funders on the side of academics and universities, or plain risk adversity on the side of the VC firm.

Wright *et al.* (2006) found that there is often a mismatch in the expectations of the Universities and their spin-off companies and the VC firms. Whilst USOs hope to attract funding for the seed stage (pre-start-up) of business development, VCs are typically only willing to invest after proof of concept has been achieved, thus reducing the risk involved. This presupposes access to alternative sources of capital, which is found to be the *de facto* hurdle in successfully launching USOs. Options at the seed stage are limited therefore to angel investors, debt financing, internal funding and seeking funding from government agencies or independent entities (definitions in glossary). Seed funding from independent entities and angel investments are neither ubiquitous nor available without a prior relationship between the respective parties and are therefore subject to the competency of the university in forming the groundwork for the formation of USOs. Government agencies and funding availability/accessibility through them clearly differ between countries, and often reflect the level of development of the national economy in general. Internal funding is typically restricted due to the universal strain of university (and personal) budgets; if USOs are not a priority for the institution, this funding will not be available. Debt funding is a widely available option for seed funding purposes, but requires collateral and is therefore generally unavailable to USOs. Most countries, however, have developed guarantee schemes so that the need for collateral is backed by public money (Wright *et al.* 2006). In South Africa and other developing nations where networks of angel investors and external investors are poorly established, and government agencies tend towards the inept, these sorts of guarantee schemes could prove invaluable in accelerating economic growth. Government policy could also be used to enable public-private partnerships, which has been shown to be highly effective in the UK (Shane, 2002; Wright *et al.* 2006). In Sweden and Finland government initiatives provide pre-seed and seed capital, networking, and even coaching for technology start-ups and USOs (Wright *et al.* 2006). The acceptance of public money to be used for these purposes in some countries would pose a problem, however. It is possible that people may not understand that the providence of public services is dependent on the national economy's ability to provide them.

Research into technology transfer in intermediate economies found that highly qualified young scientists were most effective in achieving a match between technological knowledge and market needs by capitalising on their technological competencies and relational assets (Fontes, 2001). The country's ability to exploit new opportunities in biotechnology is related to the level and nature of the scientific knowledge base, the institutional set-up and the policies instituted by government (Fontes, 2001).

Jain *et al.* (2009) argue that in order to gain insight into the recent disposition of the top US and UK universities towards commercialisation activity as opposed to publication, it is critical to focus on the scientists. They argue that policies pertaining to the creation of hybrid scientist-entrepreneur positions in university spin-off technology companies must enable scientists to retain academic activities as psychological analysis suggests that role modification, rather than role displacement occurs when scientists engage in entrepreneurial activities.

Nair and Ahlstrom (2003) discuss the 'battle' between emergent disruptive technologies and incumbent technologies for supremacy, and consider factors that extend the period over which the technologies compete, and even factors that allow for the coexistence of both technologies. The authors conclude that as the complexity of the system in question increases, the capacity for innovation and system overhaul becomes diminished. Christensen (1997) stated that 'whilst products and subsystems may display a process of evolution, revolution, maturation, destruction and replacement, the system in its entirety may show a remarkable persistence. This is an apt characterisation for the traditional batch methods of beverage production still used globally today. The reason for the persistence and resilience of the incumbent system is that the localised evolution of subsystems is usually sufficient to achieve the requisite parity for survival (Nair and Ahlstrom, 2003). This suggests that a marginally superior system will be able to coexist with the traditional system, but to completely, or substantially displace it (so called creative destruction); the emergent technology must represent a significant advance. Thus rendering the traditional system economically non-viable.

Mendonca (2009) discusses how 'low-tech' industries such as paper, food and drink have also significantly contributed to the development of technologies that are at the core of todays economy, thus demonstrating that it is not just USOs that meaningfully contribute to technological advancement, but also established businesses in the both the high, and low-tech areas, small businesses (Kassicieh *et al.* 2002) and non-USO technology start-ups. In the case of established businesses however, the predominance in development trends is creative accumulation over creative destruction, that is;

developments tend to complement existing methods and equipment, rather than displace it (as disruptive technologies do).

In the following chapters a bioreactor system that may enable the 'creative destruction' of the incumbent entrenched technologies will be developed. It will be tested at the bench scale and then scaled-up to the commercial scale. The resulting bioprocess will then be analysed and discussed from an applied financial perspective.

Chapter 2

Bioreactor Design

2.1 Introduction

A bioreactor is any device that is used to carry out one or more biochemical reactions to convert a substance to some product(s) (Ratledge and Kristiansen, 2006). Bioreactors are a necessary part of any biotechnology based production process whether it is for producing biomass or metabolites, biotransforming one compound into another, or degrading unwanted wastes. The reactions occurring in a bioreactor are driven by biocatalysts which can be enzymes, microbial cells, the cells of plants or animals, subcellular structures or other cellular components (Towler and Sinnot, 2008). The bioreactor provides an environment that is conducive to the optimal functioning of the biocatalyst. Bioreactors are typically operated under sterile or monoseptic conditions.

Atkinson and Mavituna (1991) list the exploration of the following aspects of process development as the core of the reactor design methodology:

1. The utilisation of the extensive literature on process engineering.
2. The selection of appropriate reactor systems after consideration of the process requirements.
3. Effective laboratory and pilot scale experimentation.
4. Rational extrapolation of experimental data to plant on a commercial scale.
5. The development of design procedures and cost models.
6. Minimisation of the delay between initial concept and full-scale production.

These aspects will be covered over the remaining chapters of this thesis.

In general there are three modes of bioreactor operation: the batch reactor, where $F = F_{out} = 0$ (the volume is constant); the continuous reactor, where $F = F_{out} > 0$ (the volume is constant); and the fed-batch reactor, where $F > 0$ and $F_{out} = 0$ (the volume increases).

2.2 Objectives

The objective of this chapter is to conceptualise a bioreactor using a 'bottom-up' strategy, which is appropriate for ethanolic fermentation for beverage alcohol production using very basic physical and biological principles. As discussed in Chapter 1, requirements for entering the beverage alcohol market today include low capital equipment costs, low energy and personnel requirements (simplicity of operation) and high productivities. The bioreactor should also be able to operate over long periods without the need for cleaning, sterilisation, reinoculation or the need to reculture the catalytic microorganism to compensate for genetic drift. In addition to these requirements, the bioreactor must also be able to operate with a low risk of contamination. The combination of these advantages should yield favourable process economics, and a production system suited to a decentralised production strategy.

2.3 Bioreactor Configurations

There exists an array of bioreactor configurations and several different methods for categorising them. The major types of bioreactors configurations discussed below are those used by Ratledge and Kristiansen (2006) in their description of the bioreactors used in industrial processes. This categorisation includes: stirred tank reactors, bubble columns, airlift devices, packed beds, and fluidised beds.

2.3.1 Stirred Tank Reactors

Stirred tank bioreactors (STRs) or more generally, bioreactors with an internal method of agitation, usually consist of a cylindrical vessel with a motor driven central shaft that supports one or more agitators (Rossi, 2001). Gas is supplied to the vessel via a 'sparger' and agitation of the culture allows for high values for gas transfer to be achieved. Microbial culture vessels are typically equipped with baffles projecting into the vessel from the walls to prevent vortexing of the fluid around the central axis. The aspect ratio (height to diameter ratio) of these types of vessels normally ranges from 3 to 5, with lower ratios used for the culture of cells sensitive to shear, in addition these are typically unbaffled in order to reduce turbulence (Ratledge and Kristiansen, 2006). The number of impellers used to maintain homogenous conditions is dependent on the aspect ratio of the vessel for easily discernible reasons. As gas is sparged it is the responsibility of the impeller to maintain gas dispersion, not of the sparger (Shuler and Kargi, 1992). For this reason gas enters the reactor below the bottom impeller and across a smaller diameter then that of the impeller. The superficial aeration velocity (the volumetric gas flow rate over the cross-sectional area of the vessel) in

stirred vessels must remain lower than the value needed to flood the impeller (an impeller is flooded when it receives more gas than it can effectively disperse), which can lead to cavitation. STRs can be used singly as a batch fermenter, or continuously (CSTRs) as a single fermentor or in series (Atkinson and Mavituna, 1991). Stirred tanks are commonly used in the production of antibiotics and organic acids (Ratledge and Kristiansen, 2006).

In fermentation a low cost alternative to STRs are cylindro-conical bioreactors commonly used in the beverage alcohol industry (Stanbury and Whitaker, 1984). These bioreactors typically do not have internal agitation apparatus and therefore suffer from relatively poor performance, with a focus more on minimising biomass retrieval costs via flocculation.

2.3.2 Bubble Columns

A bubble column bioreactor is cylindrical and has gas sparged at the base of the column through perforated pipes, plates, sintered glass or metal microporous spargers (Ratledge and Kristiansen, 2006; Towler and Sinnot, 2008). They typically have higher aspect ratios (commonly ranging from 4-8) (Ratledge and Kristiansen, 2006). Oxygen transfer, mixing and other performance determining factors are influenced primarily by the gas flow rate and the rheological properties of the fluid (Garcia-Ochoa and Gomez, 2009). Internal structures such as horizontal perforated plates, vertical baffles and packing materials may be placed in the vessel to improve the reactors kinetic behaviour (Ratledge and Kristiansen, 2006). For a given gas flow rate, the mixing improves with increasing vessel diameter as pipe flow behaviour recedes and axial mixing becomes more complete. Shearing effects, mass and heat transfer all increase with increased gas flow rate. Bubble columns are established for use in low viscosity aerobic fermentations such as in the treatment of wastewater (Shuler and Kargi, 1992).

2.3.3 Airlift Bioreactors

Airlift bioreactors are generally appropriate for aerobic reactions; their fluid volume is divided into two vertical connected areas, separated by means of a baffle or sometimes some other method such as a draft tube (i.e. there is empty space between the two areas). Only one of the two areas is sparged and is known as the riser, and the area not receiving any gas is called the downcomer (Verbelen *et al.* 2006). The average density of the gas-liquid dispersion in the riser tends to be lower than that in the downcomer as gas displaces liquid and consequently the mixture flows upwards in the riser and downwards in the

downcomer (Ratledge and Kristiansen, 2006). Airlift bioreactors are extremely energy efficient compared to stirred bioreactors, and their productivity rates tend to be comparable volumetrically. Airlift reactors are commonly employed in the manufacture of proteins obtained from shear-sensitive cells for the biopharmaceutical industry and other low viscosity because of their inherent low shear rates. Airlift reactors are also more effective at suspending solids than bubble columns as the dispersion has an overall velocity, not just gas bubbles (Shuler and Kargi, 1992; Towler and Sinnot, 2008).

All performance characteristics of airlift bioreactors are linked to the gas injection rate and resulting rate of liquid circulation. Airlift bioreactors with high aspect ratios, and with greater differences in the gas hold-up levels between the riser and downcomer, have higher circulation rates. Circulation is therefore enhanced if there is little or no gas in the downcomer, thus sometime gas-liquid separators are used at the top of the reactor to reduce gas carry-over to the downcomer (Atkinson and Mavituna, 1991; Ratledge and Kristiansen, 2006).

2.3.4 Fluidised Beds

Fluidised bed bioreactors are suited to reactions involving a fluid with suspended particulate biocatalysts such as immobilised enzymes, cell particles or microbial flocs (Taillandier *et al.* 1994). An upward flowing stream of liquid is used to suspend (*fluidise*) the solids. Geometrically the reactor is similar to a bubble column except that the top section of the reactor is expanded to reduce the superficial velocity of the fluidising liquid to a level below that required to keep the solids in suspension, the solids therefore sediment in the flared area of the column and sink returning to the more narrow section of the reactor (Ratledge and Kristiansen, 2006). In this way, solids are retained and liquids flow out of the column. A high density of solids improves solid-liquid mass transfer by increasing the relative velocity between the two phases, and so solid particles are sometimes artificially weighted increasing their density. Fluidisation velocity is dependent on the relative densities of the solid and liquid phases, the diameter of the particles and the viscosity of the liquid (Atkinson and Mavituna, 1991).

Additional agitation can be achieved through the addition of gas when necessary. For example, sometimes even very light particles require a high superficial liquid velocity for particle suspension, which would result in a flow rate which could negatively impact the productivity of the reactor as the solid-liquid contact time is too low. The recycling of fluid can also be used to allow for a large enough cumulative contact time, however this could result in problems with product inhibition (Ratledge and Kristiansen, 2006).

2.3.5 Packed/Fixed Beds

A bed of solid particles, sometimes with confining walls in the axial dimension (separating the length into sections) constitutes a packed bed. The biocatalyst is supported on or entrapped within the matrix of solids, which may be particles of a polymeric material or a solid carrier (Kourkoutas, 2004). A fluid flows continuously though the bed to supply the reaction substrate and the nutritional requirements of the immobilised biocatalyst (Ratledge and Kritsiansen, 2006). The biocatalyst releases metabolites (usually including the reaction product) into the fluid in the immediate environment and these are removed from the bioreactor in the outflow. The flow may be upward or downward, but down-flow under gravity is more common as it is more energy efficient. If the fluid flows up through the bed, the flow rate is limited because the velocity cannot exceed the minimum fluidisation velocity of the particles forming the bed (Shuler and Kargi, 1992; Towler and Sinnot, 2008). This is could be unfavourable if the biocatalyst is sensitive to shear stress as it can cause damage, and if the bed is fluidised axial mixing occurs which is highly unfavourable where stratification is desired (Leib *et al.* 2001; Levenspeil, 1999).

The depth of the bed is limited by a number of factors including the density, and the compressibility of the solid matrix, the need to maintain a minimum level of substrates and nutrients throughout the entire depth of the packed bed, and the flow rate that is required for a given pressure drop through the reactor. If the packing material is compressible, its weight may compress the bed unless the volume is kept low. For downward flow, the flow rate through the packed bed declines as the depth of the bed increases (Ratledge and Kristiansen, 2006). The concentration of nutrients decreases as the fluid moves through the bed, and concentrations of products and metabolites increases. The environment within a packed bed is therefore non-homogenous and accurate pH control is difficult due to inherent low mixing. Beds with greater void volumes allow for greater flow velocities though them, but the possible concentration of biocatalyst within any given bed volume decreases with increased voidage (smaller particle size = larger available surface area) (Leib *et al.* 2001).

Packed beds are used extensively in reactions involving immobilised enzymes and waste water treatment (Phugare *et al.* 2010), and are very attractive for product inhibited reactions as the product concentration varies from a low value near the inlet of the bioreactor, to a high value at the exit, and therefore only part of the bioreactor is exposed to inhibitory product levels, instead of most, as in more homogenous bioreactors. Several studies making use of packed bed reactors for potable alcohol production have been conducted

(Kourkoutas *et al.* 2004; Bekatorou *et al.* 2002; Nedovic *et al.* 2000) Historically packed bed reactors were used for vinegar production (Mitchell, 1926).

2.4 Bioreactor Operation Modes and Configurations

As mentioned at the beginning of this chapter there are only three general modes of operation for any bioreactor: batch, where the volume is constant, continuous, mode where the volume is constant, and the fed-batch mode, where the volume increases.

Each of these modes of operation has inherent advantages and disadvantages. The batch mode of bioreactor operation has the advantage of being versatile, as it is applicable, with some level of success, to almost any bioprocess. It has a relatively low risk of contamination due to its closed nature, and it also allows for the complete conversion of the substrate given enough time. Batch reactions have the disadvantages of being less productive than other models due to substrate and product inhibition common in many biological reactions. Batch reactors also suffer from long down times between reactions and the need for sterilisation after each reaction. This has the additional effect of requiring either sophisticated systems and equipment, or personnel, to control the cleaning process.

Continuous modes of bioreactor operation have the advantages of relatively high efficiencies on the reactor capacity; they also allow high productivities to be maintained for long stretches of time (Shuler and Kargi, 1992). Automation of continuous processes tends to be simple relative to other models, and the output of the reactor is constant and consistent, meaning there are no large variations in the characteristics of the product over time, as could be the case between batches in the other models. The disadvantages of continuous processing include the major implications associated with infection risks; long downtimes mean the economic impacts are likely to be higher than an infection in a batch process (Atkinson and Mavituna, 1991). The possible appearance of mutant populations over very long periods of operation means that in some bioprocesses there will be an upper limit on the period of operation without reinoculating/reloading the reactor with the biocatalyst. Another disadvantage of continuous processing is that the reactors are usually very specialised and are therefore inflexible; different reactions most probably cannot be completed in the same reactors without refitting. Another potential disadvantage of continuous processing is the need for downstream processing to be either adapted to a continuous model, or for additional holding tanks.

Fed-batch reactions have the advantage of constant operation under known conditions through the control of the feed flow. The fed-batch model also allows for very high cell densities and by extension, high final growth associated product concentrations. The fed-batch model suffers from a combination of disadvantages from the alternative models, including; batch reactors' problems of being less productive due to product inhibition, although substrate inhibition is eliminated by the gradual addition of substrate. They have the same long down times between reactions, the need for sterilisation after each, and the need for sophisticated control systems. Fed-batch processing shares continuous reactors' need for infection protection in the inlet feed over an extended period, although not to the same extent, obviously.

The bioreactor configurations discussed in section 2.4 can be operated in one or more of these modes. Table 2.1 indicates which modes are applicable to each configuration.

Table 2.1 Bioreactor configurations and applicable modes of operation.

Bioreactor Configuration	Batch	Continuous	Fed-Batch	Reference
Stirred Tank	X	X	X	Tang *et al.* 2010; Arrua *et al.* 1990; Clarke *et al.* 2006
Bubble Column	X	X	X	Letzel *et al.* 1999
Airlift Bioreactor	X	X		Smogrovicova *et al.* 1998; Smogrovicova *et al.* 1997; Kilonzo *et al.* 2004
Fluidised Bed	X	X		Tailandier *et al.* 1994; Cardona and Sanchez, 2007
Packed Bed	X	X		Iconomopoulou *et al.* 2002; Roca, 1996; Kumar *et al.* 2011

Ethanolic fermentations are substrate and product inhibited, suggesting that a rational model for an efficient fermentation process would likely be continuous (Kumar, 2011). Table 2.1 shows that all of the reactor configurations are compatible with a continuous processing model, however they do not all offer equally attractive solutions.

A continuous stirred tank model for fermentation would allow for a mid-point equilibrium where product and substrate inhibition is eliminated, however complete conversion of the substrate would not be possible unless several CSTRs were used in series; a 'cascade' system, in which several bioreactors operate in sequence, each with an appropriate product-substrate equilibrium (Levenspeil, 1999). This allows the resident microorganism population to adapt, and operate as efficiently as conditions allow. As an effective solution to the problem of the high equipment requirements and low efficiencies of the prevailing industry methods, this is not an acceptable solution, as it solves the efficiency problem to some extent, but only though the use of even more equipment.

A continuous bubble column for ethanolic fermentation is inappropriate due the bubble column's optimal performance under conditions of low viscosity and high gas flow rates due to mass transfer limitations (Ratledge and Kristiansen, 2006). The high level of mixing would result in the same reaction inhibition problems encountered with the stirred tank model. Reducing mixing by lowering aspect ratio or by decreasing the gas flow rate would result in a batch fermenter-like system with increased mass transfer problems. The gas inflow (which would have to be nitrogen of carbon dioxide to maintain anaerobic conditions) (Alfenore *et al.* 2004), in combination with the effervescence of the fermentation would likely damage the microorganisms through shear effects, and at the high substrate concentration area of a bubble column, the non-Newtonian characteristics of high density sugar solutions would result in further mass transfer implications as viscosity increases though the bubble inflicted shear (Shuler and Kargi, 1992; Towler and Sinnot, 2008). An ethanolic fermentation using a continuous airlift bioreactor is also not practical as the gas used would again have to be nitrogen or carbon dioxide, and in order to avoid product inhibition fluid recycling would not be advisable – making any ideal airlift bioreactor for ethanolic fermentation a *de facto* bubble column.

All of the three scenarios above suffer from the same problem; the fluid containing the products is not permanently removed from the biocatalyst after the required contact time. It is merely recycled through mixing or recirculation. The fluidised bed bioreactor configuration is not attractive for use with NewFlo type yeast species (Royston, 1966), because the combination of the carbon dioxide produced by the fermentation and the superficial fluid velocity would ensure that the retention of significant biomass is problematic (Atkinson and Mavituna, 1991). The use of a FLO1 yeast species would mean that higher flow rates would be possible without the loss of bioreactor productivity over time as the biomass sedimentation rate would be significantly higher (Zhao and Bai, 2009). The addition of a solid phase carrier would further improve the sedimentation rate of biomass retained though adsorption to the carrier surface (Rossi, 2001).

The combination of yeast flocculation and immobilisation on carrier surface, enabling increased relative velocities between the biocatalysts and the liquid phase has the potential to allow for a very productive bioreactor. There will be some mixing, however, which will have a negative effect on the efficiency of substrate conversion.

The use of a packed bed is ideal for product inhibited reactions, because as the bioreactor operates in a perfusion mode, the concentration varies in a linear fashion along the axial dimension of the packed bed, meaning that only part of the total biocatalyst population is exposed to inhibitory effects of high substrate concentrations at the inlet, and high product concentrations at the outlet (San, 1989; Leib, 2001). Adsorption to a solid carrier surface as a mechanism of biomass immobilisation enables the support matrix to be rigid, and therefore does not set a limit on the packed bed volume. In addition to this, a non-uniform but small solid structure comprising the support matrix will convey a substantial void volume and large surface area exposed to the liquid phase; allowing for higher flow rates without sacrificing concentration of biocatalyst within the packed bed.

FLO1 yeast would be the preferred species genotype for this kind of cell immobilisation, as this would maximise cell loading providing benefits such as cell reuse and the elimination of cell recovery steps (Shuler and Kargi, 1992). Given this; yeast flocculation would be a beneficial corollary if up-flow were used rather than a down-flow. In a down-flow situation using adsorption as the primary immobilisation method the flow rate would have to be limited in order to prevent excess biomass washout. In an up-flow situation the superficial flow velocity is too low to fluidise the packed bed, which would be composed of cells immobilised by both flocculation and adsorption (cell immobilisation is discussed in more detail in in section 2.5.4, and in chapter 4). However, given that in any ethanolic fermentation, the amount of carbon dioxide evolved by a population of yeast cells in any period (assuming that fermentable sugar concentration is not limiting) is directly proportional to the size of the population. Therefore as the volume of the packed bed increases, the volume of gas evolved within the packed bed is amplified. All gas evolved within the packed bed moves upward causing agitation on the bed components vertically above the originating point as a function of its superficial velocity (U_G). The agitation increases in the axial dimension of the packed bed logarithmically.

$$U_G = h^{r_G}$$

where:

U_G = Superficial gas velocity

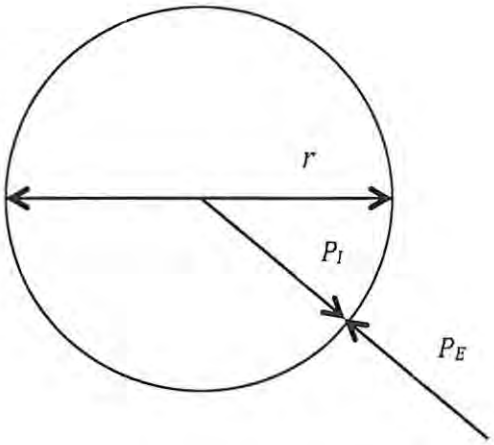
r_G = Gas production rate

h = Height

Thus, as the aspect ratio of the packed bed increases, at some critical height (h_{crit}), the upward superficial gas velocity will exceed minimum fluidisation velocity of the particles comprising the packed bed. The particles positioned at heights greater than this will be fluidised, and will be more homogenous.

A reduction in diffusion in the axial plane of the bioreactor resulting in increased heterogeneousness (plug flow/tube flow/stratification) can be achieved through a lower superficial liquid flow rate, conferred from either a decrease in aspect ratio or a reduced feed flow rate (Ratledge and Kristiansen, 2006; Towler and Sinnot, 2008). This means that a reduction in the height of the packed bed is achieved and incorporates a corresponding reduction in the total fraction of the population exposed to the inhibitory effects of high substrate or product concentrations (Shuler and Kargi, 1992). This suggests that a simple packed bed reactor is the most appropriate model for ethanolic fermentations and will achieve the highest volumetric performance. There are reasons, despite this, for designing the bioreactor with a higher aspect ratio, sacrificing some of the productivity of the volume above the h_{crit} using a hybrid (packed bed/fluidised bed) bioreactor, even when the aspect ratio of the column could be adjusted to retain all of the catalytic biomass within the packed bed.

Agitation caused by the gas bubbles will at some height (h_{crit}) disrupt the packed bed. This height, however, will be greater if the bubbles are of a smaller size.



$$P_I = P_E + \frac{4\gamma}{r}$$

$$P_I V = nRT$$

$$V = {}^4/_3 \pi r^2$$

$$P_E = \frac{\rho A h g}{g_c}$$

Figure 2.1 The size of a liquid bubble (Adapted from Yeh and Yang, 1968).

From the fundamental equations for the behaviour of gases, where:

P_E = External pressure
P_I = Internal pressure
γ = Surface tension of bubble
r = Radius of bubble
n = Number of moles gas (CO_2)
R = Gas constant
T = Temperature
V = Gas volume
ρ = Density of external fluid
h = Depth of bubble
A = Cross sectional area of fluid above bubble
g = Acceleration due to gravity
g_c = Specific gravity of fluid

we can obtain (Appendix D) the following relationship between the depth of the bubble and its radius:

$$h \propto \frac{1}{r^2}$$

Therefore, an increase in specific pressure within the packed bed will reduce the size of gas bubbles at the top of the packed bed height. It follows that there are two options for achieving a given volume for a packed bed:

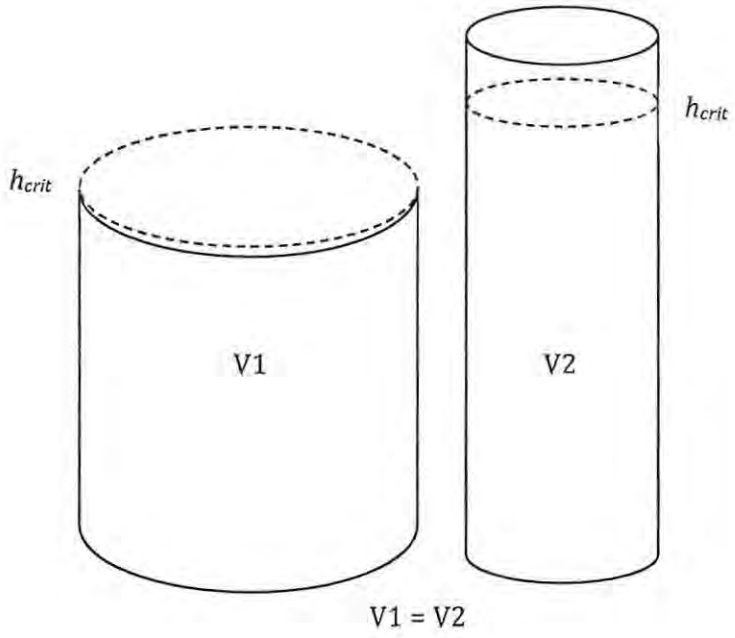


Figure 2.2 Two packed bed bioreactor models; (A) low aspect ratio packed bed, (B) high aspect ratio packed bed with greater specific pressure.

For the production of ethanolic beverages, output gravity variation is required, and so volumetric flow rates must be flexible (Stanbury and Whitaker, 1984). The additional total size of immobilisation matrix conferred by the fluidised bed region (above h_{crit} in figure 2.2 B) allows for flexibility in dilution rates and additional durability of the packed bed. This is because the limit on the total biomass able to remain immobilised in the reactor is greater, and so the feed rate can be adjusted to obtain the desired product gravity. The addition of an area with an expanded diameter at the top of the bioreactor would lower the superficial flow velocity in this area, allowing the settling of fluidised support matrix particles and microbial flocs.

When this model is compared to a packed bed with no fluidised zone allowance (figure 2.2 A), in which increasing the dilution rate to create higher gravity output would likely cause the washout of biomass and a loss of productivity, the advantages of model (B) in figure 2.2 can be seen.

Another reason to use the increased aspect ratio, allowing for a fluidised region, is that the bioreactor is more versatile in its operational parameters. This means that the substrate concentration and composition, feed flow rates and kinetic values can all vary to a certain extent without the need for refitting the bioreactor or a complete redesign, thus conferring some extent of adaptability that the CSTR possesses, without significantly impairing bioreactor performance.

The bioreactor to be designed for the purpose of beverage alcohol manufacture in this study will therefore be a packed/fluidised bed bioreactor to primarily be operated in a perfusion mode – a perfusion tower bioreactor (PTB).

Shuler and Kargi (1992) describe how a bioreactor that has very high fluid recirculation rates will approach CSTR behaviour. At the other extreme, when fluid recirculation rates are very low, or equal to zero, the bioreactor must be treated as a plug/tubular flow reactor. The packed bed region of a bioreactor acts like a tubular reactor. The feed enters at one end of the cylindrical tube and the product stream leaves at the other end. The length of the tube (h) and the minimisation of axial stirring caused by irregularities in flow and gas evolution through the design of the bioreactor act to prevent complete mixing of the fluid in the tube. The properties of the fluid will therefore vary from one point to another in the axial dimension significantly, but not in the radial dimension (Ballyk and Smith, 1999). In the plug flow model, which describes these properties, the flowing assumptions are made (for the ideal case):

1. No mixing in the axial dimension
2. Compete mixing in the radial dimension
3. A uniform velocity profile across the radius of the tube

(Atkinson and Mavituna, 1991)

The absence of longitudinal mixing is the defining characteristic of this type of bioreactor; the assumption is the opposite to that of complete mixing in the stirred tank reactor (Leib, 2001; Towler and Sinnot, 2008). The validity of the plug flow assumptions depends on the geometry of the reactor and the flow conditions (figure 2.3). Deviations will arise due to mixing in the axial dimension due to vortices and turbulence caused by gas and obstructions, and incomplete mixing in the radial dimension caused by laminar flow conditions. These deviations in real bioreactors are common (Mijnbeek *et al.* 1992b), but are not always important (Atkinson and Mavituna, 1991).

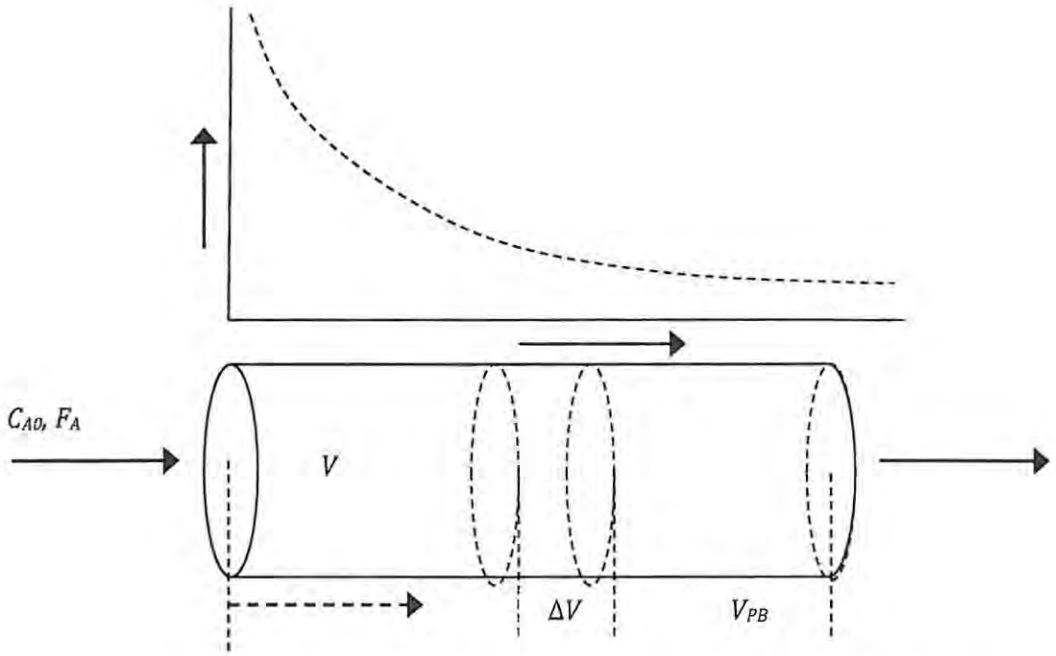


Figure 2.3 The plug flow reactor model characterised by a concentration gradient in the axial dimension of the reactor (Adapted from Mijnbeek *et al.* 1992b).

The general mass balance for a single reactant system can be described thus:

$$\textit{Feed flow of reactant} - \textit{Outflow of reactant} + \textit{Generation of reactant} = \textit{Accumulation of reactant}$$

At steady state the accumulation term of the above equation = 0, the mass balance can therefore be expressed as:

$$F_A . \Delta C_A - r_A . \Delta V = 0$$

Where:

F_A = Reactant A feed rate

C_A = Concentration of reactant A

C_{A0} = Initial concentration of reactant A

r_A = Rate of disappearance of reactant A

ΔV = Volume element

V_{PB} = Total packed bed volume

To find the relationship between the concentration and the position within the reactor we take the limit as $\Delta V \rightarrow 0$ and divide by ΔV.

$$\frac{dC_A}{dV} = \frac{r_A}{F_A}$$

Here the rate is variable but is a function of longitudinal position within the reactor rather than time. Using this equation the concentration of reactant A can be predicted at different heights within the bioreactor. Integrating:

$$\int_0^{V_{PB}} \frac{dV}{F_A} = \int_{C_{A0}}^{C_A} \frac{dC_A}{r_A}$$

At the entrance of the bioreactor $V = 0$, and $C_A = C_{A0}$. At the end of the packed bed $V = V_{PB}$ (total packed bed volume) and $C_A = C_A$ (packed bed conversion). Where F_A is constant:

$$-\frac{V_{PB}}{F_A} = \int_{C_{A0}}^{C_A} \frac{dC_A}{r_A}$$

Using this equation the concentration of reactant A can be predicted at the end of the packed bed region of the bioreactor (San, 1989; Ballyk and Smith, 1999).

2.5 Key Considerations in Bioreactor Design

Depending on the bioreactor model used and operational configuration, a given application of a bioreactor demands attention to be focused on several aspects. These considerations may include the need to maintain monoseptic operation, sufficient mixing to ensure suspension of the biocatalyst, the maintenance of a homogenous or heterogeneous environment within the bioreactor, the supply of nutrients including specific carbon and nitrogen sources, micronutrients, and the supply of oxygen or carbon dioxide in a way

that does not limit the performance of the biocatalyst (Mijnbeek *et al.* 1992). The removal of products, temperature control, heat transfer, and the control of shear stresses in the bioreactor environment such that the biocatalyst does not suffer damage as a result of hydrodynamic forces are all potential factors that require consideration in the design of the bioreactor. Often these considerations can be addressed through the physical design of the bioreactor, including fittings, outer jackets and other hardware which can be controlled by sophisticated software and feedback loops, coupled to sensitive measurement equipment (Ratledge and Kristiansen, 2006). The objectives of this study, however, implicitly state that as economic performance is as much a consideration as physical performance, the more extravagant equipment is to be avoided.

There are three overarching influences, or phenomena that underpin the overall physical performance of a bioreactor system, and therefore the decision-making when it comes to bioreactor design. These are: thermodynamics, micro kinetics and transport (Mijnbeek *et al.* 1992). Thermodynamics and micro kinetics are independent of scale, and are concerned with the microorganisms, their metabolism and their immediate environments: a typical thermodynamic property is the solubility of oxygen in a given solution, and a typical micro kinetic property is growth and product formation by the organism (Ratledge and Kristiansen, 2006). However the actual oxygen concentration and the actual kinetic behaviour of microorganisms in a bioreactor system are dependent on scale (Ratledge and Kristiansen, 2006). The reason for this is that nutrients involved in metabolic processes are consumed constantly and must be supplied by transport processes, which are scale dependent (Leib, *et al.* 2001). These three will be briefly discussed here for completeness, but will not be used as direct considerations in the design of the bioreactor as this would result in a difficult and laborious design by first principles.

2.5.1 Thermodynamics

The microbial growth within a system can be described with an overall chemical reaction in which one C-mol of biomass is formed (One C-mol of biomass is the amount of biomass that contains 12 grams of carbon; this typically amounts to 25 grams of dry matter, as the carbon content of biomass is around 45% by mass) (Ratledge and Kristiansen, 2006), which accounts for the roles of the water, the nitrogen source, electron donors and accepters, protons *etcetera*. In addition to the stoichiometric considerations, heat and Gibbs energy are involved. Energy must be generated for microbial growth to enable the construction of the complex biomass molecules from simple carbon compounds (Towler and Sinnot, 2008). This energy is generated in redox reactions between electron donors and electron accepters. The correct measurement of

the energy spent in a growth process is not the released heat but Gibbs energy (ΔG), as ΔG combines both the heat from enthalpic (ΔH) and entropic (ΔS) contributions (Ratledge and Kristiansen ,2006).

$$\Delta G = \Delta H - T\,\Delta S$$

The stoichiometric coefficients for a reaction are related to known yield coefficients:

Y_{dx} = the yield of biomass (C-mol) on the electron donor (C-mol for organic donors, mol for inorganic donors.)

Y_{ax} = the yield of biomass on the electron acceptor (mol).

Y_{hx} = the yield of biomass on heat (kJ).

Y_{gx} = the yield of biomass on Gibbs energy (kJ).

2.5.2 Microkinetics

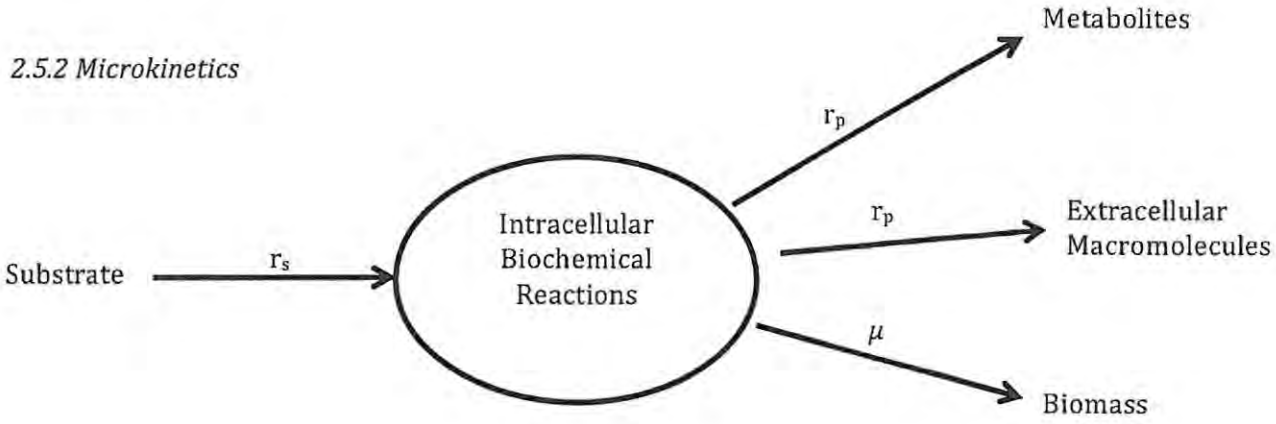


Figure 2.4 Representation of microkinentics of cellular growth and product formation.

A typical operation of a continuous bioreactor in kinetic terms is that of a chemostat, where the added medium is designed such that one single substrate is limiting. This allows for controlled variation in the specific growth rate of the biomass (Shuler and Kargi, 1992).

Through feed flow variation, the environmental conditions can be changed and thereby information concerning the influence of the environmental conditions on the cellular physiology of the catalytic biomass can be obtained (Wang and Hatzimanikatis, 2006). From the biomass mass balance equation below; in steady-state continuous reactor conditions, the specific growth rate equals the dilution rate.

$$\frac{dx}{dt} = (\mu - D)x$$

$$\therefore \mu = D$$

Where:

x = Biomass

μ = Specific growth rate

D = Dilution rate

Thus, by varying the dilution rate in a continuous culture, different specific growth rates can be obtained. At steady state the substrate mass balance gives:

$$0 = -r_s x + D\,(C_s^f - C_s)$$

Where:

r_s = Specific substrate uptake rate

C_s^f = Feed substrate concentration

C_s = Substrate concentration (Outlet substrate concentration)

which in combination with the above equation , and the definition of yield coefficient gives:

$$x = Y_{SX}\,(C_s^f - C_s)$$

Where:

Y_{SX} = Substrate yield coefficient

The yield coefficient can be determined from measurement of the biomass and the substrate concentrations in the bioreactor. If the Monod model (Atkinson and Mavituna, 1991) can be applied to the system for the mass balance, this gives:

$$D = \mu_{max}\,\frac{C_S}{C_s + K_S}$$

μ_{max} = Maximum specific growth rate

K_S = Affinity constant (Microbe/enzyme for substrate)

Therefore the concentration of the limiting substrate increases with the dilution rate. When substrate concentration in the bioreactor (in homogenous bioreactors) equals the substrate concentration in the feed flow, the dilution rate attains its maximum value, which is called the *critical dilution rate* (D_{CRIT}). When the dilution rate becomes equal to, or larger than this value, biomass is washed from the bioreactor (Mijnbeek *et al.* 1992). As the biomass concentration decreases at low specific growth rates (low dilution rates), the substrate consumption utilised for biomass maintenance is greater than that used for growth. At high dilution rates maintenance is negligible and the yield coefficient becomes equal to the true yield coefficient. Since $\mu = D$ at steady state, there is a linear relationship between the specific substrate uptake and the dilution rate, and the true yield coefficient and the maintenance coefficient can be estimated using a linear regression (Waites *et al.* 2001).

For the production of biomass such as yeast, and growth related products such as ethanol, the chemostat is very well suited since it is possible to maintain high productivity over long periods of operation.

2.5.3 Transport

To achieve the optimal rate of reaction, the transfer of substrates to the site of reaction, and the removal or products away from it must occur as rapidly as possible. These transfers should also not be rate limiting (Birol *et al.* 1998). Usually the transfer of one product/substrate involves a chain of transfer steps, for example: diffusion of sugar by diffusion or turbulence to the boundary layer surrounding a cell, followed by diffusive transport across the boundary layer to the cell surface, followed by active transport through the cell membrane to the cytoplasm where catabolism can take place. The opposite path (or close to the opposite path) is likely to be taken by the products; in this case ethanol (Ratledge and Kristiansen, 2006). The slowest step in one of these transfers determines the overall rate of the transfer; it is the rate-limiting step (Shuler and Kargi, 1992). The rate value of the rate-limiting step of a transfer can be compared to the slowest microbial kinetic reactions rate value, if it is lower, then this transfer will limit the overall process performance. If this is the case, the usual effect is a reduction in the desired product from the substrate (Leib, 2001). This can have two manifestations: firstly, the overall reaction *rate* will be below the theoretical maximum, and the process output will be slower than desired. The second effect can be a

change in the selectivity of the reaction; i.e. the organism may create a different product than the one desired.

It is therefore important to understand the rate limiting steps in the mass transfer chains for a bioprocess and ensure that mass transfer is not a limiting factor. Generally speaking, in ethanolic fermentations the transport of catabolites to the active sites of catabolic enzymes is not rate limiting (Birol, 1998).

2.5 Attached Growth

Attached growth is the term given to reactor systems in which the biocatalyst is attached to, or adsorbed onto, a solid or some suspended substratum. Attachment is normally the primary means by which the biocatalyst is retained within the bioreactor. Although most microorganisms have the potential to adsorb onto a substratum; the technological application of immobilised cells is a relatively recent large-scale trend in biotechnology (Baptista *et al.* 2007). Attached growth is not a man-made phenomenon; biofilms are common in natural waters such as rivers and streams, especially close to the source, where nutrient content in the liquid phase is lower due to low levels of suspended particulate matter and dissolved nutrients. Even in the case where there is ample food supply for microorganisms in later stages of natural waters, it is common to find microbial cells attached to suspended particles rather than as free cells because in some cases immobilisation can provide genetic stability, favourable microenvironments and protection against shear damage (Shuler and Kargi, 1992).

Continuous suspended growth bioreactors such as the bubble column and the stirred tank reactor typically operate at a dilution rate below D_{CRIT}. Under these conditions the cells can complete at least one cell division before being washed out, and consequently there is no advantage to the cells being attached (Mijnbeek *et al.* 1992a). However at dilution rates above D_{CRIT} for a given reactor, only attached cells will remain within the reactor, effectively raising the value of D_{CRIT}. The advantage of this becomes clear when comparing the cell mass productivity (as in section 2.5.2) between attached and suspended growth systems: the rate of substrate consumption relative to the rate of cell mass production in a suspended growth bioreactor is determined by the availability of substrate and by the duration of time the cells spend within the bioreactor (Birol, 1998). Figure 2.5 shows a schematic plot of cell mass productivity rate (Q_μ) against dilution rate.

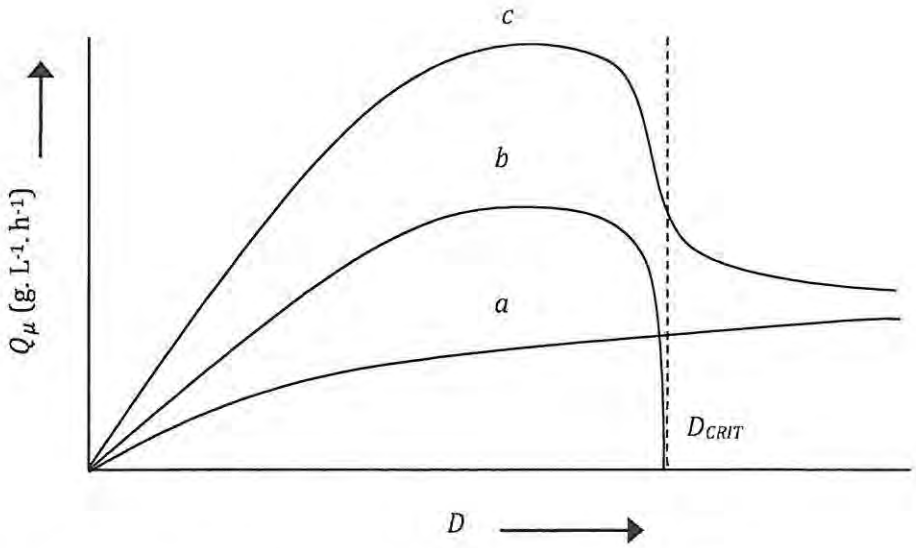


Figure 2.5 Cell mass productivity against dilution rate for attached growth (*a*), suspended growth (*b*) and a combination of the two methods (*c*) (Adapted from Mijnbeek *et al.* 1992a).

Where:

Q_μ = Cell Mass Productivity Rate

D_{CRIT} = Critical Dilution Rate

The plot for suspended growth (figure 2.5, *b*) shows a gradual rise in cell mass production rate Q_μ until a maximum is reached, followed by a drop to zero at the critical dilution rate. With attached growth systems (Figure 2.5, *a*) this increase in Q_μ with increasing dilution rate also occurs, but remains stable beyond D_{CRIT}. Adding the two curves together gives the cell growth to dilution rate model expected with the hybrid packed/fluidised bed bioreactor configuration described in section 2.4 (Figure 2.5, *c*).

The effect of reduced biomass growth due to low washout rates has been shown to improve ethanol productivity (Kargupta *et al.* 1998), even though rates of ethanolic fermentation in yeast cells that are not growing are lower than those of the growing cells because the accumulation of ATP inhibits the activity of PFK during glycolysis (Ingledew, 1999). If a limited amount of growth is enabled, the productivity rates are enhanced. This supports the decision to make use of a fluidised bed region where cell productivity rates will be higher than those of the packed bed.

Other kinds of cell immobilisation are discussed in chapter 4, however the process of spontaneous cell adsorption is the method that the cells used in the bioreactor designed are predicted to attach to the solid carrier, forming the packed bed and fluidised regions within the PTB. Spontaneous cell adsorption and the subsequent biofilm formation is the result of a combination of microbial, chemical and physical processes occurring in the liquid phase and at the substratum. The first phase involves the formation of a conditioning film over the surface of the substratum. Adsorption of organic macromolecules and other small particles in suspension occurs naturally due to hydrodynamic and chemical processes. The initial attachment creates a concentration gradient towards the substratum, contributing to the growth of the conditioning film (Mijnbeek *et al.* 1992a; Shuler and Kargi, 1992; Smukalla, 2008).

Microorganisms in the liquid phase are then moved spontaneously through a variety of mechanisms including Brownian motion, gravity, fluid dynamic forces (inertia, lift, drag, drainage and downsweep), and in some cases, taxis (Mijnbeek *et al.* 1992a). The microorganisms then adsorb onto the conditioning film either reversibly or irreversibly through weak chemical bonds (hydrogen, Van Der Waals) or though the binding of extracellular fibres or transmembrane proteins. Once the cells are adsorbed to the substratum surface the expenditure of energy and uptake of nutrients from the surrounding fluid sustains the osmotic gradient towards the biofilm, resulting in the diffusion of nutrients towards and into the biofilm. Once the biofilm is established, more cells will continue to attach until a critical biofilm depth is reached determined by the prevailing local conditions within the bioreactor such as shear effects and hydrodynamic forces (Shuler and Kargi, 1992, Atkinson and Mavituna, 1991). Cells and parts of the biofilm may detach spontaneously (with reversible adsorption) or under stress (irreversible), the detachment of single cells is termed erosion, the separation of parts of the biofilm is termed sloughing (Mijnbeek *et al.* 1992a).

The older and more established the biofilm is, the more likely it is that these processes will occur simultaneously (Mijnbeek *et al.* 1992a).

2.7 Design of a Continuous Fermenter

Continuous fermentation is useful where substrate can be stored, such as barley for beer (Royston, 1966) and honey for mead (Snowden and Cliver, 1996). Fruit, however, such as grapes and apples have comparatively short shelf lives, typically measured in weeks. The production of wine or cider requires the juicing of the fruit, and the resulting must has to be fermented almost immediately due to high microbial loads associated with the fruit and juicing equipment. Consequently wines and ciders have historically been

produced in batch systems in which large volumes of time-sensitive must can be processed simultaneously. Only recently have efforts at the continuous fermentation of these products arisen (Nedovic *et al.* 2000; Durieux *et al.* 2000; Kourkoutas *et al*, 2002). Modern techniques associated with the development of the market for mass produced fruit juices have opened up new possibilities regarding the continuous fermentation of traditionally non-storable substrates. It is now possible, for example, to acquire fruit juice concentrates, which have been enzymatically treated, and are able to be stored for several years with no loss of quality for a lower or similar cost to that of fresh pressed juice. This makes the continuous production of wines and ciders possible and potentially economically viable.

As discussed in chapter 1, in the United Kingdom during the 20th century continuous fermentation technologies for beer production enjoyed a brief period in which they were actively researched and in some case even made it into the commercial production of some beers (Royston, 1966, Coutts, 1966; Dunbar *et al.* 1988). These systems were abandoned over time however, in favour of batch fermentations in stirred tank or simple cylindro-conical reactors due to their relative low cost.

Once the basic bioreactor configuration has been selected (section 2.4), the details of the bioprocess need to be considered, this is typically a complex engineering exercise, however the lack of any need for stirring means that many considerations such as *dimensionless numbers* are eliminated (Shuler and Kargi, 1992), as are other shear effect related considerations and heat transfer problems due to the high aspect ratio of the reactor; controlling the room temperature is a domestic method of controlling the temperature of the fermentation. Reducing contamination risk is the primary concern remaining to the considered in the design of the PTB.

To this end the bioreactor should have as few openings as possible in order to minimise contamination risk, and to simplify any clean-in-place (CIP) procedures that are required before inoculation. As ethanol has antimicrobial properties, the contamination of a primary fermentation, in which $D > D_{CRIT}$, with a high gravity substrate is unlikely (Nagodowithana, 1976). Especially so if the exterior environment is clean, and the feed flow is sterilised. An in-line pasteurisation of the substrate feed between the substrate holding tank and the reactor should ensure that the risk of contamination is negligible.

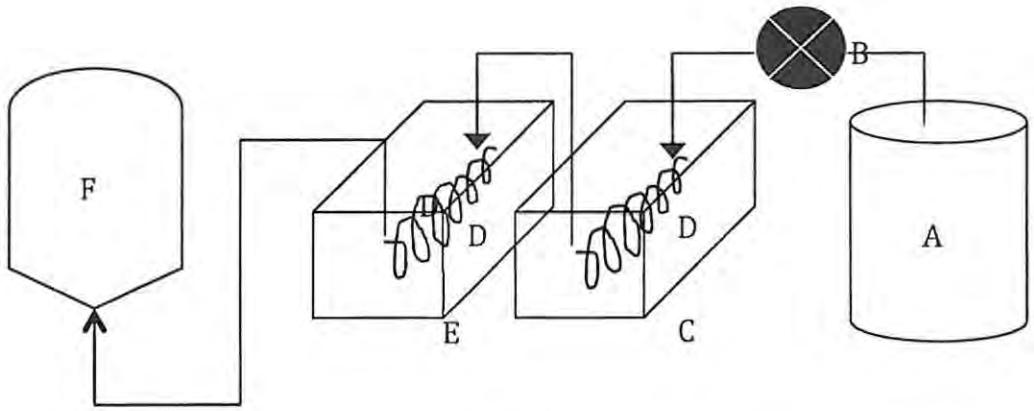


Figure 2.6 Schematic representation of the feed flow pasteurisation apparatus; (A) substrate holding vessel, (B) peristaltic pump, (C) heat bath at 70°C, (D) stainless steel coiled tube, (E) room temperature water bath, (F) bioreactor.

A headspace volume will be required for evolved gas to escape to. If this headspace is above the fluid outlet, flow from the reactor can be gravity fed and a positive pressure will be maintained within the reactor system reducing the risk of contamination from the outlet port, and preventing blockages.

The selection pressures within the reactor will favour efficient conversion of sugars to ethanol, and tolerance of high sugar concentrations near the inlet and high ethanol concentrations near the outlet. An additional effect of the plug flow like model, in combination with the immobilised biofilm and flocs within the packed bed is that local conditions are almost constant, thus creating a range of selection pressures, each 'disc' of a certain height within the packed bed contains a population of microorganisms under a slightly different set of selection pressures to those in the adjacent 'discs'. The long-term effect this diversification might have includes an incremental improvement in reactor performance over time. Additionally, as cells are selected for their ability to metabolise available sugars efficiently; the likelihood of a mutant colony, which creates 'off flavours', is low, and so any genetic drift will tend toward a 'cleaner' product, with fewer metabolites.

The ideal continuous process should have the following characteristics:

1) It should accurately reproduce the same conditions that occur in traditionally used systems so that the same minor by-products are produced as these are important for flavour.
2) The process should be more rapid than the batch process so that even if more sophisticated plant requirements are created, a net improvement in economy of operation is achieved.
3) The process should be able to run for at least 6 months free from mechanical problems or infection.

The PTB model discussed in this chapter should have some of the above characteristics.

2.8 Conclusions: The Perfusion Tower Bioreactor

A continuous hybrid packed/fluidised bed bioreactor was conceptualised, with a variable feed flow rate and simple attached growth model. The plug flow-like kinetics in the packed bed region minimise product and substrate inhibition and the fluidised bed region minimises biomass loss. High dilution rates are enabled through the immobilisation of catalytic biomass, thus allowing for high productivity rates. The lack of heat transfer problems makes the system energy efficient, requiring energy input only for a pump and a pasteurisation unit for the substrate feed. Due to the up-flow configuration of the bioreactor, the packed bed is permeated from the bottom, and the shape of the reactor resembles the tower shape of a fluidised bed reactor. Modelling of the bioreactor for performance and scale-up considerations will be simplified by plug-flow like behaviour in the packed bed region of the bioreactor.

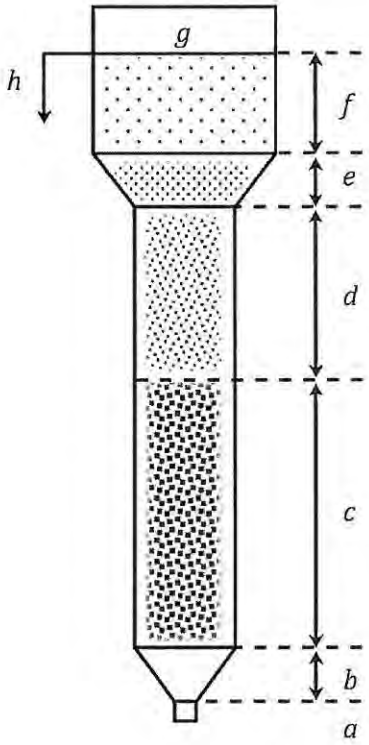


Figure 2.7 Schematic diagram of the PTB; substrate feed inlet (*a*), inlet flare (*b*), packed bed region (*c*), fluidised bed region (*d*), expander flare (*e*), expander region (*f*), headspace (*g*), outlet (*h*).

The bioreactor configuration chosen, and its additional features combine to create system appropriate for ethanolic fermentation. The reactor has low capital equipment costs; low energy and personnel requirements due to its simplicity of structure and operation, and the potential for high productivities exist due to the stratified flow and reduction of inhibitory effects. The bioreactor should also be able to operate over long periods without contamination. The success of this design will be tested in the following chapters.

Chapter 3

Fermentation Metabolite HPLC Method Development and Validation

3.1 Introduction

A method for quantifying compounds in solution must first be validated in order to have confidence in the results. In the validation of the method, the accuracy of the method must be shown, as well as it's ability to be reproduced successfully, the goal being that, hypothetically, multiple analysts in multiple laboratories could run the same samples and yield statistically equivalent results (Sadek, 2000). The fermentation metabolites of interest included sugars and alcohols, it was necessary therefore to demonstrate that the method for resolving and detecting them allowed for complete resolution and detection within the expected range of concentrations.

The method validation process requires a series of criterion to be fulfilled, these criterion are used to determine the acceptability of the method for its intended use. The criteria consist of the following parameters: reproducibility, accuracy, precision, linearity, robustness, specificity, limit of detection (LOD), limit of quantification (LOQ). Each of these parameters must be determined and scrutinised for the method to be shown to be acceptable (Edwardson *et al.*, 1990).

The purpose of this chapter is to discuss the parameters above and apply them to an analytical method, which could be used to simultaneously quantify five fermentation metabolites in the product of the PTB, and demonstrate that the proposed method was suitable for its intended purpose. This chapter demonstrated that the development and validation of an HPLC method to successfully and simultaneously separate and quantify five common fermentation metabolites.

3.2 Validation Parameters

Before proceeding with the validation process, authentic reference standards with known purity, of HPLC grade, were obtained. Standards of known concentration were prepared and the decomposition over time under specific storage conditions was observed.

3.2.1 Reproducibility

The reproducibility of a method is tested by multiple sample injections of the pure standard under the same operational parameters and observing the fluctuations in retention time, peak height or peak area. According to the International Convention on Harmonization (ICH) the number of injections should be a minimum of five injections over a specific range (Sadek, 2000; Edwardson *et al.* 1990).

3.2.2 Accuracy

Accuracy is the closeness of agreement between the true or reference value that is found by the test method. This is an absolute measurement and is dependent upon precision and specificity. To report accuracy, data must be collected from a minimum of five determinations over at least three concentration levels over a specified range (Sadek, 2000).

3.2.3 Precision

Precision is an indication of reproducibility. Under the same analytical conditions precision is a measure of closeness of agreement of the data values from multiple sampling or sample injections onto an HPLC system. This must be from the same homogenous sample and at a minimum of three different concentrations. Precision is calculated as the relative standard deviation (RSD) of the peak areas or peak heights (Edwardson *et al.*, 1990; Sadek, 2000).

3.2.4 Range

The range of a method is the concentration intervals between the upper and lower levels of the analyte that have been demonstrated to be analysed with accuracy, precision and linearity (Edwardson *et al.*, 1990).

3.2.5 Linearity

The linearity of a method is demonstrated when the response is directly proportional to a set of response-concentration points over the data set working range (Sadek, 2000). The range at which the experiments are performed should be between 25% of the minimum detection concentration of the analyte and 200% of the maximum of the nominal concentration of analyte (Edwardson *et al.*, 1990). The proportional relationship between the concentration of the analyte and the response ensures confidence in the method and confirms that a single reference standard can be used to perform calculations, rather than using the equation of the calibration line. Linearity is the variance of the slope of the regression line, an r^2 value of 0.995 is the minimal acceptable correlation coefficient for critical analytical work (Sadek, 2000). The ICH guidelines state that a minimum of five concentration levels should be used.

3.2.6 Specificity

Specificity is the ability of the chromatographic equipment to detect and resolve the peaks of interest; it is a measure of a method's sensitivity to potential sample-related hindrance. It is the ability of the method's ability to detect the analyte of interest in the presence of other compounds in the same sample. In this study the method developed to detect the five compounds should demonstrate its specificity for each compound. It is critical that the peak of interest is due to a single component response and not due to co-elution of two or more compounds (Sadek, 2000).

3.2.7 Robustness

The ideal method shows little or no variation between results acquired with different equipment or other small changes in the method. Robustness is the term used to describe methods that generate acceptable results when parameters are deliberately changed, these parameters could include temperature or pH of mobile phase for example, robustness is usually addressed at the beginning of the validation process as critical parameters for resolution could then be noted (Sadek, 2000).

3.2.8 Limit of Detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that will be reported as detected above the baseline noise by the analyst. The ratio is set by the analyst and is usually in

the range of 2:1 to 5:1, written S/N = 5.0. This measurement is dependent on background signal that could be due to electronic noise or an endogenous sample. The noise itself is defined as the largest peak to trough deflection over a given mobile phase run time (Sadek, 2000).

3.2.9 Limit of Quantification (LOQ)

The limit of detection (LOQ) is defined as the lowest concentration of an analyte in a sample that will be reported as detected and quantifiable above the baseline noise by the analyst with acceptable reproducibility, precision and accuracy. The ratio is thus typically higher than that of the LOD and typically is set between 5 and 10 (Sadek, 2000).

3.2.10 Sensitivity

The sensitivity of a method is defined as the gradient of the standard curve for that particular analyte. Since a given detector has a fixed operating response range, the extent of the working range is inversely proportional to the sensitivity of the technique. If the sensitivity is too great, the concentration range over which the analysis is useful is very narrow. If the sensitivity is too low then there is little discrimination between analyte concentration levels. Samples must therefore be carefully diluted in order to fit into the useful concentration range (Sadek, 2000).

3.3 HPLC Method Development Procedure

Literature revealed ion-exchange high-performance liquid chromatography to be the most commonly used method for the analysis of sugar and alcohol compounds in fermentation products (Bovanova and Brandsteterova, 2000). Refractive index detection with a mobile phase at acidic pH is most applicable. The mobile phases frequently used were mostly composed of water with sulphuric acid or water with heptafluorobutiric acid (Bonn, 1985; Molnar-Perl, 1999). The analytical columns that were typically used to quantify sugars were mainly reversed phase ion-exchange columns with propriety packing material such as the Shodex SH-1011 column and the Bio-Rad AMINEX HPX-87C column and the Transgenomic IC Sep ICE-Coregel 87H3 column (Bonn, 1985). These columns were used variously to detect only sugars and organic acids, with volitiles being analysed using GC (Tang *et al.* 2010; Vidrith and Hiribar. 1999; Nedovic *et al.* 2000). A number of studies, however, utilised RP-organic acid columns to detect and quantify both sugars and volitiles (Zhao and Xia, 2010; Hanko and Rohrer, 2000)

In this chapter the development of a reproducible, reliable method with a reasonable retention time for the simultaneous detection of all five fermentation metabolites was developed and described.

3.4 Instrumentation and Methods

3.4.1 Analytical Column Selection

Literature revealed that the type of analytical columns used for the separation of compounds in fermentation mixtures usually consisted of organic acid ion-exchange and size exclusion column packing material with 5 to 10 µm particle size (Zhao and Xia, 2010; Hanko and Rohrer, 2000; Tang *et al.* 2009; Vidrith and Hiribar, 1999; Nedovic *et al.* 2000). This description fits a variety of different sugar and organic acid analysis columns such as Bio-Rad® AMINEX® columns, Restek® Pinnalce II® Amino columns and Waters® SugarPak I® columns. Previous work with CFR product analysis had been conducted using a Phenomenex® REZEX® analytical column (Cambray, 2005), and it was decided to use a column known to resolve the compounds of interest when working with CFR systems.

The column selected for this specific study was a Phenomenex® REZEX® ROA Organic Acid H^+ column with 8% cross-linked sulphonated styrene-divinylbenzene (SDVB) packing with a 8 µm particle size. The column dimensions were 7.8 x 150 mm. This was obtained from Phenomenex, Terrance, California, USA.

The guard column used was a Phenomenex® SecurityGuard™ analytical guard cartridge system Carbo-H^+ (4.0 x 3.0 mm), which was also obtained from Phenomenex®.

3.4.2 Mobile Phase Selection

The optimum mobile phase selected for the simultaneous detection of the five fermentation analytes, in the present study, consisted of water in an isocratic elution. Purified Milli-Q HPLC grade water was purified through a Milli-Q system which utilised reverse osmosis process (Millipore Corporation, Bedford, USA).

The initial mobile phase that was selected consisted of 0.025 M H_2SO_4, however no significant change in resolution, run time or peak sharpness was observed when the acid was omitted.

3.4.3 Hardware

The HPLC system consisted of the following Shimadzu Corporation (Kyoto, Japan) components: a Prominence SIL-20A Autosampler, a Prominence DGU-20A_5 Degasser, a Prominence LC-20AT Liquid Chromatograph pump, the detector was an RID-10A Refractive Index detector which was chosen because all of the analytes are optically active, and finally a CTO-10AS $_{VP}$ column oven. The software used to analyse the chromatograms was the Shimadzu LC Solution™ software version 1.25.

3.4.4 Flow Rate

A standard flow rate of 0.6 ml.min^{-1} was employed to preserve the column, as it is highly pressure sensitive. The column back pressure at this flow rate was maintained at 750 psi. The longest retention time for the compounds detected in the fermentation substrate and product mixtures was ethanol at 20 min.

3.4.5 Temperature Selection

The column was maintained at ambient temperature in a controlled air-conditioned room and within a column oven at 22°C.

3.4.6 Sample Dilution and Injection Volume

Samples were diluted 200 times (maximum expected sugar concentration in unknowns was 140 mg.mL^{-1} and a maximum of 1 mg.mL^{-1} sugar concentration should be injected onto column in order to preserve analytical hardware). The volume of diluted sample injected onto the column was 20 µL.

3.5 Standard Preparation

The standards used were made up form the following stock chemicals; acetic acid (Merck, BB100017P), D-(+)-glucose (Merck, SAAR2676020EM), ethanol absolute (Merck, SAAR2233540LP), glycerol (Merck, SAAR2676520LC), sucrose (Merck, SAAR5881500EM), and D-(-)- fructose (Sigma-Aldrich, 1000591212).

Individual stock solutions of 1 mg.mL^{-1} of the above six compounds were prepared by weighing approximately 10 mg in "A" grade 100 mL volumetric flasks and dissolved in 100 mL filtered Milli-Q water.

From these stocks appropriate dilutions were made for the range of concentrations required; for the three sugars a range of 0.1 $\mu g.mL^{-1}$ to 1000 $\mu g.mL^{-1}$ were made, representing a pre-dilution range from 0.02 $g.L^{-1}$ to 200 $g.L^{-1}$. For ethanol a range of 5 $\mu L.mL^{-1}$ to 60 $\mu L.mL^{-1}$ was made up representing a pre-dilution range of 1% ABV to 12% ABV. For glycerol a range of 0.05 $\mu L.mL^{-1}$ to 10 $\mu L.mL^{-1}$ representing a sample concentration range of 0.01% V/V to 2% V/V. Working solutions of 250 $\mu g.mL^{-1}$ of the three sugars, 30 $\mu L.mL^{-1}$ ethanol and 1 $\mu L.mL^{-1}$ glycerol were prepared in HPLC grade water by performing serial dilutions of the stock solutions, this mixture was prepared again for each batch of samples and was used as the external standard for sample analysis. Samples were injected onto the column to determine the retention time of each compound. After the retention time of each compound was determined, a mixture was prepared and used as the standard for all six compounds. The acetic acid retention time (not shown) was determined in order to identify the peak qualitatively should it appear on a chromatogram; acetic acid is an indication of contamination in fermentation.

3.6 Results

The chromatogram below (Figure 3.1) shows the simultaneous detection and separation of the five fermentation metabolites, using the optimum parameters developed and validated for this research.

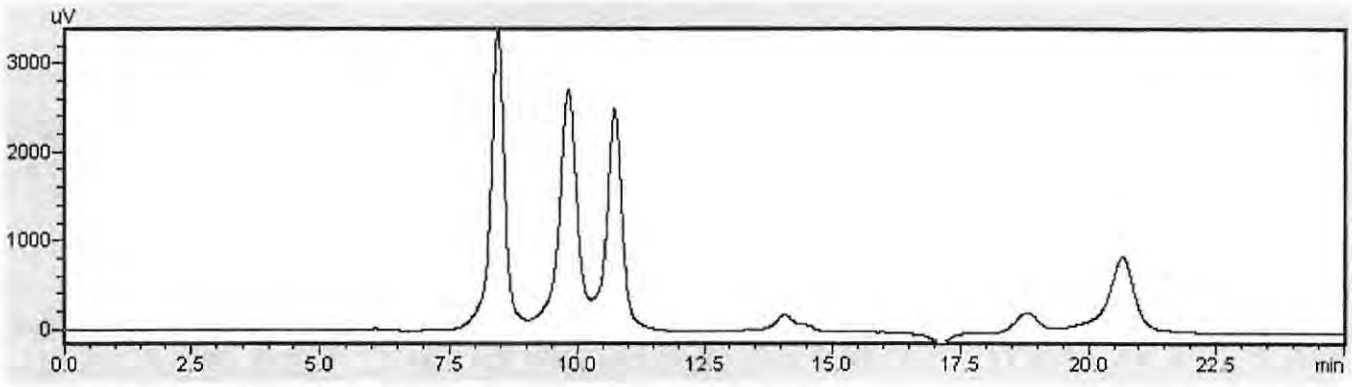


Figure 3.1 HPLC.RI chromatogram showing separation of five fermentation metabolites, retention times: sucrose (8.40 min), glucose (9.75 min), fructose (10.75 min), glycerol (14.20 min), and ethanol (20.75 min).

3.6.1 Linearity

For this study the linearity of the fermentation analytes were demonstrated by injecting standards in the concentration range of 5 $\mu g.mL^{-1}$ - 1000 $\mu g.L^{-1}$ for the three sugars, 10 $\mu L.mL^{-1}$ - 60 $\mu L.mL^{-1}$ for ethanol and 1 $\mu L.mL^{-1}$ - 10 $\mu L.mL^{-1}$ for glycerol. Plotting the peak area versus standard concentration provided the calibration plots. Straight lines were always obtained as demonstrated by the regression parameters (Table 3.2).

As can be seen in Table 3.2 below, all 5 of the compounds of interest showed good linearity with squared regression coefficients (r^2) ranging from 0.9997 for fructose to 0.9888 for glycerol.

Table 3.1 Linear range, regression and correlation coefficient of analytes.

Compound	Range	Gradient	Correlation Coefficient (r^2)
Sucrose	5 $\mu g.mL^{-1}$ - 1000 $\mu g.L^{-1}$	232.76	0.9995
Glucose	5 $\mu g.mL^{-1}$ - 1000 $\mu g.L^{-1}$	281.79	0.99696
Fructose	5 $\mu g.mL^{-1}$ - 1000 $\mu g.L^{-1}$	234.84	0.99975
Glycerol	0.1 $\mu L.mL^{-1}$ - 10 $\mu L.mL^{-1}$	2228.7	0.98882
Ethanol	1 $\mu L.mL^{-1}$ - 60 $\mu L.mL^{-1}$	636.08	0.99747

The correlation coefficients for these linear ranges are sufficiently close to 1 indicating that it can be stated that detector response is directly proportional to the concentration of the tested analytes. Additionally the sensitivity (gradient of standard curves) of the system to the analytes is great enough for the range in which the method is required to operate and not so steep that there is no discrimination between analyte concentrations (Sadek, 2000).

3.6.2 Limits of Detection (LOD) and Limits of Quantification (LOQ)

The limits of detection (LOD) and limits of quantification (LOQ) were obtained during the method validation process. The values were acceptable for the range of concentrations over which the method was developed to quantify the respective analytes. Table 3.3 below details the values obtained. The largest peak to trough deflection over a 60 min mobile phase run time was 0.0005 μRIU (Refractive Index Units). The

LOD, defined as the concentration where S/N = 2.0 (or 0.001μRIU). The LOQ, defined as the concentration where S/N = 5.0.

Table 3.2 LOD and LOQ of fermentation analytes.

Compound	LOD	LOQ
Sucrose	$1x10^{-4}$ mg/mL	$2.5x10^{-4}$ mg/mL
Glucose	$1x10^{-4}$ mg/mL	$2.5x10^{-4}$ mg/mL
Fructose	$5.4x10^{-5}$ mg/mL	$1.35x10^{-4}$ mg/mL
Glycerol	$6.6x10^{-4}$ mL/mL	$1.6x10^{-3}$ mL/mL
Ethanol	$9.5x10^{-3}$ mL/mL	$2.4x10^{-2}$ mL/mL

3.6.3 Reproducibility, Accuracy and Precision

The reproducibility of the method was demonstrated using three injections over multiple concentrations. The results represented as %RSD of peak area in Table 3.4 below demonstrated adequate reproducibility, accuracy and precision. All compounds at all levels showed %RSD below 4% (Sadek, 2000).

Table 3.3 % RSD accuracy and precision studies, (mean n=5).

Compound	%RSD
Sucrose	0.64
Glucose	0.44
Fructose	1.20
Glycerol	3.33
Ethanol	0.44

3.6.4 Specificity

This validation of the HPLC method demonstrated the ability to detect and quantify the analytes of interest. The ability to fully resolve each analyte from a mixture of fermentation metabolites and the demonstration of linearity of detector response showed this. Each individual compound showed a linear relationship between peak area and concentration. Furthermore, no interfering peaks were observed when HPLC grade water, which was used for preparation of samples was injected onto the HPLC system.

3.6.5 Robustness

The robustness of the method was tested by varying the operating temperature of the column and the pH of the mobile phase. A change of pH from pH 7 to pH 3 (0.05 M H_2SO_4) showed no significant change for the retention times of the analytes, it was assumed this is because the pH of the solution will have little effect on the polarity of the analytes of interest as they are not able to be protonated. A change of temperature 3°C above and below the optimal temperature also showed no noticeable change in retention times or resolution of compounds in a mixture. Additional variables that may be changed in order to test robustness may include the flow rate or pressure, however these variables were not tampered with due to the pressure sensitive nature of the column. The ruggedness of the method has not yet been tested.

3.7 Conclusions

A reproducible, accurate, precise and sensitive method was developed and validated for the detection and quantification of five key fermentation reactants. This method could thus be used with confidence for its intended purpose.

In the next chapter (Chapter 4), this HPLC method was employed for the analysis of fermentation substrates and products from continuous perfusion tower bioreactors.

Chapter 4

Bench-Scale Continuous Fermentation of Apple Must

4.1. Introduction

Continuous fermentations are those in which added medium displaces an equal volume of culture/broth from the fermentation vessel at a suitable rate, creating a continuous culture of cells (Shuler and Kargi, 1992). This culture will eventually reach a steady state; the formation of new biomass is balanced by the loss of cells from the vessel. The production of cider is a two-stage process, the first of which is the primary or ethanolic fermentation (Nedovic *et al.* 2000). The primary fermentation is typically the high-risk part of the production process, where the high value substrate is susceptible to contamination by spoilage bacteria such as *Acetobacter* (Bauduin *et al.* 2006); and for this reason it is essential that the production of bactericidal ethanol in the reactor system occurs as rapidly as possible. In addition to the mitigated spoilage risks, it can be argued that the economic benefits of rapid fermentations due to a reduced time-to-market are axiomatic. The secondary, or malolactic fermentation traditionally occurs spontaneously in storage due to wild yeast and lactic acid bacteria originating from fruit skins, which become associated with production equipment (Nedovic *et al.* 2000).

The support-based immobilisation of cells in continuous fermentation systems has been extensively studied for use in potable alcohol production (Kourkoutas *et al.* 2004). The localised retention of catalytic biomass affords a significant advantage in the process productivity per unit bioreactor volume over traditional fermentation systems (Verbelen *et al.* 2006). In general, immobilisation strategies tested to date have had limited commercial application either due to the limited periods over which the bioreactors can retain their fermentative capacity and the economic expense of renewing immobilised biomass, or due to the absence of cost effective immobilisation matrices that are capable of operating over extended periods of time without net biomass loss resulting in a loss of productivity (Kourkoutas *et al.* 2004).

Four broad immobilisation strategies have been used in continuous fermentation systems in the past; the entrapment of cells within a porous matrix such as alginate or chitosan (Nedovic *et al.* 2000; Ghorbani *et al.* 2011), the attachment or adsorption of cells to a solid carrier surface (Verbelen *et al.* 2006; Kourkoutas *et al.* 2002; Scott and O'Reilly 1996), the containment of cells behind a barrier or membrane (Kargupta *et al.*

1998; O'Brien *et al.* 2000) and by natural or artificially induced flocculation (Kourkoutas et al. 2004; Bauer *et al.* 2010).

In terms of productivity very small diameter gel entrapment beads have been able to demonstrate very high performance (Nedovic *et al.* 2000; Kourkoutas *et al.* 2004; Verbelen *et al.* 2006). The cost to benefit ratio of gel entrapment remains unattractive for low value-added products such as mass produced alcoholic beverages due to the prohibitive cost of high performance membranes used to overcome some of the problems associated with cell entrapment such as membrane fouling, mass transfer limitations and non-regeneration associated with membrane entrapment (Kourkoutas *et al.* 2004). Even with continued research aimed at overcoming these problems, the true economic costs of operating such reactors may remain insurmountable given the inherent physical and sometimes chemical instability of the entrapment matrix (Verbelen *et al.* 2006).

When lignocellulosic materials act as immobilisation supports, most yeast cells that become immobilised do so by adsorption to the support surface (Yu *et al.* 2007). Yeast cells tend to bind to cellulosic materials through electrostatic forces or by covalent binding between the cell membrane and the carrier (Santos *et al.* 2008). Pacheco *et al.* (2010) immobilised yeast cells to cashew apple bagasse; a cellulosic material. During adsorption, as there are no barriers between the cells and the solution, cell detachment and relocation is possible and there is most likely equilibrium between adsorbed and freely suspended cells. This balance would be influenced by the growth and detachment rates of immobilised cells, and the washout rates of free and flocculated cells.

Yeast flocculation is a reversible, asexual and calcium-dependent process in which yeast cells aggregate into clumps and sediment rapidly in the medium, or in the case of so called 'top fermenting yeast', float to the top (Verstrepen *et al.* 2003). Mainly based on sugar sensitivity, flocculation phenotypes can be classified into two categories: the Flo1-type, for which flocculation is constitutive and sensitive only to mannose, and the NewFlo-type, which flocculate in the stationary phase, commonly in the presence of high ethanol concentrations, and for which the flocculation process is sensitive to mannose, glucose, sucrose and maltose (Stratford and Assinder, 1991). The brewing industry has historically taken advantage of NewFlo type yeast flocculation for the clarification of products and the recovery/removal of biomass. Premature flocculation leads to 'hanging' fermentations (sluggish due to reduced catalytic surface area) and so Flo-1 type yeast species are rarely used in the brewing industry (Bauer *et al.* 2010).

Yeast flocculation involves flocculins, which are lectin-like cell wall proteins encoded by the *FLO* gene family, that interact with mannose residues on the surface of adjacent cells. Flocculation allows the convenient separation of cells from the fermentation products at the end of batch fermentations and the retention of biomass in immobilised cell systems. For this reason, in continuous fermentations with immobilised cells, yeast strains exhibiting strong flocculation at a wide range of sugar and ethanol concentrations are desirable. The use of flocculation to immobilise cells within a fermentation system was proposed by Hsiao *et al.* (1983) and received attention during the 1980s and 1990s, but has only recently been used successfully to self-immobilise due to new developments in the understanding of the environmental and genetic regulation of flocculation. This new understanding led eventually to the construction of yeast strains such as CHFY0321 and SPFC01 which are able to remain immobilised through conditions of high CO_2 productivity, which tend to deflocculate yeast through shearing (Zhao and Bai, 2009).

The use of flocculation as an immobilisation strategy in continuous bioreactors allows for theoretically indefinite periods of operation assuming the absence of undesirable genetic drift over time. Non-viable cells don't express adhesins; and therefore are unable to aggregate, sediment, and are thus washed out of the system. Microenvironments within the immobilisation matrix harbour their own niche metabolically adapted subgroup of microorganisms ranging from the highly ethanol tolerant to those adapted to withstand high gravity environments. Flocculation also allows operation at a high cell density, facilitates down stream processing and conveys a higher overall productivity to the reactor system (Choi *et al.* 2010).

Yeast flocs vary in size, a smaller average floc size means a greater catalytic surface area in contact with the substrate and therefore greater ethanol productivity. Zhao *et al.* (2009) reported the effect of zinc on the ethanol tolerance and yield of flocculating yeast in continuous fermentation. It was determined that all tested concentrations of zinc additions resulted in reduced floc size. The average floc size was found to be smallest when 0.05 g. L^{-1} $ZnSO_4.7H_2O$ was added to the feeding medium; a floc size average of 263.9±9 μm resulted compared with 326.2±6 μm for the control.

This chapter aims to demonstrate that the continuous fermentation system designed in chapter 2 could be operated without the need for complex and expensive immobilisation strategies, or over-engineered bioreactor systems. A continuous fermentation system with adsorption and flocculation immobilised

biomass producing cider would demonstrate that this is a possibility for commercial potable alcohol production.

4.2 Materials and Methods

4.2.1 Yeast Strain

An alcohol producing strain of *Saccharomyces cerevisiae* supplied by Makana Meadery (Grahamstown, South Africa). This strain naturally occurs on *Trichodiadema intonsum* plant roots, and had previously been determined to be FLO-1 type, or inherently flocculent. Previous studies involving the PCR amplification and subsequent sequencing of the 18s rRNA and D1D2 domain sequence of the 26s ribosomal subunit gave alignment with both the MUCL 51208 and CHF0321 strain using the 18S sequence.

4.2.2 Substrate

Concentrated apple juice (700 $g.L^{-1}$) from Granorpassi (Langkloof region, Eastern Cape, South Africa) was diluted to a concentration of 170 $g.L^{-1}$. The medium consisted of an average of 22.5±0.58 $g.L^{-1}$ glucose and 147.4±10.12 $g.L^{-1}$ fructose, and the apple juice had undergone a pectinase pre-treatment. To this was added 0.25 $g.L^{-1}$ Nutriferm (Ecolab, Johannesburg, South Africa), 0.05 $g.L^{-1}$ $CaCl_2.2H_2O$ (Merck, SAAR1524920EM), 0.25 $g.L^{-1}$ $MgSO_4.7H_2O$ (Merck, SAAR4124000EM), 0.25 g. L^{-1} KH_2PO_4 (Merck, 105108), 0.50 $g.L^{-1}$ $(NH_4)_2.SO_4$ (Merck, SAAR1124020EM) and 0.05 $g.L^{-1}$ $ZnSO_4.7H_2O$ (Merck, SAAR7582860EM). The substrate after the addition of salts was at a pH of 3.6 and was not buffered or adjusted prior to the fermentation. The media were autoclaved at 121°C for 20 min prior to use. Two cell populations for inoculation were each grown up at 30°C in 500 mL of this media on an orbital shaker at 150 rpm for 48 h.

4.2.3 Support Matrix

A displaced volume of 250 mL of 2 mm^3 cubes of ginger root was prepared and repeatedly boiled to remove ginger flavour. Ginger was selected as the immobilisation matrix due to its low cost, resistance to degradation and density.

4.2.4 Scanning Electron Microscopy

In order to investigate the cell adhesion to the ligneous material and floc size formed by cell aggregation, several samples were removed from the bioreactor and fixed with 2.5% glutaraldehyde in a 0.1M phosphate buffer overnight at 4°C. The samples were then washed in a 0.1 M phosphate buffer for 10 min and dehydrated in 30%, 50%, 60%, 80%, 90% and twice in 100% ethanol solutions. The material was then frozen in liquid carbon dioxide and fractured. After fracturing, sections were mounted and gold coated for 6 min (Balzers Union Sputtering Device) prior to scanning electron microscopy (SEM) examination (Vega LMU Tescan).

4.2.5 Reactor Inoculation and Fermentation Process

Two bench-scale glass tower perfusion bioreactors (Figure 4.1), each with a total volume of 0.930 L were sterilised and loaded with 250 cm^2 ginger root cubes. The cell growth cultures were then pumped at 30 $mL.min^{-1}$ into the tower bioreactors from the inlet. The inoculum was allowed to settle undisturbed on the immobilisation matrix for two hours to promote absorption before substrate was introduced to the column. Apple must was then pumped continuously through the inlet at a flow rate of 1.0 $mL.min^{-1}$ (dilution rate of 0.088 h^{-1}) using a Watson-Marlow variable speed peristaltic pump. The fermentation was conducted at room temperature in an air-conditioned room.

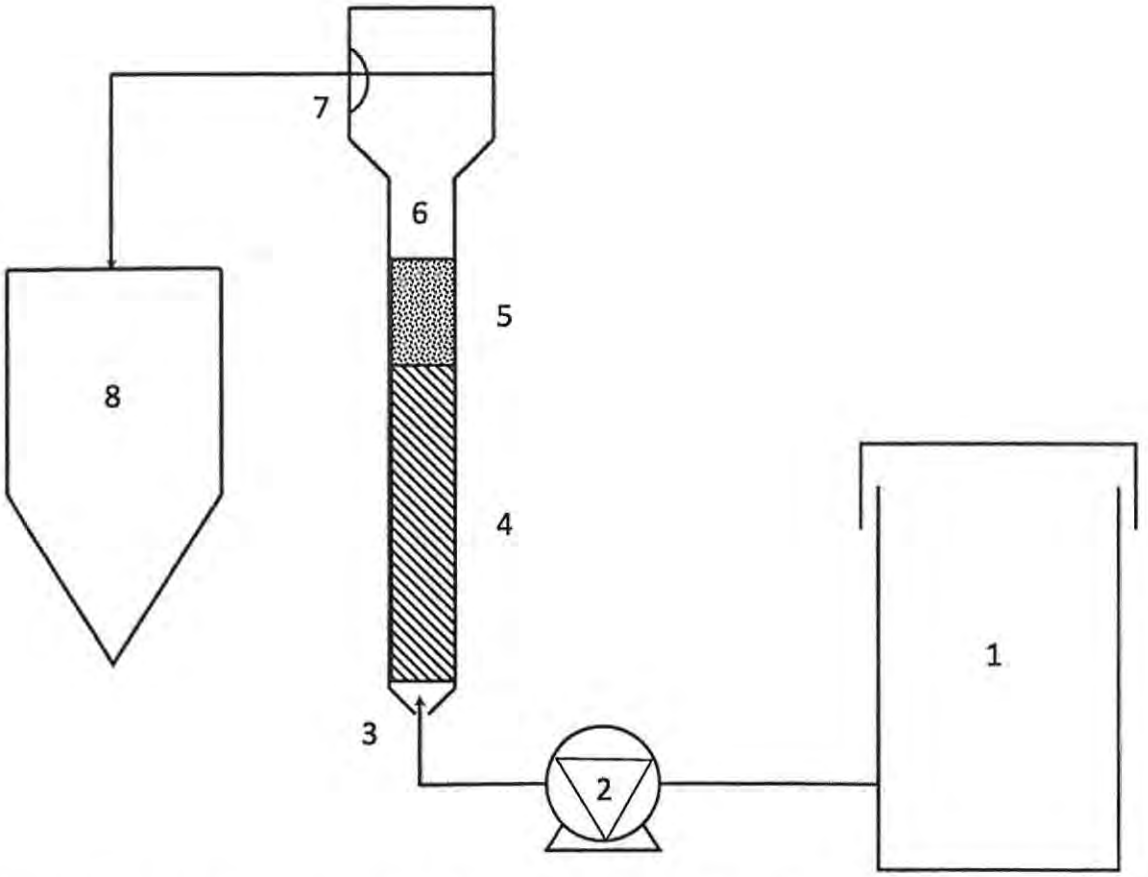


Figure 4.1 Schematic representation of the experimental set up for a single continuous perfusion tower bioreactor; (1) feed tank, (2) peristaltic pump, (3) inlet, (4) packed bed region, (5) fluidised bed region, (6) bioreactor, (7) collection outlet, (8) product tank.

4.2.6 Analytical Methods

Samples were taken in triplicate from the outlet of the reactor system and filtered using 0.22 μm syringe filters. These were stored inverted at -20°C prior to dilution and analysis. High performance liquid chromatography (HPLC) was used to quantitatively analyse carbohydrate and alcohol content in the substrate and product samples according to the method developed in chapter 3.

4.2.7 Fermentation Parameters

The fermentation parameters and yields were calculated at the steady-state average of the fermentation. The ethanol volumetric productivity for the full reactor volume (Q_P, $g.L^{-1}.h^{-1}$) and the ethanol hydraulic

volumetric productivity (Q_{hp}, $g.L^{-1}.h^{-1}$) were calculated as the ratio between the mass of ethanol in the product (P_f, $g.L^{-1}$) to the duration of the fermentation (t, h).

$$Q_p = \frac{P_f}{t}$$

The yield of ethanol on consumed sugar ($Y_{P/S}$, $g.\ g^{-1}$) was defined as

$$Y_{P/S} = \frac{P_f}{(S_0 - S_f)}$$

where S_0 ($g.L^{-1}$) and S_f ($g.L^{-1}$) are the starting and the final sugar concentrations, respectively.

The sugar conversion percentage is defined as

$$Conversion\ (\%) = \frac{(S_0 - S_f)}{S_0}\ X\ 100$$

The efficiency of sugar conversion to ethanol (η, %) was estimated using the relationship

$$\eta = \frac{Y_{P/S}}{Y_{th}}\ X\ 100$$

where Y_{th} is the theoretical maximum value of $Y_{P/S}$ (0.51 $g.\ g^{-1}$) (Pacheco *et al.* 2010). Residual sugar concentration (S_f) is defined as the sum total sugar concentration remaining in the fermentation broth immediately at the collection outlet.

4.2.8 Fermentation Modelling

Samples (1 mL) were collected from 15 points near the central axis of both of the tower perfusion bioreactors in triplicate (separated by 1 hour between sets of samples to allow standard conditions to re-establish following the disturbance caused by the sampling pipe) at different heights using a syringe and a

length of plastic tubing with an internal diameter measuring 0.2 mm. Samples were taken slowly, at 5cm height intervals (beginning at 75 cm), from the top downwards so as to minimise axial mixing. These samples were each analysed in according to the method developed in chapter 3.

4.3 Results

4.3.1 Yeast Immobilisation

The ginger root used as the carrier had an irregularly shaped surface (Figure 4.2), exposing a large surface area to the fermentation fluid, and it was observed that significant populations of yeast cells adhered to its surface.

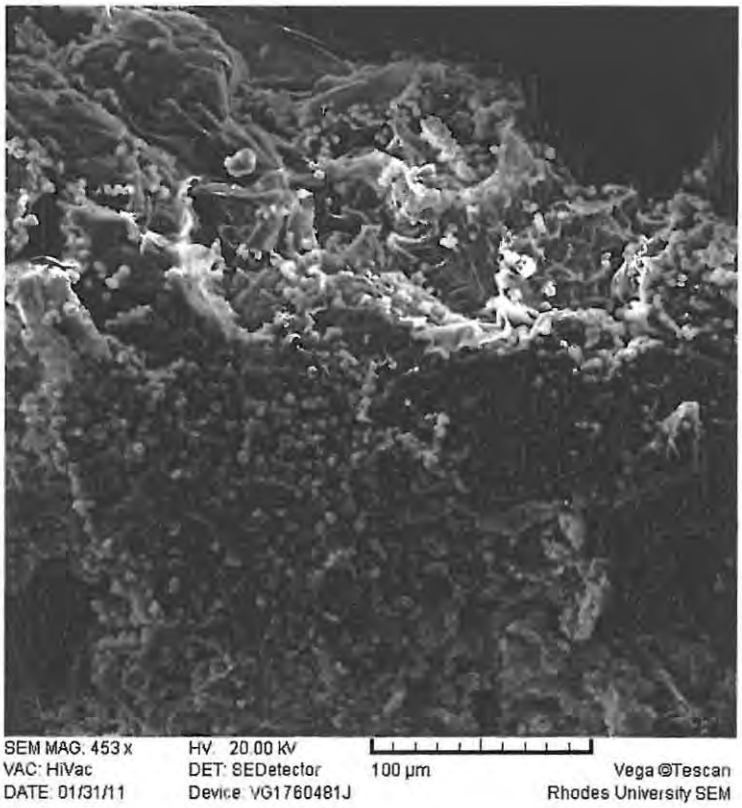


Figure 4.2 Scanning electron micrograph of *Saccharomyces cerevisiae* cells adsorbed to the carrier surface.

Flocculated cells visibly formed in the spaces between the ginger cubes, and cell aggregation was therefore a factor in the retention of biomass within the bioreactor in this study. Figure 4.3 is a micrograph of a sample floc from a perfusion bioreactor.

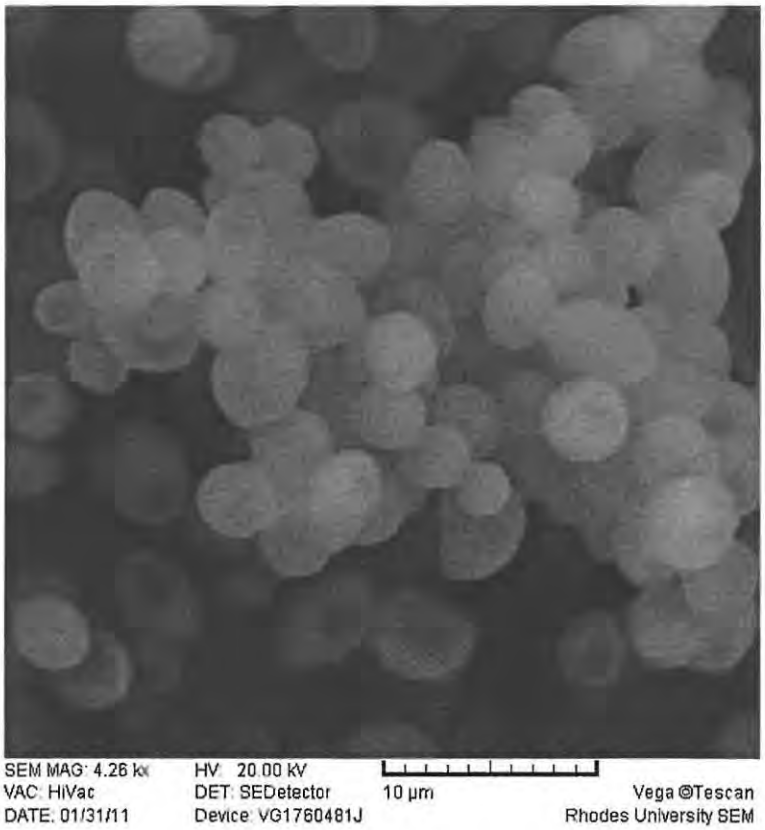


Figure 4.3 Scanning electron micrograph of *Saccharomyces cerevisiae* cells aggregated into a small floc.

SEM data obtained during this study was largely inconclusive with regards to floc size distribution, as it appeared as though multiple flocs, ranging from approximately 30 µm to 300 µm in diameter had loosely aggregated to form larger units.

4.3.2 Continuous Fermentation

Continuous fermentation was carried out to investigate the operational performance and stability of the yeast cells in the perfusion tower bioreactor system. The yeast strain used was inherently flocculent, alcohol resistant and retained fermentative capacity at room temperature. Yeast immobilisation was

confirmed using SEM, yeast cells were immobilised via adsorption to the ligneous cubes and also through the formation of flocs with a sedimentation rate greater than the upward flow velocity.

The bioreactor systems were operated in duplicate continuously for twenty days, during this time daily samples of product were collected in triplicate and analysed using HPLC. The maximum attenuation of sugar achieved during the period of operation was 95.22±1.99%. Figure 4.4 shows the percentage of residual sugar and ethanol percentage by volume (ABV) after the residence time in the reactor system. Figures 4-6 show that the system achieved steady-state-like conditions after an operational period of 18 days.

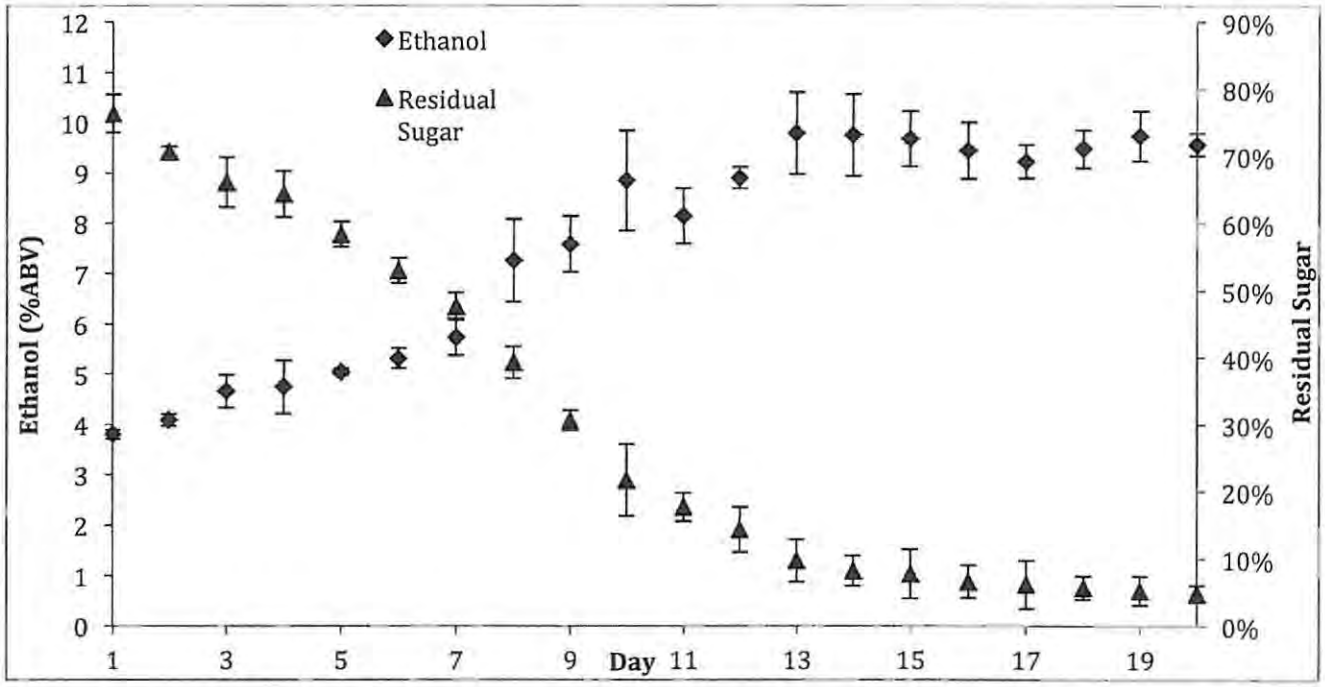


Figure 4.4 Average total residual sugar and % ABV ethanol over the operational period of the perfusion tower bioreactors. (±) SD (n=6).

The maximum alcohol by volume percentage achieved during the operational period was 9.79±0.81% ABV and an average of 9.58±0.52% was the determined ethanol concentration for the steady state achieved after 14 days of reactor operation.

Average glucose depletion during the first 24 h of operation after inoculation was more than 50%, from 22.5 $g.L^{-1}$ to 10.4 $g.L^{-1}$, whilst the average fructose depletion was approximately 20%, from 147.4 $g.L^{-1}$ to

119.4 g.L^{-1} (Figure 4.5). The trend favouring the depletion of glucose continues towards steady state, whilst fructose follows a more linear trend over time.

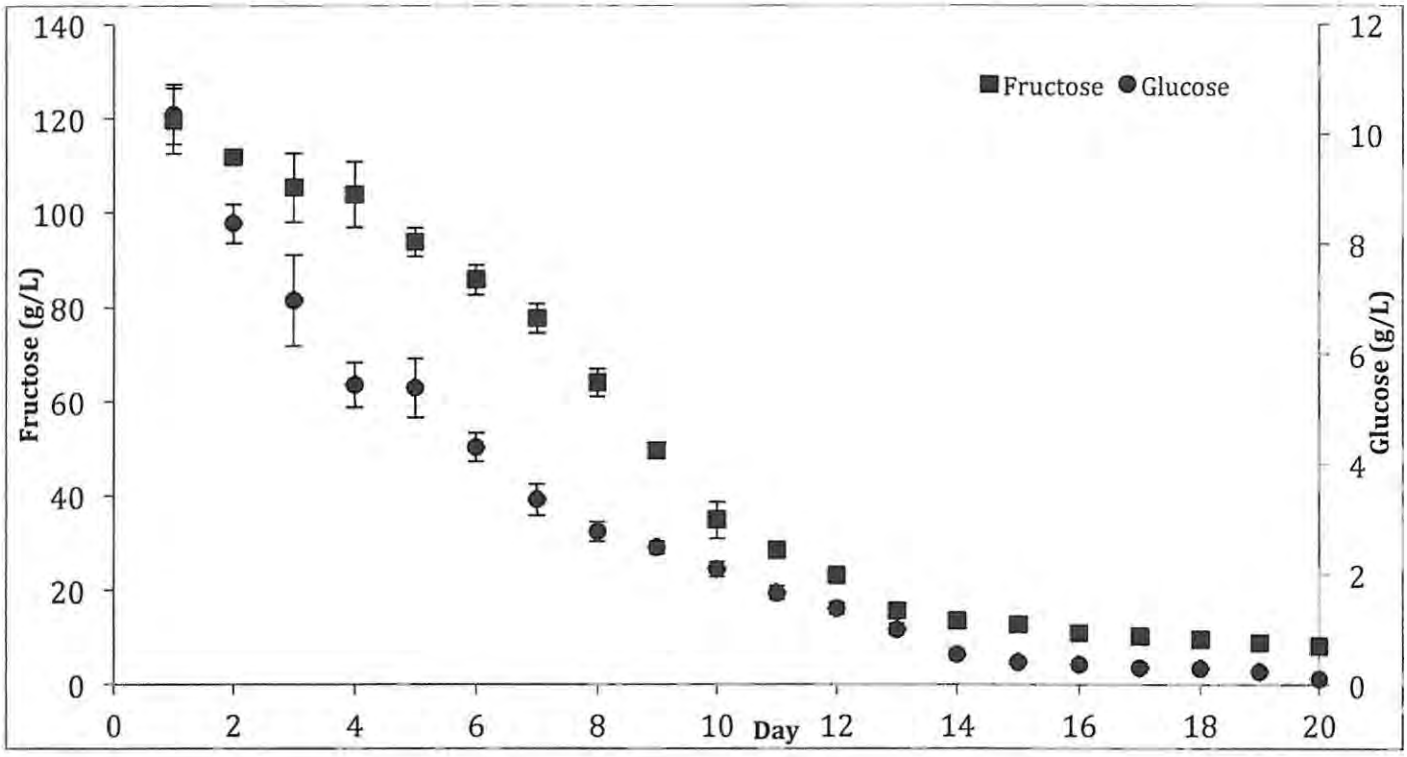


Figure 4.5 Average residual sugar concentrations of glucose and fructose in fermentation product over bioreactor operational period; (±) SD (n=6), initial sugar concentrations: 22.5 ±0.58 g. L^{-1} glucose and 147.4 ±10.12 g. L^{-1} fructose.

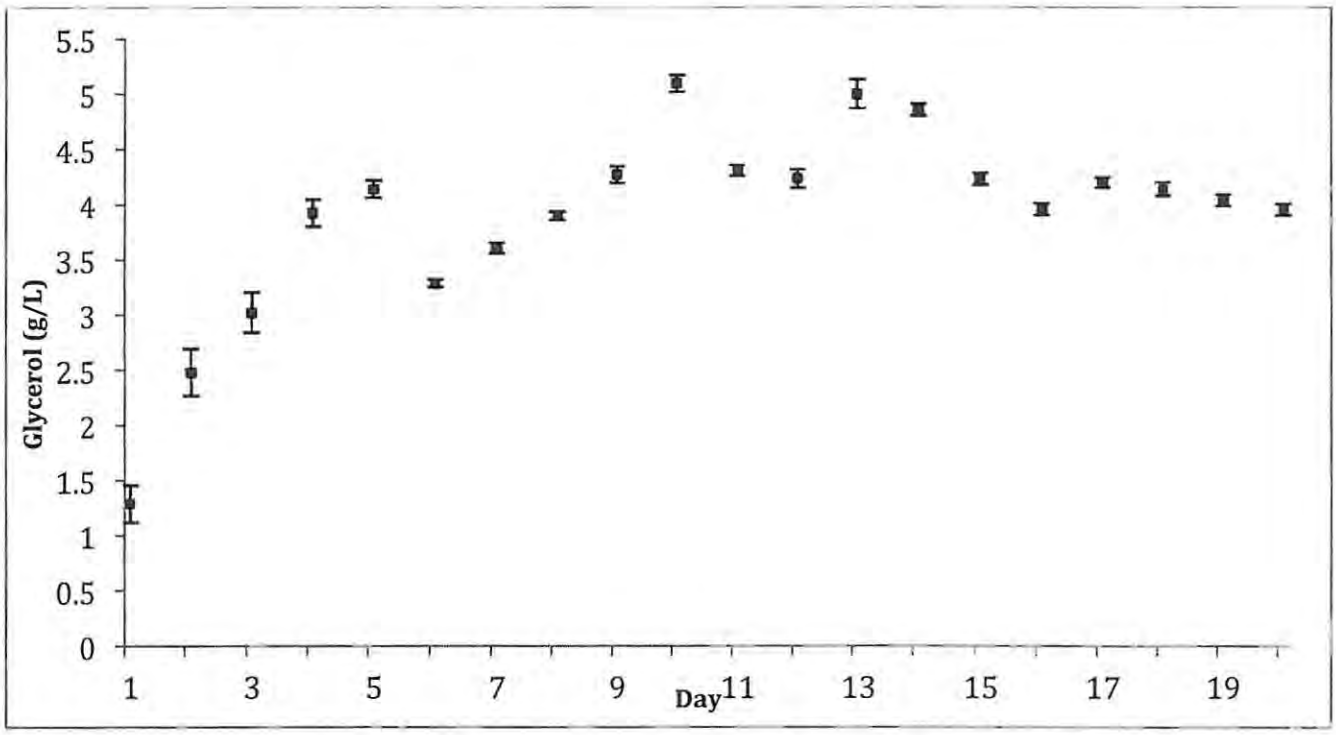


Figure 4.6 Average effluent glycerol concentration over PTB operational period. (±) Relative SD (n=6).

The average glycerol content at steady state was 4.29±0.27 $g.L^{-1}$ (Figure 4.6).

4.3.3 Bioreactor Performance

The fermentation performance and yields were calculated at steady state, after 18 days. Each bioreactor produced 1, 450 mL of product containing 75.57±4.11 $g.L^{-1}$ ethanol in 24 hours. The ethanol volumetric productivity for the full reactor volume was therefore 3.41 g. $L^{-1}.h^{-1}$ and the ethanol hydraulic volumetric productivity was 4.95 g. $L^{-1}.h^{-1}$. The average yield of ethanol for consumed sugar ($Y_{P/S}$, g. g^{-1}) was 0.470 g. g^{-1}, with an average sugar conversion percentage average of 94.52±2.17%. The efficiency of sugar conversion to ethanol was 92.23% of the maximum theoretical yield.

4.3.4 Fermentation Modelling

Samples taken at height intervals within the packed bed bioreactor showed a non-linear depreciation in total sugar concentration and ethanol concentration increase across the axial dimension of the packed bed (Figure 4.7 and Figure 4.8).

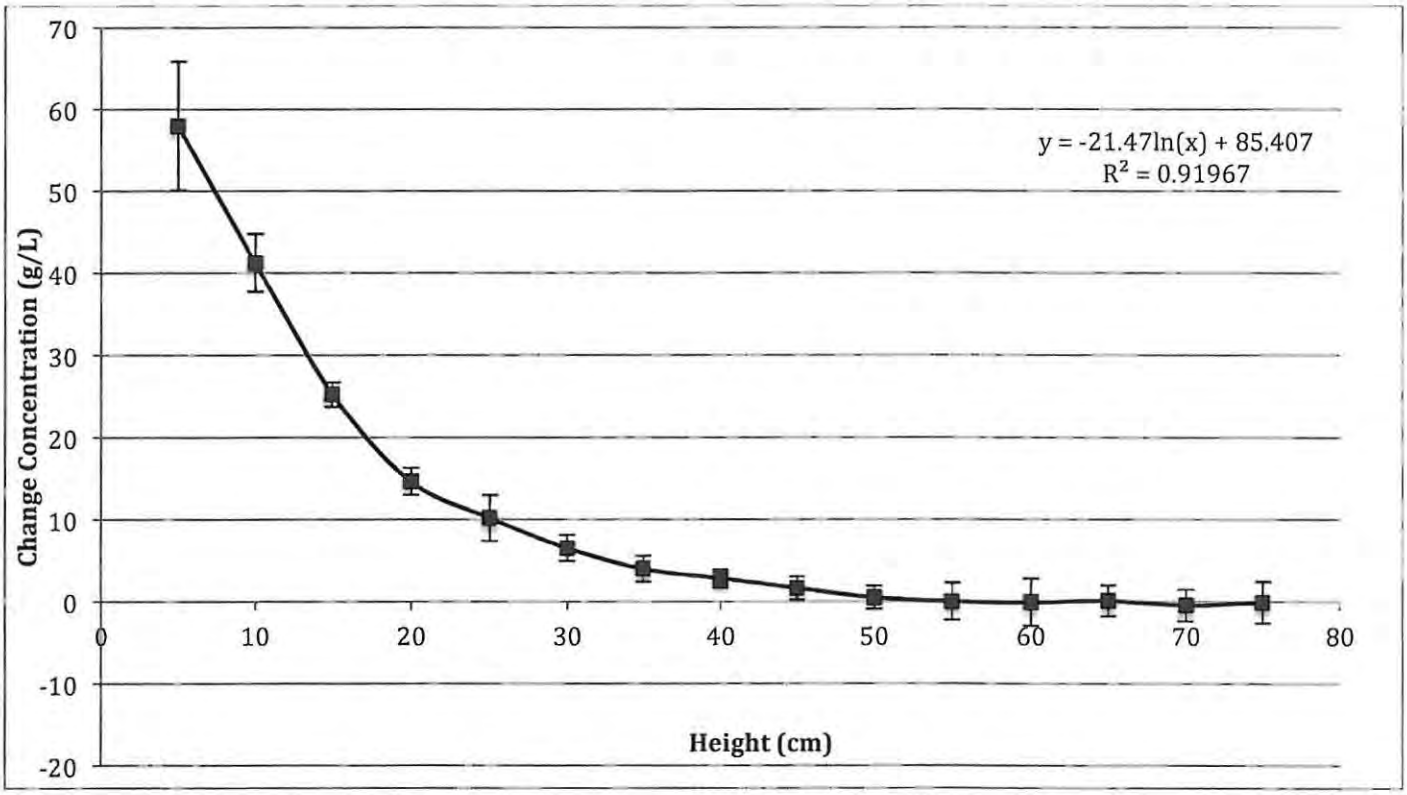


Figure 4.7 Average change in total sugar concentration against height in the packed bed region of the PTBs. (±) Average SD (n=6).

The rate of disappearance ($-r_A$) of sugars reduces in a negative logarithmic trend and approaches zero at height 55 cm in the packed bed region of the perfusion tower bioreactor.

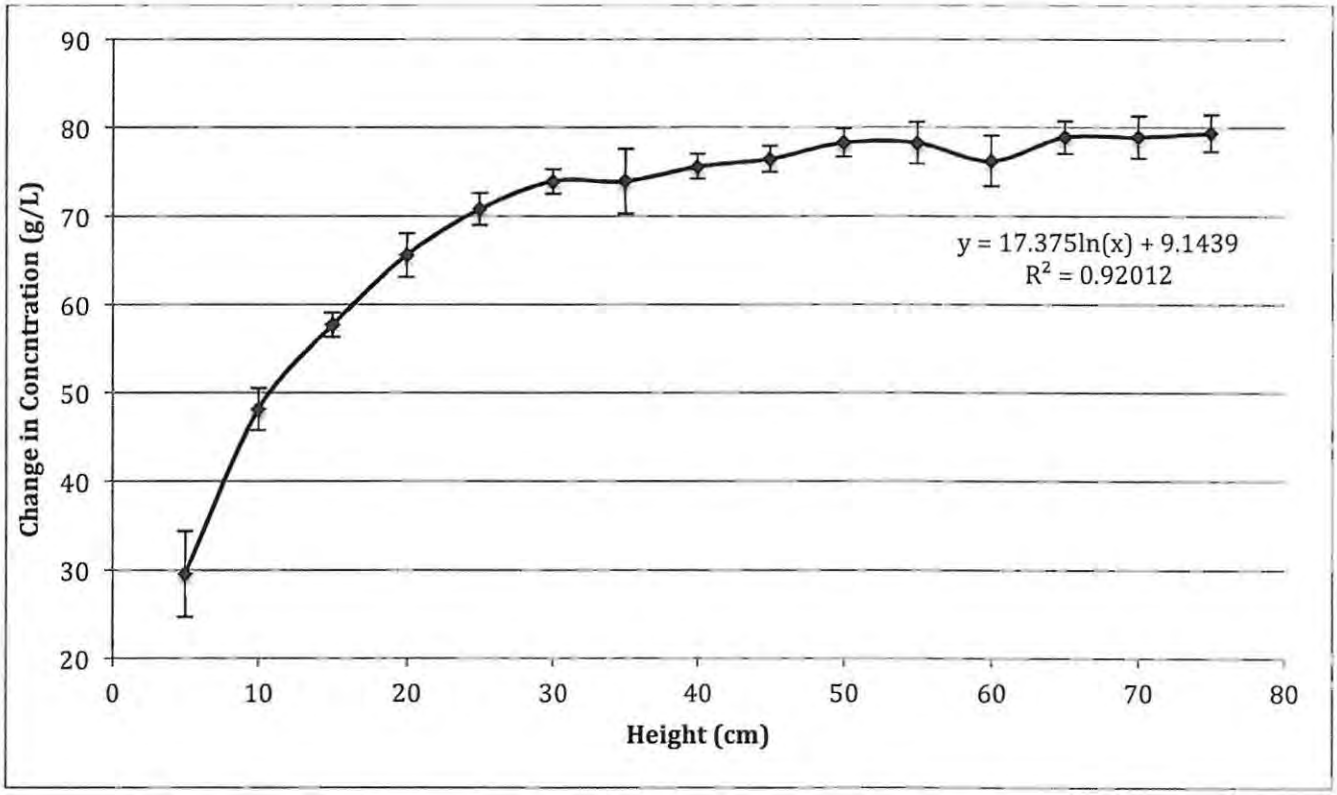


Figure 4.8 Average change in ethanol concentration against height in the packed bed region of the PTBs; (±) Average SD (n=6).

The rate of production (r_A) of ethanol decreases in a logarithmic trend and approaches zero at height 50 cm in the packed bed region of the perfusion tower bioreactor.

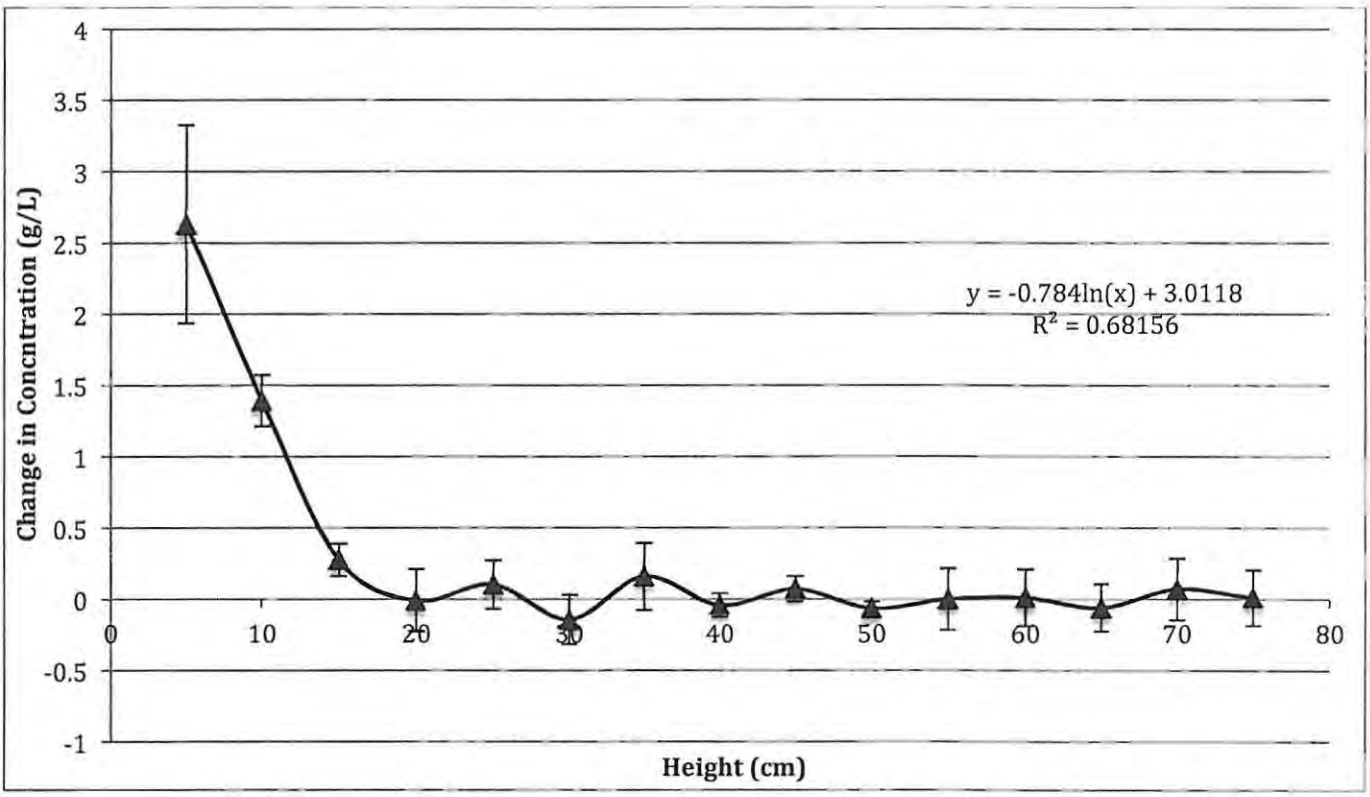


Figure 4.9 Average change in glycerol concentration against height in the packed bed region of the PTBs; (±) Average SD (n=6).

The rate of production (r_A) of glycerol decreases more rapidly than that of ethanol, but retains a loosely logarithmic trend and approaches zero at height 20 cm in the packed bed region of the perfusion tower bioreactor.

4.4 Discussion

4.4.1 Yeast Immobilisation

Choi *et al.* (2010) constructed the flocculent yeast strain CHFY0321 via protoplast fusion between the non-flocculent, high ethanol fermentative *S. cerevisiae* CHY1011 and flocculent, low ethanol fermentative *S. bayanus* KCCM12633. The naturally occurring local yeast used in this study shared 95% identity to CHF0321 in 18S rRNA sequencing (not shown), and shared similar characteristics with regards to flocculation behaviour and ethanol production.

Figure 4.2 demonstrates that significant populations of yeast cells adhered to the carrier surface. This demonstrates the advantages solid carriers convey; adhesion allows improved cell stability and recycling. Samples analysed had a similar distribution of immobilized biomass. In addition to this, the irregular flow patterns created by the presence of the carrier matrix, reduces the shearing effects caused by rapid movement of carbon dioxide produced during cellular metabolism. This plays a role in maintaining the population of aggregated cells that are not adsorbed to the carrier surface.

4.4.2 Continuous Fermentation

During bioreactor operation, the attenuation of carbohydrates became more complete over time. This is most likely due to the increase in catalytic biomass hold-up within the system from yeast population growth, and the metabolic adaptation of yeast sub-populations to their microenvironments over time. This increase in attenuation slows as the biomass approaches balance between population growth and biomass washout. By adjusting the flow rate the residual sugar content could in practice be controlled as required. The concentration of ethanol in the fermentation product is the key variable; as ethanol is typically considered the primary value add of primary fermentation processes. Commercially available ciders have a broad range of ethanol concentrations depending on national tradition, style and market preference. The acceptability of the ethanol concentration achieved in this study is therefore dependent on a variety of factors, but is appropriate for the purposes of demonstrating the system's operation.

Average glucose depletion during the first 24 h of operation after inoculation was more than 50% whilst the average fructose depletion was approximately 20% in the same period (Figure 4.5). This disparity is typical as glucose metabolism is energetically favoured over fructose consumption and glucose uptake is active, whilst fructose uptake is passive (Sussman *et al.* 1980). As the yeast population grows the product glucose concentration falls disproportionately to that of fructose for the same reason. Although the specific sugar concentrations at different points within the reactor were not measured, it is hypothesised that the yeast sub-populations closest to the bioreactor inlet consume the majority of the glucose, leaving the remaining biomass to adapt to fructose consumption. In a free cell system where biomass can easily translocate, the overall sugar attenuation might be significantly lower than that exhibited because cells will preferentially metabolise available glucose and remain unable to adapt to take advantage of accessible fructose. The data represented in figure 4.5 supports the proposition that this immobilised cell system's

biomass population is able to become more efficient at processing available energy sources over time through the development of niche metabolic regulation strategies for specific microenvironments.

This rapid evolution of ethanol and the hydraulic velocity within the column (21 $cm.h^{-1}$) theoretically offers significant protection to the bioreactor system against contamination. Any spoilage microorganisms would require remarkable adaptations to remain viable within the bioreactor system.

The product pH (typically 3.2) of a fermentation system was consistently lower that that of the substrate (typically 3.6) (data not shown), this is likely due to the uptake of ammonia supplied to the fermentation mixture by the ammonium sulphate nitrogen source. The pH of the effluent from this bioreactor system decreased on average by 0.4 units.

The production of glycerol during ethanolic fermentations is well documented (Bideaux *et al.* 2006). Glycerol plays a role in osmoregulation (Nevoigt and Stahl, 1997) and in the cellular redox balance (Bakker *et al.* 2001). In potable alcohol, a certain amount of glycerol is a desirable product of fermentation (Pretorius, 2000). Although the glycerol is produced at the expense of ethanol yield, the so-called 'body' of a beverage is conferred primarily by its glycerol content. In addition to this; glycerol production has been shown to augment yeast stress tolerance (Bideaux *et al.* 2006). The product glycerol concentration at steady state was 4.29 $g.L^{-1}$. The glycerol production levels of yeast cells isolated from high quality commercial ciders from Spain produced using local cider yeast strains range from 4.05 $g.L^{-1}$ to 4.65 $g.L^{-1}$ (Valles *et al.* 2005). The glycerol concentration determined in this investigation falls within the lower part of this range, but within the acceptable range for alcoholic ciders in general. Zhao *et al.* (2009) found that the addition of 0.05 $g.L^{-1}$ $ZnSO_4.7H_2O$ decreased the glycerol yield in continuous fermentation to 3.21 $g.L^{-1}$, a reduction of 2.32 $g.L^{-1}$ from the control. The addition of zinc to this reactor system may have reduced the glycerol yield.

Nevoight and Stahl (1997) showed that when yeast cells were placed in a hypertonic solution, the response to the resulting efflux of water from yeast cells was to intracellularly accumulate compatible solutes, which enable enzymes and other cellular components to perform functions in the low intracellular water concentrations. This would explain the relative increase in glycerol seen in the high sugar concentration of the media (Figure 4.7). The exposure of yeast to high gravity sugar solutions is one method by which the metabolic balance can be shifted to favour glycerol production in commercial wine production and other commercial fermentation systems (Sahoo and Agarwal, 2001, Wang *et al.* 2001, Bali *et al.* 2003).

The production of ethanol is a redox neutral process, and therefore has no demand for NADH, but the production of glycerol consumes NADH (Wang *et al.* 2001). Ansell *et al.* (1997) detail how in anaerobic environments the synthesis of glycerol is crucial to the balancing of the cellular energy equation; the packed bed region of the tower perfusion bioreactor is an anaerobic region. It is therefore likely that the synthesis of glycerol in the lower regions of the tower bioreactor, where sugar concentrations are highest, is an extremely important survival mechanism for the yeast cells. Even as the carbohydrate concentration of the substrate declines in the higher regions of the tower bioreactor, glycerol would still be important to balance the cellular energy budget of the yeast. When not required intracellularly, glycerol is excreted by export protein channels and by diffusion across the cell membrane (Wang *et al.* 2001).

4.4.3 Bioreactor Performance

Scott and O'Reilly (1996) demonstrated the fermentation of an alcoholic cider beverage using co-immobilised yeast and bacteria strains through electrostatic surface attachment of cells, making use of the predominant negative charge of the yeast cell surface. Whilst successful in the objectives of the experiment, full attenuation was only obtained after 18 to 21 days. Nedovic *et al.* (2000) demonstrated an efficient continuous cider fermentation using Ca-alginate gel entrapment of yeast and *Leuconostoc oenos* cells within a conical glass bioreactor. The data presented indicated that a hydraulic volume of 0.985 L (total volume of 2.5 L) achieved 95% attenuation of the 71 $g.L^{-1}$ total sugar at a flow rate of 138 $mL.h^{-1}$. A nominal value of 5% ABV was reported, this translates to a volumetric productivity of 2.73 g. $L^{-1}.h^{-1}$ and a hydraulic volumetric productivity of 6.92 $g.L^{-1}.h^{-1}$. The reported performance of the conical reactor system is above values reported by other studies (Ghorbani *et al.* 2011; Scott and O'Reilly, 1996; Bauer *et al.* 2010). The problems such a bioreactor would present at scale-up, and the economic costs of operating such a bioreactor commercially would be prohibitive; one significant obstacle is the long-term reactor productivity, as new cells will need to be immobilised frequently at a substantial cost as cell growth causes gel capsules to burst with subsequent loss of catalytic biomass.

Pacheco *et al.* (2010) immobilised *S. cerevisiae* cells on cashew apple bagasse and achieved 95% total sugar consumed after 6 h of fermentation and an ethanol yield of 0.46 g. g^{-1} on average, with an average ethanol productivity of 5.49 g $.L^{-1}$ $.h^{-1}$. It is important to note that these fermentations were carried out in 500 mL Erlenmeyer flasks with only 125 mL hydraulic volume; should the fermentation volumes have been greater, the productivity would most likely have dropped due to mass transfer problems. Additionally the

starting sugar concentrations were low (79-90 $g.L^{-1}$) and therefore the systems were not subject to substrate and product inhibition, ethanol stress or osmotic shock, which would become factors in larger scale applications. Nevertheless, the experiment confirms that adsorption-based immobilisation is effective in biomass retention for ethanolic fermentation with the following advantages: low-cost of carrier, low operating costs and good operational stability. Kumar *et al.* (2011) demonstrated a continuous ethanol production system using thermotolerent *Kluyveromyces* species yeast immobilised on bagasse chips at high temperatures inside a column bioreactor. Ethanol volumetric productivity rates ranging from 21.87±0.75 $g.L^{-1}.h^{-1}$ from 50 $g.L^{-1}$ glucose at a dilution rate of 1.25 h^{-1}, to 18.65±0.75 $g.L^{-1}.h^{-1}$ from 150 $g.L^{-1}$ glucose at a dilution rate of 0.5 h^{-1}. These productivity rates are very high when compared to the performance of the tower perfusion reactor system, however it is clear that the system is intended for biofuels production and not for drinking alcohol. Potential problems with scalability, substrate costs, energy expenditure of maintaining a 50°C operational temperature, and the yeast strain used might exclude the reactor system from application in commercial beverage manufacture.

Ghorbani *et al.* (2011) investigated ethanol production in an immobilized cell reactor (gel entrapment), with sugar cane molasses as substrate. The maximum ethanol production, the theoretical ethanol conversion efficiency and volumetric productivity were 19.15 $g.L^{-1}$, 46.23% and 2.39 $g.L^{-1}.h^{-1}$, respectively. The total sugar consumption in the fermentation column was between 85 and 96%. The comparatively low productivity and conversion efficiency in this investigation was likely due to the presence of sucrose which requires a high invertase activity and often becomes a rate limiting factor in continuous fermentation systems (Islam and Lampen, 1962). This comparison was against fermentation for fuel ethanol production; where reactor performance is more important due to the low value-add nature of the fermentation. Regardless it is clear that the system performance of the perfusion tower bioreactor is extremely competitive.

4.4.4 Fermentation Modelling

As discussed in chapter 2, the perfusion tower bioreactor may behave in many respects as a 'plug-flow' reactor. In an ideal plug flow reactor the concentration of a given reactant is a function of its position in the axial dimension of the reactor and of time (C (x, t)) (San, 1989). The given system's reactants follow logarithmic trends ($R^2 > 0.91$ for ethanol and sugars), and are also a function of the feed and outflow rates (and outflow = inflow), and hence, time. Variables can therefore be substituted into the plug flow model to

attain approximate unknown values. For example where V_X is the volume/distance along the axial dimension:

$$\frac{F_A \cdot dC_A}{dV} = r_A$$

Substituting to give:

$$\int_0^{V_X} \frac{dC_A}{dV} = \int_0^{V_X} \frac{r_A}{F_A} dV$$

$$C_A(V_x) = C_{A0} + \frac{1}{F_A}\int_0^{V_X} r_A(V_X)\, dV$$

Where:

A	= Sugar	
F_A	= Reactant A feed rate	= 5.1 g.min^{-1}*
C_A	= Concentration of reactant A	= Unknown
C_{A0}	= Initial concentration of reactant A	= 0.17 g.mL^{-1}*
r_A	= Rate of disappearance of reactant A	
h	= Height in column	
V	= Volume element	= (dV = (h)(0.56 mL.cm^{-1}))*
V_{PB}	= Packed bed volume (active)	= 30.80 mL*

*(See Appendix A)

From figures 4.7 and 4.8, it can be seen that the active volume of the packed bed region is considerably less than the total volume of the packed bed region. The rate of change in concentration of both sugar and ethanol approaches zero at 50 cm in height, however the total height of the packed bed region is 87cm, giving an inactive volume of 20.72 mL within the bed itself, and a further 10 cm of height in the column

before the expanded region. This is not detrimental to the operation of the bioreactor as the additional pressure allows for an h_{CRIT} that occurs higher within the column. Indeed, the point of fluidisation occurred at a height of 82 cm in the column, well above from the active region of the column, and therefore does not contribute to mixing in the axial dimension. This 'wasted' volume does however mean that the productivity of the bioreactor is not optimised. Either the substrate feed rate can be increased until the ethanol production rate approaches zero at h_{CRIT} or the height of the column can be reduced to achieve the same outcome. As discussed in chapter 2, however, as the behaviour of the biocatalyst is expected to change over time, a loss in apparent volumetric productivity is a pragmatic sacrifice when the benefit is a bioreactor with increased versatility.

Figure 4.9 indicates that the change in glycerol concentration rate production approaches zero at a height of approximately 20 cm and then oscillates around a concentration of 4.35 $g.L^{-1}$ but with substantial deviation from the mean. The majority of the glycerol production occurs in the bottom of the column where conditions of high osmotic potential prevail. This is in agreement with literature (Wang *et al.* 2001; Nevoight and Stahl, 1997). The apparent oscillation of glycerol concentrations at higher regions of the packed bed regions of the columns is likely due to the random diffusion and accumulation of glycerol from cell populations at different heights.

Sampling longitudinal mixing would be another test of plug flow behaviour as the model assumes perfect mixing in the longitudinal dimension, this test was not conducted, however, as the retrieval of samples would be very difficult without corrupting the outcome of the experiment.

4.5 Conclusions

This chapter demonstrated that a bench-scale continuous perfusion fermentation system could be operated for an extended period of time without the need for complex and expensive immobilisation strategies. The immobilised cell system produced cider with acceptable post-primary fermentation organoleptic properties and at a high performance; a volumetric productivity of 3.41 $g.L^{-1}.h^{-1}$, 94.52 ±2.17% attenuation of 170 $g.L^{-1}$ sugar, and a yield of 0.470 $g.g^{-1}$, was achieved, demonstrating that the perfusion tower bioreactor is an effective fermentation system. Considering the low construction and operating costs, this immobilisation strategy is therefore an attractive option for continuous potable ethanol production at the commercial-scale. The next challenge in completing the stated objectives of this study is the scale up of the

bench scale perfusion tower bioreactor to the pilot commercial scale, and subsequently to a scale producing volumes appropriate for commercial beverage production.

Chapter 5

Bioprocess Scale-Up: Pilot and Commercial Scale Mead Production Using Continuous Perfusion Tower Bioreactors

5.1 Introduction

The production of mead probably predates human existence (Acton and Duncan, 1984), and at one point was one of the most commonly consumed beverages in much of Eurasia (Gayre, 1948), its consumption in China and Tibet performing both cultural and medicinal functions (Yaochun, 1984). The primary factor thought to be responsible for the decline in mead's popularity is price, caused largely by increasing scarcity in honey caused by beekeeping technology not advancing rapidly enough to match honey supply with population growth (Gayre, 1948). As a consequence, eventually mead was not able to compete economically with wine. Mead ethanolic fermentations observed by Steinkraus and Morse (1966) were generally slow, hence the development of a nutrient and vitamin solution which could be added to new mead batches to encourage fermentation. This increased the rate of fermentation allowed mead to be produced in weeks rather than months. The fermentation of mead can also be hastened by agitation to improve mass transfer between yeast and the must (Steinkraus and Morse, 1966). Agitation of bioreactors can be costly and involves careful bioreactor design in order to avoid mass transfer problems (Ratledge and Kristiansen, 2006) and shearing effects.

Current mead research is focused on alleviating the time consumption involved in mead fermentations. Roldan *et al.* (2011) attempted to alleviate honeys low nutrient content with the addition of varying concentrations of pollen to the must ranging from 10 to 50 $g.L^{-1}$. The results demonstrated that pollen improved fermentation rates, ethanol yields, and the organoleptic characteristics of the mead produced. An increase in the volatile organic compound levels in products was also observed. A study was conducted by Mendez-Ferreira *et al.* (2010) aimed at the optimisation of must formulation in order to achieve greater productivity rates and product quality augmentation. The must was adjusted using a number of additives including nitrogen supplementation and the addition of organic acids. It was found that the addition of diammonium phosphate increased the fermentation rate but did not ensure complete attenuation of

available sugars, from which the authors concluded that other factors than nitrogen availability could account for reduced yeast activity in mead fermentations. Observed levels of volatile aromatic compounds were also higher in nitrogen-supplemented fermentations (Mendez-Ferreira *et al.* 2010). Pereira *et al.* (2009) conducted a study to evaluate the capacity of a variety of local *Saccharomyces cerevisiae* strains isolated from wild honey to survive the adverse stress conditions regularly found in mead fermentations. The isolated yeast strains were evaluated in terms of their respective fermentation performance under ethanol, SO_2 and osmotic stress. The results obtained indicated that wild yeast performed similarly to the commercial control strains used.

The significant lack of active research into mead production techniques when compared to other beverages, and the largely non-peer reviewed nature of mead knowledge generation, has meant that the majority of information transfer regarding mead production fell into the category of *word of mouth* advice between one mead maker and another.

The primary fermentation is typically the high-risk portion of the production process, where the high value substrate is susceptible to contamination by spoilage bacteria such as *Acetobacter* (Bauduin *et al.* 2006); and for this reason it is essential that the production of ethanol in the reactor system occurs as rapidly as possible. In addition to the mitigated spoilage risks, it can be argued that the economic benefits of rapid fermentations due to a reduced time-to-market are axiomatic.

The process of taking data derived from a bioreactor of a specific size and using it to design a larger bioreactor is termed scale-up, the primary objective of scale-up is to achieve better process economics (Atkinson and Mavituna, 1991).

As mentioned in chapter 2, transport processes are influenced by the scale of a bioreactor, and therefore by the scale-up process itself. This can result in changes in actual thermodynamic and microkinetic properties of the same process at different scales. In addition to this, microorganisms are subjected to shear forces, which can either damage the cells or have an influence on the formation of aggregations of biomass.

The production of beverages at a large scale is typically difficult due to the requirement of heating and cooling large volumes of must. These problems typically magnify exponentially with scale, resulting in long heat exchange times, and often cause the denaturation of flavour compounds (Kime *et al.* 1991 a). Kime *et*

al. (1991 a and b) tested different methods to overcome this problem, such as flash heating in a tubular heat exchanger and ultra filtration, and indeed, most modern fermentation facilities make use of continuous, rather than batch, heat exchange set-ups.

At any scale the problem remains that, in batches, substrate ferments slowly due to mass transfer limitations and inhibitory conditions, these factors were overcome to an extent for mead production by Steinkraus and Morse (1966) though the addition of nutrients and growth factors, and which Karuwanna *et al.* (1993) overcame through the addition of grape juice to the must. However, the inherent limitations of batch processing remain.

Atkinson and Mavituna (1991) indicate that scale-up experimentation is expensive and it is important to perform adequate initial theoretical modelling of a process before pilot and commercial scale systems are constructed. A bioreactor provides a biological, chemical and physical environment that is favourable to the production of the product and should be maintained such that the desired organisms grow, and the growth of undesirable organisms should ideally be inhibited (Wiseman, 1983). Maintenance of the chemical environment involves the maintenance of pH and the optimisation of mass transfer (provision of substrate, gasses and the removal of inhibitory substances) (Wiseman, 1983). The physical environment, specifically temperature and pressure (Atkinson and Mavituna, 1991), are also considerations.

In scaling a process up, these factors should change as little as possible to ensure that processes evaluated at laboratory-scale perform similarly at commercial scale (Atkinson and Mavituna, 1991).

The objective of this chapter is to demonstrate a scale-up of the laboratory bioprocess demonstrated in chapter 4 to the pilot and commercial-scale.

5.2 Scale-Up Strategies

A number of scale up strategies exist, and are well documented (Shuler and Kargi, 1992; Atkinson and Mavituna, 1991). Some of the most common methods used for the scale-up of a bioprocess is using 'fundamental methods', which involves solving all the microbalance equations for momentum, mass and heat transfer in the system which is most suitable to homogenous systems and simple fluids where flow is well defined (or absent) (Atkinson and Mavituna, 1991; Mijnbeek and Oosterhuis, 1992). Semi-fundamental methods involve the use of simplified flow model equations in order to avoid the use of

momentum balance equations, which are typically impossible to solve; resulting in the need for approximations. This method does not give information about regions that are difficult to model such as stirrer blades, vessel walls and cooling coils (Towler and Sinnot, 2008). This method is usually only applicable to bulk flow, plug flow, plug flow with dispersion and well-mixed systems because scale-up is based on the similarity of the larger system to the smaller system in principle (extrapolation). Dimensional analysis deals with the values of dimensionless groups of numbers, such as the Reynolds number governing flow and the Nusselt number governing heat transfer, which are to be kept constant during scale up (Atkinson and Mavituna, 1991; Demain and Davies, 1999). Inexact methods such as rules of thumb and trial and error are also used in certain situations, however the likelihood that the resulting bioreactor operates optimally is relatively low (Atkinson and Mavituna, 1991).

Regime analysis is the method selected to scale-up the laboratory scale fermentation system described in chapter 4. The reason for this is that although plug flow like behaviour was demonstrated, the presence of an irregular support matrix and gas evolution means that the modelling of key variables such as mass transfer and shear is very difficult, if not impossible. However, parameters that are important to the operation of the bioreactor can be identified. These parameters are termed 'regimes'. Atkinson and Mavituna (1991), define 'regime' in this context to mean the dominance of a mechanism in the functioning of a system and mention that a regime can be a pure one with only one mechanism controlling it, or mixed with multiple mechanisms influencing the system in comparable ways. Where dimensionless numbers and other scale up criteria cannot be kept constant, regime analysis can determine which regimes are important to keep constant (Atkinson and Mavituna, 1991). In this way, for instance in a bioreactor where oxygen mass transfer is determined to be a limiting factor, this regime would be dominant. It is possible that when a scale transition is performed with respect to one particular regime, another regime is rate limiting at a different scale; there is a change in regime.

The volume of the packed bed, in relation to the overall size of the bioreactor was identified as a dominant regime in the perfusion tower bioreactor (as the critical height and mass transfer of substrate to biocatalyst in the upper regions of the packed bed are both independent of the specific packed bed volume but linked to substrate feed rate; as in the plug flow model).

5.3 Scale-Up Methodology

Analysis of the profile data across the packed bed region showed a negative logarithmic decline in sugars and a logarithmic increase in ethanol concentration within the active section of the packed bed region, after which these values did not change significantly. As discussed in section 4.4.4, the active region exhibited plug flow characteristics; non-mixing in the axial dimension (Figure 4.6 and Figure 4.7).

The packed bed regions of continuous bioreactors can be described by material balance equations (Shuler and Kargi, 1992). In an ethanol yielding yeast based fermentation system, with a low biomass production rate and no significant mass transfer limitations, the packed bed can be described by the following formula (Shuler and Kargi, 1992):

$$S_{0i} - S_0 = \frac{q_p X}{Y_{P/S}} . \frac{A}{F} h$$

Where:

S_{0i}	=	initial sugar concentration
S_0	=	final sugar concentration
$q_p X$	=	g ethanol per g dry cells per hour
$Y_{P/S}$	=	yield (gram ethanol per gram of sugar)
A	=	cross sectional area in cm^2
h	=	height of packed bed in meters
F	=	flow rate

This formula is appropriate for predicting the height of the packed bed region for the bench-scale bioreactor (chapter 4), thus providing an indication of the dimensions of a packed bed for the flow rates required for the scaled up bioreactor. This formula was solved (Appendix D) for the bench-scale bioreactor and used to derive the dimensions of the packed bed region for a bioreactor with a maximum daily flow rate of 26 litres, and one with a maximum daily flow rate of 300 litres, with an internal diameter of 0.10 meters and 0.25 meters (Appendix B and C contain key parameter values for each bioreactor).

Some parameters and their effects are not described by formula 5.1, these include the effect of the flow rate on productivity rates, the effects of gas evolution within the packed bed region of the column and the dilution rate/contact time between the catalytic biomass and substrate available for bioconversion.

The process of biofilm formation can describe the immobilisation of biomass onto a solid carrier surface. According to Kwok *et al.* (1998) biofilm formation is determined by the growth rate of immobilised cells and the detachment forces removing the biomass. Shear, as defined by Atkinson and Mavituna (1991), refers to the movement of one layer in relation to an adjacent layer. Ranjan *et al.* (2004) state that fruit juices, which have similar sugar concentrations to most must solutions, are non-Newtonian fluids (dilatant fluids), i.e. their apparent viscosity increases with shear rate.

In the case of complete attenuation within the packed bed region, the must will have been converted from a dilatant fluid at the base of the bioreactor to a near Newtonian fluid at the end/top of the packed bed. In the context of bioreactor scale-up, within the packed bed, one surface in the definition of shear (Atkinson and Mavituna, 1991; above) is the biofilm-coated support and the other surface is the must passing over it. As flow rate increased, so would shear, leading to detachment of biofilm and loss of biomass. Towards the end of the packed bed however, the shear proportional to flow rate would decrease as the solution behaved more like a Newtonian fluid.

The flow through the packed bed is either laminar or turbulent, which has an impact on mixing within the bed, on mass transfer and biomass stability. The break-up of yeast floc and biofilm is governed by two factors; namely the rate at which the biofilm section and floc split, and the rate at which individual cells are eroded (van Hamersveldt *et al.* 1997). High flow rates leading to high shear due to turbulent flow can cause biomass to be split, detached, eroded and removed from the bioreactor leading to a decrease in bioreactor performance due to the loss of biocatalyst. Hsu and Wu (2002) modelled the effect of shear on flocculated and immobilised yeast cells and showed that with reduced shear they were able to maintain larger biofilms than at higher rates of shear. The perfusion tower bioreactor, however, will have only one directly controllable variable that influences the level of shear experienced by the immobilised biocatalyst: the flow rate. Reducing the aspect ratio for the packed bed region can reduce the superficial liquid velocity, and the aspect ratio of the packed bed was changed from 17.5 in the pilot scale bioreactor to 4.9 in the commercial scale bioreactor. This must be balanced against the loss in plug-flow behaviour at lower aspect ratios, and for this reason the superficial liquid velocity was increased during scale up despite the aspect ratio change (Appendix B and C).

From our research it is evident that the bench-scale perfusion bioreactor rapidly converted sugars into ethanol, glycerol; the associated production of carbon dioxide was observed. The carbon dioxide left the

packed bed region in the form of small bubbles, which fluidised the upper section of the packed bed and rose to the liquid surface at the top of the tower.

Eruptions were observed on a small scale in the bench-scale bioreactor (chapter 4) and for the operation of the scaled up perfusion tower bioreactor it is important that these eruptions do not dislodge significant quantities of biomass at the top of the packed bed. The reduced aspect ratio incorporated to minimise shear also reduced the critical fluidisation height of the packed bed column. It was also important that large amounts of the carrier particles do not become fluidised as this could result in the blockage of the outlet flow. If this were to occur, and the fluid level subsequently rose above the outlet, pressure would rapidly build up in the bioreactor headspace, the results of this eventuality would include the pressurisation of the reactor headspace which could cause an explosion. For this reason a pressure blowout valve was incorporated into the design of the bioreactor ensuring safety. The gas holdup volume for the bench scale bioreactor system was scaled up by the same factor as the increase in packed bed, this estimate, with the addition of a large margin for error, was used to determine the size of the headspace region. As with the bench scale bioreactor, both the evolved gas and the fluid product exit the reactor though the same outlet for simplicity, and to maintain a positive pressure in both the headspace and capture tank to prevent contamination and remove the need for additional pumps.

Contact time between biomass and substrate solution could be considered a key regime to consider in scaling up the tower bioreactor. If the aspect ratio is adjusted to increase contact time with the packed bed region of the bioreactor, then gas distribution becomes an important factor, as discussed above. It is preferable therefore to work with the packed bed volume to dilution rate ratio as the key regime, as this confers more freedom regarding the dimensions of the scaled up bioreactor within the limits identified, thus yielding a more versatile bioreactor.

Many of the problems associated with the scale up of bioreactor systems are eliminated by the use of a continuous system, such as mass transfer, upstream and downstream processing and physical space. The continuous bioreactor with identical output to a batch bioreactor requires significantly less physical space, and due to the low working volumes, would need only low throughput processing equipment for associated processing.

One key upstream process in all controlled (non spontaneous) beverage alcohol fermentations is the upstream sterilisation of substrate. To sterilise the media for a process one could either use batch

sterilisation, heating the entire fluid volume to the desired temperature and then cooling it, or a continuous system to fill a tank with processed media (Atkinson and Mavituna, 1991). If a batch bioreactor is used and filled using a continuous process, a number of engineering and microbiological considerations have to be taken into account, such as presterilisation of the bioreactor and filling of the bioreactor rapidly enough to prevent contamination. This is not a major consideration the case of the continuous bioprocess as substrate can be continuously sterilised in small volumes. The continuous perfusion bioreactor does not require that the must be mixed during fermentation, therefore engineering problems concerning agitation that may become necessary with larger volumes are removed. The system operates continuously and so the substrate can conveniently be prepared in small volumes and continuously pre-processed for fermentation.

Other practical scale up considerations such as the supply of substrate and other adjuncts, are not limiting factors due to the commercial availability of these products. There are no wild inputs or other necessities which are variable or in any way limiting. This chapter details the performance characteristics of scaled-up perfusion tower bioreactors and discusses the impact of the scale-up process.

5.4 Experimental Methods

5.4.1 Yeast Strain

An alcohol producing strain of *Saccharomyces cerevisiae* supplied by Makana Meadery (Grahamstown, South Africa) as in chapter 4.

5.4.2 Substrate

Wildflower honey was procured from Swans (Albany, Maine) and was diluted to a concentration of 220 $g.L^{-1}$. The medium typically consisted of 14.0 ±1.3 $g.L^{-1}$ sucrose, 80.1±7.5 $g.L^{-1}$ glucose and 125.7 ±11.3 $g.L^{-1}$ fructose. To this was added 0.25 $g.L^{-1}$ yeast nutrient (Crosby and Baker, Westport, Massachusetts, United States, 7344B), 0.05 $g.L^{-1}$ $CaCl_2.2H_2O$, 0.25 g. L^{-1} $MgSO_4.7H_2O$, 0.25 g. L^{-1} KH_2PO_4 and 0.50 g. L^{-1} $(NH_4)_2.SO_4$. The medium was then adjusted to pH 4.5 with $CaCO_3$ (Crosby and Baker, Westport, Massachusetts, United States, 7325B) and tartaric acid (Crosby and Baker, Westport, Massachusetts, United States, 7395B).

5.4.3 Support Matrix

Thirty-five litres displaced volume of 2 mm^3 ginger root cubes were prepared and repeatedly boiled in water until ginger flavour could no longer be tasted in the water as in 4.2.4.

5.4.4 Pilot-Scale Reactor Inoculation and Fermentation Process

Four pilot-scale glass tower perfusion bioreactors, each with a total volume of 13 L were sterilised and loaded with 1, 960 mL displaced volume of ginger root cubes. Cell growth cultures were then pumped into the tower bioreactors through the inlet. Substrate was then pumped continuously through the inlet at a flow rate of 18.0 $mL.min^{-1}$ ($D = 0.105\ h^{-1}$) using Watson-Marlow variable speed peristaltic pumps. The fermentation took place in a climate controlled room at a temperature of 22°C.

Figure 5.1 Photograph of the experimental apparatus; (A) pilot-scale PTBs, (B) commercial-Scale PTB.

5.4.5 Commercial-Scale Reactor Inoculation and Fermentation Process

A commercial scale stainless steel tower perfusion bioreactor with a total volume 210 L was constructed from 316 stainless steel. This was loaded with biomass collected from four pilot-scale bioreactors, and 26.8 L displaced volume of ginger cubes. Substrate was then pumped continuously through the inlet at a flow rate of 155.0 $mL.min^{-1}$ ($D = 0.054\ h^{-1}$) using a Watson-Marlow variable speed peristaltic pump.

5.4.6 Analytical Methods

From the pilot-scale reactors, 500 mL samples of product were taken from the reactor outlets every 24 h for 37 days. These samples were tested for sugar content through specific density measurement using a hydrometer (Richter and Tralles), and alcohol through ebulliometric analysis (Richter and Tralles Spirit Hydrometer). Samples of product (1.5 mL) were also taken from the outlet in triplicate for HPLC analysis from the pilot scale reactors and the commercial scale reactor. The final 10 days of samples, close to steady state conditions, were filtered using 0.22 μm syringe filters and stored inverted at -20°C prior to dilution and analysis. High performance liquid chromatography was used to quantitatively analyse carbohydrate and alcohol content in the substrate and product samples. The HPLC methodology and equipment used for analysis was that developed in chapter 3.

5.4.7 Fermentation Parameters

The fermentation parameters and yields were calculated at the steady-state average of the fermentation. The ethanol volumetric productivity for the full reactor volume (Q_P, $g.L^{-1}.h^{-1}$) and the ethanol hydraulic volumetric productivity (Q_{hp}, $g.L^{-1}.h^{-1}$) were calculated as the ratio between the concentration of ethanol in the product (P_f $g.L^{-1}$) to the duration of the fermentation (t, h). The yield of ethanol on consumed sugar ($Y_{P/S}$, $g.g^{-1}$) was defined as the concentration of ethanol in the product (P_f $g.L^{-1}$) over the change in the sugar concentration between S_0 ($g.L^{-1}$) and S_f ($g.L^{-1}$); the starting and the final sugar concentrations, respectively. The sugar conversion percentage is defined as change in the sugar concentration between S_0 ($g.L^{-1}$) and S_f ($g.L^{-1}$) over S_0 ($g.L^{-1}$). The efficiency of sugar conversion to ethanol (η, %) was defined as the yield of ethanol on consumed sugar ($Y_{P/S}$, $g.g^{-1}$) over Y_{th} is the theoretical maximum value of $Y_{P/S}$ (0.51 g. g^{-1}) (Pacheco *et al.* 2010).

5.5 Results

5.5.1 Continuous Fermentation

Continuous fermentations were carried out to investigate the operational performance and stability of the yeast cells in the perfusion tower bioreactor systems. Figure 5.2 shows the increase over time in ethanol concentration and the corresponding reduction in product density in the pilot-scale bioreactors as the biomass grows from the inoculation culture to take advantage of the high level of available sugars.

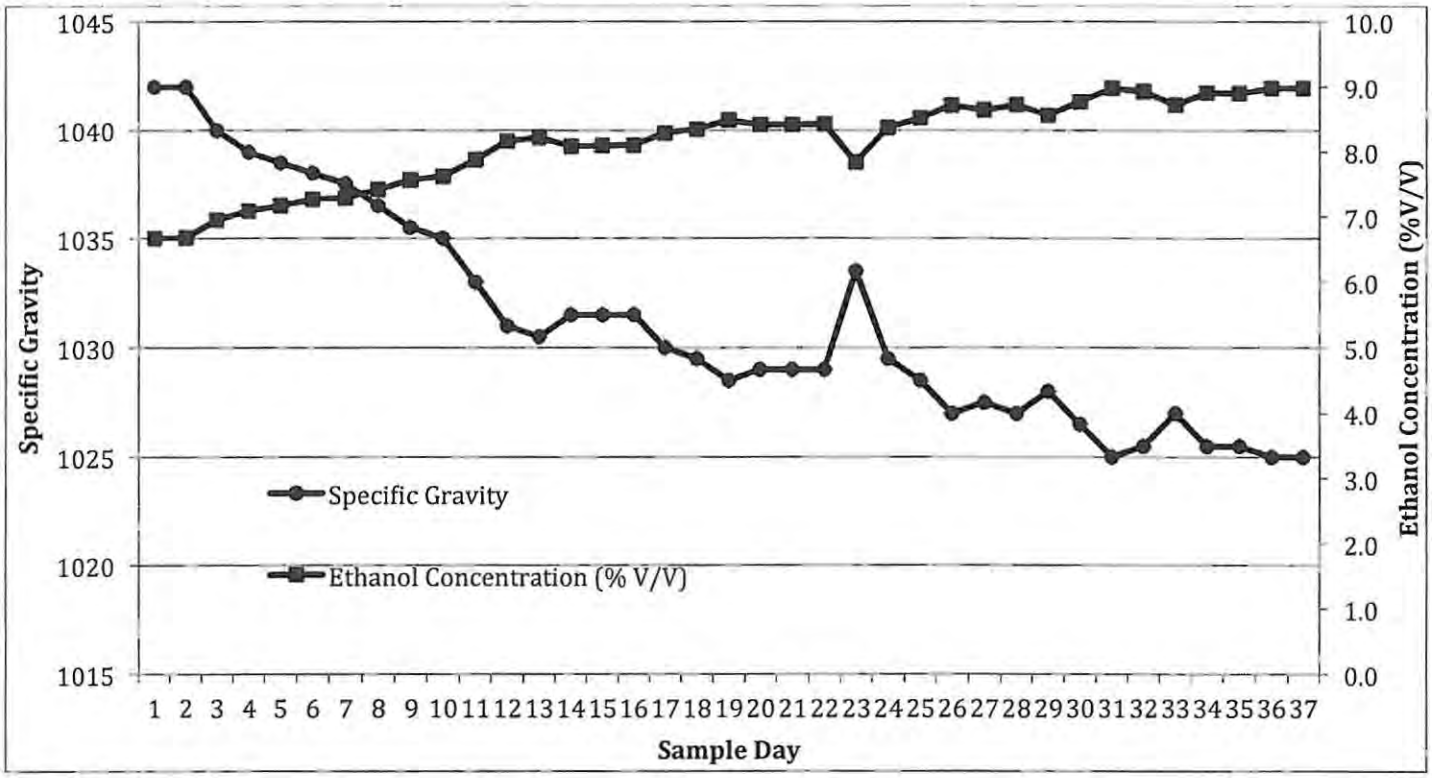


Figure 5.2 Continuous fermentation average (n=4) outlet specific density and ethanol concentration averages (n=4); SG decreases over time ethanol content by volume increases over time. $\frac{dy}{dx} \rightarrow 0$ for ethanol concentration and for specific gravity as steady state conditions are approached.

The maximum attenuation of sugar achieved by the pilot-scale bioreactor systems during the period of analysis was 202.02 ±6 $\times 10^{-3}$ g.L^{-1}, or 97.75%. The average percentage attenuation across the four pilot-scale reactors at steady state was 96.82 ±0.18%, or 200.10 ±0.36 g.L^{-1}. The highest ethanol concentration achieved in the outflow from a single pilot scale fermentation reactor was 76.32 ±0.02 g.L^{-1}, and a 10 day average at steady state operation across all four pilot-scale reactors was 70.02 ±0.99 g.L^{-1}.

The maximum attenuation of sugar achieved by the commercial-scale bioreactor system during the period of analysis was 200.25 ±0.17 $g.L^{-1}$, or 91.02%, with the average attenuation at steady state being 198.33 ±1.20 g. L^{-1}. The maximum ethanol concentration achieved during the operational period was 64.44 ±1.33 $g.L^{-1}$. The average ethanol concentration from the commercial-scale bioreactor at steady state was 61.40 ±1.75 $g.L^{-1}$.

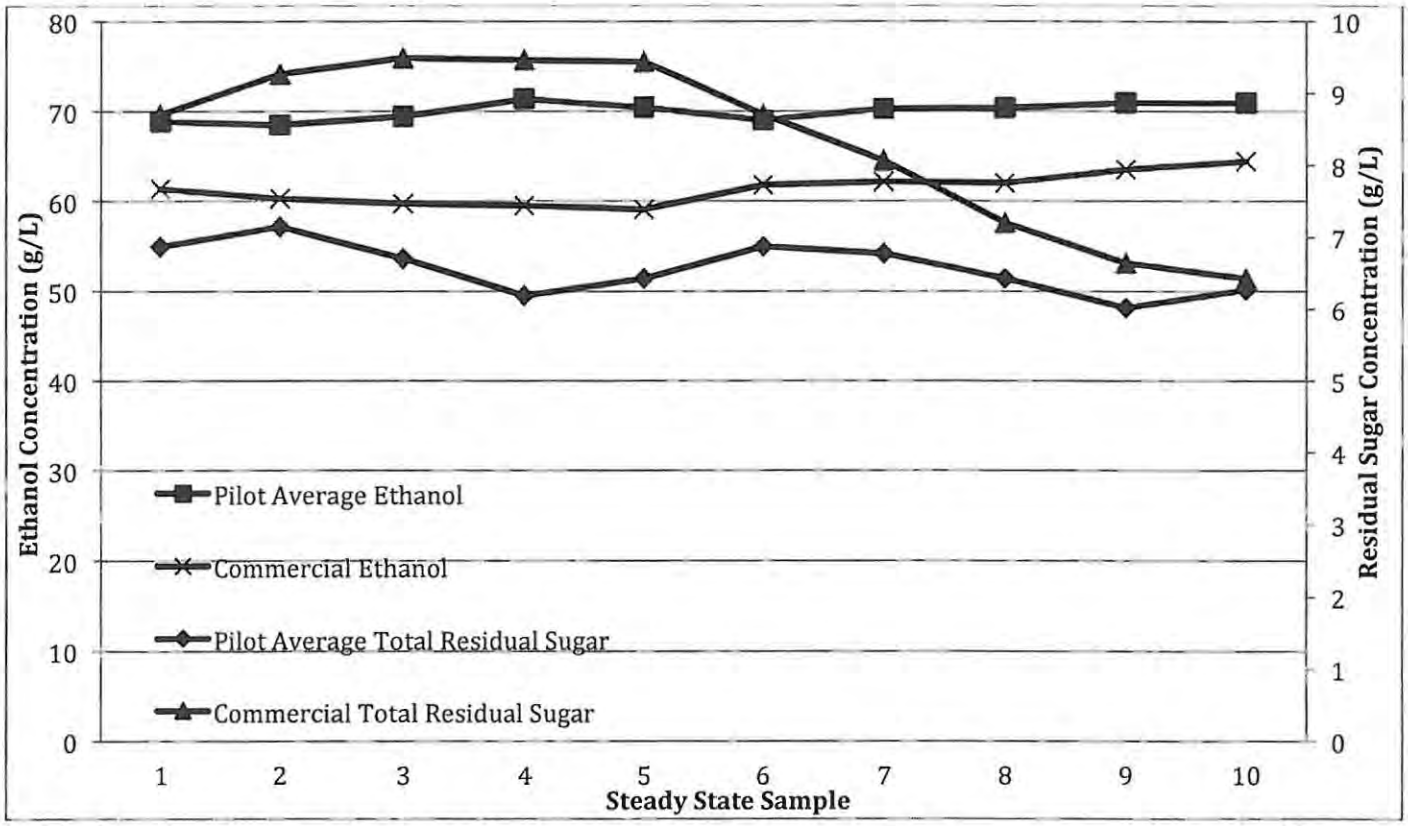


Figure 5.3 Steady state average ethanol concentration and residual total sugar concentration for pilot and commercial PTBs.

From the data shown in figure 5.3 it was evident that the average yield of ethanol was consistently higher in the pilot scale fermentation systems than in the commercial-scale system. The commercial scale fermentation system also appears to have a wider fluctuation in residual sugar concentration, however, the pilot-scale fermentation systems may undergo similar fluctuations in total residual sugar concentrations but as only an average is shown, this effect is concealed. There is an average standard deviation of 1.17 g. L^{-1} between the residual sugar concentrations of the four pilot-scale bioreactors, so a greater a degree of

fluctuation does exist in the residual total sugar concentrations of the pilot scale bioreactors than can be seen in figure 5.3 and figure 5.4.

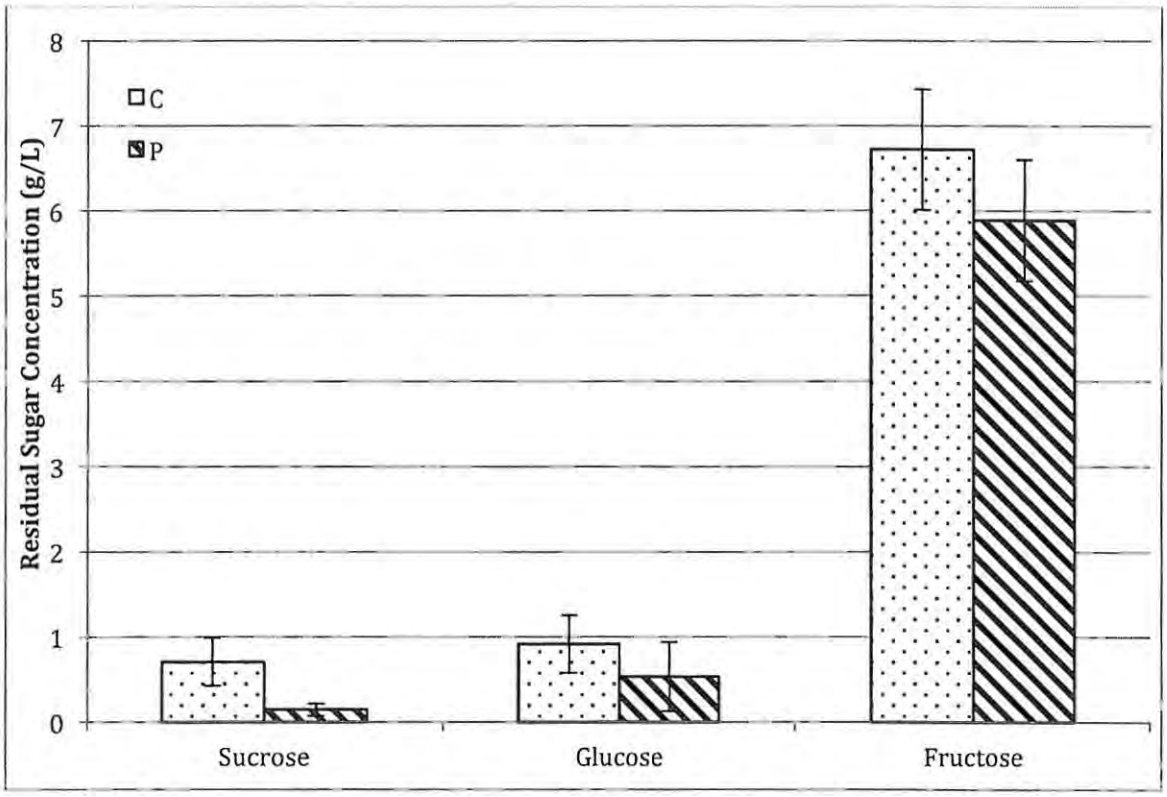


Figure 5.4 Residual sugar concentrations at steady state for pilot-scale bioreactors (P) (average, n=4), and for the commercial-scale bioreactor (C).

The data displayed in figure 5.4 shows that the residual levels of fructose were significantly higher than those of sucrose or glucose for both process scales. Residual sucrose levels in the commercial scale bioreactor were slightly elevated compared to the residual sucrose concentrations in the pilot scale bioreactor systems. Residual glucose levels were also marginally higher in the commercial scale fermentation in comparison to the residual glucose levels in the pilot scale system.

5.5.2 Scale-Up and Bioreactor Performance

The fermentation performance and yields were calculated at steady state, after continuous operation of both systems. Table 1 summarises the values obtained for key performance ratios for steady-state operation of the respective bioreactor systems.

Table 5.1 Key performance values for steady state bioreactor operation.

Parameter	Bench-Scale Average (n=2)	Δ	Pilot-Scale Average (n=4)	Δ	Commercial Scale
P_f (g. L^{-1})	75.57	-5.55	70.02	-8.62	61.40
Q_p (g. $L^{-1}.h^{-1}$)	3.41	2.43	5.84	-3.16	2.68
Q_{hp} (g. $L^{-1}.h^{-1}$)	4.95	2.42	7.37	-4.11	3.26
$((S_0 - S_f) / S_0) \times 100$ (%)	94.52	3.57	90.95	-0.80	90.15
$Y_{P/S}$ (g. g^{-1})	0.470	0.121	0.349	-0.040	0.309
η (%)	92.23	23.65	68.58	-8.12	60.70

Each pilot-scale bioreactor at steady-state produced 26 L of product containing an average of 70.02 ±0.99 $g.L^{-1}$ ethanol in 24 hours. The average ethanol volumetric productivity for the full pilot scale reactor volume was therefore 5.84 $g.L^{-1}.h^{-1}$ and the average ethanol hydraulic volumetric productivity was 7.37 $g.L^{-1}.h^{-1}$. The average yield of ethanol on consumed sugar ($Y_{P/S}$, $g.g^{-1}$) was 0.349 $g.g^{-1}$, with an average sugar conversion percentage average of 90.95%. The efficiency of sugar conversion to ethanol was 68.58% of the maximum theoretical yield.

The commercial scale bioreactor produced 220 L of product containing an average of 61.40 ±1.75 $g.L^{-1}$ ethanol in 24 hours. The average ethanol volumetric productivity for the full pilot scale reactor volume was therefore 2.68 $g.L^{-1}.h^{-1}$ and the average ethanol hydraulic volumetric productivity was 3.26 g. $L^{-1}.h^{-1}$. The average yield of ethanol for consumed sugar ($Y_{P/S}$, $g.g^{-1}$) was 0.309 $g.g^{-1}$, with an average sugar conversion percentage average of 90.15%. The efficiency of sugar conversion to ethanol was 60.70% of the maximum theoretical yield.

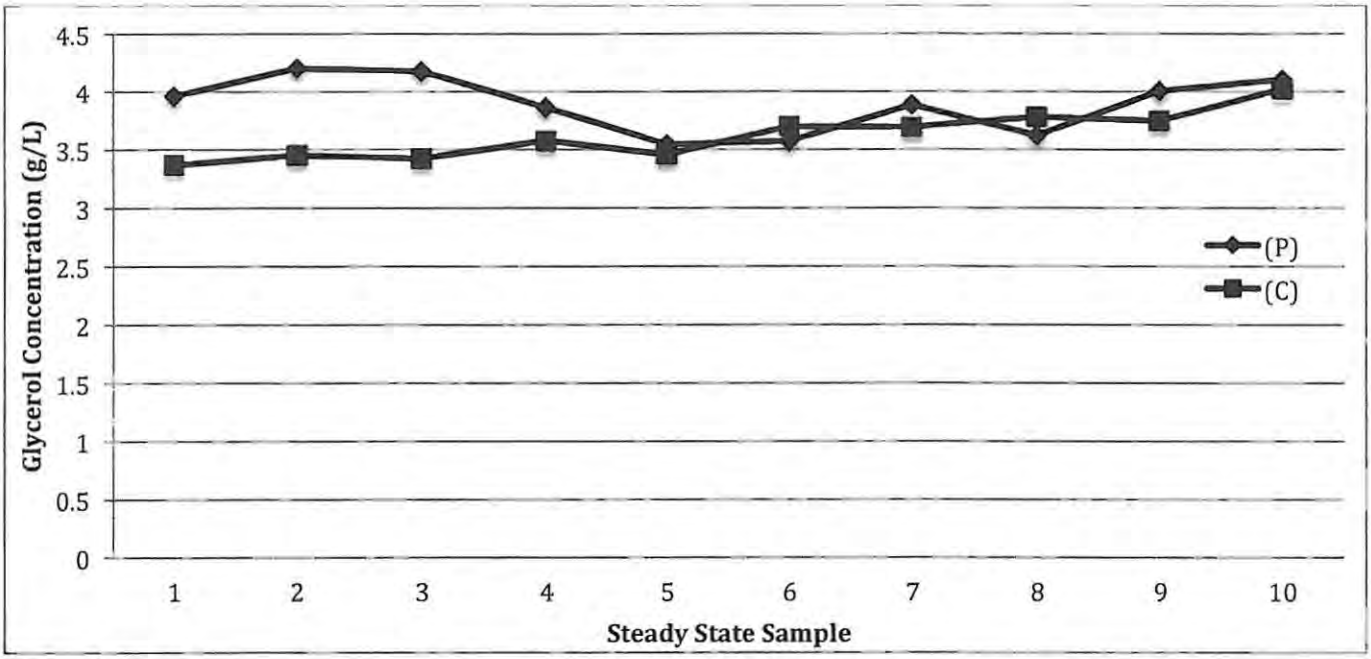


Figure 5.5 Average effluent glycerol concentrations at steady state for pilot scale bioreactors (P) (n=4), and for the commercial scale bioreactor (C).

The average glycerol content at steady state for the pilot scale bioreactors was 3.89 ±0.24 $g.L^{-1}$, and the glycerol content at steady state for the commercial scale bioreactor configuration was 3.62 ±0.20 $g.L^{-1}$ (Figure 5.5).

5.6 Discussion

5.6.1 Continuous Fermentation

During bioreactor operation, the attenuation of carbohydrates becomes more complete over time (Figure 5.2). This is predicted by the increase in catalytic biomass hold-up within the system from yeast population growth towards a maximum level determined by shear and biofilm/floc stability, ultimately determining the balance between yeast growth and washout rate.

Relative average glucose depletion in the pilot-scale fermenters was 7.06% greater than fructose depletion, in the commercial scale reactor this difference was 7.22%. The disparity between fructose and glucose utilisation is typical as glucose metabolism is energetically favoured over fructose consumption and glucose uptake is active, whilst fructose uptake is passive (Sussman *et al.* 1980). The specific attenuation of

fructose however was greater at 112.51 g. L^{-1}, than glucose at 85.81 $g.L^{-1}$ at the commercial-scale, and 113.62 $g.L^{-1}$ and 86.48 $g.L^{-1}$ respectively for the pilot-scale bioreactors. This supports the model predicting that the metabolic adaptation of yeast sub-populations to their microenvironments over time results in more efficient sugar conversion. It is hypothesised that the yeast sub-populations closest to the bioreactor inlet consume the majority of the glucose, leaving the remaining biomass to adapt to fructose and sucrose consumption. In a free cell system where biomass can easily translocate, the overall sugar attenuation might be significantly lower given a similar active volume and dilution rate than that exhibited in the perfusion tower bioreactor because cells will preferentially metabolise available glucose and remain unable to adapt to take advantage of all accessible carbohydrates; diauxic growth would be observed given an appropriately low dilution rate. It is proposed that this immobilised cell system's biomass population is able to become more efficient at processing available energy sources over time through the development of niche metabolic regulation strategies for specific microenvironments along the axial dimension of the packed bed volume, and should therefore conform closely to the plug-flow model for a three reactant system. In order to test this hypothesis it would be necessary to sample the fermentation broth at various heights in the paced bed region, data collection to test this hypothesis was not completed for these reactors, however. Smukalla *et al.* (2008) demonstrated that populations of yeast cells do adapt to become more efficient at processing available nutrient sources.

The highest product ethanol concentrations were associated with low residual sugar in the product. These sugars are primarily converted to ethanol during fermentation, and thus an increase in utilisation of sugars should lead to an increased product ethanol concentration. The results shown in figure 5.3 do not always show a direct correlation however, and the lack of correlation is more pronounced when the results of individual bioreactor are observed (Appendix E). A study conducted by Bai *et al.* (2004) found that during extremely active fermentation, ethanol accumulation in the immediate environment of yeast cells causes a loss in activity due to ethanol's toxic effect on yeast cells. Dombek and Ingram (1986) suggested that yeast cells do not necessarily accumulate ethanol internally, but that because of diffusion, internal and external ethanol concentrations are normally similar to each other. It is plausible to expect that the specific productivity of a bioreactor will fluctuate as the yeast bed exhibits periods of very active ethanol production, followed by periods of inactivity when ethanol diffuses rapidly from the cells to the passing media. The decrease in activity would lead to an increase in substrate concentrations which is observed (Figure 5.3) The high substrate conditions in higher regions of the packed bed of a given bioreactor in addition to the increased ethanol concentration due to its rapid diffusion from cells could accentuate this effect.

Beuse *et al.* (1998) studied the oscillatory behaviour of *S. cerevisiae* cells in continuous culture. They observed that at certain points in the cell cycle, yeast cells store carbohydrates, which they later catabolise. The presence of ethanol, together with changes in available fermentable sugars, initiate changes between resting states and cell growth states in continuous culture (Beuse *et al.* 1998).

A potential metabolic explanation for a higher than theoretically possible ethanol yield occasionally observed in tower bioreactors is that during oscillations in cell growth and stored carbohydrate metabolism, ethanol is produced from these stored sugars. Higher than 0.51 g. g^{-1} yield was not observed in any of the experiments conducted in this research, most probably due to the large number of equivalent samples taken which exerted a moderating effect on the consolidated data. Nedovic *et al.* (2000) demonstrated an efficient continuous cider fermentation using Ca-alginate gel entrapment of yeast and *Leuconostoc oenos* cells within a conical glass bioreactor. The data presented indicated a 95% attenuation of the 71g.L^{-1} total sugar and a nominal ethanol concentration value of 5% ABV (Figure 3A) was reported, this translates to 39.45 g.L^{-1}, 14% higher than the theoretical maximum yield showing that in some cases it is possible to observe extraordinary ethanol yields.

In addition to the positive effects of metabolic regulation on reactor productivity, the selection pressure exerted in favour of yeast strain variants that are better able to metabolise available carbon sources and tolerate high ethanol concentrations will result in a steady improvement of the productivity of the bioreactor system over time. A corollary of this effect is that the resulting product will change over time as new genetic variants of the microorganism colonise the bioreactor. The assumption made here is that change in the product must have a negative effect, but in reality, if an increase in ethanol productivity is the direction selection pressure dictates, a decrease in the production of secondary metabolites is another effect. Regardless, this is a very long-term consideration, and the overall benefits obtained in productivity more than offsets any downtime required to re-inoculate a bioreactor with a stock microorganism.

5.6.2 Scale-Up and Bioreactor Performance

The values obtained for performance values were significantly reduced for the commercial-scale compared to with the pilot-scale system. In particular the productivity values obtained for the respective systems were markedly different (Table 1). A loss of 54.11% in productivity per unit total volume of the bioreactor in the 16× scale-up is observed, suggesting that regimes other than the volume of the packed bed are

limiting factors in the productivity determination of this bioreactor set-up. The ethanol yields and conversion efficiencies, however, are very similar across both systems considering the large change in the bioprocess scale, indicating that a significant fraction of the scaled up bioreactor volume is redundant, or non active in the bioprocess. This supports the conclusions drawn from the fermentation modelling data in chapter 4 (4.3.4). This additional non-packed bed volume plays an important role in gas capture and biomass retention, however. It is highly probable therefore that the productivity figures in this case are misleading when taken as an indication of successful scale up, as the feed rate is not at its capacity. Loss in productivity as scale increases may have been due to a loss in relative radial mixing (lower aspect ratio), effectively reducing the activity of biomass immobilised close to the bioreactors walls. This could be alleviated somewhat with an improved inlet design which disperses the substrate more uniformly across the radial dimension. Challenging this hypothesis however is the feed inlet to packed bed diameter ratio for the respective bioreactors, which was reduced from 10:1 to 6.25:1 during scale up. It is more likely that the reduction in productivity was linked to the increased superficial flow rates experienced in the commercial scale bioreactor compared with those of the pilot scale bioreactor (Appendix B and C).

The similar yield, sugar conversion and specific product ethanol concentration figures indicate that the micro-kinetics of the bioprocess have not been significantly adversely affected by the scale-up process. The performance results from the bench-scale fermentations (Chapter 4) are interesting as comparisons to the larger scale fermentations (Table 5.1), however considering that a different substrate was used for the experiments of chapter 4, it may not be meaningful to compare these results.

The observed reduction in ethanol yield is not in accordance with findings by de la Rosa *et al.* (2003). They found that the yield increased by a small amount from the pilot to industrial scale. However it is worth noting that the reactors used at each respective scale in their study were not of the same design, and therefore it is possible that the industrial bioreactor design was superior to that of the pilot bioreactor in that instance.

The average glycerol production in the pilot scale bioreactors over the observed period was 0.27 $g.L^{-1}$ higher than the production of glycerol in the commercial scale fermentation over the same period. The variation between the average glycerol production between the four pilot scale bioreactors was not substantial (Appendix E), with a relative standard deviation of only 4.74%. Additionally the trend of reduced glycerol output is supported by the data obtained in chapter 4, although the use of a different substrate limits this comparison. Glycerol plays a role in osmoregulation (Nevoigt and Stahl, 1997) and in

the cellular redox balance (Bakker *et al.* 2001) and has been shown to augment yeast stress tolerance (Bideaux *et al.* 2006). Nevoight and Stahl (1997) showed that when yeast cells were placed in a hypertonic solution, the response to the resulting water loss was to accumulate compatible solutes within the cell, allowing enzymes and other cellular components to continue functioning.

As discussed in chapter 4 it is likely that the synthesis of glycerol in the lower regions of the tower bioreactor is an important survival mechanism for the yeast cells, and the lower glycerol production rates observed may be attributable to a proportionally smaller population of biomass exposed to osmotic stress at larger scales. This could be caused by a larger total biocatalyst population enabled by the surplus carrier matrix and/or the lower superficial liquid velocity experienced within the commercial scale bioreactor (Appendix C).

The virtues of continuous fermentation have been widely discussed and investigated for use in biofuel production (Najafpour *et al.* 2004; Kumar *et al.* 2011; O'Brien and Craig, 1996), however it has not been seriously considered for use in potable ethanol production for some time. Clement *et al.* (2011) demonstrated a multistage continuous bioreactor set-up as a model for the traditional fermentation process in which the physiology of the microorganisms at different stages in their lifecycles could be studied. Takaya *et al.* (2002), Kourkourtas *et al.* (2002), Nedovic *et al.* (2000) and Okabe *et al.* (1994), have all demonstrated small-scale continuous fermentation systems of various types for potable alcohol production, none of these models has yet seen widespread use in the beverage industry. One form of the continuous fermentation model has seen successful commercial usage; the continuous stirred tank reactor model (chemostat) has been used extensively in the sugar industry (Guidoboni, 1984; de Vasconcelos *et al.* 2004). The use of the tower fermentation model remains rare, however.

A major objection to the use of continuous tower fermentation bioreactor, as first proposed by Royston (1966) is that these towers could only operate for a definite but undefined period of time due to the mutation of the organism used, creating better adapted populations that will eventually displace the parental type. Ultimately this would lead to a product that had different characteristics and so re-culturing would be necessary at some point. Restarting a tower fermenter involves careful sterilisation and a period during which non-ideal product is being produced for up to 3 weeks. This substandard product would need to be blended with the later 'standard' product. The apparent assumption that this would result in a non-viable process has not been assessed. As previously discussed, it is unlikely that this consideration would adversely affect the fermentation system.

Stanbury and Whitaker (1984), list the following as the major disadvantages of continuous tower fermentation: The complexity of the plant required, the need for more highly skilled personnel relative to traditional methods, the long time required for changing from the production of one beer to another, and finally the difficulty of taste matching. Each of these assumptions can be challenged, and a number of them are axiomatically flawed. The first two disadvantages are clearly one and the same. Complex equipment requires skilled staffing. However it is difficult to follow the logical pathway that suggests that somehow a single pump and heat exchanger is in some manner more complex than the equivalent in batch fermentation. Even in the case of beer production, continuous fermentation from a commercial perspective does not require any additional skills or complex equipment, indeed at any scale, one could argue that it simplifies the process of beverage production.

The second issue, that of needing long periods of time to shutdown and restart the process to make a different beer is self-evidently false: if one were manufacturing two beers, one would have a minimum of two continuous fermenters. Why produce in series, when the continuous system clearly lends itself to producing in parallel?

Many of the problems associated with the scale up of bioreactor systems such as mass and heat transfer are eliminated by the use of a continuous system. The well-known philosophical tool Occam's razor can be rephrased into a biotechnological context as, "systems should be no more complicated than really necessary" (Wiseman, 1983). The bioreactor system used in this study may be mathematically too complex to model, but it is fundamentally very simple. Many of the scale up problems typically encountered in industrial processes are avoided though the use of such a simple solution.

5.6 Conclusions

This chapter demonstrated that a high performing pilot-scale continuous fermentation system could be successfully scaled-up to commercial-scale, using a simple regime analysis and plug-flow model. An immobilised continuous tower fermentation system suitable for commercialisation producing mead with acceptable post-primary fermentation organoleptic properties was demonstrated. Considering the low construction and operating costs, its scalability and simplicity, this fermentation strategy is an attractive option for the production of potable ethanol for the market place. The technology transfer considerations of the perfusion tower bioreactor will be considered in chapter 6.

Chapter 6

Perfusion Tower Bioreactor Technology Commercialisation

6.1 Introduction

An array of business models might be appropriate in the commercialisation of the perfusion tower bioreactor developed in chapters 2, 4 and 5. The PTB to some extent manifests all of the characteristics listed below:

- Low capital equipment cost per unit capacity
- Low energy requirements
- Ease of operation
- Low risk of contamination
- High ethanol productivity
- Versatility
- Low physical footprint
- Low maintenance

A fermentation technology with these characteristics has the potential to disrupt the beverage alcohol market. In this chapter the potential of the technology and an appropriate business model will be discussed, and a commercialisation strategy allowing for the transfer of this technology into the marketplace will be considered.

For the purposes of this discussion the production of a cider beverage for the South African market in the manner described in chapter 4 will be considered. The reason for this (as discussed in section 6.3) is that a draught cider beverage has been identified as a product with an unexploited market. All inputs, costs and markets discussed will be local. Due to this the results and conclusions drawn will be anecdotal; but it is hoped that the example is translatable to other markets. Currency will be shown in South Africa Rand (1 ZAR ≈ 0.125 USD).

6.2 Process Economics

The information relating to a process' potential economic viability and profitability is determined through an analysis of the bioprocess, its inputs and products (Ratledge and Kristiansen, 2006):

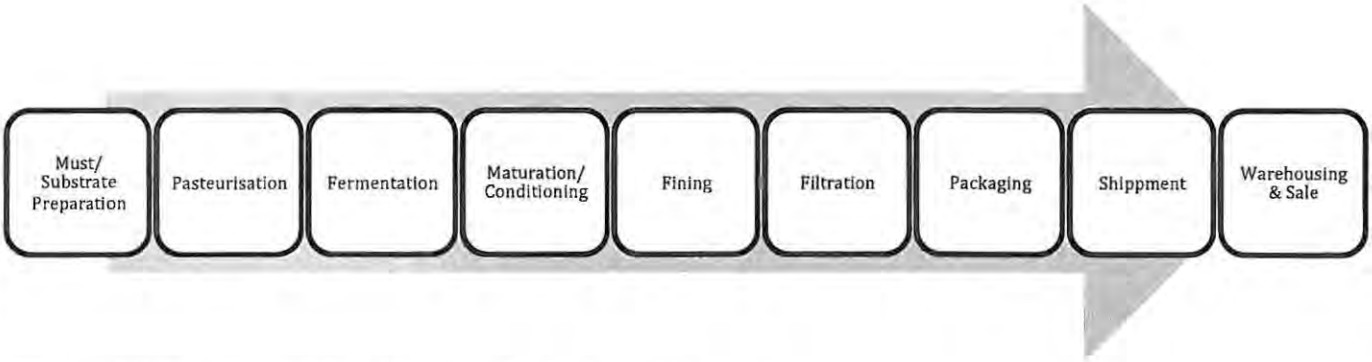


Figure 6.1 Overview of the cider manufacturing process.

Each of these steps require capital equipment, inputs and a specific period of time to be completed.

Table 6.1 Equipment and input requirements for stages in the continuous cider production process.

Stage	Capital Equipment	Rate/Time	Inputs
Substrate Preparation	Storage Tank(s)	1 h (per tank)	Apple Conc., Water, Yeast Nutrient, Conditioning Salts.
Pasteurisation	Peristaltic Pump; In-Line Heat Exchanger	12.5 L/h (× no of lines)	Energy (2 kW), Water
Fermentation	PTB(s)	12.5 L/h (× no of fermenters)	Energy (Temp Control)(6 kW)
Maturation	Storage Tank(s)	168 h	Energy (Temp Control)(6 kW)
Fining	Storage Tank(s) at 4°C	24 h	Energy (Temp Control)(6 kW), Finings.
Filtration	In-Line 0.5 μm Plate & Frame Filter.	200 L/h (× no of units)	Filter Pads (3 per 200 L), Energy (0.5 kW)
Packaging	Beverage Kegs	240 L/h (× no of lines)	Cleaning Chemicals
Shipment	Pallet Jack/Forklift		

Different scales of the filtration, packaging and storage tanks are available. It is rational to use equipment that will have a capacity greater than the maximum volume demands initially expected, as these items have a gentler price curve and are designed for batch throughput (periodic high volume throughput) rather than continuous. Additional fermenters may then be added to meet increased demand with limited additional capital expenditure (CAPEX). A single peristaltic pump and pasteurisation unit can feed up to 8 fermenters. The number of tanks required for post fermentation storage is equal to the number of tanks required to hold the fermentation product for 168 h and 24 h for fining (plus 24 h for cleaning). Therefore (12.5 L × 216 h) = 2, 700 L of storage required, and if 300 L storage tanks are used 9 tanks would be required per PTB.

Table 6.2 Capital equipment cost breakdown.

Equipment	Cost Per Unit (ZAR)*	Capacity Per Unit (L/24 h)	Units Required For 1 L/24 h Capacity
PTB	45, 000	300	3.3333×10^{-3}
Peristaltic Pump	18, 000	2, 400	4.1666×10^{-4}
Pasteurisation Unit	3, 000	1, 200	8.3333×10^{-4}
Storage Tank	1,100	33.3	0.03
Filtration Unit	23, 000	3, 530	2.0833×10^{-4}
Filling Pump	3, 500	5, 700	1.7544×10^{-4}

* Includes VAT, rounded up for simplicity.

The capital equipment cost per unit capacity is shown in figure 6.2.

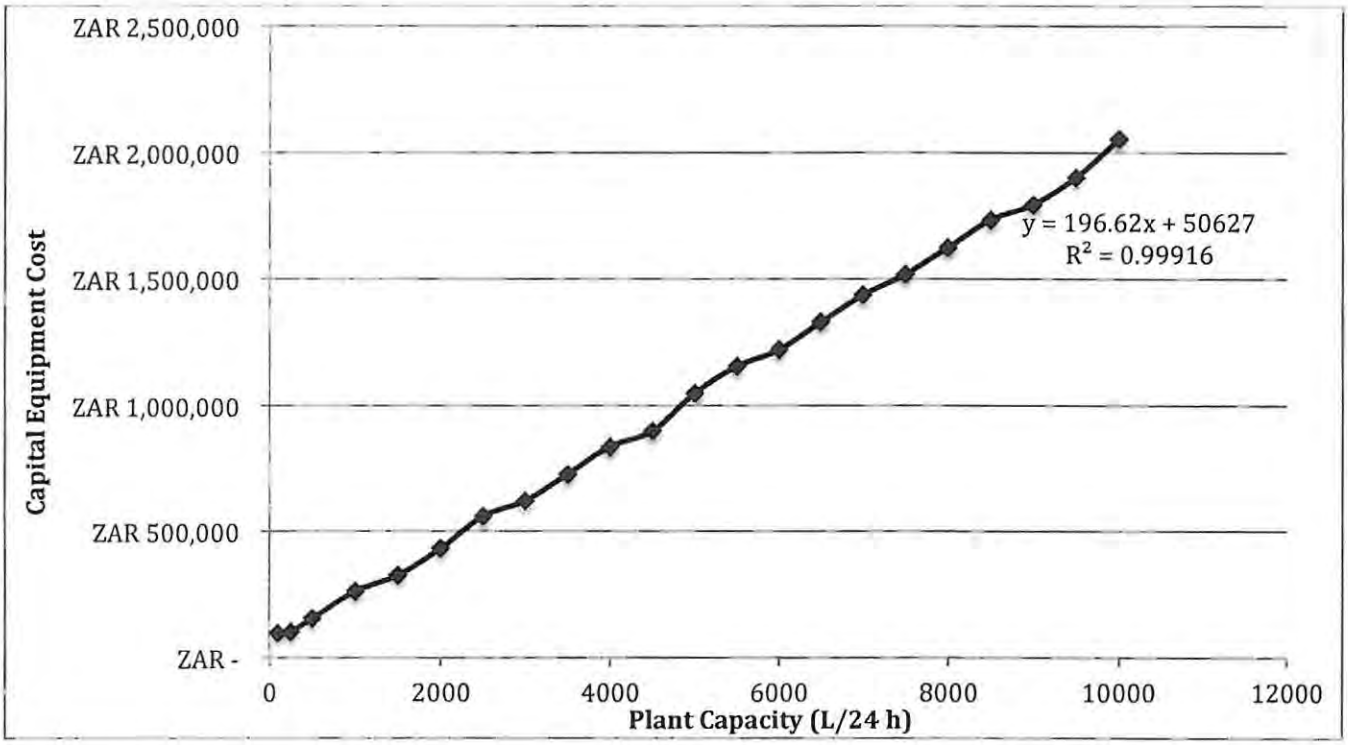


Figure 6.2 Capital equipment cost against required plant capacity.

Prefabricated batch fermentation plants which are marketed to microbreweries typically cost in excess of R900, 000 for a capacity of 100, 000 L p.a. (280 L/ 24 h) (Galitsky *et al.* 2003; Brauhaus-Austria, 2011). This is due to the need for high throughput heat exchange systems and other infrastructure (excluding beer

specific vessels such as mash & lauter tuns), heated vessels for wort boiling, cooling vessels for heat exchange and more tank space than required by the PTB system due to the additional fermentation time. Figure 6.2 shows that for a similar capacity PTB plant only R100, 000 would be required for the equivalent production output; 11% of the cost.

Operational costs include variable costs and fixed costs (Froeb and McCann, 2010). Costs not directly associated with the bioprocess such as legal, administrative, marketing and other fixed costs will be addressed separately in the business development section. Variable costs include those that are directly proportional to volume of product manufactured.

Table 6.3 Bioprocess operational cost breakdown.

Input	Cost Per Unit (ZAR)	Unit Per L	Cost Per L (ZAR)
Apple Concentrate (mL)	0.0105	200	2.10
Conditioning Salts (g)	KH_2PO_4 – 0.78 $(NH_4)_2SO_4$ – 0.26 $CaCl_2$ – 0.022 $MgSO_4$ – 0.018	0.25 0.50 0.05 0.25	0.33*
Yeast Nutrient (g)	0.028	0.25	0.007
Water (L)	0.88	0.03	0.026
Filter Pads	1.03	0.02	0.021
Finings (Kg)	51	0.005	0.255

* Total conditioning salts.

Total variable cost per litre is therefore R 2.74. The largest component of the raw materials cost is the sugar source, followed by the water (Cape Town rates used). Water usage in batch plants is significantly higher, due to the large number of vessels that require cleaning.

Other important operational costs that are not directly proportional to the volume of product produced, include cleaning chemicals, electricity, insurance, staffing and maintenance costs. Cleaning chemicals used will include peroxyacetic acid, and caustic soda as these are generally recognised as safe (GRAS) for use in food manufacturing processes. These will be used in the cleaning and sterilisation of vessels before use, as chemical sterilisation is considerably more energy efficient than steam sterilisation. The total electricity usage will vary depending on operational activity, season and other non-process related factors. However it

is estimated that a facility with related activities will use around 10 KW, costing R3, 450 per month (Cape Town area: R 0.454 per KWh). Wort boiling/must sterilisation and temperature control for larger stored volumes (and potentially computer control systems) translates into higher energy usage.

Maintenance costs are mainly restricted to associated activities, not the process itself as there are very few 'moving parts'. Insurance of all assets including loss of inventory coverage would protect the financial performance of the process in the event of a breakdown.

A batch of substrate mixture will be converted to product and be ready to be shipped within 10 days. Potential 'bottlenecks' are the fermentation stage and the maturation in storage tanks. The volume through both of these rate-limiting steps can be increased with the addition of more fermentation towers and storage tanks.

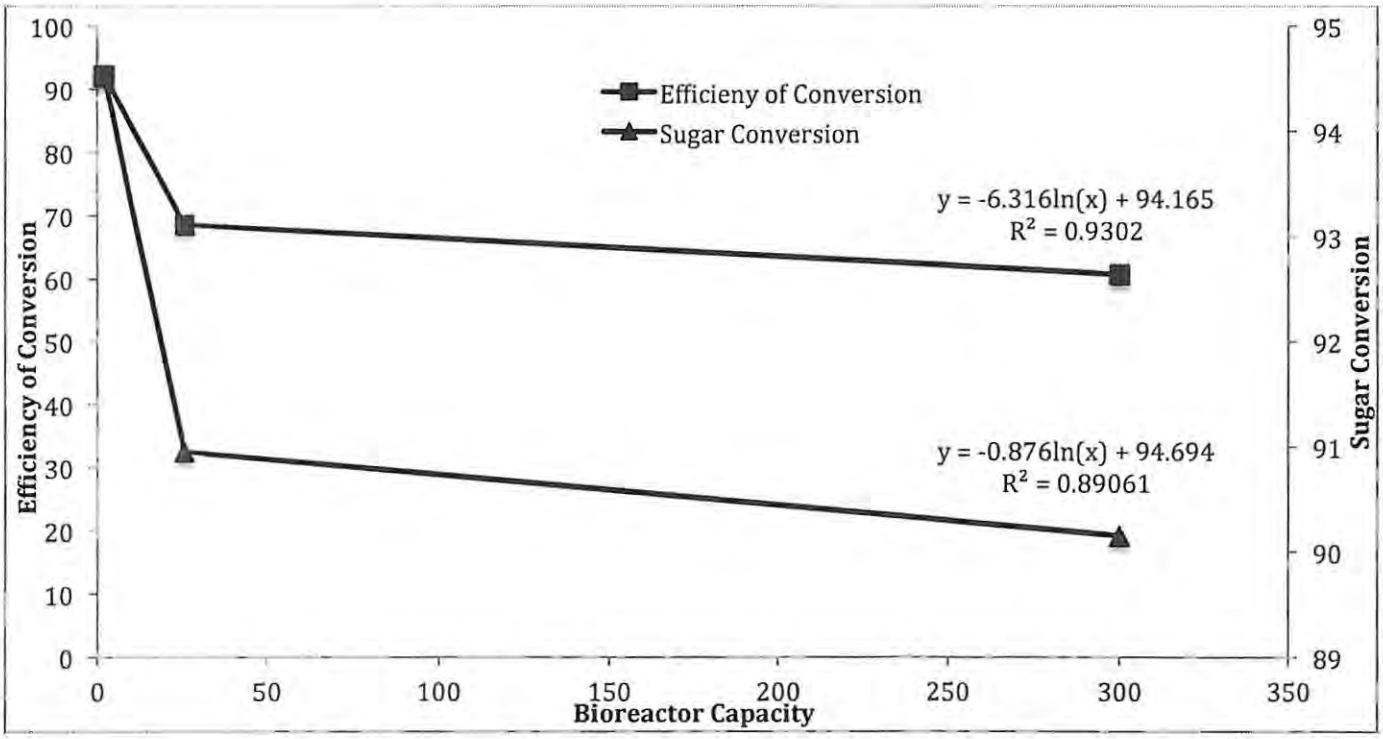


Figure 6.3 Bioreactor conversion efficiency and sugar conversion rates at increasing production capacity.

During the scale up the operational efficiency was reduced at higher scales, most probably due to lack of radial homogenisation. Figure 6.3 indicates that the relative effect of this lack of mixing on performance is inversely related to the size of the bioreactor. We can use this relationship and consider it with the cost of the bioreactor at different scales in order to determine the optimal size of a bioreactor. The cost of a

bioreactor is related to the amount of materials used (assuming a linear cost-volume relationship for the packed bed). The cost of the materials (ignoring any quantity discounts) used can be predicated using the square-cubed law to predict bioreactor surface area, the relationship between area and bioreactor volume is depicted in Figure 6.4. (NB: this uses only information from reactors used in this study; design calculations for larger bioreactors will provide more accurate relationship).

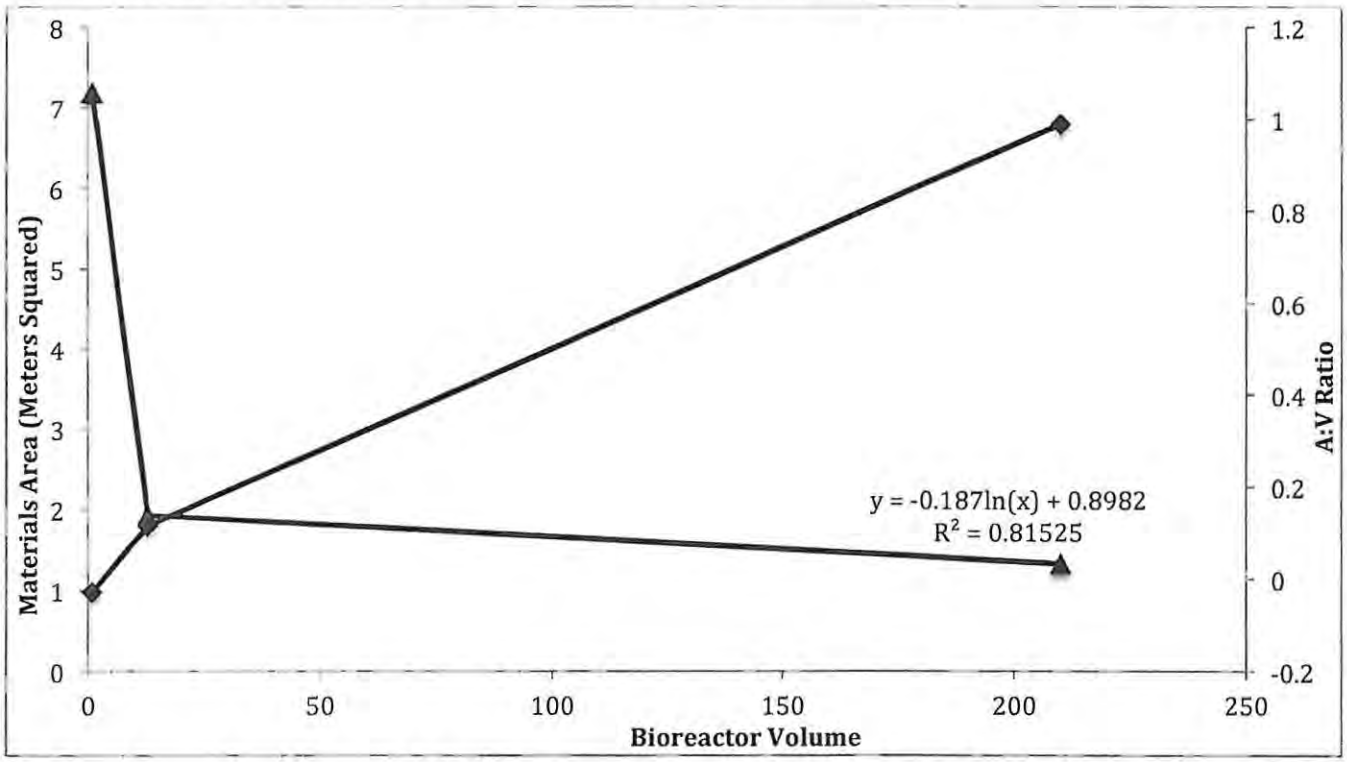


Figure 6.4 Area to volume ratio for PTBs against physical volume.

Area to volume ratio is roughly equal to:

$$-0.187ln(reactor\ volume) + 0.9 \text{ (Figure 6.5)}$$

and reactor capacity is equal to:

$$1.41(reactor\ volume) + 4.08 \text{ (Not shown)}$$

Therefore area of stainless steel materials equals:

$$-0.187 \ln\left(\frac{Capacity - 4.08}{1.41}\right) + 0.9$$

And as the cost of the commercial scale bioreactor (A = 6.79 m²) is R 45, 000 (Table 6.2):

$$Cost\ of\ PTB = \left(-0.187 \ln\left(\frac{Capacity}{1.41} - 2.89\right) + 0.9\right) \times 6627.4$$

Using this rough equation we can show an approximate cost of the bioreactor against scale. Appropriate reactor size will depend on the overall required plant capacity, which will be determined by market demand for the product.

Oversizing is the use of facility processing components that are able to accommodate multiples of the initial plant capacity (Towler and Sinnot, 2008). This saves on capital equipment, as the process components do not require replacing in order to accommodate greater capacity. For example; the use of eight-line peristaltic pumps means production can increase by eight-fold before more equipment is required. The combined total of eight single-line pumps, or two-four line pumps is higher than a single eight-line pump. Thus start-up costs are inflated to a certain extent, but the marginal cost of growth is reduced. Product loss is the reduction of product volume relative to the substrate volume over the course of the process. During this process the steps contributing to significant volumes of product loss are fining and filtration. This loss is inevitable, and amounts to approximately 1.5-2% of total input volume. The initial process is designed such that the total capacity of the process is lower than the predicted market demand, and that sufficient capacity is allowed for fluctuations in demand. The amount of the total capacity in use is referred to the process' occupancy. The occupancy of the initial system might be in the region of 75% (Ratledge and Christensen, 2006), but this figure is subject to the projected demand fluctuation.

Accurate figures for cost effectiveness comparisons between the PTB and batch systems will vary significantly depending on the level of sophistication, scale and specific practices of a given batch brewery. However a plant using the PTB system, with very little optimisation, achieves more favourable CAPEX and energy requirements, it has a lower physical footprint, and requires very little in the way of skilled personnel and maintenance. Therefore the barriers of entry into the brewing industry are lowered.

6.3 Business Development

The business development strategy will briefly be discussed with reference to the general instance of launching one of these local breweries, but a full business plan for the spinout company is available.

A model for a bioprocess has been developed. In order to commercialise it, external factors such as demand, pricing, competition, product positioning and business strategy must be considered. As discussed in chapter 1, 'it is difficult to gain any significant market share in the SA beverage market'. The PTB production process enables the cost effective manufacture of beverages in smaller volumes than was previously viable. This opportunity is best exploited using non-traditional marketing methods, by re-establishing local breweries and 'giving ownership' of the product to the local population.

The overarching key strategic positioning strategy for the business entity arising from this research, will be nurturing localism and allowing the market to take ownership of the brand (what Wipperfurth (2005) called 'brand hijack'). People take pride in their local beverage just as they would their local sports team. Associating the brand with the local culture and its values can further encourage this localism. Strategic considerations have been identified as key to the determination of the success of disruptive technologies (Padgett and Mulvey, 2007; Ronteltap *et al.* 2007).

The product development strategy outlined by Ernst *et al.* (2010), Hart (1996), and Clark and Wheelwright (1994) was followed, managing the technical, marketing and small-scale evaluation breakdown through the duration of the cider experiments (chapter 4) and extending beyond them.

Table 6.4 SWOT analyses for PTB microbreweries

Strengths	**Weaknesses**
-Knowledge specific to each local market -Lower production and capital equipment costs -Unique draught cider product, novel to SA market	- Market information is in part qualitative in the absence of quantitative data. - Potential lack of control over retail pricing.
Opportunities	**Threats**
- Tailor products to local markets - Build on localism - Generate high returns with greater margins - National and globaly applicable model - Building on global trends	- Habitual buyer purchase decisions - Limited control over raw materials supply and production costs - Powerful competition

An increase in the volume of product sold raises net profit almost linearly because increases in fixed costs are not directly proportional to production rates. As fixed costs of individual breweries do not rise exponentially due to the absence of the inefficiencies and value destroyers inherent in large organisations (over-governance, internal politics, lack of proprietorship, risk aversion, legacy issues, embedded mediocrities, HR departments, inflated traditional marketing budgets, sunken cost fallacy, attachment to commodity products, group think etc. i.e. the cost of complexity), the aggregate financial performance tends towards a value far higher than that achieved by traditional competitors. These inefficiencies, which tend to build up over time within large organisations often, outweigh economies of scale seriously damaging the companies' financial performance (diminishing marginal returns) (Froeb and McCann, 2010). These problems are typically absent from small companies. Given that each local PTB plant will not require more than 5-6 staff it may be possible to build a high revenue company with 'small enterprise' efficiency levels. The PTB also enables superior returns on capital expenditure compared to smaller scale traditional breweries and thus outperforms them.

Equity financing is the route most appropriate for this venture. This will be achieved stepwise through the acquisition of a small amount of capital (around R100, 000) to test the product within key representative potential markets which can act as indicators. This capital will be sought though angel investors for a small stake in the future business, and the operations are not intended to achieve break-even. Pilot scale

equipment will be used to produce around 5, 000 L of a product, and brands will be seeded and sold for a trial period.

If sales reach predetermined targets and market acceptance is deemed to be likely, this financial track record and additional information acquired during trials will be used to secure venture capital in the amount required to achieve the required internal rate of return (IRR), i.e. if higher IRR rates are required more facilities/consumers will be required initially. Once capitalisation reaches a sufficient level in domestic markets, foreign markets might be considered and private equity may be sought to finance growth.

6.4 Conclusions

A brewing company based on the PTB system developed during this research would be a daring economically innovative and highly profitable venture. The strategies outlined during this chapter dramatically reduce traditional barriers of entry; they are less capital intensive, use significantly less energy, wasting fewer resources and enable high efficiencies and financial performance. These advantages allow for exceptional financial performance and rapid growth – potentially into new markets and sectors.

The business model and concept is transportable, capable of replication and scalable. The production process and strategy may be able to be duplicated for other niche markets, products and locations both nationally and internationally.

Chapter 7

Conclusions and Future Work

7.1 Conclusions

It was hypothesised in chapter 1 that a cost effective fermentation strategy, able to disrupt the beverage alcohol market, could be strategically developed and capitalised upon in an effective manner using a combination of interdisciplinary proficiencies. As the work progressed, several objectives were met: the identification of key characteristics of a disruptive technology able to break the monopolies that exist in the alcoholic beverage market were identified. Bioreactor configurations and fermentation modes were considered and an appropriate design with the potential to enable the required characteristics was developed. This design was tested at a bench scale and was found to be easily operated and had acceptable performance characteristics. This bioprocess was then scaled up to a commercial capacity and did not lose any of its favourable characteristics. The economics pertaining to the process developed were then characterised and discussed, and an effective business model and strategy were compiled for the commercialisation of the PTB process.

Basic chemical engineering concepts, biological principles and business development skills are required for the development of this biotechnological and bioentrepreneurial process. It has been shown that the procedure of technology transfer from universities to industry need not be difficult to achieve, as long as an interdisciplinary skills are nurtured within the sciences.

7.2 Future Work

The PTB ethanol production process makes use of a *Saccharomyces cerevisiae* population as the catalytic biomass with which to affect a bioconversion from simple carbohydrates to ethanol and carbon dioxide. There are two key characteristics of the natural yeast used in the PTB that make it operate favourably: inherent flocculation and a high degree of ethanol tolerance.

Each industrial application of yeast has traditionally required years of selection and breeding in order to create a strain highly suited for that particular purpose, following the biotechnology revolution however, recombinant DNA techniques meant that new strains could be created specifically for a purpose in a matter of months. Following Bidard *et al.*, (1995) who identified *FLO1* as the gene encoding the cell surface adhesin protein (or flocculin) responsible for yeast flocculation it has become increasingly feasible to manipulate this behaviour of yeast cells. Until very recently researchers have been primarily interested in creating strains that were strongly NewFlo type flocculent, that is; flocculent only in high ethanol concentrations or in low sugar conditions which would be beneficial for the clarification of batch fermented beverages or reducing downstream processing costs in batch fuel ethanol production.

For the PTB however, strongly Flo1 type flocculent strains are of interest, as this retains catalytic biomass within the PTB system and reduces post-fermentation processing costs. A review by Zhao and Bai (2009) touched on the potential benefits of the combination of inherent flocculation and continuous culture, and suggested that additional research into this area should be conducted. Furthermore, Choi *et al.* (2010) used recombinant DNA techniques in order to create a yeast strain (CHFY0321) with characteristics tending towards those of the yeast used in this study.

The yeast used in this research had the benefit of several years of artificial selection within the reactor system and is therefore has good genetics to build upon. Future work that may be conducted would expand our knowledge of this yeast's genotype variations and regulatory differences involved in the expression of the observed phenotypes within the bioreactor, and investigate augmenting the performance of the PTB system to maximise economic performance perhaps for application in the fuel ethanol industry. Additionally, genetic manipulation opens up more IP options for the protection of the PTB.

Proposed work would include the characterisation of the FLO gene suite and upstream regulatory regions for all observed phenotypic variations within the PTB, the investigation of the effects of increased copy numbers of FLO1 (on pUG6), observing the higher biomass aggregation effect on washout and reactor productivity. The insertion of variably inducible artificial regulators (gDNA and pDNA) in order to artificially regulate the ethanol productivity and downstream processing costs. The conversion of highly ethanol tolerant NewFlo batch *S.cerevisiae* strains to Flo1 type and effects on biomass retention and ethanol productivity and the conversion of commercial wine and beer yeast to Flo1 type through recombinant techniques and hybridisation perhaps allowing the adaptation of the PTB technology to a wide rage of beverages (beverages produced in this manner could not be sold as GMOs in food products is

currently outlawed be US federal, EU and South African legislation). Also completing thorough economic analysis of the process could be completed and refined with more information.

Some other simpler questions could also be answered with additional work, such as: what effect would different immobilisation matrices have on the reactor performance? How long does the adaptation of a commercial yeast strain to one capable of remaining immobilised within the bioreactor take? What is the flocculation rate of the yeast used in the PTB and how does it compare to other yeasts? How did biomass levels vary under growth conditions in the bioreactor, and at steady state?

The possible implications of such research would be interesting and perhaps widespread. The technology developed during this research has the potential to enable the democratisation of the beverage alcohol and potentially, many other industries. The PTB system allows for drastically reduced capital expenditure for beverage alcohol manufacture and the business strategy using decentralised low capacity production facilities enables extremely favourable economic performance. With further research and development, this technology could make a significant and positive impact on one of the worlds largest industries.

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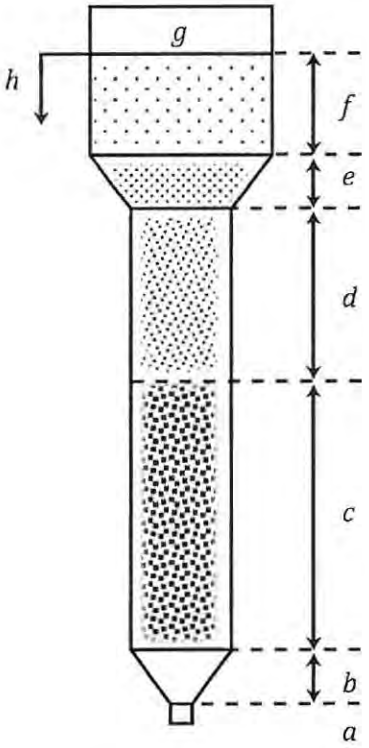
Appendix A

Key Parameter Values for Bench Scale PTB



Section	Height (cm)	Diameter (Internal)(cm)
a	3	1
b	2	1-3
c	87	3
d	10	3
e	4	3-10
f	13	10
g	4	10

Flare angles:

b = 45°

e = 60.25°

Volumes :

Packed bed (fluid) = 48.72 mL

Headspace = 324.16 mL

Total Internal = 0.923 L

Packed Bed Aspect Ratio = 29:1

Flow :

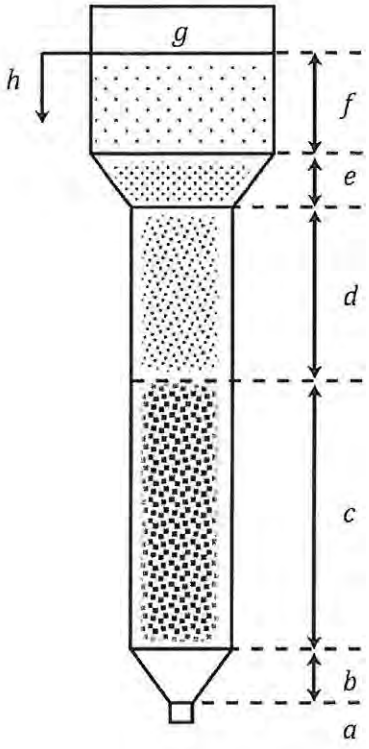
Max Volumetric Flow = 1.388 $mL.min^{-1}$

Superficial Flow Before Expander = 0.196 $cm.min^{-1}$

Superficial Flow After Expander = 0.018 $cm.min^{-1}$

Appendix B

Key Parameter Values for Pilot Scale PTB



Section	Height (cm)	Diameter (Internal)(cm)
a	3	1
b	4	1-10
c	130	10
d	10	10
e	6	10-20
f	21	20
g	4	20

Flare angles:

b = 46.04°

e = 59.04°

Volumes :

Packed bed (fluid) = 326.62 mL

Headspace = 1256.64 mL

Total Internal = 13 L

Packed Bed Aspect Ratio = 17.5:1

Flow :

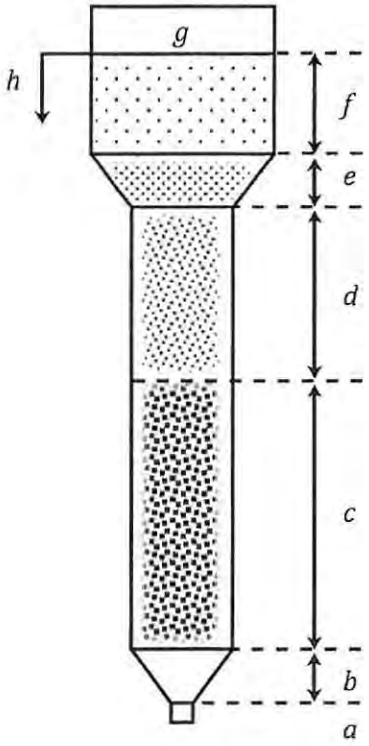
Max Volumetric Flow = 18.056 $mL.min^{-1}$

Superficial Flow Before Expander = 0.229 $cm.min^{-1}$

Superficial Flow After Expander = 0.057 $cm.min^{-1}$

Appendix C

Key Parameter Values for Commercial Scale PTB



Section	Height (cm)	Diameter (Internal)(cm)
a	5	4
b	19	4-25
c	122	25
d	76	25
e	24	25-56
f	33	56
g	20	56

Flare angles:

b = 47.86°

e = 52.25°

Volumes :

Packed bed (fluid) = 33, 086 mL

Headspace = 19.7 L

Total Internal = 210 L

Packed Bed Aspect Ratio = 4.9:1

Flow :

Max Volumetric Flow = 208.33 $mL.min^{-1}$

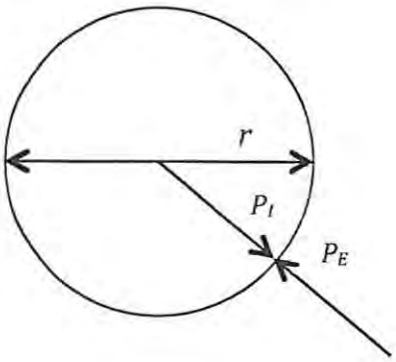
Superficial Flow Before Expander = 0.424 $cm.min^{-1}$

Superficial Flow After Expander = 0.021 $cm.min^{-1}$

Appendix D

Additional Calculations

Size of Liquid Bubble



$$P_I = P_E + \frac{4\gamma}{r} \qquad P_E = \frac{\rho A h g}{g_c}$$

$$P_I V = nRT \qquad V = {}^{4}/_{3}\,\pi r^2$$

$$P_I = \frac{\rho A h g}{g_c} + \frac{4\gamma}{r}$$

Qualitatively, where γ, n, R, T, ρ and g_c are constant and are equal to C

$$(C)(h) + \frac{C}{r} = P_I$$

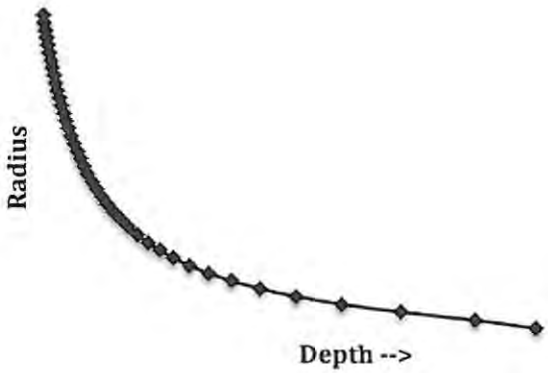
$$\left(Ch + \frac{C}{r}\right) V = C$$

$$\left(Ch + \frac{C}{r}\right)(Cr^2) = C$$

$$Ch + \frac{C}{r} = \frac{1}{r^2}$$

$$h + \frac{1}{r} = \frac{1}{r^2 C}$$

$$h = \frac{1}{r^2 C} - \frac{1}{r}$$



Scale-Up Packed Bed Volume Determination (Regime Analysis)

$$S_{0i} - S_0 = \frac{q_p X}{Y_{P/S}} . \frac{A}{F} h$$

Where:				Bench Scale Value
S_{0i}	=	initial sugar concentration	=	170 $g.L^{-1}$
S_0	=	final sugar concentration	=	8.1 $g.L^{-1}$
q_pX	=	g ethanol per g dry cells per hour	=	?
$Y_{P/S}$	=	yield (gram ethanol per gram of sugar)	=	0.470 $g.g^{-1}$
A	=	cross sectional area	=	7.07 cm^2
h	=	height of active packed bed	=	55 cm
F	=	flow rate	=	0.06 $L.h^{-1}$

q_pX = 0.01174 g eth.g $cells^{-1}.h^{-1}$

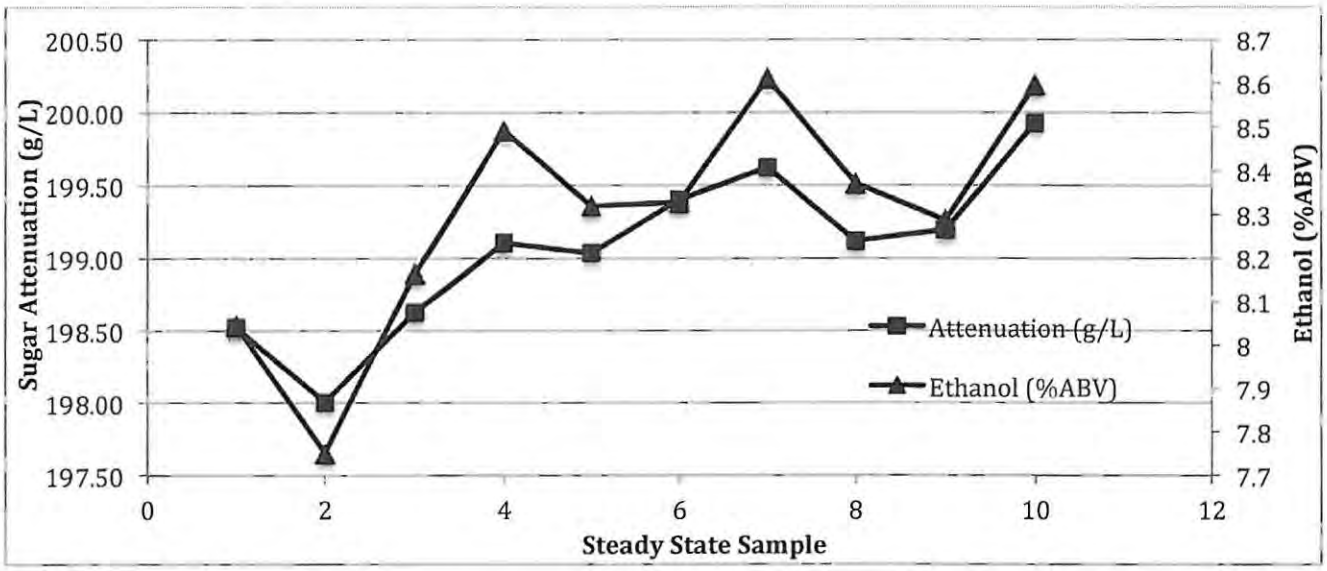
Solving for h where A = 78.54 cm^2 and F = 1.083 $L.h^{-1}$ (pilot scale), and where A = 490.87 cm^2 and F = 12.5 $L.h^{-1}$ (commercial scale) gives:

Pilot: h = 89.37 cm

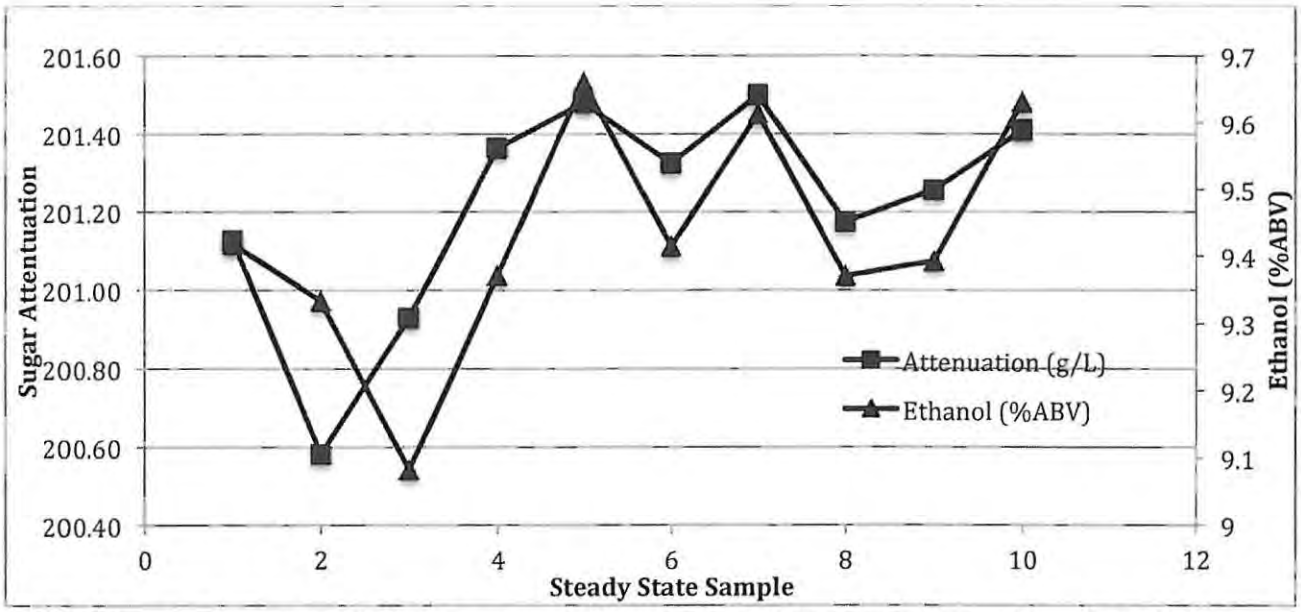
Commercial: h = 165 cm

Appendix E

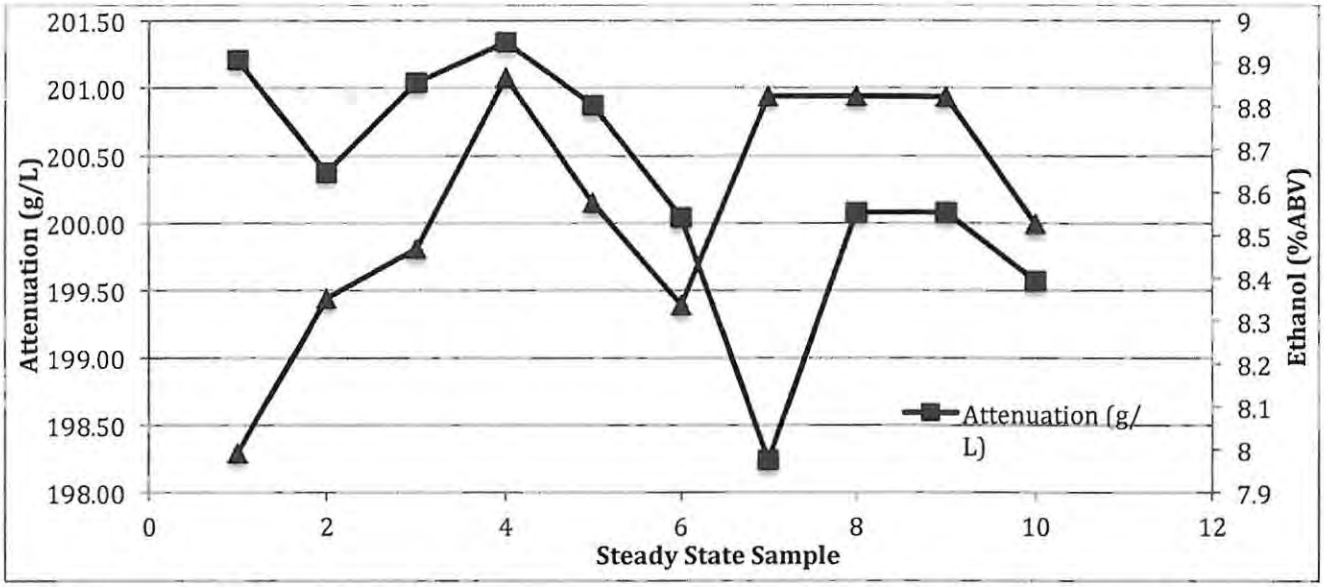
Pilot Scale PTB Individual Performance Data



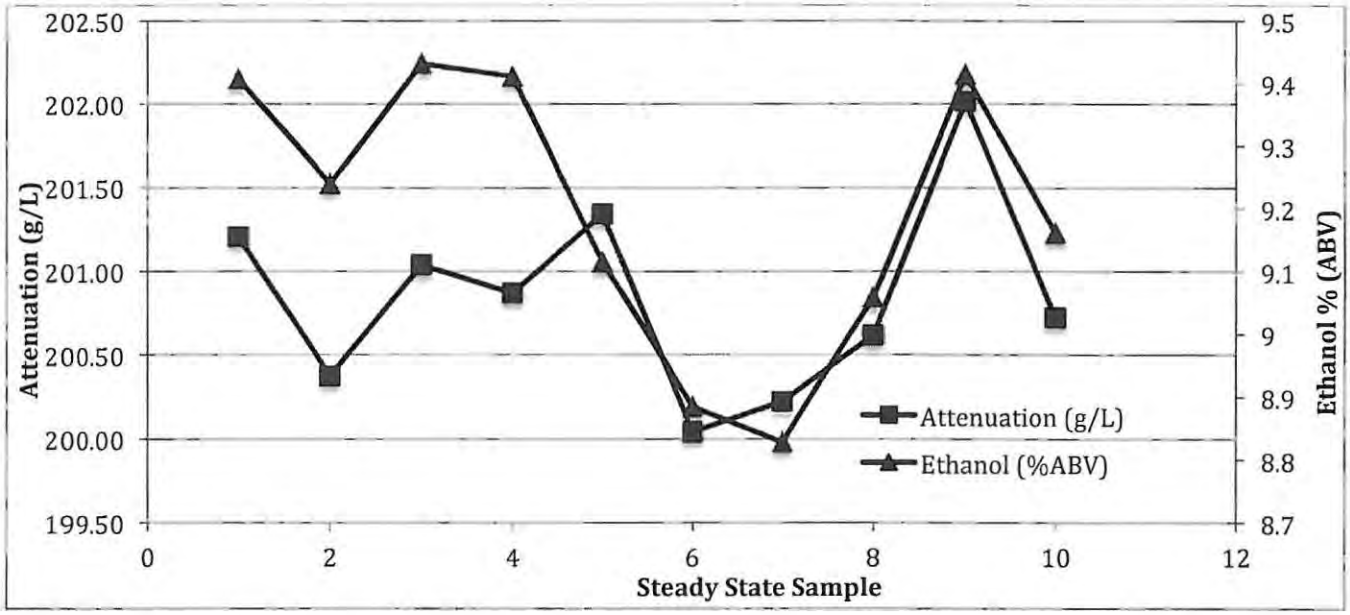
Pilot scale fermenter 1: Average sugar attenuation and % ethanol by volume for 10 day period during early steady state, (n=2).



Pilot scale fermenter 2: Average sugar attenuation and % ethanol by volume for 10 day period during early steady state, (n=2).



Pilot scale fermenter 3: Average sugar attenuation and % ethanol by volume for 10 day period during early steady state, (n=2).



Pilot scale fermenter 4: Average sugar attenuation and % ethanol by volume for 10 day period during early steady state, (n=2).